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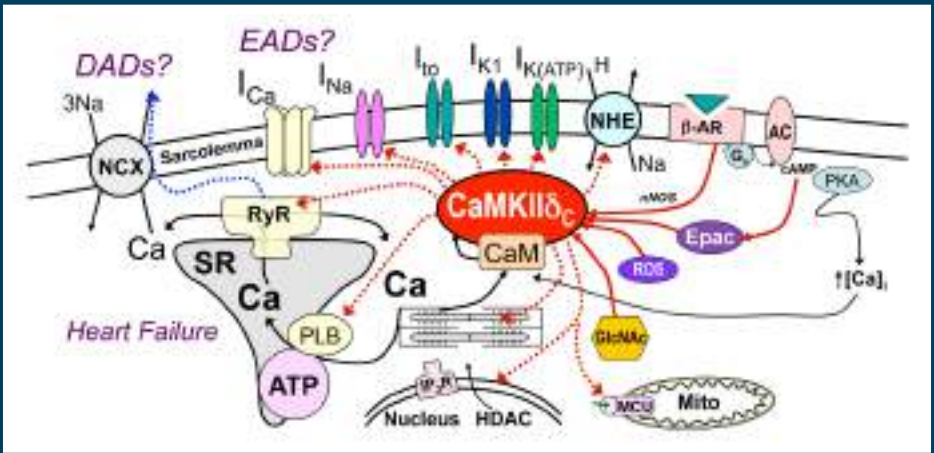

CaMKII IN CARDIAC HEALTH AND DISEASE

Topic Editors
Eleonora Grandi, Andrew G. Edwards
Anthony W. Herren and Donald M. Bers

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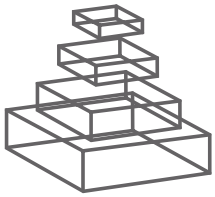

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CaMKII IN CARDIAC HEALTH AND DISEASE

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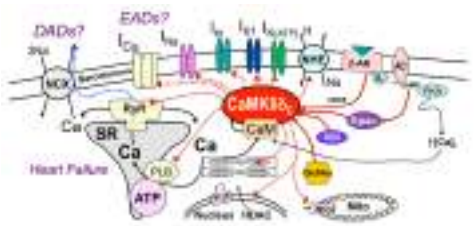

Mechanisms by which CaMKII regulates cardiomyocyte electrophysiology, calcium handling, transcription and mitochondrial function.

The calcium-calmodulin dependent protein kinases (CaMKs) are a broadly expressed family of calcium-sensitive intracellular kinases, which are responsible for transducing cytosolic calcium signals into phosphorylation-based regulation of proteins and physiological functions. As the multifunctional member of the family, CaMKII has become the most prominent for its roles in the central nervous system and heart, where it controls a diverse range of calcium-dependent processes; from learning and memory at the neuronal synapse, to cellular growth and death in the myocardium. In the heart, CaMKII directly regulates many of the most important ion channels and calcium handling proteins, and controls the expression of an ever-increasing number of transcripts and their downstream products. Functionally, these actions are thought to orchestrate many of the electrophysiologic and contractile adaptations to common cardiac stressors, such as rapid pacing, chronic adrenergic stimulation, and oxidative challenge.

In the context of disease, CaMKII has been shown to contribute to a remarkably wide variety of cardiac pathologies, of which heart failure (HF) is the most conspicuous. Hyperactivity of CaMKII is an established contributor to pathological cardiac remodeling, and is widely thought to directly promote arrhythmia and contractile dysfunction during HF. Moreover, several non-failing arrhythmia-susceptible phenotypes, which result from specific genetic channelopathies, functionally mimic constitutive channel phosphorylation by CaMKII. Because CaMKII contributes to both the acute and chronic manifestations of major cardiac diseases, but may be only minimally required for homeostasis in the absence of chronic stress, it has come to be one of the most promising therapeutic drug targets in cardiac biology. Thus, development of more specific and deliverable small molecule antagonists remains a key priority for the field.

Here we provide a selection of articles to summarize the state of our knowledge regarding CaMKII in cardiac health and disease, with a particular view to highlighting recent developments in CaMKII activation, and new targets in CaMKII-mediated control of myocyte physiology.

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EDITORIAL
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CaMKII comes of age in cardiac health and disease

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TRANSLATIONAL PERSPECTIVE

Almost four decades since its initial discovery in brain (Schulman and Greengard, 1978), the multifunctional Ca^{2+} and calmodulin-dependent protein kinase II (CaMKII) has now emerged as a key signaling molecule in the heart. The isoform that predominates in heart, CaMKIIδ, directly regulates expression and function of several of the main cardiac ion channels and Ca^{2+} handling proteins (Bers and Grandi, 2009). CaMKII-dependent effects are thought to orchestrate many of the electrophysiologic and contractile adaptations to common cardiac stressors, such as rapid pacing, adrenergic stimulation, and oxidative challenge. In the context of disease, CaMKII has been shown to contribute to a remarkably wide variety of cardiac pathologies, including myocardial hypertrophy, ischemia, heart failure (HF), and arrhythmia. CaMKII expression is increased in patients with HF (Hoch et al., 1999), and elevated CaMKII expression and activity have been implicated in the transition to HF (Zhang et al., 2003). Indeed, inhibiting CaMKII appears to reduce arrhythmias and pathological signaling, which makes this kinase a promising new therapeutic target (Anderson et al., 2011).

OVERVIEW OF THE RESEARCH TOPIC

This series reviews the molecular physiology of CaMKII and discusses the impact of CaMKII on heart function. The subjects were chosen to summarize the current state of our knowledge of various aspects of CaMKII-mediated control of myocyte physiology, with in-depth focused reviews by recognized leaders in these areas.

To start off, a group of articles examines the biochemistry of CaMKII activation and the pharmacology of its inhibitors. Recent efforts to characterize the individual pathways of CaMKII activation in the heart are reviewed. These studies improved our understanding of the downstream pathological consequences of these specific pathways (Erickson, 2014). Indeed, the promise of CaMKII inhibition for simultaneous prevention of HF progression and development of arrhythmias justifies the development of more specific CaMKII inhibitors that will further both basic research studies and drug development (Westenbrink et al., 2013). Thus, the kinase structure and possible sites for its inhibition (Pellicena and Schulman, 2014) are illustrated. It is also becoming clear that design of therapeutic intervention requires an improved understanding of the specific roles and function of the CaMKIIδ subtypes, δB and δC, as discussed by Gray and Heller Brown (2014).

A second group of articles reviews the large body of work describing CaMKII-specific interaction with its numerous intracellular targets, many of which play important roles in modulating cardiac excitation contraction coupling (ECC). CaMKII involvement at multiple levels in ECC indicates that it is an important modulator of both electrophysiological and contractile properties in the heart. Active CaMKII phosphorylates several Ca^{2+} handling proteins including ryanodine receptors (RyR2) (Camors and Valdivia, 2014), phospholamban (Mattiazzi and Kranias, 2014), and L-type Ca^{2+} channels (Bers and Morotti, 2014). In addition, non-Ca^{2+} transporters such as sarcolemmal Na^{+} (Grandi and Herren, 2014) and K^{+} (Mustroph et al., 2014) channels are regulated by CaMKII. This in turn influences myocyte Ca^{2+} regulation and also confers further Ca^{2+}-dependence to a variety of electrophysiological processes. In combination these effects create a complex and non-linear feedback system that necessitates quantitative computational approaches. As such, multiscale models have played an important role in advancing our understanding of CaMKII function in cardiac ECC, particularly by being able to both reconstruct the details of local signaling events within the cardiac dyad, and predict their functional consequences at the level of the whole cell (Greenstein et al., 2014). CaMKII also regulates Ca^{2+} handling proteins that are thought to contribute relatively little to ECC, the best example of which is the inositol 1,4,5-trisphosphate receptor (IP3R), as reviewed by Camors and Valdivia (2014). This regulatory role is thought to be key in modulating IP3R-mediated Ca^{2+} release in the regulation of cytosolic and nuclear Ca^{2+} signals (Hohendanner et al., 2014). These mechanisms are suspected to be involved in coupling cytosolic and SR Ca^{2+} handling to adaptive transcriptional and epigenetic processes. Indeed, in addition to the above-mentioned acute effects due to phosphorylation of cytosolic proteins, CaMKII has been shown to influence chronic physiological and pathological processes, particularly those contributing to the ventricular remodeling that leads to HF (Kreusser and Backs, 2014).

Finally, the integrative role of this enzyme in cardiac physiology and disease is discussed. As described above, the multifunctional nature of CaMKII causes this integration to be very challenging, and the development of detailed multiscale computational models has greatly improved our ability to describe how molecular actions can impact tissue and organ function. The review by Onal et al. (2014) dissects how these systems biology approaches have contributed to linking CaMKII activity in single

myocytes to observable tissue-level arrhythmogenic outcomes in cardiac disease. Experimental disease models that incorporate CaMKII overexpression clearly demonstrate a link between its excessive activity and arrhythmias associated with congenital and acquired heart disease. Vincent et al. (2014) review CaMKII involvement in both types of disease, and argue that the importance of CaMKII phosphorylation at RyR2 across a variety of disease models suggests that this molecular interaction may be a point of mechanistic convergence in many forms of cardiac arrhythmia. In addition to its abundantly studied ventricular consequences, such as in HF and ischemia/reperfusion challenge (Bell et al., 2014), CaMKII has emerged as a key determinant of sinoatrial node dysfunction (as well as a central regulator of physiological sinoatrial node responses) (Wu and Anderson, 2014; Yaniv and Maltsev, 2014). Recently, a significant effort has also been invested in describing the role played by CaMKII in atrial fibrillation, where the many actions of CaMKII are superimposed upon the unique electrophysiologic and structural characteristics of the diseased atrium (Heijman et al., 2014).

CONCLUSIONS AND FUTURE DIRECTIONS

CaMKII is an abundant signaling molecule in myocardium, where it integrates and transduces cellular Ca^{2+} signals into physiological responses in heart. Under various pathological stresses, CaMKII hyperactivity appears to be an important component of disease signaling. However, in the context of complex cardiac disease, such as HF, multiple signaling pathways are likely to be altered, many of which might crosstalk with CaMKII signaling. Thus, the interplay of various mechanisms of CaMKII activation, particularly during the development of cardiovascular diseases, is an important open avenue of investigation. As a complicating factor, CaMKII itself contributes to the regulation of intracellular signaling processes, such as mitochondrial function (Joiner and Koval, 2014), thus forming potential feedback loops leading to further CaMKII activation.

Recent findings that CaMKII inhibition ameliorates HF and suppresses arrhythmias suggest that developing specific CaMKII inhibitory drugs may be a new therapeutic approach to these diseases. However, current pharmacological tools are limited. Development of new inhibitors (Pellicena and Schulman, 2014) will enable preclinical proof-of-concept tests and clinical development of successful lead compounds, as well as improved research tools to more accurately examine and extend knowledge of the role of CaMKII in cardiac health and disease.

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Mechanisms of CaMKII activation in the heart

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Calcium/calmodulin (Ca^{2+}/CaM) dependent protein kinase II (CaMKII) has emerged as a key nodal protein in the regulation of cardiac physiology and pathology. Due to the particularly elegant relationship between the structure and function of the kinase, CaMKII is able to translate a diverse set of signaling events into downstream physiological effects. While CaMKII is typically autoinhibited at basal conditions, prolonged rapid Ca^{2+} cycling can activate the kinase and allow post-translational modifications that depend critically on the biochemical environment of the heart. These modifications result in sustained, autonomous CaMKII activation and have been associated with pathological cardiac signaling. Indeed, improved understanding of CaMKII activation mechanisms could potentially lead to new clinical therapies for the treatment or prevention of cardiovascular disease. Here we review the known mechanisms of CaMKII activation and discuss some of the pathological signaling pathways in which they play a role.

Keywords: CaMKII, heart failure, oxidative stress, diabetes, *O*-GlcNAc modification

CaMKII STRUCTURE/FUNCTION RELATIONSHIP

Calcium/calmodulin (Ca^{2+}/CaM) dependent protein kinase II (CaMKII) is expressed as a multimeric protein, typically comprised of 12 subunits in most commonly observed physiological conditions (Hoelz et al., 2003). Individual monomers assemble into a dodecameric mulitmer via association at the *C*-terminal domain of each subunit. This association takes the form of a pair of hexameric rings arranged in parallel with one another (Rellos et al., 2010). This CaMKII structural motif is sometimes termed a "wagon wheel" arrangement, as individual monomers form the spokes of the wheel around a central core.

Each monomer is itself comprised of three domains (**Figure 1**). The *C*-terminal association domain directs assembly of the CaMKII multimer, while the *N*-terminal catalytic domain binds to potential substrates and performs the serine/threonine kinase function of CaMKII. In the intervening linker region (the composition of which varies greatly depending on isoform and splice variant) lies the regulatory domain, which has two primary roles. First, the regulatory domain acts as a substrate for the catalytic domain within each CaMKII monomer, while adjacent regulatory domains within the multimer block both substrate and ATP binding to the catalytic domain itself (Rosenberg et al., 2005). Thus, the regulatory and catalytic domains are tightly associated at basal conditions, resulting in autoinhibition of the kinase. Secondly, the regulatory domain binds CaM with a K_D of 10–70 nM (Gaertner et al., 2004) when intracellular [Ca^{2+}] is elevated (half maximal CaM occupancy requires approximately 1.0 μM Ca^{2+}; Rostas and Dunkley, 1992). When CaM binds to CaMKII, a conformational shift occurs that disrupts the association between the catalytic and regulatory domains, exposing the catalytic domain for substrate binding and relieving autoinhibition of the kinase. CaMKII function is critically linked to this CaM binding function of the regulatory domain; indeed, all known mechanisms of CaMKII activation require Ca^{2+}/CaM binding as an initiating step. Thus, the extent of CaMKII activation within a cell is likely to be at least partly dependent on local CaM concentrations, as has been proposed in cardiac myocytes (Saucerman and Bers, 2008). The role of the regulatory domain to CaMKII function is so integral, it is not surprising to discover that the known isoforms and splice variants of mammalian CaMKII have nearly identical primary structure within the regulatory domain (**Figure 2**).

CaMKII PHOSPHORYLATION AND AUTONOMOUS ACTIVITY

The duration of CaMKII activation by Ca^{2+}/CaM binding is dependent on the frequency of Ca^{2+} release events (De Koninck and Schulman, 1998). Conditions of prolonged Ca^{2+}/CaM association with CaMKII, as would be observed during lengthy or high frequency calcium transients, allow for intersubunit autophosphorylation of CaMKII monomers at the T287 (T286 in the α isoform) site. The addition of a phosphate group at T287 has two critical effects on CaMKII function. First, the binding of affinity of CaM for the CaMKII regulatory domain increases by more than 1000-fold (Meyer et al., 1992). Second, the presence of a negatively charged phosphate group at the T287 site precludes reassociation of the catalytic and regulatory domains, preventing autoinhibion even if $[Ca^{2+}]_i$ falls and CaM dissociates from CaMKII (Lai et al., 1987). Subsequently, autonomous activation of CaMKII by T287 phosphorylation will persist until the phosphate group is removed by a protein phosphatase (PP1 or PP2A; Stack et al., 1997). The extent of CaMKII activation in the heart after autophosphorylation is not known, though evidence in the neuronal form of CaMKII points to autonomous activity that corresponded to less than 25% of full CaMKII activity (Coultrap et al., 2010).

CaMKII has been called the "memory molecule" in part for its function in the processing of neural information from short-term to long-term memory, but also due to the ability of CaMKII to translate Ca^{2+} transient frequency into autonomous activation of the kinase (Hudmon and Schulman, 2002; Coultrap and

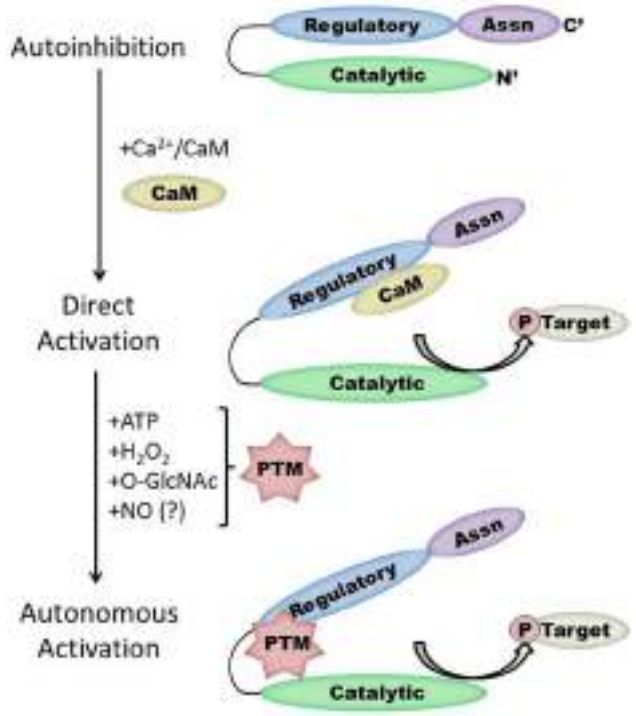

FIGURE 1 | Schematic representation of the CaMKII structure/function relationship in the heart. Under basal conditions, the kinase is autoinhibited due to interaction between the regulatory and catalytic domains. Direct activation occurs when Ca^{2+}/CaM binds to CaMKII, while subsequent post-translational modification (PTM) results in autonomous kinase activity. Protein interactions with CaMKII autonomously activate CaMKII in the brain but have not yet been demonstrated in the heart. Note that the size of each region is not to scale and will vary by isoform and splice variant of CaMKII.

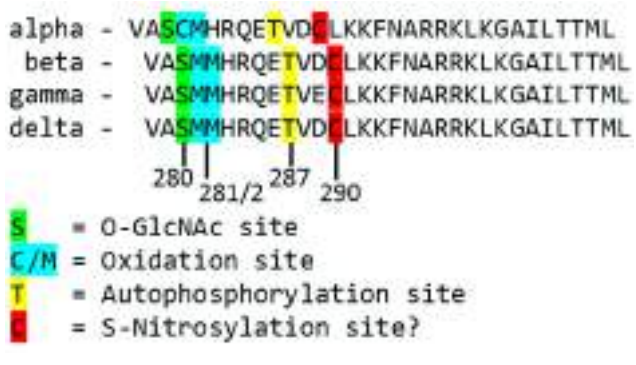

FIGURE 2 | Alignment of the primary structure of regulatory domains from *Mus musculus* CaMKII isoforms. Specific residues associated with autonomous activation by post-translational modifications have been labeled.

Bayer, 2012). Indeed, the discovery that post-translational modification of CaMKII resulted in a shift from Ca^{2+}-dependent to Ca^{2+}-independent states gave rise to the now popular belief that CaMKII functions as a nodal cardiac signaling molecule that translates upstream cellular events into downstream physiological effects. In the heart, autophosphorylation of CaMKII is particularly prevalent during β-adrenergic signaling (Erickson et al., 2011) and is thought to play a key role in the development of cardiac hypertrophy and dilated cardiomyopathy (Zhang et al., 2003).

CaMKII OXIDATION

While direct Ca^{2+}/CaM-binding and T287 autophosphorylation have been recognized for over two decades, more recently published results have demonstrated new pathways of CaMKII activation. For example, CaMKII can be directly modified by reactive oxygen species (ROS), resulting in autonomous activation (Erickson et al., 2008). The regulatory domain of CaMKII contains a pair of redox-sensitive amino acids (C280/M281 in the α isoform, M281/M282 in the β, γ, and δ isoforms) that can be oxidized when exposed to elevated levels of oxidative stress. As is the case in T287 phosphorylation, redox modification of CaMKII only occurs after Ca^{2+}/CaM binding initiates a conformational shift of the inhibited kinase and exposes the target sites. An examination of the crystal structure of CaMKII suggests that the addition of an oxidative modification at the M282 site preserves kinase activity by preventing reassociation between the regulatory and catalytic domains (Rellos et al., 2010). Additionally, this study suggests that M282 oxidation should preclude T287 phosphorylation, though this hypothesis has not been confirmed experimentally. Mutation of the M281 and M282 residues does not disrupt phosphorylation-dependent CaMKII activation (Erickson et al., 2008). We can therefore conclude that while the phosphorylation- and oxidation-dependent mechanisms share similarities, they constitute parallel pathways for maintaining CaMKII activation.

Since the redox-dependent mechanism was reported, oxidation of CaMKII has been found to play a role in a number of cardiac pathologies. For example, the renin-angiotensin-aldosterone signaling (RAAS) pathway promotes enhanced oxidative stress in the heart. Angiotensin II (AngII) induced apoptosis of cardiac myocytes is ablated in isolated neonatal mouse myocytes expressing the oxidant-resistant mutant of CaMKII (Erickson et al., 2008). Similarly, elevated levels of circulating aldosterone enhance CaMKII oxidation, leading to apoptosis, impaired cardiac function, and potential cardiac rupture (He et al., 2011; Velez Rueda et al., 2012). Redox-dependent CaMKII activation also enhances electrical remodeling in the heart, including enhanced Na^+ current (and subsequent $[Na^+]_i$ overload; Wagner et al., 2011), apoptosis of sinoatrial nodal cells (Swaminathan et al., 2011), and eventual impaired conduction of transients (Christensen et al., 2009), all of which contribute to life-threatening arrhythmias. Moreover, a diabetic mouse model expressing oxidation resistant CaMKII (MM281/282VV) was found to be resistant to sinoatrial nodal cell death, fibrosis, and mortality after myocardial infarction compared to diabetic animals expressing wild type CaMKII (Luo et al., 2013). These observations suggest that oxidation-dependent CaMKII activity plays a critical role in numerous pathological processes in the heart.

Just as T287 phosphorylation of CaMKII is reversed by phosphatases, the enzyme methionine sulfoxide reductase A (MsrA)

reduces oxidized methionine residues to inactivate the kinase (Erickson et al., 2008). Thus, MsrA has emerged as a potential cardioprotective molecule against the deleterious effects of oxidative stress. For example, a genetic knockout mouse model lacking MsrA was found to have significantly increased redox-activated CaMKII, myocyte apoptosis, structural remodeling, and mortality 4 weeks after myocardial infarction compared to wild type littermates (Erickson et al., 2008). Conversely, a transgenic mouse model that overexpresses MsrA was found to be less susceptible to aldosterone-mediated CaMKII oxidation and cardiac remodeling (He et al., 2011), and was also more resistant to AngII-induced atrial fibrillation (Purohit et al., 2013). Interestingly, the expression and activity of MsrA is reduced in brain tissue from aging human patients (Petropoulos et al., 2001), and this age-dependent reduction in MsrA activity is linked with the development of Alzheimer's disease (Gabbita et al., 1999). However, the age-dependence of MsrA expression and activity in the human heart has not been studied.

CaMKII ACTIVATION IN DIABETES

Diabetes mellitus is marked by a number of altered cellular signaling pathways, creating physiological stress that can activate CaMKII. For example, diabetic patients have a significantly greater proportion of oxidized to total CaMKII compared to non-diabetic patients (Luo et al., 2013), consistent with observations that altered cellular metabolism in diabetics results in enhanced oxidative stress. A recently published study points to a novel mechanism for CaMKII activation during hyperglycemia and diabetes through the addition of an *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) modification (Erickson et al., 2013). Post-translational modification by *O*-GlcNAc ("*O*-GlcNAcylation") is an emerging field with important regulatory implications in disease states characterized by altered glucose signaling, such as myocardial infarction and diabetes (Hart et al., 2007; Chatham and Marchase, 2010). *O*-GlcNAc modification can alter protein function (Dias et al., 2009), and such regulation is known to play a role in both the heart and brain (Gao et al., 2001). *O*-GlcNAcylation is catalyzed by the enzyme *O*-GlcNAc transferase in the presence of the substrate UDP-*N*-acetylglucosamine, which is produced in conditions of elevated glucose as a product of the hexosamine biosynthesis pathway (Hart et al., 2007).

When CaMKII is activated by Ca^{2+}/CaM binding in the presence of elevated [glucose], an *O*-GlcNAc modification is added to the regulatory domain at S280 (S279 in the alpha form), resulting in autonomous activation of the kinase (Erickson et al., 2013). The extent of both *O*-GlcNAc modification and activation of CaMKII varies with glucose availability in a dose-dependent manner and is reversible by the action of *O*-GlcNAcase, an enzyme that removes *O*-GlcNAc modifications from proteins. These observations point to a potential regulatory role for *O*-GlcNAcylation of CaMKII, consistent with the observation that the ratio of *O*-GlcNAc modified CaMKII to total CaMKII is greatly enhanced in the heart and brain from diabetic patients. Further, glucose induced Ca^{2+} leak from the sarcoplasmic reticulum (measured as Ca^{2+} sparks) is both CaMKII and *O*-GlcNAc dependent, suggesting a connection between *O*-GlcNAc mediated CaMKII activity and arrhythmogenesis in the diabetic heart. Indeed, pharmacological inhibition of the hexosamine biosynthesis pathway (and therefore the production of *O*-GlcNAc precursors) prevented ventricular tachycardia in hearts from diabetic rats challenged with dobutamine and caffeine (Erickson et al., 2013). Taken together, these observations suggest that *O*-GlcNAc modification of CaMKII could play a critical role in structural and electrical remodeling in the diabetic heart.

ALTERNATE MECHANISMS OF CaMKII ACTIVATION IN THE HEART

The preceding sections describe several established mechanisms of CaMKII activation in the heart, but it is unlikely that they are exhaustive. Indeed, emerging evidence points to other potential mechanisms that are not fully described for cardiac CaMKII. For example, several recent studies have provided new evidence suggesting the existence of an undescribed mechanism of CaMKII activation mediated by intracellular nitric oxide (NO). For example, isoproterenol-induced Ca^{2+} leak from the sarcoplasmic reticulum is determined in part by the activity of both NO synthase and CaMKII (Curran et al., 2009). ATP-sensitive potassium channels are also modulated by NO, and these NO-dependent effects on K_{ATP} were ablated in knockout mice lacking the cardiac isoform of CaMKII (Zhang et al., 2013). CaMKII activity is enhanced in the presence of the NO donors NOC-18 (Zhang et al., 2013) and GSNO [Gutierrez et al., 2013; though another study found inactivation of CaMKII by NO donors (Song et al., 2008)], while a non-site specific antibody suggests that, at least *in vitro*, CaMKII contains S-nitrosylated cysteine residues. Computational prediction of S-nitrosylation sites on CaMKII indicate three potential target sites, including the C290 site present in the regulatory domain (Gutierrez et al., 2013). While none of the potential sites have been confirmed and the mechanism by which nitrosylation induces CaMKII activity has not been described, the evidence in favor of this novel pathway of CaMKII activation is compelling.

Additional potential mechanism for activation of CaMKII in the heart is through interaction of the kinase with protein partners. The first and best described such mechanism was identified in the α (neuronal) isoform of CaMKII, which can become autonomously activated through association with the NMDA receptor (Strack and Colbran, 1998; Bayer et al., 2001), contributing to long term potentiation (Barria and Malinow, 2005), and memory consolidation (Halt et al., 2012). While this topic extends beyond the scope of the current, cardiac-focused review [please see references Coultrap and Bayer (2012) and Colbran (2004) for a closer examination of CaMKII/NMDA receptor binding], it does suggest the possibility that interactions between CaMKII and other protein partners could alter kinase activity. For example, binding of α-actinin to the α isoform of CaMKII can mimic Ca^{2+}/CaM binding and activate the kinase in HEK293 cells (Jalan-Sakrikar et al., 2012). In cardiac cells, CaMKII association with the $K_V4.3$ potassium channel blocks Ca^{2+}/CaM binding and reduces kinase activity (Keskanokwong et al., 2011), but whether protein binding can enhance CaMKII activity in the heart is an important open question.

SYNERGISTIC ACTIVATION OF CaMKII: AN OPEN QUESTION

While our understanding of the underlying mechanisms that determine CaMKII function has grown rapidly of late, numerous questions remain unanswered. Recent efforts have focused on the characterization of individual pathways of CaMKII activation and on the downstream pathological consequences of these specific pathways. In the context of cardiac disease states with complex pathophysiologies, such as in heart failure or diabetes, multiple signaling pathways are likely to be altered. Complicating matters is the issue that the various pharmacological inhibitors of CaMKII currently in use rely on different mechanisms. For example, the CaMKII inhibitor KN-93 (but not the inactive analog KN-92) competes with CaM binding to the CaMKII regulatory domain and prevents activation of the kinase (Hudmon and Schulman, 2002). However, KN-93 has no such CaMKII inhibitory effect after autonomous activation (Buard et al., 2010). Conversely, peptide inhibitors such as AIP and AC3-I, which mimic the regulatory domain sequence and bind to the catalytic domain of CaMKII, prevent substrate binding regardless of the mode of CaMKII activation. Thus, careful consideration must be given to the selection and development of inhibitors in studies focused on CaMKII (Coultrap and Bayer, 2011). For a more thorough review of CaMKII inhibitors, please see the review by Dr. Howard Schulman in this Research Topic.

Moreover, CaMKII itself contributes to the regulation of intracellular signaling processes such as mitochondrial function (Joiner et al., 2012) and insulin secretion (Dixit et al., 2013), many of which could form potential feedback loops through additional CaMKII modification. Thus, the interplay between various mechanisms of CaMKII activation, particularly during the development of cardiovascular diseases, is an important open line of inquiry. Moreover, models based on data generated from examination of the crystal structure of CaMKII predict that intersubunit phosphorylation does not occur as a random, coincidence-based process, but rather occurs via cooperative modification of individual subunits (Chao et al., 2010). This observation suggests that the activity of each CaMKII monomer is mediated acutely by the post-translational modifications present on adjacent monomers.

Does the presence of one type of modification enhance the probability of other modifications occurring on the same or on neighboring subunits? In one study enhanced *O*-GlcNAc modification of CaMKII was associated with an increase in phosphorylation of CaMKII in isolated rat myocytes exposed to hyperglycemia (Erickson et al., 2013). In summary, these results provide compelling evidence for potential cross-talk between signaling mechanisms, but more work will be required to elucidate a fully integrative model of CaMKII activation.

CONCLUSION

Calcium/calmodulin dependent protein kinase II activity is dependent on a number of intracellular signaling pathways, either independently or as part of an integrative system. Thus, CaMKII represents an important nodal point in the translation of a broad range of cellular stresses into physiological and pathological pathways in the heart and other organ systems. Consequently, CaMKII has emerged as a potential therapeutic target in the clinical treatment of cardiac and neurological diseases. If we hope to develop a new generation of therapies based on modulation of CaMKII, we must understand the mechanisms that regulate CaMKII activity.

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CaMKII inhibitors: from research tools to therapeutic agents

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The cardiac field has benefited from the availability of several CaMKII inhibitors serving as research tools to test putative CaMKII pathways associated with cardiovascular physiology and pathophysiology. Successful demonstrations of its critical pathophysiological roles have elevated CaMKII as a key target in heart failure, arrhythmia, and other forms of heart disease. This has caught the attention of the pharmaceutical industry, which is now racing to develop CaMKII inhibitors as safe and effective therapeutic agents. While the first generation of CaMKII inhibitor development is focused on blocking its activity based on ATP binding to its catalytic site, future inhibitors can also target sites affecting its regulation by Ca^{2+}/CaM or translocation to some of its protein substrates. The recent availability of crystal structures of the kinase in the autoinhibited and activated state, and of the dodecameric holoenzyme, provides insights into the mechanism of action of existing inhibitors. It is also accelerating the design and development of better pharmacological inhibitors. This review examines the structure of the kinase and suggests possible sites for its inhibition. It also analyzes the uses and limitations of current research tools. Development of new inhibitors will enable preclinical proof of concept tests and clinical development of successful lead compounds, as well as improved research tools to more accurately examine and extend knowledge of the role of CaMKII in cardiac health and disease.

Keywords: CaMKII, kinase inhibitors, cardiovascular disease, CaMKII inhibitors, AC3-I, KN-93, CaMKIINtide, KN-62

INTRODUCTION

The search for a multifunctional Ca^{2+}-stimulated protein kinase serving to coordinate the actions of Ca^{2+}-linked signals, in analogy to the cAMP-dependent protein kinase (PKA) already known to coordinate the actions of cAMP, led to the discovery and characterization of multifunctional Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII; reviewed in Hudmon and Schulman, 2002; Swulius and Waxham, 2008). Delineation of its functions and relevant substrates was initially complicated by the fact that its activator, Ca^{2+}/CaM, regulates many other enzymes. It has therefore been pharmacological inhibitors and genetic ablation or suppression of CaMKII activity that have served to define its functions. This review aims to provide the context for understanding protein kinase inhibition and specifically to describe the types of inhibitors used, their advantages, and their disadvantages. There is now the potential for better inhibitors as therapeutic agents and research tools stemming from industry interest in pursuing CaMKII-based therapeutics, due in no small measure to the cardiovascular scientists in this issue who have identified its critical role in cardiac disease.

CaMKII STRUCTURE

Among Ca^{2+}/CaM-dependent kinases CaMKII can be best claimed as the multifunctional CaM kinase because it has broad substrate specificity and is ubiquitous, with the γ and δ isoforms present in heart, brain, and other tissues and α and β present at very high levels in brain. Each of these four genes give rise to multiple isoforms, primarily by alternatively spliced sequences (Hudmon and Schulman, 2002; Tombes et al., 2003). The most distinctive feature of CaMKII among protein kinases is that it self-assembles into supramolecular structures of twelve subunits. Each subunit contains an N-terminal catalytic domain followed by a regulatory segment of approximately 40 residues that serves an autoinhibitory function by blocking access to the catalytic site. This domain organization is typical of CaM-regulated protein kinases. The regulatory segment (or autoinhibitory domain) contains most of the elements that are critical for regulation of activity; the posttranslational modification (PTM) segment for regulation by autophosphorylation (Thr287), O-GlcNAC modification (Ser280), and oxidation (Met281/Met282), and the CaM-recognition sequence (**Figure 1**). We will use the amino acid numbering based on the sequence of CaMKIIδ, which are one higher than for the α isoform. Unique to CaMKII is the C-terminal hub or association domain, which is responsible for subunit oligomerization into dodecameric holoenzymes. A flexible linker of variable length connects the regulatory segment to the association domain and it is where most variability resides.

The high resolution crystal structures of the autoinhibited kinase domain and regulatory segment of *C. elegans* CaMKII (Rosenberg et al., 2005) and of all four human isoforms (Rellos et al., 2010) have been elucidated. The structures show a canonical kinase fold with an N-terminal lobe (N-lobe) connected by a "hinge" segment to a C-terminal lobe (C-lobe), where the peptide or protein substrate binding site resides. The ATP-binding site is located at the interface between the two lobes in close proximity to the peptide substrate binding site. In these autoinhibited structures the regulatory segment forms an α-helix of various lengths and folds back onto the kinase domain blocking access to the catalytic site (**Figure 1**). The critical autophosphorylation site, Thr287,

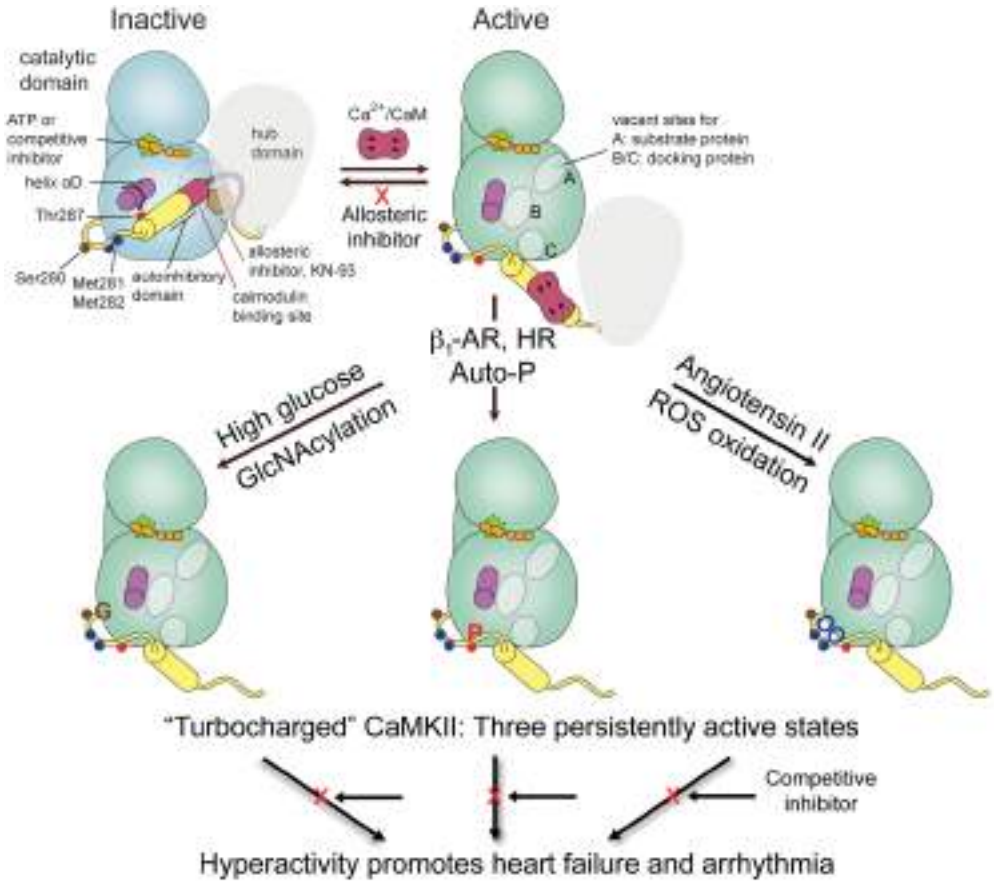

FIGURE 1 | CaMKII domain organization and schematic of activation states. One subunit of a holoenzyme composed of hub and bi-lobed catalytic domain is shown in the inactive conformation. The autoinhibitory domain or regulatory segment contains the PTM segment, important for regulation by phosphorylation, oxidation, and GlcNAcylation, followed by the CaM binding domain. Binding of Ca^{2+}/CaM displaces the autoinhibitory domain leading to rearrangement of the lower or C-lobe of the kinase allowing substrate access to the catalytic cleft and the phosphoacceptor site A, and formation of docking sites B/C that can also be used for substrate specificity. Regulatory modifications at PTM segment by either Ca^{2+}/CaM-stimulated autophosphorylation, e.g., by β-adrenergic stimulation or rapid heart rate (HR), oxidation by ROS, e.g., by Angiotensin II signaling, or GlcNAcylation by hyperglycemia disable the autoinhibitory domain to generate a persistently active or "turbocharged" CaMKII leading to cardiac pathophysiology.

is buried at the base of the regulatory segment and inaccessible for phosphorylation. Ca^{2+}/CaM binding to the regulatory segment has therefore the dual purpose of first facilitating access to the active site of the kinase by displacing the regulatory segment, and second, to make Thr287 available for phosphorylation *in trans* by a neighboring activated kinase subunit (Hanson et al., 1994). Phosphorylation of Thr287 likely impairs the rebinding of the autoinhibitory domain (Colbran et al., 1989) rendering the kinase "autonomous" of Ca^{2+}/CaM and constitutively active until dephosphorylated (reviewed in Hudmon and Schulman, 2002).

The activated state seen in a crystal structure of the kinase domain with the regulatory segment displaced from the kinase domain and bound to Ca^{2+}/CaM sheds light on the process of activation by CaM (Rellos et al., 2010). The most notable structural rearrangement is a major reorganization of a helical segment in the C-lobe of the kinase, helix αD (**Figure 1**), impeding the rebinding of the CaM-displaced regulatory segment. The positional shift in helix αD results in the reorientation of Glu97, an important ATP-coordinating residue, leading to a conformation improved for ATP-binding and catalysis (Rosenberg et al., 2005; Rellos et al., 2010). An interesting feature of this "activated" structure is that the regulatory segment adopts an extended conformation and positions Thr287 for capture and autophosphorylation by the active site of a neighboring kinase, as similarly seen in some of the *C. elegans* structures (Chao et al., 2010).

Studying activation states can give insights to additional strategies for inhibitor design (see below). The phosphoacceptor sequence in substrates is positioned at docking site A (previously termed S-site; **Figure 1**; Chao et al., 2010) and has been used in the design of peptide substrates and of "pseudosubstrate" peptides used as inhibitors. An important consequence of helix αD reorientation is the creation of a hydrophobic pocket (first identified and termed docking site B by Chao et al., 2010) that is absent in the autoinhibited form of the kinase. This site anchors hydrophobic residues located five to eight residues N-terminal to the phosphoacceptor site of some substrates for added specificity, and is used for intracellular targeting of the kinase and by

peptide inhibitors such as CaMKIINtide (see below). Similarly, an acidic pocket at the base of the C-lobe designated docking site C provides additional interactions for orienting interacting proteins (Chao et al., 2010; **Figure 1**). Docking sites B/C correspond functionally to the region of the molecule referred to as the T-site in previous studies of the autoinhibited state (Hudmon and Schulman, 2002 and references therein). Referring to these as docking sites B and C is now preferred because the site is not just vacated by the regulatory segment during activation but is altered in the process.

The holoenzyme is assembled as two hexameric rings symmetrically stacked one on top of the other with the kinase domains arranged peripherally around a central hub (Woodgett et al., 1983; Kolodziej et al., 2000; Morris and Torok, 2001; Chao et al., 2011). In an isoform lacking the linker domain, the kinase domains nestle between two hub domains with their active sites and regulatory segments completely inaccessible to Ca^{2+}/CaM. It is proposed that a dynamic equilibrium governed by the linker length between the kinase and the association domains regulates exposure to CaM-binding sites facilitating the process of holoenzyme activation (Chao et al., 2011).

The PTM segment that enables autonomous activity following autophosphorylation evolved to extend such regulation to reactive oxygen species (ROS) and glucose-linked signaling (**Figure 1**). Increased ROS leads to oxidation of Met 281/282 at the base of the regulatory segment (Erickson et al., 2008). Elevated glucose leads to covalent modification of CaMKII nearby at Ser280 by O-linked N-acetylglucosamine (GlcNAcylation; Erickson et al., 2013). Introduction of bulky groups to the region normally interacting with the surface of the C-lobe is expected to weaken the interaction between the two and, like Thr287 phosphorylation, keep the autoinhibitory domain displaced and the kinase persistently active (**Figure 1**). All three modifications, individually and together, can produce a "turbocharged" kinase with consequences for arrhythmia (Chelu et al., 2009; Purohit et al., 2013), heart failure (Anderson et al., 2011; Luo et al., 2013), asthma (Sanders et al., 2013), and diabetes (Erickson et al., 2013). There may also be additional PTM of CaMKII involving nitrosation of Cys (Gutierrez et al., 2013).

Taken together, the recent accumulation of structural information offers a clearer understanding of CaMKII regulation. These structures not only provide a foundation for the rational design and optimization of CaMKII-specific inhibitors but may also present the opportunity for novel inhibitor-design strategies that extend beyond ATP-binding sites.

CaMKII INHIBITORS: FROM BENCH TO CLINIC

KN-93/KN-62

The most widely used inhibitor for study of cellular and *in vivo* functions of CaMKII has been KN-93 (Sumi et al., 1991; **Figure 2A**). It is one of a remarkable number of tool inhibitors developed by Hidaka and his colleagues for PKA, protein kinase C (PKC), CaMKII, and MLCK, many of which became commercially available and widely used. However, these were not on the path to therapeutic use and are therefore not optimized for potency, selectivity, or pharmacokinetics. KN-93 supplanted KN-62 which shares structural elements and mechanism of action (Tokumitsu et al., 1990). Inhibition by both is competitive with Ca^{2+}/CaM and not competitive with ATP. KN-62 binds to the holoenzyme and interferes with the ability of Ca^{2+}/CaM to activate it, but does not directly bind to CaM, i.e., it is not a CaM antagonist at effective inhibitory concentrations. It is worth noting, however, that a "predecessor" of KN-93 with a very similar structure, HMN-709, is a CaM antagonist (Yokokura et al., 1996; **Figure 2A**). Neither KN-62 nor KN-93 inhibits the activity of autophosphorylated CaMKII, consistent with a block of activation but not of catalysis (Tokumitsu et al., 1990; Sumi et al., 1991). Such inhibition can be classified as "ATP non-competitive" or "allosteric" as binding likely occurs outside the active site. Based on an ischemic stroke model, it has been suggested previously that blocking catalytic activity is the more effective approach because autonomous activity is resistant to allosteric inhibition by KN-93 (Vest et al., 2010). The observed differing efficacies, however, may have been due to differences in inhibitor concentrations at the site of action.

KN-93 (and KN-62) likely blocks the ability of Ca^{2+}/CaM to wrap around the CaM-binding segment and free it from the catalytic domain. The displacement of the autoinhibitory regulatory segment can be monitored by appropriate FRET pairs and indeed KN-93 blocks the change in FRET signal induced by Ca^{2+}/CaM and the change promoted by either autophosphorylation, oxidation, or GlcNAcylation (Erickson et al., 2011, 2013). KN-93 blocks Ca^{2+}/CaM from displacing and "presenting" Thr287 to an active neighboring subunit for phosphorylation (Rich and Schulman, 1998). KN-93 similarly blocks presentation of Ser280 for GlcNAcylation (Erickson et al., 2013). Once activated and autophosphorylated, however, KN-93 does not inhibit the kinase, nor is it likely to inhibit kinase made autonomously active by oxidation or GlcNAcylation.

The initial characterization of KN-93 and KN-62 showed them to be selective for CaMKII relative to PKA, PKC and MLCK (Tokumitsu et al., 1990; Sumi et al., 1991), but they were later shown to inhibit CaMKI and CaMKIV equally well (Mochizuki et al., 1993; Enslen et al., 1994). KN-93 is not very potent, inhibiting CaMKII with an IC50 ~1–4 μM depending on level of CaM and other assay conditions (Sumi et al., 1991; Anderson et al., 1998; Rezazadeh et al., 2006). A recent screen against 234 protein kinases shows that KN-93 is indeed very selective, but its targets now include Fyn, Haspin, Hck, Lck, MLCK, Tec, and TrkA (Gao et al., 2013).

Tool inhibitors are rarely optimized for potency or off-target effects and indeed KN-62 and KN-93, while inhibiting only a few protein kinases, do inhibit many of the ion channels that have been tested. KN-62 and KN-93 block modulation of the L-type Ca^{2+} channel by CaMKII but also have direct effects on the channel (Li et al., 1992; Anderson et al., 1998) so it is important to use their kinase-inactive controls (KN-04 and KN-92). KN-62 and KN-93 block macroscopic voltage-dependent K^+ current (K_v) in smooth muscle cells at concentrations (0.3–3 μM) used to inhibit CaMKII (Ledoux et al., 1999). KN-92 similarly blocks the channel and is therefore useful in excluding K^+ channel effects. A detailed analysis demonstrated that KN-93, but not KN-92, blocked members of the Kv1, Kv2, Kv3, Kv4, and Kv7 (hERG) voltage-gated potassium

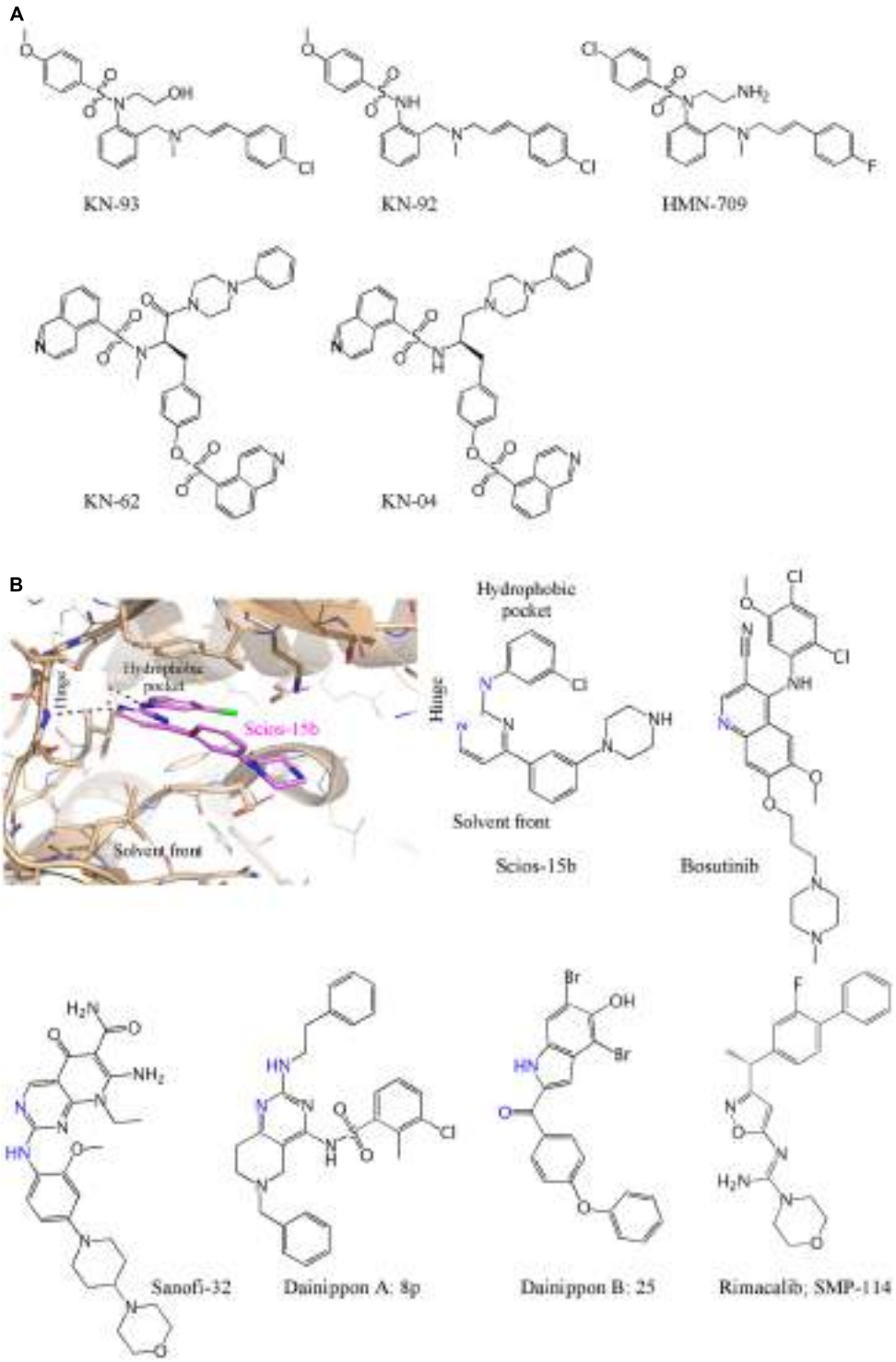

FIGURE 2 | Chemical structures of CaMKII inhibitors. (A) The ATP non-competitive inhibitors and controls are: KN-93 (Sumi et al., 1991); KN-92 (Tombes et al., 1995); KN-62 and KN-04 (Ishikawa et al., 1990) HMN-709 (Yokokura et al., 1996). **(B)** Computational docking of Compound 15b (Mavunkel et al., 2008) illustrates interaction of an ATP competitive inhibitor viewed from the solvent front and shown docked at the kinase "hinge" and interaction at the hydrophobic pocket. The structure of Compound 15b is shown, with the same orientation, with residues that interact at the hinge in blue based on either a crystal structures (Bosutinib) or based on modeling when a reasonable docked structure could be obtained. The compounds above are: Scios-15b (Mavunkel et al., 2008); Bosutinib (Chao et al., 2011); Sanofi-32 (Beauverger et al., 2012); Dainippon A: 8p (Asano et al., 2010); Dainippon B: 25 (Komiya et al., 2012); Rimacalib/SMP-114 (Westra et al., 2010).

channel families at concentrations that also block CaMKII (Rezazadeh et al., 2006). To be broadly useful as a control, it would be ideal if KN-93 and KN-92 had the same pharmacology other than for CaMKII, but this is unfortunately not the case. For example, KN-92 is not as potent in direct inhibition of L-type Ca^{2+} channels as is KN-93 (Anderson et al., 1998) or as potent in direct inhibition of IP_3-induced Ca^{2+} release via IP_3R-1 (Smyth et al., 2002) and thus not every effect blocked by KN-93 and not by KN-92 can be ascribed to CaMKII. Despite these caveats, these inhibitors have been extremely useful as initial evidence for CaMKII action, as illustrated by several examples in **Table 1**.

It is a bit surprising, given the channel blocking effects of KN-93, that it has not been as problematic to use it for *in situ* inhibition of CaMKII, when compared to peptide inhibitors below. The interplay between its effect on K^+ channels and L-type Ca^{2+} channels and inhibition of CaMKII are not well understood and complicate arrhythmia studies. Clearly, modification of K currents, such as in genetic mutations, can lead to arrhythmia, whereas ablation or peptide inhibition of CaMKII are anti-arrhythmic. KN-93 may therefore be anti-arrhythmic despite its K^+ channel blockade because concurrent inhibition of CaMKII serves as an arrhythmia shield that blocks the pro-arrhythmic consequence of the K^+ channel inhibition. Alternatively, a significant component of the anti-arrhythmic effect of KN-93 could be to reduce CaMKII activation by inhibiting the L-type Ca^{2+} channel and lowering free Ca^{2+} levels. Ultimately, inhibition of CaMKII with small molecule allosteric or ATP competitive inhibitors can be achieved without a significant K^+ channel component, something that is harder to achieve with channel blockers used as anti-arrhythmic agents.

SUBSTRATE-BASED INHIBITORS: AUTOCAMTIDE-3 INHIBITOR (AC3-I)/AUTOCAMTIDE-2 INHIBITOR PROTEINS (AIP)

Identification of the autoinhibitory regulatory segment of CaMKIIα led to development of long inhibitory peptides lacking the CaM binding sequence (residues 273–302) that could be injected into cells, e.g., implicating CaMKII in functions such as long-term potentiation (Payne et al., 1988; Malinow et al., 1989). The N-terminal end of this peptide contains the autophosphorylation site that was the basis for peptides substrates such as autocamtide-2 and autocamtide-3 (Hanson et al., 1989) and substitution of the phosphorylatable Thr to an Ala generated the

Table 1 | CaMKII inhibitors and related compounds.

Inhibitor type	Controls/verification	Action of inhibitor
KN-93/KN-62	KN-92; GFP-AC3-I transgene	KN-93 blocked pacing induced atrial fibrillation in the $Ryr2^{R176Q/+}$ mouse model (Chelu et al., 2009).
	KN-92; AIP; CaMKIIδ knockout	KN-93, AIP, and knockout block cardiac arrhythmogenesis and sarcoplasmic reticulum Ca^{2+} leak (Sag et al., 2009).
	KN-92	KN-93 and AIP were used to demonstrate that CaMKII is linked to SAN cell bioenergetics, affecting both ATP consumption and ATP generation (Yaniv et al., 2013).
	KN-92; CaMKII*δ* knockout	KN-93 blocks increase in GlcNAcylation-dependent Ca^{2+} spark frequency and prevents premature ventricular complexes also seen in diabetes (Erickson et al., 2013).
AC3-I/AIP	GFP-AC3-C	Myocardial GFP-AC3-I transgene blocked maladaptive remodeling following chronic *β*-adrenergic stimulation or myocardial infarct with GFP-AC3-I (Zhang et al., 2005).
	GFP-AC3-C;KN-93/KN-92	Myocardial GFP-AC3-I transgene in calcineurin hypertrophy model primarily reduced ventricular arrhythmias, improved mechanical function, and decreased mortality with minimal effect on the hypertrophic phenotype (Khoo et al., 2006).
	GFP-AC3-C	AngII promoted AF was blocked by GFP-AC3-I and prevented by knockins with oxidation resistant CaMKII(MM > VV) or RyR2 lacking CaMKII phosphorylation site (RyR2S2814A; Purohit et al., 2013).
	GFP-AC3C; CaMKIIN	Myocardial GFP-AC3-I and blocked increase mortality of diabetic mice after myocardial infarction via reactive oxygen species and confirmed with CaMKII(MM > VV) mice (Luo et al., 2013).
CaMKIIN	Myocardial GFP-AC3-I and -AC3-C	GFP-CaMKIIN (sinoatrial node expressed) blocked isoproterenol-stimulated CaMKII activation and reduced the fight or flight heart rate response to stress or isoproterenol (Wu et al., 2009).
	GFP; AC3-I	GFP-CaMKIIN (sinoatrial node expressed) blocked AngII and ROS activation of CaMKII and cell death contributing to sinus node dysfunction (Swaminathan et al., 2011).
	AC3-I; shRNA; KN-93	HA-CaMKIIN targeted to cytoplasmic membranes acts outside the nucleus to mediate induction of complement factor B following myocardial infarct (Singh et al., 2009).
	CaMKII (Thr287Asp)	mtCaMKIIN (with mitochondrial localization sequence) and palmitoyl-CaMKIIN for membrane localization support a role of mitochondrial CaMKII in ischemia reperfusion injury, MI and neurohumoral injury due to increased inner membrane mitochondrial Ca^{2+} uniporter current (Joiner et al., 2012).

peptide inhibitors AIP (Ishida et al., 1995) and AC3-I (Braun and Schulman, 1995). They inhibit CaMKII with >100-fold selectivity relative to PKC, PKA and CaMKIV, although their selectivity has not been broadly profiled, e.g., green fluorescent protein (GFP)-AC3-I was found to inhibit cellular actions of protein kinase D1 (PKD1) as well as those of CaMKII (Backs et al., 2009). As exemplified in **Table 1**, peptide inhibitors with internalization sequences for cellular studies or the transgenic expression of the peptides in mice have been critical first steps in delineating cardiovascular functions of CaMKII. Transgenic expression of inhibitor/control pairs can also be used to delineate molecular pathways of gene expression (Singh et al., 2009) and phosphorylation initiated by CaMKII activation (Scholten et al., 2013).

Some caution is warranted in the use of peptide (or small molecule) inhibitors that are often optimistically described as "highly specific inhibitors" when experience or data should suggest otherwise. Useful first generation tool inhibitors are typically developed by academic labs with limited resources, so selectivity is based on a handful of kinases available to the lab rather than on the 50–300 kinases that should be tested. As a minimum, the off-target effects of AC3-I should be checked by use of a control peptide (AC3-C; Patel et al., 1999; Wu et al., 2009). Altered selectivity can also occur when peptides are fused to GFP in order to increase expression and metabolic stability, or modified by lipids or internalization sequences for cell permeation. For example, addition of Ant (Antennapedia) to another peptide inhibitor generated a direct CaM antagonistic sequence (Buard et al., 2010) and myristoylated AIP and AC3-C were shown to have some effects unrelated to CaMKII inhibition (Wu et al., 2009).

CaMKIIN (CaM-KIIN)

CaMKIIN or CaM-KIIN designates small endogenous proteins that inhibit CaMKII with high affinity that can be applied pharmacologically or genetically. CaMKIIN was discovered by use of a yeast two-hybrid screen whereby the catalytic domain of CaMKIIβ served as bait to clone interacting proteins from a rat neuronal library (Chang et al., 1998, 2001). Two small proteins sharing high homology were identified and termed CaM-KIINβ (79 amino acids) and CaM-KIINα(78 amino acids) to designate them as inhibitors, and often spelled CaMKIIN. The α and β in their names are unrelated to the CaMKII isoform, as either of these inhibits all CaMKII isoforms with IC50 of 50 nM (Chang et al., 2001). The protein has not been detected in heart although a related mRNA was detected (Zhang et al., 2001).

Identification of the core inhibitory domain of CaMKIIN led to generation of a 28 amino acid peptide inhibitor termed CaMKIINtide (Chang et al., 1998) that was subsequently shortened and modified to improve potency (Vest et al., 2007; Coultrap and Bayer, 2011; Gomez-Monterrey et al., 2013). CaMKIIN and CaMKIINtide only bind to the activated conformation of CaMKII, suggesting that they dock to the kinase surface exposed and shaped only after displacement of the autoinhibitory domain during activation. They should therefore inhibit CaMKII activated by bound Ca^{2+}/CaM or autonomously active due to autophosphorylation, methionine oxidation, or GlcNAcylation. A 21-residue segment of CaMKIINtide was co-crystallized with CaMKII and shown to dock at the B/C sites using hydrophobic and basic residues at its N-terminal region to support potency and specificity and to extend to the A site where it precludes substrate from binding in a manner similar to protein kinase inhibitor (PKI) binding to PKA (Chao et al., 2010; **Figure 1**).

CaMIINtide has been modified to increase potency (Coultrap and Bayer, 2011; Gomez-Monterrey et al., 2013). In one series of optimizations, a shorter sequence of 21 amino acids (CN21a) was found to retain the potency of CaMKIINtide (Vest et al., 2007). A 19 amino acid sequence was then subjected to Ala scanning substitutions to identify critical residues and subsequent modifications produced a more potent and selective CaMKII inhibitor termed CN19o (Coultrap and Bayer, 2011). CN19o inhibited CaMKIIα with IC50 < 0.4 nM and improved selectivity for tested kinases, showing minimal or no inhibition at 5 μM against CaMKI, CaMKIV, DAPK1, AMPK, PKC, and PKA. A similar study generated a smaller optimized 17 amino acid peptide, CN17β, with IC50 of 30 nM and little inhibition of CaMKI or CaMKIV (Gomez-Monterrey et al., 2013).

CaMKIIN and CaMKIINtides are excellent experimental tools being adopted by the field but their use can result in additional effects by blocking interaction of CaMKII with some anchoring proteins and substrates that share the B/C docking sites (**Figure 1**). Translocation and docking to anchoring proteins aids in fidelity of signal transduction that would be disrupted by CaMKIIN and may generate secondary effects because anchoring proteins often cluster several signaling proteins that might be affected (Colbran, 2004; Schulman, 2004; Tsui et al., 2005). Mutations at the B/C sites block both binding of CN21a and CaMKII translocation/docking to the glutamate NR2B receptor (Leonard et al., 1999; Strack et al., 2000; Bayer and Schulman, 2001; Bayer et al., 2006; Vest et al., 2007) suggesting overlapping binding sites. Application of TatCN21 on neurons inhibits the kinase but also reduces the level of kinase at synaptic sites (Sanhueza et al., 2011), decreases clustering in dendrites, and produces aggregates with polyribosomes (Tao-Cheng et al., 2013). CaMKIINtides also block interaction with densin (Jiao et al., 2011) and Cav2.1 calcium channels (Magupalli et al., 2013) and possibly with β_{IV}-spectrin and other proteins (Hund et al., 2010). Finally, the biological function of CaMKIIN is not fully understood and it may directly affect cellular pathways unrelated to CaMKII inhibition or interference with its translocation.

The cardiovascular field has appropriately advanced from the AC3-I/AIP- to CaMKIIN-based inhibitors to delineate *in situ* and *in vivo* functions (**Table 1**). The inhibitor can be directly introduced in a regionally selective manner via locally applied adenoviral constructs, as a transgene targeted to selective tissues, and even directed to distinct intracellular sites with appropriate targeting sequences.

ERA OF SMALL MOLECULAR THERAPEUTICS

The inhibitory agents and approaches described above have been essential in identifying key roles of CaMKII in health and disease and make a compelling case for targeting CaMKII for several cardiovascular indications, so as one of us asked previously, "where are the drugs?" (Anderson et al., 2006). While it is possible that

a CaMKIIN-based inhibitor could be developed as a therapeutic, e.g., for local expression at the sinoatrial node (SAN), new small molecule inhibitors will be needed for treating cardiovascular disease, and these will, in turn, provide better tools for advancing CaMKII research. The period covered since the review above has seen a large increase in the number of protein kinases targeted in oncology, with over 100 hundred in clinical development and many in clinical practice (Cohen and Alessi, 2013). The cardiovascular field, largely because of the greater safety requirements but also because of decades-long investments in ion channel blockers, has been slower in turning to this target class. The message emerging from data published by the CaMKII research field has been heard and now there are several programs targeting CaMKII for cardiovascular indications.

One of the early programs was initiated at Scios, expanded following its acquisition by Johnson and Johnson, and discontinued along with more advanced programs for strategic reasons when Scios was closed. The program did provide some potent ATP competitive inhibitors, along with structure – activity relationships that enables an understanding of how to inhibit CaMKII (Lu et al., 2008; Mavunkel et al., 2008). One of the lead compounds, a pyrimidine (Scios 15b) with IC50 of 9 nM *in vitro* and 320 nM *in situ*, is shown in **Figure 2B**. Bosutinib (and sunitinib) were developed as ATP competitive inhibitors of protein tyrosine kinases but have surprising potency at inhibiting CaMKII and a co-crystal of CaMKII with bosutinib has been published (Chao et al., 2011). Dainippon Sumitomo Pharma has had the most advanced CaMKII program and developed Rimacalib (SMP-114) for treatment of rheumatoid arthritis. It passed the Phase I safety trial, but appears not to have shown efficacy in a 24-week Phase II trial (Tagashira and Fukushima, 2008; Westra et al., 2010). The other compounds are all potent (2–60 nM) inhibitors generated during CaMKII inhibition programs at Sanofi (Beauverger et al., 2012) and Dainippon (Asano et al., 2010; Komiya et al., 2012). Allosteros Therapeutics is developing both ATP-competitive and allosteric or ATP non-competitive inhibitors for cardiovascular and other indications and a CaMKII program at Myogen (with Novartis) was part of an acquisition by Gilead with no publications of structures so far. We are aware of several other early stage CaMKII inhibitor programs and anticipate that both tool inhibitors for academic research as well as new chemical entities for treating cardiovascular disease will arise from several of these programs.

The biochemical properties of some the best characterized CaMKII inhibitors are summarized in **Table 2**.

INHIBITOR DESIGN

CaMKII is now accepted as a key target in cardiovascular disease and the focus is shifting to creation of selective inhibitors that are safe and effective for therapeutic use. The global market for kinase inhibitors is over $30B, mostly targeting protein tyrosine kinases with both biologics and small molecules. Structure-guided drug design and virtual library and fragment screening are likely to benefit from the recent availability of high resolution crystal structures of CaMKII in various conformations. Targeting the ATP-binding site is the most common approach with small molecule inhibitors; however, specificity becomes a challenge because there are over 500 kinases whose active conformation of the site have a similar shape and amino acid composition. Their potency must also be very high in order to compete with millimolar levels of cellular ATP. Successful development of ATP competitive inhibitors for oncology indications has demonstrated that appropriate selectivity is achievable. The first generation of CaMKII therapeutics will likely target the ATP-binding site because of the large body of structural information and medicinal chemistry experience that facilitates the design of relatively selective ATP competitive inhibitors.

The role of CaMKII in cognitive memory and neuronal plasticity that involves its α and β isoforms in brain necessitates development of inhibitors do not block these isoforms in brain, certainly when intended for long-term or chronic use. One approach is gene therapy with viral vectors for expression of peptides or proteins, such as SERCA2a or S100A1 to the heart or specific or very localized regions of heart, such as SAN by intracoronary application or endocardial injection by catheter (Pleger et al., 2013). This type of approach for CaMKII inhibitory proteins, coupled with cardiac-selective promoters, would minimize expression outside the heart and would certainly avoid expression in brain. However, widespread adoption will need to await successful and safe demonstration of exemplary cardiac gene therapy

Table 2 | Biochemical properties of best characterized CaMKII inhibitors.

Inhibitor	Mechanism of action	Autonomous[a] kinase inhibition	Off-target effects
KN-93, KN-62	Allosteric, CaM-competitive	No	CaMKI, CaMKIV, ion channels
AC3I/AIP	Peptide substrate-competitive	Yes	PKD-1 in cells
CaMKIIN	Peptide substrate/regulatory domain-competitive	Yes	None identified
Small molecule inhibitors (Scios 15b, SMP-114, Bosutinib)	ATP-competitive	Yes	Inhibit other ser/thr and tyr kinases *in vivo*

[a] *Autonomy is based on Thr287 autophosphorylation but results are likely the same for autonomy generated by regulatory domain methionine oxidation or by GlcNAcylation.*

such gene therapy on a larger scale. Gene therapy still holds great promise. For CaMKII inhibition, it promises a lower threshold for success in attaining limiting exposure to heart and avoiding effects in brain and other organs, but it still has a much higher threshold in the development and regulatory path than development of small molecule inhibitors.

For small molecule inhibitors inhibition of α- and β-CaMKII in brain can be minimized by reducing CNS penetration through optimization of their pharmacokinetic properties. There is a large body of literature of physical and structural properties that either promote or limit CNS penetration and typically there are sites on the molecules not involved in target binding that are optimized for solubility, plasma protein binding, and CNS penetration. In addition, while the ATP-binding pockets of the four CaMKII isoforms are similar, it should be possible to further reduce CNS action by perhaps a 10-fold selectivity for δ- over α- and β-CaMKII. The β isoform, in particular, has a slight folding down of the phosphate-binding loop as well as a bulkier amino acid side chain not seen in the other three isoforms making it possible to achieve significant discrimination for this isoform.

A strategy designed to circumvent the high redundancy associated with kinases in the active conformation is to target the more diverse inactive conformation (Huse and Kuriyan, 2002). The anti-cancer drug imatinib (Gleevec®) exhibits a high degree of selectivity for its target Bcr-Abl because, in addition to partially occupying the ATP-binding site, it recognizes a distinctive conformation of the "activation" loop seen only in the inactive state (Schindler et al., 2000; Nagar et al., 2002). It is more difficult to use this strategy for CaMKII because the ATP-binding site is not so different in the active and inactive states. However, because CaMKII is subjected to complex and multilayered mechanisms of regulation, inhibition strategies extending beyond the ATP-binding pocket may be possible.

Allosteric inhibitors, molecules that inhibit enzyme function by binding outside of the active site, show higher selectivity profiles for their targets because such sites are not conserved or even broadly present in the kinome. KN-93 and KN-62 appear to be allosteric inhibitors that may stabilize the interaction between the autoinhibitory regulatory segment and kinase domain and may thereby hinder activation by Ca^{2+}/CaM (**Figure 1**). Although the novelty of their unidentified binding sites makes it more challenging to optimize allosteric inhibitors, they offer the advantage of greater selectivity and binding unaffected by high cellular ATP concentrations.

Selectivity would be most easily achieved at unique sites on the kinase. For example, docking sites B/C uncovered in the recent crystal structures may be amenable to medicinal chemistry (Chao et al., 2010; **Figure 1**). It may be possible to block some CaMKII signaling by blocking its targeting to anchoring proteins via the B/C sites as blocking anchoring can decrease phosphorylation even if catalytic activity is not blocked (Tsui et al., 2005). Additionally, it may be possible to achieve target specificity by blocking exposure of the site on substrates or anchoring proteins that interacts with the B/C site. In that case only one or a subset of the multiple CaMKII substrates would be affected. In many cases, however, the phosphorylation site on kinase substrates tends to be exposed, making it difficult to block with a small molecule inhibitor.

The most unique region of CaMKII is the linker region between the catalytic and hub domains (Tombes et al., 2003). Linker length affects the equilibrium between compact and extended holoenzyme conformations and the sensitivity of the kinase to the frequency of Ca^{2+} pulses (Bayer et al., 2002; Chao et al., 2011). Thus, in principle, CaMKII inhibition can be achieved by favoring a more compact holoenzyme and reducing its Ca^{2+} sensitivity. It is likely that the first generation of CaMKII therapeutics will be ATP competitive inhibitors with others following that exploit more unique sites thus enabling fewer off-target effects and safer use for more chronic and less severe indications.

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CaMKIIdelta subtypes: localization and function

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In this review we discuss the localization and function of the known subtypes of calcium/calmodulin dependent protein kinase IIδ (CaMKIIδ) and their role in cardiac physiology and pathophysiology. The CaMKII holoenzyme is comprised of multiple subunits that are encoded by four different genes called CaMKIIα, β, γ, and δ. While these four genes have a high degree of sequence homology, they are expressed in different tissues. CaMKIIα and β are expressed in neuronal tissue while γ and δ are present throughout the body, including in the heart. Both CaMKIIγ and δ are alternatively spliced in the heart to generate multiple subtypes. CaMKIIδ is the predominant cardiac isoform and is alternatively spliced in the heart to generate the CaMKIIδ$_B$ subtype or the slightly less abundant δ$_C$ subtype. The CaMKIIδ$_B$ mRNA sequence contains a 33bp insert not present in δ$_C$ that codes for an 11-amino acid nuclear localization sequence. This review focuses on the localization and function of the CaMKIIδ subtypes δ$_B$ and δ$_C$ and the role of these subtypes in arrhythmias, contractile dysfunction, gene transcription, and the regulation of Ca^{2+} handling.

Keywords: Ca^{2+}/calmodulin-dependent protein kinase II, heart, splice variants, nuclear localization, transgenic mice

EXPRESSION AND LOCALIZATION

Calcium/calmodulin dependent protein kinase II (CaMKII) is a multimeric enzyme consisting of distinct subunits encoded by four different genes known as CaMKIIα, β, γ, and δ. These genes have a high degree of sequence homology but show differential tissue expression. CaMKIIα and β are predominantly expressed in neuronal tissue while γ and δ are present throughout the body, including the heart (Bennett et al., 1983; Tobimatsu and Fujisawa, 1989). CaMKIIδ is the predominant cardiac isoform and is alternatively spliced to generate multiple subtypes (Edman and Schulman, 1994).

Schworer et al. (1993) were the first to demonstrate that there are different subtypes of CaMKIIδ expressed in various tissues. The authors reported four distinct proteins with differential expression patterns and named them CaMKIIδ$_{1-4}$. CaMKIIδ$_2$, and CaMKIIδ$_3$ were shown to be identical except for the insertion of an 11-amino acid sequence in the variable domain of CaMKIIδ$_3$, the more abundant of the two subtypes in the heart (Schworer et al., 1993). Around the same time, Edman and Schulman (1994) identified these same CaMKIIδ subtypes in rat heart and characterized their catalytic activity and regulation by calcium-liganded calmodulin (Ca^{2+}/CaM). They refer to the predominant cardiac subtypes as CaMKIIδ$_B$ and CaMKIIδ$_C$ (the convention that will be used in this review), which correspond to the δ$_3$ and δ$_2$ subtypes, respectively. The structure of these proteins is shown in **Figure 1**. CaMKIIδ$_B$ and δ$_C$ possess similar catalytic activity and sensitivity to Ca^{2+}/CaM. Furthermore, both subtypes can undergo autophosphorylation and acquire a similar degree of Ca^{2+}-independent or autonomous activity (Edman and Schulman, 1994). In the years that followed, seven additional splice variants of the CaMKIIδ gene, referred to as CaMKIIδ$_{5-11}$, were identified. Only one of these, CaMKIIδ$_9$, is expressed in the adult heart (**Figure 1**; Mayer et al., 1994, 1995; Hoch et al., 1998, 1999).

The 11-amino acid insert in CaMKIIδ$_B$ (^{328}KKRKSSSSVQMM) is also present in some splice variants of CaMKIIα and γ; this conservation suggests an important function (Schworer et al., 1993). Srinivasan et al. (1994) showed that when constructs of CaMKIIδ$_B$ are transfected into fibroblasts the expressed protein is localized to the nucleus. This is not the case for constructs of CaMKIIδ$_C$, implying that that the additional amino acid sequence present in CaMKIIδ$_B$ is responsible for nuclear localization (Srinivasan et al., 1994). A similar differential localization pattern was also observed when CaMKIIδ subtypes were expressed neonatal rat ventricular myocytes (NRVMs; Ramirez et al., 1997). Further studies showed that the 11-amino acid insert in CaMKIIδ$_B$ can confer nuclear localization when inserted into the variable domain of CaMKIIα and that mutagenesis of the first two lysines in the insert abrogates the nuclear localization of these constructs. Thus it is widely accepted that the CaMKIIδ$_B$ variable domain contains a nuclear localization signal (NLS).

CaMKII heteromultimerization is permissive in that the CaMKII holoenzyme can include subunits from multiple CaMKII genes and multiple splice variants of those genes (Bennett et al., 1983; Yamauchi et al., 1989). It seems likely that more than a single CaMKIIδ subtype is present in a single CaMKIIδ multimer and accordingly the ratio of δ$_B$ to δ$_C$ in a multimer could regulate the localization of the holoenzyme. This has been demonstrated experimentally. When CaMKIIδ$_B$ and δ$_C$ are cotransfected into fibroblasts or NRVMs, the localization of the expressed protein can be shifted in accordance with the ratio of the expressed CaMKIIδ subtypes, i.e., highly expressed δ$_C$ sequesters δ$_B$ in the cytosol and blocks its nuclear localization (Srinivasan et al., 1994; Ramirez et al., 1997). The opposite is also true: high relative expression

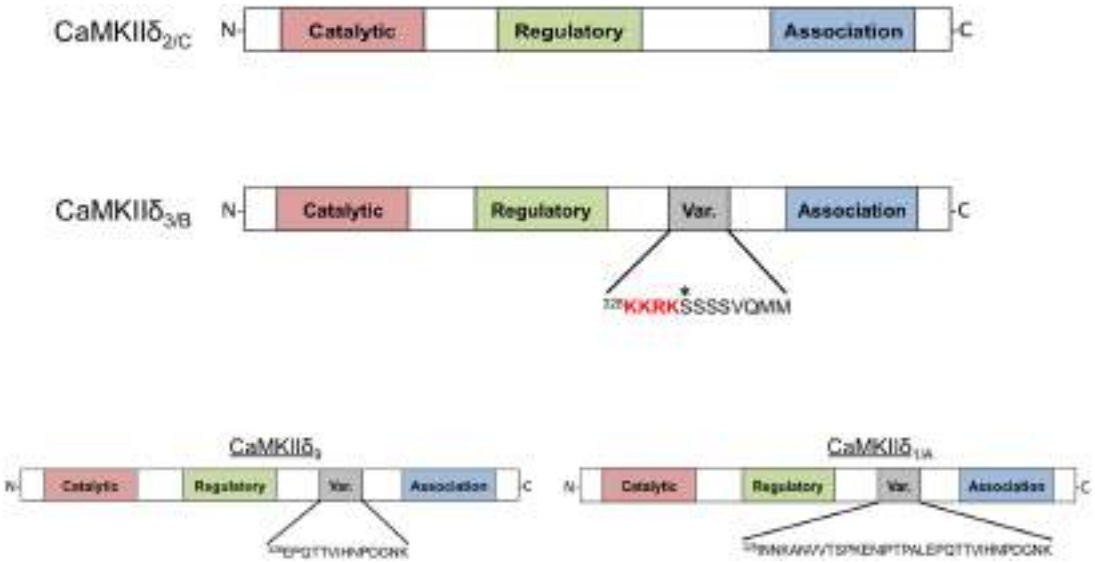

FIGURE 1 | Domain scheme of CaMKIIδ monomers. The four subtypes shown are distinguished by the amino acid sequence of a variable region between the regulatory and association domains. CaMKII$\delta_{2/C}$ lacks this variable domain while CaMKII$\delta_{3/B}$ contains an 11-amino acid insertion that contains the canonical nuclear localization signal (NLS) sequence KKRK. An asterisk denotes a phosphorylation site (Ser^{332}) in this domain that affects NLS accessibility. CaMKIIδ_9 contains a different variable domain sequence that is also present in the longer variable region of CaMKII$\delta_{1/A}$. All of these subtypes can be detected in adult murine heart with the exception of CaMKII$\delta_{1/A}$, which is normally only present in neonatal mouse heart.

of δ_B can localize δ_C to the nucleus. While not well appreciated, CaMKIIδ_B localization can also be regulated by phosphorylation. A serine (Ser^{332}) immediately adjacent to the NLS of CaMKIIδ_B was shown to be a site of phosphorylation by CaMKI and CaMKIV *in vitro*. Phosphorylation prevents association of δ_B with the NLS receptor m-pendulin and thus limits localization of CaMKIIδ_B to the nucleus (Heist et al., 1998). Remarkably this mode of regulation is also seen when the NLS is moved from the middle of the protein to the N-terminus, suggesting that conformational changes are not required for phosphorylation to block the NLS.

Relative expression of CaMKIIδ subtypes is altered during cardiomyocyte differentiation and maturation and in association with the development of heart failure and ischemia/reperfusion (I/R) injury (Hoch et al., 1998, 1999; Colomer et al., 2003; Peng et al., 2010). In humans CaMKIIδ_B mRNA is selectively upregulated during heart failure (Hoch et al., 1999). The altered expression of particular subtypes suggests the possibility of a regulated process governing CaMKIIδ mRNA splicing because transcriptional regulation would not be expected to alter the ratio of CaMKIIδ subtypes. Alternative splicing factor/pre-mRNA-splicing factor SF2 (ASF/SF2) was initially described by Krainer and Maniatis (1985) and subsequently mice lacking ASF/SF2 expression were demonstrated to have incomplete processing of CaMKIIδ mRNA (Krainer and Maniatis, 1985; Xu et al., 2005). Specifically, enhanced expression of the δ_A subtype [δ_1 in the nomenclature of Schworer et al. (1993)] was observed while expression of CaMKIIδ_B and δ_C was diminished. **Figure 1** also depicts the structure of the δ_A subtype, which is expressed in the fetal heart. ASF/SF2 can be regulated by phosphorylation. Protein kinase A (PKA)-mediated ASF/SF2 phosphorylation has been correlated with alternative splicing of CaMKIIδ in heart and brain (Gu et al., 2011). Additionally, regulation of ASF/SF2 by Protein phosphatase 1 γ (PP1γ) has been demonstrated to affect CaMKIIδ splicing (Huang et al., 2013). CaMKIIδ_A expression is increased in models of isoproterenol-induced cardiac hypertrophy and thus regulation of CaMKIIδ splicing by PKA and PP1γ may be relevant in the context of chronic β-adrenergic stimulation (Li et al., 2011). The RNA binding proteins Fox 1 (RBFOX1) and 2 (RBFOX2) collaborate with ASF/SF2 to induce proper CaMKIIδ splicing (Han et al., 2011) and factors that regulate these proteins could also influence the expression of CaMKIIδ subtypes. Thus, CaMKIIδ splicing is a dynamic and regulated process. The role of this system in the heart has not been extensively explored but could be of major importance since regulation of CaMKIIδ splicing may account for altered subtype expression and CaMKIIδ signaling in physiological and pathophysiological settings.

CaMKIIδ_B TRANSGENIC MICE

The differential localization and function of CaMKIIδ subtypes could be of considerable importance to understanding the role of this enzyme in normal physiology and disease states. Early studies demonstrated that expression of CaMKIIδ_B in NRVMs induced atrial natriuretic factor (ANF) expression and led to increased myofilament organization, both hallmarks of cardiac hypertrophy, while expression of CaMKIIδ_C did not (Ramirez et al., 1997). This finding suggested that nuclear CaMKIIδ localization is required to regulate gene expression. Consistent with this notion are data indicating that CaMKIIδ_B signaling activates several transcription factors including myocyte enhancer factor 2 (MEF2), GATA4, and heat shock factor 1 (HSF1; Little et al., 2009; Lu et al., 2010; Peng et al., 2010). The significance of the hypertrophic responses elicited by δ_B *in vitro* was explored further

by generation of CaMKIIδ_B transgenic (TG) mice (Zhang et al., 2002). These animals, which overexpress δ_B under the control of the cardiac-specific α-myosin heavy chain (α-MHC) promoter, demonstrate the enhanced expression of hypertrophic markers observed in NRVMs expressing CaMKIIδ_B. CaMKIIδ_BTG animals develop hypertrophy and moderate cardiac dysfunction by 4 months of age. Thus, CaMKIIδ_B expression appears to be sufficient to induce cardiac hypertrophy. Surprisingly, despite the increased CaMKII activity in the CaMKIIδ_BTG mouse heart, phosphorylation of the canonical cardiac CaMKII substrate phospholamban (PLN) at its CaMKII site (Thr^{17}) was not increased but rather was decreased relative to WT mice. PLN phosphorylation at the PKA site (Ser^{16}) was similarly reduced. These data were related to increases in phosphatase activity (Zhang et al., 2002), but also implied that CaMKIIδ_B did not lead to robust phosphorylation of PLN. A subsequent paper that examined CaMKIIδ_BTG animals at a younger age to avoid changes in phosphatase activity confirmed that phosphorylation of PLN and another cardiac CaMKII substrate, the cardiac ryanodine receptor (RyR2), was not increased by cardiac CaMKIIδ_B expression (Zhang et al., 2007). This finding is consistent with a predominantly nuclear localization and function of the δ_B subtype.

CaMKIIδ_B has also been suggested to regulate expression of the Na^+/Ca^{2+} exchanger (NCX1) during the development of cardiac dysfunction following trans-aortic constriction (TAC; Lu et al., 2011). The conclusion that δ_B was the subtype involved in NCX1 regulation relied on the use of a constitutively active construct of CaMKIIδ_B in which a Thr to Asp mutation (T287D) simulates autophosphorylation. Interestingly, the authors found that this construct was excluded from the nucleus (Lu et al., 2010). This differs from the localization pattern described above (Srinivasan et al., 1994; Ramirez et al., 1997) and can be explained as a result of phosphorylation of Ser^{332} in the 11-amino acid insert of δ_B (**Figure 1**). The observation that mutation of Ser^{332} to Ala restores nuclear localization of constitutively active CaMKIIδ_B (Backs et al., 2006) confirms the role of this site in the cytosolic localization of the active construct. The possibility that phosphorylation of Ser^{332} might regulate CaMKIIδ_B localization in the intact heart has not been evaluated, but such a mechanism could contribute to the observation that CaMKIIδ_B is found outside the nucleus even in the absence of multimerization with δ_C (Mishra et al., 2011).

CaMKIIδ_C TRANSGENIC MICE

CaMKIIδ_C transgenic mice have also been generated and demonstrate a strikingly different phenotype from mice that express CaMKIIδ_B. While cardiac dysfunction is relatively moderate and takes months to develop in CaMKIIδ_BTG animals, mice expressing δ_C rapidly progress to heart failure and premature death (Zhang et al., 2003). By 6 weeks of age CaMKIIδ_CTG animals display marked changes in cardiac morphology and by 12 weeks these animals display severe cardiac dysfunction and upregulation of hypertrophic genes.

Ca^{2+} HANDING AND ARRHYTHMIA

Expression of the cardiac sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is diminished in δ_CTG mice as occurs in other models of heart failure. Since SERCA regulates Ca^{2+} reuptake into the sarcoplasmic reticulum (SR), this decrease would diminish SR Ca^{2+} loading. On the other hand, the CaMKIIδ_CTG mice show hyperphosphorylation of PLN at Thr^{17}, which should improve SERCA function. In addition δ_CTG animals display marked increases in phosphorylation of the RyR2, the channel through which Ca^{2+} exits the SR. Taken together, these changes would predict dysregulation of SR Ca^{2+} cycling and excitation–contraction coupling. This was substantiated in an accompanying paper that systematically analyzed and demonstrated dysregulation of cardiac Ca^{2+} handling in mice expressing δ_C (Maier et al., 2003). Specifically it was shown that SR Ca^{2+} stores were depleted in myocytes from these animals, explaining the observation that isolated myocytes displayed diminished twitch shortening amplitude. Furthermore, Maier et al. (2003) showed that the frequency and duration of Ca^{2+} sparks, or spontaneous intracellular Ca^{2+}-release events, was markedly increased in myocytes from animals expressing δ_C. Hyperphosphorylation of RyR2 by CaMKIIδ_C was hypothesized to underly the enhanced leak of Ca^{2+} from the SR, and this was verified by the demonstration that acute inhibition of CaMKII in δ_CTG myocytes rescues the altered Ca^{2+} handling (Maier et al., 2003). In other experiments, acute expression of δ_C in rabbit cardiomyocytes was shown to be sufficient to induce SR Ca^{2+} sparks and diminished SR Ca^{2+} loading (Kohlhaas et al., 2006). These findings imply that direct regulation of Ca^{2+} handling targets including RyR2 by CaMKIIδ_C can account for the dysregulation of Ca^{2+} and contractile function seen in myocytes from δ_CTG animals (**Figure 2**).

Dysregulation of excitation–contraction coupling by CaMKII is thought to contribute to arrythmogenesis in a variety of contexts, as supported by the increased incidence of arrhythmogenic events in CaMKIIδ_CTG mice (Anderson et al., 1998; Wu et al., 2002; Wagner et al., 2006). Overexpression of CaMKIIδ_C not only induces more spontaneous arrhythmias but also enhances the susceptibility of mice to arrhythmogenic challenge by β-adrenergic stimulation. Sag et al. (2009) found that much of the proarrhythmogenic effects of β-adrenergic stimulation on SR Ca^{2+} leak were significantly inhibited by treatment of myocytes with KN-93, an inhibitor of CaMKII. Furthermore the SR Ca^{2+} leak induced by isoproterenol did not occur in myocytes from mice lacking CaMKIIδ. These findings collectively implicate SR Ca^{2+} leak as one of the key mechanisms in δ_C-mediated arrhythmias (Sag et al., 2009). The notion that hyperphosphorylation of RyR2 at the CaMKII site (Ser^{2814}) contributes to arrhythmias and SR Ca^{2+} leak is supported by the finding that mutation of Ser^{2814} to Ala (S2814A) blocks the ability of CaMKII to induce Ca^{2+} sparks (van Oort et al., 2010). The autosomal dominant form of catecholaminergic polymorphic ventricular tachycardia (CPVT) can be caused by the RyR2 mutation R4496C and mice carrying this mutation are predisposed to arrhythmia and ventricular fibrillation. Enhanced CaMKIIδ_C expression and activity are implicated in the etiology of premature death in CPVT as expression of CaMKIIδ_C exacerbates the effects of the R4496C mutation (Dybkova et al., 2011). As mentioned earlier RyR2 Ser^{2814} phosphorylation is increased by expression of CaMKIIδ_C (but not by δ_B) *in vivo* (Zhang et al., 2007) and the effects of mutating this site (van Oort et al., 2010) emphasize the importance of RyR2 phosphorylation by CaMKII in SR Ca^{2+} leak and arrhythmia.

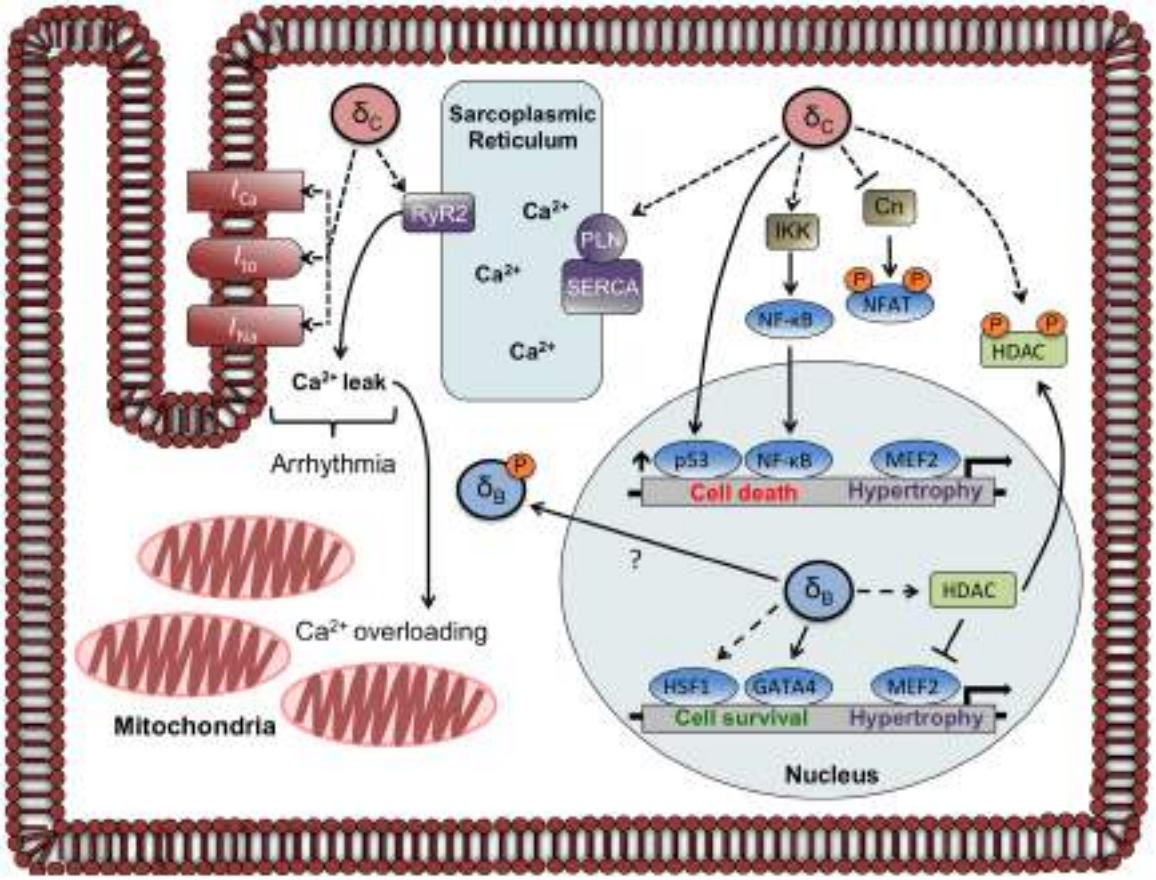

FIGURE 2 | Localization and function of CaMKIIδ subtypes in the adult cardiomyocyte. The circles labeled δ_C and δ_B represent CaMKIIδ multimers that are composed primarily of δ_C and δ_B subunits, respectively. Documented phosphorylation events are indicated by dashed lines. CaMKIIδ_C regulates Ca^{2+} homeostasis and currents involved in arrhythmogenesis through phosphorylation of Ca^{2+} handling proteins and channels. CaMKIIδ_C can also affect gene transcription through direct and indirect mechanisms including phosphorylation of NFAT and HDAC (sequestering them in the cytosol), increases in p53, and increased nuclear import of NF-κB. The CaMKIIδ_B subtype has little effect on phosphorylation of Ca^{2+} handling proteins but increases gene expression through HDAC phosphorylation and nuclear export and activation of HSF1 and GATA4. A putative mechanism for δ_B redistribution is depicted, showing δ_B exiting or being excluded from the nucleus due to phosphorylation at a site (Ser^{332}) adjacent to its NLS.

Other targets besides those at the SR may contribute to the arrhythmogenic phenotype of CaMKIIδ_C mice. The cardiac sodium channel $Na_V1.5$ is physically associated with CaMKIIδ_C based on coimmunoprecipitation of these proteins from CaMKIIδ_CTG animals and $Na_V1.5$ is phosphorylated in mice expressing δ_C (Wagner et al., 2006). CaMKIIδ_C phosphorylates $Na_V1.5$ at multiple sites and phosphorylation appears to elicit the loss-of-function changes in $Na_V1.5$ gating that are observed in the context of CaMKIIδ_C expression *in vitro* (Ashpole et al., 2012; Koval et al., 2012). Incomplete inactivation of $Na_V1.5$ generates a late Na^+ current (I_{Na}), which can prolong the duration of the action potential and contribute to arrhythmias. Additionally, increased I_{Na} can lead to Na^+-overloading of the cardiomyocyte, which contributes to diminished diastolic contractile performance (Maltsev et al., 1998). Late I_{Na} is observed in CaMKIIδ_CTG mice and inhibition of this current ameliorates arrhythmia and diastolic dysfunction in these animals (Sossalla et al., 2011). Modulation of I_{Na} therefore appears to contribute to the phenotype of CaMKIIδ_C mice with respect to arrhythmia development; additionally the CaMKIIδ_C subtype likely regulates the L-type Ca^{2+} channel (LTCC) and repolarizing potassium currents (I_{to} and I_{K1}; McCarron et al., 1992; Wagner et al., 2009). Thus, a multitude of mechanisms link CaMKIIδ_C to arrhythmogenesis.

CONTRACTILE DYSFUNCTION

Arrhythmias may contribute to the premature death of CaMKIIδ_CTG mice but there are also marked decreases in contractile function in these animals. Since alterations to cardiomyocyte Ca^{2+} handling are seen in relatively young CaMKIIδ_CTG mice and precede the development of heart failure, it is possible that dysregulated Ca^{2+} homeostasis (specifically SR Ca^{2+} leak) is an initiating event in δ_C-induced heart failure. Specifically, as a consequence of SR Ca^{2+} leak and SERCA downregulation, the SR Ca^{2+} load is diminished which would compromise contractile function. To determine whether diminished SR Ca^{2+} load is the primary causal event leading to contractile dysfunction and premature death in response to δ_C overexpression, we crossed the

δ_CTG mice with mice in which the SERCA regulatory protein PLN was deleted (PLN-KO). Deletion of PLN in the context of δ_C overexpression normalized SR Ca^{2+} levels and the contractile function of isolated myocytes was restored (Zhang et al., 2010). Remarkably the development of cardiac dysfunction *in vivo* was not rescued but instead was accelerated in the δ_CTG/PLN-KO mice. In addition SR Ca^{2+} leak was enhanced. It was hypothesized that the increased SR Ca^{2+} load, in the context of RyR2 hyperphosphorylation, precipitated greater Ca^{2+} leak and further suggested that the accelerated development of cardiac dysfunction was due to mitochondrial Ca^{2+} overloading (Zhang et al., 2010). These observations and their interpretation places central importance on the Ca^{2+} leak elicited by δ_C-mediated phosphorylation of RyR2 in the development of heart failure. Further support for this hypothesis comes from the finding that CaMKIIδ knockout mice have attenuated contractile dysfunction in response to pressure overload induced by TAC and myocytes from these animals show diminished SR Ca^{2+} leak in response to TAC (Ling et al., 2009). Additionally, mice expressing the RyR2 S2814A mutation are protected from the development of heart failure in response to pressure overload (Respress et al., 2012) consistent with a critical role for CaMKII-mediated RyR2 phosphorylation. We recently crossed CaMKIIδ_C mice with those expressing RyR2 S2814A; if the hypothesis is correct these mice will show diminished SR Ca^{2+} leak and improved contractile function when compared to CaMKIIδ_CTG mice.

Another approach used to determine the role of RyR2 phosphorylation and SR Ca^{2+} leak in the phenotype of CaMKIIδ_CTG mice was to cross the CaMKIIδ_CTG mice with mice expressing SR-targeted autocamtide-2-related inhibitory peptide (SR-AIP; Huke et al., 2011). AIP simulates the regulatory domain of CaMKII and inhibits the kinase, and SR-AIP mice have been shown to display diminished phosphorylation of CaMKII substrates at the SR (Ji et al., 2003). A reduction in the extent of PLN and RyR2 hyperphosphorylation observed in CaMKIIδ_CTG mice was conferred by SR-AIP. There were associated changes in Ca^{2+} handling that indicated a modest improvement in SR Ca^{2+} leak. Despite the salutary effects of SR-AIP in cells from δ_CTG mice, *in vivo* cardiac function was not improved. One possible explanation for these findings is that the degree of inhibition of RyR2 phosphorylation conferred by SR-AIP was insufficient to prevent the effects of CaMKIIδ_C overexpression. Alternatively, while δ_C-mediated phosphorylation of targets at the SR including RyR2 and PLN is of considerable consequence, targets of CaMKII elsewhere in the cell may also contribute to the pathogenesis of cardiac dysfunction induced by CaMKIIδ_C.

Mitochondrial Ca^{2+} is elevated in mice overexpressing δ_C in the context of intact SR Ca^{2+} load (Zhang et al., 2010) and increases in mitochondrial Ca^{2+} are known to induce opening of the mitochondrial permeability transition pore (MPTP) and cell death (Halestrap and Davidson, 1990). Considering the central importance of mitochondria in the regulation of cell death and of cell death in the development of heart failure (Wencker et al., 2003), any pathway by which CaMKIIδ_C induces mitochondrial Ca^{2+} overloading and subsequent loss of mitochondrial integrity would be predicted to contribute to the development of contractile dysfunction and heart failure. To test the role of mitochondrial dysregulation in the cardiomyopathy that develops in δ_CTG animals, CaMKIIδ_CTG mice were crossed with mice lacking expression of cyclophilin D, a mitochondrial protein required for the formation of the MPTP. The ability of high Ca^{2+} to induce swelling of isolated mitochondria, an index of MPTP opening, was impaired in the CaMKIIδ_CTG mice lacking cyclophilin D, but development of dilated cardiomyopathy and premature death of these mice was not diminished. Indeed these responses were exacerbated when compared to δ_CTG mice with intact cyclophilin D expression. The authors suggest that cyclophilin D may actually play a beneficial role in stress responses, as they observed that TAC-induced heart failure development was also made more severe by genetic deletion of cyclophilin D (Elrod et al., 2010). However, CaMKIIδ_C is found at mitochondria and a recent seminal study by Joiner et al. (2012) identified the mitochondrial Ca^{2+} uniporter (MCU) as a potential target of CaMKII (Mishra et al., 2011; Joiner et al., 2012). While phosphorylation of the MCU by CaMKII was not shown to occur *in vivo*, a CaMKII-dependent change in the function of the MCU was evidenced by data demonstrating that a CaMKII inhibitory peptide targeted to the mitochondria diminished mitochondrial Ca^{2+} uptake and inhibited apoptosis in mice subjected to myocardial infarction and I/R injury.

CaMKIIδ SUBTYPES IN GENE TRANSCRIPTION

The discussion above, and indeed most of the literature, considers the role of CaMKIIδ-mediated phosphorylation and regulation of Ca^{2+} handling proteins and ion channels. Chronic elevations in CaMKIIδ expression and activity are observed in humans with heart failure (Hoch et al., 1999) and these long-term changes are likely to elicit altered gene expression. As discussed earlier, CaMKIIδ_B induces the expression of hypertrophic genes in myocytes and transgenic mice, consistent with its primarily nuclear localization (Ramirez et al., 1997; Zhang et al., 2002). Other work showed that the CaMKIIδ_B subtype is required for GATA-4 binding to the B cell lymphoma 2 (Bcl-2) promoter and subsequent gene expression (Little et al., 2009). Furthermore, CaMKIIδ_B was shown to phosphorylate the transcription factor HSF1 thereby increasing its transcriptional activity (Peng et al., 2010). Taken together, these observations imply that it is the CaMKIIδ_B subtype that regulates gene expression as a result of its actions in the nucleus.

It is not necessarily the case, however, that gene regulation requires CaMKIIδ to be localized to the nuclear compartment. Despite its primarily cytosolic localization, CaMKIIδ_C overexpressed in mouse heart increased phosphorylation of histone deacetylase 4 (HDAC4), resulting in activation of the transcription factor MEF2 (Zhang et al., 2007). CaMKIIδ_C has also been demonstrated to regulate nuclear localization of nuclear factor of activated T cells (NFATs) in NRVM. The ability of CaMKIIδ_C to decrease nuclear NFAT was blocked by coexpression of a dominant-negative construct of CaMKIIδ_C and was shown to be elicited by phosphorylation and inhibition of the Ca^{2+}/CaM dependent phosphatase calcineurin (Cn; MacDonnell et al., 2009), presumably in the cytosol. Alteration of Ca^{2+} homeostasis by cytosolic CaMKIIδ_C expression may indirectly affect gene expression and additionally the constitutively active CaMKIIδ_B utilized in the studies discussed

above (Lu et al., 2011) is cytosolic and yet regulates expression of NCX1.

Regulation of gene expression by CaMKIIδ_B has been demonstrated to promote cardiomyocyte survival while the opposite is true for CaMKIIδ_C. CaMKIIδ_B was shown to protect cardiomyocytes from doxorubicin-induced cell death via transcriptional upregulation of Bcl-2 (Little et al., 2009). Along similar lines, CaMKIIδ_B contributes to cardioprotection from H_2O_2 by increasing inducible heat shock protein 70 (iHSP70) expression (Peng et al., 2010). Conversely, CaMKIIδ_C activation is implicated in cell death elicited by a variety of stimuli (Zhu et al., 2007). It has been suggested that CaMKIIδ_C (but not δ_B) upregulates the proapoptotic transcription factor p53 (Toko et al., 2010), and recent work from our laboratory demonstrates that CaMKIIδ_C expression in NRVMs activates the proinflammatory transcription factor nuclear factor κB (NF-κB; Ling et al., 2013). We demonstrated that CaMKIIδ_C increased phosphorylation of IκB Kinase (IKK) and since IKK activation can also upregulate p53(Jia et al., 2013), this pathway may contribute to the proapoptotic response reported by Toko et al. (2010).

FUTURE DIRECTIONS

There is compelling evidence that the CaMKIIδ_B and δ_C subtypes differentially regulate cardiomyocyte Ca^{2+} handling and survival *in vitro*. Whether this occurs *in vivo* under physiological or pathophysiological conditions, and whether δ_B and δ_C subserve different functions based on their localization or selective activation, remains to be determined.

It seems likely that the relative levels of endogenous δ_B and δ_C determine localization and could therefore impact CaMKIIδ signaling. Hypothetically, a selective increase in CaMKIIδ_C would result in accumulation of cytosolic CaMKIIδ and depletion of nuclear CaMKIIδ while a selective increase in CaMKIIδ_B would have the opposite effect. CaMKIIδ redistribution in this manner may contribute to the phenotype of mice that overexpress δ_B and δ_C and importantly there are changes in the relative expression of δ_B and δ_C in models of heart failure and I/R injury. In both models δ_C expression is enhanced relative to that of δ_B (Zhang et al., 2003; Peng et al., 2010). It is not known how this occurs but it is of interest to postulate that in heart failure and during I/R regulation of CaMKIIδ splicing is altered. ASF/SF2 and RBFOX1/2 regulate the splicing of the CaMKIIδ gene and thus expression of δ_B and δ_C, but whether changes in splicing occur in and contribute to the development of heart failure or I/R injury remains to be determined. It is likely that the increased δ_C expression observed in these models is pathogenic.

While CaMKIIδ_B contains an NLS, this subtype is not completely sequestered in the nucleus (Mishra et al., 2011). As mentioned previously the NLS within the variable domain of δ_B can be regulated by phosphorylation, which prevents nuclear localization. This type of regulation could be of considerable importance since the nuclear localization of δ_B appears to correlate with enhanced expression of protective genes and cell survival while cytosolic localization does not (Little et al., 2009; Peng et al., 2010; Lu et al., 2011).

Of additional interest is the neglected CaMKIIδ_9. The pioneering work of (Hoch et al., 1998; Mayer et al., 1995) identified δ_9 as one of the three subtypes of CaMKIIδ in the adult heart and showed that it is expressed at similar levels to those of CaMKIIδ_B. δ_9 contains a sequence (^{328}EPQTTVIHNPDGNK) not present in δ_B or δ_C and thus may possess unique properties that merit further investigation, as the function and localization of δ_9 *in vivo* has not been explored. Along similar lines, CaMKIIδ_A expression is increased in a model of cardiac hypertrophy (Li et al., 2011), but the possibility that this splice variant is upregulated in and contributes to cardiovascular disease has not been investigated.

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CaMKII regulation of cardiac ryanodine receptors and inositol triphosphate receptors

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Ryanodine receptors (RyRs) and inositol triphosphate receptors ($InsP_3Rs$) are structurally related intracellular calcium release channels that participate in multiple primary or secondary amplified Ca^{2+} signals, triggering muscle contraction and oscillatory Ca^{2+} waves, or activating transcription factors. In the heart, RyRs play an indisputable role in the process of excitation–contraction coupling as the main pathway for Ca^{2+} release from sarcoplasmic reticulum (SR), and a less prominent role in the process of excitation–transcription coupling. Conversely, $InsP_3Rs$ are believed to contribute in subtle ways, only, to contraction of the heart, and in more important ways to regulation of transcription factors. Because uncontrolled activity of either RyRs or $InsP_3Rs$ may elicit life-threatening arrhythmogenic and/or remodeling Ca^{2+} signals, regulation of their activity is of paramount importance for normal cardiac function. Due to their structural similarity, many regulatory factors, accessory proteins, and post-translational processes are equivalent for RyRs and $InsP_3Rs$. Here we discuss regulation of RyRs and $InsP_3Rs$ by CaMKII phosphorylation, but touch on other kinases whenever appropriate. CaMKII is emerging as a powerful modulator of RyR and $InsP_3R$ activity but interestingly, some of the complexities and controversies surrounding phosphorylation of RyRs also apply to $InsP_3Rs$, and a clear-cut effect of CaMKII on either channel eludes investigators for now. Nevertheless, some effects of CaMKII on global cellular activity, such as SR Ca^{2+} leak or force-frequency potentiation, appear clear now, and this constrains the limits of the controversies and permits a more tractable approach to elucidate the effects of phosphorylation at the single channel level.

Keywords: ryanodine receptors, inositol triphosphate receptor, CaMKII, sarcoplasmic reticulum, Ca^{2+} leak, myocardium

CaMKII REGULATION OF CARDIAC RYANODINE RECEPTORS

GENERAL CONSIDERATIONS

Ryanodine receptors (RyRs) are the calcium release channels of sarcoplasmic reticulum (SR) that provide the majority of the calcium ions (Ca^{2+}) that are needed for contraction of cardiac and skeletal muscle. In the heart, these intracellular Ca^{2+} release channels are essential for normal cell development and indispensable for life, as demonstrated by the fact that genetic ablation of *RYR2*, the gene encoding for the cardiac isoform of the RyR channel (RyR2), causes death at early embryonic stage in mice (Takeshima et al., 1998). In humans, single amino acid mutations in RyR2 lead to life-threatening cardiac arrhythmias, also as demonstrated by the ∼160 mutations that have been linked so far to catecholaminergic polymorphic ventricular tachycardia (CPVT; Priori and Chen, 2011). Thus, in their intracellular environment, RyR2 channels must be finely regulated so that their output signal (Ca^{2+}) may induce finely graded cell contraction without igniting cellular processes that may lead to aberrant electrical activity or pathological cellular remodeling. This review will concentrate on regulation of RyR2 by *phosphorylation*, a common post-translational process that confers dynamic functional modulation to a myriad of ion channels and transporters. In the majority of cases, phosphorylation of ion channels or transporters provides an additional layer of regulation without actually being the effector or trigger of activity, i.e., in the case of RyR channels, phosphorylation *modulates* the effect of Ca^{2+} on cardiac RyR (RyR2) or depolarization on skeletal RyR (RyR1) without having the inherent ability to open or close the channel *per se*. In a useful analogy, phosphorylation and Ca^{2+}/depolarization would be similar to the "volume" and "on/off" switches, respectively, of the radio in which RyR songs are played. However, as we will see on several sections of this review, this mode of regulation is so controversial that a definitive role for RyR2 phosphorylation has not been reached yet, despite the paramount importance of this process in physiological and pathophysiological conditions. RyR2 may be phosphorylated *in vitro* and *in vivo* by several kinases, but here we will concentrate on CaMKIIδ (the most abundant CaMKII expressed in the heart and referred to here simply as "CaMKII"), and incorporate protein kinase A (PKA) whenever relevant, as these two kinases share some common transduction pathways, bear the most relevance in cardiac diseases and have been studied the most. Similarly, at least two splice variants of CaMKIIδ are expressed in cardiomyocytes, $CaMKII\delta_C$ (cytosolic) and $CaMKII\delta_B$ (nuclear), and we will identify their differential effect whenever appropriate.

RyR2 CHANNEL PROTEIN: COMPLEX STRUCTURE AND MULTIPLE PHOSPHORYLATION SITES

The RyR2 channel is a homotetrameric protein of ∼2 million Da that is often complexed with several accessory proteins (see below), forming an intricate multi-protein array (Marx et al., 2000; Bers, 2004; Valdivia, 2013). The RyR2 monomer (almost 5,000 amino acids) is organized as a series of discrete domains, with the carboxy-terminal segment crossing the SR membrane as few as four and as many as ten times (depending on the model) and forming the Ca^{2+}-permeable pore, whereas the bulk of the protein (∼90%) protrudes into the cytosol to bridge a ∼15–20 nm gap between the SR and t-tubule membranes (Hamilton and Serysheva, 2009; Capes et al., 2011; Van Petegem, 2012). The cytosolic portion of the channel contains multiple regulatory domains, most prominently Ca^{2+} activation and inactivation sites, but also binding sites for energy sensors such as nucleotides (ATP, ADP, and AMP) and inorganic phosphate, metabolites such as pyruvate, fatty acids and polyamines, and ions such as Mg^{2+}, H^+, and Cl^- (Zucchi and Ronca-Testoni, 1997; Fill and Copello, 2002; Meissner, 2004). The cytoplasmic site harbors also multiple phosphorylation epitopes (see below). Therefore, the RyR2 channel may act as a molecular switchboard that integrates a multitude of cytosolic signals such as dynamic and steady Ca^{2+} fluctuations, oxidation, metabolic states, β- adrenergic stimulation (phosphorylation), etc. and transduces these cytosolic signals to the channel pore to release appropriate amounts of Ca^{2+}. Furthermore, Ca^{2+} release is critically influenced by *luminal* (intra-SR) factors such as Ca^{2+} content and protein interactions, thus conferring RyR2 channels an additional role as integrative switch-valves that restrict luminal Ca^{2+} overload, e.g., during sympathetic stimulation. Most of the signal-decoding structures are integral domains of the RyR2 protein, but as if this huge structural assembly were not sufficiently complex, RyR2 channels are also capable of protein–protein interactions that allow them to bind, in some cases steadily and in other cases in a time- and Ca^{2+}-dependent manner, to smaller and independently regulated accessory proteins that add another layer of versatility (and complexity) to regulation of Ca^{2+} release *in vivo*. The best known RyR2-interacting proteins are calmodulin (CaM), which tonically inhibits Ca^{2+} release (Balshaw et al., 2002; Yang et al., 2014) FKBP12.6, which stabilizes RyR2 closures (Marx et al., 2000; Kushnir and Marks, 2010; but see also Timerman et al., 1996; Xiao et al., 2007), sorcin, which inhibits Ca^{2+} release in a Ca^{2+}-dependent manner (Farrell et al., 2003), and the ternary complex triadin-junctin-calsequestrin, which "senses" luminal Ca^{2+} content and modulates RyR2 activity, probably by acting as direct channel ligands (Gyorke et al., 2004). More recently, RyR2 has been found to hold anchoring sites for PKA, protein phosphatase 1 (PP1) and 2A (PP1 and PP2A), phosphodiesterase 4D3 (PDE4D3) and CaMKII (Marx et al., 2000; Currie et al., 2004; Lehnart et al., 2005), underscoring the importance of RyR2 regulation by *phosphorylation*.

RyR2 AS SUBSTRATE FOR CaMKII AND PKA

The multi-protein complex described above that includes kinases and phosphatases associated to the RyR2 strongly suggest that this channel is an avid target of downstream signaling effectors of the β-adrenergic system. PKA is a classical effector of the β-adrenergic pathway and, although the extent of activation of CaMKII by β-adrenergic stimulation and the precise signaling pathways involved are still incompletely understood, it is also accepted that CaMKII is involved in the inotropic and lusitropic effects of sympathetic stimulation (Grimm and Brown, 2010). Therefore, RyR2 proteins are natural targets of PKA and CaMKII and indeed, stimulation of beating hearts with β-adrenergic agonists readily triggers phosphorylation of RyR2 (Takasago et al., 1989; Witcher et al., 1991; Benkusky et al., 2007). Which kinase phosphorylates RyR2 to a greater extent? As early as 1989 (and before the controversy surrounding the stoichiometry of CaMKII and PKA phosphorylation of RyR2 that started in 2000, see below), Takasago et al. (1989) reported that the extent of RyR2 phosphorylation by CaMKII was ∼4 times greater than that of PKA in dog hearts. This was confirmed by Witcher et al. (1991) in the same animal species. Later, using phospho-specific antibodies and autoradiograms, Rodriguez et al. (2003) confirmed that CaMKII phosphorylated at least four sites per each PKA-phosphorylated site. Hence, there is significant evidence indicating that RyR2 are far better substrates for CaMKII than they are for PKA, which is not entirely surprising given that the phosphorylation consensus for CaMKII: R/K-X-X-S/T (where X is any amino acid residue) is less stringent than that for PKA: R-R-X-S/T (Pinna and Ruzzene, 1996; however, as we will see below, a CaMKII or PKA phosphorylation consensus does not necessarily result in CaMKII or PKA phosphorylation).

Despite the presence of multiple phosphorylation consensuses in each RyR2 subunit (George, 2008) and the fact that RyR2 channels are eager substrates for several kinases (see above), only three phospho-sites have been discovered to date. Let's discuss the most salient features of each of these sites.

Serine 2808 (S2808, mouse and human nomenclature) was first discovered by Witcher et al. (1991) as a CaMKII site but later Marx et al. (2000), Wehrens et al. (2006) labeled it as an exclusive PKA site, despite the fact that RyR2 channels with specific ablation of this phospho-epitope (RyR2-S2808A) were still phosphorylated by PKA (Benkusky et al., 2007). So, a major issue with this site is whether it's a preferred substrate for CaMKII or PKA. Since the study by Marx et al. (2000) reporting that S2808 was hyperphosphorylated in heart failure patients and that its phosphorylation enhanced dramatically the activity of RyR2 channels, several groups have studied this phospho-site in detail (Jiang et al., 2002; Rodriguez et al., 2003; Stange et al., 2003; Currie et al., 2004; Ai et al., 2005; Xiao et al., 2005; Carter et al., 2006; Kohlhaas et al., 2006; Huke and Bers, 2008; MacDonnell et al., 2008; Fischer et al., 2013), with the majority of evidence pointing to S2808 being a target for PKA, CaMKII and possibly PKG. Also, most studies find S2808 constitutively phosphorylated [basal phosphorylation ∼50–75% in several animal species and humans (Jiang et al., 2002; Rodriguez et al., 2003; Carter et al., 2006; Huke and Bers, 2008)], thus raising doubts that this site may be a reliable index of abnormal PKA-phosphorylation in heart failure patients. Indeed, Benkusky et al. (2007) found that mice with genetic ablation of the S2808 phospho-epitope (RyR2-S2808A) do not alter their β-adrenergic response, have cardiomyocyte function almost unchanged, and are not significantly protected against the maladaptive cardiac remodeling induced by chronic

stress. Further, although PKA phosphorylation of RyR2 modified single-channel activity, its effect was modest and occurred at activating (systolic) [Ca^{2+}], only, not at the expected low (diastolic) [Ca^{2+}], where it would cause significant Ca^{2+} leak (Bers et al., 2003). The lack of protection against heart failure dysfunction as well as the normal β-adrenergic response of the RyR2-S2808A mice were confirmed by MacDonnell et al. (2008), Zhang et al. (2012) using the same transgenic mouse line. In the scheme of Marx et al. (2000), which is continuously validated and extended by additional reports by the same group (for example, Wehrens et al., 2006; Shan et al., 2010a,b), PKA phosphorylation of S2808 led to dissociation of FKBP12.6, which in turn destabilized the closed state of the channel and induced multiple subconductance states, overall increasing RyR2 Ca^{2+} fluxes, especially at diastolic [Ca^{2+}]. However, most of the central tenets of this scheme have not been confirmed by others (reviewed by George, 2008; Currie, 2009; Eschenhagen, 2010; Bers, 2012; Valdivia, 2012). Specifically, Stange et al. (2003) found no effect of ablating the S2808 phospho-site (RyR2-S2808A) or simulating constitutive activation (RyR2-S2808D) in the activity of the channel or its affinity for FKBP12.6. That phosphorylation of RyR2 does not appear to dissociate FKBP12.6 was also found by Xiao et al. (2004), Guo et al. (2010). Also, there are no reports, except by the Marks' group, indicating that RyR2 phosphorylation induces subconductance states, presumably the hallmark of FKBP12.6 dissociation. Lastly, several groups have independently reported that FKBP12.6 does not affect RyR2 activity at all (Timerman et al., 1996; Xiao et al., 2007) or that it has modest effects, only (Guo et al., 2010). Thus, although it is indisputable that S2808 is phosphorylated by PKA, CaMKII and possibly other kinases, the functional output of such reaction has been difficult to pin down and is the subject of intense debate. Further studies are needed and should continue to provide insights as drugs designed to prevent FKBP12.6 dissociation enter clinical trials and testing in humans.

Serine 2814 (S2814) was discovered by Wehrens et al. (2004) as a CaMKII site and, although there is consensus that CaMKII is the preferential kinase for this site, there is less agreement that it is *the only* CaMKII site in RyR2. As mentioned above, S2808 is also a target for CaMKII, and since there are at least ∼4 CaMKII sites for each PKA site, this suggests that there are still other CaMKII sites yet to be discovered. Although S2814 is only six amino acid residues downstream of S2808 and forms part of the same RyR2 "phosphorylation hot spot" (Valdivia, 2012; Yuchi et al., 2012) the role of S2814 has been less controversial because so far it appears to be a more specific substrate for CaMKII, despite the fact that it forms part of a non-canonical CaMKII phosphorylation consensus: ^{2805}RRIS<u>Q</u>TSQV<u>S</u>V^{2815} (S2808 and S2814 are underlined). Interestingly, another serine residue within that hot spot, S2811, is also part of a non-canonical CaMKII consensus and yet, it appears to be phosphorylated *in vitro* by PKA and CaMKII (Yuchi et al., 2012) and *in vivo* in mice stimulated by β-adrenergic agonists (Huttlin et al., 2010). Whether S2811 contributes to the effect of CaMKII phosphorylation of RyR2, or distorts the signal of phospho-specific antibodies pS2808 and pS2814 (Huke and Bers, 2008), making it difficult to discern the specificity of kinases for these phospho-epitopes, is still unclear. In quiescent cardiomyocytes, S2814 is barely phosphorylated (unlike S2808), and although its activity-dependent phosphorylation may be prevented by CaMKII inhibitors, the basal phosphorylation at rest is maintained by a Ca^{2+}-dependent kinase other than CaMKII (Huke and Bers, 2008). Thus, at least two Ca^{2+}-dependent kinases phosphorylate S2814, possibly leading to the same functional output (discussed below). Whether S2814 phosphorylation contributes to the inotropic effects induced by β-adrenergic stimulation has not been firmly established. However, mice with germline ablation of the S2814 phospho-epitope (RyR2-S2814A) appear more resilient than WT against a variety of cardiac insults. van Oort et al. (2010) found that RyR2-S2814A mice were protected from catecholaminergic- and pacing-induced tachyarrhythmias, whereas Ather et al. (2013) reported that arrhythmogenic spontaneous Ca^{2+} waves that were prevalent in the *mdx* mice (Duchenne muscular dystrophy model), were suppressed by crossbreeding with the RyR2-S2814A mice. In a protocol of ischemia-reperfusion injury, the RyR2-S2814A mice exhibited significantly fewer premature beats (that could be ascribed to delayed afterdepolarizations) than WT, a protection that was not seen in mice with ablation of two phospholamban phospho-sites (PLB-DM; Said et al., 2011). Respress et al. (2012) reported by RyR2-S2814 mice fared much better than WT after TAC (transverse aortic constriction)-induced heart failure, but interestingly, were not protected against MI (myocardial infarction)-induced heart failure, proposing that CaMKII phosphorylation of RyR2 plays a role in non-ischemic forms of heart failure, only. Purohit et al. (2013) reported that CaMKII activation and phosphorylation of S2814 were required to induce atrial fibrillation in angiotensin-infused mice. Thus, several laboratories (albeit all of them using the same mouse line) have independently bestowed on S2814 a preponderant role in cardiac protection, wherein inhibition of S2814 phosphorylation averts the functional and structural damage to the heart induced by heart failure, atrial fibrillation, and other insults. Since it appears illogical that S2814 phosphorylation, a seemingly common reaction, was naturally designed to wreak havoc in the heart's function and structure, it is therefore important to demarcate the limits in which S2814 phosphorylation turns pathogenic. Interestingly, mice with constitutive activation of S2814 (S2814D), have structurally and functionally normal hearts without arrhythmias (van Oort et al., 2010) which is surprising in the context of the presumably malicious role played by S2814 phosphorylation described above.

Finally, Serine 2030 (S2030) was found by Xiao et al. (2005) using classical phospho-epitope mapping. Although S2030 is squarely within a CaMKII phosphorylation consensus (^{2027}R-L-L-S^{2030}), oddly this site is preferentially phosphorylated by PKA, at least *in vitro*. This site, therefore, like the other sites discussed above, does not follow *in silico* predictions of kinase specificity and conforms instead to the cryptic rules of steric hindrance, topological association to specific kinases, substrate availability, etc. that operate *in vivo* and separate *predicted* from *actual* phosphorylation sites. Experiments *in vitro* revealed a major role for S2030 in the control of RyR2 activity, and in heart failure patients, it appeared as a reliable marker of RyR2 dysfunction (Xiao et al., 2006), presumably even superseding S2808 (Marx et al., 2000). The RyR2

channel appears to be completely unphosphorylated at S2030 in quiescent cardiac myocytes, and phosphorylation is promoted by β-adrenergic stimulation (Huke and Bers, 2008), in line with *in vitro* experiments that indicate PKA phosphorylation of this site. However, the precise role of S2030 in intracellular Ca^{2+} homeostasis and cardiac performance, and its involvement in pathological states of the heart are not well understood yet. Mouse lines with genetic ablation of this site (RyR2-S2030A) have just been generated (Camors et al., 2014) and should shed light on the functional role of this novel phopho-site.

FACTORS THAT COMPLICATE INTERPRETATION OF RyR2 PHOSPHORYLATION EFFECTS

Many lines of evidence demonstrate that RyR2 channels are phosphorylated *in vitro* and *in vivo*, but a fundamental question still pervades the field: what is the functional effect of RyR2 phosphorylation? This appears as a simple question, set for a straightforward answer, but examining the diverse and apparently opposite effects that have been published in the topic in the last ∼2 decades, the only safe response seems to be that phosphorylation does *something* to RyR2 activity. All potential functional outcomes for RyR2 phosphorylation (increase, decrease, and no effect on activity) have been reported, with tantalizing hints toward, but no clear factors responsible for, the radical differences. Also, the question needs to be framed in a specific integrative context for a defined response to hold some truth. For example, whereas there is compelling evidence that PKA can alter RyR2 activity *at the single channel level* (Hain et al., 1995; Valdivia et al., 1995; Marx et al., 2000; Jiang et al., 2002; Uehara et al., 2002; Carter et al., 2006; Wehrens et al., 2006; Benkusky et al., 2007; Li et al., 2013), multiple self-regulatory systems operating in intact cells (not to mention the whole heart) may bring down this response to undetectable levels so that the answer from the cellular viewpoint would appear to be "no effect."

Although not always sure foretellers of a defined outcome, there are some factors that modify the activity of RyR2s and are likely to complicate their response to phosphorylation. First, as stated above, RyR2 channels contain multiple phosphorylation sites that, depending on their phospho-state, may attenuate or synergize the effect of the other sites, or may require prior phosphorylation to activate the whole protein. This has become evident in experiments in which it has been possible to link variable levels of phosphorylation with defined single-channel activity (Carter et al., 2006) and also where it is clear that *phosphatases* activate RyR2 channels to higher levels than either PKA or CaMKII alone (Lokuta et al., 1995; Terentyev et al., 2003), suggesting that dephosphorylation uncovers a set of phospho-sites that modulate RyR2 activity but are not affected by either kinase. Second, RyR2 activity has long been established to be dependent on the speed of Ca^{2+} application [as inferred by Fabiato in his classical experiments that characterized CICR (Fabiato, 1985) and demonstrated in single channel experiments (Gyorke and Fill, 1993)], and this in turn may greatly influence the overall effect of phosphorylation (Valdivia et al., 1995; Jiang et al., 2002). For example, PKA phosphorylation of RyR2 channels increases their transient component of activity (peak activation) and accelerates their rate of adaptation to a steady level of activity. In cellular settings, this effect would translate into faster rates of Ca^{2+} release in response to a fast and transient Ca^{2+} entry (such as I_{Ca}) and into little effects on steady RyR2 activity. In fact, experiments in which SR Ca^{2+} load and I_{Ca} were kept constant showed that β-adrenergic stimulation of ventricular myocytes accelerated the rate of Ca^{2+} release (Ginsburg and Bers, 2004), and in permeabilized cells with constant [Ca^{2+}], PKA had little effect on RyR2 activity (Li et al., 2002). Thus, not all effects of RyR2 phosphorylation are detectable at constant [Ca^{2+}], the preferred method of testing for such effects; fast and transient Ca^{2+} stimuli are required to unveil some of its most critical effects. Third, there exist self-correcting mechanisms that preclude persistent activation (or inhibition) of RyR2s in intact cells and can mitigate effects of phosphorylation in a few beats (Eisner et al., 1998). These mechanisms invoke restoration of steady-state Ca^{2+} fluxes when a single component of the excitation–contraction coupling machinery is perturbed or malfunctions. Thus, in its simplest terms, if the effect of PKA phosphorylation of RyR2 channels was to cause a persistent Ca^{2+} leak (as postulated by Marx et al., 2000), then the persistent leak of Ca^{2+} would necessarily cause at least partial SR Ca^{2+} depletion, which in turn would re-tune Ca^{2+} release and stop the leak. Thus, again, RyR2 responses to phosphorylation that are discretely detected at the single channel level for a relatively long period of time, may be short-lived or undetectable in cellular settings due to self-correcting mechanisms. Lastly, other factors such as Mg^{2+} (Li et al., 2013) and luminal [Ca^{2+}] (Xiao et al., 2005) seem to be required in just about the right quantity for phosphorylation to exert maximal effects. Overall then, the response of RyRs to phosphorylation is neither simple nor monotonous; it is complicated by factors intrinsic and extrinsic to the channel protein and depends critically on the context (molecular, cellular, whole heart) in which it is examined.

OVERVIEW OF CURRENT MODEL OF RyR2 PHOSPHORYLATION

Despite the recognized difficulty in linking RyR2 phosphorylation with a defined functional output, the current model of RyR2 phosphorylation is extremely simple, and needs revision. In its simplest version, PKA phosphorylation of S2808 dissociates FKBP12.6 and activates the channel by inducing long-lived subconducting states, whereas CaMKII phosphorylation of S2814 activates the channel by a different mechanism (Marx et al., 2000; Wehrens et al., 2004). However, this model disregards the S2030 phospho-site, a *bona fide* PKA site (Xiao et al., 2006), and does not give weight to the potential contribution of *other* phospho-sites, hitherto uncovered but convincingly supported by several lines of evidence. This model also does not bode well with recent structural data, wherein S2808 and S2814, by virtue of its immediacy (only six residues apart in a ∼5000 amino acid subunit), form part of a larger "phosphorylation hotspot" that encompasses S2811 and possibly T2810 (^{2805}RRI**S**Q**TS**QV**S**V^{2815}; Yuchi et al., 2012). This phosphorylation hotspot is harbored in a flexible loop connecting two symmetrical repeats interacting with one another through β strand interactions (Yuchi et al., 2012). Because the flexible loop is exposed to solvent and protrudes prominently on the RyR2 surface, it is not surprising that the whole phosphorylation hotspot may be easily accessed by several kinases. Because of the tight clustering of phospho-sites in this hotspot, there emerges the interesting possibility that they all provide *functional redundancy*, i.e., phosphorylation of S2808

may lead to the same downstream effects as phosphorylation of S2811, or S2814. In this scenario, the sequential addition of phosphate groups to the hotspot would lead to a graded (instead of all-or-none) response of RyR2 to kinases, which could explain the widely variable response detected by investigators to a specific phosphorylation maneuver. Of course, this alternative model based on the phosphorylation hotspot alone does not explain the distinct effect of CaMKII and PKA on RyR2 activity (see below), and to fill this void it is necessary to invoke the participation of S2030 and likely other as-yet-unrecognized phospho-sites. Thus, in an obligatorily more complex model (**Figure 1**) that we call "multi-phosphosite model" (Valdivia, 2012), the differential effect of PKA and CaMKII on RyR2 activity is dictated by the integrated response of the phosphorylation hotspot *and* of additional phosphorylation sites. For example, phosphorylation of S2808 and S2030 by PKA could coordinate channel openings in response to fast calcium stimuli (Valdivia et al., 1995; Ginsburg and Bers, 2004), and phosphorylation of S2814 and other CaMKII site(s) could open RyR2s at diastolic [Ca^{2+}], which would translate in Ca^{2+} leak. Certainly, until we understand the molecular basis by which the phosphorylation hotspot and other phospho-sites talk to the channel's gating domains, this structurally-based model will remain speculative and incomplete. Nonetheless, it takes into consideration compelling evidence on the existence of various phosphorylation sites and departs substantially from the simplified notion that one kinase phosphorylates one site and produces one effect.

CaMKII PHOSPHORYLATION OF ISOLATED RyR2 CHANNELS

Ryanodine receptor 2 channels have long been known to be a suitable substrate for CaMKII. In fact, in 1984, long before the controversies surrounding RyR2 phosphorylation and before the purification of RyR2, Seiler et al. (1984) observed that "high molecular weight proteins," later identified as RyR2 channels, were excellent substrates for CaMKII. In the experiments of Marx et al. (2000), where RyR2 was initially presented as a structural scaffold for multiple accessory proteins, CaMKII was *not* identified as part of that macromolecular complex. However, Currie et al. (2004) and J.H. Brown's group (reviewed in Grimm and Brown, 2010), have provided ample evidence that CaMKII is intimately associated to RyR2 (although the actual biding site is not known), logically portending an important effect of CaMKII on

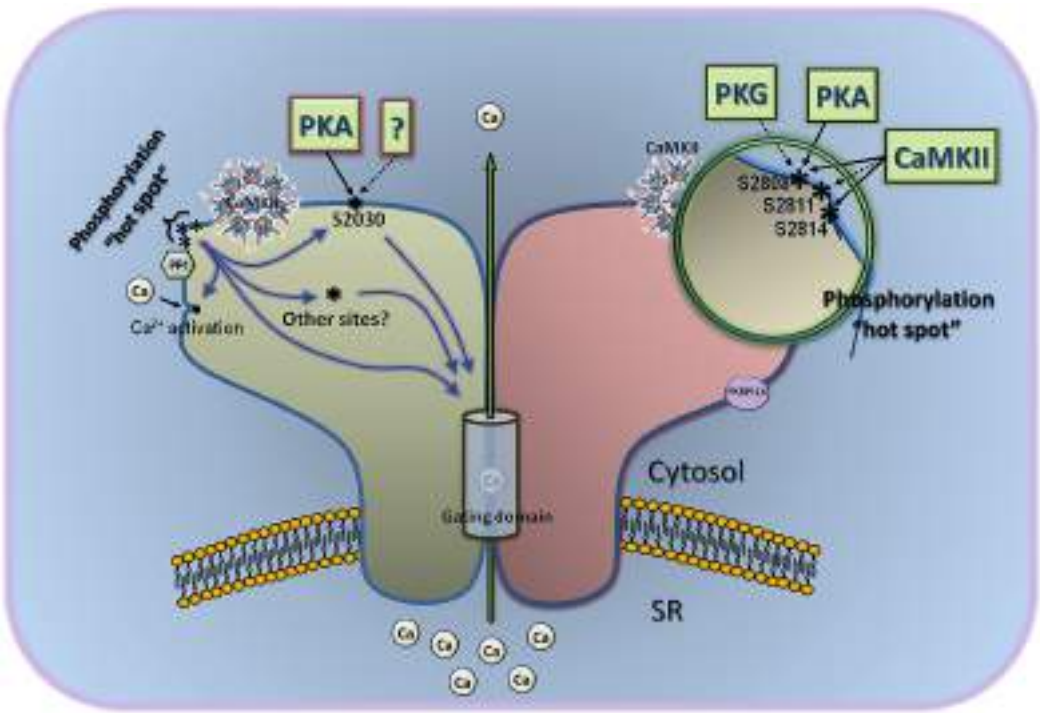

FIGURE 1 | Multi-site model of RyR2 phosphorylation. This model considers the three phospho-sites known to date, and gives also significant weight to other as-yet-uncovered sites. The classical sites S2808 and S2814 are part of a "phosphorylation hotspot" that is located in a protruding part of the channel, is targeted by several kinases, and may contain other phospho-epitopes not yet characterized (for example, S2811). Phosphorylation of individual residues within this hotspot may be undistinguishable by the channel's gating domain, but gradual addition of phosphate groups here may contribute to a tunable effect instead of an all-or-none response. Assuming that the phosphorylation hotspot works collectively toward a single effect, the differential regulation of PKA and CaMKII on channel gating may come about by the combined effect of each kinase on phospho-residues of the hotspot and other phosphorylation sites, such as S2030. This model also accommodates solo effects of S2030 or the phosphorylation hotspot on gating domains of the channel, as well as indirect effect on gating via interaction with classical Ca^{2+} activation sites. CaMKII and protein phosphatase 1 (PP1) are depicted close to the phosphorylation hotspot because the latter is readily phosphorylated/dephosphorylated by endogenous CaMKII and PP1. Demonstrated effect of kinases on S2808, S2814, and S2030 in intact cells or hearts is shown with a solid line, and *in vitro* effect is shown with a broken line.

modulation of RyR2 activity. At the single channel level, the preponderance of results suggests that CaMKII *activates* RyR2 channels, but again, the results are not unanimous. Let's briefly review the reports.

Although indirect evidence of RyR2 modulation by CaMKII was initially obtained by [^{3}H]ryanodine binding assays (Takasago et al., 1989), the first direct demonstration was provided by Witcher et al. (1991) using single channel recordings of canine RyR2 channels reconstituted in lipid bilayers. These authors observed *activation* of RyR2 channels by both, endogenous (cardiac SR-resident) and exogenous (purified from brain homogenates) CaMKII, and correlated their results with biochemical assays showing additive levels of phosphorylation by endogenous and exogenous CaMKII. This was also the report that unveiled S2808 (S2809 in dogs) as a major CaMKII site, although the proportion of the total RyR2 phosphorylation for which S2808 was solely responsible could not be determined. Thus, if the endogenous CaMKII of Witcher et al. (1991) is the same CaMKII intimately attached to RyR2 (Currie et al., 2004; Grimm and Brown, 2010), it appears that this CaMKII phosphorylates a subset of RyR2 phospho-sites only, fewer than what is actually possible by exogenous CaMKII. Thus, the recurring point that emerges is that multiple CaMKII sites concur in the RyR2 protein, each potentially exerting a defined level of control in the channel and not always phosphorylated by a given experimental condition. This might have been why Lokuta et al. (1995) detected *inhibition* of single RyR2 channel activity by exogenous CaMKII, similar to Takasago et al. (1989), who used exogenous CaMKII in [^{3}H]ryanodine binding experiments. Nevertheless, multiple CaMKII sites as justification for apparent discrepancies may not be universally applied, as Hain et al. (1995) found activation of RyR2 channels by CaMKII, using methods and animal species (dog) similar to Lokuta et al. (1995). The four studies mentioned above [Witcher et al. (1991), Lokuta et al. (1995), Takasago et al. (1989), Hain et al. (1995)] all used high (μM) [Ca^{2+}] to keep the RyR2 channels open, and none of them frontally addressed the question of whether CaMKII activates RyR2 at low (diastolic) [Ca^{2+}], a question of paramount importance given the current thinking that CaMKII increases SR Ca^{2+} leak (see below). This was technically difficult at the time because CaMKII itself was known to require Ca^{2+} as a cofactor for activation, and the Ca^{2+}-free, auto-phosphorylated active form of the enzyme was not widely known. Hence, more studies are needed to clarify the Ca^{2+}-dependence of CaMKII effect on isolated RyR2 channels. Lastly, in an oversimplified scheme that largely ignored the overwhelming evidence for the aforementioned multiple CaMKII sites, Wehrens et al. (2004) found that CaMKII activated single RyR2 channels and postulated that ablation of a single phospho-site (S2815) was sufficient to inhibit CaMKII phosphorylation completely. In summary, CaMKII phosphorylation of isolated RyR2 channels is readily detected at the biochemical level, and the preponderance of results indicate that CaMKII activates RyR2 channels by targeting multiple sites; however, some studies find that CaMKII may inhibit RyR2 channel activity and the nature of this apparent discrepancy is not easily explained. Furthermore, whether CaMKII phosphorylation activates individual RyR2 channels at low (diastolic) [Ca^{2+}] has not been firmly established yet and needs more refined studies.

EFFECT OF CaMKII ON SR Ca^{2+} RELEASE

Many studies have addressed the role of CaMKII phosphorylation in SR Ca^{2+} release and excitation–contraction coupling of intact ventricular myocytes, and most of them are detailed in excellent reviews (George, 2008; Currie, 2009; Grimm and Brown, 2010; Currie et al., 2011; Luo and Anderson, 2013; Bers, 2014). Here we will simplify the discussion by concentrating on the studies that have addressed the role of CaMKII phosphorylation on the most direct indicators of RyR2 function, namely, SR Ca^{2+} leak, spontaneous Ca^{2+} waves, and Ca^{2+} sparks. This circumvents the problem of interpreting CaMKII effects on RyR2 based on whole cell results or global Ca^{2+} transients, which are the product of multiple nodes of activity interacting in complex ways.

Most studies find that β-adrenergic stimulation increases SR Ca^{2+} leak, and that chronic adrenergic stimulation of ventricular myocytes such as that occurring in heart failure produces a cellular substrate favorable for generation of Ca^{2+}-triggered arrhythmias. To what extent are CaMKII and RyR2 channels responsible for these effects? We examined in preceding paragraphs that, although the precise transduction pathways have not been completely elucidated yet, it is clear that CaMKII is activated upon β-adrenergic stimulation of the heart (Grimm and Brown, 2010). In addition, a significant number of studies [but not all (Yang et al., 2007)] find that CaMKII activation increases SR Ca^{2+} leak (Maier et al., 2003; Currie et al., 2004; Guo et al., 2006; Curran et al., 2007) and that the SR Ca^{2+} leak that is characteristically increased in heart failure (Kirchhefer et al., 1999; Marx et al., 2000; Ai et al., 2005) may be prevented by specific CaMKII inhibition (Wu et al., 2002; Ai et al., 2005; Curran et al., 2010; Sossalla et al., 2010; Respress et al., 2012), but not by PKA inhibition (Curran et al., 2010). Hence, making the reasonable assumption that RyR2 channels are the main pathway for SR Ca^{2+} leak, and deriving from the reports above that CaMKII activation evokes arrhythmogenic SR Ca^{2+} leak, then it is fair to conclude that CaMKII phosphorylation *activates* RyR2 at diastolic [Ca^{2+}] to produce unchecked SR Ca^{2+} release that is capable of bringing membrane potential to threshold (delayed afterdepolarizations) and quite possibly ignite cellular pathways that lead to cardiac remodeling. From this perspective, CaMKII is presented as an arrhythmogenic, deleterious kinase, and RyR2 its main instrument of deraignment. Obviously, the seemingly belittled positive effect of CaMKII in normal cell function cannot be discounted, and a balance between physiological and pathological effects of CaMKII activation must exist *in vivo*. Thus, an emerging notion is that normally, *acute* CaMKII and PKA activation result in an increased magnitude and rate of Ca^{2+} release, respectively (Ginsburg and Bers, 2004), which account in no small part for the inotropic effects of β-adrenergic stimulation. This hypothesis is supported by studies that find that CaMKII increases fractional Ca^{2+} release (reviewed in Anderson et al., 2011) and that PKA increases the rate of Ca^{2+} release, only, in cells with controlled L-type Ca^{2+} channel trigger and SR Ca^{2+} content (Ginsburg and Bers, 2004). The role of CaMKII in force-frequency stimulation is also well known (Krishna et al., 2013). On the other hand, it appears that most of the deleterious effects of CaMKII are exerted under conditions that allow its *chronic* activation. It has become evident that CaMKII is not

only a sensor of Ca^{2+} signals, but it is also exquisitely sensitive to oxidative stress (Luczak and Anderson, 2014). Oxidative stress is an important ingredient of the pathogenic recipe that deranges cardiomyocytes in atrial fibrillation, heart failure, sinus node dysfunction, and other cardiomyopathies. Thus, persistent activation of CaMKII by reactive oxygen species (ROS), is an expected (and demonstrated) side effect of many cardiac insults, and a constitutively activated CaMKII has an ample range of action (hence the name multi-functional), including several ion channels and transporters that control membrane excitability and excitation–contraction coupling (Bers and Grandi, 2009), and others that control excitation–transcription coupling (Bers, 2011). Since, as we noted above, RyR2 channels are natural and avid substrates of CaMKII and are themselves affected by oxidative stress (Donoso et al., 2011), the contribution of RyR2 to altered Ca^{2+} homeostasis in these cardiac pathologies is almost assured. Overall, then, CaMKII walks a fine line separating "good" from "evil," and a great part of this dichotomy is dictated by its effect on RyR2 channels and its capacity to induce (potentially excessive) SR Ca^{2+} leak. Studies aimed at demarcating the pivotal point in which CaMKII contributes to health or disease continues at great strides. Until then, inhibiting CaMKII phosphorylation of RyR2 channels as targeted approach to prevent the excessive Ca^{2+} leak and the spontaneous Ca^{2+} waves that undergird several cardiomyopathies appears enticing, but needs further studies.

CaMKII REGULATION OF INOSITOL 1,4,5-TRIPHOSPHATE RECEPTORS

GENERAL CONSIDERATIONS

In the majority of mammalian cells, complex intracellular Ca^{2+} signals elicited by neurohormonal stimuli are mediated through the generation of inositol 1,4,5-triphosphate ($InsP_3$) and the activation of its receptor, the $InsP_3R$. $InsP_3$ originates from the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C (PLC; reviewed in Foskett et al., 2007; Taylor and Tovey, 2010). Concomitant binding of $InsP_3$ and Ca^{2+} is necessary for $InsP_3R$ activation, and this in turn leads to Ca^{2+} release within the cytoplasm. Multiple stimuli by a given agonist result in repetitive $InsP_3R$-mediated Ca^{2+} releases (Ca^{2+} oscillations) that propagate as Ca^{2+} waves through the entire cell first and may ultimately stimulate neighboring cells to form inter-cellular Ca^{2+} waves. In cardiac myocytes, localization of $InsP_3Rs$ in the nuclear envelop underlies their role in excitation–transcription coupling, but recent evidence (mostly from atrial cells) suggest that although their abundance is low in the t-tubules and the sarcolemma, $InsP_3Rs$ can also rev up excitation–contraction coupling and eventually trigger cellular arrhythmias (Mackenzie et al., 2002; Zima and Blatter, 2004; Li et al., 2005). Similar to RyR2 channels, $InsP_3Rs$ are scaffolding proteins highly regulated by ions (Ca^{2+}, H^+), nucleotides (ATP), accessory proteins (FKBP12, calmodulin), and also undergo major post-translational modifications such as phosphorylation (Taylor and Tovey, 2010). The first evidence for $InsP_3Rs$ phosphorylation was obtained in the late 80s by Greengard's and Sydner's groups from rat cerebella (Supattapone et al., 1988; Walaas et al., 1988). Today, at least 15 different kinases and phosphatases, including CaMKII, are postulated to target the $InsP_3Rs$ and regulate their activity in an isoform- and tissue-specific manner. This second part of this review will focus on the newly discovered CaMKII site (Ser150), on the physiological consequences of CaMKII phosphorylation of $InsP_3R$, and on the mechanisms by which this event may participate in cardiac Ca^{2+} signaling. Further details on the general properties of $InsP_3R$ and their role in cardiac myocytes may be found in excellent reviews (Foskett et al., 2007; Kockskämper et al., 2008; Vanderheyden et al., 2009; Taylor and Tovey, 2010).

$InsP_3R$ CHANNEL: FROM STRUCTURE TO FUNCTION

In mammals, three different genes (*ITPR1*, *ITPR2*, and *IPTR3*) encode for ∼300 kDa subunits that assemble as homo- or hetero-tetramers to form a functional $InsP_3R$ channel. Similar to RyR2, the low resolution of $InsP_3Rs$ crystals shows a mushroom-like structure with a large cytosolic *cap* constituted by the N-terminal and central regions of the protein and a short *stem*, inserted into the membrane by the C-terminal domain (Da Fonseca et al., 2003). The $InsP_3$-binding domain (or "core") is located within the first amino acids of the channel, while up to eight Ca^{2+} binding sites are distributed throughout its entire sequence (including two sites within the $InsP_3$-binding core; Mignery et al., 1990; Mignery and Sudhof, 1990; Pietri et al., 1990; Sienaert et al., 1996, 1997). The Ca^{2+} pore is formed by a classic P-loop between the membrane-spanning segments 5 and 6. Altogether the organization of $InsP_3Rs$ suggests that the opening of the channel depends on large allosteric movements that, by analogy with the RyR2s, may be finely tuned by phosphorylation (Van Petegem, 2012).

Depending on the subtype, the apparent *Kd* of the receptors for $InsP_3$ varies from 10 to 80 nM (with $InsP_3R$-2>>$InsP_3R$-1>$InsP_3R$-3; Newton et al., 1994). Remarkably, the binding of $InsP_3$ to the receptor channel is stoichiometric and regulates the properties of the $InsP_3R$-mediated Ca^{2+} release. At low intracellular concentrations, $InsP_3$-binding induces the opening of single $InsP_3R$ channel promoting a unitary Ca^{2+} release (dubbed "Ca^{2+} blip"). Increasing $InsP_3$ concentrations facilitate the opening of clusters of $InsP_3Rs$, allowing CICR within a cluster. At high concentration, $InsP_3$ promotes CICR between the clusters and intracellular Ca^{2+} waves (Bootman et al., 1997). A noteworthy property of the $InsP_3Rs$ is their high affinity for Ca^{2+}, causing maximal activity at diastolic $[Ca^{2+}]_i$ (100 nM) while in similar conditions RyR2 are closed (Ramos-Franco et al., 1998). Therefore in myocytes stimulated by Gq–protein coupled receptor (GqPCR) agonists, $InsP_3Rs$-mediated Ca^{2+} release may occur during diastole and "prime" the RyR2 to open, thus potentially increasing RyR2-mediated SR Ca^{2+} leak and triggering of arrhythmias (Mackenzie et al., 2002; Zima and Blatter, 2004; Li et al., 2005).

EFFECT OF CaMKII PHOSPHORYLATION ON $InsP_3R$ ACTIVITY

Since the first reports of $InsP_3R$ phosphorylation more than two decades ago (Supattapone et al., 1988; Walaas et al., 1988), the growing number of kinases that target $InsP_3Rs$ and either decrease or increase their activity have uncovered the complexity of $InsP_3Rs$ regulation. Among those enzymes, recent observations demonstrate that CaMKII plays a critical role for $InsP_3R$ function.

The consequences of CaMKII phosphorylation of $InsP_3R$ were first ascertained in permeabilized embryonic mouse fibroblasts (Zhang et al., 1993). In these cells, CaMKII phosphorylation permits the activation of the $InsP_3R$ channel upon addition of $InsP_3$ or an agonist cocktail (bradykinin + GTPγS). This activation takes place at intracellular [Ca^{2+}] ranging from 30 to 100 nM. At higher $[Ca^{2+}]_i$, calcineurin activity prevails and dephosphorylation of the channel occurs. In agreement with these results, Cameron et al. (1995) observed that CaMKII enhanced the $InsP_3$-sensitive Ca^{2+} flux in rat cerebellum microsomes. However, these outcomes were challenged by observations made in intact HeLa cells stimulated by histamine (Zhu et al., 1996). In this model, it is the Ca^{2+} released by the $InsP_3R$ that activates CaMKII, which phosphorylates back $InsP_3R$ to terminate the release. In a second step, the activation of a phosphatase (likely PP1/PP2A) dephosphorylates $InsP_3Rs$ to restore its activity. This model is particularly elegant since the authors also demonstrated that activated CaMKII facilitates the ER Ca^{2+} refilling by increasing SERCA activity (likely through PLN phosphorylation). As a consequence, the alternation between phosphorylated and dephosphorylated states of $InsP_3Rs$ seemed to be the basic mechanism for histamine-induced intracellular Ca^{2+} oscillations (**Figure 2**, *left panel*). Later, similar inhibitory effects of $InsP_3Rs$ phosphorylation by CaMKII were reported in channels reconstituted in lipid bilayers (Bare et al., 2005) as well as in *Xenopus* oocytes (Matifat et al., 2001) and bovine endothelial cells (Aromolaran and Blatter, 2005). Today, the origin of these conflicting observations remains unclear but a fair approximation is that it is linked to the high variability of the $InsP_3Rs$ sequence induced by alternative splicing, or to tissue-specific expression of accessory proteins (Foskett et al., 2007).

Recently, Mignery et al. (1990), Maxwell et al. (2012) identified Ser150 (S150) as a distinct CaMKII phosphorylation site on $InsP_3R$-2s. They demonstrated in lipid bilayers that the replacement of S150 by the non-phosphorylatable residue alanine ($InsP_3R$-2^{S150A}) abrogates the inhibitory effects of CaMKII phosphorylation. Conversely, the phospho-mimetic mutant $InsP_3R$-2^{S150E} reproduced the blunted channel activity of phosphorylated WT receptors. Noticeably, S150 belongs to a suppressor domain located prior to the $InsP_3$-binding core. The ablation of that

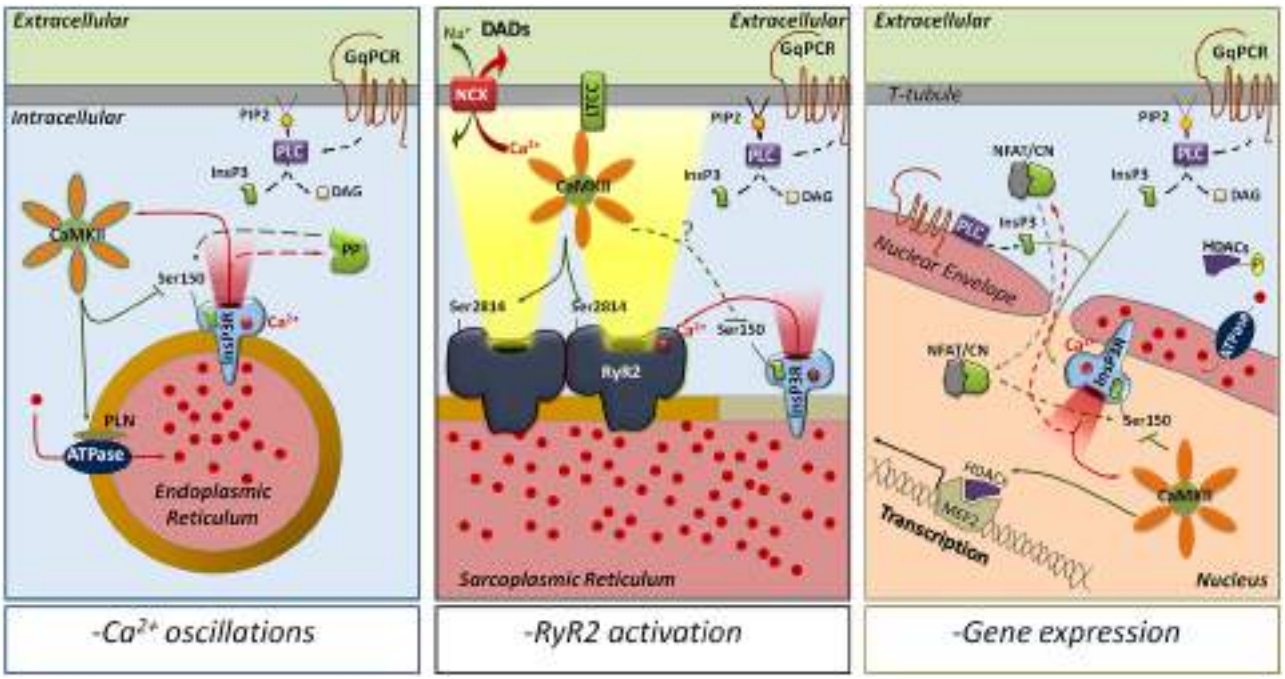

FIGURE 2 | CaMKII phosphorylation of Ser150 as a modulator of $InsP_3R$-mediated Ca^{2+} release. The activation of GqPCRs leads to the production of $InsP_3$ by PLC and initiates $InsP_3R$-mediated Ca^{2+} release. The released Ca^{2+} activates CaMKII, which in turn phosphorylates $InsP_3R$-S150 to inhibit channel function. Depending on the intracellular location, CaMKII phosphorylation of $InsP_3Rs$ could be implicated in multiple responses. In the endoplasmic reticulum of most of the mammalian cells (*left panel*), CaMKII phosphorylation of S150 alternates with protein phosphatases dephosphorylation to trigger intracellular Ca^{2+} oscillations and mediate complex Ca^{2+} signals. In the SR of cardiac myocytes (*middle panel*), $InsP_3Rs$ are able to activate RyR2s by CICR even though they seem to be excluded from the dyadic cleft. This increases Ca^{2+} transients and might trigger delayed afterdepolarizations (via NCX activation). CaMKII phosphorylation of $InsP_3R$-S150, which has not been determined yet, could then prevent the uncontrolled activation of RyR2s and decrease the incidence of arrhythmias. In the nucleus (*right panel*), $InsP_3R$ Ca^{2+} release activates CaMKII, which phosphorylates HDAC proteins to promote transcription. S150- phosphorylation by CaMKII could limit $InsP_3Rs$ activation in time and space. However, if associated with calcineurin-dephosphorylation of S150, CaMKII could favor $InsP_3R$-dependent Ca^{2+} oscillations as secondary mechanism to activate gene expression. ATPase, sarco/endoplasmic reticulum Ca^{2+} ATPase; NFAT/CN, NFAT-calcineurin complex; DAD, delayed after depolarization; DAG, diacylglycerol; GqPCR, Gq–protein coupled receptor; HDAC, histone deacetylase; LTCC, L-type Ca^{2+} channel; MEF2, myocyte enhancer factor-2; NCX, Na^+/Ca^{2+} exchanger; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PP, protein phosphatase (1, 2A, and/or 2B).

domain increases $InsP_3R$ affinity for $InsP_3$ but additionally blocks Ca^{2+} release by the channel (Uchida et al., 2003). The proposed mechanism is that the suppressor domain participates in the transduction of the allosteric movements that are necessary for channel opening (Uchida et al., 2003). Therefore, it is possible that phosphorylation of S150 by CaMKII also blocks the transmission of the activation signal to the pore domain rather than decreasing the affinity of the $InsP_3Rs$ for the $InsP_3$. This hypothesis is reinforced by the fact that high $[InsP_3]_i$ is not able to reverse the inhibition of $InsP_3Rs$ activity after CaMKII phosphorylation (Zhu et al., 1996).

$InsP_3R$ IN THE HEART: CONSEQUENCES OF CaMKII PHOSPHORYLATION

The mammalian heart expresses all three $InsP_3R$ isoforms. $InsP_3R$-1 is abundant in endothelial cells and Purkinje fibers. $InsP_3R$-2 is the main isoform of cardiac myocytes and pacemaker cells. Finally, $InsP_3R$-3s are present in all cell types but at a lower level (5–15% total $InsP_3R$ expression; Perez et al., 1997; Lipp et al., 2000; Ju et al., 2011). Although the roles of $InsP_3Rs$ in the cardiac function have been overlooked, recent evidence assigns to $InsP_3R$-2s an important role in pathologic hypertrophy and Ca^{2+}-triggered arrhythmias.

$InsP_3R$-2, CaMKII, and excitation–transcription coupling

In both atrial and ventricular myocytes, $InsP_3R$-2s are mainly present in the nuclear envelope where their implication in pathological excitation–transcription coupling has been recently uncovered (reviewed in Kockskämper et al., 2008). During cardiac hypertrophy induced by pressure overload or chronic activation of the GqPCRs, the $InsP_3R$-2s activation leads to a subsequent release of Ca^{2+} in the nucleoplasm, which activates the nuclear isoform of CaMKIIδ (CaMKIIδ_B). CaMKIIδ_B phosphorylates the histone deacetylases (HDACs) to induce their nuclear export and relieve their inhibition on the transcription factor MEF-2 (myocyte enhancer factor-2; Zhu et al., 2000; Wu et al., 2006; Ago et al., 2010). In this context the inhibition of the $InsP_3Rs$ by a CaMKIIδ_B feedback phosphorylation would limit in time and space the $InsP_3R$-mediated Ca^{2+} release and consequently be anti-hypertrophic. Conversely, a possible pro-hypertrophic role for CaMKIIδ_B phosphorylation of $InsP_3R$ is borne out from recent observations. First, $InsP_3R$-mediated Ca^{2+} release is able to activate two targets of calmodulin: CaMKIIδ_B and calcineurin, a perinuclear phosphatase that translocates to the nucleus complexed with the transcription factor NFAT (reviewed in Molkentin, 2000; Bootman et al., 2009). The inhibition of either CaMKII or calcineurin prevents the $InsP_3R$-mediated cardiac hypertrophy (Molkentin et al., 1998; Taigen et al., 2000; Zhu et al., 2000). Second, in addition to their classical localization in the sarcolemma, GqPCRs have been recently identified in the nuclear envelope as well as in t-tubules that spread near the nucleus of cardiomyocyte (Tadevosyan et al., 2012; Ibarra et al., 2013). This allows for a rapid and local production of $InsP_3$ that diffuses and signals into the nucleus independently from the cytosolic $InsP_3$ concentration (Ibarra et al., 2013). Finally, Luo et al. (2008) showed that neonatal rat cardiac myocytes stimulated by a GqPCR agonist exhibit repetitive $InsP_3R$-mediated Ca^{2+} waves (or Ca^{2+} oscillations) that take place in the nucleus autonomously from the cytosolic $[Ca^{2+}]$. Interestingly, frequency-dependent Ca^{2+} oscillations have been shown to increase gene expression via the activation of the calcineurin/NFAT pathway (Dolmetsch et al., 1998). Altogether, these observations suggest that hypertrophic signals may be generated by intra-nuclear Ca^{2+} oscillations independently (frequency, amplitude, and duration) from the excitation–contraction coupling. $InsP_3$-dependent oscillations would be created through $InsP_3Rs$ activation–inhibition cycles mediated by calcineurin dephosphorylation and CaMKIIδ_B phosphorylation of S150, respectively (**Figure 2**, *right panel*).

$InsP_3R$-2, CaMKII, and excitation–contraction coupling

In cardiac myocytes, the expression of $InsP_3R$-2s is remarkably low compared to RyR2s (for an $InsP_3R$-2:RyR2 ratio of 1:50 to 1:100 in ventricular myocytes). In addition, most of the immunofluorescence studies that have observed partial localization of $InsP_3R$-2s in the sub-sarcolemmal space of atrial myocytes, failed to detect $InsP_3R$-2s in the surface sarcolemma or t-tubules of ventricular myocytes (Bare et al., 2005; Escobar et al., 2011; except Mohler et al., 2003). Activation of $InsP_3R$-2s by a GqPCR agonist produces, in the myocyte, a small and slow cytosolic Ca^{2+} spark-like release dubbed "Ca^{2+} puffs" (Kockskämper et al., 2008). Ca^{2+} puffs can activate the neighboring RyR2s to increase the Ca^{2+} spark frequency and the Ca^{2+} transient amplitude. In pathological conditions, $InsP_3R$-2 mediated Ca^{2+} release can ultimately cause, in atrial cells, Ca^{2+} alternans and increased susceptibility to arrhythmias (Mackenzie et al., 2002; Zima and Blatter, 2004; Li et al., 2005). Moreover, although their expression is not detected in t-tubules, some reports support a similar role for $InsP_3R$-mediated release in ventricular myocyte (Domeier et al., 2008; Signore et al., 2013 but not Mackenzie et al., 2002; Zima and Blatter, 2004; Li et al., 2005). All together, these data suggest that $InsP_3R$-2s are not directly involved in excitation–contraction coupling but participate as modulators that "prime" the RyR2s to increase their sensitivity to diastolic $[Ca^{2+}]$ and LTCC current. In that context, the inhibition of $InsP_3R$-dependent Ca^{2+} release following CaMKIIδ_C phosphorylation of $InsP_3R$-Ser150 would block RyR2 potentiation and exert an anti-arrhythmic effect. However, in an extensive phosphoproteome analysis that identified the vast majority of proteins phosphorylated during β-adrenergic stimulation in mice, Hutlin et al. (2010) failed to detect phosphorylation of the cardiac $InsP_3R$-2s, including S150, while $InsP_3R$-1 and -3 were targeted by PKA at Ser1588 and Ser934, respectively. This suggests that the $InsP_3R$-2 channels expressed in the SR are not located in the vicinity of the CaMKIIδ_C/PKA microdomains, and therefore they may be excluded from the dyad containing the RyR2s (**Figure 2**, *central panel*). In agreement with that result, Signore et al. (2013) described in mouse ventricular myocytes that the $InsP_3R$-2 effects are mediated through the activation of the NCX and the increase of the action potential duration rather than a direct effect on RyR2s. Overall, these observations suggest an absence of a direct cross-talk between RyR2s and $InsP_3Rs$ and advocate the hypothesis that $InsP_3Rs$ mediate their effects on RyR2s by limited Ca^{2+} diffusion from the $InsP_3R$ release sites to the RyR2 sites.

Interestingly, Huttlin et al. (2010) also identified two cardiac-specific epitopes on $InsP_3R$-3 (Ser930 and Ser2189) for which the targeting kinase(s) remain(s) undetermined. Noticeably, S2189 of $InsP_3R$-3 contains the CaMKII consensus sequence (Pinna and Ruzzene, 1996) and is absent from $InsP_3R$-1 or -2. More studies will be necessary to determine whether this $InsP_3R$-3 phosphorylation occurs in cardiac myocytes and the mechanisms by which it affects cardiac function.

CONCLUDING REMARKS

CaMKII is a pleiotropic kinase that targets several ion channels and transporters in the heart, and RyR2 and $InsP_3R$ channels are among its principal substrates. Interestingly, despite the relatively strong structural similarity between these two intracellular Ca^{2+} release channels, the majority (but not all) of the studies indicate that CaMKII phosphorylation of RyR2s and $InsP_3Rs$ leads to antithetical outcomes. On one hand, CaMKII phosphorylation is presumed to *increase* RyR2 activity and promote SR Ca^{2+} leak that, when excessive, may trigger cardiac arrhythmias. On the other hand, CaMKII phosphorylation of nuclear $InsP_3Rs$ is presumed to *inhibit* $InsP_3Rs$-mediated Ca^{2+} release and to prevent intra-nuclear Ca^{2+} oscillations (although cytosolic $InsP_3R$-mediated Ca^{2+} release seems to promote arrhythmias, too). The magnitude of the effect of phosphorylation on these channels is also purportedly different since CaMKII *modulates* RyR2 channel activity, only, while it appears to play an on/off function on $InsP_3Rs$. The bases of both of these differences are not immediately apparent and in fact, there is no universal agreement that such differences exist. As can be derived from the preceding paragraphs, a unified scheme on the effect of phosphorylation on both, RyR2 and $InsP_3Rs$ is yet to be forged. Nonetheless, independent of its precise mechanism of action on these channels, an emerging notion is that *excessive* CaMKII activity is detrimental for cardiac performance, and the potential salutary effect of blocking its chronic effects appears worth pursuing since its action on RyR2s and $InsP_3Rs$ might prevent both Ca^{2+}-mediated arrhythmias and Ca^{2+}-dependent activation of hypertrophic gene programs.

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The role of CaMKII regulation of phospholamban activity in heart disease

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Phospholamban (PLN) is a phosphoprotein in cardiac sarcoplasmic reticulum (SR) that is a reversible regulator of the Ca^{2+}-ATPase (SERCA2a) activity and cardiac contractility. Dephosphorylated PLN inhibits SERCA2a and PLN phosphorylation, at either Ser^{16} by PKA or Thr^{17} by Ca^{2+}-calmodulin-dependent protein kinase (CaMKII), reverses this inhibition. Through this mechanism, PLN is a key modulator of SR Ca^{2+} uptake, Ca^{2+} load, contractility, and relaxation. PLN phosphorylation is also the main determinant of β1-adrenergic responses in the heart. Although phosphorylation of Thr^{17} by CaMKII contributes to this effect, its role is subordinate to the PKA-dependent increase in cytosolic Ca^{2+}, necessary to activate CaMKII. Furthermore, the effects of PLN and its phosphorylation on cardiac function are subject to additional regulation by its interacting partners, the anti-apoptotic HAX-1 protein and Gm or the anchoring unit of protein phosphatase 1. Regulation of PLN activity by this multimeric complex becomes even more important in pathological conditions, characterized by aberrant Ca^{2+}-cycling. In this scenario, CaMKII-dependent PLN phosphorylation has been associated with protective effects in both acidosis and ischemia/reperfusion. However, the beneficial effects of increasing SR Ca^{2+} uptake through PLN phosphorylation may be lost or even become deleterious, when these occur in association with alterations in SR Ca^{2+} leak. Moreover, a major characteristic in human and experimental heart failure (HF) is depressed SR Ca^{2+} uptake, associated with decreased SERCA2a levels and dephosphorylation of PLN, leading to decreased SR Ca^{2+} load and impaired contractility. Thus, the strategy of altering SERCA2a and/or PLN levels or activity to restore perturbed SR Ca^{2+} uptake is a potential therapeutic tool for HF treatment. We will review here the role of CaMKII-dependent phosphorylation of PLN at Thr^{17} on cardiac function under physiological and pathological conditions.

Keywords: myocardium, CaMKII, PLN regulation, acidosis, ischemia/reperfusion injury, heart failure

INTRODUCTION

A major characteristic of human and experimental heart failure (HF) is altered Ca^{2+} cycling, associated with decreased contractility, which partially reflects the impaired function of the sarcoplasmic reticulum (SR) membrane. During a normal excitation-contraction-coupling cycle (ECC), Ca^{2+} enters the cell through the L-type Ca^{2+} channels leading to activation of the ryanodine receptors (RyR2) in the SR and release of Ca^{2+} from this membrane system. This Ca^{2+}-induced-Ca^{2+}-release mechanism (Fabiato and Fabiato, 1977) underlies a fine-tuned synchronization of Ca^{2+} cycling in the heart, coordinating contraction and relaxation. Relaxation is mediated mainly by the activity of the SR Ca^{2+}-ATPase (SERCA2a) and to a lesser extent by the Na^{+}/Ca^{2+} exchanger (NCX). Thus, the SR is the major regulator of Ca^{2+}-handling during the cardiac excitation-contraction-relaxation cycle (Bers, 2001).

The activity of SERCA2a is under the reversible control of phospholamban (PLN), an SR associated protein (Tada et al., 1975). PLN is a 52 amino acid phosphoprotein, which, in the dephosphorylated state, inhibits the apparent Ca^{2+}-affinity of SERCA2a (James et al., 1989; Kim et al., 1990). PLN can be phosphorylated at three distinct sites in vitro: Ser^{16} by cyclic AMP (cAMP)- and cGMP-dependent protein kinases, Thr^{17} by Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII), and Ser^{16} by protein kinase C (Movsesian et al., 1984; Simmerman et al., 1986; Huggins et al., 1989). Phosphorylation of these sites *in vitro* relieves the inhibition of PLN on SERCA2a and increases SR Ca^{2+} uptake. Whereas Ser^{10} phosphorylation by PKC does not occur in intact hearts (Edes and Kranias, 1990), cGMP phosphorylation of PLN has been described in isolated myocytes (Bartel et al., 1995). However, the physiological significance of this pathway is still unclear. In contrast, phosphorylation of Ser^{16} and Thr^{17} by PKA and CaMKII has been shown to be a key mediator of the positive inotropic and relaxant effects of ß1-adrenergic stimulation in the heart. The increase in SERCA2a activity and Ca^{2+} uptake rate elicited by the phosphorylation of these sites, leads to an increase in the velocity of relaxation, SR Ca^{2+} load and SR Ca^{2+} release which, in association with L-type Ca^{2+} channel and RyR2 phosphorylation, mediate the enhanced contractility produced by ß1-stimulation (Lindemann et al., 1983; Lindemann and Watanabe, 1985; Vittone et al., 1990; Napolitano et al., 1992; Mundiña-Weilenmann et al., 1996; Kuschel et al.,

1999). Dephosphorylation of PLN, occurring by a SR-associated type 1 phosphatase (PP1; MacDougall et al., 1991), reverses the activation of SERCA2a and the stimulatory effects of β1-agonists. This article will discuss the role of Thr^{17} phosphorylation of PLN and address its significance under physiological and pathological processes.

THE PHOSPHOLAMBAN REGULATOME

Phospholamban was first described as a cAMP-dependent protein kinase substrate in the early 1970s. The phosphorylated amino acid was shown to be Ser^{16} and phosphorylation enhanced SERCA2a activity and Ca^{2+}-uptake (Kirchberger et al., 1972). Subsequently, PLN was shown to be also phosphorylated by a SR-associated Ca^{2+}-CaM-kinase (CaMKII) at Thr^{17} and this phosphorylation occurred independently of its PKA-phosphorylation (Bilezikjian et al., 1981; Davis et al., 1990). Phosphorylation by CaMKII also enhances SR Ca^{2+}-transport through an increase in the apparent affinity of the SERCA2a for Ca^{2+} (K_{Ca}). Thus, it was initially proposed that phosphorylated PLN acts as a stimulator of cardiac SERCA2a activity. However, in the late 1980s and early 1990s, there were two significant breakthroughs: (a) *in vitro* studies of reconstituted SR membrane systems (James et al., 1989; Kim et al., 1990); and (b) *in vivo* studies in mouse models with ablation or overexpression of PLN (Luo et al., 1994, 1996; Kadambi et al., 1996), which demonstrated that dephosphorylated PLN is actually an inhibitor of SERCA2 and phosphorylation relieves this inhibition, giving the appearance of phosphorylation-induced stimulation. These findings, together with the characterization and identification of a cardiac SR–associated protein phosphatase that can dephosphorylate PLN (Kranias, 1985), has led to our current understanding of PLN as a reversible inhibitor of cardiac SR Ca^{2+}-ATPase activity.

Furthermore, recent studies showed that the activity of PLN can itself be regulated by the HS-1 associated protein X-1 (HAX-1), which is ubiquitously expressed in mitochondria and SR. HAX-1 physically interacts with PLN and the binding region of PLN includes amino acids 16–22 with both Ser^{16} and Thr^{17} phosphorylation sites. Interestingly, phosphorylation of PLN diminishes its binding to HAX-1, indicating that this interaction may be physiologically relevant in the heart (Vafiadaki et al., 2007). Indeed, HAX-1 has been found to increase PLN inhibition of SR Ca^{2+} cycling and cardiac contractility *in vivo*, whereas β1-adrenergic stimulation relieves this inhibition (Zhao et al., 2009; Lam et al., 2013).

Besides HAX-1, other regulatory proteins such as PKA, CAMKII and PP1 are also associated with PLN, achieving an efficient and compartmentalized complex that regulates SR Ca^{2+}-cycling and cardiac function. PP1 is a negative regulator of PLN activity through its dephosphorylation and increased inhibition of SERCA2a. Interestingly, the type 1 enzyme is modulated by its endogenous inhibitors, Inhibitor-1 (I-1) and Inhibitor-2 (I-2). Inhibitor-1 gets activated upon its PKA phosphorylation at Thr-35 resulting in potent inhibition of PP1 activity and amplification of the β1-adrenergic receptor stimulatory effects (Iyer et al., 1988; Neumann et al., 1991; Gupta et al., 1996). More recently, the small heat shock protein 20 (Hsp20) was also identified as a novel interacting partner of PP1 and inhibitor of its enzymatic activity, resulting in diminished PLN inhibition and enhanced cardiac function (Qian et al., 2011). Thus, there is a multimeric functionally coupled signaling complex, which reversibly regulates SR Ca^{2+} cycling in the cell, composed of SERCA, PLN, HAX-1, PKA, CAMKII, PP1, I-1, and Hsp20 (**Figure 1**).

PHOSPHORYLATION OF PLN BY CaMKII UNDER PHYSIOLOGICAL CONDITIONS

ß1-ADRENERGIC STIMULATION

It is well established that stimulation by β1-agonists at the cell membrane, initiates a signal-transduction pathway that involves the Gs proteins to stimulate cAMP formation by adenylate cyclase, followed by PKA activation (**Figure 2**). PKA then phosphorylates several proteins in the cardiac myocytes to induce positive chronotropic, inotropic, and relaxant effects, the so-called "fight or flight response," which is considered the most effective mechanism to acutely increase cardiac output. The underlying phosphoproteins include PLN and RyR2 at the SR level, the L-type Ca^{2+} channel and phospholemman, at the sarcolemma level, and troponin I (TnI), C protein, and myosin light chain, at the level of the myofibrils (Bers, 2001).

The role of PLN phosphorylation vs. the phosphorylation of other proteins, which are also involved in ECC, was demonstrated by the generation and characterization of gene knockout and transgenic models with ablation, reduction or overexpression of PLN in the heart. Ablation of PLN was associated with enhanced affinity of SERCA2a for Ca^{2+}, contractility and relaxation (Luo et al., 1994, 1996). In contrast, overexpression of PLN decreased Ca^{2+}affinity of SERCA2a and diminished SR Ca^{2+}-load and cardiac contractility (Kadambi et al., 1996). Importantly, studies at the cardiomyocyte, organ and intact animal levels from PLN-deficient mice, indicated a significant attenuation of the inotropic and lusitropic effects of isoproterenol, compared

FIGURE 1 | Phospholamban regulatome. Scheme of the multimeric protein complex constituted by SERCA2a, PLN, HAX-1, PKA, CAMKII, PP1, Inhibitor-1 (I-1), and Hsp20, which reversibly regulates SR Ca^{2+} transport in the cell. SERCA2a activity is regulated by its reversible inhibitor PLN and the histidine rich Ca^{2+}-binding protein (HRC). Phosphorylation of PLN is mediated by cAMP-dependent protein kinase (PKA) at Ser^{16} site and Ca^{2+}-calmodulin-dependent protein kinase (CaMKII) at Thr^{17} site. Dephosphorylation of these sites occurs by protein phosphatase 1 (PP1). The activity of PP1 is regulated by inhibitor-1 (I-1) and Hsp20.

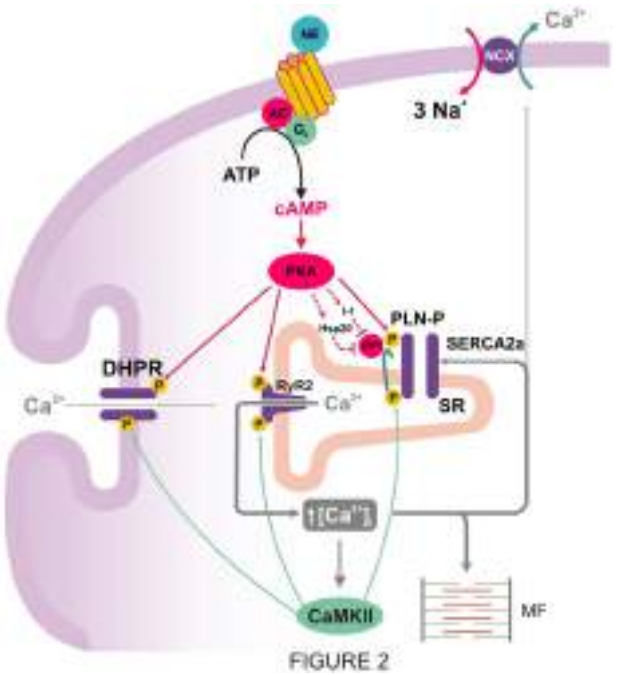

FIGURE 2 | PKA mediated increase in cytosolic Ca^{2+} and inhibition of PP1: two prerequisites for CaMKII-dependent phosphorylation of PLN during β1-adrenergic stimulation. PKA-dependent phosphorylation of Ca^{2+} handling proteins, particularly L-type Ca^{2+} channel and PLN, produces an increase in cytosolic Ca^{2+} that is necessary to activate CaMKII and produce CaMKII-dependent phosphorylation. PKA also increases inhibitor-1 and Hsp20 phosphorylation, amplifying the stimulatory effects of β1–adrenergic stimulation on SR Ca^{2+}-transport, relaxation, and contractility.

with wild types (Luo et al., 1994; Hoit et al., 1995), and revealed that PLN is a major mediator of the β1-adrenergic response in the mammalian heart. However, as noted above, PLN is not only phosphorylated by PKA, at Ser^{16}, but also by CaMKII at Thr^{17}, during β1-adrenergic stimulation. These phosphorylations appear to occur independently of each other *in vitro* (Bilezikjian et al., 1981; Davis et al., 1990). However, *in vivo* attempts to phosphorylate PLN by CaMKII indicated that an increase in cAMP levels was a requirement for CaMKII activation (Lindemann et al., 1983; Lindemann and Watanabe, 1985; Vittone et al., 1990; Napolitano et al., 1992; Mundiña-Weilenmann et al., 1996; Kuschel et al., 1999). This may be due to required PKA-phosphorylation of PLN, as well as L-type Ca^{2+} channels and RyR2, to enhance cytosolic Ca^{2+}, necessary to activate CaMKII. Indeed the availability of transgenic models, expressing either wild type PLN (PLN-WT), $Ser^{16} \rightarrow$ Ala mutant PLN (PLN-S16A) or $Thr^{17} \rightarrow$ Ala mutant PLN (PLN-T17A) in the cardiac compartment of PLN knockout mice, indicated that the phosphorylation of Ser^{16} of PLN is a prerequisite for the phosphorylation of Thr^{17} (Luo et al., 1998). In addition, these studies showed that Ser^{16} can be phosphorylated independently of Thr^{17} *in vivo* and that phosphorylation of Ser^{16} was sufficient for mediating the maximal cardiac responses to β1-adrenergic stimulation (Chu et al., 2000).

The role of CaMKII-phosphorylation of PLN was also addressed in a model with expression of a CaMKII inhibitory peptide targeted to the longitudinal SR (AIP4-LSR TG; Ji et al., 2006). The results indicated that Thr^{17} PLN-phosphorylation as well as SR Ca^{2+}-uptake and contractile parameters were decreased. However, the response to isoproterenol remained unaltered. Similarly, transgenic mice with CaMKII inhibition (AC3-I mice), decreased SR Ca^{2+}-content without changes in the myocyte response to isoproterenol (Zhang et al., 2005). These findings suggested a predominant role of Ser^{16}phosphorylation over that of Thr^{17} in the β1-adrenergic response. Furthermore, experiments in perfused rat hearts using the PKA inhibitor H-89, confirmed that PKA activation is required for β1-adreniceptor mediated phosphorylation of the Thr^{17} site in PLN (Said et al., 2002). It was further demonstrated that, when both PLN phosphorylation sites are present, the CaMKII site contributes to PLN phosphorylation and enhanced mechanical effects only at relatively high levels of β1-adrenergic stimulation, i.e., isoproterenol concentrations $\geq$10 nM. The lack of contribution of Thr^{17}site to PLN phosphorylation at lower isoproterenol concentrations was attributed to a moderate increase in PKA activity, which would raise intracellular Ca^{2+} to a level not sufficient to activate CaMKII and phosphorylate Thr^{17} site (Mundiña-Weilenmann et al., 1996; Said et al., 2002). Taken together, these findings support the notion that CaMKII is a contributor in the stimulatory effects of β1-adrenergic receptor in the heart. However, PKA activation is required to create the necessary conditions for CaMKII activation and Thr^{17} phosphorylation (**Figure 2**). A similar conclusion should hold true for the different Ca^{2+} handling proteins which are phosphorylated by both kinases, like L-type Ca^{2+} channels or RyR2. Interestingly, sustained β1-adrenergic receptor stimulation enhanced cell contraction and Ca^{2+} transients by a mechanism which is largely PKA-independent but sensitive to CaMKII-inhibitors. In these studies, a shift from Ser^{16} to Thr^{17} phosphorylation pathway was observed, underscoring the role of CaMKII during prolonged β1-adrenergic stimulation (Wang et al., 2004). In addition, β1-adrenoceptors activate the guanine nucleotide exchange protein that is directly activated by cAMP (Epac), independently of, and in parallel with, PKA. Indeed, Oestreich et al. (2009) identified RyR2 and PLN as two effector targets of a pathway mediated by Epac-PLCϵ-PKCϵ-CaMKII. These authors described an increase in Ca^{2+} transient mainly attributed to an increase in RyR2 sensitivity by Ca^{2+} influx activation. Although the specific role of Thr^{17} phosphorylation of PLN in these effects was not directly tested, they showed that β-adrenergic stimulation-mediated enhancement of SR Ca^{2+} load and myoplasmic Ca^{2+} clearance were not significantly altered by PLCϵ ablation, suggesting a poor role of the pathway described, on SR Ca^{2+} uptake (Oestreich et al., 2007). Moreover, other results showed that Epac activation decreases the amplitude of evoked Ca^{2+} transient due to Epac-induced SR Ca^{2+} leak by CaMKIIδ-phosphorylation of RyR2 and SR depletion (Pereira et al., 2007, 2013). The different outcomes of the effects of Epac on Ca^{2+} transient amplitude may be due to different experimental protocols, since Epac activation produces an initial increase in Ca^{2+} transients before reaching a steady state, in which Ca^{2+} transients are decreased. Yet, both results are consistent with an increase

in RyR2 activation produced by Epac. The more recent study by Pereira et al. (2013) further showed that inhibition of PKA-dependent effects of isoproterenol by H-89 pretreatment blocked the isoproterenol-induced increase of Ca^{2+} transient amplitude, speed of relaxation and SR Ca^{2+} load. In contrast, isoproterenol still greatly increased SR Ca^{2+} spark frequency and decreased Ca^{2+} transient amplitude. Both of these effects were similar to the steady state responses produced by Epac activation. These results would imply that: (1) most of the isoproterenol-induced PKA-independent Ca^{2+} leak enhancement is mediated by Epac; and (2) the contribution of Epac to isoproterenol–induced SR Ca^{2+} reuptake through PLN phosphorylation, is very modest, if any, since no relaxant effects of isoproterenol could be detected after PKA inhibition.

PHOSPHORYLATION OF Thr^{17} OF PLN IN THE ABSENCE OF β1-ADRENERGIC STIMULATION

As indicated above, several studies showed that CaMKII-dependent PLN phosphorylation can only occur in the intact beating heart in the presence of ß1-adrenergic stimulation, while it occurs independently of cAMP-PKA activation *in vitro*. To address this apparent discrepancy, the phosphatase inhibitor okadaic acid was used in the presence of high extracellular Ca^{2+}. Under phosphatase inhibition, increasing Ca^{2+}, increased contractility, relaxation and phosphorylation of Thr^{17} of PLN, without significantly changing either cAMP or Ser^{16}phosphorylation (Mundiña-Weilenmann et al., 1996). These findings indicated that Thr^{17} can be phosphorylated independently of Ser^{16} of PLN in the intact heart, in accordance with the *in vitro* studies. Thus, the relative balance of protein kinase (PKA and CaMKII) and phosphatase activities appears to regulate phosphorylation of Thr^{17} and Ser^{16} in PLN.

Stimulation frequency (SF), a fundamental physiological modulator of myocardial performance, is another example in which Thr^{17} phosphorylation of PLN can occur in the absence of prior Ser^{16} phosphorylation (Hagemann et al., 2000; Zhao et al., 2004; Valverde et al., 2005). These findings are in concert with the fact that CaMKII can decode the frequency of Ca^{2+} spikes into distinct amounts of kinase activity (De Koninck and Schulman, 1998), and indicate that SF can produce a sustained increase in CaMKII, which leads to the phosphorylation of Thr^{17} in PLN, without the requirement of phosphatase inhibition. Moreover, these results prompted the link between the observed Thr^{17} phosphorylation and the relaxant effect of increasing SF (frequency-dependent acceleration of relaxation or FDAR, Bers, 2001; Hagemann et al., 2000). Indeed, FDAR was inhibited in the presence of CaMKII-inhibitors and in cardiomyocytes expressing the mutant T17A-PLN (Zhao et al., 2004). However, although the involvement of SR and CaMKII in FDAR was supported by several studies (Bassani et al., 1995; DeSantiago et al., 2002; Picht et al., 2007; Wu et al., 2012), a recent report challenged these previous findings by showing that FDAR was still present in CaMKIIδ-KO mice (Neef et al., 2013). These results would suggest that either a CaMKIIδ-independent mechanism or another CaMKII isoform, like CaMKIIγ, is playing a role in FDAR.

The role of Thr^{17} phosphorylation of PLN on FDAR was also questioned on the basis of three main findings: (1) FDAR precedes the phosphorylation of Thr^{17} site of PLN (Valverde et al., 2005; Huke and Bers, 2007); (2) Most studies concur that the main regulatory effect of PLN phosphorylation is to increase the apparent Ca^{2+} affinity of SERCA2a (Simmerman and Jones, 1998), while FDAR is associated with an increase in the maximal velocity of SR Ca^{2+} uptake (Picht et al., 2007); and (3) FDAR has been also detected in PLNKO mice in one study (DeSantiago et al., 2002), although this finding was not observed in other studies (Bluhm et al., 2000; Wu et al., 2012).

Taken together, the underlying molecular steps that encompass the FDAR process are currently unclear. Although most of the experimental evidence indicates that CaMKII is involved in FDAR, some studies have challenged this possibility and the participation of PLN in FDAR. Thus, it is likely that several rather than a single mechanism, are associated with this phenomenon.

PHOSPHORYLATION OF PLN BY CaMKII UNDER PATHOLOGICAL CONDITIONS

ACIDOSIS

Mechanical recovery during acidosis

An understanding of how pH changes alter cardiac function is important for a better comprehension of some cardiac pathological situations, which are important in the clinical setting. Myocardial ischemia is particularly relevant along these lines: in human, acidosis can be detected 15 s after the occlusion of the coronary artery and is a major mechanism for the loss of contractility during ischemia (Poole-Wilson, 1989). Substantial changes in intracellular pH may also occur in disorders of different origins which affect cardiac function, like sleep apnea/hypopnea syndrome, diabetic ketoacidosis or in patients on dialysis.

Acidosis produces a rapid decrease in the strength of contraction (Cingolani et al., 1970; Allen and Orchard, 1983), which is largely due to a decrease in myofilament Ca^{2+} responsiveness (Fabiato and Fabiato, 1978). This decrease displaces Ca^{2+} from troponin C, and would be the main mechanism responsible for the early increase in diastolic Ca^{2+} during acidosis. The initial fall in contractility is followed by an increase in the amplitude of intracellular Ca^{2+} transients and contractile force (Mattiazzi and Cingolani, 1977a,b; Allen and Orchard, 1983, the mechanism of which is not intuitively obvious, because acidosis inhibits most of the steps of excitation-contraction-coupling (Orchard and Kentish, 1990). Earlier experiments suggested that acidosis-induced activation of Na^{+}-H^{+} exchanger (NHE), by increasing cytosolic Na^{+} and then Ca^{2+} through the NCX, was sufficient to overcome the inhibitory effect of acidosis on SERCA2a, increasing SR Ca^{2+} and intracellular Ca^{2+} transients (Harrison et al., 1992). However, inhibition of NHE does not always prevent intracellular Ca^{2+} and mechanical recovery (Choi et al., 2000; DeSantiago et al., 2004), indicating that additional mechanisms may play a role (**Figure 3A**). A major clue supporting this possibility was given by experiments showing that Ca^{2+} and contractile recovery during acidosis require an intact SR and CaMKII activity, suggesting that CaMKII-dependent phosphorylation at the SR level is involved in the recovery mechanism (Pérez et al., 1995; Komukai et al., 2001; Nomura et al., 2002; DeSantiago et al., 2004; Mundiña-Weilenmann et al., 2005; Neef et al., 2013). Indeed, it was shown that phosphorylation of the Thr^{17} site of PLN transiently increased

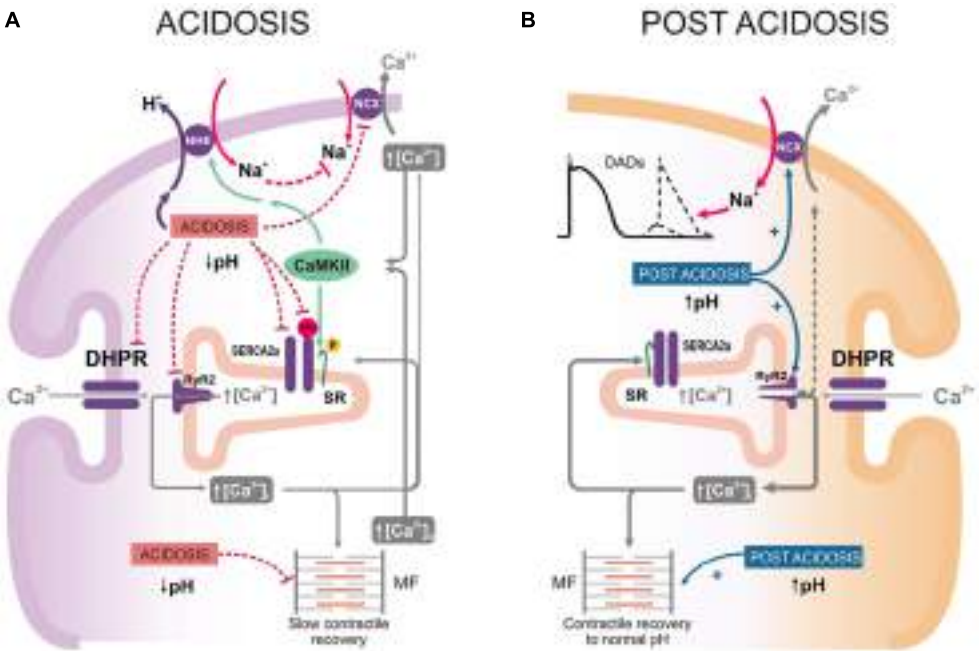

FIGURE 3 | (A) Intracellular mechanisms that may contribute to the mechanical recovery during acidosis. Acidosis produces a decrease in myofilament Ca^{2+} responsiveness which increases diastolic Ca^{2+} ($[Ca^{2+}]_d$). Activation of NHE and direct acidosis inhibition of Na^+-Ca^{2+} exchanger (NCX), would contribute to the increase in cytosolic Ca^{2+}. Acidosis also inhibits PP1. The simultaneous increase in cytosolic Ca^{2+} and inhibition of PP1 activates CaMKII and enhances PLN phosphorylation at Thr^{17} site. As a consequence, there is an increase in SR Ca^{2+} uptake, able to offset the direct acidosis-induced inhibition of SERCA2a activity. This would lead to enhanced SR Ca^{2+} release and Ca^{2+} transients, which counteract the negative effect of acidosis on contractile proteins and supply the substrate for the slow mechanical recovery during acidosis. **(B)**. Putative intracellular mechanisms of post-acidosis induced-arrhythmias. Upon returning to control pH, the inhibitory effects of acidosis are rapidly removed, favoring the increase in Ca^{2+} cycling and the contractile recovery towards control levels. However, the relief of RyR2 from the previous constrain produced by acidosis, evokes also an increase in diastolic Ca^{2+} leak from the (Ca^{2+}-loaded) SR. Such release may activate inward currents through the NCX, also relieved from the inhibition evoked by acidosis. The inward Na^+ current, if large enough, can trigger arrhythmias.

at the onset of acidosis, possibly favored by the increase in intracellular (diastolic) Ca^{2+} and the inhibition of phosphatase induced by acidosis (Allen and Orchard, 1983; Vittone et al., 1998). This phosphorylation was associated with the initial and most significant portion of the contractile/relaxation recovery, and both were blunted by CaMKII-inhibition (Mundiña-Weilenmann et al., 2005). Thus, CaMKII-dependent PLN phosphorylation provides a mechanism to overcome the depressant effect of acidosis on SERCA2a (Mandel et al., 1982). These increases in SR Ca^{2+} content and release also counteract the effect of acidosis on contractile proteins, thereby helping to maintain contractile force. More recent experiments showed that CaMKII also activates NHE which may add to the direct activation of the exchanger induced by acidosis (Vila-Petroff et al., 2010; **Figure 3A**). Interestingly, experiments by DeSantiago et al. (2004) showed absence of mechanical recovery in myocytes lacking PLN (PLNKO). This finding may be taken to indicate that PLN is essential for SR Ca^{2+} and mechanical recovery during acidosis. However, the effects of PLN ablation mimic maximal PLN phosphorylation. Therefore the results of DeSantiago et al. (2004) actually raise the question of whether accelerating SR Ca^{2+} reuptake during acidosis is beneficial, favoring mechanical recovery as discussed above, or harmful, hindering it. An explanation to these apparent contradictory results may lie on the fact that intracellular Ca^{2+} and mechanical recovery during acidosis require an increase in SR Ca^{2+} uptake above steady state, a condition that cannot be accomplished in PLNKO mice in which basal SR Ca^{2+} uptake is already at maximal levels. In line with DeSantiago's results, Nomura et al. (2002) showed that the mechanical recovery from acidosis did not occur in highly phosphorylated myocytes treated with isoproterenol and a phosphatase inhibitor.

Acidosis and post-acidosis arrhythmias

The increase in SR Ca^{2+} load during acidosis, responsible for the mechanical recovery, may also increase spontaneous SR Ca^{2+} release and produce extra-systoles (Orchard et al., 1987). Moreover, returning to normal pH after acidosis is also arrhythmogenic: recovery of pH induces an increase in SR Ca^{2+} leak. This effect was attributed to the increase in the opening probability of RyR2 due to the pH increase after acidosis and the acidosis-induced increase in SR Ca^{2+} content, still present at the beginning of post-acidosis (Said et al., 2008). The return to normal pH also leads to recovery of the previous acidosis-induced inhibition of NCX (Philipson et al., 1982), favoring Ca^{2+} extrusion and Na^+ gain into the cell, membrane depolarization and eventually triggered arrhythmias (Said et al., 2008; **Figure 3B**). Together, these results indicate that post-acidosis CaMKII-dependent DADs are triggered by two concurrent factors: (1) acidosis-induced increase

in SR Ca^{2+} content; and (2) relief of RyR2 and NCX, previously inhibited by acidosis.

ISCHEMIA/REPERFUSION (I/R)

Stunning

The role of CaMKII in I/R will be addressed in detail elsewhere in this issue. We will briefly refer here to the role of CaMKII-dependent PLN phosphorylation in this pathological situation. In the last few years, a dual effect of CaMKII-dependent protein phosphorylation (beneficial and detrimental) has been described in the scenario of I/R in the intact heart. The beneficial effect of CaMKII usually refers to the intracellular Ca^{2+} and contractile recovery that occurs during stunning, a fully reversible post-ischemic dysfunction (Braunwald and Kloner, 1982). Initially, this beneficial effect was associated with an increase in the phosphorylation of Thr^{17} site in PLN at the onset of reperfusion (Vittone et al., 2002). Further experiments in transgenic mice in which Thr^{17} and/or Ser^{16} sites of PLN were mutated to Ala and direct measurements of intracellular Ca^{2+}, demonstrated that Thr^{17} phosphorylation was essential for the recovery of Ca^{2+} transients and contractility in the stunned heart (Said et al., 2003; Valverde et al., 2006). These findings confirmed that the increase in Thr^{17} phosphorylation of PLN upon reperfusion, although transient, offers a mechanism that helps to limit cytosolic Ca^{2+} overload, by accelerating SR Ca^{2+} reuptake and thereby ameliorating intracellular Ca^{2+} handling (**Figure 4**). In contrast, when SR Ca^{2+} reuptake is highly enhanced by ablation of PLN, post-ischemic recovery of contractile function was negligible (Cross et al., 2003). A possible explanation for this apparent paradox is the higher ATP consumption of PLNKO hyperactive hearts relative to WTs, which may greatly influence contractile recovery. A second possibility that does not exclude the first one, is that under conditions in which RyR2 are altered, a persistent and exacerbated SR Ca^{2+} uptake, would greatly elevate SR Ca^{2+} content and enhance the propensity for SR Ca^{2+} leak, which may conspire against contractile recovery and favor reperfusion arrhythmias. Indeed, a rise in CaMKII phosphorylation of Ser2814 in RyR2 and an abrupt increase in SR Ca^{2+} release at the onset of reflow were recently associated with early reperfusion arrhythmias. This occurs in spite of the fact that Thr^{17} site of PLN was also phosphorylated (Said et al., 2011; Valverde et al., 2010). These results strongly suggest that the beneficial effects of increasing SR Ca^{2+} uptake in I/R, may turn to be deleterious under conditions in which the balance between SR Ca^{2+} uptake and leak is lost (**Figure 4**).

As will be discussed below, this unbalance may constitute a major cause of the detrimental effect of CaMKII activation in the irreversible I/R.

Irreversible ischemia/reperfusion

After a prolonged ischemic period, reperfusion evokes irreversible cardiac injury. Under these conditions, myocytes die by apoptosis, autophagy and necrosis. The rise in Ca^{2+} during ischemia and reperfusion leads to mitochondrial Ca^{2+} accumulation, which is greatly favored by the close association between mitochondria and

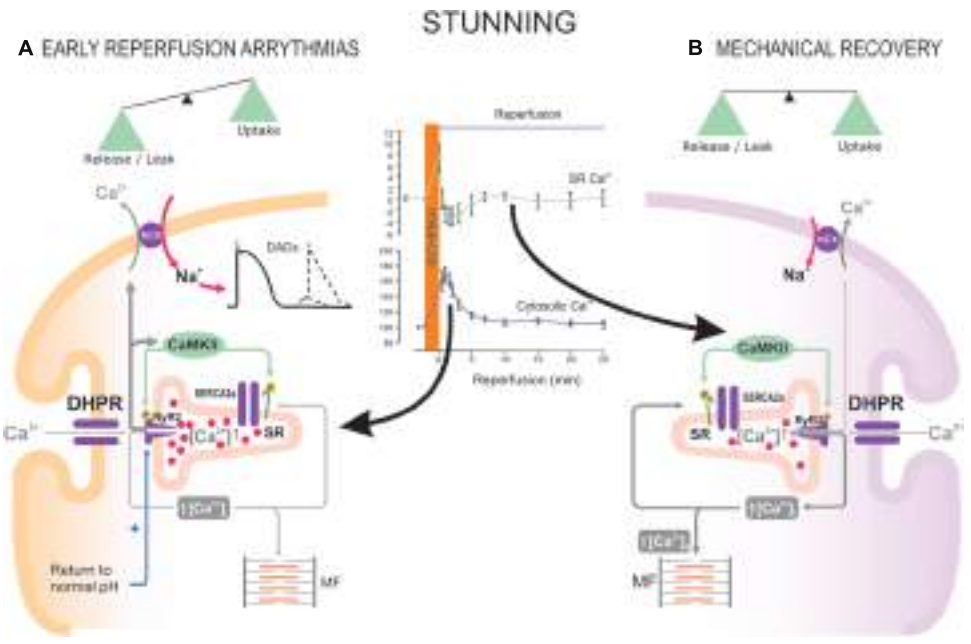

FIGURE 4 | The beneficial or detrimental effects of CaMKII activation and PLN phosphorylation in I/R depend on a tight balance between SR Ca^{2+} reuptake and leak. Reperfusion after a short ischemic period (stunning) is associated with an increase in CaMKII-dependent PLN and RyR2 phosphorylation. During early reperfusion **(A)**, there is: abrupt release of SR Ca^{2+} (inset of the Figure, Valverde et al., 2010), possibly favored by the ischemia-induced increase in SR Ca^{2+} content; relief of RyR2 inhibition exerted by the ischemic acidosis; and increase in Ser2814 phosphorylation of RyR2 (Said et al., 2011). CaMKII-dependent phosphorylation of PLN does not counteract SR Ca^{2+} release, which is partially responsible for early reperfusion arrhythmias. **(B)** After the first minutes of reperfusion, the increase in Thr^{17} phosphorylation of PLN appears to successfully counteract SR Ca^{2+} leak, leading to Ca^{2+} transients and mechanical recovery.

SR and constitutes a main event in the initiation of cell death (Rizzuto and Pozzan, 2006).

Experimental evidence consistently indicates that CaMKII-inhibition is protective in the irreversible I/R injury (Zhang et al., 2005; Vila-Petroff et al., 2007; Salas et al., 2010). Although the mechanisms for myocardial protection by CaMKII inhibition are still unclear, the CaMKII deleterious pathway in I/R certainly involves the SR and the mitochondria (Vila-Petroff et al., 2007; Salas et al., 2010; Joiner et al., 2012; Valverde et al., 2013). Phosphorylation of Thr^{17}, the CaMKII site of PLN, has been shown to increase at the onset of reperfusion (Vila-Petroff et al., 2007; Salas et al., 2010). This finding may suggest either that the effect of PLN phosphorylation is part of the deleterious cascade of CaMKII activation, or that this phosphorylation is beneficial, although insufficient to counteract the effect of simultaneous detrimental mechanisms. The experimental outcome of testing these possibilities has remained controversial. Referent to the first one, Yang et al. (2006) demonstrated that the protective effect of chronic CaMKII inhibition in AC3-I mice was lost, when they were interbred with PLNKO mice and submitted to myocardial infarction, supporting a detrimental effect of enhancing of SR Ca^{2+} uptake. Referent to the second possibility, several studies demonstrated that accelerating SR Ca^{2+} uptake by different means (i.e., overexpressing SERCA1a, with higher kinetics than SERCA2a, or expressing a repressor of PLN activity, PP1 inhibitor-1), alleviated post-ischemic cardiac injury (Talukder et al., 2007, 2008; Nicolaou et al., 2009), supporting a beneficial effect of accelerating SR Ca^{2+} uptake. A possible clue to explain these controversial findings is given by results showing that proteins, different from PLN, may be involved in the cascade by which CaMKII activity is deleterious in I/R. A decrease in the expression of RyR2 has been described in I/R (Salas et al., 2010), compatible with a degradation/damage of these channels by the concerted action of calpains and proteasomes (Pedrozo et al., 2010), that would lead to an increase in the opening probability of RyR2 (Domenech et al., 2003). Moreover, redox alterations or CaMKII-dependent phosphorylation might also influence the activity of RyR2 and SR Ca^{2+} leak in I/R (Hidalgo et al., 2004; Said et al., 2011; Valverde et al., 2013). These alterations would favor the unbalance between SR Ca^{2+}uptake and leak, promoting mitochondrial Ca^{2+} overload and cell death. This cascade would be further stimulated by the recently described CaMKII-dependent phosphorylation of mitochondrial Ca^{2+} uniporter (Joiner et al., 2012). Taken together, these findings suggest that the progression toward a beneficial or detrimental effect of CaMKII activation and PLN phosphorylation in I/R would critically depend on the balance between the extent of SR Ca^{2+} reuptake and SR Ca^{2+} leak, largely given by the status/characteristics of other proteins, also involved in SR Ca^{2+} handling, like RyR2 (**Figure 4**).

HEART FAILURE

Heart failure develops when the heart is unable to provide an adequate cardiac output to meet the metabolic needs of the organism. Mechanical dysfunction and arrhythmias are hallmark features of HF, being aberrant Ca^{2+} handling a main cause of these two characteristic alterations. Indeed, there is evidence supporting a decrease in intracellular Ca^{2+}-transient and diminished SR Ca^{2+} content, an outcome that constitutes the major origin of the altered contractility in HF (O'Rourke et al., 1999; Piacentino et al., 2003), and that can be attributed to alterations in the expression/activity of different Ca^{2+} regulatory proteins. In particular, a decrease in SERCA2a and an increase in NCX expressions have been described in different HF models and species, including human (Hasenfuss, 1998). An enhanced SR Ca^{2+} leak, through hyperphosphorylated RyR2, would also contribute to the decrease in SR Ca^{2+} content and Ca^{2+} release, typical of HF (Ai et al., 2005; Shan et al., 2010; Respress et al., 2012).

The decrease in SERCA2a expression is not associated with a parallel decrease in PLN, which would produce an increase in the functional stoichiometry PLN/SERCA, with a decrease in SERCA2a Ca^{2+} affinity and SR Ca^{2+} uptake rate and a prolongation of relaxation times (Meyer et al., 1995). Moreover, phosphorylation of PLN has been found to be decreased, either at Ser^{16} (Schwinger et al., 1999; Sande et al., 2002), Thr^{17} (Netticadan et al., 2000), or both (Huang et al., 1999; Mishra et al., 2003), accounting for increased inhibition of SERCA2a. These findings may be due to the attenuation of β1-adrenergic cascade, due to receptor desensitization, down-regulation and uncoupling, typical of the disease progression (Bristow et al., 1982; Dash et al., 2001; Port and Bristow, 2001), and/or the increase in PP1 activity, described in HF (Bibb et al., 2001; Carr et al., 2002; Gupta et al., 2003). Indeed, in human failing myocardium, phosphorylation of Ser^{16}in PLN decreased because of increases in PP1 activity (Schwinger et al., 1999), whereas phosphorylation of Thr^{17} decreased due to increased activity of PP2B (calcineurin; Münch et al., 2002). Interestingly, this decrease occurred despite an increase in CaMKII activity characteristic of HF. Taken together, these results indicate that the increase in SERCA2a/PLN ratio and the diminished phosphorylation of PLN, are key determinants of the depressed SR Ca^{2+} uptake in HF, leading to an increase in diastolic Ca^{2+}, a decrease in SR Ca^{2+} stores and therefore in Ca^{2+} available for contraction. This results in reduced contractile force, impaired relaxation and altered force-frequency relationship.

Given this central role of SERCA2a and PLN in the defective Ca^{2+} handling typical of HF, the strategy of altering SERCA2a and/or PLN levels or activity to restore perturbed Ca^{2+} uptake into the SR are potential therapeutic strategies for HF treatment (del Monte and Hajjar, 2003). Indeed, overexpression of SERCA2a can restore Ca^{2+} handling and contractile function in animal models (Cutler et al., 2012) and in human HF (del Monte et al., 1999; Jaski et al., 2009), suggesting that repairing SERCA2a expression may be a viable therapy. Moreover PLN ablation prevented HF in a mouse model of dilated cardiomyopathy caused by deficiency of the muscle-specific LIM protein (Arber et al., 1997; Minamisawa et al., 1999). In isolated human HF myocytes, gene therapy with antisense against PLN improved contractile and diastolic function (del Monte et al., 2002). In contrast, PLN ablation increased SR Ca^{2+} filling and contractility in mice with cardiomyopathy attributable to overexpression of CaMKII. This led to premature death and mitochondrial Ca^{2+} overload, suggesting that accelerating SR Ca^{2+} uptake and increasing SR Ca^{2+} load, is disadvantageous at least in the presence of excessive CaMKII activity (Zhang et al., 2010). These findings are consistent with the idea

already discussed for I/R: in the face of phosphorylated RyR2 channels, as is the case of CaMKII overexpressing mice, repletion of Ca^{2+} stores through PLN ablation could further worsen overall heart function, via mitochondrial Ca^{2+} loading, cell death, and arrhythmias.

CONCLUDING REMARKS

We have described that PLN and its CaMKII-dependent phosphorylation are part of a multimeric functionally coupled signaling complex, composed of SERCA, PLN, HAX-1, PKA, CaMKII, PP1, I-1, and Hsp20, which reversibly regulates SR Ca^{2+} cycling. Although CaMKII-dependent PLN phosphorylation contributes to β1-adrenergic mechanical response, its role is subordinate to the PKA-dependent increase in cytosolic Ca^{2+} and inhibition of phosphatase, necessary to activate CaMKII and phosphorylate Thr^{17} of PLN. These requirements are also achieved under different pathological situations, like acidosis and I/R, independent of PKA activation. Under these conditions, CaMKII-dependent PLN phosphorylation may paradoxically produce either favorable or harmful cardiac effects. The findings summarized in this review also suggest that the beneficial or detrimental effects associated with CaMKII activation and PLN phosphorylation depend on a tight balance between SR Ca^{2+} reuptake and leak, determined by the status/characteristics of other SR proteins, among which the RyR2 is a main candidate. A moderate or even high increase in SR Ca^{2+} uptake (and content) due to PLN phosphorylation, would enhance RyR2 opening due to the regulatory effect of intra-SR Ca^{2+}. However, in the absence of additional RyR2 modifications, the increased SERCA2a activity, produced by PLN phosphorylation, may cope with the enhanced diastolic SR Ca^{2+} release/leak. In contrast, even moderate increases in SR Ca^{2+} may increase diastolic SR Ca^{2+} release under conditions where RyR2 activity is altered independently of intra-SR Ca^{2+}-induced modifications, enhancing the propensity to arrhythmias and leading to mitochondrial Ca^{2+} overload, which favors apoptosis and necrosis. Thus, increasing SERCA2a activity by PLN phosphorylation seems to have the potential of producing salutary effects in a number of diseases, as long as these effects are achieved under conditions in which diastolic Ca^{2+} release is satisfactorily controlled. Future research in this area is needed to parse the contribution of different players involved in the balance/interaction between SR Ca^{2+} reuptake and leak, including the RyR2 regulators or the L-type Ca^{2+} channels. More specifically to the subject of this review, the recently described multimeric SERCA/PLN-ensemble may represent a nodal point in the interaction of several protein partners, regulating and modifying the fine-tuned control of Ca^{2+} cycling achieved by the duo SERCA-PLN. An intensive scrutiny of the various proteins of this new pathway will give new insights into their role in SR Ca^{2+} uptake control and may provide novel therapeutic avenues which can contribute to solve the abnormalities in Ca^{2+} handling underlying different pathological process.

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Ca^{2+} current facilitation is CaMKII-dependent and has arrhythmogenic consequences

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The cardiac voltage gated Ca^{2+} current (I_{Ca}) is critical to the electrophysiological properties, excitation-contraction coupling, mitochondrial energetics, and transcriptional regulation in heart. Thus, it is not surprising that cardiac I_{Ca} is regulated by numerous pathways. This review will focus on changes in I_{Ca} that occur during the cardiac action potential (AP), with particular attention to Ca^{2+}-dependent inactivation (CDI), Ca^{2+}-dependent facilitation (CDF) and how calmodulin (CaM) and Ca^{2+}-CaM dependent protein kinase (CaMKII) participate in the regulation of Ca^{2+} current during the cardiac AP. CDI depends on CaM pre-bound to the C-terminal of the L-type Ca^{2+} channel, such that Ca^{2+} influx and Ca^{2+} released from the sarcoplasmic reticulum bind to that CaM and cause CDI. In cardiac myocytes CDI normally pre-dominates over voltage-dependent inactivation. The decrease in I_{Ca} via CDI provides direct negative feedback on the overall Ca^{2+} influx during a single beat, when myocyte Ca^{2+} loading is high. CDF builds up over several beats, depends on CaMKII-dependent Ca^{2+} channel phosphorylation, and results in a staircase of increasing I_{Ca} peak, with progressively slower inactivation. CDF and CDI co-exist and in combination may fine-tune the I_{Ca} waveform during the cardiac AP. CDF may partially compensate for the tendency for Ca^{2+} channel availability to decrease at higher heart rates because of accumulating inactivation. CDF may also allow some reactivation of I_{Ca} during long duration cardiac APs, and contribute to early afterdepolarizations, a form of triggered arrhythmias.

Keywords: CaMKII, calcium channel, calcium current inactivation, calcium current facilitation, calcium current staircase

INTRODUCTION

The cardiac L-type Ca^{2+} channel (LTCC) current (I_{Ca}) is an important contributor to overall cardiac electrophysiology and arrhythmias, excitation-contraction coupling (ECC; it causes further intracellular Ca^{2+} release and activation of the myofilaments), mitochondrial energy regulation, cell death and transcriptional regulation (Bers, 2008). I_{Ca} is mainly via the Cav1.2 α1 LTCC isoform, although the Cav1.3 isoform is expressed in some atrial cells (especially pacemaker cells). That pore-forming α1 subunit also carries the intrinsic voltage-dependent gating properties (Perez-Reyes et al., 1989) and many key regulatory sites. However, the mature LTCC in heart is a complex containing also a β as well as an α2-δ subunit that influence LTCC trafficking and gating (Shirokov et al., 1998; Bichet et al., 2000; Wei et al., 2000; Dzhura and Neely, 2003). Cav1.2 has four major domains (I-IV), each of which contains six transmembrane segments (S1-S6), where positive charges in the S4 segments participate as voltage sensors and the S5-S6 loop is the locus of the ion-conducting pore (Bers, 2001).

The rapid upstroke or phase 0 of the cardiac action potential (AP) is driven by Na^+ current (I_{Na}) in most cardiac myocytes, and causes voltage-dependent activation of I_{Ca}. In pacemaker cells in the sino-atrial and atrio-ventricular node, it is I_{Ca} activation that is responsible for the rapid upstroke of the AP. I_{Ca} activation is a bit slower than I_{Na} activation, but starts early during the cardiac AP. The early repolarization phase of the AP (phase 1) can enhance I_{Ca} because of an increase in electrochemical driving force, i.e., membrane potential (E_m) is further from the Ca^{2+} equilibrium potential (E_{Ca}; Sah et al., 2002). However, both depolarization and the rise in local intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) begin the processes of voltage- and Ca^{2+}-dependent inactivation (VDI and CDI), which continues during the plateau phase of the AP (phase 2) causing a progressive decrease in I_{Ca}. As rapid and terminal AP repolarization ensue (phase 3) the LTCC undergoes de-activation, but then recovery from inactivation is both time and E_m-dependent. Thus, for LTCC to recover full availability between beats, some time must elapse and that recovery time depends on E_m (e.g., at −80 and −50 mV the time constant is about 100 and 400 ms, respectively).

I_{Ca} amplitude and gating properties are influenced by myriad regulatory pathways, but here we will focus on the Ca^{2+}-dependent mechanisms that shape the I_{Ca} occurring during the AP in ventricular myocytes. Hence, this review will describe how the Ca^{2+} sensing protein calmodulin (CaM) mediates CDI, and is involved in the activation of CaMKII, a serine/threonine-specific protein kinase which is a key mediator of ECC. Note that, although CaMKII activation can also be Ca^{2+}-independent (see accompanying article by Erickson, 2014), here we will focus

on the main activation mechanism, which is Ca^{2+}/CaM dependent. Moreover, the particular structure of this kinase (well described in this series by Pellicena and Schulman, 2014) confers to CaMKII the ability to integrate oscillatory Ca^{2+} signals, because CaMKII activity depends on both frequency and duration of previous Ca^{2+}/CaM pulses (De Koninck and Schulman, 1998; Saucerman and Bers, 2008). We will show how the CaMKII-dependent LTCC phosphorylation mediates the Ca^{2+}-dependent facilitation (CDF) of I_{Ca}, and how this process can eventually lead to E_m or Ca^{2+} instabilities in ventricular myocytes.

Ca^{2+}- vs. E_m-DEPENDENT INACTIVATION OF I_{Ca}

Inactivation of I_{Ca} is driven by VDI and CDI (Kass and Sanguinetti, 1984; Lee et al., 1985; Hadley and Hume, 1987). Several studies have shown that the Ca^{2+}-sensing protein CaM mediates CDI by interacting with the carboxyl tail of the LTCC α1 subunit (Zuhlke and Reuter, 1998; Peterson et al., 1999; Qin et al., 1999; Zuhlke et al., 1999; Pate et al., 2000), a cytoplasmic region that contains an EF-hand region and an IQ motif. At rest, CaM is pre-bound to the LTCC at or near the IQ motif (Erickson et al., 2001; Pitt et al., 2001). Upon I_{Ca} activation and consequent Ca^{2+} release from the sarcoplasmic reticulum (SR), local $[Ca^{2+}]_i$ rises, causing Ca^{2+} to bind to CaM and induce inactivation. The details of the CDI process are not totally resolved, and may involve multiple regions of the channel, including the I-II loop that is thought to be key for VDI (Kim et al., 2004; Cens et al., 2006). An intriguing new hypothesis has emerged from detailed studies from the Yue lab (Ben Johny et al., 2013). During diastole, the C-lobe of apoCaM (CaM without any Ca^{2+} bound) would be associated with the IQ domain, and its N-lobe associated with the pre-IQ domain (between the IQ locus and the upstream EF-hand domain). Ca^{2+} binding to the N-lobe of CaM (the faster, low-affinity site) would cause the N-lobe to shift and bind to part of the LTCC N-terminal domain (which they call the NSCaTE module), and thereby trigger N-lobe CDI. Then when Ca^{2+} also binds to the C-lobe of CaM (the higher affinity, slower binding lobe) the C-lobe shifts its binding from the IQ domain to a position just upstream of the Pre-IQ region where the N-lobe had been bound. If Ca^{2+} binds only to the C-lobe (e.g., if the N-lobe is unavailable) then the C-lobe does a similar sort of shift on its own, and mediates C-lobe CDI. For cardiac Cav1.2 channels, overall CDI and C-lobe-CDI are relatively similar, while N-lobe CDI alone was not apparent (Peterson et al., 1999). That differs from some neuronal P/Q, N or R type Ca^{2+} channels, where N-lobe CDI seems to be dominant (Liang et al., 2003).

Figure 1A shows I_{Ca} inactivation kinetics in a rabbit ventricular myocyte under different Ca^{2+} conditions. The time to half inactivation ($t_{1/2}$) increases from 17 to 37 ms when normal Ca^{2+} transients are abolished (e.g., by buffering the intracellular Ca^{2+} with 10 mM EGTA). Note that EGTA is a relatively slow buffer and cannot abolish very local $[Ca^{2+}]$ elevation around the mouth of the channel (although in this case SR Ca^{2+} release is prevented). In absence of extracellular Ca^{2+}, LTCC are permeable to Ba^{2+}, and this current (I_{Ba}) has been often studied to differentiate VDI and CDI (Lee et al., 1985; Peterson et al., 2000; Cens et al., 2006), despite a modest ability of Ba^{2+} to induce inactivation (Ferreira et al., 1997). When Ba^{2+} is the charge carrier (and intracellular Ca^{2+} is buffered), I_{Ba} inactivation is further slowed ($t_{1/2} = 161$ ms).

In the absence of divalent ionic species, LTCC is permeable to monovalent cations and is referred to as non-specific monovalent current (I_{NS}, mostly carried by Na^+ and Cs^+). I_{NS} inactivates only very slowly at this voltage at room temperature ($t_{1/2} > 500$ ms; **Figure 1A**), but exhibits VDI, which becomes faster at more positive voltages (Hadley and Hume, 1987; Grandi et al., 2010). I_{NS} inactivation is incomplete (after 500 ms) even at more positive E_m (**Figure 1B**). The additional I_{Ca} inactivation at intermediate E_m has an U-shaped E_m-dependence (as does inward I_{Ca} amplitude, maximal at about 0 mV), reflecting the contribution of CDI. Note that at +50–60 mV little Ca^{2+} enters during I_{Ca}, and the extent of I_{Ca} and I_{NS} inactivation is similar. It is tempting to speculate that I_{NS} inactivation properties might provide pure VDI characteristics that are relevant for I_{Ca}. However, I_{NS} can actually inactivate faster than I_{Ba} at positive voltages, so we think that using I_{NS} to assess VDI characteristics for I_{Ca} is likely to be invalid (Grandi et al., 2010). However, I_{Ba} inactivation is also not purely VDI, because inactivation is I_{Ba}-amplitude dependent (Brunet et al., 2009) and Ba^{2+} can partially substitute for Ca^{2+} in CDI (Ferreira et al., 1997). To resolve this we have attempted to carefully account for the weak Ba^{2+}-dependent inactivation and refine the characteristics of VDI vs. CDI in cardiac myocytes in a computational analysis (Morotti et al., 2012). That is, most prior work using I_{Ba} to characterize VDI had slightly overestimated VDI. This is certainly not meant to discourage the use of I_{Ba} vs. I_{Ca} as a means to study CDI, just that this I_{Ba} is not entirely devoid of divalent-dependent inactivation.

Given the role of I_{Ca} in sustaining the AP plateau, CDI and VDI are important determinant for AP duration (APD) regulation. Inhibition of I_{Ca} inactivation induces AP prolongation, and has pro-arrhythmic consequences (see section "Arrhythmogenic consequences of CaMKII-dependent I_{Ca} effects"). For example, impaired VDI has been observed in Timothy syndrome (Splawski et al., 2004, 2005; Brunet et al., 2009), an inherited disease characterized by severe ventricular arrhythmias and sudden cardiac death. The expression of mutant Ca^{2+}-insensitive CaM (via adenovirus) in adult guinea-pig cardiomyocytes also prevents CDI and causes dramatic AP prolongation (Alseikhan et al., 2002). Moreover, some human patients with arrhythmias resembling long QT syndrome have linked mutations in the Ca^{2+} binding domains in one of the three CaM genes (which otherwise encode the identical CaM protein; Crotti et al., 2013). A loss of CDI also characterizes the more common pathologic condition of heart failure (HF), where marked AP prolongation and associated defective Ca^{2+} cycling have been reported (Beuckelmann et al., 1992). It is interesting to note that, at first, the down-regulation of repolarizing K^+ currents (I_{to} and I_{K1}) was thought to be responsible for the increased APD seen in HF. Only in the late 1990s the pivotal role of CDI became clear, when it was first proposed in a theoretical study in dog (Winslow et al., 1999), and then experimentally observed in a guinea pig model of HF (Ahmmed et al., 2000). So clearly defective I_{Ca} CDI can be arrhythmogenic in people.

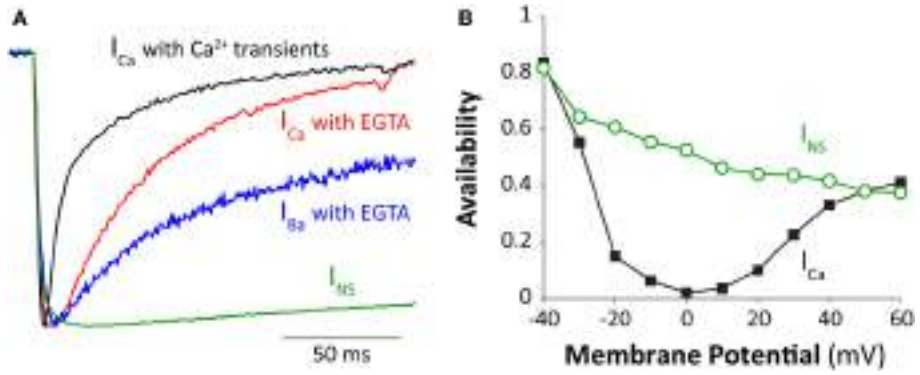

FIGURE 1 | Inactivation of cardiac Ca^{2+} channel. (A) Normalized I_{Ca}, I_{Ba}, and I_{NS} elicited by a square voltage pulse at room temperature to 0 mV (except I_{NS} at −30 mV to obtain comparable activation state). I_{Ca} was recorded under both perforated patch (where normal SR Ca^{2+} release and Ca^{2+} transients occur) and ruptured patch conditions with cells dialyzed with 10 mM EGTA (to prevent global Ca^{2+} transients). I_{Ba} was also recorded with ruptured patch (with 10 mM EGTA in the pipette). Extracellular [Ca^{2+}] and [Ba^{2+}] were both 2 mM and I_{NS} was measured in divalent-free conditions (10 mM EDTA inside and out) with extracellular [Na^+] at 20 mM and intracellular [Na^+] at 10 mM. Peak currents were 1370, 808, 780, and 5200 pA and were attained at 5, 7, 10, and 14 ms for I_{Ca} (perforated), I_{Ca} (ruptured), I_{Ba} and I_{NS} respectively, with $t_{1/2}$ of current decline of 17, 37, 161, and > 500 ms respectively. **(B)** Amplitude of I_{NS} and I_{Ca} through LTCC (at −10 mV) after 500 ms pulses to the indicated E_m in guinea-pig ventricular myocytes (modified from Bers, 2001 with permission, data from Hadley and Hume, 1987).

I_{Ca} DURING THE AP CHANGES WITH INCREASING FREQUENCY AND Ca^{2+} LOADING

APD regulation is fundamental to control the Ca^{2+} level in myocytes, which is functionally important with respect to the Ca^{2+} requirements for myofilament activation, and thus contractility. Indeed, CDI is a physiological negative feedback mechanism that limits excessive Ca^{2+} entry in myocytes. When the myocyte has relatively high Ca^{2+} load, a large Ca^{2+} transient enhances I_{Ca} inactivation (limiting further Ca^{2+} influx). Conversely, when myocyte Ca^{2+} is low and SR Ca^{2+}release is small, there is less CDI and enhanced Ca^{2+} entry that increases intracellular Ca^{2+} content (Puglisi et al., 1999; Eisner et al., 2000; Bers and Grandi, 2009). Notably, Na^+/Ca^{2+} exchange also participates in this negative feedback (i.e., higher Ca^{2+} transients limit Ca^{2+} entry and increase Ca^{2+} extrusion from the myocyte via Na^+/Ca^{2+} exchange).

The time course of I_{Ca} during the AP is significantly different compared to that seen during a square voltage pulse [**Figure 2A**, rabbit ventricular myocyte, 25°C, with 10 mM EGTA to prevent Ca^{2+} transients (Yuan et al., 1996)]. Peak I_{Ca} during the AP is lower and occurs later than during a square pulse, with larger I_{Ca} late in the AP. The later I_{Ca} peak is because at the AP peak (+50 mV) Ca^{2+} channels activate rapidly, but the driving force for Ca^{2+} (E_m–E_{Ca}) is initially low, because E_m is close to the reversal potential for I_{Ca} (E_{Ca} ∼ +60 mV). As E_m repolarizes, the driving force increases faster than channel inactivation, producing a larger current at later times during the AP (Sah et al., 2002). Sipido et al. (1995) first investigated how Ca^{2+} released from the SR modulates I_{Ca} performing "classic" voltage-clamp experiments, and observed that CDI increases as SR Ca^{2+} release gets larger. Our group confirmed this observation in a more "physiological" condition, as shown in **Figure 2B**, where repeated AP-clamps are performed as the SR Ca^{2+} stores are reloaded, such that contractions get progressively larger (beat 1–10; Puglisi et al., 1999). One can see the contribution of SR Ca^{2+} release to CDI as the Ca^{2+} transients and contractions get larger. Integration of the Ca^{2+} influx via I_{Ca} during these ten pulses (which approach the steady state) shows that the I_{Ca}-dependent influx decreases from 12 to 6 μmol/L cytosol, indicating that I_{Ca} inactivation due to SR Ca^{2+} release decreases net Ca^{2+} influx by about 50%. These experiments were done at both 25 and 35°C. At 35°C peak I_{Ca} occurs earlier and is higher, but also inactivates faster and the AP duration is also shorter. The net result is that there is very little difference between these temperatures for the integral of Ca^{2+} influx during the AP (with SR Ca^{2+} release fully functional).

Using a combination of AP and square voltage-clamp protocols, Linz and Meyer (1998) assessed the time-course of I_{Ca} inactivation during the AP in different Ca^{2+} homeostasis conditions. Their analysis pointed out that, in physiological condition, CDI is the overwhelmingly dominant inactivation on the time scale of an AP, as recapitulated in the theoretical study by Greenstein and Winslow (2002). Moreover, Linz and Meyer (1998) showed that CDI is mostly controlled by Ca^{2+} released from the SR during the initial part of the AP, then by Ca^{2+} entered through the LTCCs. These results are well described by our recent computational study that updated the balance of VDI and CDI in the context of a detailed Ca^{2+} cycling electrophysiological myocyte model (Morotti et al., 2012).

At increased heart rates, there is typically an increase in Ca^{2+} transient amplitude (known sometimes as the positive force-frequency relationship) in normal hearts in species other than rat and mouse (Bers, 2001). The higher Ca^{2+} transients also typically decline faster at high heart rates (known a frequency-dependent acceleration of relaxation; Bers, 2001). Thus, I_{Ca} inactivation is expected to be faster, based on the above discussion. The higher heart rate could also shorten the diastolic interval and increase diastolic $[Ca^{2+}]_i$, which might reduce I_{Ca} availability.

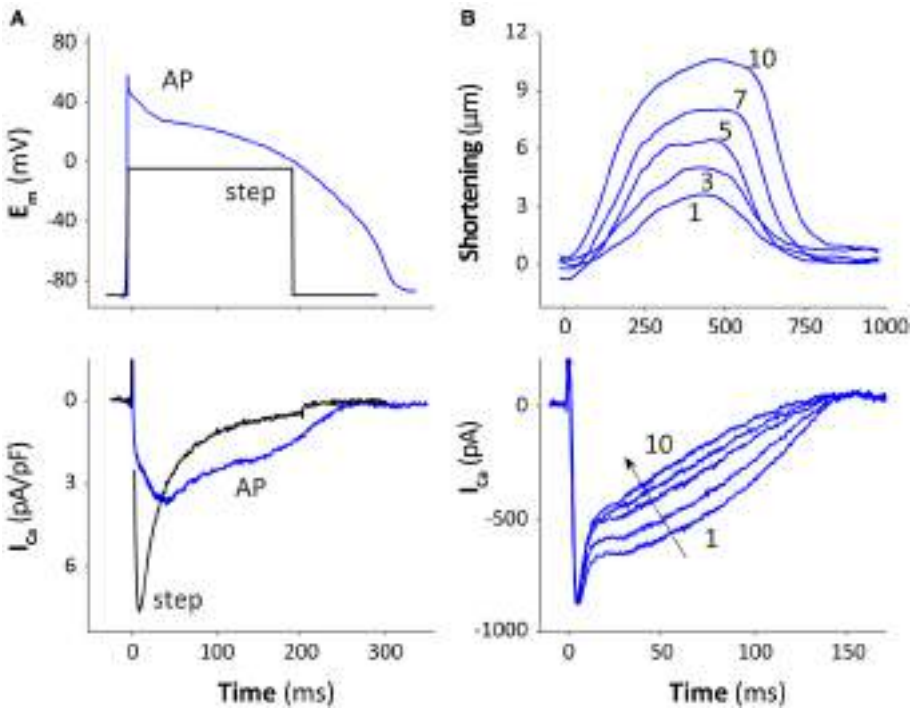

FIGURE 2 | I_{Ca} inactivation during the AP. (A) Rabbit ventricular myocytes (at 25°C) were voltage-clamped with either a square voltage step or an AP waveform (measured from 5 other cells under physiological conditions). All other currents were blocked, e.g., by replacement of K^+ with Cs^+ and Na^+ with TEA (inside and out) and cells were dialyzed with 10 mM EGTA to prevent Ca^{2+} transients (data from Yuan et al., 1996, modified from Bers, 2001 with permission). **(B)** After SR Ca^{2+} was depleted by a brief caffeine-application (with Na^+), a series of AP-clamps were given, and contraction and I_{Ca} recovered to steady state over 10 sequential pulses at 25°C in rabbit ventricular myocyte (modified from Bers, 2001 with permission, data from Puglisi et al., 1999).

Indeed, while I_{Ca} recovery from inactivation is classically time and E_m-dependent (Hadley and Hume, 1987), we showed that elevations of $[Ca^{2+}]_i$ could slow recovery from inactivation, especially under conditions where SR Ca^{2+} uptake is depressed and diastolic E_m is slightly depolarized (Altamirano and Bers, 2007), as can be the case in human HF (Sipido et al., 1998). This sort of diastolic $[Ca^{2+}]_i$ effect on LTCC availability is probably of only minor relevance under normal physiological conditions and heart rates in healthy hearts, but may be more of a factor under pathophysiological conditions. That is, in HF there is an increased likelihood that peak I_{Ca} will decrease at high heart rates, and that might contribute to limiting the more negative force-frequency relationship observed in HF (Sipido et al., 1998).

I_{Ca} FACILITATION IS CaMKII-DEPENDENT

Ca^{2+}-DEPENDENT FACILITATION OF I_{Ca}: EARLY FUNCTIONAL CHARACTERISTICS

Several early studies reported progressive increases in I_{Ca} amplitude and prominent slowing of inactivation that was observed during increased frequency of voltage-clamp pulses from physiological holding potentials (∼ −80 mV), as shown in the example in **Figure 3** (Lee, 1987; Boyett and Fedida, 1988; Tseng, 1988; Hryshko and Bers, 1990). This phenomenon is not reproduced if holding E_m is more depolarized (e.g., −40 mV) where a negative staircase is observed, or in the absence of Ca^{2+} (e.g., when Ba^{2+} is the charge carrier). This I_{Ca} staircase was also stronger when local Ca^{2+} influx was amplified by SR Ca^{2+} release. Thus, this phenomenon is termed Ca^{2+}-dependent facilitation of I_{Ca}.

CDF and CDI co-exist under physiological conditions, and this may be why I_{Ca} facilitation was masked by holding E_m near −40 mV. That is, recovery from inactivation at that E_m is slow, so the records were dominated by a negative I_{Ca} staircase that was attributable to CDI and incomplete I_{Ca} recovery from inactivation. It has been proposed that the facilitatory mechanism may partly offset reduced Ca^{2+} channel availability at high heart rates (caused by direct CDI), contributing to improving cardiac performance during exercise (Ross et al., 1995). While CDI responds rapidly (in response to local $[Ca^{2+}]_i$ during the same beat), CDF occurs more slowly (over several beats). Indeed, biphasic effects of $[Ca^{2+}]_i$ on unitary I_{Ca} have been reported (Hirano and Hiraoka, 1994). Some studies even claimed that progressive decrease in SR Ca^{2+} release (negative staircase in rat) and CDI are responsible for the observed CDF (Guo and Duff, 2003, 2006). However, because CDF is quite similar in species that exhibit positive Ca^{2+} transients staircases and even when SR Ca^{2+} release is blocked this seems unlikely to be the case (Hryshko and Bers, 1990).

CDF IS CaMKII-DEPENDENT: MECHANISTIC STUDIES

About 20 years ago three groups independently demonstrated that Ca^{2+}-dependent I_{Ca} facilitation is mediated by CaMKII-dependent phosphorylation of LTCC (Anderson et al., 1994; Xiao

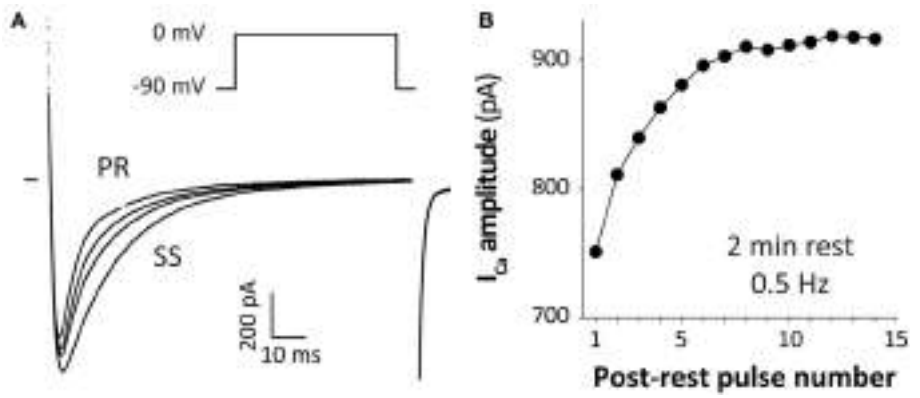

FIGURE 3 | Ca^{2+}-dependent facilitation of I_{Ca}. I_{Ca} at 0.5 Hz (from −90 to 0 mV) after a 2 min rest period in a ferret ventricular myocyte. The first post-rest pulse (PR) and the second, third and steady state (SS) pulses are shown in **(A)** and the whole post-rest I_{Ca} "staircase" in **(B)** (modified from Bers, 2001 with permission, data from Hryshko and Bers, 1990).

et al., 1994; Yuan and Bers, 1994). Xiao et al. (1994) also observed that sarcolemmal CaMKII activation correlates qualitatively with the changes in I_{Ca}. All three studies reported that pharmacological inhibition of CaMKII abolishes CDF in mammalian cardiomyocytes (**Figures 4A–D**). Anderson's group extended this work by characterizing the CaMKII-dependent effect on single channel I_{Ca} recorded in excised inside-out patches (Dzhura et al., 2000). They showed that addition of activated CaMKII to the cytoplasmic side of the sarcolemma results in phosphorylation of the LTCC complex, inducing high-activity (mode 2) gating that is characterized by long frequent openings (**Figures 4E,F**), consistently with the macroscopic effect of CDF.

Since CDF is observed when cells are dialyzed with 10 mM EGTA (but is abrogated by 20 mM BAPTA), the active CaMKII must be highly localized near the channels (Hryshko and Bers, 1990). Although the CaMKII-dependent phosphorylation of LTCC has been studied for a long time, the molecular bases of this phenomenon are not still completely understood. In particular, it is debated which LTCC subunit is involved, since multiple candidate phosphorylation sites have been identified in both the pore-forming α1C subunit and the auxiliary β2 subunit (Sun and Pitt, 2011).

Some early studies suggested that the IQ motif on the α1C subunit is involved in CDF (Wu et al., 2001). Wu et al. showed that in rabbit ventricular myocytes I_{Ca} facilitation could be nearly abolished by the CaMKII inhibitory peptide AC3-I, but could then be rescued by cell dialysis with a peptide resembling the Ca^{2+} channel IQ domain, called "IQ-mimetic peptide." This may also relate to early studies of CDI with wild-type and mutant α1C in Xenopus oocytes, where it was found that isoleucine point mutations in the IQ domain could either enhance (Ile to Ala) or abolish (Ile to Glu) CDF (Zuhlke et al., 1999).

More recent studies in heterologous cells indicate that CaMKII may directly bind and phosphorylate the α1C subunit. In oocytes CaMKII could phosphorylate the α1C subunit (Hudmon et al., 2005). Hudmon et al. (2005) also showed that tethering of CaMKII to the Cav1.2 C-terminus is an essential molecular feature of CDF, because mutations to a putative C-terminus binding site prevent CDF. Other recent studies support the idea of CaMKII-dependent phosphorylation of the pore-forming α1C subunit, and propose possible phosphorylation sites. Erxleben et al. (2006) studied the increase in mode 2 activity of rabbit Cav1.2 channels seen in neurons in two pathologic conditions of cyclosporin neurotoxicity and Timothy syndrome. They found that mode 2 activity increases through a CaMKII-dependent mechanism involving respectively Ser-1517 (at the end of the S6 helix in domain IV), and Ser-439 (at the end of the S6 helix in domain I). Wang et al. (2009) expressed guinea pig Cav1.2 channel in Chinese hamster ovary, and found that CaMKII phosphorylates Thr-1603 residue (Thr-1604 in rabbit) within the pre-IQ region in the C-terminal tail of the Cav1.2 channel. In HEK cells I_{Ca} facilitation was decreased by the single mutations (to Ala) in Ser-1512 and Ser-1570 (two serines that flank the C-terminal EF-hand motif), and abolished by the double mutation S1512A/S1570A (Lee et al., 2006). Furthermore, Blaich et al. (2010) observed impaired I_{Ca} facilitation in mice with knockin mutations at the Ser-1512 and Ser-1570 (to Ala) phosphorylation sites, and confirmed that Cav1.2 channel is modulated by CaMKII-dependent phosphorylation in the murine heart.

In contrast to that data implicating sites on the pore-forming α1 subunit, other results point to CaMKII-dependent phosphorylation of regulatory β subunits. In particular, it was reported that CDF is mediated by phosphorylation of the β2a subunit, at Thr-498 in isolated adult rat (Grueter et al., 2006) and rabbit (Koval et al., 2010) ventricular myocytes. Grueter et al. (2006) first investigated whether, and in which conditions, CaMKII can directly bind to a β2a subunit (expressed as a glutathione S-transferase, GST, fusion protein). They found such high affinity binding when CaMKII was in the active (i.e., autophosphorylated) state. By screening a library of GST-fusion proteins, they identified the β2a region that bound to CaMKII, and verified that CaMKII would phosphorylate this region. Among the different possible phosphorylation sites present in this region, only the mutation of Thr-498 to Ala (T498A) impaired CaMKII-phosphorylation. Expressing T498A β2a with Cav1.2 in tsA201 cells resulted in impaired CaMKII-dependent increase in channel

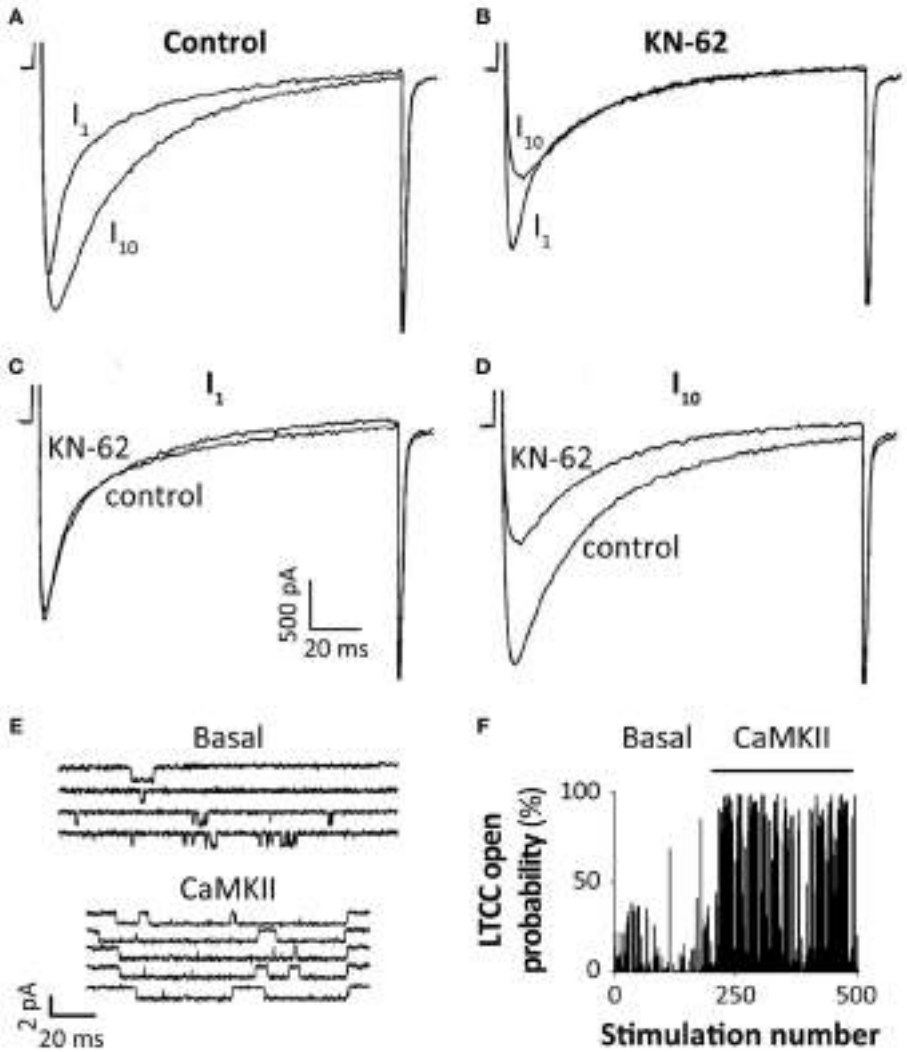

FIGURE 4 | CaMKII-dependent regulation of I_{Ca}. Superimposed I_{Ca} traces from the first (I_1) and tenth (I_{10}) voltage-clamp pulse from −90 to 0 mV at 2 Hz in a single rabbit ventricular myocyte obtained in control condition **(A)** or after 10 min equilibration with the CaMKII inhibitor KN-62 (1 μM) **(B)**; I_1 and I_{10} obtained in the two conditions are respectively shown (superimposed) in panels **(C,D)** (modified from Yuan and Bers, 1994 with permission). **(E)** A single LTCC current (channel openings are seen as downward deflections from baseline) is elicited by repetitive depolarizing voltage-clamp steps (from −70 to 0 mV) and reveals infrequent, brief openings under basal conditions (upper panel). CaMKII (bottom) causes frequent and prolonged LTCC openings compared with baseline. Panel **(F)** shows that the probability of LTCC opening during a depolarizing voltage-clamp step is dramatically increased upon addition of CaMKII, compared with basal conditions (modified from Anderson, 2004 with permission, data from Dzhura et al., 2002).

open probability, and ablation of CaMKII-mediated whole cell I_{Ca} facilitation has been observed in rat cardiomyocytes (Grueter et al., 2006). It was also shown that Leu-493 present in the β2a and β1a (but not present in β3 and β4) subunits was important for high affinity CaMKII binding, and that mutation of Leu-493 to Ala (L493A) substantially reduced CaMKII binding, but did not interfere with β2a phosphorylation at Thr-498 (Grueter et al., 2008; Abiria and Colbran, 2010). Other studies have shown that overexpression of β2a, which can dramatically increase I_{Ca}, causes cellular Ca^{2+} overload, and facilitates arrhythmogenesis, apoptosis and hypertrophic signaling (Chen et al., 2005; Koval et al., 2010; Chen et al., 2011). Koval et al. (2010) showed that prevention of intracellular Ca^{2+} release by ryanodine, by inhibition of CaMKII activity, or expression of β2a T498A or L493A mutants could reduce Ca^{2+} entry and improved cell survival.

Despite much effort aimed at the detailed molecular mechanism for CaMKII-dependent I_{Ca} facilitation, more work will be required to develop a fully satisfying explanation. It may be that sites on both the α and β subunit are important, that the α-β subunit interaction is critical, and there may also be more than one CaMKII binding domain and phosphorylation target. The CaM involved in activating the CaMKII that is associated with the LTCC seems unlikely to be the same CaM that is involved in CDI, since that CaM appears dedicated and bound strongly even at low $[Ca^{2+}]_i$ not to fully dissociate from the CDI regulatory sites.

ARRHYTHMOGENIC CONSEQUENCES OF CaMKII-DEPENDENT I_{Ca} EFFECTS

CaMKII-dependent modulation of I_{Ca} is characterized by both increased current amplitude and slowed inactivation, and can result in an overall increase in Ca^{2+} entry, which can be pro-arrhythmic. Intracellular Ca^{2+} overload is associated with increased propensity of spontaneous SR Ca^{2+} release, which can lead to delayed afterdepolarizations (DADs) because of the transient inward current carried by the Na^+/Ca^{2+} exchanger (in the Ca^{2+} extrusion mode). In a theoretical study (Morotti et al., 2012), we also showed that, when CDI is dramatically impaired, the same mechanism can be responsible for the development of early afterdepolarizations (EADs) during the prolonged AP plateau. It has also been shown that the CaMKII-dependent shift of LTCC into mode 2 gating can explain the global I_{Ca} facilitation typically measured (Hashambhoy et al., 2009). That group also showed that higher mode 2 activity can favor the development of EADs because of I_{Ca} reactivation during the AP plateau (Tanskanen et al., 2005; Hashambhoy et al., 2010). For a further detailed review about mathematical modeling of CaMKII-mediated regulation of LTCC see the accompanying article in this series by Greenstein et al. (2014).

Studying different conditions in which the AP is forcibly prolonged, Anderson's group obtained the first experimental evidence for the role of CaMKII in the development of afterdepolarizations in rabbit ventricular myocytes. They showed that the development of EADs (due to I_{Ca} reactivation during the prolonged plateau) is prevented by CaMKII inhibition (with KN-93 or AC3-I) (Anderson et al., 1998; Wu et al., 1999a), and that AC3-I also prevents the development of DADs caused by the increased Na^+/Ca^{2+} exchanger current (Wu et al., 1999b). They observed the development of EADs due to CaMKII-dependent enhancement of LTCC open probability in a transgenic mouse model of cardiac hypertrophy as well (Wu et al., 2002). This model, together with increased CaMKII, showed an increased propensity for ventricular arrhythmias, which can be prevented by CaMKII-inhibition. Increased CaMKII levels have been observed also in a murine model of pressure overload HF (Wang et al., 2008). In this model, CaMKII-dependent activation of I_{Ca} is already maximal and CDF cannot be induced, suggesting an important role of CaMKII in remodeling in failing myocytes.

It is now well known that CaMKII is hyperactive in several forms of cardiac diseases (Anderson et al., 2011; Swaminathan et al., 2012; Vincent et al., 2014), and interesting insights about I_{Ca} modulation have been provided by studies on animal models in which CaMKII is overexpressed or inhibited. Both chronic CaMKII overexpression in transgenic mouse myocytes and acute overexpression in rabbit myocytes cause increase in I_{Ca} amplitude and slowing in inactivation (consistent with CDF), and I_{Ca} could be reduced back to control levels by blocking CaMKII with KN-93 or AIP (Maier et al., 2003; Kohlhaas et al., 2006). Conversely, two different mouse models with CaMKII inhibition (Zhang et al., 2005; Picht et al., 2007) are characterized by complete inhibition of I_{Ca} facilitation. Notably, Picht et al. used a CaMKII inhibitory peptide (AIP) genetically targeted to the SR, consistent with the notion that CaMKII involved in I_{Ca} facilitation being localized at junctions between the SR and sarcolemma. Interestingly, Xu et al. (2010) showed that I_{Ca} facilitation was significantly reduced in a CaMKII-knockout mouse model. They also found an increase in Cav1.2 expression, which may be due to a compensatory mechanism for the reduced CaMKII-dependent facilitation over the long-term CaMKII inhibition.

In fact, other studies suggest that CaMKII activity can influence LTCC expression (Meffert et al., 2003; Shi et al., 2005; Ishiguro et al., 2006), based on the evidence that CaMKII phosphorylates the nuclear factor-kappaB (NFkB) component p65, causing its nuclear translocation, and consequent release of NFkB-dependent inhibition of Cav1.2 channel expression. Xu et al. (2010) found a significant reduction of p65 nuclear translocation in their transgenic myocytes.

Beyond LTCC, CaMKII influences many other targets within the cell (Bers and Grandi, 2009), many of which play important roles in modulating the cardiac ECC. An accurate analysis of the arrhythmogenic consequences of CaMKII-dependent LTCC phosphorylation cannot neglect, among the various targets, the effects on phospholamban (PLB) and ryanodine receptors (RyRs). CaMKII phosphorylation of PLB releases its inhibition on Ca^{2+}-sensitivity of SR Ca^{2+} pump (Simmerman and Jones, 1998), thus causing an increase in the pump affinity for Ca^{2+}. When RyRs are phosphorylated, their sensitivity for cytosolic Ca^{2+} (Li et al., 1997; Wehrens et al., 2004) and passive leak (Ai et al., 2005; Guo et al., 2006) are enhanced. Thus, consequences of CaMKII-dependent phosphorylation of RyRs and PLB are increased SR Ca^{2+} uptake and release, resulting in an increase in Ca^{2+} transient amplitude, which further activates CaMKII, and this can have arrhythmogenic consequences. Integrated mathematical models have been helpful in quantitatively understanding the complex interactions among these players. Soltis and Saucerman (2010) demonstrated the key role of RyR phosphorylation in the prominent positive feedback that associates the CaMKII-dependent increase in Ca^{2+} signal to a further increase in CaMKII activity. They also showed that the CaMKII-Ca^{2+}-CaMKII feedback is enhanced by β-adrenergic stimulation (which further enhances Ca^{2+} signal). We recently extended their work, by studying the synergy of Na^+ handling with Ca^{2+} and CaMKII signaling, since CaMKII hyperactivity in HF has also been associated with late I_{Na} and intracellular $[Na^+]$ ($[Na^+]_i$) overload (Wagner et al., 2006; Grandi and Herren, 2014). We found that a significant gain in $[Na^+]_i$ ($\sim$ 3–4 mM), which is what happens in HF (Despa et al., 2002), induces an increase in Ca^{2+} and consequent Ca^{2+}-dependent CaMKII activation, which in turn enhances Na^+ and Ca^{2+} signals, leading to a pro-arrhythmic condition. We also showed that, in condition of CaMKII overexpression, the CaMKII-Na^+-Ca^{2+}-CaMKII feedback is predominant, and leads to a hyper-phosphorylation of RyRs responsible for spontaneous SR Ca^{2+} release and DADs development (Morotti et al., 2014).

CONCLUDING REMARKS

CaMKII has numerous targets in cardiac myocytes, and we must assume that under normal physiological conditions this orchestrates a response that is acutely adaptive. However, when CaMKII becomes chronically activated in disease, by autophosphorylation and oxidation (Anderson et al., 2011;

Swaminathan et al., 2012), O-GlcNAcylation (Erickson et al., 2013) or possibly nitrosylation (Gutierrez et al., 2013), these regulatory systems may become maladaptive. The key CaMKII-dependent regulation of LTCC is I_{Ca} facilitation, a moderate increase in I_{Ca} amplitude and slowing of I_{Ca} inactivation in response to changes in heart rate. It seems likely that I_{Ca} facilitation is a normal adaptation to increased heart rate, to ensure Ca^{2+} channel availability and the integrity of ECC (which might otherwise be depressed by CDI or encroachment into recovery from inactivation). However, when this system is chronically on in pathological states it may contribute to inappropriate Ca^{2+} loading of the myocytes, and contribute to worsening pathology via poor diastolic function or arrhythmias triggered by EADs or DADs, altered I_{Ca} restitution or cardiac alternans. The detailed molecular mechanisms remain to be fully resolved, but work over the past 10–20 years has paved the way for further clarification in the near future.

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Modeling CaMKII-mediated regulation of L-type Ca^{2+} channels and ryanodine receptors in the heart

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Excitation-contraction coupling (ECC) in the cardiac myocyte is mediated by a number of highly integrated mechanisms of intracellular Ca^{2+} transport. Voltage- and Ca^{2+}-dependent L-type Ca^{2+} channels (LCCs) allow for Ca^{2+} entry into the myocyte, which then binds to nearby ryanodine receptors (RyRs) and triggers Ca^{2+} release from the sarcoplasmic reticulum in a process known as Ca^{2+}-induced Ca^{2+} release. The highly coordinated Ca^{2+}-mediated interaction between LCCs and RyRs is further regulated by the cardiac isoform of the Ca^{2+}/calmodulin-dependent protein kinase (CaMKII). Because CaMKII targets and modulates the function of many ECC proteins, elucidation of its role in ECC and integrative cellular function is challenging and much insight has been gained through the use of detailed computational models. Multiscale models that can both reconstruct the detailed nature of local signaling events within the cardiac dyad and predict their functional consequences at the level of the whole cell have played an important role in advancing our understanding of CaMKII function in ECC. Here, we review experimentally based models of CaMKII function with a focus on LCC and RyR regulation, and the mechanistic insights that have been gained through their application.

Keywords: cardiac myocyte, excitation-contraction coupling, cell signaling, CaMKII, computational modeling

INTRODUCTION

Cardiac electrophysiology is a discipline with a rich and deep history dating back more than a half-century. Much of our understanding of fundamental biological mechanisms in this field comes from experimental research coupled with integrative mathematical modeling. The goal of this modeling has been to achieve a quantitative understanding of the functional mechanisms and relationships that span from the level of molecular structure and function to integrated cardiac myocyte behavior in health and disease. Advances in computational techniques and hardware have led to the recent development of many sophisticated large-scale models of heart tissue, in which these electrophysiological myocyte models are the fundamental building blocks. Some of the most fundamental advances in computational cell biology, including formulation of dynamic models of voltage-gated ion channels, mitochondrial energy production, membrane transporters, intracellular Ca^{2+} dynamics, and signal transduction pathways have emerged from this field. One such signaling pathway is that involving the Ca^{2+}/calmodulin (CaM)-dependent protein kinase (CaMKII), which targets and functionally regulates a number of proteins that are central to cardiac excitation-contraction coupling (ECC), and has been identified as a promising new target for antiarrhythmic therapy in patients with heart failure (Rokita and Anderson, 2012). This review focuses on the role of experimentally based models of CaMKII function with a focus on L-type Ca^{2+} channel (LCC) and ryanodine receptor (RyR) regulation, and the mechanistic insights that have been gained through their application.

The nature of ECC is linked closely to the micro-anatomical structure of the cell. Sarcomeres, the basic units of contractile proteins, are bounded on both ends by the t-tubular system (Soeller and Cannell, 1999; Bers, 2001). The t-tubules extend deep into the cell and approach the sarcoplasmic reticulum (SR), an intracellular luminal organelle involved in the uptake, sequestration and release of Ca^{2+}. The junctional SR (JSR) is the portion of the SR most closely approximating (within 12–15 nm Franzini-Armstrong et al., 1999) the t-tubules. The close proximity of these two structures forms a microdomain known as the dyad. RyRs are Ca^{2+}-sensitive Ca^{2+}-release channels which are preferentially located in the dyadic region of the JSR membrane. In addition, LCCs are preferentially located within the dyadic region of the t-tubules, where they are in close opposition to the RyRs. The process by which Ca^{2+} enters the myocyte during the initial depolarization stages of the action potential (AP) via voltage-gated LCCs and triggers the opening of RyRs, and hence JSR Ca^{2+} release, is known as Ca^{2+}-induced Ca^{2+} release (CICR) and comprises the initial transduction event of ECC. Recent imaging studies have demonstrated that dyadic clefts are small, where junctions contain an average of 14 RyRs, with some clusters incompletely filled with RyRs, and with a large fraction of clusters closely spaced within 20–50 nm, suggesting that smaller clusters may act together to function as a single site of CICR (Baddeley et al., 2009; Hayashi et al., 2009).

The elucidation of CICR mechanisms became possible with the development of experimental techniques for simultaneous measurement of L-type Ca^{2+} current (I_{CaL}) and Ca^{2+} transients,

and detection of local Ca^{2+} release events known as Ca^{2+} sparks (Cheng et al., 1993). The evidence for tight regulation of SR Ca^{2+} release by triggering LCC current (for a review see Soeller and Cannell, 2004) gave rise to and later verified the local control theory of ECC (Stern, 1992). The mechanism of local control predicts that tight regulation of CICR is achieved because LCCs and RyRs are regulated by local dyadic Ca^{2+}. It is now widely accepted that CaMKII, a ubiquitous Ca^{2+}-dependent protein that can become highly activated in the dyad (Currie et al., 2004; Hudmon et al., 2005; Maier, 2005), can modulate CICR via phosphorylation of a number of ECC proteins including LCCs and RyRs (Maier and Bers, 2007). CaMKII modulates LCCs via a Ca^{2+}-dependent positive-feedback regulatory mechanism known as I_{CaL} facilitation (Anderson et al., 1994; Xiao et al., 1994; Yuan and Bers, 1994). This is observed as a positive staircase in current amplitude in combination with a slower rate of I_{CaL} inactivation upon repeated membrane depolarization. Although multiple mechanisms have been suggested to underlie I_{CaL} facilitation, experiments from Dzhura et al. (2000) demonstrated that CaMKII-dependent I_{CaL} facilitation alters the behavior of LCCs such that high activity gating modes with prolonged open times are more likely to occur. The specific functional effects of CaMKII phosphorylation of RyRs remain controversial. CaMKII has been shown to increase (e.g., Wehrens et al., 2004) or decrease (e.g., Lokuta et al., 1995) the channel open probability. Inconsistencies in experimental findings regarding the role of CaMKII on RyR function may not be surprising given the methodological differences between the studies. In addition, the high sensitivity of RyR gating to both cytosolic and SR Ca^{2+} makes it difficult to interpret experiments in which these Ca^{2+}concentrations are not tightly controlled. In one well designed study, Guo et al. (2006) developed an experimental protocol that overcame this challenge and provided strong evidence that CaMKII mediated phosphorylation of RyRs increases channel open probability. Such an increase is also consistent with transgenic studies of CaMKII overexpression which report increased Ca^{2+} spark frequency in response to elevated CaMKII activity (Maier et al., 2003; Kohlhaas et al., 2006).

The CaMKII holoenzyme exists as an elaborate macromolecular complex consisting of two stacked ring-shaped hexamers (Hudmon and Schulman, 2002). Each of the holoenzyme's 12 subunits can be activated through the binding of Ca^{2+}-bound CaM (Ca^{2+}/CaM) to the CaMKII regulatory domain in response to beat-to-beat transient increases of intracellular Ca^{2+} concentration. In cardiac myocytes, an activated CaMKII molecule can undergo autophosphorylation by neighboring subunits at threonine amino acid residues in its regulatory domain, which allows the kinase to retain activity even upon dissociation of Ca^{2+}/CaM (Meyer et al., 1992). An alternative mechanism of oxidative CaMKII activation has also been identified (Erickson et al., 2008). As a result of the fact that CaMKII regulates multiple protein targets (both directly and indirectly involved in ECC), and that the effect of CaMKII mediated phosphorylation on any particular target protein may involve complex alterations in biophysical function (e.g., mode-switching behavior of LCCs), the task of elucidating its role in cardiac function in both normal and failing hearts continues to present great challenges and cannot be accomplished via experiments alone. Some recent advances in understanding the mechanisms of CaMKII-dependent function in cardiac myocytes have been discovered by coupling mathematical models to experimental observations. In this review we will focus on key models of CaMKII-mediated regulation of LCCs and RyRs arising from both Ca^{2+}/CaM-dependent as well as oxidative activation pathways, the integration of such models into whole-cell models, and the mechanistic insights that have been obtained from this work. A summary of the models covered here is provided in **Table 1**.

MODELS OF Ca^{2+}/CaM-DEPENDENT CaMKII ACTIVATION AND REGULATION

The first model of CaMKII signaling within the context of ECC integrated into the cardiac myocyte was presented by Hund and Rudy (2004). This model, known as the HRd model, incorporates a scheme based on the work of Hanson et al. (1994), where a single population of CaMKII transitions from an inactive to active state in response to elevated subspace Ca^{2+} levels. CaMKII activity is assumed to modify the function of LCCs and RyRs by slowing their inactivation kinetics, thereby enhancing I_{CaL} and JSR Ca^{2+} release, respectively. SR Ca^{2+} uptake was also enhanced by CaMKII activity via its action on phospholamban (PLB) and the SR Ca^{2+}-ATPase (SERCA). The model predicted that CaMKII plays a critical role in the rate-dependent increase of the cytosolic Ca^{2+} transient by increasing ECC gain, but that it does not play a significant role in rate-dependent changes of AP duration. Similar results were obtained in the human cardiac AP model of O'hara et al. (2011), which incorporates the role of CaMKII phosphorylation on LCCs, the fast Na^+ current (I_{Na}), and the transient outward K^+ current (I_{to1}). The HRd model was later updated and used to investigate the role of altered CaMKII signaling in the canine infarct border zone (Hund et al., 2008). Experimentally measured elevation of CaMKII autophosphorylation in this region is reproduced by the model, which indicates that hyperactive CaMKII impairs Ca^{2+} homeostasis by increasing Ca^{2+} leak from the SR. A number of additional modeling studies have focused on the role of CaMKII on various aspects of intracellular Ca^{2+} dynamics. Iribe et al. (2006) incorporated a conceptual CaMKII model into their previous myocyte model (Noble et al., 1991) combined with a model of mechanics (Rice et al., 1999) in order to investigate its role on SR Ca^{2+} handling and interval-force relations. They found that a relatively slow time-dependent inactivation of CaMKII allowed for the reconstruction of a variety of interval-force relations, including alternans. Koivumaki et al. (2009) built a model of the murine cardiac myocyte to analyze genetically engineered heart models in which CaMKII-mediated phosphorylation of LCCs is disrupted or CaMKII is overexpressed. They demonstrated how these genetic manipulations lead to the observed experimental phenotypes as a result of autoregulatory mechanisms that are inherent in intracellular Ca^{2+} cycling (e.g., steady-state regulation of SR content via Ca^{2+} release dependent inactivation of LCCs), and that disruption of the regulatory system itself (e.g., via CaMKII overexpression) leads to the most aberrant physiological phenotypes. Livshitz and Rudy (2007) formulated a new model of SR Ca^{2+} release kinetics and incorporated it into the HRd model in order to better understand regulation of Ca^{2+} and electrical alternans under various pacing protocols. They found that increased CaMKII activity

Table 1 | Key features of CaMKII models in cardiac ECC.

Cell model	Species	Parent ECC model	CaMKII activation model	Included CaMKII targets
Hund and Rudy, 2004	Dog	Luo and Rudy, 1994	Hanson et al., 1994	LCCs, RyRs, SERCA/PLB
Iribe et al., 2006	Guinea pig	Noble et al., 1991	Conceptual kinetic model	LCCs, RyRs, SERCA
Livshitz and Rudy, 2007	Guinea pig/Dog	Luo and Rudy, 1994; Hund and Rudy, 2004	Hanson et al., 1994	LCCs, RyRs, SERCA/PLB
Grandi et al., 2007	Rabbit	Shannon et al., 2004	Static formulation Target parameters adjusted	I_{CaL}, I_{Na}, I_{to}
Saucerman and Bers, 2008	Rabbit	Shannon et al., 2004	Adapted from Dupont et al. (2003), Gaertner et al. (2004)	no target
Hund et al., 2008	Dog	Hund and Rudy, 2004	Hanson et al., 1994	LCCs, RyRs, SERCA/PLB, I_{Na}
Koivumaki et al., 2009	Mouse	Bondarenko et al., 2004	Bhalla and Iyengar, 1999	LCCs, RyRs, SERCA/PLB
Hashambhoy et al., 2009	Dog	Greenstein and Winslow, 2002	Dupont et al., 2003	LCCs
Christensen et al., 2009	Dog	Hund et al., 2008	Hanson et al., 1994	LCCs, RyRs, SERCA/PLB, I_{Na}
Soltis and Saucerman, 2010	Rabbit	Saucerman and Bers, 2008	Dupont et al., 2003; Gaertner et al., 2004	LCCs, RyRs, PLB, I_{Na}, I_{to}
Hashambhoy et al., 2010	Dog	Hashambhoy et al., 2009	Dupont et al., 2003	LCCs, RyRs, PLB
O'hara et al., 2011	Human	Hund and Rudy, 2004; Decker et al., 2009	Hund and Rudy, 2004	LCCs, PLB, I_{Na}, I_{to}
Hashambhoy et al., 2011	Dog	Hashambhoy et al., 2010	Dupont et al., 2003	LCCs, RyRs, PLB, I_{Na}
Zang et al., 2013	Dog	Hund and Rudy, 2004	Hanson et al., 1994	LCCs, RyRs, I_{Na}, I_{to}, I_{K1}, SERCA
Morotti et al., 2014	Mouse	Soltis and Saucerman, 2010	Dupont et al., 2003; Gaertner et al., 2004	LCCs, RyRs, PLB, I_{Na}, I_{to},I_{K1}, I_{NCX}

leads to increased alternans magnitude as well as the appearance of both Ca^{2+} and electrical alternans at lower pacing rates (where they would not normally occur). This model identifies Ca^{2+} alternans as the underlying mechanism for electrical alternans, both of which were eliminated with CaMKII inhibition, suggesting this as an antiarrhythmic strategy. Zang et al. (2013) recently developed a new canine myocyte model, also based on the HRd model, to study the role of upregulated CaMKII in heart failure. Similarly, they find that enhanced RyR Ca^{2+}-sensitivity and SR Ca^{2+} leak mediated by CaMKII overexpression alters Ca^{2+} handling in a manner that promotes alternans, while AP prolongation occurs primarily due to an associated down regulation of K^+ currents. Interestingly, they find that blocking SR Ca^{2+} leak restores contraction and relaxation function, but does not eliminate alternans completely.

Grandi et al. (2007) developed a model of CaMKII overexpression in the rabbit ventricular myocyte, which incorporates the functional effects of CaMKII-mediated phosphorylation of LCCs, as well as I_{Na} and I_{to1}. This model shows that while CaMKII-mediated action on LCCs prolongs the AP, the combined effect of CaMKII on all three of the targets studied leads to AP shortening. While this study was primarily motivated by and focused on the functional role of CaMKII-mediated regulation of I_{Na} (see accompanying article in this series by Grandi and Herren), it clearly demonstrates that the multiple targets of CaMKII interact to produce net alterations of integrated cellular function which are difficult to predict from knowledge if its functional effects on isolated individual targets. Saucerman and Bers (2008) developed and incorporated models of CaM, CaMKII, and calcineurin (CaN) into the rabbit ventricular myocyte model of Shannon et al. (2004) in order to better understand the functional consequences of the different affinities of CaM for CaMKII and CaN during APs. The model predicts that the relatively high Ca^{2+} levels that are achieved in the cardiac dyad lead to a high degree of CaM activity which results in frequency-dependent CaMKII activation and constitutive CaN activation, whereas the lower Ca^{2+} levels in the cytosol only minimally activate CaM, which allows for gradual CaN activation, but no significant activation of CaMKII. The prediction that robust beat-to-beat oscillations of CaMKII activity occur in the dyad (i.e. in the vicinity of RyRs and LCCs) but not in the cytosol is a key factor that would influence the way in which local signaling mechanisms would be incorporated into models that followed that included detailed reconstruction of dyadic Ca^{2+} dynamics. In one such model, Soltis and Saucerman (2010) integrated dynamic CaMKII-dependent regulation of LCCs, RyRs, and PLB with models of cardiac ECC, CaMKII activation, and β-adrenergic activation of protein kinase A (PKA). In this model phosphorylation of all CaMKII substrates exhibits positive frequency dependence similar to that observed in experiments (De Koninck and Schulman, 1998). However, both CaMKII activity and target protein phosphorylation levels adapt to changes in pacing rate with distinct kinetics (see Figure 2 of Soltis and Saucerman, 2010). CaMKII-mediated phosphorylation is relatively fast at LCCs, slower at RyRs, and very slow at PLB. Additionally, the model predicts a high degree of phosphorylation of LCCs in control conditions, but a moderate amount of phosphorylation of RyRs and PLB (e.g., PLB phosphorylation $<$10% at all frequencies). In addition, this study predicts a novel mechanism in which CaMKII and PKA synergize to form a positive feedback loop of CaMKII-Ca^{2+}-CaMKII regeneration.

Furthermore, this model predicts that CaMKII-mediated hyperphosphorylation of RyRs, which renders them leaky, may be a proarrhythmic trigger via induction of delayed afterdepolarizations (DADs). Recently, Morotti et al. (2014) modified the Soltis and Saucerman (2010) rabbit model to further investigate the arrhythmogenic role of another synergistic interaction in mouse ventricular myocytes. This synergism is that of the positive feedback loop of CaMKII-Na^+-Ca^{2+}-CaMKII in which CaMKII-dependent increases in intracellular Na^+ level perturbs Ca^{2+} homeostasis and CaMKII activation. Simulation results from Morotti et al. (2014) demonstrate that the feedback between disrupted Na^+ fluxes and CaMKII signaling is exaggerated when CaMKII is overexpressed. Under this condition, and upon an increase in intracellular Na^+ concentration,the model predicts Ca^{2+} overload and enhancement of CaMKII activity which in turn increases spontaneous Ca^{2+} release events via an increase in RyR phosphorylation. This CaMKII-dependent hyperphosphorylation of RyRs exacerbates the associated electrophysiological instability as simulated by the occurrence of DADs (see Figure 7 of Morotti et al., 2014). In this model, the DADs do not occur when CaMKII target phosphorylation is clamped to basal levels.

Recently, Hashambhoy et al. (2009) developed a biochemically and biophysically detailed model of CaMKII-mediated phosphorylation of LCCs. The model includes descriptions of the dynamic interactions among CaMKII, LCCs, and phosphatases as a function of dyadic Ca^{2+} and CaM levels, and has been incorporated into an integrative model of the canine ventricular myocyte with stochastic simulation of LCC and RyR channel gating within a population of release sites based on the theory of local control of ECC (Greenstein and Winslow, 2002). A schematic representation of one such model Ca^{2+}-release site is shown in **Figure 1A**. This CaMKII-LCC model is formed by the integration of three modules: a CaMKII activity model which reflects the different structural and functional states of the kinase based on the work of Hudmon and Schulman (2002) and Dupont et al. (2003), an LCC phosphorylation model derived from studies of facilitation in LCC mutants (Grueter et al., 2006; Lee et al., 2006), and a previously developed LCC gating model (Jafri et al., 1998; Greenstein and Winslow, 2002). In this model it is assumed that there is one 12-subunit CaMKII holoenzyme tethered to each LCC (Hudmon et al., 2005), each CaMKII monomer can transition among a variety of activity states (see Figure 2 of Hashambhoy et al., 2009), and CaMKII monomers can catalyze phosphorylation of individual LCCs. The LCC gating model reflects two forms of channel gating, mode 1 (normal activity) and mode 2 (high activity with long openings). In the model, LCC phosphorylation promotes transitions of LCCs from mode 1 to mode 2 gating, based on experimental observations with constitutively active CaMKII (Dzhura et al., 2000). This model demonstrates that these CaMKII-dependent shifts of LCC gating patterns into high activity gating modes may be the underlying mechanism of I_{CaL} facilitation. In addition, the model predicts that this CaMKII-mediated shift in LCC gating leads to an apparent increase in the speed of both I_{CaL} inactivation and recovery from inactivation, both of which are experimentally associated with I_{CaL} facilitation, with no change to the underlying intrinsic LCC inactivation kinetics. Hashambhoy et al. (2010) further expanded this model to include CaMKII-dependent regulation of RyRs. In this model update, RyR phosphorylation is modeled as a function of dyadic CaMKII activity and it is assumed that RyR sensitivity to dyadic Ca^{2+} is increased upon phosphorylation, the degree to which is constrained by experimental measurements of Ca^{2+} spark frequency and steady state RyR phosphorylation (Guo et al., 2006). This study demonstrated that under physiological conditions, CaMKII phosphorylation of LCCs ultimately has a greater effect on ECC gain, RyR leak flux, and AP duration as compared with phosphorylation of RyRs (**Figure 1B**). AP duration at 90% repolarization (APD_{90}) correlates well with a CaMKII-mediated shift in modal gating of LCCs (**Figure 1C**). A modest additional increase in LCC phosphorylation, and hence mode 2 gating, beyond that shown in **Figure 1C** leads to the appearance of early afterdepolarizations (EADs) in simulated APs (see Figure 5 of Hashambhoy et al., 2010). The results of these model analyses suggest that LCC phosphorylation sites may in fact prove to be a more effective target than those on the RyR for modulating diastolic RyR-mediated Ca^{2+} leak and preventing abnormal proarrhythmic cellular depolarization.

MODELS OF OXIDATIVE CaMKII ACTIVATION AND REGULATION

The modeling studies described above focus on the role of CaMKII-mediated regulation of its targets via the classical Ca^{2+}/CaM-dependent activation pathway which involves CaMKII autophosphorylation. Recently a novel mechanism for oxidative CaMKII activation was discovered in the heart (Erickson et al., 2008). This alternative oxidation-dependent pathway involves the oxidation of CaMKII at specific methionine residues, which has been shown to produce persistent kinase activity and increase the likelihood of arrhythmias (Xie et al., 2009; Wagner et al., 2011). These new findings implicate oxidative CaMKII activation as a putative mechanistic link between the accumulation of reactive oxygen species (ROS) and life-threatening cardiac arrhythmias (Erickson et al., 2011). Therefore, developing a quantitative understanding of the role of CaMKII oxidation in regulating cardiac ECC requires the integration of computational models that link cellular ROS and redox balance, CaMKII activity and function, ECC, and whole-cell electrophysiology. Such models will prove to be powerful tools for teasing out important mechanisms of arrhythmia in heart disease.

In one recent study, Christensen et al. (2009) developed a deterministic model of oxidative CaMKII activation based on their own experimental findings to study the role of this signaling pathway in the canine infarct border zone following myocardial infarction. A new simplified model of CaMKII activation was developed based on the work of Dupont et al. (2003), and incorporated into a model of a single cardiac fiber. Each myocyte within the fiber model was represented by the Hund et al. (2008) model, which as noted above, incorporates CaMKII effects on I_{Na}, I_{CaL}, SR Ca^{2+} leak and uptake, as well as ion channel remodeling associated with the infarct border zone. Simulation results demonstrate that enhanced oxidative CaMKII activity is associated with reduced conduction velocity, increased refractory

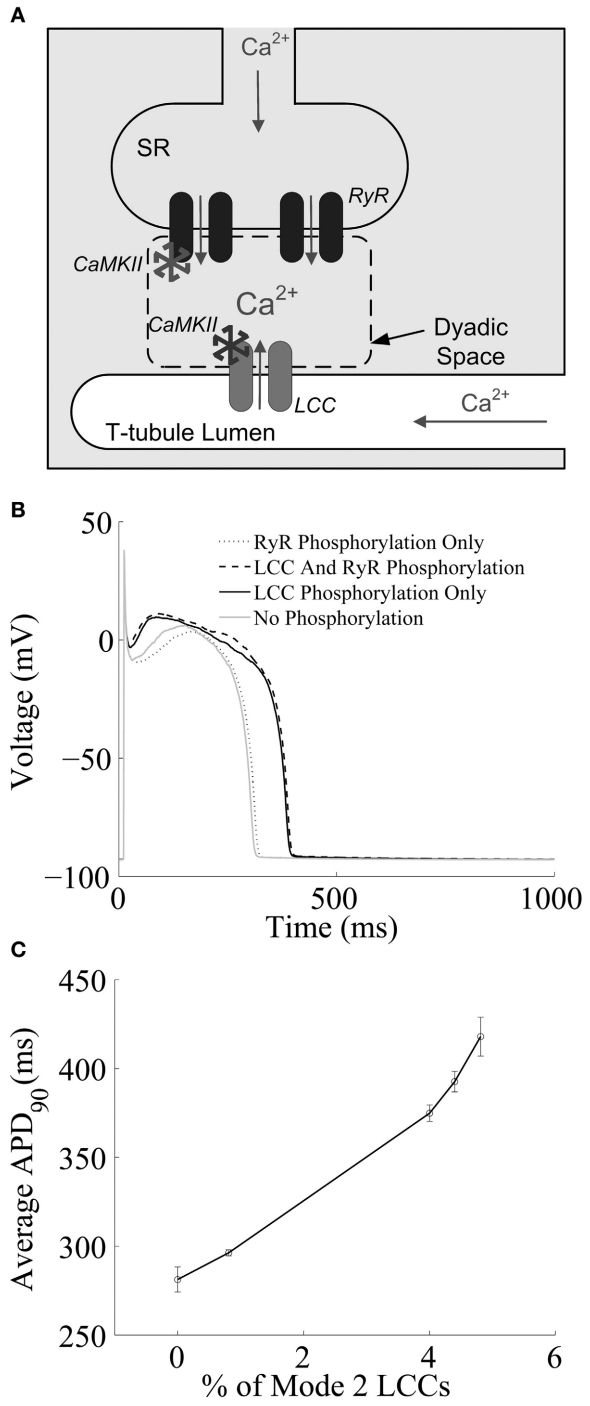

FIGURE 1 | (A) Schematic representation of the cardiac dyad. Ca^{2+} ions pass through the LCC and enter the dyad, where they bind to RyRs, triggering CICR. The activity of each CaMKII monomer is a function of dyadic Ca^{2+} levels. Ca^{2+} ions released from the SR diffuse out of the dyad into the bulk myoplasm. **(B)** Simulated results from a 1-Hz AP-pacing protocol under different LCC and/or RyR phosphorylation conditions. **(C)** APD_{90} as a function of average LCC phosphorylation levels. Fully phosphorylated LCCs gate in mode 2, which exibit long-duration openings. **(A)** Reprinted with permission from Hashambhoy et al. (2009). Copyright 2009 Elsevier. **(B,C)** Reprinted with permission from Hashambhoy et al. (2010). Copyright 2010 Elsevier.

periods, and a greater likelihood of conduction block formation. Furthermore, CaMKII inhibition in the model improves conduction and reduces vulnerability to conduction block and reentry. The results of Christensen et al. (2009) are attributed primarily to CaMKII-mediated regulation of I_{Na} kinetics and availability. They note that CaMKII activation will also impact ECC proteins in ways that may promote arrhythmias, but these mechanisms were not explored in detail in this model.

Along these lines, Foteinou et al. (2013) have recently developed a novel stochastic model of CaMKII activation (**Figure 2A**) that reflects the functional properties of the cardiac isoform including both the canonical phosphorylation-dependent activation pathway and the newly identified oxidation-dependent pathway. This model builds upon the work of Hashambhoy et al. (2010, 2011) in order to incorporate recent experimental data for CaM affinity and autophosphorylation/oxidation rates measured specifically for the cardiac isoform of CaMKII (Gaertner et al., 2004; Erickson et al., 2008). Predicated upon this, the authors implemented the four-state deterministic activation model of Chiba et al. (2008) within a stochastic framework that is constrained by the geometry of the CaMKII holoenzyme. This was accomplished by restricting CaMKII autophoshorylation events to occur only between adjacent CaMKII subunits. The stochastic activation model was further modified by including oxidized active states in addition to a Ca^{2+}/CaM-bound active state, an autophosphorylated Ca^{2+}/CaM-bound state, and an autophosphorylated Ca^{2+}/CaM-dissociated state (i.e., an autonomous active state). Following incorporation into the canine cardiac myocyte model (Hashambhoy et al., 2011), Foteinou et al. (2013) obtain steady state APs at a pacing cycle length (PCL) of 2 s as illustrated in **Figure 2B**. This model predicts increased likelihood of EADs under this protocol upon increased oxidative stress (application of 200 μM H_2O_2, which is at the high end of pathophysiological levels), as shown in **Figure 2C**. Notably, the model simulates no EADs in the presence of ROS at a PCL of 1 s, which is consistent with the experimentally measured rate-dependence of EADs in the presence of 200 μM H_2O_2 (Zhao et al., 2012). The model predicts that EADs result from both enhanced CaMKII-dependent shifts of LCCs into highly active mode 2 gating and a progressively increased late component of Na^+ current (I_{NaL}). The simulated increase in I_{CaL} via oxidized CaMKII corroborates the experimental findings of Song et al. (2010) who demonstrated that oxidative CaMKII activation is involved in the regulation of LCCs. In particular, the *in silico* model of H_2O_2 exposure discussed here predicts that the fraction of LCCs gaiting in mode 2 will shift from 5% in control (absence of ROS) to 7% upon this increase in ROS. Using this model, the simulated diastolic RyR leak also increases in response to oxidative stress (∼44%) primarily as an indirect consequence of increased Ca^{2+} influx via LCCs. Wagner et al. (2011) recently observed a ∼15-fold H_2O_2-mediated increase in SR Ca^{2+} leak, which is far greater than that produced in this model (<2-fold). They, however, provide additional evidence indicating that their observed increase in Ca^{2+} leak does not require the presence of CaMKII, suggesting an important role for CaMKII-independent mechanisms of ROS-mediated alteration of cardiac ECC as well.

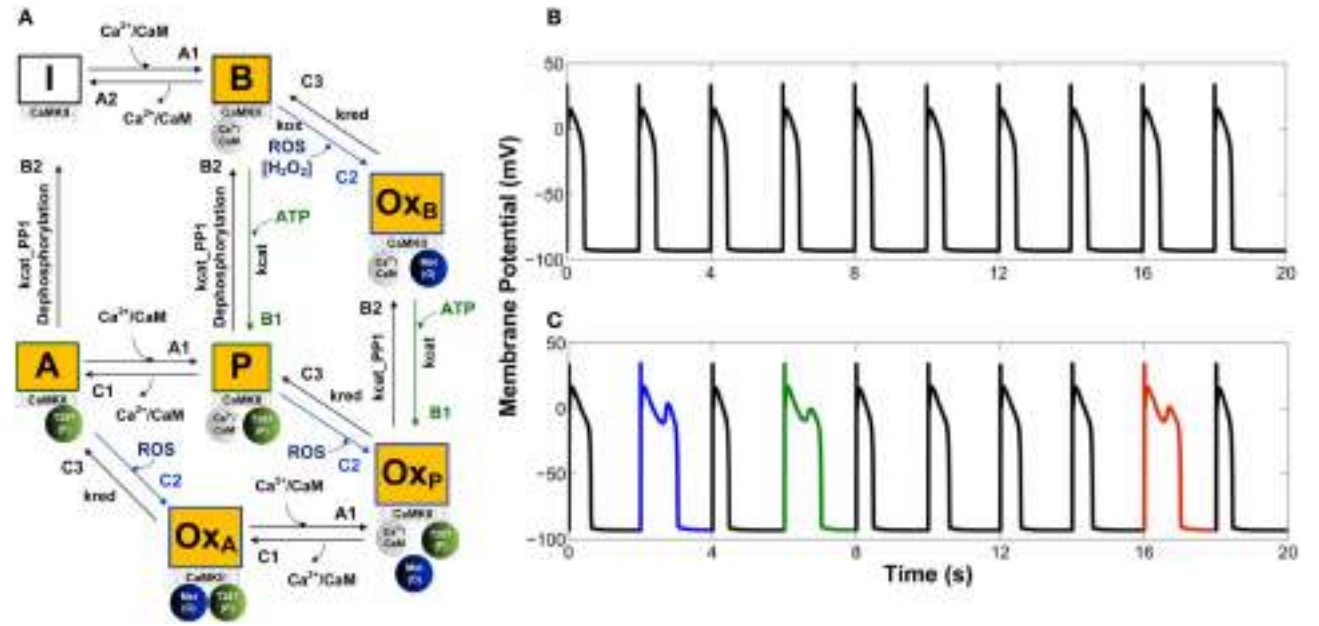

FIGURE 2 | (A) State diagram of the stochastic CaMKII activation model of Foteinou et al. (2013). Prior to the introduction of Ca^{2+}/CaM, all of the CaMKII subunits are in the inactive form (state I). Activation occurs upon binding of Ca^{2+}/CaM (state B), autophosphorylation (state P), and oxidation (state Ox_B). Autonomous active states (Ca^{2+}/CaM-unbound) can be either autophosphorylated (state A) or oxidized (state Ox_A). The model also includes an active state that is both oxidized and phosphorylated (state Ox_P). **(B)** Simulated steady state APs under control conditions (0 μM H_2O_2). **(C)** Simulated APs, some of which exhibit EADs, under conditions of elevated oxidant stress (200 μM H_2O_2). Simulated APs are from a 2 s PCL pacing protocol (ensemble of 12500 calcium release units). For each condition, results for 10 consecutive APs following an initial 10 s of pacing are shown.

As these ROS-mediated effects need to be further examined, future studies will focus on establishing quantitative links between cellular ROS and redox balance, CaMKII activity and function, ECC, and whole-cell electrophysiology. Recently, Gauthier et al. (2013a,b) developed mechanistic models of ROS production and scavenging to investigate how these two competing processes control ROS levels in cardiac mitochondria. Simulations confirm the hypothesis that mitochondrial Ca^{2+} mismanagement leads to decreased scavenging resources and accounts for ROS overflow as is believed to occur in heart failure (Hill and Singal, 1996). Notably, the ROS regulation module of Gauthier et al. (2013b) enables its use in larger scale heart models designed to simulate and study how mitochondrial ROS and the functional consequences of its accumulation, such as CaMKII oxidation, regulate cellular physiological function, AP properties, and arrhythmogenesis.

CONCLUSION

Integrative modeling of cardiac ECC, cell signaling, and myocyte physiology has played a critical role in revealing mechanistic insights across a range of biological scales. With respect to CaMKII function at the smallest scale, models have shed light on how Ca^{2+} ions, CaM, CaMKII, LCCs, and RyRs interact in the cardiac dyadic junction both at rest and during triggered ECC events. On an intermediate scale, models have predicted the consequences of normal and abnormal CaMKII signaling on whole-cell Ca^{2+} cycling, effects of ROS imbalance, AP shape, and the generation of cellular arrhythmias such as EADs. Incorporation of these cellular models into higher scale tissue simulations has provided important insight into the relationship between CaMKII function, electrical wave conduction velocity, and the emergence of arrhythmogenic substrates in diseased tissue. The continuum of biological scales spanned by these models allows for the development of multiscale approaches whereby we can predict and understand the emergence of macroscale phenotypes as a consequence of CaMKII-mediated molecular signaling events. A great deal of evidence now implicates CaMKII as a nexus point linking heart failure and arrhythmias (Swaminathan et al., 2012), identifying it as a prime target for antiarrhythmic therapies (Fischer et al., 2013). Despite this growing body of evidence, the modeling studies presented here demonstrate that it remains difficult to identify which of the many CaMKII target proteins are primarily responsible for the functional changes that increase the likelihood of arrhythmia development. Some studies (Hund et al., 2008; Soltis and Saucerman, 2010; Zang et al., 2013; Morotti et al., 2014) identify phosphorylation of RyRs, which leads to elevated SR Ca^{2+} leak and/or increased likelihood of spontaneous Ca^{2+} release events and DADs, as the key mechanism underlying arrhythmogenesis. Others (Hashambhoy et al., 2010; Foteinou et al., 2013) predict that LCC phosphorylation, which leads to elevated channel open probability via high activity gating, plays the predominant role by altering whole cell Ca^{2+} levels and AP properties, including the appearance of EADs. These mechanisms may co-exist in heart failure, and it is likely that both play an important role. Moreover, CaMKII modulation of other targets such as PLB, I_{Na}, and K^+ currents, many of which have been incorporated

into the above cell models, adds additional layers of complexity to the task of interpreting and predicting the mechanisms by which CaMKII signaling alters cardiac function in health and disease. As a result of the breadth and diversity of CaMKII targets, strategies that involve broad inhibition of CaMKII activity will impact and alter the function of many cellular subsystems, with complex consequences that may not all be beneficial. As mathematical models of CaMKII signaling in the cardiac dyad and beyond are further developed, they will play a key role in identifying target-specific therapeutic strategies for improving myocardial function and reducing arrhythmias.

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CaMKII-dependent regulation of cardiac Na^+ homeostasis

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Na^+ homeostasis is a key regulator of cardiac excitation and contraction. The cardiac voltage-gated Na^+ channel, $Na_V1.5$, critically controls cell excitability, and altered channel gating has been implicated in both inherited and acquired arrhythmias. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a serine/threonine kinase important in cardiac physiology and disease, phosphorylates $Na_V1.5$ at multiple sites within the first intracellular linker loop to regulate channel gating. Although CaMKII sites on the channel have been identified (S516, T594, S571), the relative role of each of these phospho-sites in channel gating properties remains unclear, whereby both loss-of-function (reduced availability) and gain-of-function (late Na^+ current, I_{NaL}) effects have been reported. Our review highlights investigating the complex multi-site phospho-regulation of $Na_V1.5$ gating is crucial to understanding the genesis of acquired arrhythmias in heart failure (HF) and CaMKII activated conditions. In addition, the increased Na^+ influx accompanying I_{NaL} may also indirectly contribute to arrhythmia by promoting Ca^{2+} overload. While the precise mechanisms of Na^+ loading during HF remain unclear, and quantitative analyses of the contribution of I_{NaL} are lacking, disrupted Na^+ homeostasis is a consistent feature of HF. Computational and experimental observations suggest that both increased diastolic Na^+ influx and action potential prolongation due to systolic I_{NaL} contribute to disruption of Ca^{2+} handling in failing hearts. Furthermore, simulations reveal a synergistic interaction between perturbed Na^+ fluxes and CaMKII, and confirm recent experimental findings of an arrhythmogenic feedback loop, whereby CaMKII activation is at once a cause and a consequence of Na^+ loading.

Keywords: CaMKII, Na^+ channel, DADs, Na^+ overload, arrhythmia

CARDIAC Na^+ HANDLING

In cardiac myocytes, intracellular Na^+ concentration ($[Na^+]_i$) is a key modulator of Ca^{2+} cycling, contractility and metabolism, and is controlled by the balance between Na^+ influx and extrusion (**Figure 1**). The major contributors to Na^+ entry during the cardiac cycle are the Na^+/Ca^{2+} exchanger (NCX), the voltage-dependent Na^+ channel (Na_V), and the Na^+/H^+ exchanger (NHE) with relative contributions of NCX > Na_V > NHE (see **Figure 1**). Smaller amounts of Na^+ also enter the cell via the Na^+/HCO_3^- and the $Na^+/K^+/2Cl^-$ cotransporters, and the Na^+/Mg^{2+} exchanger. Na^+ efflux is controlled primarily through the Na^+/K^+ ATPase (NKA) that keeps $[Na^+]_i$ constant at steady-state [reviewed in (Despa and Bers, 2013)].

An integrative approach to cellular Na^+ handling is critical to understand how these pathways interact spatially and temporally to affect cardiac cell function. Indeed, compelling evidence has accumulated that local $[Ca^{2+}]_i$ and $[Na^+]_i$ gradients exist close to the cell membrane (Carmeliet, 1992) that depend on the spatial localization of specific Na^+ and Ca^{2+} handling proteins and their isoforms (**Figure 1**). For example, NKA-α2 is more concentrated at the t-tubules (whereas NKA-α1 is homogenously distributed), and this could be important in regulating local cleft $[Na^+]_i$ and $[Ca^{2+}]_i$ (Berry et al., 2007; Despa and Bers, 2007). NCX is concentrated at the t-tubules, but only a small NCX fraction colocalizes with proteins specific to the dyadic cleft [i.e., L-type Ca^{2+} channels (LTCCs) and ryanodine receptors (RyRs; Jayasinghe et al., 2009)]. Nevertheless, functional data indicate that NCX senses an early and high rise in local vs. bulk $[Ca^{2+}]_i$ (Weber et al., 2003a) and Ca^{2+} entry via reverse mode NCX can even trigger sarcoplasmic reticulum (SR) Ca^{2+} release (Litwin et al., 1998). Moreover, a loss-of-function mutation in ankyrin-B results in reduced NKA and NCX protein levels and impaired targeting to the t-tubules, which causes altered Ca^{2+} signaling and after contractions (Mohler et al., 2003) and can affect local $[Na^+]$, cellular and SR Ca^{2+} cycling (Camors et al., 2012), and kinase/phosphatase balance (DeGrande et al., 2012).

Cardiac Na^+ channel ($Na_V1.5$) activity also requires proper sarcolemmal localization. For example, defective membrane targeting by impaired interaction of $Na_V1.5$ and ankyrin-G causes Brugada syndrome (BrS; Mohler et al., 2004). $Na_V1.5$ are found roughly homogenously distributed in the t-tubules and external sarcolemma, whereas non-cardiac Na_V isoforms (whose role is still poorly understood) are more concentrated at the t-tubules (Brette and Orchard, 2006). However, immunofluorescence studies show $Na_V1.5$ mostly at the intercalated disks, and it has been proposed that different pools of Na^+ channels are located in the external sarcolemma vs. intercalated disks, where they interact within different macromolecular complexes and are regulated differently (Lin et al., 2011; Petitprez et al., 2011; Sato et al., 2011).

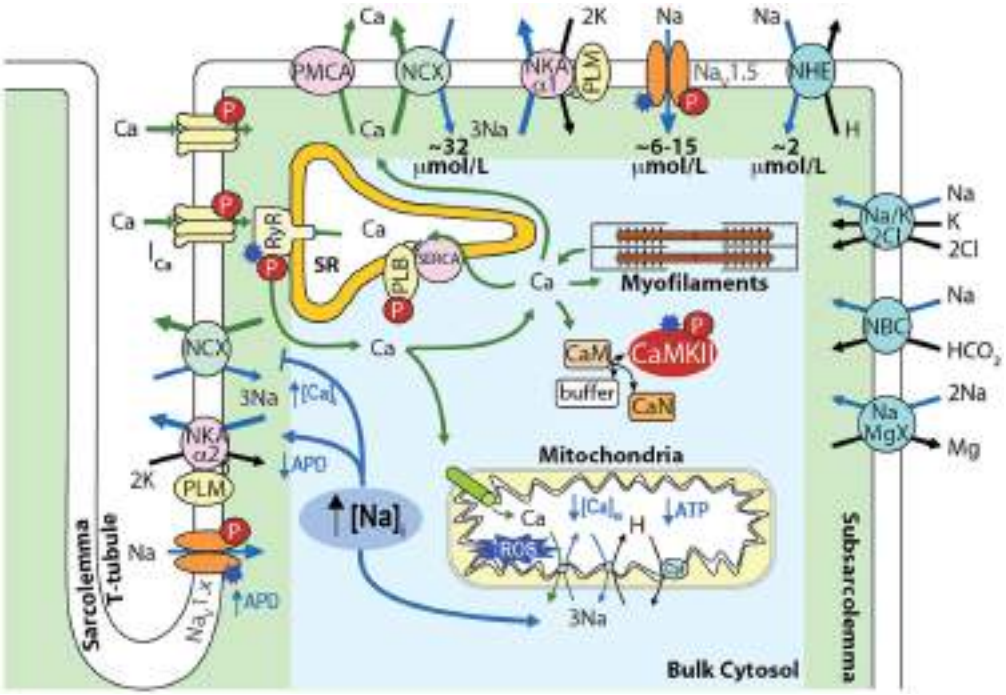

FIGURE 1 | Schematic showing the main processes regulating $[Na^+]_i$ and $[Ca^{2+}]_i$ homeostasis in cardiac myocytes and the mechanisms by which an increase in $[Na^+]_i$ affects $[Ca^{2+}]_i$, contractility, and metabolism. Upon cardiac myocyte electrical excitation, Na_V opening initiates the AP upstroke and allows Na^+ entry (limited to 6–15 μmol/L by rapid inactivation). E_m depolarization causes LTCC openings and consequent Ca^{2+}-induced SR Ca^{2+} release, which triggers contraction. During relaxation, Ca^{2+} is reuptaken into the SR by SERCA, and extruded out of the cytosol through NCX, which extrudes the ~10 μmol/L Ca^{2+} that enters via LTCCs and leads to an increase in $[Na^+]_i$ by ~32 μmol/L during each AP. NHE brings in ~2 μmol/L Na^+ at physiological intracellular pH (~16 μmol/L during intracellular acidosis). The Na^+/HCO_3^- and the $Na^+/K^+/2Cl^-$ cotransporters, and the Na^+/Mg^{2+} exchanger contribute minimally to the total Na^+ entry (~40–45 μmol/L), which is then extruded by NKA [reviewed in (Despa and Bers, 2013)].

Altered Na^+ homeostasis through the mechanisms described above (and in **Figure 1**) contributes to action potential (AP) and $[Ca^{2+}]$ cycling dysregulation, leading to arrhythmia, metabolic imbalance, remodeling and cell death, and is a hallmark of various cardiac diseases. The search for a common denominator has pointed to the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). CaMKII is a basophilic serine/threonine kinase that plays critical roles in cardiac physiology and disease (where it is often found hyperactive) through phosphorylation of several Ca^{2+} handling proteins and ion channels (**Figure 1**; Bers and Grandi, 2009; Anderson et al., 2011). $Na_V1.5$, in particular, has emerged as a principle CaMKII target. Herein, we summarize growing evidence indicating an important role for CaMKII in regulating $Na_V1.5$ gating and cardiomyocyte Na^+ homeostasis, with an emphasis on the important interconnection between Na^+ and Ca^{2+} handling, and consequences for arrhythmia.

CARDIAC Na^+ CHANNEL STRUCTURE AND FUNCTION

The cardiac Na^+ channel is responsible for the upstroke of the cardiac AP and is a critical determinant of cardiac electrical excitability. The pore forming α-subunit, $Na_V1.5$, is composed of four core domains (DI–DIV), each containing six transmembrane segments (S1–S6; ~230 kD; **Figure 2**). The positively charged S4 segments serve as the channel voltage sensors and the S5–S6 segments comprise the ion-conducting pore. Upon depolarization, the channel rapidly activates (<1 ms) producing a large (tens of nA), transient inward Na^+-current, I_{Na}, and then undergoes fast inactivation (within a few ms) through interactions of the well-described DIII–IV linker IFM motif with the pore (similar to N-type inactivation in K^+ channels). This is followed by poorly understood slower modes of inactivation (hundreds of ms to a few s) that likely involve rearrangements of the pore structure (similar to C-type inactivation in K^+ channels). Channels that fail to completely inactivate, or close and then reopen, give rise to a persistent, or late Na^+ current (I_{NaL}). $Na_V1.5\alpha$ forms macromolecular complexes with β-subunits (β1 to β4) and many other accessory and regulatory proteins that modify channel gating [extensively reviewed by Abriel (2010); Wilde and Brugada (2011)]. The intracellular N- and C-termini and linker loops connecting DI–IV are also all involved in channel gating. In particular, the $Na_V1.5$ I–II cytoplasmic linker loop is the subject of extensive post-translational regulation, and this region has received much attention as a hot spot for phosphorylation by CaMKII [reviewed in (Herren et al., 2013)].

PHOSPHORYLATION OF $Na_V1.5$ by CaMKII

CaMKII activation has been shown to alter the gating properties of the Na^+ channel, as summarized in **Table 1**. Specifically, CaMKII phosphorylation of $Na_V1.5$ shifts the voltage

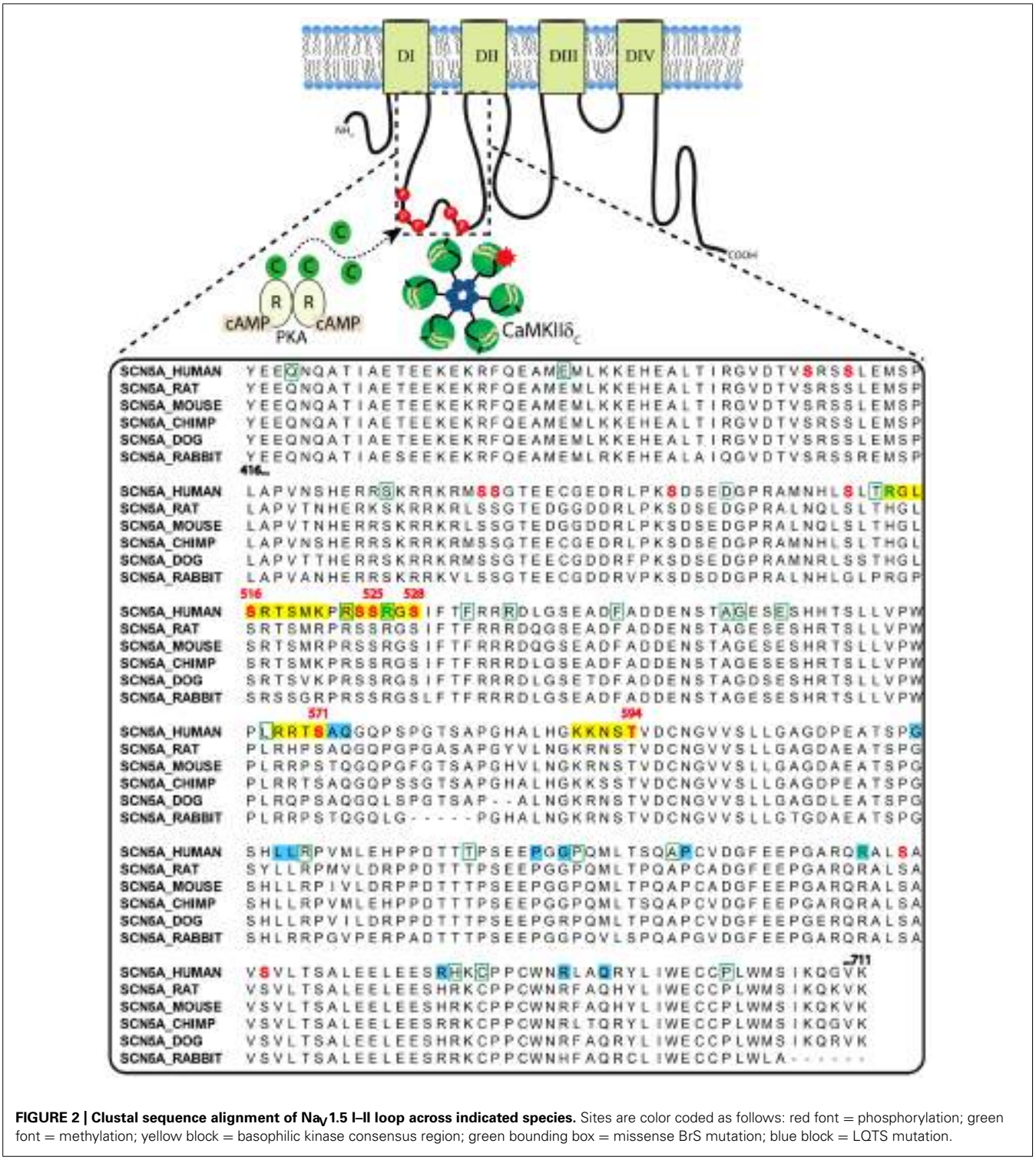

FIGURE 2 | Clustal sequence alignment of $Na_V1.5$ I–II loop across indicated species. Sites are color coded as follows: red font = phosphorylation; green font = methylation; yellow block = basophilic kinase consensus region; green bounding box = missense BrS mutation; blue block = LQTS mutation.

dependence of inactivation to negative potentials without affecting channel activation, slows recovery from and enhances entry into slower forms of inactivation, and increases I_{NaL} (Wagner et al., 2006).

The $Na_V1.5$ I–II linker loop interacts with CaMKII (Ashpole et al., 2012) and contains multiple basophilic kinase consensus sequences. Mass spectrometry analysis of wild type $Na_V1.5$ (purified from mouse heart lysates) revealed several sites within this loop that are basally phosphorylated (Marionneau et al., 2012; **Figure 2**, red color, and **Table 1**). Hund et al. (2010) mutated putative CaMKII consensus sites to non-phosphorylatable alanine residues and determined that CaMKII

specifically phosphorylates S571. When expressed in a heterologous expression system, the S571A non-phosphorylatable mutant abolished constitutively active co-expressed CaMKII enhancement of channel inactivation and I_{NaL}. On the other hand, phosphomimetic S571E recapitulated CaMKII effects (but still in the presence of constitutively active CaMKII). S571 phosphorylation was increased in murine (Toischer et al., 2013) and human heart failure (HF) and canine ischemic cardiomyopathy (Koval et al., 2012).

Because CaMKII (and other kinases) can phosphorylate non-canonical sequences, we screened the entire $Na_V1.5$ I–II loop for CaMKII phosphorylation with an *in vitro* kinase assay system (Ashpole et al., 2012). This assay consisted of a tiled peptide array spanning the entire loop region followed by *in vitro* phosphorylation with recombinant CaMKII and revealed that site S516 was phosphorylated most efficiently by CaMKII (Ashpole et al., 2012). Immobilized peptides containing S483/S484 and S593/T594 were also phosphorylated in this assay at much lower efficiency. It is important to consider peptide conformation may change when a peptide is immobilized or soluble, which can affect kinase access to phospho-acceptor sites (Bayer et al., 2006). In fact, subsequent studies using soluble peptides showed that T594 but not S483/4 could be phosphorylated, although only at low efficiencies. Furthermore, peptide conformation and kinase binding may be different for the full-length channel, thus affecting its ability to be phosphorylated. Therefore, additional phosphorylation studies using full-length $Na_V1.5$ are needed.

Wild type or non-phosphorylatable mutant channels (S516A, T594A, S571A) were coexpressed with CaMKIIδC in HEK293 cells and voltage-clamped under pipette conditions to acutely activate CaMKII (with Ca^{2+} and calmodulin), with or without CaMKII inhibition [by autocamtide-2 inhibitory peptide (AIP); Ashpole et al., 2012]. CaMKII shifted steady-state inactivation (SSI) to hyperpolarizing potentials and increased entry into inactivation, and this was blocked with AIP or by mutating these sites to non-phosphorylatable alanine. Phosphomimetic S516E and T594E mutants recapitulated the hyperpolarizing shift in SSI even in the absence of CaMKII and presence of AIP. In our hands, however, S571E phosphomimetic mutants showed no statistically significant negative shift in SSI. Moreover, we observed no enhancement of late I_{NaL} in any of the mutants tested, but we did not coexpress β-subunits, which some have indicated to be important for I_{NaL} (Maltsev et al., 2009). Importantly, the phosphorylation status of S516 and T594 in native myocytes is yet to be determined, and more studies are needed to determine the relative contribution of these sites to specific channel gating properties.

FUNCTIONAL CONSEQUENCES OF I–II LOOP PHOSPHORYLATION: INSIGHT FROM INHERITED MUTATIONS

Structure-function studies of *SCN5A* channelopathies/inherited mutations may further an understanding of the functional consequences of $Na_V1.5$ phosphorylation. CaMKII-dependent alterations of $Na_V1.5$ gating are remarkably similar to those caused by the $Na_V1.5$ mutation 1795insD (Bezzina et al., 1999), which is associated to patients with mixed long QT syndrome (LQTS) and BrS phenotypes. DelK1500 is another overlap mutation that causes both loss and gain of function channel effects and is functionally similar to the effects of CaMKII phosphorylation on $Na_V1.5$ (Grant et al., 2002). Experiments and simulations have shown how the heart rate acts as a switch imparting LQTS or BrS phenotypes to the same genotype (Veldkamp et al., 2000; Clancy and Rudy, 2002), and we have hypothesized a similar scenario for CaMKII effects (Grandi et al., 2007). However, 1795insD and delK1500 are present on the $Na_V1.5$ C-terminus and III–IV loop respectively, making structural correlation with CaMKII phosphorylation on the I–II loop difficult. To date, no overlap mutations have been identified anywhere within the I–II loop (Remme et al., 2008).

While no overlap mutations are present, several mutations or polymorphisms have been identified within the I–II loop phosphorylation hot spot through studies of LQTS and BrS patient cohorts. Examination of these mutations may be useful in dissecting out the structure-function relationship of kinase phosphorylation within this region. More than 30 putative BrS mutations have been identified in the I–II loop from residues 416–711 (Kapplinger et al., 2010; **Figure 2**). Unfortunately, not all of these have been followed up with functional studies. One mutation, L567Q, was identified in a family exhibiting BrS and sudden infant death syndrome (Priori et al., 2000). When expressed heterologously, the mutant $Na_V1.5$ channel displayed a negative shift in inactivation and enhanced entry into inactivation that was not dependent on coexpression of β-subunits

Table 1 | $Na_V1.5$ phosphorylation sites, their associated kinases, and biophysical effects.

Residue	Kinase	SSI	I_{NaL}	Comments	Reference
S516	CaMKII	←	↔		Ashpole et al. (2012)
T594	CaMKII	←	↔		Ashpole et al. (2012)
S571	CaMKII	←	↑	Basally phosphorylated by MS	Hund et al. (2010), Koval et al. (2012), Marionneau et al. (2012)
S525	PKA	←	ND	EP effects are indirect; mutant EP studies ND	Zhou et al. (2000)
S528	PKA	←	ND	EP effects are indirect; mutant EP studies ND	Zhou et al. (2000)
S457, S460, S483/4, S497, S510, S524/5, S664, S667	ND	ND	ND	MS identification; basal phosphorylation in untreated mouse heart lysates; responsible kinases ND	Marionneau et al. (2012)

EP = electrophysiological; ND = not determined; MS = mass spectrometry.

(Wan et al., 2001). Another mutation, T512I, was identified in a patient exhibiting cardiac conduction disease, and resulted in hyperpolarizing shifts in SSI and activation and enhanced slow inactivation (Viswanathan et al., 2003). Not only is this mutation juxtaposed to one of the known CaMKII phosphorylation consensus regions identified at R513 and phosphorylated at S516 [see **Figure 2** and (Ashpole et al., 2012)], it also functionally mirrors the enhanced inactivation conferred by CaMKII phosphorylation at S516. Furthermore, PKA phosphorylation of nearby S525 and S528 (Murphy et al., 1996) has been previously described to similarly shift the voltage dependence of inactivation to negative potentials (Zhou et al., 2000). Thus, phosphorylation by either PKA (S525, S528) or CaMKII (S516) within a short ∼10 amino acid stretch results in similar channel biophysics compared with the loss of function BrS mutation identified at T512. Moreover, a recent proteomics study demonstrated that residues R513 and R526 within this same region can be methylated, but the functional effect of this post-translational modification is unknown (Beltran-Alvarez et al., 2011). Another mutation, G514C, was identified in a family with cardiac conduction disease. Under voltage clamp, this mutation displayed a mixed biophysical phenotype of destabilized inactivation and decreased activation (Tan et al., 2001). These observations cement the importance of this short region in voltage-dependent inactivation of the channel.

CaMKII-dependent phosphorylation at S571 has been shown to result in both loss and gain of channel function (Hund et al., 2010). The functional effects of a negative charge at this site from CaMKII phosphorylation were suggested to phenocopy the adjacent inherited LQTS charge mutations at A572D and Q573E (Koval et al., 2012). Although A572D was initially identified as a causative LQTS mutation (Tester et al., 2005), a subsequent study showed that this mutation is actually a benign variant and does not cause LQTS in and of itself (Tester et al., 2010). Similarly, a mutation identified in a LQTS patient at 619 was also attributed to an observed LQTS phenotype in this patient. This L619F mutation was found to induce I_{NaL} when expressed in a heterologous expression system (Wehrens et al., 2003). However, a LQTS screening study from the Roden group failed to detect any late I_{NaL} (or gating changes) from L618F mutants expressed in heterologous cells and concluded this mutation is also a benign variant (Yang et al., 2002). Indeed, mutations within the $Na_V1.5$ I–II loop have poor disease predictive value stemming from the high incidence of benign variants within this region (Kapa et al., 2009).

Thus, a clear and consistent role for the $Na_V1.5$ I–II loop in loss of function inactivation gating emerges from studies of kinase phosphorylation (both CaMKII and PKA) and inherited mutations within this hotspot. There is less evidence for this loop region mediating I_{NaL} effects, with different groups providing contradictory results. It remains to be determined whether other unidentified CaMKII phospho-sites exist on other intracellular loops of $Na_V1.5$ that may mediate (contribute to) enhancement of I_{NaL} (e.g., the C-terminus (Coyan et al., 2014) or III–IV loop where overlap mutations have been identified). It is also conceivable that I_{NaL} enhancement in pathological conditions could be due to an increase in neuronal Na^+ channel isoforms with higher fractional I_{NaL} (Xi et al., 2009; Biet et al., 2012; Yang et al., 2012; Toischer et al., 2013). Alternatively, the effect of CaMKII to augment I_{NaL} may require accessory proteins not present in heterologous cell systems [such as regulatory β subunits (Maltsev et al., 2009)]. Indeed, this emphasizes the need for studies of these phosphorylation sites in native adult cardiomyocytes. Human induced pluripotent stem cells may also be used as suitable models, as has been done for some inherited mutations, such as 1795insD (Davis et al., 2012).

Enhanced inactivation and consequent reduction in channel availability due to CaMKII phosphorylation is expected to contribute to re-entrant arrhythmias from slowed conduction and enhanced dispersion of repolarization [as we have extensively reviewed previously (Herren et al., 2013)]. On the other hand, CaMKII enhancement of I_{NaL}, while not as well understood mechanistically, can also lead to arrhythmias arising from prolonged AP duration (APD) that makes the cell more vulnerable to triggered activity [via early after-depolarizations (EADs)]. EADs are favored by conditions leading to prolongation of the AP plateau within a voltage range permitting recovery from inactivation and reactivation of LTCC, or can be the consequence of SR Ca^{2+} release and consequent augmentation of NCX current. A novel and unique mechanism underlying phase 3 EADs in ventricular myocytes from CaMKIIδC overexpressing mice has been recently described (Edwards et al., 2012), which involves isoproterenol-induced exaggerated Ca^{2+} release, increased inward NCX current, and non-equilibrium reactivation of fast I_{Na}.

I_{NaL} AND CELLULAR Na^+ LOADING IN HF

In addition to directly affecting myocytes electrical stability, I_{Na} is a major pathway of Na^+ influx into cardiac myocytes, although NCX plays the most dominant role both at rest and in contracting cells [see **Figure 1** and (Despa and Bers, 2013)]. In normal myocytes I_{NaL} contribution to Na^+ entry is limited, but when I_{NaL} is enhanced in diseased conditions (such as cardiac hypertrophy and HF) it carries as much Na^+ as the fast I_{Na} transient, thus increasing total cellular Na^+ influx during a cardiac cycle and potentially contributing to Na^+ overload (Makielski and Farley, 2006; Despa and Bers, 2013). Indeed, intracellular Na^+ concentration ($[Na^+]_i$) is increased in myocytes from failing hearts compared to non-failing myocytes by 2–6 mM (Pogwizd et al., 2003; Shryock et al., 2013), but the precise mechanism(s) remains unclear (**Figure 3**). It has been suggested that CaMKII and enhanced I_{NaL} contribute to $[Na^+]_i$ elevation in HF, as CaMKIIδC overexpressing mice with HF exhibit prominent I_{NaL} and have increased $[Na^+]_i$ (Wagner et al., 2006).

While the increase in I_{NaL} may play a role in the observed $[Na^+]_i$ loading in these transgenic mice (Wagner et al., 2006), we demonstrated that CaMKII-dependent enhancement of I_{NaL} is not quantitatively sufficient to account for the $[Na^+]_i$ elevation observed in HF (Grandi et al., 2007; Wagner et al., 2011; Moreno et al., 2013; Morotti et al., 2014). Additionally, in a rabbit model of pressure- and volume-overload-induced HF, Despa et al. (2002) reported an increased Na^+ influx resulting primarily from a TTX-sensitive pathway, which accounted for a gain in $[Na^+]_i$ of ∼3 mM, and was present both in resting and electrically stimulated cells. This supports the existence of a diastolic Na^+ influx in failing myocytes responsible for $[Na^+]_i$ elevation, although it is currently

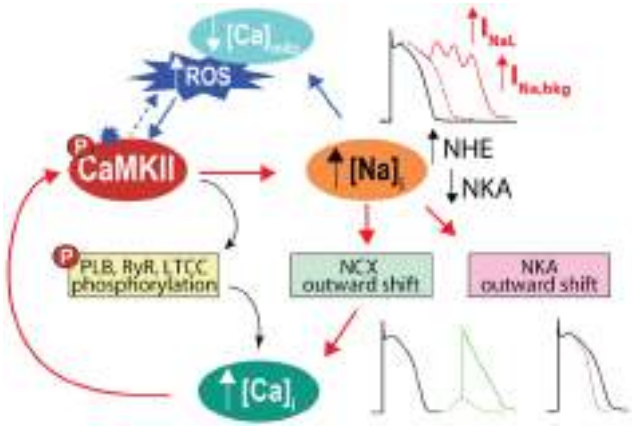

FIGURE 3 | CaMKII activation and Na^+ homeostasis are intimately related in the regulation of cellular Ca^{2+}, contractility and electrical stability. With CaMKII hyperactivity, increase in late I_{Na} favors AP prolongation and EADs. Cellular Na^+ loading increases Ca^{2+}, via outward shift in NCX, which further activates CaMKII, fueling a vicious cycle that favors spontaneous SR Ca^{2+} release and predisposes to Ca^{2+}-related arrhythmia. Elevated $[Na^+]_i$ can also influence ROS production by affecting mitochondrial $[Ca^{2+}]_i$. A less appreciated observation is that high $[Na^+]_i$ by causing outward shifts in both NCX and NKA will tend to shorten the AP, as predicted by computational AP models (Grandi et al., 2010, 2011). We speculate that the increased $[Na^+]_i$ in HF may limit AP prolongation caused by reduced K^+ channel conductance and increased I_{NaL}.

unclear whether this diastolic influx is carried by Na_Vs [whether they be of cardiac, neuronal, or skeletal muscle isoforms (Biet et al., 2012; Yang et al., 2012)] or by which gating mechanism. Increases of Na^+ window and/or background current can potentially contribute to increased Na^+ influx in failing vs. normal myocytes [and could be modified by drugs affecting the voltage dependence of Na^+ channel gating (Shryock et al., 2013)]. We showed that simulation of an increased sarcolemmal Na^+ leak current allows recapitulating the $[Na^+]_i$ gain seen in HF (Wagner et al., 2011; Moreno et al., 2013; Morotti et al., 2014). Baartscheer et al. (2003) using the same pressure- and volume-overload rabbit HF model in Despa et al. (2002) found that increased Na^+ influx via NHE was the largest contributor to the elevated Na^+ influx rate and $[Na^+]_i$ in paced myocytes. Interestingly, CaMKII activates NHE (Vila-Petroff et al., 2010). Reduced Na^+ extrusion could also contribute to the observed intracellular Na^+ gain during HF. Despite reduced expression, NKA function is unchanged in HF (Despa et al., 2002; Baartscheer et al., 2003), which is possibly due to higher NKA function secondary to reduced relative expression and elevated phosphorylation of phosholemman (Bossuyt et al., 2005).

CONSEQUENCES OF Na^+ LOADING ON EXCITATION-CONTRACTION COUPLING

$[Na^+]_i$ elevation in HF is expected to limit Ca^{2+} extrusion via forward mode NCX, and could even favor Ca^{2+} entry via reverse mode NCX (Weber et al., 2003b; **Figures 1** and **3**). Slowing Ca^{2+} extrusion via NCX will tend to elevate diastolic $[Ca^{2+}]_i$, thereby contributing to diastolic dysfunction. The slowed $[Ca^{2+}]_i$ decline and elevated diastolic $[Ca^{2+}]_i$ will also tend to increase SR Ca^{2+} content, thus exerting a positive inotropic effect and enhancing contractility. This explains the efficacy of cardiac glycosides in the treatment of congestive HF. These compounds promote inotropy by selectively inhibiting NKA and thereby impairing Na^+ extrusion and weakening the NCX Ca^{2+} extrusion gradient. However, they are well known for having undesired arrhythmic consequences (Altamirano et al., 2006) by increasing the propensity for spontaneous SR Ca^{2+} release and delayed after-depolarizations (DADs). DADs arise from a transient inward current I_{ti} through forward mode NCX, which is evoked by the sudden increase in $[Ca^{2+}]_i$. It has been recently proposed that CaMKII is mechanistically involved in glycoside-induced arrhythmogenesis, as ouabain increased CaMKII activity, and CaMKII inhibition significantly reduced ouabain-induced spontaneous contractile activity and Ca^{2+} waves (Gonano et al., 2011). CaMKII overexpression exacerbated ouabain-induced spontaneous contractile activity (Gonano et al., 2011), possibly by favoring spontaneous SR Ca^{2+} release and DADs, as demonstrated in a recent computational mouse model (Morotti et al., 2014). Ouabain-induced $[Na^+]_i$ loading has also been shown to result in apoptosis (Sapia et al., 2010), mediated by reverse mode NCX-dependent activation of CaMKII. Thus, CaMKII inhibition may have potential therapeutic benefit during glycoside treatment to prevent arrhythmia and cell death while minimally impacting the positive inotropic effect. Analogously, the Na^+ channel opener ATX-II induces spontaneous diastolic Ca^{2+} release from the SR and DADs in myocytes (Song et al., 2008) and arrhythmia in Langendorff perfused hearts (Yao et al., 2011), which is attenuated with CaMKII or I_{NaL} inhibition.

Our recent model of the failing human ventricular myocyte confirmed that a 20% increase in $[Na^+]_i$ in HF compared to control conditions slows forward mode inward NCX (Ca^{2+} extrusion) and enhances reverse mode NCX (Ca^{2+} entry, at the beginning of the AP; Moreno et al., 2013). This, coupled with AP prolongation due to extensive ionic remodeling in HF (including enhanced I_{NaL}), increased diastolic $[Ca^{2+}]_i$ while maintaining adequate SR Ca^{2+} load and Ca^{2+} transient despite reduced SR Ca^{2+}-ATPase (SERCA) function. However, $[Na^+]_i$-induced Ca^{2+} enhancement, in combination with hyperphosphorylated RyRs, causes diastolic SR Ca^{2+} release, I_{ti}, and triggered APs [**Figure 3**, also favored by a more depolarized resting membrane potential (E_m) in HF, due to decreased I_{K1} and increased background Na^+ current]. Simulations showed that by targeting pathological late Na^+ current and diastolic Na^+ influx, ranolazine (1) limits $[Na^+]_i$ thus restoring normal NCX forward mode that speeds up Ca^{2+} extrusion, (2) shortens APD, thus further limiting Ca^{2+} entry, and (3) hyperpolarizes the E_m, which elevates the threshold of triggered APs. These simulation results are consistent with recent experimental data in human failing myocytes from hypertrophic cardiomyopathy samples (Coppini et al., 2013). Similarly, CaMKII-dependent $[Na^+]_i$ elevation (normalized by ranolazine) has been associated with diastolic dysfunction and arrhythmias in CaMKIIδC overexpressing mice with HF (Sossalla et al., 2011). Taken together, these observations suggest that limiting $[Na^+]_i$ overload may be an appropriate antiarrhythmic therapeutic for the prevention of diastolic tension and arrhythmia triggers driven by $[Ca^{2+}]_i$ loading.

ARRHYTHMOGENIC CaMKII–Na^+–Ca^{2+}–CaMKII FEEDBACK

It has been proposed that $[Ca^{2+}]_i$ loading caused by elevated $[Na^+]_i$ in HF may positively feed back to further activate CaMKII (and enhance target phosphorylation) thus creating an arrhythmogenic vicious circle. This positive feedback from Na^+ to Ca^{2+} to CaMKII to Na^+ has been qualitatively described for Na^+ loading induced by ATX-II or accompanying a LQT3 mutation associated with increased I_{NaL} (Yao et al., 2011). Both of these conditions increased $[Ca^{2+}]_i$, induced CaMKII activation, increased CaMKII-dependent phosphorylation of phosholamban and RyRs, and favored arrhythmias. Although a CaMKII-dependent increase in Na_V phosphorylation was not directly confirmed in that study, a $[Ca^{2+}]_i$-dependent increase in I_{NaL} involving both CaMKII and PKC has previously been demonstrated (Ma et al., 2012). Accordingly, blockade of I_{Na} or I_{NaL} with TTX or ranolazine reversed all these effects, as did CaMKII inhibition via AIP or KN-93 (Yao et al., 2011). Our recently developed mouse myocyte model showed that alterations in Na^+ handling accompanying transgenic CaMKII overexpression (namely, increase in systolic I_{NaL} and diastolic Na^+ leak) can increase intracellular Na^+ gain to initiate Ca^{2+} overload and CaMKII activation. Furthermore, this mechanism proved to be quantitatively sufficient to further disrupt Ca^{2+} (and Na^+) homeostasis and promote cellular arrhythmias (Morotti et al., 2014). The simulated effect of Na^+ loading to fuel CaMKII-Na^+-Ca^{2+}-CaMKII feedback was even more striking when CaMKII was further activated by isoproterenol. This is consistent with the observation that β-adrenergic stimulation of myocytes isolated from mice overexpressing CaMKII (Sag et al., 2009) or subjected to TAC-induced HF (Toischer et al., 2013) increased the number of DADs, which were largely prevented by either ranolazine or AIP (Toischer et al., 2013).

CONSEQUENCES OF $[Na^+]_i$ LOADING ON CARDIAC ENERGETICS

$[Na^+]_i$ is also important in regulating cardiac myocyte bioenergetics, by controlling mitochondrial $[Ca^{2+}]$ via the mitochondrial NCX, and critically regulating the production of mitochondrial reactive oxygen species (ROS, **Figure 1**). High $[Na^+]_i$ in HF may negatively affect cardiac metabolism during rapid pacing, as elevated $[Na^+]_i$ has been shown to impair frequency-induced mitochondrial $[Ca^{2+}]$ accumulation thereby decreasing NADH/NAD^+ redox potential, and increasing H_2O_2 formation in myocytes from failing hearts (Liu and O'Rourke, 2008). Notably, higher oxidative stress may further exacerbate $[Na^+]_i$ loading by enhancing I_{NaL} through direct effects on $Na_V1.5$ or secondarily by activating CaMKII (Wagner et al., 2011). This suggests the interesting notion that ROS, like Na^+, is both a cause and consequence of CaMKII activation. Not only does oxidative stress activate CaMKII directly (Erickson et al., 2008), but CaMKII-induced $[Na^+]_i$ loading can induce an increase in ROS (**Figures 1** and **3**). This parallels recent evidence indicating that CaMKII regulates ROS production (Pandey et al., 2011; Sepulveda et al., 2013; Zhu et al., 2014). Li et al. (2012) put forward an intriguing model-based hypothesis that Na^+ accumulation in the SERCA knock-out mouse, which occurs secondary to marked NCX upregulation and intracellular acidosis, might play a role in the development of HF in these animals. This mechanism acts by initiating a reinforcing cycle involving a mismatch between ATP supply and demand, increasingly compromised metabolism, decreased intracellular pH, and further elevation of $[Na^+]_i$ due to NHE, which may shift the time of transition from compensated to decompensated function.

CONCLUDING REMARKS

CaMKII-dependent loss and gain of function effects on $Na_V1.5$ gating are pro-arrhythmic and functionally phenocopy *SCN5A* inherited mutations causing LQTS and BrS. Analogous to BrS mutations, CaMKII-dependent loss of Na^+ channel function and consequent reduction in Na^+ channel availability slows cardiac conduction and increases the propensity for conduction block and re-entry. Evidence points to the I–II loop phosphorylation hot spot as a likely mediator of these loss-of-function effects. Conversely, CaMKII enhancement of I_{NaL}, while not as well understood, may prolong the APD and predispose to lethal ventricular tachyarrhythmia (as in LQTS). Further study of these mechanisms may provide important advances for rational design of novel antiarrhythmic therapeutics. Additionally, experimental data and quantitative findings support the intriguing hypothesis that drugs designed to correct aberrant Na^+ channel gating behavior, causing I_{NaL} and cellular Na^+ loading, may act importantly by reducing Ca^{2+} overload. Growing evidence suggests that prevention of Na^+-dependent Ca^{2+} overload is a key mechanism of action for these compounds (Sossalla et al., 2011; Yao et al., 2011). Inhibition of intracellular Na^+ loading can contribute to normalizing Ca^{2+} and E_m homeostasis in HF, but should not be achieved at the expense of systolic function. Inhibition of CaMKII or its targets may be an attractive means of facilitating these outcomes.

AUTHOR CONTRIBUTIONS

Eleonora Grandi and Anthony W. Herren wrote the article.

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CaMKII regulation of cardiac K channels

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Cardiac K channels are critical determinants of cardiac excitability. In hypertrophied and failing myocardium, alterations in the expression and activity of voltage-gated K channels are frequently observed and contribute to the increased propensity for life-threatening arrhythmias. Thus, understanding the mechanisms of disturbed K channel regulation in heart failure (HF) is of critical importance. Amongst others, Ca/calmodulin-dependent protein kinase II (CaMKII) has been identified as an important regulator of K channel activity. In human HF but also various animal models, increased CaMKII expression and activity has been linked to deteriorated contractile function and arrhythmias. This review will discuss the current knowledge about CaMKII regulation of several K channels, its influence on action potential properties, dispersion of repolarization, and arrhythmias with special focus on HF.

Keywords: CaMKII, K channel, heart failure, action potential, arrhythmias

INTRODUCTION

Heart failure (HF) is a leading cause of death in western countries (United States and Europe), (Neumann et al., 2009; Go et al., 2012; Nichols et al., 2013) but also in developing countries like China (Hu et al., 2012). Morbidity in HF is characterized by contractile dysfunction and an increased propensity for arrhythmias (Luo and Anderson, 2013). Both are known consequences of the electromechanical remodeling of the cardiomyocyte. It is well established that reduced expression of K channels in hypertrophied and failing myocardium (Kääb et al., 1996) can lead to action potential (AP) prolongation, which is known to be pro-arrhythmogenic. Moreover, AP prolongation also leads to greater systolic Ca entry through voltage-gated L-type Ca channels ($Ca_V1.2$) and impairs the Ca export function of cardiac Na/Ca exchange (NCX, Bers, 2002a), which results in cytosolic Ca overload and dramatically impairs diastolic contractile function (**Figure 1**).

Thus, understanding the mechanisms that are involved in the regulation of cardiac K channel expression and function in HF could greatly improve patient treatment.

Ca/calmodulin-dependent protein kinase II (CaMKII) has been identified as an important regulator of ion channels and transporters involved in cardiac excitation–contraction coupling under physiological but also pathophysiological conditions (Maier and Bers, 2007). Increased CaMKII expression and function was found in HF and is linked to contractile dysfunction and arrhythmias. Interestingly, there is substantial evidence that CaMKII is also involved in K channel regulation (Nerbonne, 2011). This review will discuss CaMKII-dependent regulation of several cardiac potassium channels and its significance for arrhythmogenesis and contractile function in HF.

K CHANNELS ARE IMPORTANT REGULATORS OF CARDIAC EXCITABILITY

The cardiac AP is initiated by activation of voltage-gated Na channels ($Na_V1.5$). The resulting Na current (I_{Na}) leads to a rapid depolarization, i.e., the AP upstroke (phase 0; Bers, 2002b). The upstroke is limited by inactivation of I_{Na} and voltage-dependent activation of transient outward K channels ($K_V4.2$, $K_V4.3$, and $K_V1.4$ generating I_{to}). I_{to} activation results in an early repolarization (notch, phase 1), thus setting the voltage plateau of the AP. Activation of L-type Ca channels generates a depolarizing Ca current (I_{Ca}) that stabilizes the membrane potential during the plateau phase (phase 2). Repolarization in phase 3 is mainly caused by activation of delayed rectifying K channels [hERG (*KCNH2*), Kv7.1 (*KCNQ1*), and Kv1.5 (*KCNA5*) responsible for I_{Kr}, I_{Ks}, and I_{Kur}, respectively]. Additionally, activation of inward rectifying K channels (Kir2.x, generating I_{K1}) contributes to late phase repolarization. The resting membrane potential (phase 4) is stabilized by I_{K1}, but ion conductance in phase 4 is also influenced by the Na/K-ATPase and NCX.

In pacemaker cells, the absence of a stabilizing I_{K1} is responsible for a more positive resting membrane potential (Cho et al., 2003). The non-specific cation current I_f (channel protein HCN) can thus generate diastolic depolarization leading to the generation of APs (Bers, 2002b).

Several mechanisms of arrhythmogenesis involving K channels have been described. Reduced function of Kv7.1 and hERG are the hallmark of congential long QT syndrome 1 and 2, respectively (Brenyo et al., 2012). A smaller I_{Ks} and I_{Kr} results in prolonged repolarization that is associated with torsade de pointes and sudden cardiac death (Roden, 2008). The underlying arrhythmic mechanisms involve increased triggered activity due to early afterdepolarizations (EADs) or reentry due to increased spatial heterogeneities in repolarization (see below). Recently, a mutation of an ATP-sensitive K channel (Medeiros-Domingo et al., 2010) has been identified in a patient with early repolarization syndrome, which is characterized by a prominent J wave on the ECG (see below) and is associated with an increased risk of ventricular fibrillation (VF) and cardiac death (Tikkanen et al., 2009).

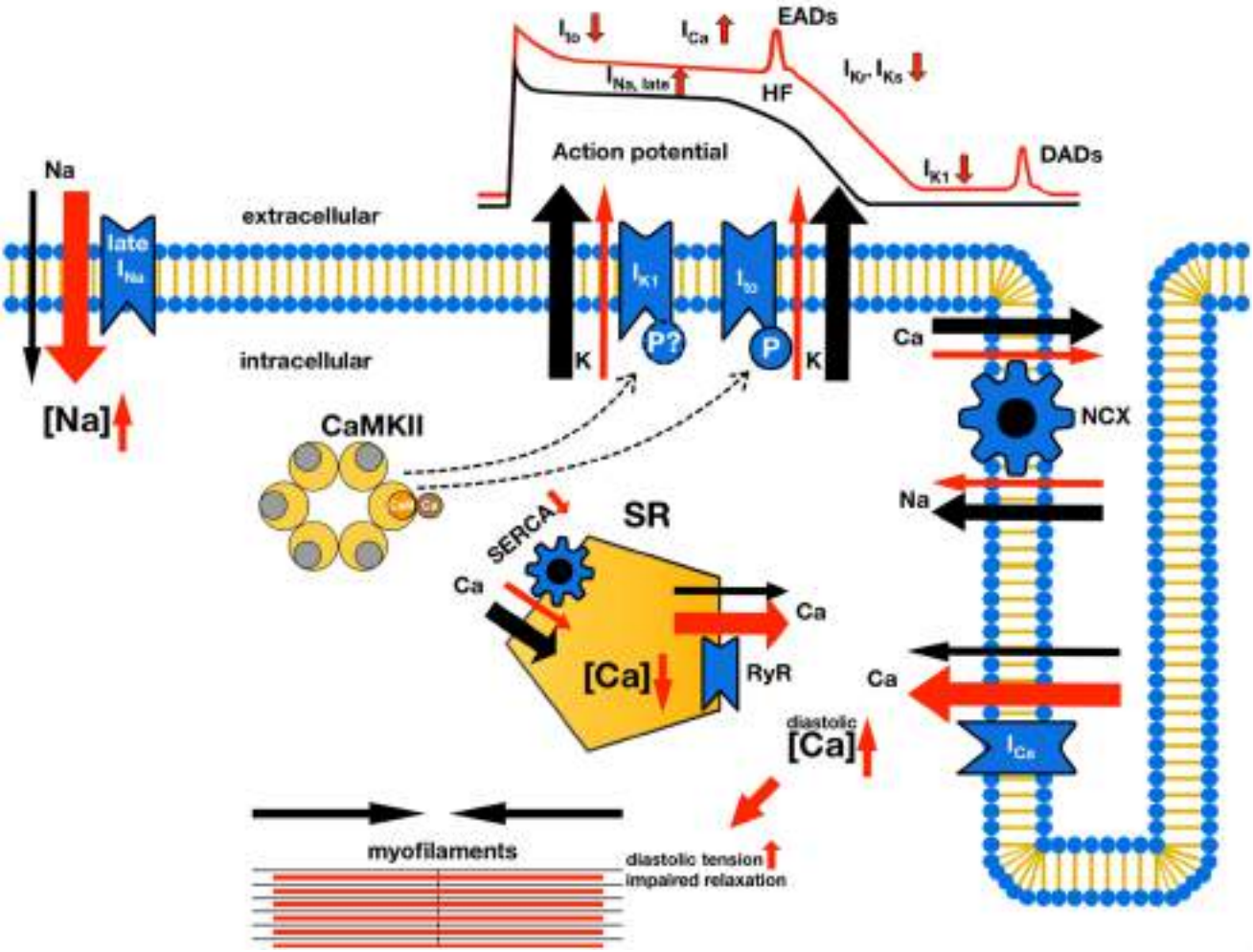

FIGURE 1 | Electro-mechanical remodeling in ventricular myocytes in HF. Normal currents are indicated by black arrows, while changes in HF are indicated by red arrows and changes in size to indicate an increase or decrease in current density. CaMKII effects on potassium currents are indicated by bar-headed lines (in this figure only CaMKII effects on K currents are shown, for a detailed review of CaMKII effects refer to Maier and Bers, 2007). Decreased expression and function of repolarizing K currents (I_{to}, I_{Kr}, I_{Ks}, I_{K1}), for instance due CaMKII-mediated effects leads to prolongation of the AP duration. This can result in greater systolic Ca entry through voltage-gated Ca channels, but also Na entry through increased late Na current (via voltage-gated Na channels, Shryock et al., 2013). Increased cytosolic Na concentrations, a feature also observed in HF (Pieske et al., 2002), together with prolonged AP duration also impairs the Ca export function of cardiac Na/Ca exchanger (Bers, 2002a), which further aggravates the net gain in cytosolic Ca. In the face of a reduced function of the sarcoplasmic reticulum (SR) Ca ATPase in HF **(SERCA; Hasenfuss, 1998)**, this Ca remains in the cytosol thereby dramatically impairing diastolic function. Moreover, increased depolarizing currents (Na and Ca currents) during the plateau phase of the AP could lead to early afterdepolarizations (EADs), while increased diastolic SR Ca leak through ryanodine-receptor 2 (RyR) facilitates delayed afterdepolarizations (DADs).

It was shown that this mutation results in gain of function in K_{ATP} (Medeiros-Domingo et al., 2010), consequently resulting in increased transmural heterogeneity of repolarization (see below).

Interestingly, besides rare congenital disease, altered K channel function has also been described for HF. It was shown that decreased I_{K1} and I_{to} density could lead to AP prolongation (Kaab et al., 1998).

Increased triggered activity is an important consequence of prolonged repolarization. The longer phase 2 of the AP results in reactivation of Ca channels that generate a depolarizing current possibly resulting in an EAD and ultimately leading to a triggered AP (Weiss et al., 2010). On the other hand, K channels have been also been shown to be involved in the generation of delayed afterdepolarizations (DADs) that are a consequence of cytosolic and sarcoplasmic reticulum (SR) Ca overload. The latter causes an increased propensity of spontaneous ryanodine-receptor (RyR) activation leading to a depolarizing inward NCX current (Kääb et al., 1996). Interestingly, this inward NCX current is more likely to induce DADs if I_{K1} is functionally downregulated, causing an unstable resting membrane potential (Dhamoon and Jalife, 2005).

Differential K channel expression across the ventricular wall is the basis for transmural dispersion of repolarization (TDR,

Antzelevitch and Fish, 2001). Physiologically, the endocardial myocyte has a smaller I_{to} amplitude compared to the epicardial myocyte. This, together with increased depolarizing currents, contributes to a more positive AP plateau and a longer AP duration in the endocardial compared to the epicardial myocyte. The result is a physiological TDR that also determines the positive T wave on the surface ECG. However, under pathophysiological conditions this fine balanced regional difference in K channel function can be substantially altered. A preferential shortening of the epicardial AP by enhanced I_{to}, for instance, together with a preferential prolongation of the endocardial AP by enhanced late I_{Na} and minor changes in the small I_{to} would increase the TDR. While a TDR increase in phase 1 and 2 of the AP results in the occurance of a J wave (positive deflection at the QRS-ST junction; Yan and Antzelevitch, 1996), increased TDR in phase 3 and 4 can cause abnormal T waves. If the increase in TDR in phase 3 and 4 reached a threshold, abnormal electrical activity would find excitable myocytes, resulting in reentry and leading to torsade de pointes (Yan and Antzelevitch, 1996). Computational modeling of a rabbit ventricular myocyte overexpressing CaMKII was used to investigate the importance of the expression level of I_{to} for AP duration (Grandi et al., 2007). If 100% I_{to} expression was used (= epicardial myocytes), CaMKII overexpression resulted in a shortening of the AP duration mainly due to a CaMKII-dependent enhancement of I_{to}. With 10% I_{to} expression (= endocardial myocytes), however, AP duration increased because CaMKII-enhanced late I_{Na} and L-type Ca current outweighed the effect on the smaller I_{to}. The mechanisms by which CaMKII alters potassium channel expression and function will be discussed in this review.

CaMKII AND HF

Calcium-Calmodulin-dependent kinase II is a serine/threonine kinase that can regulate multiple ion channels and transporters including K channels (see below). Currently, four isoforms and up to 30 splice-variants of the serine/threonine CaMKII have been identified, with CaMKIIδ as the predominant cardiac isoform (Maier and Bers, 2007). CaMKII contains an N-terminal catalytic kinase-domain with an ATP-binding site as well as substrate binding sites. Adjacent to the catalytic subunit, an autoregulatory domain with a calmodulin (CaM)-binding site and important regulatory threonine (T287, T306, T307) and methionine residues (M281/282) precedes the C-terminal association-domain, which is critical for the assembly of the holoenzyme. *In vivo*, self-association of CaMKII holoenzymes forms two ring-like CaMKII-hexamers which are stacked on top of each other (dodecameric configuration; Rellos et al., 2010). CaMKII is activated by binding of a Ca/CaM complex to its autoregulatory domain, resulting in conformational changes which expose the catalytic subunit, enabling ATP and substrate binding. An important substrate is the autoregulatory domain of an adjacent subunit, resulting in inter-subunit phosphorylation at T287 (auto-phosphorylation). The latter enables CaM-independent activity after the dissociation of Ca/CaM (Maier and Bers, 2007). Novel alternative activation pathways have also been described involving oxidation or glycosylation at M281/282, both of which result in Ca-independent activity similar to auto-phosphorylation (Erickson et al., 2008, 2013).

CaMKII has been associated with HF development. In human HF, expression and activity of CaMKII is increased (Hoch et al., 1999; Kirchhefer et al., 1999; Ai et al., 2005). Moreover, CaMKIIδ-transgenic mice develop HF with increased AP duration, disturbed Ca handling, and are prone to ventricular arrhythmias (Maier, 2003; Wagner et al., 2011). In contrast, transgenic CaMKII inhibition or CaMKII knockout prevents cardiac remodeling and HF development after myocardial infarction or increased afterload (Zhang et al., 2005; Backs et al., 2009; Ling et al., 2009).

TRANSIENT OUTWARD K CURRENT

I_{to} is generated by a pore-forming α-subunit with six transmembrane segments (S1–S6). Accessory β-subunits can associate with this α-subunit (**Figure 2A**, Niwa and Nerbonne, 2010). In their either homologous or heterologous tetrameric assembly, the subunits' S5 and S6 segments face each other to form the pore, while segment S4 senses voltage (Snyders, 1999; Niwa and Nerbonne, 2010). I_{to} is critical for the early repolarization ("notch") immediately following the upstroke in phase 0 of the cardiac AP. There are at least two components of I_{to} generated by different channel isoforms, that can be distinguished according to their recovery and inactivation kinetics (**Figure 2B**; Brahmajothi et al., 1999). The fast component ($I_{to,fast}$) inactivates and recovers with time constants (τ) of less than 100 ms, whereas the slow component ($I_{to,slow}$) inactivates with τ of about 200 ms and recovers with τ ranging from hundreds of milliseconds up to several seconds (Brahmajothi et al., 1999; Xu et al., 1999).

In human, rat, and canine tissue, I_{to} is generated mostly by the rapidly recovering channel population Kv4.3 (KCND3, Dixon et al., 1996). In a tachycardia-induced canine model of HF, a reduced ventricular Kv4.3 protein expression has been reported along with decreased I_{to} density (Zicha et al., 2004). It has also been shown that Kv4.3 expression is significantly reduced in human HF and that this is associated with a significant decrease in I_{to} density (Kaab et al., 1998; Zicha et al., 2004). Reduced I_{to} density is known to contribute to AP prolongation and prolonged QT intervals (Barry et al., 1998).

Despite this important role of Kv4.3 for I_{to} in human cardiac tissue, many animals species show a rather heterogeneous channel population comprised of Kv4.3, Kv1.4 (*KCNA4*), Kv4.2 (*KCND2*), and accessory KChIP subunits. In these species, I_{to} can be separated into the fast and slow component with varying relative contributions to total I_{to}. In rabbit and mouse cardiac myocytes, for instance, Kv1.4 has been shown to be responsible for the slow component while a complex of Kv4.2, Kv4.3, and KChIPs is responsible for the fast component (Niwa and Nerbonne, 2010). Similar to the dog model of HF, tachycardia-induced HF in rabbits showed reduced total I_{to} density while AP duration was prolonged (Rose, 2005). Interestingly, mRNA levels of Kv1.4 and Kv4.2, as well as KChIP2, were significantly reduced, while Kv4.3 mRNA was unchanged. Protein expression of Kv4.2 and KChIP2 was significantly reduced, though Kv4.3 and Kv1.4 were unchanged (Rose, 2005). In a TNF-α-overexpressing mouse model of HF, $I_{to,fast}$ density and Kv4.2 protein expression was significantly reduced (Petkova-Kirova, 2006). Other mouse models

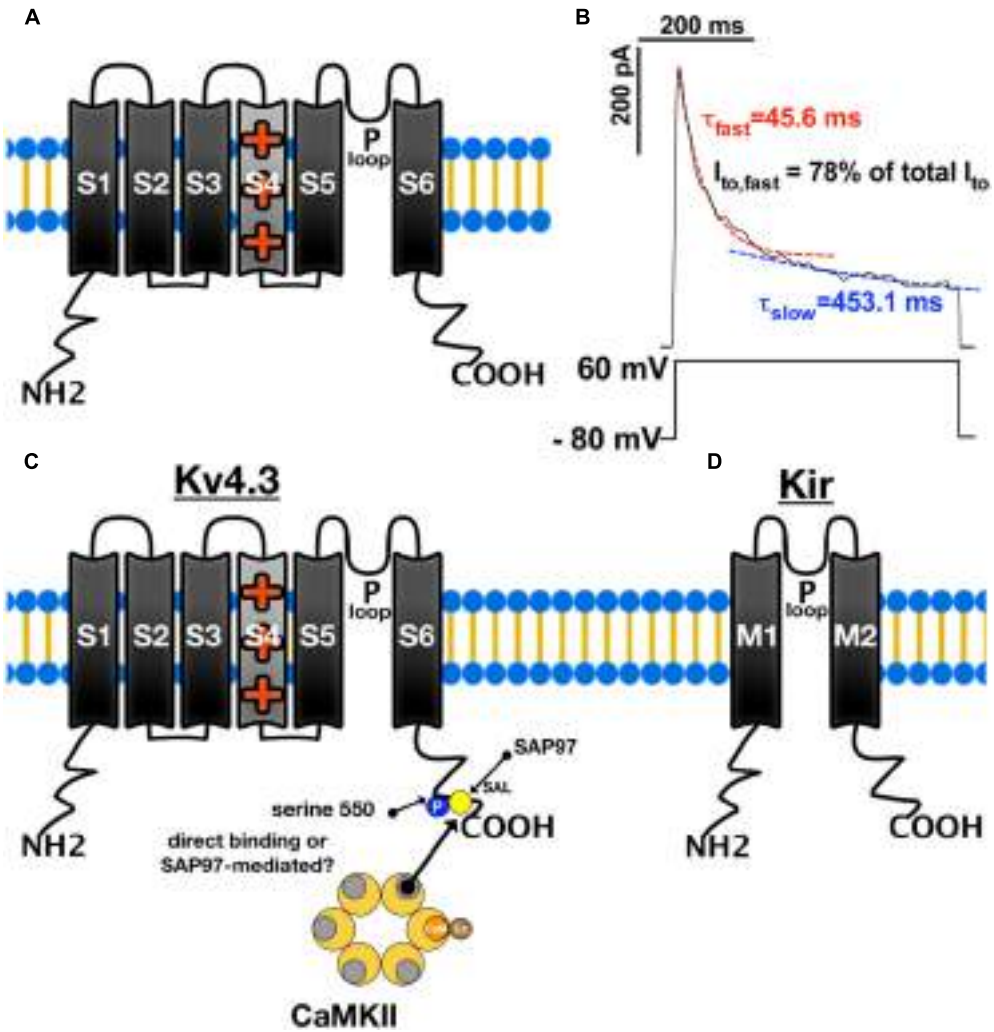

FIGURE 2 | Structure and function of K channels. (A) Structure of voltage-gated K channel α-sunbunit (Kv) with six transmembrane segments (S1–S6). The S5–S6 segments face each other to form the central pore. The P-loop between the S5 and S6 segments acts as an ion conductance pathway and its signature motif G(Y/F)G functions as a K ion selectivity filter. Segment S4 senses voltage and moves outward during cell membrane depolarization resulting in conformational changes which open the pore. **(B)** There are two current components of I_{to} generated that can be distinguished according to their inactivation kinetics. $I_{to,fast}$ inactivates with time constants (τ) of less than 100 ms, whereas the $I_{to,slow}$ inactivates with τ of about 200 ms. **(C)** CaMKII can bind to Kv4.3 and phosphorylate serine 550 of its C terminus, which leads to altered current kinetics. SAP97 can also bind to Kv4.3 [at its Ser-Ala-Leu (SAL) segment] and possibly mediates the CaMKII-Kv4.3 interaction. **(D)** Inward rectifying potassium channels are formed by four α-subunits containing only two transmembrane segments (M1–M2) with a central P-loop as ion conductance pathway.

of HF exhibited similar reductions in I_{to} density and increased AP duration (Knollmann et al., 2000; Mitarai, 2000).

The differential regulation in the expression and function of the various channel isoforms underlying I_{to} suggests that the two components $I_{to,fast}$ and $I_{to,slow}$ are functionally and structurally independent ion currents.

CaMKII-DEPENDENT REGULATION OF I_{to} EXPRESSION

CaMKII has been shown to influence the expression of channel isoforms underlying I_{to}. In mice overexpressing CaMKIIδ, it was shown that total I_{to} density is significantly reduced (Wagner et al., 2009). This reduction was secondary to a reduced expression of Kv4.2 with reduced $I_{to,fast}$ and accompanied by a prolongation of the cardiac AP (Wagner et al., 2009). In contrast, expression of Kv1.4 and $I_{to,slow}$ were increased but this increase could not fully compensate the reduction in $I_{to,fast}$ (Wagner et al., 2009). Interestingly, chronic CaMKII inhibition in mice by transgenic expression of the specific CaMKII inhibitory peptide AC3-I, a derivative of CaMKII substrate autocamtide-3, resulted in an increase in $I_{to,fast}$ and shorter AP duration. On the other hand, the increase in I_{to} density was absent in crossbred mice expressing AC3-I but lacking phospholamban (PLN; Li et al., 2006). Since mice overexpressing CaMKIIδ also develop HF and chronic CaMKII inhibition may also affect the SR, it is not clear whether these changes are CaMKII-specific or secondary to remodeling or interference with other pathways. This is supported by the fact

that short-term overexpression of CaMKIIδ in rabbit myocytes increases I_{to} (Wagner et al., 2009). Similary, in silico experiments with simulated CaMKII overexpression in rabbit myocytes also led to an increase in I_{to} along with faster $I_{to,slow}$ recovery from inactivation (Grandi et al., 2007). Furthermore, Li et al. (2006) found no change in the expression of pore-forming subunits Kv4.2/Kv4.3 underlying increased I_{to} but only a downregulation of the accessory subunit KChIP2, suggesting that the regulation of I_{to} is complex, involving many interacting partners. In this respect it is not surprising that Kv4.3 and Kv4.2 form large macromolecular complexes with other proteins such as diaminopeptidyl transferase-like protein 6 (DPP6) and Eps15 homology domain-containing protein 4 (EHD4) (Marionneau et al., 2011). Recent evidence suggests that these proteins are important for endocytosis, vesicular recycling and trafficking (Cai et al., 2013). Perhaps more importantly, it has been shown that KChIP1 clamps two adjacent Kv4.3 α-subunits together via two contact interfaces that interact with the N-termini of Kv4.3 (Pioletti et al., 2006; Wang et al., 2006b). This stabilizes K_V4.3 tetramers and also exerts an influence on current kinetics with current density being increased, inactivation slowed, and recovery from inactivation enhanced (Wang et al., 2006b). In addition, KChIP1 has been shown to be essential for proper Kv4 trafficking to the membrane (Cui et al., 2008).

More evidence that the downregulation of Kv4 in HF after CaMKII overexpression may be secondary and not directly mediated by CaMKII is derived from experiments investigating the interaction of the MAGUK (membrane-associated guanylate kinase) protein SAP97 with Kv4.

In neurons, the interaction of the C-terminal Ser-Ala-Leu (SAL)-sequence of Kv4.2 with SAP97 has been shown to be crucial for trafficking of Kv4.2 to the synaptic membrane (Gardoni et al., 2007). Interestingly, this trafficking has been shown to be enhanced by CaMKII phosphorylation of SAP97 at Serin-39 (Gardoni et al., 2007). Furthermore, it was shown in cardiac myocytes that Kv4.2/Kv4.3 channels form complexes with SAP97 and CaMKII (El-Haou et al., 2009). In the same publication, suppression of SAP97 in rat atrial myocytes via shRNA led to a decrease in I_{to}, whereas SAP97 overexpression resulted in enhanced I_{to}. Moreover, expression of Kv4.3 lacking the C-terminal SAL-sequence or SAP97 silencing via shRNA abolished the co-precipitation with CaMKII (El-Haou et al., 2009). Also, inhibition of CaMKII with autocamtide-2 related inhibitory peptide (AIP) resulted in reduced I_{to} and the inhibition was more pronounced after SAP97 overexpression (El-Haou et al., 2009). Interestingly, recent evidence suggests that SAP97 is downregulated in patients with dilated cardiomyopathy (Szuts et al., 2013).

CaMKII-DEPENDENT REGULATION OF I_{to} GATING

The first evidence for a CaMKII-dependent regulation of cardiac potassium channel gating came from a study investigating human atrial myocytes (Tessier et al., 1999). The myocytes were isolated from donors with either chronic atrial dilation or chronic atrial fibrillation (AF). Patch–clamp experiments showed that inactivation of I_{to} was accelerated by CaMKII inhibition with either KN-93 or AIP (Tessier et al., 1999). Moreover, Tessier et al. (1999) also showed an increased expression level of CaMKII in the atrium of donors with chronic atrial dilation or chronic AF. More evidence for CaMKII regulating I_{to} came from experiments using transgenic mice overexpressing CaMKIIδ$_c$ and also short-term CaMKIIδ$_c$ overexpression in rabbit myocytes. It was shown that the recovery from inactivation of $I_{to,fast}$ and $I_{to,slow}$ was enhanced by CaMKII overexpression (Wagner et al., 2009). This enhancement could be blocked by acute CaMKII inhibition suggesting that this was not secondary to HF development (Wagner et al., 2009). The underlying mechanism of CaMKII-dependent regulation of I_{to} may involve direct CaMKII-dependent phosphorylation of Kv4.2 at serine 438/459, and of Kv1.4 at serine 123 (Roeper, 1997; Varga, 2004). Patch-clamp experiments in HEK-293 cells transfected with Kv4.3 showed that addition of autothiophosphorylated (pre-activated) CaMKII slowed I_{to} inactivation consistent with the results obtained by Tessier et al. (1999) and enhanced recovery from inactivation (Sergeant, 2005). Conversely, HEK cells treated with CaMKII-inhibitor KN-93 or CaMKII-inhibitory-peptide displayed significantly accelerated current inactivation and slowed recovery from inactivation (Sergeant, 2005). Moreover, if the C-terminal Kv4.3 mutant (serine 550 to alanine) was expressed, I_{to} inactivation was enhanced and I_{to} recovery was slowed (Sergeant, 2005). Neither addition of autothiophosphorylated CaMKII nor dialysis of CaMKII inhibitors could affect I_{to} recovery in HEK cells expressing this Kv4.3 S550A mutant (Sergeant, 2005), supporting the concept that the C-terminus of Kv4.3 is a hotspot for CaMKII-dependent association and regulation of $I_{to,fast}$ (**Figure 2C**). Further evidence for a direct regulation of $I_{to,fast}$ by CaMKII comes from studies in rat ventricular myocyte lysates showing that CaMKII co-immunoprecipitates with both Kv4.3 and Kv4.2 (Colinas, 2006), and inhibition of CaMKII with KN-93 resulted in a significant acceleration of I_{to} inactivation even through recovery from inactivation was unaffected (Colinas, 2006).

Interestingly, this CaMKII-dependent enhancement of I_{to} may also be important for reactive oxygen-species (ROS) induced arrhythmogenesis. ROS are known to oxidize and activate CaMKII (Erickson et al., 2008; Wagner et al., 2011) and ROS-induced arrhythmias are known to be CaMKII-dependent (Wagner et al., 2011). Recently, it was proposed that ROS-dependent activation of I_{to} favors EADs by facilitating I_{Ca} reactivation (Zhao et al., 2012).

Thus, CaMKIIδ$_c$ appears to regulate both channel expression and/or trafficking, but also acutely regulates channel gating properties. In both cases, acute regulation results in an enhancement of I_{to}. In contrast to this, chronic CaMKII overexpression that leads to HF development results in a reduction of I_{to} but this appears to be a secondary effect.

Kv4.3 AS AN IMPORTANT REGULATOR OF CaMKII ACTIVITY

While Kv4.3 is an important target for CaMKII, it may also influence CaMKII localization and activity. Recently, in HEK-293 cells transfected with Kv4.3 and His-tagged CaMKII, it was shown that Kv4.3 binds to CaM-dissociated CaMKII competitively at its CaM binding site (residues 301 and 307; Keskanokwong et al., 2010). This binding was independent from the auto-phosphorylation status of CaMKII, since both constitutively active (T-287D) or inactive (T-287A) CaMKII-mutants could also bind to Kv4.3 (Keskanokwong et al., 2010).

Since the CaMKII inhibitor KN93 also binds CaMKII at the CaM binding site (Sumi, 1991), it is conceivable that KN-93 disturbs the interaction of CaMKII and Kv4.3. Consistent with this idea, Keskanokwong et al. showed that co-purification of Kv4.3 and CaMKII is abolished upon addition of KN-93. Furthermore, it was shown that application of the Kv4.3 blocker 4-aminopyridine (4-AP) disturbes the co-purification of CaMKII and Kv4.3 in HEK-293 cells, while CaMKII auto-phosphorylation is increased (Keskanokwong et al., 2010). Similarly, increased CaMKII activity was found in guinea pig ventricular myocytes treated with 4-AP (Wang et al., 2006a). Moreover, 4-AP-induced blockade of Kv4.3 in HEK-293 cells has been shown to result in increased apoptosis and enhanced CaMKII-auto-phosphorylation, while the authors were able to prevent apoptosis by inhibition of CaMKII with KN-93 (Zhang et al., 2012).

This suggests that Kv4.3 may function as a reservoir for inactive CaMKII-units and exert an influence on CaMKII activation levels (**Figure 2C**). In accordance with this hypothesis, *in vivo* overexpression of Kv4.3 in mouse ventricular myocardium via multiple-site virus injection decreased the level of phosphorylated CaMKII, while CaMKII expression was not affected. CaMKII bound to Kv4.3 was also shown to be protected from activation by systolic Ca transients (Keskanokwong et al., 2010). The Kv4.3-CaMKII interaction may also be important for the regulation of other CaMKII target proteins. For instance, it was shown that blockade of Kv4.3 with 4-AP results in increased I_{Ca} that could be blocked by buffering cytosolic Ca with BAPTA or application of AIP (Wang et al., 2006a).

As previously mentioned, Kv4.3 is downregulated in HF (Kaab et al., 1998) while CaMKII is upregulated (Hoch et al., 1999). The CaMKII-Kv4.3 interaction may thus be severely altered in HF, contributing to higher CaMKII activity. In this context, the previously mentioned role for SAP97 in the regulation of Kv4.3 expression and Kv4.3-CaMKII interaction may be important. Downregulation of SAP97 in HF (Szuts et al., 2013) may underlie reduced Kv4.3 and may contribute to increased CaMKII activity.

INWARDLY RECTIFYING CURRENT I_{K1}

In contrast to the voltage-gated K channels, inwardly rectifying potassium channels [Kir2.1 (*KCNJ2*), Kir2.2 (*KCNJ12*), Kir2.3 (*KCNJ4*), and Kir2.4 (*KCNJ14*)] are formed by four α-subunits containing only two transmembrane segments (M1–M2) with a central P-loop but without a voltage-sensor (**Figure 2D**; Hibino et al., 2010). The main characteristic of this class of potassium channels is inward rectification, which features a strong potassium conductance during hyperpolarization, but a decrease in ion conductance upon depolarization due to blockade of the pore by Mg, Ca and cell membrane polyamines (Matsuda et al., 1987; Matsuda and Cruz, 1993; Hibino et al., 2010).

This peculiar inward rectifying property of the Kir2.x channels that generate I_{K1} renders these channels important stabilizers of the resting membrane potential by neutralizing resting influx of positive ions (Fauconnier et al., 2005). In addition, Kir2.x channels also contribute to late-phase (phase 4) repolarization (Dhamoon, 2004; Dhamoon and Jalife, 2005; Fauconnier et al., 2005).

In sinoatrial myocytes, the expression of channels forming I_{K1} is notably reduced, which allows for an unstable resting membrane potential that can be depolarized by I_f, thus inducing diastolic depolarization (Bers, 2002b).

There is evidence that Kir2.x isoforms can assemble as homo- or heterotetrameres (Zobel et al., 2003). The functional characteristics of I_{K1} depend very much on the Kir isoforms that comprise I_{K1} (Panama et al., 2010), since rectification of current at depolarized membrane potentials (> -30 mV) is complete for Kir2.1 and Kir2.2, but incomplete for Kir2.3 (Dhamoon, 2004). There is great variability in the expression of these isoforms between left and right ventricle (Warren et al., 2003) but also atrium and ventricle (Gaborit et al., 2007). Similar to channel subunits generating I_{to}, expression of the Kir isoforms appears to be strongly species-dependent (Jost et al., 2013).

Ca OR CaMKII-DEPENDENT REGULATION OF I_{K1}

I_{K1} functional expression also seems to be regulated differently under pathophysiological conditions. It was shown that I_{K1} density is reduced in failing rat ventricular myocytes (Fauconnier et al., 2005). Interestingly, this reduction was attenuated in the presence of high EGTA (10 mmol/L) and abolished if intracellular Ca was buffered with BAPTA (20 mM; Fauconnier et al., 2005). Moreover, activation of RyR by application of ryanodine or FK506 led to a similar reduction of I_{K1} density in non-failing wild-type rat ventricular cells and this effect could be blocked by Ca-buffering with BAPTA (Fauconnier et al., 2005). Whether this reduction occurs via direct Ca-dependent blockade of I_{K1} via the mechanism described (Matsuda and Cruz, 1993) or mechanisms involving altered expression/trafficking of the underlying Kir isoforms is, however, completely unknown. Fauconnier et al. (2005) also suggested the involvement of protein kinase C (PKC), since the PKC inhibitor staurosporine antagonized the effect of ryanodine on I_{K1}. PKC has been shown to phosphorylate Kir2.1 at serine 64 and threonine 353, leading to reduced I_{K1} in human atrial myocytes (Karle, 2002). On the other hand, opposite results have recently been shown in canine ventricular myocytes. Addition of 900 nmol/L Ca in the patch pipette significantly increased I_{K1} current compared to measurements with 160 nM Ca (Nagy et al., 2013). Therefore, the effect of Ca on I_{K1} may be species-dependent. Supporting evidence comes from intact field-stimulated (1 Hz) canine right ventricular papillary muscle. Increasing extracellular Ca from 2 to 4 mmol/L, resulted in increased Ca transient amplitude and significantly shortened AP duration. This Ca-dependent AP shortening could be prevented by inhibition of I_{K1} using $BaCl_2$. Moreover, $BaCl_2$ preferentially prolonged AP duration at 4 mM $[Ca]_o$ vs. 2 mM $[Ca]_o$. The authors conclude that Ca-dependent enhancement of I_{K1}, at least in canine myocytes, may be an important contributor to repolarization reserve and an endogenous negative feedback mechanism inhibiting the generation of DADs due to high Ca levels (Nagy et al., 2013). Moreover, Nagy et al. (2013) also showed that CaMKII inhibition with KN-93 abolished the Ca-induced activation of I_{K1}, suggesting that CaMKII is also involved in I_{K1} regulation. Supporting evidence for a CaMKII-dependent activation of I_{K1} also comes from rabbit ventricular myocytes. Acute overexpression of CaMKIIδ by adenovirus-mediated gene transfer resulted in a significant increase in I_{K1} density that could be blocked by addition of CaMKII-inhibitory peptide AIP to

the pipette (Wagner et al., 2009). In the same, study, transgenic CaMKIIδ overexpression in mice that develop HF, however, resulted in a reduced I_{K1} density and reduced expression of Kir2.1 (Wagner et al., 2009). Thus, the discrepancy between the studies showing either increased or decreased I_{K1} may be due to species-differences but this remains speculative. In accordance, mouse ventricular myocytes with transgenic inhibition of CaMKII showed an increased I_{K1} and a shorter AP duration without a significant change in Kir2.1 and Kir2.2 expression levels (Li et al., 2006).

Aside from the species, it could also be relevant if the studied model results in HF. It was shown that SAP97 co-immunoprecipitates with Kir2.2 in rat hearts (Leonoudakis et al., 2004). Interestingly, in human dilated cardiomyopathy, the co-localization of SAP97 with Kir2.x was shown to be disturbed (Szuts et al., 2013). This suggests that a mechanism similar to the above mentioned Kv4.x-SAP97 interaction may be present. Therefore, further studies are greatly needed to clarify the importance of Ca and CaMKII for the regulation of I_{K1} in different animal models and in human disease (**Table 1**).

I_{K1} AND ARRHYTHMIAS

I_{K1} is generally regarded as anti-arrhythmic by stabilizing resting membrane potential. In a canine model of tachycardia-induced HF, reduced I_{K1} has been shown to increase the propensity for sudden cardiac death and ventricular tachycardia (Kääb et al., 1996). Also, loss of function mutations in *KCNJ2* have been associated with long QT syndrome (LQT7), in which increased AP duration and increased propensity for arrhythmias can be observed (Tsuboi and Antzelevitch, 2006).

On the other hand, contrasting results have been shown for wild-type Kir2.1 overexpressing mice that have an increased propensity for ventricular arrhythmias (Noujaim et al., 2006; Piao et al., 2007) or AF (Li, 2004). Kir2.1 knock-down in mice was associated with longer AP duration and a reduced incidence of premature ventricular contractions before and after AV node ablation, reduced arrhythmias due to extracellular hypokalemia, and a reduced incidence of halothane-induced ventricular tachycardia (Piao et al., 2007).

This discrepancy may be solved by the fact that both increase or decrease of I_{K1} can be pro-arrhythmic if there is a substantial spatial heterogeneity in the functional expression profile (Sekar et al., 2009). In accordance with this, gain-of-function mutations in *KCNJ2* can result in short QT syndrome (SQT3), which is also pro-arrhythmogenic (Brenyo et al., 2012).

DELAYED RECTIFYING K CHANNELS

The three channels Kv1.5 (*KCNA5*), hERG (*KCNH2*), and Kv7.1 (*KCNQ1*) comprise the group of the delayed rectifying K channels. They generate I_{Kur} (ultra rapid), I_{Kr} (rapid), and I_{Ks} (slow), respectively. Together, they are important currents for phase 3 repolarization.

I_{Kur} is only present in atrial myocardium. In chronic human AF, it was shown that AP duration is reduced, possibly contributing to the arrhythmogenic mechanisms (Wettwer, 2004). Evidence for a role of Kv1.5 in AF came from a study investigating pharmacological Kv1.5 inhibition in a canine model of AF (Regan et al., 2007). They could show that AF terminates if Kv1.5 is inhibited. Furthermore, SAP97 was reported to co-immunoprecipitate with Kv1.5 (Murata et al., 2001) resulting in increased I_{Kur} (Godreau et al., 2002; Eldstrom et al., 2003).

Since SAP97 and CaMKII have been shown to interact (El-Haou et al., 2009), CaMKII expression is increased in AF (Tessier et al., 1999; Neef et al., 2010), and given the similarities between SAP97-dependent Kv4.3 and Kv1.5 regulation (Tessier et al., 1999; Godreau et al., 2002; El-Haou et al., 2009), it seems tempting to speculate that CaMKII could also regulate Kv1.5. Interestingly, in human atrial myocytes it was shown that CaMKIIδ is especially localized at intercalated disks, the region where Kv1.5 is also located (Tessier et al., 1999). Furthermore, Tessier et al. (1999) showed that selective inhibition of CaMKII with KN-93 or AIP reduced the amplitude of the sustained component of outward K current (I_{sus}), whereas inhibition of phosphatases with okadaic acid increased I_{sus} (Tessier et al., 1999; Tessier, 2001). This I_{sus} is regarded as mainly generated by Kv1.5 (Fedida et al., 1993), suggesting that CaMKII, possibly by phosphorylation, regulates Kv1.5 (Tessier et al., 1999).

Besides I_{Kur}, other K currents may also be involved in AF. I_{K1}, for instance, has been shown to be upregulated in AF possibly contributing to shortening of AP duration (Dobrev and Ravens, 2003).

I_{Ks} is comprised of the pore-forming α-subunit Kv7.1, but also the auxiliary β-subunit KCNE1 (Ruscic et al., 2013). Loss-of-function mutations in *KCNQ1* are linked to an increase in AP duration associated with long QT-syndrome type I, whereas gain-of-function mutations in *KCNQ1* are associated with short QT-syndrome (SQT2) (Brenyo et al., 2012) and familial AF (Chen et al., 2003). Additionally, loss-of-function mutations in *KCNE1* have been associated with long QT-syndrome 5 (Splawski et al., 1997), which points out the important role of KCNE1 for the generation of I_{Ks}. Indeed it has been shown that KCNE1 is important in slowing down the movement of the voltage-sensor S4 of Kv7.1 upon depolarization, thus explaining the slow activation kinetics of I_{Ks} (Ruscic et al., 2013). Similar mechanisms may very well regulate other voltage-gated channels and underlie their distinct activation kinetics.

Interestingly, co-immunoprecipitation experiments in yeast cells expressing wild-type Kv7.1 or mutated Kv7.1 with truncated α-helices showed that calmodulin can bind to the C-terminus of Kv7.1 (Shamgar, 2006). This IQ-motif appears to be a hot spot for mutations: yeast 2-hybrid experiments indicated that *KCNQ1* mutations A371T and S373P, which are associated with LQTS, lose their calmodulin-Kv7.1 interaction.

Moreover, agarose-pulldown assays in HEK-293 cells revealed that LQTS-associated Kv7.1 mutants W392R, S373P, and A371T bound significantly less calmodulin than wild-type Kv7.1 (Shamgar, 2006). This disturbed calmodulin- Kv7.1 interaction may be important for channel expression. Cell surface expression experiments with biotinylated channel proteins showed that mutants with impaired CaM-binding are significantly less expressed than wild-type Kv7.1 (Shamgar, 2006). Interestingly, overexpression of calmodulin in HEK-293 cells either expressing wild-type Kv7.1 or mutant S373P showed significant increases in Kv7.1 (5x) as well as S373P (100x) cell surface and protein expression, which highlights

Table 1 | Synopsis of studies investigating I_{K1} and arrhythmias.

Species	Model	I_{K1} Current	Phenotype	Reference
Rat ventricular myocytes	Myocardial infarction	↓		Fauconnier et al. (2005)
Canine ventricular myocytes	High intracellular calcium	↑		Nagy et al. (2013)
Rabbit ventricular myocytes	Adenoviral CaMKII overexpression	↑		Wagner et al. (2009)
Mouse ventricular myocytes	Transgenic CaMKII overexpression	↓	Polymorphic and monomorphic VTs	Wagner et al. (2006, 2009)
Mouse ventricular myocytes	Transgenic CaMKII inhibition (AC3-I expression)	↑		Li et al. (2006)
Mouse ventricular myocytes	Acute CaMKII inhibition by AC3-I dialysis	→		Li et al. (2006)
Mouse ventricular myocytes	Kir2.1 knock-down	↓	Less ventricular arrhythmias	Piao et al. (2007)
Mouse ventricular myocytes	Kir2.1 overexpression	↑	More ventricular arrhythmias	Piao et al. (2007)
Kir2.1-overexpressing [ESC]-derived myocytes	Transplantation of ESC-derived myocytes into mouse ventricles after MI	↑	Less spontaneous VTs, less inducible VTs	Liao et al. (2013)
Rabbit ventricular myocytes	Tachycardia-induced HF			Rose (2005)
Mouse ventricular myocytes	Calsequestrin-overexpression-induced HF	↓	QRS and QTc prolongation	Knollmann et al. (2000)
Mouse ventricular myocytes	Gαq-overexpression-induced hypertrophy	↓		Mitarai (2000)
Mouse ventricular myocytes	Calcineurin overexpression	→		Petrashevskaya et al. (2002)
Mouse ventricular myocytes	Dominant-negative Kv4.2 expression - induced HF	↓		Wickenden et al. (1999)
Mouse ventricular myocytes	Dominant-negative Kv4.2 expression	↓	QRS and QTc prolongation	McLerie (2003)
Mouse ventricular myocytes	Kir2.1 overexpression	↑	More inducible, more stable VTs	Noujaim et al. (2006)
Mouse ventricular myocytes	Kir2.1 overexpression	↑	Bradycardia, AF, AV-Block, PVC, short QT	Li (2004)
Guinea pig ventricular myocytes	Kir2.1 overexpression	↑	QTc shortening	Miake et al. (2003)
Guinea pig ventricular myocytes	Dominant-negative Kir2.1 expression (downregulation)	↓	QTc prolongation	Miake et al. (2003)
Human ventricular myocytes	Dilated or ischemic cardiomyopathy	↓	APD prolongation	Beuckelmann et al. (1993)
Canine ventricular myocytes	Tachycardia-induced HF	↓	APD prolongation, QTc prolongation, more VTs	Kääb et al. (1996), Pak et al. (1997)
Monolayers of cultured neo-natal rat ventricular myocytes	Homogeneous Kir2.1 overexpression	↑	No reentry arrhythmias inducible	Sekar et al. (2009)
Monolayers of cultured neo-natal rat ventricular myocytes	Heterogeneous Kir2.1 overexpression	↑	Inducible reentry arrhythmias	Sekar et al. (2009)
Monolayers of cultured neo-natal rat ventricular myocytes	Homogeneous Kir2.1 suppression	↓	No reentry arrhythmias inducible	Sekar et al. (2009)
Monolayers of cultured neo-natal rat ventricular myocytes	Heterogeneous Kir2.1 suppression	↓	Inducible reentry arrhythmias	Sekar et al. (2009)
Canine atrial myocytes	Tachycardia-induced HF	→	Inducible atrial fibrillation (AF)	Li et al. (2002)

the important role of calmodulin for I_{Ks} assembly and cell surface expression (Shamgar, 2006).

In addition, the CaM- Kv7.1 interaction may also be relevant for the regulation of I_{Ks} gating. Patch-clamp experiments of inside-out membrane from *Xenopus* oocytes showed that application of calmodulin antagonist W7 significantly reduced current density of Kv7.1/KCNE1, while an increase in Ca significantly shifted voltage-dependence of channel activation toward more hyperpolarized membrane potentials (Shamgar, 2006).

Thus, the interaction of calmodulin and Kv7.1 appears to be critical for expression and function of I_{Ks}, with the intriguing possibility that regulatory mechanisms could also involve some form of CaMKII interaction with calmodulin and Kv7.1 or KCNE1.

ATP-SENSITIVE POTASSIUM CURRENT K_{ATP}

The ATP-sensitive potassium current K_{ATP}, comprising of Kir6.1 (*KCNJ8*) and Kir6.2 (*KCNJ11*) α-subunits, plays an important role in ischemic preconditioning (Li et al., 2007). K_{ATP} can be a substrate for CaMKII: in mice expressing CaMKII-inhibitory peptide AC3-I, an increased K_{ATP} current density has been shown along with an increase in the sarcolemmal Kir6.2 membrane surface expression (Li et al., 2007). Also, recent evidence from pancreatic β-cells suggests that Kir6.2 can be phosphorylated by CaMKII at threonine 224 (Kline et al., 2013). Co-expression of CaMKII and Kir6.2 in COS-cells resulted in a decreased K_{ATP} current.

The significance of K_{ATP} in HF and arrhythmogenesis is still largely unknown. There is evidence suggesting that K_{ATP}-channel opening with cromakalim produces more stable ventricular arrhythmias (Quintanilla et al., 2013). In addition, Langendorff-perfused canine failing hearts with induced VF showed an increased rate of spontaneous VF termination, if K_{ATP} was blocked with glibenclamide (Taylor et al., 2012). Also, recently, a mutation in cardiac Kir6.1 that is associated with gain of function has been identified in a patient with early repolarization syndrome (see above; Medeiros-Domingo et al., 2010).

On the other hand, K_{ATP}-blockade with glibenclamide in non-failing canine hearts with induced VF delayed the termination of VF (Taylor et al., 2012). Thus, the role of cardiac K_{ATP} and its regulation by CaMKII has yet to be evaluated.

SUMMARY

While there is increasing evidence for an involvement of CaMKII in the regulation of K channels, many discrepancies are not yet understood. These discrepancies result from the great variability in the expression profile of K channels in different species and disease models. The greatest evidence so far exists for CaMKII-dependent regulation of Kv4.x expression, trafficking and function. Most intriguingly, the Kv4.x macromolecular complex appears to serve as a hotspot and reservoir for CaMKII, which may have profound impact on the regulation of various other CaMKII targets like Ca channels. CaMKII expression and activity has been shown to be increased in many animal models of HF, but also in human HF. Increased CaMKII activity has been shown to induce contractile dysfunction and arrhythmias. Therefore, a more detailed understanding of the mechanisms of K channel regulation by CaMKII is warranted.

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Calcium and IP3 dynamics in cardiac myocytes: experimental and computational perspectives and approaches

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Calcium plays a crucial role in excitation-contraction coupling (ECC), but it is also a pivotal second messenger activating Ca^{2+}-dependent transcription factors in a process termed excitation-transcription coupling (ETC). Evidence accumulated over the past decade indicates a pivotal role of inositol 1,4,5-trisphosphate receptor (IP_3R)-mediated Ca^{2+} release in the regulation of cytosolic and nuclear Ca^{2+} signals. IP_3 is generated by stimulation of plasma membrane receptors that couple to phospholipase C (PLC), liberating IP_3 from phosphatidylinositol 4,5-bisphosphate (PIP_2). An intriguing aspect of IP_3 signaling is the presence of the entire PIP_2-PLC-IP_3 signaling cascade as well as the presence of IP_3Rs at the inner and outer membranes of the nuclear envelope (NE) which functions as a Ca^{2+} store. The observation that the nucleus is surrounded by its own putative Ca^{2+} store raises the possibility that nuclear IP_3-dependent Ca^{2+} release plays a critical role in ETC. This provides a potential mechanism of regulation that acts locally and autonomously from the global cytosolic Ca^{2+} signal underlying ECC. Moreover, there is evidence that: (i) the sarcoplasmic reticulum (SR) and NE are a single contiguous Ca^{2+} store; (ii) the nuclear pore complex is the major gateway for Ca^{2+} and macromolecules to pass between the cytosol and the nucleoplasm; (iii) the inner membrane of the NE hosts key Ca^{2+} handling proteins including the Na^+/Ca^{2+} exchanger (NCX)/GM1 complex, ryanodine receptors (RyRs), nicotinic acid adenine dinucleotide phosphate receptors (NAADPRs), Na^+/K^+ ATPase, and Na^+/H^+ exchanger. Thus, it appears that the nucleus represents a Ca^{2+} signaling domain equipped with its own ion channels and transporters that allow for complex local Ca^{2+} signals. Many experimental and modeling approaches have been used for the study of intracellular Ca^{2+} signaling but the key to the understanding of the dual role of Ca^{2+} mediating ECC and ECT lays in quantitative differences of local $[Ca^{2+}]$ in the nuclear and cytosolic compartment. In this review, we discuss the state of knowledge regarding the origin and the physiological implications of nuclear Ca^{2+} transients in different cardiac cell types (adult atrial and ventricular myocytes) as well as experimental and mathematical approaches to study Ca^{2+} and IP_3 signaling in the cytosol and nucleus. In particular, we focus on the concept that highly localized Ca^{2+} signals are required to translocate and activate Ca^{2+}-dependent transcription factors (e.g., nuclear factor of activated T-cells, NFAT; histone deacetylase, HDAC) through phosphorylation/dephosphorylation processes.

Keywords: Ca^{2+}, IP_3, excitation-contraction coupling, excitation-transcription coupling, cardiomyocyte

Calcium is a pivotal signaling molecule and its intracellular concentration ($[Ca^{2+}]_i$) is precisely regulated in different subcellular domains. The modulation of $[Ca^{2+}]$is a crucial factor for a variety of physiological functions of living cells. In cardiac myocytes, including ventricular and atrial cells, Ca^{2+} release through channels located in the sarcoplasmic reticulum (SR) membrane and termed ryanodine receptors (RyRs), is a key event linking membrane depolarization and mechanical activity during excitation-contraction coupling (ECC) (Bers, 2001). The amount of Ca^{2+} release with each heart beat and by that the force of contraction is also modulated by hormonal action, e.g., by Endothelin I and Angiotensin II (Proven et al., 2006). These two hormones stimulate plasma membrane receptors (G protein coupled receptors, GPCRs) that couple to phospholipase C (PLC), liberating IP_3 from phosphatidylinositol 4,5-bisphosphate (PIP_2). IP_3 freely diffuses within the cytoplasm to bind to a second type of SR Ca^{2+} release channels, the inositol 1,4,5-trisphosphate receptor (IP_3R) (Roderick and Bootman, 2003; Kockskämper et al., 2008; Berridge, 2009). IP_3Rs, albeit at a much smaller density compared to ryanodine receptors (RyR:IP_3R ~100:1), are expressed

in the SR membrane and nuclear envelope (NE) (Bootman et al., 2009). The activation of IP_3Rs upon binding of IP_3 can modulate ECC by sensitizing nearby RyRs leading to positive inotropic but also pro-arrhythmic effects (Petersen et al., 1994; Vogelsand et al., 1994; Zima and Blatter, 2004; Harzheim et al., 2009). Experimental evidence accumulated over the past decade also indicates an important role of IP_3R-mediated Ca^{2+} release in excitation-transcription coupling (ETC) and pro-hypertrophic signaling (Arantes et al., 2012). The entire PIP_2-PLC-IP_3 cascade, including GPCRs and IP_3Rs, can be found in the NE (Bkaily et al., 2011; Vaniotis et al., 2011; Tadevosyan et al., 2012). The presence of nuclear GPCRs in combination with highly localized nuclear IP_3R-mediated Ca^{2+} release and Ca^{2+} removal might provide for a putative distinct signaling domain that regulates nuclear Ca^{2+} dynamics (e.g., for autocrine signaling), whereas the cytosolic Ca^{2+} is regulated separately via sarcolemmal GPCR signaling and IP_3R-mediated SR Ca^{2+} release in conjunction with Ca^{2+} release and removal by the set of proteins involved in ECC (e.g., RyR, SERCA, troponin C). Sarcolemmal GPCRs allow for paracrine signaling and positive inotropic effects mediated by hormonal stimulation (e.g., with Angiotensin II or Endothelin I), (Kockskämper et al., 2008; Bootman et al., 2009). A comprehensive understanding of the mechanisms regulating nuclear IP_3 and Ca^{2+} signals and the impact of alterations of cytosolic Ca^{2+} and IP_3 signals on nuclear functions requires well-characterized experimental approaches, but also whole-cell system mathematical models. In this review, we discuss quantitative aspects of IP_3-dependent Ca^{2+} homeostasis in adult ventricular and atrial myocytes. In particular, we focus on novel modeling and experimental approaches to support the concept that IP_3R-mediated Ca^{2+}-release and the Ca^{2+} removal machinery in the SR and NE allow for highly localized and independent cellular signaling.

EXCITATION-CONTRACTION COUPLING IN VENTRICULAR AND ATRIAL MYOCYTES AND THE ROLE OF IP_3

In cardiomyocytes, ECC describes the process of action potential (AP) triggered Ca^{2+}-induced Ca^{2+} release (CICR) providing sufficient Ca^{2+} for the activation of the proteins regulating muscle contraction and to induce active muscle force (Bers, 2001). Membrane depolarization during an AP allows Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels (LTCC) which triggers CICR and thereby amplifies the cytosolic Ca^{2+} signal to levels required for the activation of the contractile proteins. An important feature of all *ventricular myocytes*, setting them apart from most atrial cells, is the presence of plasma membrane invaginations throughout the cytosol (transverse or t-tubules), putting LTCC in close vicinity to RyRs (**Figure 1**). The SR containing RyRs that oppose LTCC is called junctional SR (jSR). The jSR is crucial for the spatiotemporal homogeneity of Ca^{2+} release leading to largely uniform cytosolic Ca^{2+} transients ($[Ca^{2+}]_i$) during a *ventricular cell* twitch (**Figure 2**), (Franzini-Armstrong et al., 1999; Heinzel et al., 2002; Louch et al., 2004; Crossman et al., 2011; Hake et al., 2012; Signore et al., 2013). Unlike in ventricular cells, the t-tubular system in *atrial myocytes* is either absent (**Figure 1**) (Hüser et al., 1996; Kockskämper et al., 2001) or poorly developed (Kirk et al., 2003). However more recent work in sheep and human has provided evidence that atrial cells from larger animals tend to have a higher density of t-tubules (Dibb et al., 2009; Richards et al., 2011), and even in rodent atrial cells an irregular internal transverse-axial tubular system has been identified that affects kinetics of SR Ca^{2+} release (Kirk et al., 2003).The absence or paucity of t-tubules in atrial cells leads to great differences in the shape and kinetics of local Ca^{2+} transients and gradients in subcellular regions where Ca^{2+} is provided by release from jSR and non-junctional SR (njSR) (**Figure 2**). Subsarcolemmal Ca^{2+} transients rise faster, have a higher Ca^{2+} peak and are initiated by Ca^{2+} currents through LTCCs, followed by RyR-mediated Ca^{2+} release from the jSR. These local jSR Ca^{2+} transients resemble Ca^{2+} release in ventricular cells. Central cytosolic Ca^{2+} transients, however, have a slower rise time and a lower peak, and result from CICR that propagates in a Ca^{2+} wave-like fashion from the periphery to the center of the cell. (Blatter et al., 2003; Maxwell and Blatter, 2012). Furthermore, the specific topological organization of the plasma membrane in atrial myocytes leads not only to different spatial $[Ca^{2+}]_i$ distribution as compared to the ventricle, it also affects nuclear Ca^{2+} transients by further delaying their onset due to the wave-like propagation of Ca^{2+} toward the nucleus (**Figure 2**). Interestingly, for both atrial and ventricular cells, a role of cytosolic IP_3 ($[IP_3]_i$) has been reported for the modulation of cytosolic Ca^{2+} transients in a variety of animal models (Zima and Blatter, 2004; Proven et al., 2006; Domeier et al., 2008; Harzheim et al., 2009; Kim et al., 2010). IP_3R channel activity, with type-2 IP_3Rs as the most prevalent isoform in cardiac myocytes, depends on $[IP_3]_i$ and $[Ca^{2+}]_i$ (Michell et al., 1981; Domeier et al., 2008; Kockskämper et al., 2008). There is evidence that atrial myocytes express functional IP_3Rs at higher densities than ventricular myocytes (**Figure 1**; in ventricular cell the IP_3Rs are not shown in the junctional space due their relatively low density) (Mackenzie et al., 2004; Zima and Blatter, 2004). As shown in **Figure 2**, the acute increase in cytosolic IP_3, induced by photolytic release of IP_3 from a caged IP_3 compound, increases cytosolic Ca^{2+} transient peak amplitudes during field stimulation in atrial cells in contrast to ventricular cells. In ventricular cells only increased expression levels of IP_3R, as it occurs in cardiac hypertrophy, could experimentally be tied to enhanced cytosolic SR Ca^{2+} release (Harzheim et al., 2009). The neurohumoral stimulation with Endothelin I or Angiotensin II, however, has been shown to have similar positive inotropic effects in both ventricular and atrial cells, indicating a role of IP_3-mediated Ca^{2+} release in the enhancement of cytosolic Ca^{2+} release (Zima and Blatter, 2004).

EXCITATION-TRANSCRIPTION COUPLING IN VENTRICULAR AND ATRIAL MYOCYTES AND THE ROLE OF IP_3

Nuclear Ca^{2+} signals however are different with regards to kinetics during action potential induced Ca^{2+} transients. This can largely be attributed to the fact that the nucleus is surrounded by the nuclear envelope (Kockskämper et al., 2008; Alonso and García-Sancho, 2011), consisting of the outer and inner nuclear membranes and the space between them that is contiguous with the SR (Wu et al., 2006; Shkryl et al., 2012). The nuclear membranes fuse at many locations to form pores (diameter ~100, length ~50 nm) that harbor the nuclear pore complexes (NPCs).

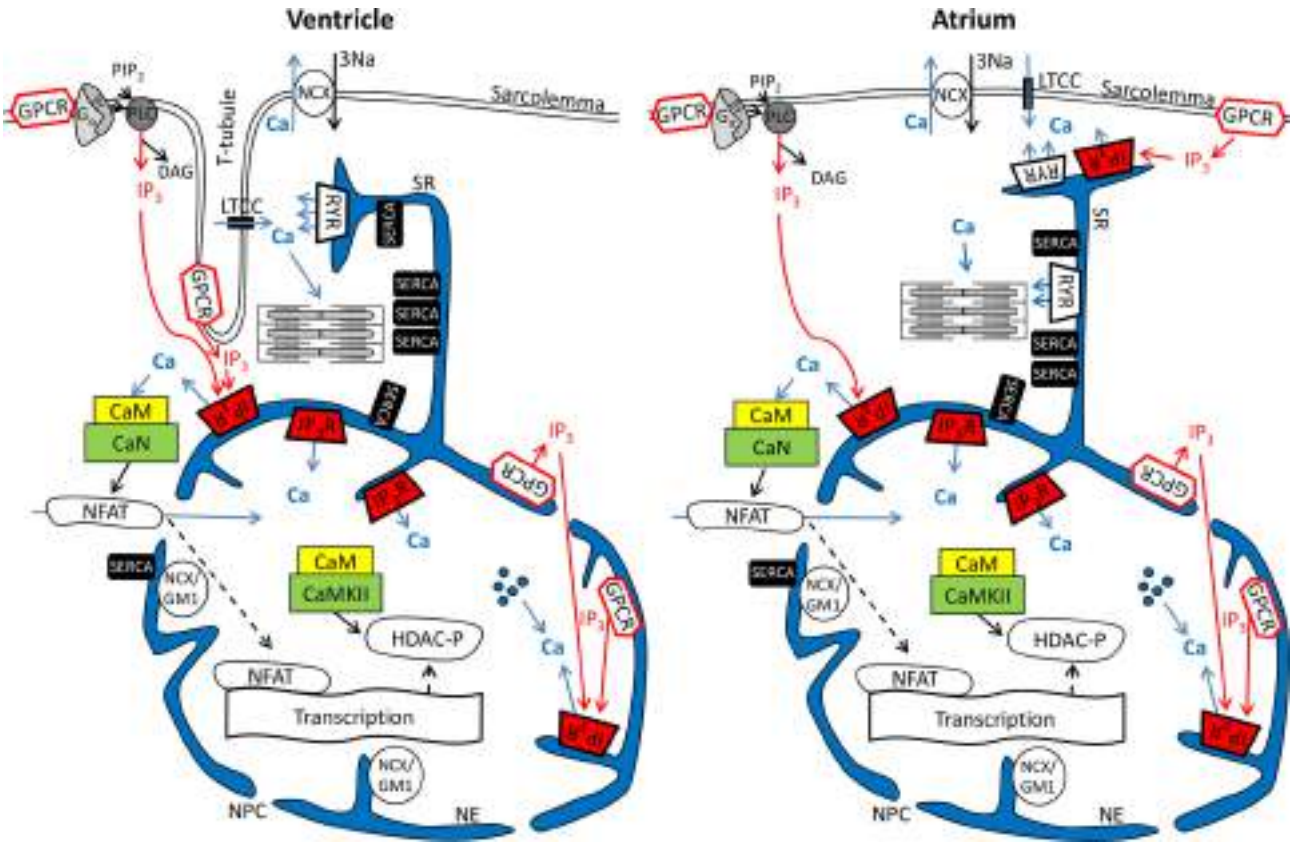

FIGURE 1 | Ca^{2+} and IP_3 and their involvement in cardiac excitation-contraction and excitation-transcription coupling in ventricular and atrial myocytes. Schematics depict parts of the sarcolemmal plasma membrane as well sarcoplasmic reticulum and nuclear envelope as a contiguous Ca^{2+} store. Abbreviations: GPCR, G protein-coupled receptor; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; LTCC, L-type Ca^{2+} channel; NCX, Na^+/Ca^{2+} exchanger; RyR, ryanodine receptor; IP_3R, IP_3 receptor; SERCA, SR Ca^{2+} ATPase; NCX/GM1, NCX ganglioside complex; CaM, Calmodulin; CaN, Calcineurin; CaMKII, Ca-Calmodulin dependent kinase; NFAT, nuclear factor of activated t-cells; DAG, Diacylglycerol; HDAC, histone deacetylase; NPC, nuclear pore complex; Ca^{2+} released from intra-nuclear pools.

The NPCs are the major gateway for ions (including Ca^{2+}) to diffuse along the gradient between the cytosol and nucleoplasm. It has been proposed that NPCs can act as diffusion filter and introduce a kinetic delay in the equilibration of nucleoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{nuc}$) and $[Ca^{2+}]_i$ (Bootman et al., 2009). The extent of the kinetic delay might be subject to modulation. Although NPCs do not close, their conductance can change in response to factors such as Ca^{2+} and ATP. The density of NPCs can vary from 1 to 5 NPCs per μm^2, depending on the cell type (Wang and Clapham, 1999). A greater expression of NPCs would allow for a more rapid equilibration of $[Ca^{2+}]_i$ and $[Ca^{2+}]_{nuc}$. Recent data from Alonso and García-Sancho (2011) also suggest a role for NE invaginations (nucleoplasmic reticulum) and intra-nuclear Ca^{2+} pools for the regulation of nuclear Ca^{2+} (**Figure 1**). More evidence that nuclear Ca^{2+} dynamics are not just a function of cytosolic Ca^{2+} transients can be found in structural and functional differences of NE Ca^{2+} handling proteins as compared to the SR. Even though the NE is an extension of the SR (Wu et al., 2006; Shkryl et al., 2012) SERCA presumably is not expressed at the inner NE membrane (Malviya and Klein, 2006; Bootman et al., 2009). Nonetheless, other putative Ca^{2+} handling and ion transporting proteins have been suggested to be present in the NE, including a splice variant of the type-1 Na^+/Ca^{2+} exchanger associated with ganglioside (NCX/GM1 complex, typical for non-excitable cells), RyRs, NAADPR (nicotinic acid adenine dinucleotide phosphate receptor), Na^+/K^+ ATPase and Na^+/H^+ exchanger (Gerasimenko et al., 2003; Irvine, 2003; Bkaily et al., 2006; Ledeen and Wu, 2007; Zima et al., 2007; Guatimosim et al., 2008; Wu et al., 2009).

Even more important seems the preferential expression of IP_3Rs in the NE (Bare et al., 2005). Using Fluo-5N Zima et al. observed a depletion of the nuclear envelope upon experimental stimulation of IP_3Rs with IP_3 in isolated nuclei (Zima et al., 2007) that was paralleled by an increase of $[Ca^{2+}]_{nuc}$. Wu and colleagues obtained similar results with Fluo-5N on IP_3 dependent NE Ca^{2+} depletion in permeabilized cells (Wu et al., 2006). The importance of IP_3 for the regulation of $[Ca^{2+}]_{nuc}$ is underscored by the results shown in **Figure 2**: Following cell-wide IP_3 uncaging, nuclear Ca^{2+} transients are consistently and preferentially altered in atrial and ventricular cells. However since IP_3 is buffered (i.e., by IP_3Rs) and degraded over time (Woodcock and Matkovich, 2005), the subcellular localization of IP_3Rs and the site of IP_3

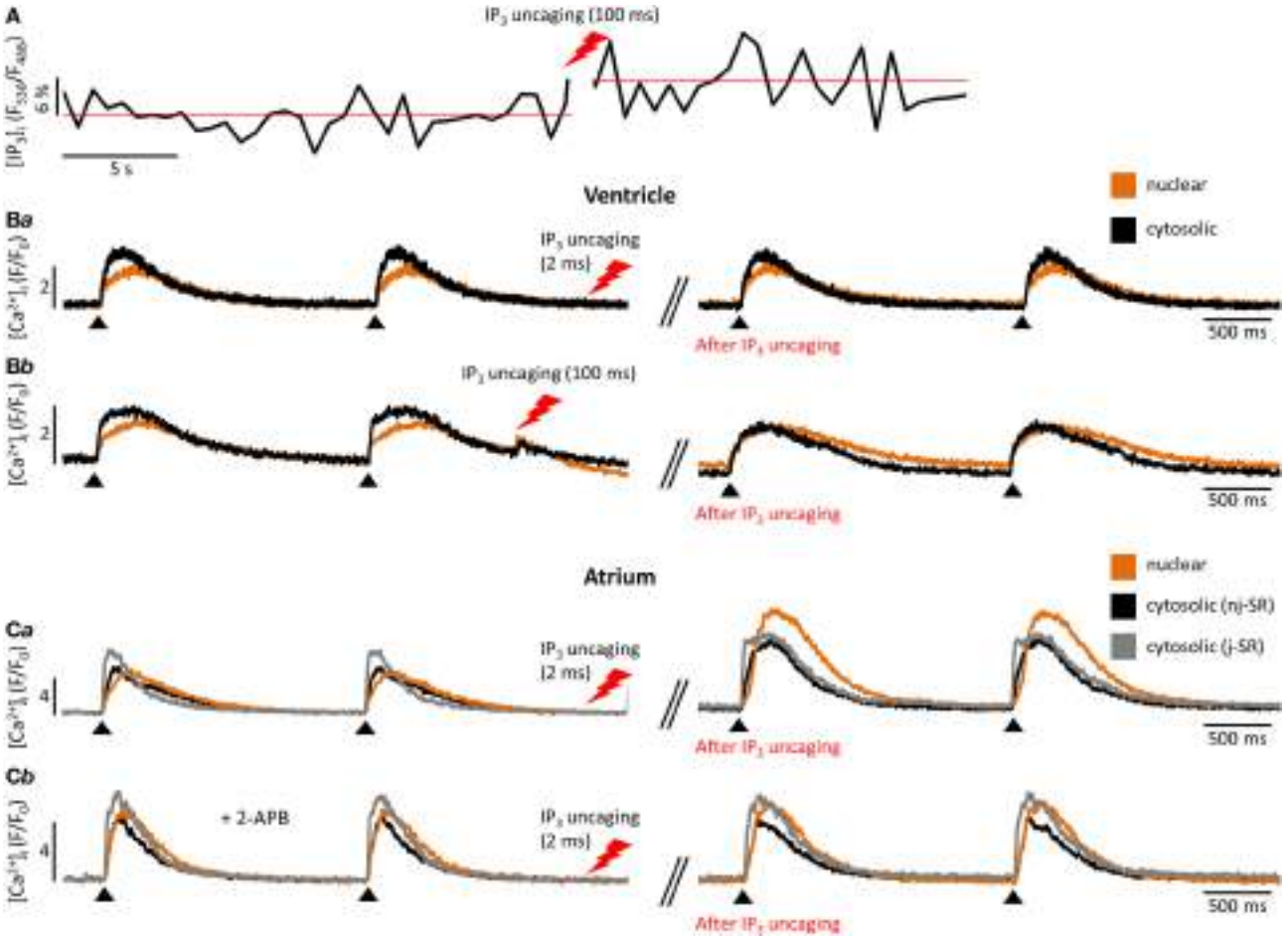

FIGURE 2 | Experimental measurement of cytosolic and nuclear Ca^{2+} and IP_3. (A) Cardiomyocyte loaded with the caged IP_3 compound cag-IP_3 PM and expressing cytosolic FIRE-1. Whole cell [IP_3] signal shown as changes in FIRE-1 FRET signal (expressed as F_{530}/F_{488}) after global IP_3 uncaging by exposure to 405 nm laser illumination for 100 ms. **(Ba,b)** Effects of global IP_3 uncaging (2 ms or 100 ms illumination) in field-stimulated ventricular myocytes on global nuclear and cytosolic Ca^{2+} transients. **(Ca)** The effect of global IP_3 uncaging (2 ms) on global nuclear, subsarcolemmal (j-SR) and central (nj-SR) Ca^{2+} transients in field stimulated atrial myocyte. **(Cb)** The effect of global IP_3 uncaging (2 ms) on global nuclear, j-SR and central Ca^{2+} transients in field stimulated atrial myocyte pre-incubated with the IP_3R blocker 2-APB. Pacing frequency 0.5 Hz. Black arrowheads indicate application of electrical stimuli.

generation (i.e., GPCR) are important to generate highly localized Ca^{2+} signals to control Ca^{2+}-dependent transcription (Bers, 2013; Ibarra et al., 2013). The traditional view on the positioning of GPCRs in cardiac myocytes sees their main site of expression in the sarcolemmal and nuclear membrane (**Figure 1**). Only recently, work from Ibarra et al. (2013) suggested a third type of localization for GPCRs in t-tubules close to the nuclear envelope (**Figure 1**, ventricular cell). The positioning of IP_3 production and IP_3Rs is important since differences in the kinetics of local [Ca^{2+}] can lead to altered activation of transcription factors. A pronounced local elevation of [Ca^{2+}] for instance, can activate calmodulin dependent-protein kinase II (CaMKII) and promote histone deacetylases (HDAC) phosphorylation (Wu et al., 2006), whereas a sustained smaller [Ca^{2+}] elevation increases nuclear factor of activated T-cells (NFAT) dephosphorylation via the Ca^{2+} sensitive phosphatase calcineurin (CaN). This ultimately leads to the activation of different sets of transcription factors, e.g., myocyte enhancer factor 2 (MEF2) for HDAC and GATA for NFAT (Molkentin et al., 1998). The separate set of Ca^{2+} release and removal proteins in the NE, with IP_3Rs as the most prominent example, as well as the specific expression of GPCRs in the sarcolemmal and nuclear membranes might be key to understanding the conundrum of Ca^{2+} being a modulator of contraction and transcription at the same time (Bootman et al., 2009). Mathematical modeling of nuclear and cytosolic Ca^{2+} homeostasis, accounting for different expression levels of sarcolemmal, cytosolic and nuclear Ca^{2+} handling proteins, paralleled by experimental approaches might provide a better understanding of functional differences of nuclear and cytosolic Ca^{2+}.

EXPERIMENTAL TOOLS FOR MEASURING CYTOSOLIC AND NUCLEAR Ca^{2+} AND IP_3 SIGNALS

Confocal laser microscopy, multiphoton imaging and conventional microscopy provide the basis for visualization of whole cell and subcellular ion concentration distributions, and the development of chemical fluorescent Ca^{2+} indicators

(Grynkiewicz et al., 1985) made imaging of Ca^{2+} movements inside living cells feasible. Nowadays a variety of ratiometric and non-ratiometric Ca^{2+} indicators, with Indo-1 and Fluo-4 among the most prominent examples, are being used. In principle, upon excitation, these indicators emit light at particular wave lengths and the emitted fluorescence intensity or the emission spectrum is changed in a Ca^{2+} bound state (Takahashi et al., 1999). The dissociation constant (K_d) as a measure of Ca^{2+} binding affinity is crucial for the selection of the appropriate Ca^{2+} dye for a particular cellular compartment of interest. Low affinity, high K_d dyes (like Fluo-5N) are used for the visualization of changes in SR $[Ca^{2+}]$ or nuclear envelope $[Ca^{2+}]$, whereas, e.g., Fluo-4 (K_d of 345 nM) is one of the preferred dyes for imaging of changes in cytosolic free $[Ca^{2+}]$, which varies roughly between 100 nM and values at times exceeding 1 μM during ECC. Since the nucleoplasm and the cytoplasm are interconnected compartments with similar global $[Ca^{2+}]$ characteristics, dyes suitable to show changes in $[Ca^{2+}]_i$ can be used for the detection of changes in $[Ca^{2+}]_{nuc}$ as well. Using Ca^{2+} sensitive dyes, Zima and Blatter (2004) were able to visualize cytosolic IP_3R-mediated Ca^{2+} release events (Ca^{2+} puffs) and show a positive inotropic effect of neurohumoral stimulation with Endothelin-1 in cardiac myocytes. As mentioned above, the same group was also able to show changes of local nuclear envelope $[Ca^{2+}]$ in isolated nuclei upon stimulation with IP_3, using Fluo-5N (Zima et al., 2007).

A variety of pharmacologic interventions can be used to influence the IP_3-dependent signaling cascade. Tools for stimulation of the neurohumoral GPCR pathway in cardiomyocytes include for example Angiotensin II and Endothelin-1. PLC-inhibitors like U73122 and IP_3R blockers like 2-Aminoethoxydiphenyl borate (2-APB) or heparin are widely used IP_3R blockers to study the GPCR/PLC/IP_3 pathway. More recent molecular techniques and the generation of transgenic animals complement these tools. Noteworthy are the generation of IP_3R knock-out and IP_3R overexpressing mice as well as the development of IP_3-sponges that allows the cellular overexpression of IP_3 buffering proteins. The generation of IP_3R overexpressing mice combined with the adenoviral expression of an IP_3 sponge provided novel insights into the importance of this pathway in cardiac physiology and pathophysiology. Ca^{2+} transients in IP_3R overexpressing mice were increased and showed a higher potential for arrhythmias after Endothelin-1 treatment. These effects were abrogated after expression of the IP_3 sponge (Nakayama et al., 2010). Insensitivity toward GPCR stimulation and IP_3R-mediated pro-arrhythmic effects were confirmed in IP_3R knock-out mice (Li et al., 2005).

An approach to directly visualize cellular $[IP_3]$ would allow for a more complete picture of cell physiology. Only recently Remus et al. (2006) developed biosensors termed FIRE to dynamically study $[IP_3]$ in living cells. Briefly, FIRE is incorporated into an adenoviral vector, expressed in target cells, and utilizes fluorescence resonance energy transfer (FRET) between cyan and yellow fluorescent protein (CFP and YFP) upon binding of IP_3. For that purpose FIRE contains a fusion protein of CFP, YFP, and the IP_3 binding domain of the IP_3 receptor type 1, 2, or 3 and can be targeted to the cytosolic or nuclear compartment. An increase in $[IP_3]$ is detected by an increase in FRET signals and a change in the YFP/CFP fluorescence ratio.

Further progress in the study of IP_3-dependent Ca^{2+} signaling became possible with the development of caged IP_3 compounds (Smith et al., 2009). Upon UV-light dependent photolysis, IP_3 is released in its biological active form and can be readily used to study this signaling pathway without possible additional effects of GPCR stimulation other than IP_3 generation (i.e., effects mediated by diacylglycerol that is generated concomitantly with IP_3 by PLC). These approaches can be used in parallel, as shown in **Figure 2A**: a cardiomyocyte expressing FIRE-1-cyt exhibits an increase in the FRET signal of ∼6% upon IP_3 uncaging, indicative of a detectable change of global cytosolic $[IP_3]$. Moreover **Figure 2** depicts the influence of IP_3 uncaging on different cellular compartments in atrial and ventricular cells. **Figure 2B** exemplifies the small impact of IP_3 uncaging on local cytosolic and nuclear Ca^{2+} transients in field stimulated (0.5 Hz) ventricular cells (Fluo-4). Only prolonged exposure to the IP_3 uncaging signal (100 ms laser illumination) has immediate visible effects on local Ca^{2+} release (**Figure 2C**). The IP_3 effects on diastolic $[Ca^{2+}]_i$ and the Ca^{2+} transient amplitude are particularly pronounced for the nuclear region. As compared to ventricular cells, atrial myocytes are more sensitive to IP_3 uncaging at smaller laser exposure durations (2 ms; i.e., smaller $[IP_3]$) and the overall effect on cytosolic, nuclear and subsarcolemmal Ca^{2+} transient amplitudes is higher upon IP_3 uncaging. Note also the altered Ca^{2+} transient kinetics with a prolongation of the Ca^{2+} transient's amplitude following IP_3 uncaging (**Figure 2C*a***). **Figure 2C*b*** shows the effect of the IP_3R blocker 2-APB (10 μM). The effect of IP_3 uncaging on Ca^{2+} transients in an atrial cell, pre-incubated with 2-APB, was abolished.

MATHEMATICAL APPROACHES FOR SIMULATING CYTOSOLIC AND NUCLEAR Ca^{2+} AND IP_3 SIGNALS

Computational modeling has proven to be a powerful approach to study cardiac physiology and its implications for disease. With increasing availability of biophysical and physiological data, mathematical models have also become more sophisticated. They provided new insights into how cellular structures, channels and receptor distributions or Ca^{2+}/IP_3 signaling regulate cardiac ECC. A number of *deterministic models* of ventricular and atrial myocyte electrophysiology, intracellular Ca^{2+} handling and bioenergetics have been published. For a more complete review on successes and failures in these modeling pursuits we refer the reader to some excellent recently published articles (Noble, 2011; Jafri, 2012; Noble et al., 2012; Sobie and Lederer, 2012; Poláková and Sobie, 2013; Wilhelms et al., 2013). Several *computational models* have been constructed to investigate IP_3 synthesis and the sub-cellular mechanisms regulating IP_3R-mediated Ca^{2+} signaling. The first model of an IP_3 signaling system, built to simulate IP_3 signals in response to stimulation with cardiac hypertrophic neurohumoral agonists like Endothelin-1 and Angiotensin II, was published by Cooling et al. (2007). The key controlling parameters with respect to the resultant cytosolic $[IP_3]$ in atrial cells were identified, including phosphorylation of membrane receptors, ligand strength, binding kinetics to pre-coupled (with GαGDP) receptors and kinetics associated with pre-coupling the receptors. In 1992, De Young and Keizer (1992) constructed the first simplified model of the IP_3 receptor. Subsequent theoretical studies,

based on new experimental data, have investigated the complex dynamic properties of type 1, 2, or 3 IP_3Rs (Li and Rinzel, 1994; Laurent and Claret, 1997; LeBeau et al., 1999; Moraru et al., 1999; Mak et al., 2001; Sneyd and Dufour, 2002; Dawson et al., 2003; Siekmann et al., 2012). Based on quantitative measurements of IP_3R properties, several stochastic models of the single channel and channel-clusters have been constructed (Swillens et al., 1998; Shuai and Jung, 2002; Falcke, 2003; Fraiman and Dawson, 2004; Thul and Falcke, 2004; Gin et al., 2009). Fraiman and Dawson (2004) were the first to include an explicit dependence of IP_3R gating on SR-luminal Ca^{2+}. To investigate the mechanisms underlying pacemaker cell activity, Youm et al. (2006) developed a deterministic model that includes ion channels, NCX, pumps, the intracellular machinery for Ca^{2+} regulation, cytosolic IP_3 production and IP_3-mediated Ca^{2+} release activity. Their model supports the idea that the cyclic changes in cytosolic Ca^{2+} and IP_3 play a key role in the generation of regenerative pacemaker potentials. *Spatiotemporal continuum models,* seeking to investigate the mechanisms of IP_3-mediated Ca^{2+} signaling in cells where IP_3Rs are known to be the dominant Ca^{2+} release channels, have been published as well. Jafri and Keizer, combining a realistic model of IP_3-induced Ca^{2+} oscillations with the diffusion of IP_3 and buffered diffusion of Ca^{2+}, developed a reaction-diffusion continuum model in Xenopus oocytes (Jafri and Keizer, 1994, 1995). Their results suggest that Ca^{2+} diffusion, which was much slower than that of IP_3 because of endogenous Ca^{2+} buffers, had only a small effect on predicted Ca^{2+} transients. These findings imply a possible previous undisclosed role for IP_3 in cell signaling. Means et al. (2006) used a reaction-diffusion model to simulate Ca^{2+} and IP_3 dynamics in mast cells. The model was built upon a 3D reconstruction of the endoplasmic reticulum (ER) geometry from electron-tomography series. This model simultaneously tracks the changes in cytoplasmic and ER $[Ca^{2+}]$, includes luminal and cytoplasmic Ca^{2+} buffers, plasma membrane Ca^{2+} fluxes, SERCA, ER leakage, and type-2 IP_3R. A unique feature of the model is the inclusion of the stochastic behavior of type-2 IP_3R. The results showed that IP_3Rs in close proximity modulate the activity of their neighbors through local Ca^{2+} feedback effects. Finally, in 1999 an analysis performed by fluorescence measurements of $[Ca^{2+}]_i$ and $[Ca^{2+}]_{nuc}$ in ventricular myocytes revealed that $[Ca^{2+}]_{nuc}$ increases concomitantly with $[Ca^{2+}]_i$ upon electrical stimulation, but the pattern of $[Ca^{2+}]_{nuc}$ increase was biphasic (rapid and slow) (Genka et al., 1999). Both sets of $[Ca^{2+}]_i$ and $[Ca^{2+}]_{nuc}$ data were well fitted by predictions derived from a simplified model of Ca^{2+} diffusion across the NPCs with two different Ca^{2+} diffusion constants. A plausible explanation of this finding is that the change in $[Ca^{2+}]_{nuc}$ is caused by Ca^{2+} diffusion from the cytosol to the nucleus through NPCs, but the permeability of the NPCs shifts from free to moderately restricted during contraction (Genka et al., 1999). The partial restriction of Ca^{2+} diffusion into the nucleus at high $[Ca^{2+}]_i$ may support the idea of a defense mechanism protecting the nucleus against Ca^{2+} overload during cell contraction.

Taken together, the aforementioned modeling efforts fill a number of specific gaps of knowledge with respect to cell electrophysiology and cytosolic Ca^{2+} and IP_3 signaling. To date, however, no quantitative model coupling the cell electrophysiology with Ca^{2+} and IP_3 signaling in the cytosol and nucleus in cardiomyocytes exists. The development of a new system model, coupling ECC and ETC is important because: (a) this tool would provide fundamental new information on the role of IP_3R-mediated Ca^{2+} signaling during ECC for arrhythmogenesis, for electrophysiological changes and for nuclear Ca^{2+} signaling in normal and failing cardiac cells; (b) as more experimental details on the complexity of IP_3 regulation in myocytes accumulates, the intuitive interpretation of new findings becomes increasingly impractical and sometimes controversial. In pursuing this goal we extended the Shannon-Bers model in rabbit ventricular myocytes (Shannon et al., 2004). New equations, describing nuclear Ca^{2+} dynamics and its dependence on $[Ca]_i$, nuclear Ca^{2+} buffering and transport via NPCs and NE (i.e., SR) were incorporated (see **Figure 1**; Michailova et al. unpublished data). Preliminary results (**Figures 3A,B**) show that the model predictions are in qualitative agreement with our Ca^{2+} transient measurements at 0.5 Hz electrical stimulation (see **Figure 2B**) and published experimental data (Ljubojevic et al., 2011) of global cytosolic and nuclear Ca^{2+} transients under control conditions, i.e., in absence of activation of IP_3 signaling. The predicted $[Ca^{2+}]_i$ and $[Ca^{2+}]_{nuc}$ transients (and action potentials and $[Ca^{2+}]_{SR}$; not shown) are stable during 10 min stimulation at 0.5, 1, or 2 Hz. The model mimics also the frequency-dependent increases in the diastolic $[Ca^{2+}]_i$ (Shannon et al., 2004), but no obvious differences in diastolic levels of $[Ca^{2+}]_{nuc}$ vs. $[Ca^{2+}]_i$ at any given frequency were predicted. At each frequency the systolic Ca^{2+} peaks were lower in the nuclei and positive force-frequency increases in systolic $[Ca^{2+}]_i$ and $[Ca^{2+}]_{nuc}$ were predicted. The kinetic parameters of Ca^{2+} transients (time to peak and time to 50% $[Ca^{2+}]$ relaxation; RT_{50}) were slower in the nucleus as compared to the cytosol. The physiological utility of the model was tested further by applying different frequencies to simulate the positive force-frequency relationship (**Figure 3C**). In agreement with experiments (Ljubojevic et al., 2011), upon increasing the rate from 0.5 to 2 Hz diastolic $[Ca^{2+}]$ and systolic Ca^{2+} peaks in the nucleus and cytoplasm increased in magnitude and the predicted amplitude of the Ca^{2+} transients were smaller in the nucleus compared to the cytosol.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this review we discussed the current state of experimental and modeling approaches to investigate nuclear and cytosolic Ca^{2+} homeostasis, whereby we focused on IP_3-dependent Ca^{2+} signaling in adult myocytes. We presented experimental data from ventricular and atrial cells, showing the effects of sudden increases in $[IP_3]$ on nuclear and cytosolic Ca^{2+} transients during field stimulation as well as different approaches to study IP_3-mediated Ca^{2+} release (i.e., FIRE-1-cyt as a tool to quantify $[IP_3]$, IP_3 uncaging to mimic physiological increases in $[IP_3]$ and 2-APB to block IP_3R mediated Ca^{2+} release). Moreover we compared experimentally the influence of IP_3 uncaging on different compartments (nucleoplasm, cytosol) and were able to show that ventricular cells need a stronger IP_3 stimulus to elicit a nuclear response, whereas atrial cells display substantial increases in nuclear and cytosolic Ca^{2+} transient amplitude upon a weaker IP_3 uncaging stimulus, consistent with their higher total expression of IP_3Rs as compared to ventricle. The recent development of FRET-based

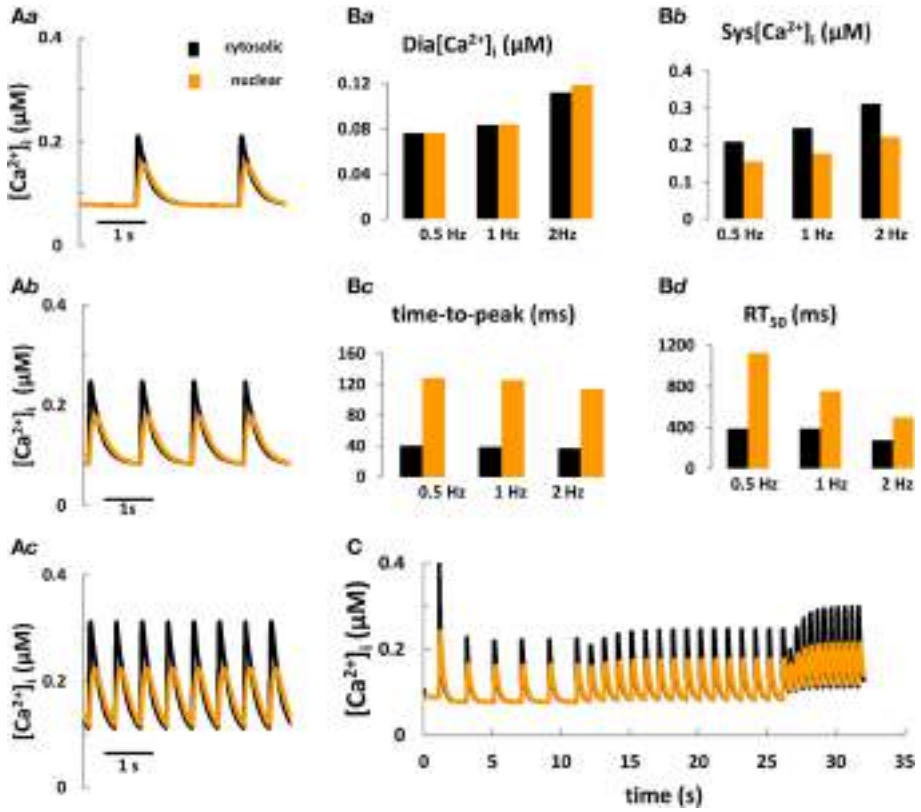

FIGURE 3 | Computer modeling of cytosolic and nuclear Ca^{2+} signals under control conditions. (**A*a*–A*c***) Predicted cytosolic and nuclear steady-state Ca^{2+} transients under control conditions in rabbit ventricular myocytes stimulated for 10 min at frequencies of 0.5, 1, and 2 Hz, respectively. **(B*a*–B*d*)** Predicted kinetic parameters of $[Ca^{2+}]_i$ and $[Ca^{2+}]_{nuc}$ transients: Dia $[Ca^{2+}]$, diastolic Ca^{2+}; Sys $[Ca^{2+}]$, systolic Ca^{2+}; RT_{50}, time to 50% $[Ca^{2+}]$ relaxation. **(C)** Frequency-dependent changes in $[Ca^{2+}]$ in the nucleus vs. the cytoplasm during stepwise increases of the stimulation frequency from 0.5 to 2 Hz.

probes used for the detection of $[IP_3]$ as well as approaches to alter nuclear and/or cytosolic $[IP_3]$ provide experimental tools for the study of IP_3-dependent Ca^{2+} release and its importance in ECC and ETC.

We also presented our recent efforts of a first attempt to develop an electrophysiological and Ca^{2+} signaling model that integrates three different cellular subsystems (cytosol, SR, nucleus) and couples Ca^{2+} dynamics in the cytosol and nucleus. This new tool is under development and will undergo further testing in its prediction of experimental $[Ca^{2+}]_{nuc}$ and $[Ca^{2+}]_i$ data in rabbit ventricular cells. The proposed model will also be extended to investigate how the complex dynamics of type-2 IP_3 receptors (Sneyd and Dufour, 2002; Siekmann et al., 2012), the stochastic behavior of IP_3R channel (Fraiman and Dawson, 2004) and/or the stimulation of IP_3 signal transduction pathway with neurohumoral agonists (Cooling et al., 2007) regulate ventricular ECC and ETC. Furthermore, the mechanisms underlying IP_3-induced positive inotropy in cardiomyocytes continue to be controversial with numerous cellular targets being implicated in the response, including L-type Ca^{2+} channels, K^+ channels, and Na^+/Ca^{2+} exchange (Lauer et al., 1992; Watanabe and Endoh, 1999; Woo and Lee, 1999; Yang et al., 1999; He et al., 2000; James et al., 2001; Zhang et al., 2001; Puglisi et al., 2011; Signore et al., 2013). The current model can be extended to investigate these effects as well. This model also provides a good quantitative framework to integrate reactions for calmodulin (CaM), calcineurin (CaN), CaMKII, and CaM buffering in the nucleus and can be coupled to the previously described and validated ECC models of CaM-CaMKII-CaN in rabbit ventricular cells (Hund and Rudy, 2004; Grandi et al., 2007; Saucerman and Bers, 2008; Bers and Grandi, 2009; Kraeuter et al., 2010; Soltis and Saucerman, 2010). This will allow testing hypotheses on how the interactions between Ca^{2+}, IP_3, and CaMKII signaling pathways contribute to heart failure phenotypes. Finally, the tools and insights our group develops will be useful to investigate how perturbations in cytosolic and nuclear Ca^{2+} and IP_3signaling affect ECC and ETC in atrial myocytes (Grandi et al., 2011; Koivumäki et al., 2011).

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Integrated mechanisms of CaMKII-dependent ventricular remodeling

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CaMKII has been shown to be activated during different cardiac pathological processes, and CaMKII-dependent mechanisms contribute to pathological cardiac remodeling, cardiac arrhythmias, and contractile dysfunction during heart failure. Activation of CaMKII during cardiac stress results in a broad number of biological effects such as, on the one hand, acute effects due to phosphorylation of distinct cellular proteins as ion channels and calcium handling proteins and, on the other hand, integrative mechanisms by changing gene expression. This review focuses on transcriptional and epigenetic effects of CaMKII activation during chronic cardiac remodeling. Multiple mechanisms have been described how CaMKII mediates changes in cardiac gene expression. CaMKII has been shown to directly phosphorylate components of the cardiac gene regulation machinery. CaMKII phosphorylates several transcription factors such as CREB that induces the activation of specific gene programs. CaMKII activates transcriptional regulators also indirectly by phosphorylating histone deacetylases, especially HDAC4, which in turn inhibits transcription factors that drive cardiac hypertrophy, fibrosis, and dysfunction. Recent studies demonstrate that CaMKII also phosphorylate directly histones, which may contribute to changes in gene expression. These findings of CaMKII-dependent gene regulation during cardiac remodeling processes suggest novel strategies for CaMKII-dependent "transcriptional or epigenetic therapies" to control cardiac gene expression and function. Manipulation of CaMKII-dependent signaling pathways in the settings of pathological cardiac growth, remodeling, and heart failure represents an auspicious therapeutic approach.

Keywords: CaMKII, epigenetics, transcription factors, HDAC4, remodeling

INTRODUCTION

Heart failure is the leading cause of death in developed countries and is characterized by adverse cardiac remodeling upon pathological stress situations such as arterial hypertension, ischemic injuries or due to genetic causes. Adverse left ventricular remodeling is usually described by a combination of myocardial hypertrophy, cell death, interstitial fibrosis and an activation of a so-called fetal gene program (Koitabashi and Kass, 2012). Calcium-dependent signaling pathways including Calcium/Calmodulin-dependent kinase II (CaMKII) signaling play pivotal roles in adverse cardiac remodeling (Heineke and Molkentin, 2006; Bers, 2008; Backs et al., 2009; Ling et al., 2009). Activation of CaMKII during cardiac stress results in a broad number of biological effects. On the one hand, CaMKII mediates immediate effects due to phosphorylation of cellular proteins such as ion channels and calcium handling proteins (Anderson et al., 2011). On the other hand, CaMKII affects structural features of the cardiac phenotype due to phosphorylation of proteins of the transcriptional machinery (Anderson et al., 2011). This review focuses on CaMKII-dependent transcriptional and epigenetic mechanisms that occur in cardiomyocytes during pathological and physiological processes. First, we will review the different CaMKII genes and splice variants that localize to different subcellular compartments.

CaMKII ISOFORMS AND SPLICE VARIANTS

In 2003, Colomer and colleagues observed an increased activity of Calcium/Calmodulin-dependent kinases upon pathological pressure overload due to transverse aortic constriction (TAC), and they described the expression patterns of the Calcium/Calmodulin-dependent kinases CaMKI, CaMKII, and CaMKIV (Colomer et al., 2003). They found CaMKI to be expressed in left ventricular tissue, but not up-regulated upon TAC. Whereas artificial overexpression of CaMKIV in a transgenic model was sufficient to induce cardiac hypertrophy in another study (Passier et al., 2000), in the model of Colomer, left ventricular CaMKIV was not detectable, and mice lacking CaMKIV did not display an altered response to TAC, indicating that CaMKIV is not required for cardiac hypertrophy. They convincingly demonstrated that CaMKII is the only multifunctional CaMK that is not only up-regulated on the expression level but also activated after TAC.

CaMKII consists of four different isoforms with distinct expression patterns. CaMKIIα and CaMKIIβ are enriched in neuronal tissue, and CaMKIIδ, and CaMKIIγ are expressed ubiquitously (Hudmon and Schulman, 2002). CaMKIIδ is the most abundant cardiac CaMKII isoform but CaMKIIγ is also expressed

in the heart (Hoch et al., 1999; Colomer et al., 2003). The first *in vivo* studies establishing CaMKII as a potential target for cardiac arrhythmias and structural heart disease were conducted by the use of a pharmacological inhibitor such as KN-62 or KN-93 and a CaMKII inhibitory peptide (Zhang et al., 2005; Vila-Petroff et al., 2007; Liu et al., 2011). Due to the unclear role of the single CaMKII isoforms and potential unspecific effects of CaMKII inhibitors, isoform-specific genetic loss of function models were generated. Mice with a global deletion of CaMKIIδ were protected against adverse cardiac remodeling (Backs et al., 2009; Ling et al., 2009). CaMKIIδ global knockout mice produced by us were protected from cardiac fibrosis and hypertrophy 3 weeks after TAC surgery. CaMKIIδ global knockout model generated by Ling and colleagues were protected from fibrosis and dysfunction. These mice were not protected from cardiac hypertrophy 2 weeks but only 6 weeks after TAC. These seemingly different phenotypes with regard to cardiac hypertrophy may be explained by different surgery techniques, different genetic backgrounds, or different knockout strategies. With regard to the latter, in the first model, no residual protein was expressed (transcriptional null due to deletion of exon 1 and 2), whereas in the second model the possible existence of a truncated protein encoding a region before exon 8 was not ruled out (exons 9–11 were deleted). The specific role of cardiac CaMKIIγ and a potential redundancy with CaMKIIδ have not been investigated yet. In human and experimental heart failure, enhanced CaMKII activity was mainly attributed to an enhanced expression of the CaMKIIδ splice variants CaMKIIδB and CaMKIIIδC (Edman and Schulman, 1994; Hoch et al., 1999). From transgenic mouse models with artificial overexpression of these splice variants it was concluded that CaMKIIδB (localizes to the nucleus) promotes cardiac hypertrophy and CaMKIIδC (localizes to the cytosol) results in dilated cardiomyopathy, respectively (Zhang et al., 2002b, 2003). Moreover, CaMKIIδA (localizes to sarcolemmal and nuclear membranes) was implied as another splice variant that is regulated at least in a model of cardiac hypertrophy due to isoproterenol treatment in mice (Xu et al., 2005; Li et al., 2011). However, to our knowledge transgenic models of CaMKIIδA have not been generated so far. An overview of available genetic mouse models related to cardiac CaMKII is given in **Table 1.**

CaMKII AND TRANSCRIPTIONAL REGULATION

Effects of CaMKII on cardiac gene expression was first reported by the group of Joan Heller Brown when transient expression of CaMKIIδB in neonatal rat ventricular myocytes induced gene expression of atrial natriuretic factor (ANF) and resulted in enhanced transcriptional activation of an ANF-luciferase reporter gene (Ramirez et al., 1997). As we know now, CaMKII is involved in the regulation of many transcription factors such as the activation protein-1 (AP-1) (Antoine et al., 1996), activating transcription factor-1 (ATF-1) (Shimomura et al., 1996), serum response factor (SRF) (Fluck et al., 2000), cAMP-response element binding protein (CREB) (Sun et al., 1994), and myocyte enhancer factor 2 (MEF2). The latter is discussed in the next paragraph. An overview about the identified transcriptional regulators is given in **Figure 1**.

A well-known transcription factor in striated muscle biology is SRF, and Calcium/Calmodulin-dependent regulation of SRF via interaction with CaMKIV and histone deacetylase 4 (HDAC4), but not CaMKII, was demonstrated to be involved in the development of cardiac hypertrophy (Davis et al., 2003). A direct phosphorylation of SRF by CaMKII has been shown in skeletal muscle at Ser-103 and Thr-160 (Fluck et al., 2000), but its relevance in cardiomyocytes remains unclear. However, data from other cell types indicate that SRF-dependent gene transcription might depend on phosphorylation by CaMKII (Ely et al., 2011). As genetic animal models provide evidence for an important role for SRF in the induction and maintenance of the cardiac myogenic program (Lin et al., 1997; Parlakian et al., 2005; Backs et al., 2011), a better understanding of CaMKII-dependent SRF regulation is urgently needed.

AP-1 elements are a group of transcription factors composed of either homodimers of the Jun family (c-Jun, JunB, and JunD) or heterodimers of the Fos and Jun families (c-Fos, Fra-1, Fra-2, and FosB) (Mechta-Grigoriou et al., 2001). There is evidence for CaMKII-dependent transcriptional activity via AP-1 in non-cardiac tissues (Mishra et al., 2005; Zayzafoon et al., 2005). Recently, CaMKII-dependent upregulation of the sodium/calcium exchanger 1 (NCX1) has been shown to depend on AP-1 transcription factors c-Jun and JunB in cardiomyocytes (Mani et al., 2010). However, it remains unclear whether CaMKII phosphorylates AP-1 elements directly or indirectly.

Table 1 | Genetic mouse models for CaMKIIδ and γ.

Gene/splicing variant	Type	Strategy	Cardiac phenotype	Location	References
CaMKIIδ*B*	Gain of function	α*MHC*-driven transgene	Cardiac hypertrophy	Nucleus	Zhang et al., 2002b
CaMKIIδ*C*	Gain of function	α*MHC*-driven transgene	Dilated cardiomyopathy	Cytosol	Zhang et al., 2003
CaMKIIδ	Loss of function	Global knockout exons 9–11	Protection from fibrosis, dysfunction, and late hypertrophy	Nucleus/Cytosol	Ling et al., 2009
CaMKIIδ	Loss of function	Global knockout exons 1–2	Protection from early hypertrophy and fibrosis	Nucleus/Cytosol	Backs et al., 2009
CaMKIIγ	Loss of function	Global knockout exons 1–2	Not investigated	Nucleus/Cytosol	Backs et al., 2010

Cardiomyocyte-specific transgenic overexpression of CaMKIIδ (splice variants B and C) are driven by the αMHC promoter. Global knockout models for CaMKIIδ were generated by two labs. The second cardiac CaMKII isoform, CaMKIIγ, has so far not been investigated with regard to cardiac stress situations.

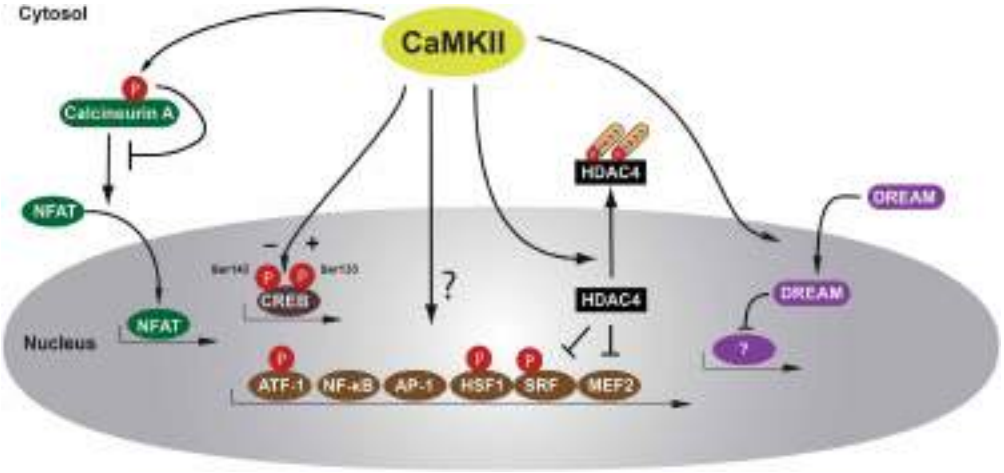

FIGURE 1 | Schematic of transcription factors and transcriptional repressors regulated by CaMKII in cardiomyocytes. CaMKII phosphorylates HDAC4 at Ser-467 and Ser-632, allowing binding of the chaperone protein 14-3-3, leading to nucleo-cytoplasmic shuttling of a phospho-HDAC4/14-3-3 complex out of the nucleus and resulting in derepression of transcription factors such as MEF2 that regulates genes responsible for adverse cardiac remodeling. Other transcription factors such as NF-κB or HSP-1 may play maladaptive or adaptive roles and these factors can be directly or indirectly regulated by CaMKII. Another transcription factor regulated after β-adrenergic stimulation is CREB that can be phosphorylated by CaMKII at two serine residues, Ser-133 and Ser-142, resulting in opposing effects in regard to activation of CREB. However, the functional effects of CREB/CaMKII interaction during cardiac remodeling remain unclear. Another recently recognized mechanism is translocation of the transcriptional repressor DREAM from the cytosol to the nucleus. Calcineurin/NFAT interaction may also be inhibited by direct phosphorylation by CaMKII at Ser-411, leading to decreased NFAT translocation to the nucleus and subsequent reduced transcriptional activity. AP-1 activation protein-1, ATF-1 activating transcription factor-1, CaMKII Calcium/Calmodulin-dependent kinase II, CREB cAMP-response element binding protein, DREAM downstream regulatory element agonist modulator, HDAC4 histone deacetylase 4, HSF-1 heat shock factor 1, MEF2 myocyte elongation factor, NFAT nuclear factor of activated T-cells, NF-κB nuclear factor κB, and SRF serum response factor.

As one of the well characterized transcription factors activated by G-protein coupled receptors, CREB has been shown to be phosphorylated by Calcium/Calmodulin-dependent kinases (Sheng et al., 1991). Several phosphorylation sites in CREB have been identified to regulate transcriptional activity and have been shown to be regulated by CaMKII in a dual way. Phosphorylation of CREB at Ser-133 by PKA and CaMKII is required for CREB activation, whereas phosphorylation of Ser-142 by CaMKII inhibits CREB activity by inhibition of CREB dimerization and protein/protein interactions that are necessary to form an active promoter complex (Wu and McMurray, 2001). *In vitro* studies using purified CaMKIV and CaMKII have demonstrated that CaMKIV phosphorylates CREB on Ser-133, whereas CaMKII has equal affinity for Ser-133 and Ser-142 (Sun et al., 1994). However, whereas nuclear calcium elevations increase CREB-dependent transcriptional activity (Kobrinsky et al., 2011), it remains unclear whether CaMKII-dependent CREB phosphorylation plays a significant role in cardiac remodeling processes (Li et al., 2006). Another member of the cAMP-responsive transcription factor family is ATF-1. ATF-1 is phosphorylated at Ser-63 by CaMKII, increasing its transcriptional activity (Shimomura et al., 1996), but the relevance of ATF-1 activity in cardiomyocytes is unknown.

Studies using KN-93, a CaMKII-inhibitory chemical compound, postulated CaMKII-dependent activation of transcription factor nuclear factor-κB (NF-κB) leading to cardiomyocyte hypertrophy (Kashiwase et al., 2005). In CaMKIIδ knockout mouse studies, NF-κB-dependent mechanisms and subsequent activation of inflammatory genes were found to play a maladaptive role in myocardial ischemia and ischemia/reperfusion injury in mice (Singh et al., 2009; Ling et al., 2013). Of note, the latter work conducted by the group of Mark Anderson was the first to perform gene expression profiling in a relevant animal model of cardiac stress, after myocardial infarction in mice. In this study, a CaMKII inhibitory peptide was used. Thus, potential off target effects need to be taken into account. For instance, it was shown that the CaMKII inhibitory peptide can also inhibit protein kinase D (PKD) (Backs et al., 2009). Gene expression arrays in genetic loss of function models might provide important additional information.

A possible physiological CaMKII-dependent transcriptional effect mediated by CaMKIIδB is the activation of heat shock factor 1 (HSF-1), a transcription factor responsible for inducible heat shock protein 70 (iHSP70) gene regulation. CaMKII is known to phosphorylate HSF-1 at Ser-230 (Holmberg et al., 2001), and Wei Peng and colleagues provided data suggesting that this might be a CaMKII-dependent antiapoptotic mechanism during cardiac ischemia and reperfusion (Peng et al., 2010). In this context, others could show that CaMKIIδB protects from doxorubicine-induced apoptosis, perhaps through GATA4-related expression of the antiapoptotic bcl-2 (B-cell lymphoma 2) gene (Little et al., 2009), although the mechanism how CaMKII induced GATA4-dependent gene expression was not shown. Taken the central role of GATA4 in cardiac hypertrophy and growth (Oka et al., 2006; Heineke et al., 2007), more data are warranted to clarify the role of GATA4 as a downstream target of CaMKII. Taken together, there are only sparse data available on how CaMKII directly interacts

with transcription factors, and for most factors a direct binding and phosphorylation by CaMKII is not yet shown (See also **Table 2**).

In an interesting recent *in vitro* study, Jarkko Ronkainen et al. describe how CaMKII potentiates the translocation of the transcriptional repressor DREAM (downstream regulatory element agonist modulator) into the nucleus and thereby promotes DREAM-induced transcriptional repression. In their study, the authors could show that this mechanism is involved in CaMKII-dependent downregulation of the pore-forming α-subunit (Cav1.2) of the *L*-type calcium channel (LTCC) and postulate this to be a physiological feedback mechanism, which enables cardiomyocytes to adjust calcium influx through the LTCC to calcium-activated CaMKII activity (Ronkainen et al., 2011).

Another "indirect" transcriptional mechanism seems to be mediated by an interaction between CaMKII and calcineurin A. The phosphatase calcineurin A dephosphorylates nuclear factor of activated T-cells (NFAT), resulting in nuclear accumulation of NFAT and consequent activation of NFAT-dependent transcriptional programs and severe cardiac hypertrophy (Molkentin et al., 1998). In an elegant study, it was demonstrated that cytosolic CaMKIIδC phosphorylates calcineurin A within its calmodulin binding domain at Ser-411 and thereby inhibits its activity (MacDonnell et al., 2009). Although the relevance of these findings needs to be proven *in vivo*, this suggest that CaMKIIδC may act as a negative modulator of calcineurin/NFAT activity.

CaMKII AND EPIGENETIC REGULATION

Besides its direct effects on transcriptional regulators, CaMKII regulates gene expression also by phosphorylation of proteins of the epigenetic machinery, especially histone deacetylases (HDACs) and in particular class II HDACs. These interesting mechanisms were initially identified upon the observation that class II HDACs interact with the transcription factor MEF2. MEF2 was introduced to depend on CaMKI and CaMKIV more than 10 years ago by the group of Eric Olson and has been established as a critical transcription factor in cardiac remodeling processes (Passier et al., 2000). MEF2 is a common target for several hypertrophic pathways, although its precise function in cardiac remodeling and the cardiac genes that are modulated by this factor are still under investigation. MEF2 proteins are responsive to calcium-controlled signaling pathways, such as CaMKI, CaMKII, CaMKIV, and Calcineurin (Passier et al., 2000; McKinsey et al., 2002; Zhang et al., 2007). Class II HDACs are expressed in the heart and contain a MEF2 binding domain in the N-terminal region, which is not present in other HDACs. This N-terminal domain binds to the chaperone 14-3-3 and is then exported from the nucleus with the consequent de-repression of MEF2 (Backs and Olson, 2006; McKinsey, 2007; Ling et al.,

Table 2 | CaMKII-dependent regulators of cardiac transcription.

Name	Abbrev.	Type	Effect	Phosphorylation site	Kinase assay	References
cAMP-response element binding protein	CREB	Transcription factor	Unknown	Ser-133, Ser-142	Yes	Sun et al., 1994
Activating transcription factor 1	ATF-1	Transcription factor	Unknown	Ser-63	Yes	Shimomura et al., 1996
Myocyte elongation factor 2	MEF2	Transcription factor	Hypertrophy/remodeling	Unknown	/	Passier et al., 2000
Serum response factor	SRF	Transcription factor	Unknown	Ser-103, Thr-160	Yes	Fluck et al., 2000
Nuclear factor κB	NF-κB	Transcription factor	Hypertrophy/remodeling	Indirect via IκB kinase	/	Kashiwase et al., 2005; Ling et al., 2013
Histone deacetylase 4	HDAC4	Transcriptional repressor	Hypertrophy/remodeling	Ser-467, Ser-632	Yes	Backs et al., 2006
Histone deacetylase 5	HDAC5	Transcriptional repressor	Hypertrophy/remodeling	Unknown	/	Wu et al., 2006; Backs et al., 2008
GATA4	/	Transcription factor	Antiapoptotic	Unknown	/	Little et al., 2009
Activation protein 1	AP-1	Transcription factor	Calcium homeostasis	Unknown	/	Mani et al., 2010
Heat shock factor 1	HSF-1	Transcription factor	Antiapoptotic	Ser-230	Yes	Holmberg et al., 2001; Peng et al., 2010
Downstream regulatory element agonist modulator	DREAM	Transcriptional repressor	Calcium homeostasis	Unknown	/	Ronkainen et al., 2011
Histone H3	H3	Histone	Hypertrophy/remodeling	Ser-10	Yes	Awad et al., 2013

CaMKII interacts with various transcription factors, transcriptional repressors, and histone 3 and thereby influences cardiac gene expression. This interaction can be a direct phosphorylation of Ser/Thr residues by CaMKII, indirect via other proteins (other kinases or cardiac repressors) or by unknown mechanisms. Known phosphorylation site and proof of direct phosphorylation are indicated. CaMKII Calcium/Calmodulin-dependent kinase II.

2013). 14-3-3 binding depends on phosphorylation of HDACs by different kinases. For example, PKD phosphorylates all class II HDAC family members (HDAC4, HDAC5, HDAC7, HDAC9) (Vega et al., 2004; Harrison et al., 2006).

We found that CaMKII selectively signals to HDAC4 via binding to a unique docking site and phosphorylation of Ser-467 and Ser-632 (Backs et al., 2006) (See also **Figure 2**). These data were confirmed by others and phosphorylation of HDAC4 by CaMKII was suggested as a central mechanism in the development of cardiac hypertrophy and remodeling (Little et al., 2007; Zhang et al., 2007; Backs et al., 2009). HDAC5 does not bind to CaMKII and can therefore only be regulated by CaMKII when it is located in close proximity to HDAC4. When HDAC5 oligomerizes with HDAC4, it can be phosphorylated and exported in a complex with HDAC4 and CaMKII (Backs et al., 2008). Accordingly, HDAC5 has been shown to be regulated by CaMKII under certain conditions. The Bers lab demonstrated that calcium in the nuclear envelope is regulated independently from the global calcium transients that cause contraction at each heartbeat. Interestingly, calcium release from the nuclear envelope activates nuclear CaMKII, which triggers nuclear export of HDAC5 (Wu and Bers, 2006; Wu et al., 2006). Whereas nuclear CaMKIIδB and cytosolic CaMKIIδC exert different effects on the phosphorylation of calcium handling proteins as the ryanodine receptor or phospholamban and on calcium homeostasis (Zhang et al., 2007), both isoforms lead to cytosolic accumulation of HDAC4 and an increase in the activity of the transcription factor MEF2 (Backs et al., 2006). Nuclear CaMKIIδB phosphorylates HDAC4 in the nucleus, leading to nucleo-cytoplasmic shuttling of HDAC4. Activation of cytoplasmic CaMKIIδC phosphorylates HDAC4 in the cytosol and prevents the import of HDAC4 from the cytosol to the nucleus (Backs et al., 2006). Thus, cytoplasmic CaMKII is also capable to regulate transcription in addition to its effects on excitation-contraction coupling. These findings strongly suggested that CaMKII indirectly regulates MEF2 by dissociating HDAC4 and HDAC5. However, HDAC4 binds to many other proteins such as other transcription factors including SRF (Davis et al., 2003), co-repressors as CtBP (C-terminal-binding protein) (Zhang et al., 2001) but also to other chromatin modifying enzymes (Zhang et al., 2002a), opening the possibility that CaMKII exerts via cytosolic accumulation of HDAC4 broader effects than simply activating MEF2 (Lehmann et al., 2013).

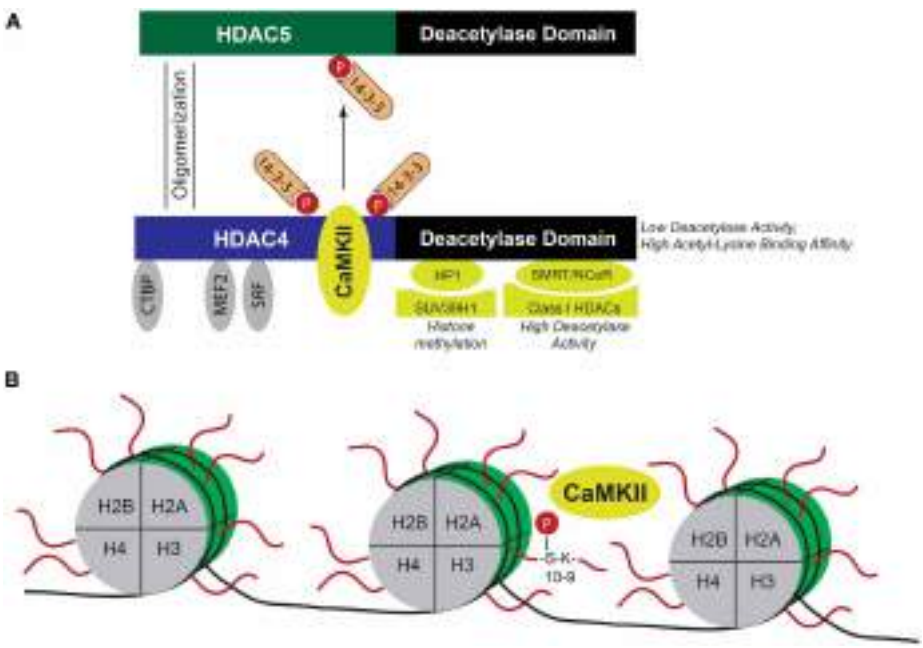

FIGURE 2 | Schematic of CaMKII-dependent epigenetic mechanisms. **(A)** HDAC4 as a nodal point for CaMKII-dependent epigenetic regulation. CaMKII binds to HDAC4 and phosphorylates HDAC4 at Ser-467 and Ser-632, leading to nucleo-cytoplasmic shuttling of HDAC4. When located in the nucleus, HDAC4 represses transcription factors such as MEF2, SRF, or the co-repressor CtBP. Binding to these transcriptional regulators directs HDAC4 to specific chromatin regions. HDAC4 recruits other chromatin modifying enzymes and direct them to the aforementioned specific chromatin regions. This results in CaMKII/HDAC4-dependent regulation of histone methylation (via interaction with HP1 and histone methyltransferase SUV39H1) and deacetylation (via interaction with SMRT/N-CoR and class I HDACs). Moreover, HDAC4 oligomerizes with HDAC5, and thereby induces 14-3-3 dependent nucleo-cytoplasmic shuttling of the HDAC4/HDAC5 complex, leading to de-repression of HDAC4/HDAC5-dependent transcription factors. Thus HDAC4 integrates CaMKII-dependent signals via epigenetic mechanisms. **(B)** Histones (H2A, H2B, H3, and H4) assemble with DNA to form nucleosomes. CaMKII directly phosphorylates Ser-10 in the N-terminal region of histone 3, which is located next to Lys-9, a major site for acetylation, and methylation. Phosphorylation at Ser-10 was suggested to result in cardiomyocyte hypertrophy and increased chromatin binding of CaMKII at specific gene loci reactivated during cardiac hypertrophy. CaMKII Calcium/Calmodulin-dependent kinase II, CtBP C-terminal binding protein, HDAC histone deacetylase, HP1 heterochromatin protein 1, MEF2 myocyte elongation factor, N-CoR nuclear receptor co-repressor, SMRT silencing mediator of retinoic acid and thyroid hormone receptor, and SRF serum response factor.

Histones are major components of chromatin and assemble with DNA to form nucleosomes, (Jenuwein and Allis, 2001). The N-terminal regions of histones are subjected to a variety of post-translational modifications, including acetylation, methylation, ubiquitination, SUMOylation, and phosphorylation (Kouzarides, 2007; Ruthenburg et al., 2007). **Figure 2** summarizes important interacting proteins of HDAC4 that direct HDAC4 to specific chromatin regions by binding to transcription factors. Of note, the deacetylase activity of class II HDACs is low but the binding affinity to acetylated lysines is high, suggesting that HDAC4 is mainly recruited to acetylated chromatin regions in close proximity to regions where MEF2 or SRF binds (Lahm et al., 2007). Although its deacetylase activity is low, HDAC4 binds indirectly via the co-repressors SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) and N-CoR (nuclear receptor co-repressor) to class I HDACs with high deacetylase activity (Fischle et al., 2002) and via HP1 (heterochromatin protein 1) to histone methyltransferases (Zhang et al., 2002a). Thus it is tempting to speculate that CaMKII effects besides transcriptional activity of MEF2 and SRF also class I HDAC-dependent histone acetylation and histone methyltransferase-dependent histone methylation. Indeed, together with the Maack lab we could show that HDAC4 controls histone methylation in a CaMKII-dependent manner (Hohl et al., 2013). ANF and brain natriuretic peptide (BNP) expression in failing hearts was accompanied by demethylation of histone 3 at lysine 9 (H3K9) and dissociation of HP1 from the promoter regions of ANF and BNP, and this was controlled by HDAC4, possibly by forming a transcriptional repressor complex with the histone methyltransferase SUV39H1 that was disrupted by CaMKII-induced phosphorylation of HDAC4. The importance of the CaMKII/HDAC4/MEF2-pathway with regard to epigenetic mechanisms in cardiac remodeling was underscored by a recent study from the Condorelli lab (Papait et al., 2013). The authors performed chromatin immunoprecipitation combined with genomic sequencing (ChIP-Seq) and RNA sequencing in isolated cardiomyocytes after TAC surgery and found a specific epigenetic signature that regulated gene expression by governing the activity of promoters and enhancers related to cardiac hypertrophy. Interestingly, they found MEF2 to be the main transcription factor to orchestrate this hypertrophic gene program by regulating the activity of transcriptional enhancers.

With regard to epigenetic mechanisms, in cardiac biology most attention so far was paid to histone acetylation and methylation. Histone phosphorylation is thought to be important for cell cycle regulation and was thus not carefully studied in the adult heart (Walter et al., 2008; Baek, 2011). Recently, it was reported by the group of Coralie Poizat that nuclear CaMKII activates cardiac transcription by direct binding to the chromatin. CaMKII was shown to phosphorylate Ser-10 of histone 3 (H3S10) which is located next to Lys-9, a major site for acetylation and methylation (Awad et al., 2013). Phosphorylation of H3S10 was accompanied by hypertrophy of primary cultured cardiomcyocytes and with increased chromatin binding of CaMKII at specific gene loci reactivated during cardiac hypertrophy under control of the transcription factor MEF2. These findings represent an interesting new epigenetic mechanism governed by CaMKII. The possibility, that ventricular remodeling can be mediated by CaMKII-dependent chromatin modifications opens a new avenue of regulatory mechanisms. Important further studies are warranted. ChIP-Seq studies may identify direct target genes of CaMKII that are important for diseases processes.

SUMMARY AND OUTLOOK

CaMKII regulates not only immediate cellular functions but also chronic processes such as ventricular remodeling leading to heart failure. In particular, CaMKII integrates several cellular pathways by inducing gene programs that are not understood in detail. Here, we reviewed the yet known transcriptional and epigenetic mechanisms by which CaMKII regulates cardiac gene expression. However, the relative importance of the different downstream mechanisms still needs to be clarified. Unbiased gene expression analyses and epigenetic profiling are warranted to define the specific gene programs that contribute to phenotypic changes induced by CaMKII.

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Modeling CaMKII in cardiac physiology: from molecule to tissue

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Post-translational modification of membrane proteins (e.g., ion channels, receptors) by protein kinases is an essential mechanism for control of excitable cell function. Importantly, loss of temporal and/or spatial control of ion channel post-translational modification is common in congenital and acquired forms of cardiac disease and arrhythmia. The multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) regulates a number of diverse cellular functions in heart, including excitation-contraction coupling, gene transcription, and apoptosis. Dysregulation of CaMKII signaling has been implicated in human and animal models of disease. Understanding of CaMKII function has been advanced by mathematical modeling approaches well-suited to the study of complex biological systems. Early kinetic models of CaMKII function in the brain characterized this holoenzyme as a bistable molecular switch capable of storing information over a long period of time. Models of CaMKII activity have been incorporated into models of the cell and tissue (particularly in the heart) to predict the role of CaMKII in regulating organ function. Disease models that incorporate CaMKII overexpression clearly demonstrate a link between its excessive activity and arrhythmias associated with congenital and acquired heart disease. This review aims at discussing systems biology approaches that have been applied to analyze CaMKII signaling from the single molecule to intact cardiac tissue. In particular, efforts to use computational biology to provide new insight into cardiac disease mechanisms are emphasized.

Keywords: calmodulin kinase II, mathematical modeling, calcium, arrhythmias, heart failure

INTRODUCTION

Signal transduction, whereby a cell receives and processes extracellular information to coordinate a cellular process, is critical for normal cell function. Signal-transduction systems are commonly perturbed in disease, making core constituents (e.g., kinases) attractive therapeutic targets (Levitzki, 2003). While we have learned a great deal about the components of key signaling pathways, the complex nature of these vast networks represents a significant obstacle to understand their dynamics, regulation, and function. Systems biology and computational modeling of biological systems have become increasingly valuable in enhancing our understanding of these complex protein interaction networks.

Systems biology involves the study of the complex interactions and associated dynamics found in biological systems. Systems biology approaches commonly involve translation of the system into a mathematical model for subsequent computer simulation and analysis. As systems-based approaches have gained favor in the study of human disease processes, so has mathematical modeling of biological systems with associated advancements in understanding complex biological phenomenon like circadian rhythms, apoptosis, synaptic plasticity, and cell communication (Herzel and Bluthgen, 2008; Kotaleski and Blackwell, 2010).

The multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has emerged as an attractive target for systems-based approaches that aim to integrate large experimental data with mathematical modeling and computational approaches across spatial and temporal scales (**Figure 1**). CaMKII serves as a nodal point for a vast signaling network that regulates critical processes like learning and memory, cardiomyocyte contractility, T-cell selection, and expression and localization of class II MHC molecules in dendritic cells (Braun and Schulman, 1995; Maier and Bers, 2002; McGargill et al., 2005; Herrmann et al., 2007; Anderson et al., 2011; Swaminathan et al., 2012). For example, CaMKII regulates multiple important functions in neurons, including synthesis and release of neurotransmitters, modulation of ion channel activity, neurite extension, synaptic plasticity, learning, and gene expression (Braun and Schulman, 1995). Similarly, in heart, CaMKII phosphorylates ion channels, transcription factors, signaling molecules, and other membrane proteins that are critical to cardiac electrical activity and structure. Abnormal CaMKII activity has been observed in human and animal models of cardiovascular disease (e.g., heart failure, myocardial infarction, arrhythmia), and is thought to promote downstream dysfunction in excitation-contraction coupling, structural remodeling, cell death, and even transcriptional activation of inflammation factors (Maier and Bers, 2002; Swaminathan et al., 2012). Current research aims at elucidating how this large effector molecule acts as a pro-cardiac disease/arrhythmogenic

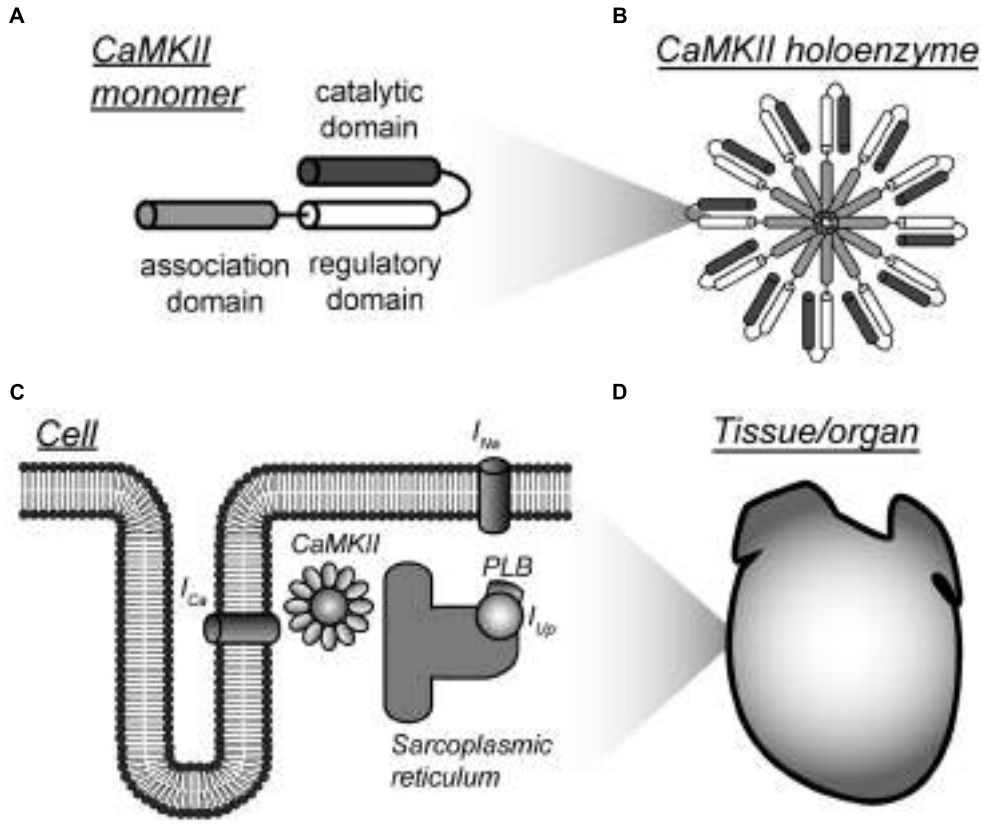

FIGURE 1 | Multi-scalar mathematical modeling of CaMKII signaling. **(A–D)** Mathematical modeling has been applied to understand CaMKII function across scales from the molecular to the tissue level. Each scale presents unique challenges and opportunities for modeling efforts. Abbreviations are as follows: I_{Ca}, L-type Ca^{2+} current; I_{Na}, fast Na^{+} current; PLB, phospholamban; I_{Up}, Ca^{2+} uptake into the sarcoplasmic reticulum.

molecule and whether it may be effectively targeted for therapy.

Mathematical modeling studies over the past three decades have elucidated important aspects of CaMKII function and signaling mechanisms. Pioneering modeling studies focused on understanding CaMKII structure and function in the brain (Lisman, 1985; Lisman and Goldring, 1988; Hanson et al., 1994; Coomber, 1998; Kubota, 1999; Dupont et al., 2003). This early work motivated later studies that incorporated models of CaMKII activity into models of the whole cell and tissue (mostly cardiac) to understand the larger role of CaMKII signaling in cell/organ function (**Figure 1**; Hund and Rudy, 2004; Iribe et al., 2006; Grandi et al., 2007; Livshitz and Rudy, 2007; Hund et al., 2008; Saucerman and Bers, 2008; Christensen et al., 2009; Koivumaki et al., 2009; Hashambhoy et al., 2010; O'Hara et al., 2011). Recently, these efforts have been expanded to gain insight into the role of CaMKII in human disease (Hund et al., 2008; Christensen et al., 2009; Koivumaki et al., 2009; Hashambhoy et al., 2010; Swaminathan et al., 2011; Lascano et al., 2013; Luo et al., 2013; Zang et al., 2013). This review aims at describing the challenges, advances and opportunities for mathematical modeling of CaMKII signaling at each stage of development across scales from the molecular to the tissue level.

MODELING THE CAMKII HOLOENZYME

The CaMKII holoenzyme possesses a number of distinguishing characteristics that pose unique challenges for modeling. Briefly (details may be found elsewhere (Couchonnal and Anderson, 2008; Anderson et al., 2011; Swaminathan et al., 2012), multiple CaMKII isoforms are expressed in cells with CaMKIIα and CaMKIIβ expressed predominantly in neurons, whereas CaMKIIγ and CaMKIIδ are more uniformly expressed in other tissues. Structurally, the CaMKII holoenzyme is organized as a hexamer of dimers arranged as two stacked rings. Each monomer is comprised of an N-terminal catalytic domain, a regulatory domain, and a C-terminal association domain. In its inactive conformation, the regulatory domain binds to the active site in catalytic domain, thereby inhibiting the activity of the enzyme. Association of Ca^{2+} bound calmodulin to the regulatory domain causes its release from the active site and exposes the active site in catalytic subunit, enabling the kinase to phosphorylate its substrates (Kolodziej et al., 2000; Rosenberg et al., 2005). Multiple residues within the regulatory domain are also exposed that may subsequently undergo post-translational regulation (e.g., phosphorylation, oxidation, glycosylation) that, in turn, alter kinase function (Lai et al., 1986; Braun and Schulman, 1995; Erickson et al., 2008; Swaminathan et al., 2012; Erickson et al., 2013). Enzyme regulation/activity depends heavily on the multimeric holoenzyme structure (Kolodziej et al.,

2000; Hoelz et al., 2003; Rellos et al., 2010; Chao et al., 2011; Stratton et al., 2013). For example, a distinguishing characteristic is the ability of CaMKII to undergo autophosphorylation where an active (Ca^{2+}/calmodulin bound) kinase subunit is phosphorylated at a specific residue (Thr286/287) by a neighboring active subunit (Lai et al., 1986; Braun and Schulman, 1995). The autophosphorylated kinase retains activity in the absence of bound Ca^{2+}/calmodulin and is thought to contribute to synaptic plasticity and learning functions as well as myocyte excitation-contraction coupling (Silva et al., 1992a,b; Dupont et al., 2003).

One of the most obvious and compelling challenges for modeling of CaMKII is autoregulation. The simplest models consider the entire population of CaMKII subunits that are subject to autophosphorylation at a rate dependent on levels of Ca^{2+}/calmodulin (Hanson et al., 1994; Dupont et al., 2003; Gaertner et al., 2004; Chiba et al., 2008). Detailed models have also been developed that incorporate structural information to account for the fact that CaMKII autophophosphorylation is constrained by physical proximity of active subunits (Lisman and Goldring, 1988; Michelson and Schulman, 1994; Zhabotinsky, 2000; Kubota and Bower, 2001; Miller et al., 2005; Lucic et al., 2008; Michalski, 2013). Recently, efforts have been made to also account for other kinase activation modes (e.g., oxidation; Christensen et al., 2009). Modeling studies at the molecular level have generated important insight into CaMKII function. In particular, models have been used to demonstrate that CaMKII activity is sensitive to changes in Ca^{2+} spike frequency and is capable of long-term storage of information at the post-synaptic density by acting as a bistable switch (Lisman, 1985; Lisman and Goldring, 1988; Hanson et al., 1994; Coomber, 1998; Kubota, 1999; Dupont et al., 2003). Furthermore, modeling studies have demonstrated the importance of autophosphorylation for bistability in CaMKII signaling, although there is some debate about the requisite conditions and physiological relevance (Zhabotinsky, 2000; Michalski, 2013). Together, these initial CaMKII modeling studies provided important insight into the link between holoenzyme structure, the ability of the kinase to encode Ca^{2+}spike information, and behavior (e.g., long-term potentiation) in neurons. Moreover, this work laid the essential foundation for subsequent multi-scale studies in other systems (e.g., heart).

MODELING CAMKII SIGNALING IN THE INTACT CELL AND TISSUE

Much work has been done, particularly in the cardiac field, to incorporate models of the CaMKII signaling pathway into models of the intact cell (**Figure 2**). Modeling of CaMKII signaling at the cellular level poses a unique set of challenges in addition to those encountered at the molecular level (**Table 1**). First, the kinase is sensitive to intracellular Ca^{2+}, whose temporal and spatial profile is tightly controlled. In the myocyte, for example, influx of Ca^{2+} through voltage-gated Ca^{2+} channels during the action potential (AP) triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) that leads to a large increase in intracellular Ca^{2+}(free and calmodulin-bound) levels. Thus, any cell model of the kinase pathway must address the dynamic nature of the input, namely Ca^{2+}-bound calmodulin. Second, once activated, the multifunctional kinase targets a large number of substrates in the cell, from membrane ion channels, pumps and transporters to contractile proteins and even transcription factors. One must consider *a priori* which targets are likely important for the phenomenon of interest. Finally, CaMKII interacts with a vast and complex signaling web that includes other proteins directly regulated by Ca^{2+}/calmodulin (e.g., ion channels, calcineurin), protein phosphatases that antagonize CaMKII phosphorylation (e.g., PP1), and other kinases that potentially synergize CaMKII effects (e.g., protein kinase A).

Despite these numerous obstacles, CaMKII signaling networks have been successfully incorporated with varying degrees of complexity into whole cell models of the myocyte (mostly ventricular) action potential and calcium transient (Hund and Rudy, 2004; Iribe et al., 2006; Grandi et al., 2007; Livshitz and Rudy, 2007; Hund et al., 2008; Saucerman and Bers, 2008; Christensen et al., 2009; Koivumaki et al., 2009; Hashambhoy et al., 2010; O'Hara et al., 2011), as well as other non-cardiac cell types (Dupont et al., 2010; Mironov, 2013). These models have employed different strategies to deal with challenges outlined above. The most common class of models incorporate a scheme where a single population of CaMKII responds to changes in bulk or subspace Ca^{2+}/calmodulin (Hund and Rudy, 2004; Iribe et al., 2006; Livshitz and Rudy, 2007; Hund et al., 2008). In other cases, a static formalism is adopted where CaMKII-dependent effects on membrane substrates are implemented in the absence of dynamic changes in CaMKII activity (Grandi et al., 2007; Thiel et al., 2008; Koval et al., 2010; Koval et al., 2012). More recently, consideration has been given to compartmentalization of CaMKII signaling within the cell (Saucerman and Bers, 2008; Song et al., 2008; Soltis and Saucerman, 2010). In general, models account for CaMKII-dependent effects on membrane ion channels and transporters important for Ca^{2+} cycling, including the ryanodine receptor (RyR), SERCA 2a (SR Ca^{2+} ATPase), phospholamban (PLB), and L-type Ca^{2+} channels. As data have emerged regarding CaMKII-dependent effects on other channels important for the action potential (e.g., I_{Na} and I_{to}), these effects have also been incorporated (Grandi et al., 2007; Hund et al., 2008; Christensen et al., 2009; Hashambhoy et al., 2010; Koval et al., 2012). It is expected that as we learn more about the specific molecular targets for CaMKII within the cell, models will adapt to account for the new findings.

What have we learned from cellular models of CaMKII signaling? Several computational studies have demonstrated the ability of CaMKII to regulate myocyte action potential, Ca^{2+} transient, and even contractile force in a rate-dependent manner (Hund and Rudy, 2004; Iribe et al., 2006; Livshitz and Rudy, 2007; Soltis and Saucerman, 2010; O'Hara et al., 2011). Interestingly, a role for CaMKII has emerged not only in normal rate dependent behavior (e.g., AP duration adaptation and force-frequency relationships), but also in promoting cellular triggers for arrhythmias such as AP alternans and afterdepolarizations (Grandi et al., 2007; Livshitz and Rudy, 2007; Thiel et al., 2008; Hashambhoy et al., 2010; Koval et al., 2010; Soltis and Saucerman, 2010). Integrated myocyte models have also been applied to increase our understanding of spatial and temporal control of CaMKII signaling (Saucerman and Bers, 2008; Song et al., 2008; Soltis and Saucerman, 2010).

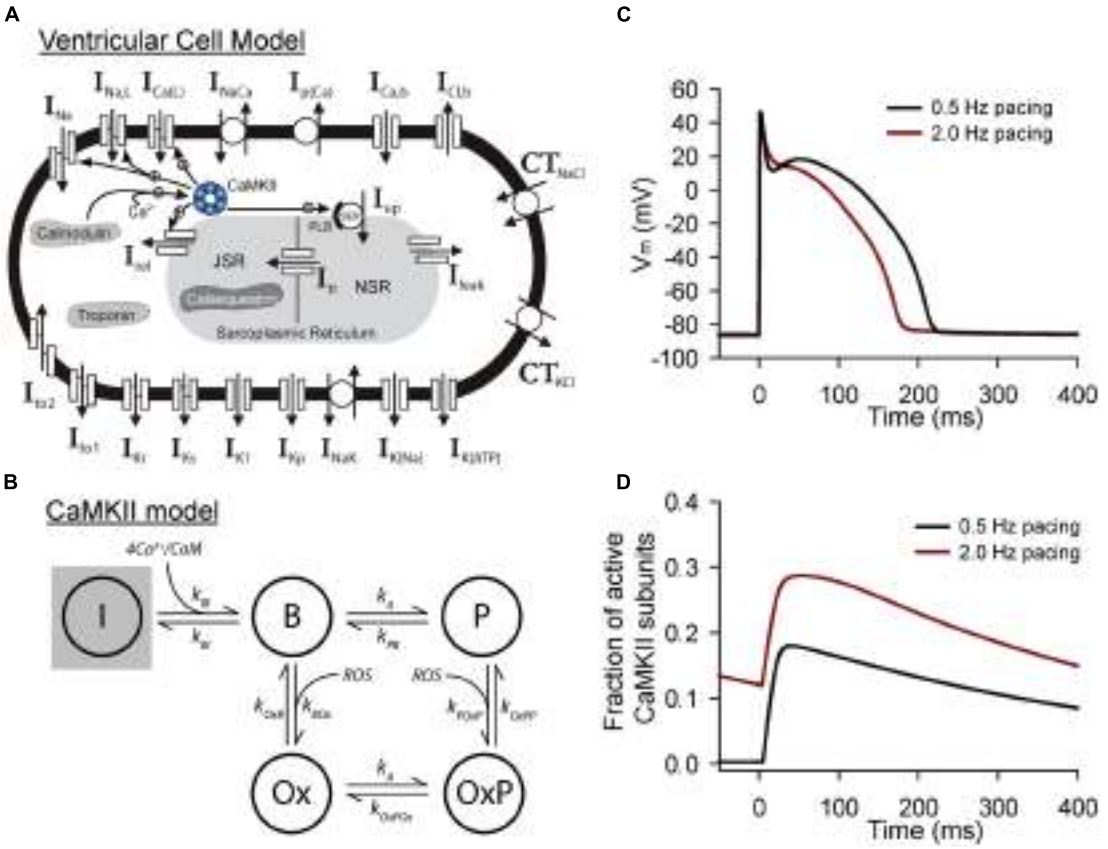

FIGURE 2 | Mathematical modeling of CaMKII signaling in the intact cell. (A) Schematic of the Hund-Rudy model of the ventricular action potential that accounts for dynamic CaMKII signaling (Christensen et al., 2009). Abbreviations are as follows: I_{Na}, fast Na^+ current; $I_{Na,L}$, late (persistent) Na^+ current; $I_{Ca(L)}$, L-type Ca^{2+} current; I_{NaCa}, Na^+/Ca^{2+} exchanger; $I_{p(Ca)}$, sarcolemmal Ca^{2+} pump; $I_{Ca,b}$, background Ca^{2+} current; $I_{Cl,b}$, background Cl^- current; CT_{NaCl}, Na^+/Cl^-cotransporter; CT_{KCl}, K^+/Cl^-cotransporter; I_{to1}, transient outward K^+ current; I_{to2}, Ca^{2+}-dependent transient outward current; I_{Kr}, rapid delayed rectifier K^+ current; I_{Ks}, slow delayed rectifier K^+ current; I_{K1}, inward rectifier K^+ current; I_{Kp}, plateau K^+ current; I_{NaK}, Na^+/K^+ ATPase; $I_{K(Na)}$, Na^+-dependent K^+ current; $I_{K(ATP)}$, ATP-sensitive K^+ current; I_{rel}, sarcoplasmic reticulum (SR) ryanodine receptor Ca^{2+} release channel; I_{Up}, SR Ca^{2+} pump; PLB, phospholamban; I_{leak}, SR Ca^{2+} leak; NSR, network SR; JSR, junctional SR; I_{tr}, Ca^{2+} transfer from NSR to JSR. **(B)** State diagram for integrated CaMKII model that includes inactive (I), Ca^{2+}/calmodulin bound (B), autophosphorylated (P, OxP) and oxidized active states (Ox, OxP). Abbreviations are as follows: k_{IB}, k_{BI}, forward and reverse rate constants, respectively, for transition from inactive state to Ca^{2+}/calmodulin bound state; k_A, k_{PB}, autophosphorylation and dephosphorylation rate constants, respectively, for transition from Ca^{2+}/calmodulin bound state to autophosphorylated state; k_A, k_{OxPOx}, autophosphorylation and dephosphorylation rate constants, respectively, for transition from oxidized active state to oxidized and autophosphorylated state; k_{OxB}, k_{BOx}, forward and reverse rate constants, respectively, for transition fromCa^{2+}/calmodulin bound state to oxidized active state; k_{POxP}, k_{OxPP}, forward and reverse rate constants, respectively, for transition from autophosphorylated state to oxidized and autophosphorylated active state. Simulated **(C)** action potentials and **(D)** CaMKII activity from the model at two different pacing frequencies to demonstrate sensitivity of CaMKII to pacing frequency.

Interestingly, studies in this area have demonstrated the importance of affinity for Ca^{2+}/calmodulin in defining the differential response of CaMKII and the protein phosphatase calcineurin to the dynamic Ca^{2+} transient (Saucerman and Bers, 2008; Song et al., 2008). Furthermore, studies that incorporate both CaMKII and PKA signaling have shown how the two networks synergize for joint regulation of excitation-contraction coupling (Soltis and Saucerman, 2010). It will be interesting, going forward, to model how other factors such as interaction with scaffolding/anchoring proteins (e.g., β_{IV}-spectrin) may contribute to spatial control of CaMKII signaling (Hund et al., 2010; Koval et al., 2010), similar to studies involving other signaling networks (Chan et al., 2012; Greenwald et al., 2014). Finally, although considerable less work has been done in this area compared to smaller scales, progress has been made to understand the role of CaMKII in coordinating function at the tissue/organ level (Livshitz and Rudy, 2007; Christensen et al., 2009; Swaminathan et al., 2011; Luo et al., 2013). These multicellular studies have identified roles for CaMKII in regulating AP heterogeneity and conduction, as well as cardiac pacemaking.

MODELING CAMKII SIGNALING IN DISEASE

CaMKII plays a critical role in regulating the substrate for both electrical and mechanical dysfunction in cardiovascular disease (Anderson et al., 2011; Swaminathan et al., 2012). Perhaps the greatest challenge for mathematical modeling of CaMKII

Table 1 | Challenges for modeling of CaMKII activity across scales from molecule to tissue.

Scale	Challenges for modeling	Representative models
Molecule	Regulation by Ca^{2+}/calmodulin and post-translational modification (including autophosphorylation).	Hanson et al. (1994), Coomber (1998), Dupont et al. (2003), Gaertner et al. (2004).
	Complex structure/function relationship.	Lisman and Goldring (1988), Michelson and Schulman (1994), Zhabotinsky (2000), Kubota and Bower (2001), Miller et al. (2005), Lucic et al. (2008), Michalski (2013).
Cell	Dynamic Ca^{2+} signaling as input.	Hund and Rudy (2004), Iribe et al. (2006), Grandi et al. (2007), Livshitz and Rudy (2007), Chiba et al. (2008), Saucerman and Bers (2008), Soltis and Saucerman (2010), Dupont et al. (2010), O'Hara et al. (2011).
	Large number of substrates.	
	Resides at center of vast signaling network.	
	Chronic vs. acute effects of CaMKII activation.	Hund et al. (2008), Koivumaki et al. (2009), Hashambhoy et al. (2010), Lascano et al. (2013), Zang et al. (2013).
Tissue/organ	Chronic and acute remodeling in disease.	Christensen et al., 2009, Swaminathan et al., 2012, Luo et al., 2013.

signaling is how to ultimately link function at the molecular level to behavior at cell/tissue level in the setting of disease. Among the difficulties for modeling in this area involves distinguishing between acute and chronic effects of CaMKII activity. For example, while acute effects of CaMKII are mostly mediated by posttranslational modification of substrates, chronic CaMKII activation may facilitate large scale remodeling changes due to effects on transcription and gene expression (Koivumaki et al., 2009; Swaminathan et al., 2012). Mathematical modeling and computer simulation have been used to generate new insights into molecular mechanisms for arrhythmia in several disease states, including myocardial ischemia/infarction, heart failure, and diabetes (Hund et al., 2008; Christensen et al., 2009; Swaminathan et al., 2011; Lascano et al., 2013; Luo et al., 2013; Zang et al., 2013).

Arrhythmia mechanisms in the canine infarct border zone have been studied extensively using a mathematical modeling approach (Cabo and Boyden, 2003; Cabo et al., 2006; Hund et al., 2008; Christensen et al., 2009). The canine infarct border zone is particularly well suited to mathematical modeling approach due to the tremendous amount of available data at the molecular, cellular, and tissue level (Pinto and Boyden, 1999). Mathematical models have been used to link defects in CaMKII signaling with ion channel remodeling, abnormal Ca^{2+} handling, and arrhythmias in the infarct border zone. Specifically these studies have demonstrated that increased autophosphorylation and oxidation of the kinase results in increased activity that both increases Ca^{2+} leak from the sarcoplasmic reticulum and compromises availability of voltagegated Na^{+} channels to create a favorable substrate for arrhythmias (Hund et al., 2008; Christensen et al., 2009). More recently, mathematical models have been used to study the role of chronic CaMKII activation in sinus node dysfunction in the setting of heart failure and diabetes (Swaminathan et al., 2011; Luo et al., 2013). A two dimensional model of the intact sinus node has been applied to demonstrate that CaMKII-induced apoptosis and associated loss of sinoatrial node cells disrupts the source–sink balance between the sinoatrial node and surrounding atrial myocardium resulting in slowed pacemaking and even failure (Swaminathan et al., 2011; Luo et al., 2013). Other studies have used mathematical modeling to determine relative importance of direct CaMKII effects and compensatory changes in gene regulation in the setting of chronic CaMKII overexpression (Koivumaki et al., 2009). Finally, in addition to common forms of acquired disease (e.g., myocardial infarction, heart failure, diabetes), mathematical models have been used to better understand the role of CaMKII in congenital disease (Thiel et al., 2008; Koval et al., 2012). A recent study used mathematical modeling to demonstrate that human variants identified in the CaMKII phosphorylation motif of $Na_v1.5$ confer arrhythmia susceptibility by mimicking the phosphorylated channel (Koval et al., 2012), while an earlier study examined the role of CaMKII regulation of SR Ca^{2+} release in increased incidence of afterdepolarizations in Timothy syndrome (Thiel et al., 2008). Together these studies demonstrate the potential for mathematical modeling and computer simulation in advancing our understanding of CaMKII biology and its role over a broad range of cardiovascular disease.

FUTURE DIRECTIONS

This review has outlined the many unique challenges and opportunities for multiscale mathematical modeling of CaMKII signaling. While great strides have been made in development and application of mathematical models of CaMKII signaling from molecule to tissue, clearly there are outstanding issues and unanswered questions to be addressed by future research in this area. At the molecular level, the recent discovery of the CaMKII crystal structure represents an exciting development with great potential for modeling (Chao et al., 2011). Similarly, it will be important for future modeling efforts to address novel pathways for regulation of CaMKII activity (e.g., glycosylation). At the cell level, a daunting challenge remains the sheer number of targets for CaMKII within the cell, with new substrates identified every year. Moreover, it remains to be understood the "tipping point" from the adaptive to the maladaptive aspects of CaMKII signaling. Finally, while most models have focused on the ventricular myocyte as a system, clearly CaMKII has important roles in other heart regions/cell types (e.g., atrial, sinoatrial node cells). Models of these different cell types that incorporate cell-specific CaMKII signaling will be of great use for studying CaMKII signaling at the organ level.

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CaMKII-dependent responses to ischemia and reperfusion challenges in the heart

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Ischemic heart disease is a leading cause of death, and there is considerable imperative to identify effective therapeutic interventions. Cardiomyocyte Ca^{2+} overload is a major cause of ischemia and reperfusion injury, initiating a cascade of events culminating in cardiomyocyte death, myocardial dysfunction, and occurrence of lethal arrhythmias. Responsive to fluctuations in intracellular Ca^{2+}, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has emerged as an enticing therapeutic target in the management of ischemic heart injury. CaMKII is activated early in ischemia and to a greater extent in the first few minutes of reperfusion, at a time when reperfusion arrhythmias are particularly prominent. CaMKII phosphorylates and upregulates many of the key proteins involved in intracellular Na^+ and Ca^{2+} loading in ischemia and reperfusion. Experimentally, selective inhibition of CaMKII activity reduces cardiomyocyte death and arrhythmic incidence post-ischemia. New evidence is emerging that CaMKII actions in ischemia and reperfusion involve specific splice variant targeted actions, selective and localized post-translational modifications, and organelle-directed substrate interactions. A more complete mechanistic understanding of CaMKII mode of action in ischemia and reperfusion is required to optimize intervention opportunities. This review summarizes the current experimentally derived understanding of CaMKII participation in mediating the pathophysiology of the heart in ischemia and in reperfusion, and highlights priority future research directions.

Keywords: CaMKII, ischemia, reperfusion, contractile function, Ca^{2+} handling, cardiomyocyte death

ISCHEMIA, REPERFUSION, AND Ca^{2+} OVERLOAD

Ischemic heart disease is a leading cause of mortality worldwide. Though considerable advances have been made in the understanding of the processes involved in the pathological consequences of an ischemic event, effective therapeutic management of this disease has been difficult to achieve. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has emerged as an enticing therapeutic target in the management of ischemic heart injury, with considerable focus on its inhibition as a means for reducing injury arising from ischemia and reperfusion.

A complex series of cardiomyocyte events occur during both ischemia and reperfusion to culminate in an intracellular milieu primed for activating CaMKII. Briefly, insufficient tissue perfusion and oxygen availability in ischemia induce a shift to increased reliance on anaerobic glycolysis for ATP generation, promoting a build-up of metabolic intermediates including lactate and protons. This ionic shift stimulates the Na^+/H^+ exchanger (NHE) to maintain physiological pH, which contributes to an accumulation of intracellular Na^+. The rise in intracellular Na^+ is exacerbated by a decrease in Na efflux, including a reduction of Na^+/K^+-ATPase (NKA) activity that may be related to lower ATP availability (van Echteld et al., 1991). Elevated cytosolic Na^+ levels reduce the capacity for Ca^{2+} efflux via the Na^+/Ca^{2+} exchanger (NCX) and promote the Ca^{2+} influx "reverse" operating mode of this transporter. Re-establishing coronary flow (reperfusion) is essential to ultimately allow any salvage of the ischemic myocardium, but involves a significant acute cardiomyocyte jeopardy. Reperfusion rapidly restores the trans-sarcolemmal proton gradient, exacerbating cellular Na^+ accumulation (concomitant with restoration of NKA activity) and eventually culminating in Ca^{2+} overload. High cytosolic Ca^{2+} levels can have profound negative effects on the cardiomyocyte in reperfusion (Murphy and Steenbergen, 2008), inducing hypercontracture, electrical instability, and contractile dysfunction. Ca^{2+} overload is a major activator of the mitochondrial permeability transition pore (mPTP) in reperfusion, a response associated with reactive oxygen species (ROS) generation and the initiation of pro-death pathways (Halestrap, 2009). As CaMKII is highly sensitive to cytosolic Ca^{2+} levels and regulates many channels and transporters implicated in the steps leading to Ca^{2+} overload, there is considerable scope for a CaMKII-mediated amplification of high Ca^{2+} related pathologies (see **Figure 1**). Furthermore, reports that CaMKII is susceptible to oxidation (ox-CaMKII(Met281/2)), promoting autonomous activation (Erickson et al., 2008; Palomeque et al., 2009), suggests the sub-cellular environment in ischemia (acidosis, high cellular Ca^{2+}) and reperfusion (Ca^{2+} overload, oxidative stress) provides an optimal setting for rapid CaMKII activation – and highlights the potential for intervention strategies targeting CaMKII.

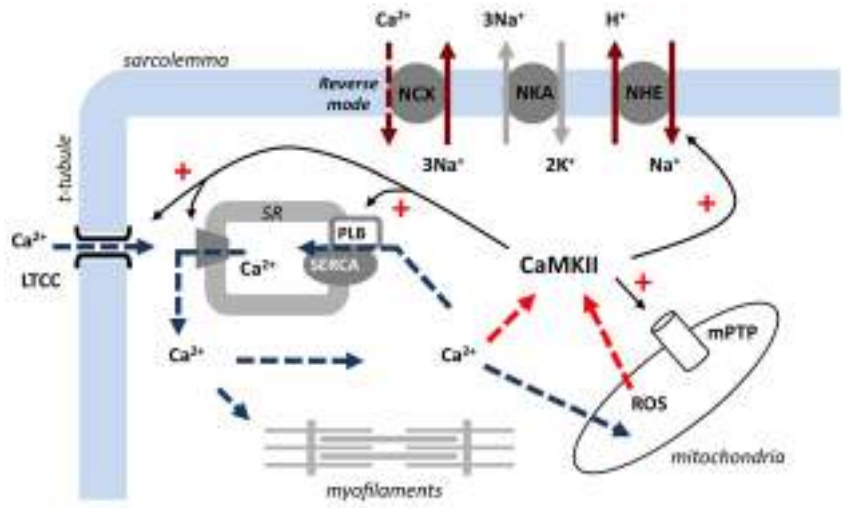

FIGURE 1 | Overview of CaMKII activation and substrate interaction in reperfusion. Post-ischemic restoration of coronary flow re-establishes the trans-sarcolemmal proton gradient and stimulates Na^+/H^+ exchange. This promotes reverse-mode Na^+/Ca^{2+} exchange and leads to intracellular Ca^{2+} overload. Ca^{2+}-activated CaMKII upregulates many Ca^{2+}-related channels/transporters, further increasing cytosolic/mitochondrial Ca^{2+} levels, and triggers the opening of the mitochondrial permeability transition pore. The increase in Ca^{2+} and reactive oxygen species generation creates a positive feedback on CaMKII and exacerbates ischemia/reperfusion injury. NCX, Na^+/Ca^{2+} exchanger; NHE, Na^+/H^+ exchanger; NKA, Na^+/K^+-ATPase; LTCC, L-type Ca^{2+} channel; SR, sarcoplasmic reticulum; SERCA2a, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; PLB, phospholamban; ROS, reactive oxygen species; mPTP, mitochondrial permeability transition pore.

CaMKII IS RAPIDLY ACTIVATED IN ISCHEMIA AND REPERFUSION

Seminal studies involving the characterization of CaMKII activation in *ex vivo* rodent hearts have mapped phospholamban (PLB) phosphorylation at the CaMKII-specific site (P-PLB(Thr17)) as a marker of CaMKII activation during acute ischemia and reperfusion (Vittone et al., 2002). PLB is a regulatory accessory protein to the sarcoplasmic reticulum (SR) Ca-ATPase (SERCA2a), which exerts an inhibitory action on SERCA2a. PLB inhibition is relieved by phosphorylation either by CaMKII or protein kinase A. Numerous subsequent studies, including our own, have shown P-PLB(Thr17) to be briefly elevated in early ischemia, peaking in the initial 1–3 min of reperfusion (Vittone et al., 2002; Said et al., 2003; Valverde et al., 2006; Vila-Petroff et al., 2007; Salas et al., 2010) at a time when hypercontracture and ventricular arrhythmias are prevalent, before rapidly returning to basal activation level. This elevated P-PLB(Thr17) occurs concomitantly with phosphorylation of other CaMKII substrates, including the SR Ca^{2+} release channel (RyR2) and titin (Said et al., 2011; Hidalgo et al., 2013), and can be blocked with CaMKII inhibitors, including KN93 and AIP (Vittone et al., 2002; Said et al., 2003; Valverde et al., 2006; Salas et al., 2010; Hidalgo et al., 2013). The source of Ca^{2+} stimulating CaMKII activity differs in ischemia and reperfusion, with Ca^{2+} entry through the L-type Ca^{2+} channel (LTCC) activating CaMKII in ischemia and the NCX in reperfusion (blocked by nifedipine and KB-R7943 respectively; Vittone et al., 2002). There is evidence that CaMKII is activated both by phasic and tonic shifts in cardiomyocyte intracellular free Ca^{2+} levels, and that local and global Ca^{2+} signals have distinct effects (Wu et al., 2006). The relative importance of each type of stimulus, and the additional influence of post-translational modifications (e.g., oxidation of the regulatory domain at Met281/282) in determining overall CaMKII activation status in ischemia and reperfusion is not fully elucidated.

CaMKII is also undoubtedly influenced by the intracellular acidosis prevalent in ischemia and early reperfusion. Both CaMKII autophosphorylation (P-CaMKII(Thr287)) and P-PLB(Thr17) rapidly increase in acidic conditions, contributing to the recovery of Ca^{2+} transients and contractile function that are initially suppressed in acidic conditions (DeSantiago et al., 2004; Mundina-Weilenmann et al., 2005; Vila-Petroff et al., 2010). Inhibiting CaMKII suppresses PLB phosphorylation (and hence SERCA activity), reducing SR Ca^{2+} uptake, causing cytsolic Ca^{2+} levels to increase (affecting both systolic and diastolic function; see (Mattiazzi and Kranias, 2014)). An increase in P-CaMKII(Thr287) has also been shown in the initial minutes of reperfusion (Said et al., 2011), consistent with an elevated autonomous CaMKII activity recently reported in *in vivo* mouse hearts subjected to 1 h ischemia and 3 min reperfusion (Ling et al., 2013). Interestingly, in contrast to the *ex vivo* studies, this elevated activity *in vivo* was associated with a maintained increase in P-PLB(Thr17) and RyR2 phosphorylation (P-RyR2(Ser2814)) throughout 120 min of subsequent reperfusion. The substantial activation of CaMKII, which occurs in ischemia and reperfusion, would be expected to be an important determinant of cardiomyocyte Ca^{2+} homeostasis and post-ischemic outcomes.

CaMKII EXACERBATES ISCHEMIC INJURY

Increased intracellular Ca^{2+} correlates with the onset of irreversible injury in ischemia (Murphy and Steenbergen, 2008). Inhibiting Ca entry through the LTCC has been shown to

prevent/delay ischemic contracture onset and arrhythmias (Henry et al., 1977; Curtis et al., 1984; Curtis and Walker, 1988). Considering CaMKII activation in early ischemia is linked with Ca entry through the LTCC (Vittone et al., 2002), it may be predicted that CaMKII contributes to the cascade of events leading to ischemic pathogenesis. We have shown that inhibiting CaMKII with KN93 significantly delays and blunts the extent of ischemic contracture in *ex vivo* hearts subjected to 20 min of global ischemia (**Figure 2**, (Bell et al., 2012)), suggesting a role for CaMKII in ischemic myocyte Ca^{2+} loading and the onset of irreversible injury. Inhibiting CaMKII also protects the heart in a chronic *in vivo* ischemic setting, as demonstrated in studies utilizing two different CaMKII inhibitor rodent expression models (CaMKII inhibitor peptide, AC3-I; mitochondrial-specific CaMKII inhibitor protein, mtCaMKIIN). These studies found that CaMKII promotes apoptosis *in vivo* (5 h post-myocardial infarction) by exacerbating SR and/or mitochondrial Ca^{2+} loading (Yang et al., 2006; Joiner et al., 2012).

CaMKII ACTIONS IN REPERFUSION RECOVERY ARE DEPENDENT ON ISCHEMIC DURATION

While initial studies assessing CaMKII in post-ischemic reperfusion suggested that CaMKII activation may improve functional recovery, subsequent studies have reported different findings and suggest that the role played by CaMKII in the dysfunction associated with reperfusion may be dependent on the duration of the preceding ischemic insult. In isolated hearts subjected to 20 min of ischemia, recovery of left ventricular developed pressure in reperfusion was lower and diastolic dysfunction exacerbated in the presence of KN93 (Vittone et al., 2002; Said et al., 2003). This poor recovery was attributed to a disruption of CaMKII actions on SR Ca^{2+} uptake, as P-PLB(Thr17) was suppressed in early reperfusion. Parallel experiments were conducted on hearts from mutant mice expressing a PLB Thr to Ala mutation at amino acid residue 17 (Said et al., 2003), such that CaMKII-mediated phosphorylation at this site could not occur. The inhibitory action of PLB on SERCA cannot be relieved in these mice. These hearts exhibited a comparable contractile dysfunction in reperfusion as was observed in wild-type hearts treated with KN93. Taken together, these observations indicate that CaMKII can be beneficial in reperfusion, augmenting SR Ca^{2+} uptake in reperfusion and enhancing cytosolic Ca^{2+} clearance. However, with a longer ischemic challenge, these benefits attributed to CaMKII activation in modulating reperfusion response are lost. Our further studies showed that extending the duration of ischemia from 20 to 45 min in *ex vivo* hearts profoundly affects how CaMKII influences reperfusion outcomes. Indeed, treating hearts with KN93 significantly reduces infarct size and apoptosis, and improves functional recovery (**Figure 3**, Vila-Petroff et al., 2007). Isolated cardiomyocyte survival is improved in simulated ischemia and reperfusion with KN93 and AIP, to an extent equivalent to the improvement achieved by inhibition either of reverse-mode NCX or SR Ca^{2+} cycling (SERCA2a and/or RyR2; (Vila-Petroff et al., 2007; Salas et al., 2010)). These data suggests that following shorter durations of ischemia, CaMKII activation augments SERCA2a activity to enhance cytosolic Ca^{2+} removal and reduce reperfusion injury/dysfunction. However, with more extensive ischemia, the influence of CaMKII on SR Ca^{2+} release mechanisms become more prominent in reperfusion, such that SR Ca^{2+} leak exceeds the capacity of SERCA2a to reuptake cytosolic Ca^{2+}, and Ca^{2+}-triggered reperfusion pathologies are exacerbated.

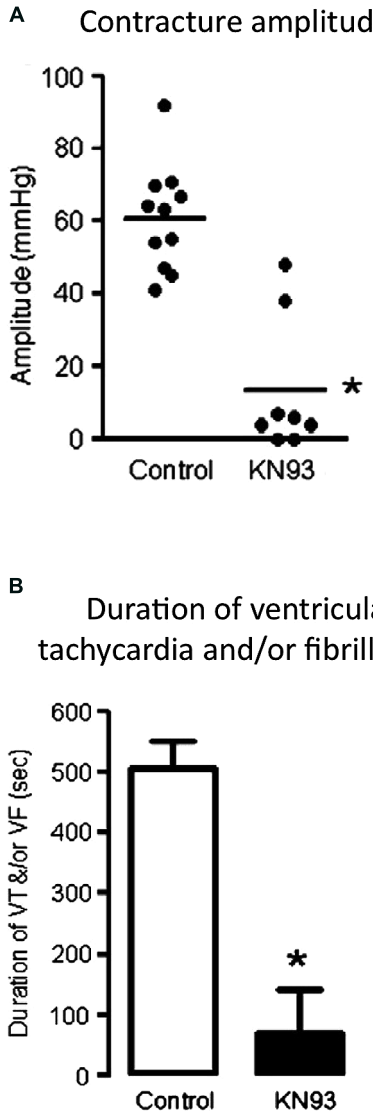

FIGURE 2 | CaMKII inhibition reduces ischemic contracture and reperfusion arrhythmias in male ex vivo hearts. Hearts subjected to ischemia/reperfusion were treated with a CaMKII inhibitor (KN93). **(A)** The amplitude of ischemic contracture was significantly lower in the presence of KN93, indicating CaMKII contributes to cardiomyocyte Ca^{2+} loading in ischemia. **(B)** Analysis of ventricular pressure traces showed a substantial reduction in the total duration of ventricular tachycardia and/or fibrillation in the first ten minutes of reperfusion in KN93-treated hearts. Data are expressed as mean ± SEM, $*p < 0.05$ vs. control. Reproduced with permission (Elsevier, (Bell et al., 2012)).

Phosphorylation of the RyR2 (P-RyR2(Ser2814)) by CaMKII increases the open probability of the SR Ca^{2+} release channel, augmenting SR Ca^{2+} leak and elevating cytosolic Ca^{2+} levels (Wehrens, 2011). A CaMKII-mediated increase in SR Ca^{2+} release channel leakiness with elevation of cytosolic

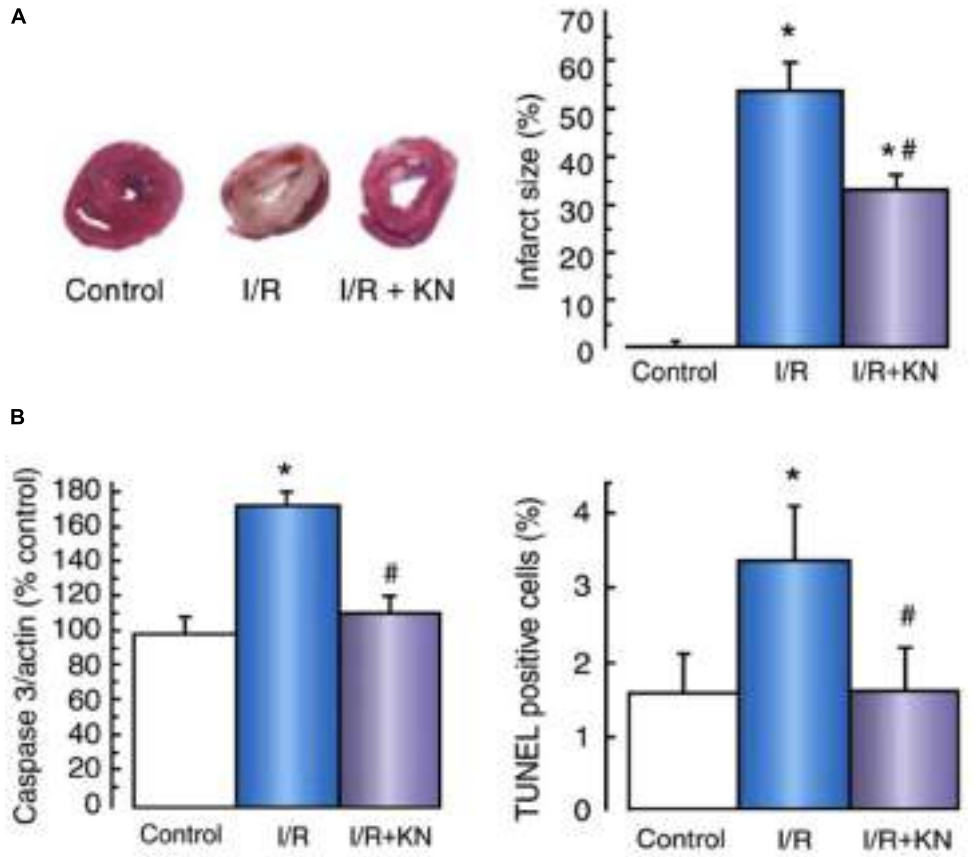

FIGURE 3 | Cell death in ischemia/reperfusion was suppressed by CaMKII inhibition. (A) Assessment of TTC stained heart cross-sections showed KN93 significantly reduced infarct size in ischemia/reperfusion. **(B)** Lower caspase 3 activation and less TUNEL positive cells in these hearts showed KN93 also reduced apoptosis in reperfusion. Data are expressed as mean ± SEM, $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. I/R. Reproduced with permission (Oxford Journals, (Vila-Petroff et al., 2007)).

Ca^{2+} levels has important implications for mitochondrial function and myocyte viability. Studies indicate that inhibiting CaMKII with KN93 may lower SR Ca^{2+} load to suppress cytochrome C release and mitochondrial swelling in reperfusion (Salas et al., 2010), both of which activate pro-death pathways in the heart. While this mitochondrial response partly reflects the close beat-to-beat relationship between cytosolic and mitochondrial Ca^{2+} levels (Robert et al., 2001), there is also evidence that CaMKII directly influences mitochondrial operation in ischemia/reperfusion (Joiner et al., 2012). Hearts from mice overexpressing a highly specific CaMKII inhibitor localized to the mitochondria (mtCaMKIIN) exhibit less mitochondrial injury and apoptosis, smaller infarcts, and recover contractile function to a greater degree (vs. wild-type). Evidence suggests the actions of CaMKII in the mitochondria are multi-faceted, including a direct phosphorylation of the mitochondrial Ca^{2+} uniporter to increase the uniporter current (Joiner et al., 2012). This would increase mitochondrial Ca levels, leading to mPTP opening and dissipation of the mitochondrial inner membrane potential. Furthermore, a resultant increase in ROS production would be expected to exacerbate CaMKII activation and actions.

Beyond these mitochondrial actions, recent data suggests that the role of CaMKII in exacerbating ischemia and reperfusion pathologies is not restricted to disrupting Ca^{2+} homeostasis at the SR and mitochondria, and may extend to direct pro-inflammatory actions. Indeed, cardiac-specific deletion of CaMKIIδ reduces chronic post-ischemic reperfusion (24 h) inflammation and injury *in vivo*, diminishing IκBα degradation and NF-κB activation (Ling et al., 2013). NF-κB is well recognized as an important pro-inflammatory mediator in ischemia and reperfusion (Van der Heiden et al., 2010), and this more recent evidence of a direct stimulatory action on NF-κB and the inflammatory response extends understanding of CaMKII as a regulator of the ischemic myocardial stress response.

CaMKII MODULATION OF RyR2 DETERMINES REPERFUSION ARRHYTHMOGENESIS

CaMKII has been implicated in generating arrhythmias in numerous different cardiopathologies (Rokita and Anderson, 2012), primarily attributed to its stimulatory action on RyR2. Augmented P-RyR2(Ser2814) increases SR Ca^{2+} leak and promotes Ca^{2+} extrusion though Na^{+}/Ca^{2+} exchange. This electrogenic process partially depolarizes the cell, increasing the likelihood

of spontaneous contraction. Experimentally, inhibiting CaMKII reduces spontaneous beats in post-acidotic and pro-oxidant environments (Said et al., 2008; Xie et al., 2009), both of which play a central role in the pathogenesis of ischemia and reperfusion injury. We have shown that CaMKII inhibition with KN93 suppresses the incidence of lethal arrhythmias (ventricular tachycardia and/or fibrillation) in early reperfusion through an unknown mechanism (**Figure 2**, (Bell et al., 2012)). There are reservations regarding the use of KN93 as a CaMKII inhibitor, due to its reported non-selective actions on other transporters including the L-type Ca and potassium channels. The occurrence of these non-selective actions depends on treatment duration and dose (Gao et al., 2006; Rezazadeh et al., 2006). Studies utilizing a similar isolated heart preparation and CaMKII inhibition protocol also report an antiarrhythmic action with KN93, that is absent in parallel studies using the inactive analog, KN92 (Said et al., 2011). This suggests a legitimate role for CaMKII in promoting arrhythmias in reperfusion. Indeed, these studies showed that inhibiting CaMKII reduces incidence of ventricular premature beats, primarily through a suppression of CaMKII mediated SR Ca release channel phosphorylation (at RyR2(Ser2814)) and associated SR Ca leak (Said et al., 2011). CaMKII-dependent phosphorylation of the SR Ca channel increases channel open probability, lowering the SR Ca threshold and increasing the propensity for Ca waves (Curran et al., 2010; Stokke et al., 2011). This ability to modulate the SR Ca channel and its influence on self-propagating SR Ca release indicates that inhibiting CaMKII at the time of reperfusion may have therapeutic potential as a first-line antiarrhythmic agent.

It should be noted that CaMKII actions are dependent on the balance of substrate phoshorylation by CaMKII and dephosphorylation by associated protein phosphatases (PPs) in the myocyte, including PP1 and PP2a. Downstream Ca handling protein targets of CaMKII are regulated by these phosphatases. Indeed, PP1 is reported to form part of a multimeric complex in the regulation of PLB (as reviewed previously, (Mattiazzi and Kranias, 2014)), which may influence CaMKII-mediated phosphorylation, cardiac contractility and the ischemic stress response (Nicolaou et al., 2009). The balance between CaMKII and phosphatase activation will therefore clearly influence the relative actions of CaMKII in the heart. Furthermore, CaMKII autophosphorylation is itself regulated by phosphatases including PP1, PP2a, and CaMK phosphatase (CaMKP; Ishida et al., 2008), though little is known about how these regulate myocardial CaMKII. Very recently, it has been shown that the CaMKP is expressed in the heart, and changes in response to systemic loading (Previlon et al., 2014). Interestingly, basal CaMKP levels were higher in females (vs. males) and loading-induced CaMKP expressions were sex-specific. It is important to highlight that all the CaMKII studies discussed above have involved experimental studies of male rodent cardiac tissues/myocytes only. There are well-described sex differences in excitation–contraction coupling and cardiomyocyte Ca^{2+} handling processes, and the myocardial response to ischemia and reperfusion (Bell et al., 2013a). Limited experimental evidence indicates that CaMKII activity is influenced by sex and sex steroids (Konhilas et al., 2004), and that estrogen may suppress CaMKII actions in ischemia and reperfusion (Ma et al., 2009). However, our very recent data show that P-CaMKII(Thr287) and P-PLB(Thr17) levels are augmented in reperfused female hearts (vs. male controls), despite these hearts exhibiting fewer arrhythmias in reperfusion (Bell et al., 2013b). These findings suggest that CaMKII is not always pro-arrhythmic in reperfusion, and support the concept that CaMKII can have both beneficial and detrimental actions in reperfusion which may depend on the sub-cellular environment in which the enzyme is activated.

EVIDENCE FOR A BENEFICIAL ROLE FOR CaMKII IN ISCHEMIA AND REPERFUSION

Though considerable evidence indicates a deleterious role for CaMKII in ischemia and reperfusion, a growing body of work suggests there may be settings where CaMKII is beneficial. CaMKII has been implicated in the cardioprotection afforded by ischemic preconditioning (Osada et al., 2000; Benter et al., 2005; Li et al., 2007) and may form part of the signaling cascade that culminates in opening of the end-effector K_{ATP} channels (Li et al., 2007; Chai et al., 2011; Zhang et al., 2014). Other data also suggest that CaMKIIδ splice variants may exert differential responses in the pathological environments prevalent in ischemia and reperfusion. Peng et al. (2010) showed that CaMKIIδ_B has been shown to protect against apoptosis in hydrogen peroxide-treated neonatal rat ventricular myocytes, through an upregulation of heat shock protein 70 (Peng et al., 2010). However, expression of CaMKIIδ_B decreases in these conditions over a period of hours, in direct contrast to CaMKIIδ_C, such that a reduction of influence on ischemic resilience would be expected to reduce with time (Peng et al., 2010). Interestingly, preliminary reports indicate that hearts from transgenic mice selectively overexpressing CaMKIIδ_B are less susceptible to ischemia and reperfusion injury (Gray et al., 2013), corroborating the notion of a cardioprotective action for this splice variant in ischemia/reperfusion.

As outlined above, CaMKII can provide important inotropic support to maintain contractile function in stunned hearts subjected to a brief ischemic challenge. An important body of experimental work supports the view that CaMKII actions can transition from conferring benefit to liability in a manner which reflects the myocyte capacity to balance relative SR Ca^{2+} uptake and leak. CaMKII "protection" is understood to be forfeited when the greater probability of spontaneous SR Ca^{2+} release outweighs the enhancement of cytosolic Ca^{2+} clearance (Mattiazzi and Kranias, 2011). The mechanisms responsible are not fully understood, but the extent of ox-CaMKII(Met281/2) generation may be implicated. With extended ischemia, it may be that an increase in ROS production in reperfusion further augments CaMKII activation and actions, possibly through greater ox-CaMKII(Met281/2) generation. As ox-CaMKII(Met281/2) increases P-RyR2(Ser2814) phosphorylation (Ho et al., 2014), an increase in SR Ca^{2+} leak would be expected. This may be a critical factor in promoting injury in reperfusion. Our very recent findings suggest that specific post-translational modifications of CaMKII determine substrate specificity, and that in contrast to P-CaMKII(Thr287), ox-CaMKII(Met281/2) selectively phosphorylates the RyR2 and not PLB (Bell et al., 2014). Augmented SR Ca uptake would be predicted to increase SR Ca^{2+} load (Shannon et al., 2002; Venetucci

et al., 2007). With a concomitant increase in P-RyR2(Ser2814), this could augment SR Ca^{2+} leak; an effect that may be offset if the Ca^{2+} levels at the RyR2 cytoplasmic domain are sufficiently reduced (Laver, 2009).

In summary, CaMKII is a crucial regulatory intermediate in ischemia and reperfusion injury. CaMKII is relatively quiescent under basal conditions, but displays considerable capacity to exacerbate Ca^{2+} mismanagement and mitochondrial dysfunction in response to ischemic changes in cellular Ca^{2+} and redox status. Thus, there is potential for CaMKII inhibitor utilization as a prophylactic therapeutic intervention for "at risk" patients with ischemic heart disease. Conversely, conflicting reports suggest that in other circumstances maintained CaMKII activation can improve reperfusion recovery. To optimize and tailor therapeutic strategies involving manipulation of CaMKII activation, it is clear that further mechanistic studies are required to fully understand the nuances of CaMKII response in a range of pathophysiologic settings.

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CaMKII in sinoatrial node physiology and dysfunction

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The calcium and calmodulin-dependent protein kinase II (CaMKII) is present in sinoatrial node (SAN) pacemaker cells and is required for physiological "fight or flight" SAN beating rate responses. Inhibition of CaMKII in SAN does not affect baseline heart rate, but reduces heart rate increases in response to physiological stress. CaMKII senses intracellular calcium (Ca^{2+}) changes, oxidation status, and hyperglycemia to phosphorylate substrates that regulate Ca^{2+}-sensitive proteins, such as L-type Ca^{2+} channels, phospholamban, and cardiac ryanodine receptors (RyR2). All of these substrates are involved in the SAN pacemaking mechanism. Excessive CaMKII activity, as occurs under pathological conditions such as heart failure, ischemia, and diabetes, can promote intracellular Ca^{2+} overload and reactive oxygen species production. Oxidation of CaMKII (ox-CaMKII) locks CaMKII into a constitutively active configuration that contributes to SAN cell apoptosis and fibrosis. This ox-CaMKII-mediated loss of functional SAN cells contributes to SAN dysfunction (SND) and sudden death. Thus, CaMKII has emerged as a central regulator of physiological SAN responses and a key determinant of SND.

Keywords: calcium/calmodulin-dependent protein kinase II, sinoatrial node, heart rate, sinoatrial node dysfunction, calcium

INTRODUCTION

The sinoatrial node (SAN) is a specialized region of heart tissue present at the junction of the right atrium and superior vena cava that extends along the cristae terminalis, where it initiates each normal heart beat. The pacemaking function of SAN cells is accomplished by generation of spontaneous action potentials. There appear to be redundant systems in SAN for generating spontaneous cell membrane potential depolarizations, which are ultimately necessary to sustain life by maintaining cardiac output. One of these systems comprises a set of cell membrane delimited ion channels. These ion channels include hyperpolarization-activated cyclic nucleotide-gated (HCN) channels that conduct an inward current, sometimes called a pacemaker current or funny current (I_f; DiFrancesco, 1991), L-type ($Ca_V1.2/1.3$; Christel et al., 2012) and T-type ($Ca_V3.1/3.2$) Ca^{2+} channels (Mangoni et al., 2006; Tanaka et al., 2008; Brahmajothi et al., 2010) and several K^+ channels, including ERG (Brahmajothi et al., 1997, 2010) and KvLQT1 (Chandler et al., 2009; Brahmajothi et al., 2010). All of these ion channels have the potential to play a role in pacemaking under different conditions. The other system involves intracellular Ca^{2+} machinery that is used for excitation–contraction coupling in mechanically purposed myocardium, but that contributes to rhythmic intracellular Ca^{2+} oscillations in SAN. This system enables SAN fight or flight heart rate increases and contributes to SAN cell death under pathological stress. These components include the sarcoplasmic reticulum (SR; Rigg and Terrar, 1996), which contains the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a), the ryanodine receptor 2 (RYR2), a large Ca^{2+} channel that releases Ca^{2+} from the SR lumen to the cytoplasm and the cell membrane spanning Na^+/Ca^{2+} exchanger (NCX1; Sanders et al., 2006). The components in both systems collaborate but are also capable of independent activity that ensures nonstop pacemaking activity (Lakatta and DiFrancesco, 2009; Lakatta et al., 2010).

We believe that the effects of the multifunctional Ca^{2+} and calmodulin-dependent protein kinase II (CaMKII) on SAN cell biology are related to actions on Ca^{2+} homeostasis. CaMKII is a multifunctional serine/threonine-specific protein kinase that is initially activated by the Ca^{2+}/calmodulin complex (Schulman and Greengard, 1978). CaMKII is present in contracting myocardium and in SAN cells (Vinogradova et al., 2000). Details of CaMKII structure, function, activation, and inactivation are contained in another chapter in this compendium (XYZ). However, the CaMKII holomeric structure allows it to perform as a precisely regulated enzyme that activates and inactivates with Ca^{2+}/calmodulin binding and unbinding but also to transition into a constitutively active conformation by post-translational modifications to the autoregulatory domain (Kuret and Schulman, 1985; Erickson et al., 2008, 2013; Chao et al., 2011; Gutierrez et al., 2013). Excessive levels of constitutively active CaMKII are linked to cardiovascular and pulmonary diseases, including SAN dysfunction (SND; Erickson et al., 2011; Sanders et al., 2013).

CaMKII IN SAN PHYSIOLOGY

Activated CaMKII can catalyze phosphorylation of multiple Ca^{2+} homeostatic proteins, including L-type, e.g., $Ca_V1.2$ (Dzhura et al., 2000; Grueter et al., 2006) and T-type, e.g., $Ca_V3.2$ (Yao et al., 2006) Ca^{2+} channels, phospholamban (PLN; Lindemann et al., 1983), a protein that negatively regulates SERCA2a in the absence of CaMKII or protein kinase A catalyzed phosphorylation (Kranias and Hajjar, 2012), and RYR2 (Witcher et al., 1991; Wehrens et al., 2004). CaMKII catalyzed phosphorylation increases Ca^{2+} entry

through Ca^{2+} channels, increases SERCA2a uptake of cytoplasmic Ca^{2+} into the SR lumen through phosphorylating PLN, which in turn increases the pool of SR releasable Ca^{2+}, increases Ca^{2+} release from RYR2 by phosphorylation of RYR2 at several sites, including serine 2814. On one hand, these effects will increase intracellular Ca^{2+} flux through the SR and RYR2 to accelerate NCX1 to increase SAN cell action potential frequency and the physiological fight or flight heart rate response. On the other hand, excessive CaMKII activity will cause Ca^{2+} overload (Wagner et al., 2011), which can induce increased reactive oxygen species (ROS) production and cause SAN cell damage or death (Swaminathan et al., 2011; Luo et al., 2013).

The role of CaMKII in SAN function has been explored since 1989 (Hagiwara and Irisawa, 1989). The major focus of this study was on the effects of calmodulin or CaMKII on I_f currents by using calmidazolium, a calmodulin inhibitor with many off target actions (Klöckner and Isenberg, 1987). They found that I_f currents were sensitive to intracellular Ca^{2+} but no evidence that I_f was regulated by CaMKII. A more recent study (Rigg et al., 2003) confirmed that I_f currents are regulated by Ca^{2+} and calmodulin but not by the CaMKII pathway. They showed that I_f current amplitude was unaffected by the CaMKII inhibitor KN-93 (1 μM) although this CaMKII inhibition did reduce L-type Ca^{2+} current by 48 ± 19% at 0 mV voltage clamp command potential. However, a more recent study challenged the concept of calmodulin regulation of I_f (Chatelier et al., 2005) based on experiments in inside-out cell membrane macro-patches excised from rabbit SAN cells. They found that "intracellular" calmodulin perfusion had no effect on HCN activity and did not change the cAMP-induced I_f activation shift. This study suggested that another calmodulin inhibitor, W-7, with well documented off target actions had direct effects on I_f that were independent of Ca^{2+} and calmodulin. The myriad off target actions on ion channels represent major obstacles to the use of CaMKII inhibitors in functional studies (Ledoux et al., 1999; Gao et al., 2006; Rezazadeh et al., 2006; Liao et al., 2011). CaMKII enhances $Ca_V1.2$ channel currents in ventricular myocytes (Anderson et al., 1994; Xiao et al., 1994; Yuan and Bers, 1994) and so could potentially affect SAN automaticity by actions on $Ca_V1.2$. A paper from the Xiao group showed that CaMKII was likely to play an important role in SAN pacemaker activity by actions at voltage-gated Ca^{2+} channels (Vinogradova et al., 2000). They were able to stop SAN cell automaticity by using CaMKII inhibitors KN-93 or myristoylated autocamtide-2-related inhibitory peptide (AIP) (a cell membrane permeant peptide inhibitor modeled after the CaMKII autoinhibitory region). The findings from the Xiao group supported an I_f-independent role for cardiac pacing. However, these studies were mostly performed using small molecule inhibitors with off target actions that complicate interpretation of the results. Taken together, these findings highlight some of the limitations of available small molecule calmodulin and CaMKII antagonists and suggest that I_f is not directly responsive to calmodulin or CaMKII but leave open the question whether CaMKII actions at $Ca_V1.2$ channels contribute to SAN automaticity. We developed a mouse with myocardial targeted transgenic expression of AC3-I, a highly selective CaMKII inhibitory peptide, under control of the α-myosin heavy chain promoter (Zhang et al., 2005). AC3-I expression was present in SAN cells and a study from our group using this mouse found that CaMKII inhibition did not affect baseline SAN pacemaking activity but selectively impaired the fight or flight response of SAN cells to isoproterenol (Wu et al., 2009). CaMKII was responsible for approximately half of the dynamic heart rate response range. We found that neither SAN cell Ca^{2+} channels nor I_f currents from AC3-I mice were different compared with their WT littermates nor control transgenic mice expressing AC3-C, an AC3-I like peptide without biological activity. We found that SR Ca^{2+} content responses to isoproterenol in those mice were reduced, potentially as a consequence of diminished CaMKII catalyzed phosphorylation of PLN. The reduced SR Ca^{2+} content likely contributed to reduced Ca^{2+} spark frequency as well as decreased Ca^{2+} release from SR (**Figure 1**). Our findings were later confirmed by studies from another group using a CaMKIIδ knock out mouse (Xu et al., 2010). Their study also showed that CaMKII is required for heart rate increases by isoproterenol stimulation or in response to a physiological fight or flight mechanism. A recent study from Terrar group (Collins and Terrar, 2012) suggested that the effect of CaMKII in atrial myocytes may be primarily on SR proteins due to different distribution of CaMKII in ventricular myocytes compare to atrial myocytes which lack of T-tube. The effects of CaMKII on atrial Ca^{2+} channels are indirectly through CaMKII enhanced SR Ca^{2+} releasee that stimulates adenylyl cyclases (ACs). Recently, one study from Lakatta group using KN-93, myristoylated AIP, and W-7 to inhibit CaMKII (Yaniv et al., 2013) suggest that CaMKII may affect SAN automaticity by actions on metabolism. In our opinion, these results are intriguing but inconclusive because of the documented off-target actions of these reagents (Ledoux et al., 1999; Chatelier et al., 2005; Gao et al., 2006; Rezazadeh et al., 2006; Liao et al., 2011). Taken together, these studies support a view that CaMKII is not required to maintain basal heart rates but plays a critical role in sustained heart rate increases during physiological stress. This selective role of CaMKII on heart rate suggests that CaMKII inhibition could protect against excessive heart rates without reducing baseline heart rate.

CaMKII IN SND

Conditions that favor SND, such as heart failure, atrial fibrillation (AF), and advanced age are marked by heightened ROS (Cesselli et al., 2001; Kim et al., 2005; Dai et al., 2009). Because CaMKII is activated by ROS (Erickson et al., 2008) in the setting of increased angiotensin II (Ang II), a circulating neurohormone present at increased levels in heart failure, we tested if oxidized CaMKII (ox-CaMKII) could contribute to SND. We found Ang II increased atrial and SAN oxidation by activating NADPH oxidase, leading to increased ox-CaMKII, SAN cell apoptosis, and SND (Swaminathan et al., 2011). In order to test whether elevated ox-CaMKII could cause SND, mice were infused with Ang II. Ang II infusion for 3 weeks caused increased SAN ox-CaMKII, SAN cell apoptosis, fibrosis, slowed atrial impulse conduction velocity, and SND. Ang II-triggered SND was prevented by transgenic myocardial and SAN cell expression of AC3-I (Zhang et al., 2005) and by SAN-targeted gene therapy (Kikuchi et al., 2005) providing ectopic SAN expression of a CaMKII inhibitory peptide, CaMKIIN, that is endogenous to neurons but absent in

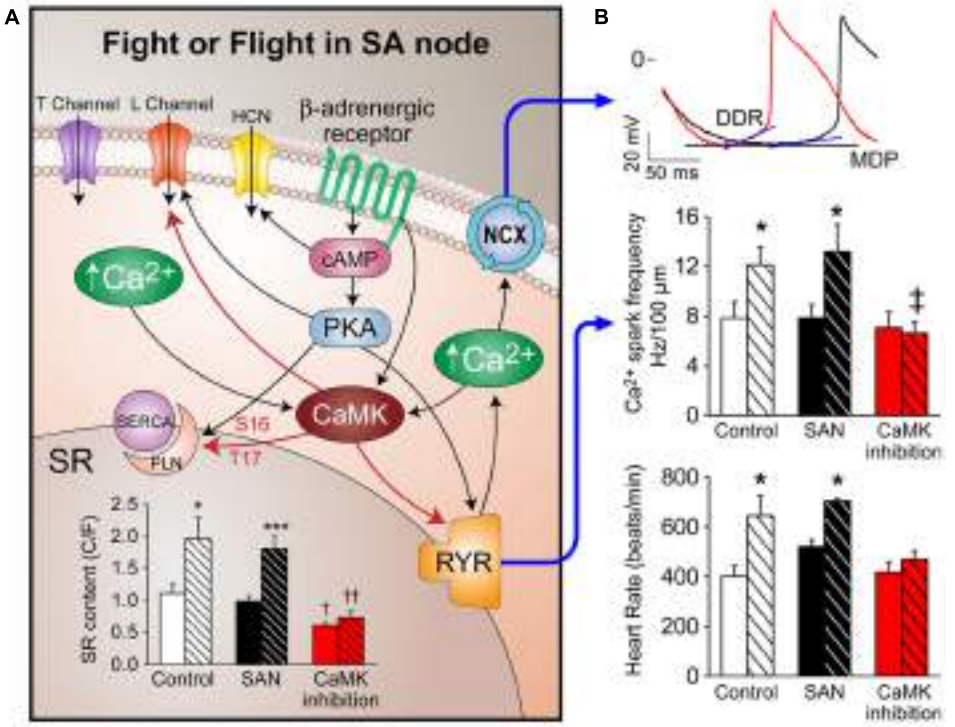

FIGURE 1 | Mechanism of CaMKII effects on Fight or Flight in SA node. **(A)** Fight or flight stimulation (e.g., isoproterenol) activates PKA and CaMKII, which phosphorylate L-type Ca^{2+} channels and PLN to increase SR Ca^{2+} content. RYR2 phosphorylation increases Ca^{2+} release from SR. Increased Ca^{2+} release accelerates NCX1 activity which increases the diastolic depolarization rate (DDR) of SAN action potentials **(B)**. SR Ca^{2+} content increases by isoproterenol are abolished by CaMKII inhibition. Upper panel shows DDR change with isoproterenol (red trace) compare to control DDR (black trace), MDP, maximal diastolic potential. Middle panel shows Ca^{2+} spark frequency increases after isoproterenol are abolished by CaMKII inhibition. The lower panel shows the heart rate increase by isoproterenol is abolished by CaMKII inhibition. All shaded bars in bar graphs represent data with isoproterenol effects. Both white bars (WT control) and black bars (transgenic control) represent data from control SAN. $*p < 0.05$, $***p < 0.001$ before vs. after isoproterenol, † $p < 0.05$ compare to control SAN groups, †† or ‡ $p < 0.01$ compare to control SAN groups.

heart (Chang et al., 1998). Neither transgenic nor gene-targeting approaches to SAN CaMKII inhibition affected the hypertensive response to Ang II, nor did they abrogate the increased SAN ROS due to Ang II infusion, indicating that CaMKII was a critical downstream signal for the pathological actions of ROS on SAN. The increase in SAN ox-CaMKII by Ang II required activation of NADPH oxidase, because it was absent in $p47^{-/-}$ mice (Huang et al., 2000) lacking functional NADPH oxidase. We developed a structural and computational model of the SAN that revealed a quantitative mechanism to explain how Ang II-induced SAN cell apoptosis resulted in SND by reducing SAN cell number and increasing electrotonic loading of surviving SAN cells to cause loss of high-fidelity impulse formation and propagation (**Figure 2**; Huke and Knollmann, 2011). We also found that right atrial tissue from patients with heart failure who required artificial pacemakers for SND or dogs with pacing-induced heart failure and SND had elevated ox-CaMKII compared with patients with heart failure alone or dogs with non-SND controls. These findings provide insights into how excessive activation of CaMKII in SAN cells causes SND, suggest ox-CaMKII is a biomarker for SND and identify what we believe to be a novel candidate approach to preventing SND in high risk settings by CaMKII inhibition.

Patients with AF are at increased risk for SND (Chang et al., 2013) and CaMKII activity and expression are increased in fibrillating human atria (Neef et al., 2010). We recently found that ox-CaMKII is increased in fibrillating compared to non-fibrillating human atria and that Ang II infusion increases AF induction in mice (Purohit et al., 2013). Mice with transgenic expression of AC3-I, mice with a knock-in mutation (MM-VV) in CaMKIIδ that prevents oxidative activation and mice with transgenic overexpression of methionine sulfoxide reductase A that reverses the first oxidation state (sulfoxide) of methionine were all resistant to Ang II-induced AF. We interpret these findings to suggest that ox-CaMKII is a unifying signal for SND and AF.

Diabetes is a risk factor for SND (Podlaha and Falk, 1992). We recently found significantly more ox-CaMKII in right atrium from patients with a history of diabetes and myocardial infarction (MI) compared with right atrial tissue from patients with MI but no diabetes, suggesting that ox-CaMKII could contribute to the increased mortality in diabetic patients after MI (Luo et al., 2013). Streptozotocin (STZ)-treated mice develop severe type I diabetes due to death of pancreatic β-cells. STZ-treated diabetic mice were twice as likely to die after MI surgery as vehicle-treated control mice, mimicking the increased mortality in diabetic patients compared with that in non-diabetic patients after MI. STZ-treated

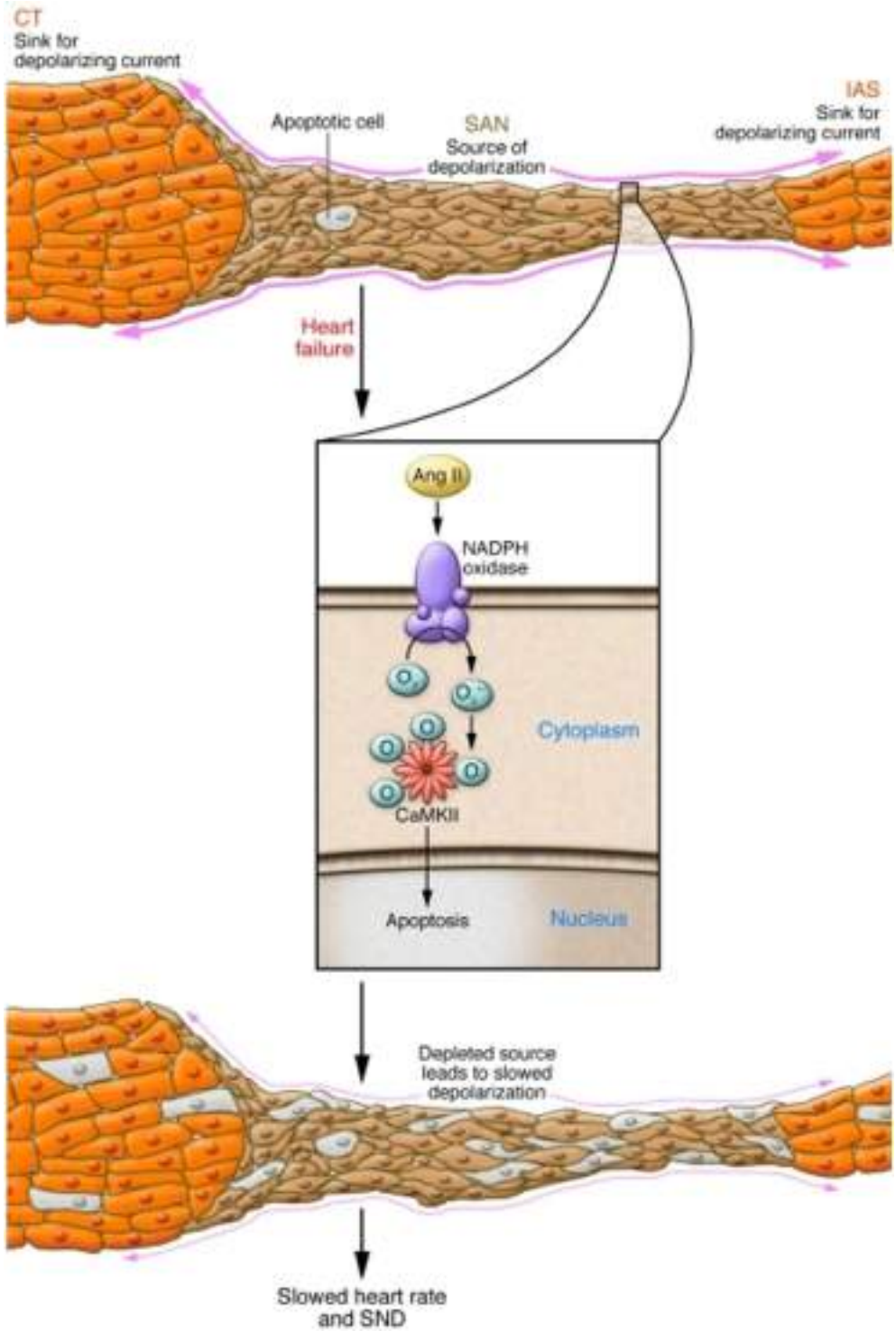

FIGURE 2 | Mechanism of Ang II-induced SND. Normally, the small volume of excited tissue in the SAN (source) depolarizes the neighboring quiescent atrial tissue (sink). In conditions with increased Ang II, NADPH oxidase is activated, leading to oxidation of two methionine residues of CaMKII, rendering the enzyme autonomously active. Elevated activity of CaMKII leads to SAN cell death, reducing the threshold volume of automatic cells of the SAN and increasing non-excitable tissue in the form of fibrosis. This increased electrotonic loading produces a source-sink mismatch slows the beating rate, and causes SND. CT, crista terminalis; IAS, inferior atrial septum. Reproduced from Huke and Knollmann (2011), with permission from JCI.

MM-VV mice and mice with transgenic myocardial and SAN expression of AC3-I (Zhang et al., 2005) were protected from increased mortality after MI, indicating that increased ox-CaMKII was essential for excess mortality after MI in STZ-treated mice. Death in STZ-treated mice after MI was due to severe bradycardia, consistent with known defects in cardiac pacemaker function in another diabetic animal model (Howarth et al., 2007). In contrast to our earlier studies with Ang II-triggered ROS by activation of NADPH oxidase (Swaminathan et al., 2011), we found that hyperglycemia-induced ROS were primarily from mitochondria. Excess mortality in STZ-treated diabetic mice after MI surgery was prevented by chronic infusion with a mitochondrial targeted antioxidant, Mito-TEMPO. Mito-TEMPO reduced ox-CaMKII, preserved heart rates, and improved survival after MI. These results provide new evidence that ox-CaMKII is a biomarker for SND and suggest that mitochondrial or CaMKII-targeted antioxidant therapies could benefit high-risk diabetic patients.

In summary, CaMKII appears to play important roles in tuning the fight or flight response and in promoting SND. It may be that the physiological benefits of CaMKII activation in early life are outweighed in later life by the tendency of CaMKII to become persistently active under conditions of high oxidative, neurohumoral and hyperglycemic stress. The tractability of CaMKII as a target for selectively controlling heart rate and preventing SND will depend upon the availability of clinically suitable CaMKII inhibitory drugs or gene therapy.

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Numerical modeling calcium and CaMKII effects in the SA node

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Sinoatrial node (SAN) is the primary heart pacemaker which initiates each heartbeat under normal conditions. Numerous experimental data have demonstrated that Ca^{2+}- and CaMKII-dependent processes are crucially important for regulation of SAN cells. However, specific mechanisms of this regulation and their relative contribution to pacemaker function remain mainly unknown. Our review summarizes available data and existing numerical modeling approaches to understand Ca^{2+} and CaMKII effects on the SAN. Data interpretation and future directions to address the problem are given within the coupled-clock theory, i.e., a modern view on the cardiac pacemaker cell function generated by a system of sarcolemmal and intracellular proteins.

Keywords: cardiac pacemaker, sinoatrial node, numerical modeling, calcium, CaMKII, ion channels

INTRODUCTION

Under normal conditions, SAN cells (SANC) generate spontaneous rhythmic action potentials (AP) that initiate the heartbeat. The evolution of thought regarding the cardiac pacemaker cell operation paradigm switched back and forth between intracellular origin [e.g., a "metabolic" intracellular clock (Bozler, 1943) or sarcoplasmic reticulum (SR)-based Ca^{2+}-clock (Maltsev et al., 2006)] and cell membrane origin [voltage membrane clock or M-clock (Noble, 1960)]. A more recent paradigm shift has been the realization that both intracellular and sarcolemmal mechanisms are tightly, dynamically coupled to each other and are indispensable for normal pacemaker function. These ideas have been summarized within a "coupled-clock" theory of interacting M-clock and Ca^{2+}-clock (Maltsev and Lakatta, 2009; **Figure 1**) that explained numerous experimental findings (Lakatta et al., 2010; Maltsev and Lakatta, 2012). The key processes of the coupled-clock system depend on Ca^{2+}, calmodulin (CaM), and CaMKII signaling (**Figure 1**, red). Interactions within the system are extremely complex and their detailed investigation requires numerical model simulations. The CaMKII function in pacemaker cells has not been systematically studied using numerical simulations. Our review summarizes major principles of the coupled-clock theory, available data, and existing numerical modeling approaches that are important to delineate future numerical integration and exploration of CaMKII within the pacemaker cell system.

INTEGRATION OF Ca^{2+} AND CaMKII SIGNALING WITHIN THE COUPLED-CLOCK SYSTEM

Operation of the coupled-clock system has been explored in recent numerical model studies (Maltsev and Lakatta, 2009, 2013; Yaniv et al., 2013a,d), and experimental evidence for the coupled-clock theory has been summarized (Lakatta et al., 2010; Maltsev and Lakatta, 2012). The system generates spontaneous, rhythmic APs separated by a slow diastolic depolarization (DD) that starts each cycle from the maximum diastolic potential (MDP $\sim$−60 mV) and brings the membrane potential (V_m) to a cell excitation threshold of $\sim$−40 mV. The coupled-clock theory postulates that the DD is generated by the two coupled oscillators, Ca^{2+}-clock and M-clock, rather than just by M-clock alone (**Figure 1**).

The first numerical model of M-clock was developed by Noble (1960), by application of Hodgkin–Huxley (HH) theory to cardiac pacemaker cells. The M-clock-based models generate the DD via time-dependent kinetics of ion channels upon AP repolarization, e.g., by inactivation of a K^+ current (Noble, 1960) or by activation of a non-selective, "funny" current (DiFrancesco and Noble, 2012). The SR, a major Ca^{2+} store in cardiac cells, can also generate spontaneous oscillations via rhythmic cycles of SR Ca^{2+} pumping (via SERCA) and release (via release channels, RyR; **Figure 1**). Ventricular muscle cells can spontaneously cycle Ca^{2+} (under conditions of high Ca^{2+} loading) via global Ca^{2+} waves via regenerative Ca^{2+}-induced Ca^{2+} release (CICR) propagating by Ca^{2+} diffusion (Fabiato, 1983). However, cardiac pacemaker cells generate rhythmic, spontaneous Ca^{2+} releases during DD under normal Ca^{2+} conditions (in the absence of Ca^{2+} overload; Huser et al., 2000; Bogdanov et al., 2001). These releases occur in the form of abrogated waves, dubbed local Ca^{2+} releases or LCRs. The synchronous occurrence of the LCRs generates a powerful, diastolic, net Ca^{2+} signal, dubbed the late diastolic Ca^{2+} elevation or LDCaE (**Figure 2**). The rhythmic LCRs are generated in the absence of M-clock, e.g., under voltage clamp or in membrane-permeabilized SANC [when $[Ca^{2+}]$ is normal, $\sim$100 nM, review (Lakatta et al., 2010)]. The Ca^{2+}-clock in SANC is driven by Ca^{2+} cycling proteins (e.g., phospholamban and RyR, **Figure 1**), whose function is

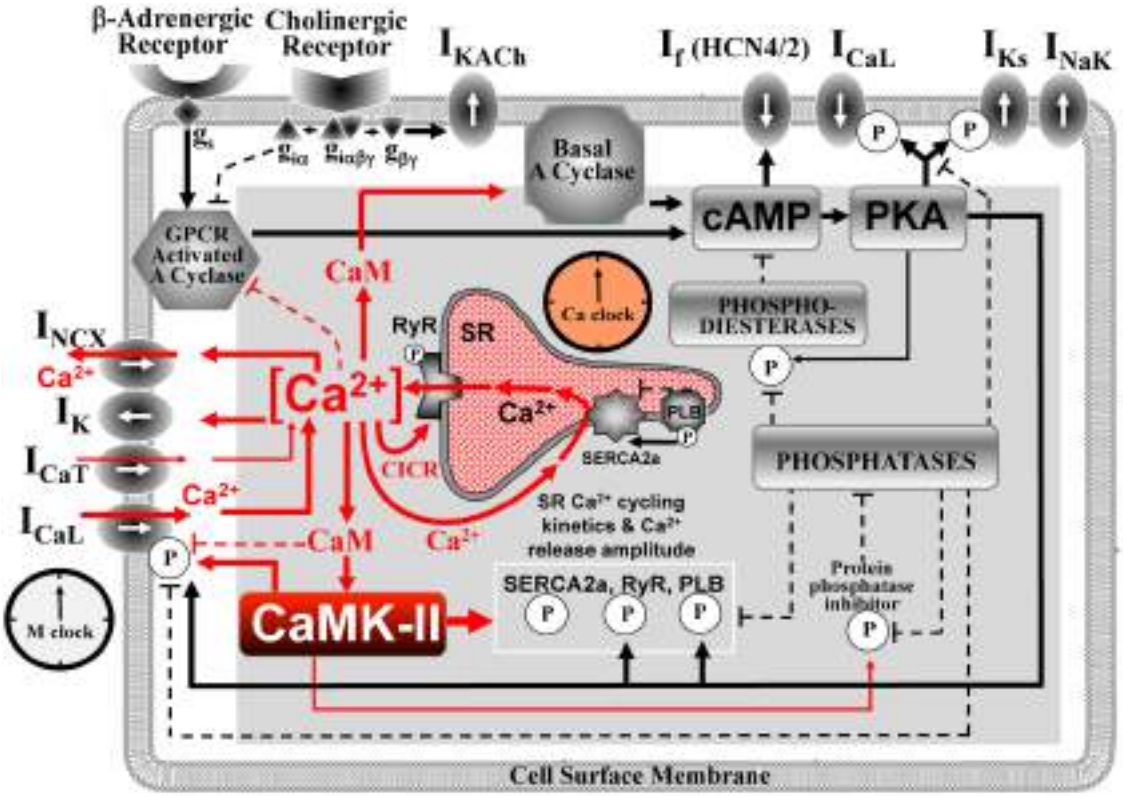

FIGURE 1 | Schematic illustration of sinoatrial node cell as a coupled-clock system of voltage membrane clock (M-clock) and an intracellular, sarcoplasmic reticulum (SR)-based Ca^{2+}-clock (gray intracellular area). Interactions of key molecules comprising the system with Ca^{2+} and CaMKII signaling are shown in red. Note that common regulatory factors govern the function of both clocks. These common factors, including CaMKII, act as nodes within the system to couple the function of both clocks activities. The system is balanced: signals accelerating action potential (AP) firing are balanced by signals suppressing AP firing. This balance determines a given steady-state level of net Ca^{2+}, cAMP, and protein phosphorylation via PKA, and CaMKII. G protein-coupled receptors (top left corner) within the cell membrane modulate both the Ca^{2+}-clock and M-clock function via the same crucial signaling nodes of the system. Modified from Lakatta et al. (2010).

enhanced by phosphorylation via basal activity of PKA (Vinogradova et al., 2006) and CaMKII (Vinogradova et al., 2000). In turn, the PKA is activated by a high basal level of cAMP produced by Ca^{2+}-activated adenylyl cyclases (ACs) which are highly expressed in SANC [particularly types 1 and 8, (Mattick et al., 2007; Younes et al., 2008)]. The high rate of cAMP production and protein phosphorylation is counterbalanced by activities of phosphatases and phosphodiesterases. Interestingly, a powerful Ca^{2+}-clock generating rhythmic LCRs (similar to that in SANC) also emerges in ventricular myocytes when the phosphorylation of Ca^{2+} cycling protein increases (e.g., via inhibition of phosphatases and/or phosphodiesterases; Sirenko et al., 2014).

The Ca^{2+}-clock and the M-clock are coupled in SANC via Na^+/Ca^{2+} exchanger (NCX; **Figure 2**) that senses the LCR ensemble (i.e., LDCaE) and, operating in the forward mode, generates a substantial inward current (I_{NCX}) during DD. M-clock, in turn, regulates Ca^{2+}-clock via L-type Ca^{2+} current (I_{CaL}) by (1) resetting phases of local Ca^{2+} oscillators that synchronizes LCR ensemble; (2) supplying Ca^{2+}, i.e., the Ca^{2+}-clock's oscillatory substrate. Both clocks are coupled not only directly via V_m and Ca^{2+}, but indirectly, enzymatically, by coupling factors, such as PKA and CaMKII, affecting multiple targets within both clocks (**Figure 1**). PKA- and CaMKII-dependent phosphorylation enhances function of the proteins comprising the system and is required for normal pacemaker function and autonomic modulation. Because of these complex interactions (which define the Ca^{2+} balance and enzymatic activity balance), each component of the system contributes to the LCR spatiotemporal characteristics, especially the LCR period, i.e., the time when LCRs emerge and accelerate the DD (**Figure 2**). Thus, the LCR period is contributed not only directly by the Ca^{2+} release channels RyR, but also indirectly by L-type Ca^{2+} channels (LCCh), SERCA, and NCX regulating Ca^{2+} fluxes (Maltsev et al., 2013), and even by K^+ channels or "funny" channels via respective V_m changes, also regulating Ca^{2+} fluxes (Yaniv et al., 2013a).

EXPERIMENTAL EVIDENCE FOR IMPORTANCE OF CaMKII SIGNALING IN PACEMAKER CELLS

CaMKII indirectly senses $[Ca^{2+}]$ by binding Ca^{2+}-CaM complex at the CaM region in its regulatory domain, which increases its activity (Anderson et al., 2011). (Of note, there are two

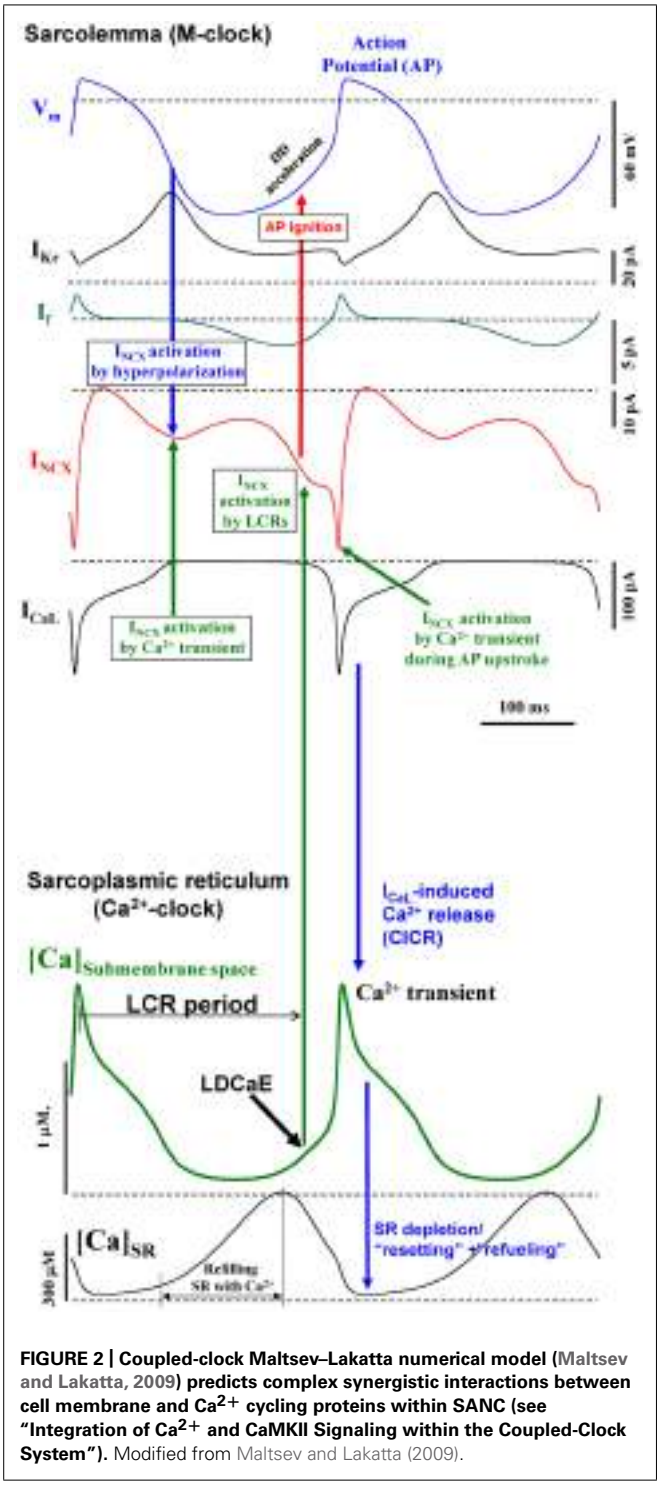

FIGURE 2 | Coupled-clock Maltsev–Lakatta numerical model (Maltsev and Lakatta, 2009) predicts complex synergistic interactions between cell membrane and Ca^{2+} cycling proteins within SANC (see "Integration of Ca^{2+} and CaMKII Signaling within the Coupled-Clock System"). Modified from Maltsev and Lakatta (2009).

predominant CaMKII isoforms in the heart: CaMKIIδ_B localizes in nuclei and CaMKIIδ_C in cytosol). While CaMKII does not regulate directly cAMP production, reduction in CaMKII activity is associated with reduction in [cAMP] in rabbit SANC (Yaniv et al., 2013b), indicating a complex interplay of the CaMKII, ACs, and PKA signaling (**Figure 1**). In this special issue Wu and Anderson discuss in detail experimental evidence for contribution of CaMKII activity to SAN function during health and heart disease (Wu and Anderson, 2014). Here we summarize the key facts with respect to the integration of CaMKII within the coupled-clock system of SANC (**Figure 1**) and its future numerical modeling.

Pharmacological inhibition of CaMKII signaling (using AIP or KN-93) depresses the basal rate and amplitude of spontaneous APs in SANC of rabbit (Vinogradova et al., 2000; Yaniv et al., 2013b) and guinea-pig (Rigg et al., 2003). Confocal imaging of immunolabeled proteins demonstrates that active CaMKII is highly localized beneath the surface membrane (Vinogradova et al., 2000). Thus, CaMKII activity is geographically associated with proteins of both M- and Ca^{2+}-clocks. CaMKII modulates several membrane ion channels in the heart: LCCh, K^+ channels, and Ca^{2+}-clock proteins: SERCA (directly and indirectly via phospholamban) and RyR. Studies in isolated rabbit SANC suggested that CaMKII regulates the pacemaker activity via modulating I_{CaL} inactivation and reactivation (Vinogradova et al., 2000) and LCR morphology (Vinogradova et al., 2011). I_f is not affected directly by CaMKII inhibition (Rigg et al., 2003).

Thus, contribution of CaMKII to basal AP generation by SANC was demonstrated for rabbit and guinea pig [but remains controversial for mice (Zhang et al., 2005; Wu et al., 2009)]. Because CaMKII is sensitive to the frequency of the Ca^{2+} transients, CaMKII is ideally suited to respond to changes in SAN rhythm. For example, electrical stimulation alone increases CaMKII-dependent phosphorylation of phospholamban at CaMKII phosphorylation site in a frequency-dependent manner in ventricular myocytes (Hagemann et al., 2000). CaMKII also mediates SAN response to β-adrenergic receptor stimulation (Wu et al., 2009). Moreover, SANC and isolated hearts from mice with CaMKII inhibition (by transgenic expression of AC3-I) were insensitive to BayK, an LCCh agonist, which increases pacemaker rate in wild type mice (Gao et al., 2011). New evidence that CaMKII is a key part of the coupled-clocked system (**Figure 1**) has been obtained in studies of specific I_f inhibitor ivabradine (Yaniv et al., 2013a; discussed below).

CaMKII activity can also be enhanced by pro-oxidant conditions (Erickson et al., 2008). Clinical studies show that right atrial tissue from patients with heart failure who also required artificial pacemakers have more Oxidize-CaMKII compared to patients with heart failure alone and patients without heart failure or severe SAN dysfunction (Swaminathan et al., 2011). Ang II infusion in mice increases Oxidize-CaMKII and elicits SAN dysfunction that is prevented by overexpression of a synthetic CaMKII inhibitory peptide (AC3-I) or by CaMKIIN, an endogenous CaMKII protein present in neurons, but absent in the heart (Swaminathan et al., 2011).

CaMKII activity appears to be increased in heart disease (e.g., arrhythmia, heart failure, atrial fibrillation; Anderson et al., 2011). Sinus sick syndrome prevails during heart failure and hypertension conditions (with both conditions exhibiting elevated angiotensin II levels). Because CaMKII inhibition is sufficient to protect against angiotensin II-induced sick sinus syndrome in aforementioned mouse model (Swaminathan et al., 2012), CaMKII inhibition may be a useful approach to prevent sinus sick syndrome.

It was demonstrated that basal AC-cAMP/PKA signaling directly, and Ca^{2+} indirectly, regulate mitochondrial ATP production (Yaniv et al., 2011, 2013c). As a crucial element of normal automaticity in rabbit SANC, CaMKII signaling is also involved in SANC bioenergetics. When ATP demand is reduced by interfering with CaMKII or CaM activity, SANC become depleted of ATP, indicating reduction in ATP generation with lower demand (Yaniv et al., 2013b).

NUMERICAL MODELING STUDIES THAT SHOW IMPORTANCE OF CaMKII SIGNALING FOR SAN FUNCTION

Although CaMKII signaling, *per se*, has not been systematically studied in pacemaker cell models, at least two recent numerical model studies point to a key functional importance of CaMKII signaling in pacemaker cells and tissues.

Yaniv et al. (2013a) have recently demonstrated that CaMKII likely serves as a key functional integrator of M-clock and Ca^{2+}-clock signals (**Figure 1**) by testing effects of specific perturbations of either clock in rabbit SANC. The M-clock was specifically perturbed by ivabradine that at low concentrations (<3 μM) specifically inhibits I_f, i.e., it does not suppress I_{CaL} (Yaniv et al., 2012a), other membrane ion currents (Bois et al., 1996), or Ca^{2+} cycling in permeabilized SANC (Yaniv et al., 2013a).

Numerical simulations (Yaniv et al., 2013a) using a modified coupled-clock Maltsev–Lakatta model (Yaniv et al., 2012b), provided new insights in ivabradine-induced bradycardia. An initial I_f reduction slows AP rate that, in turn, reduces the number of I_{CaL} activations/unit time, average Ca^{2+} influx, and Ca^{2+} available for SR pumping. This results in lower SR Ca^{2+} load and longer LCR period (both effects were also found experimentally). Later activation of diastolic I_{NCX} by the LCRs (and I_{NCX}-linked DD acceleration) leads to a delayed activation of I_{CaL}, i.e., M-clock slowing. Thus, inhibition of the M-clock inhibits (indirectly) Ca^{2+}-clock that further suppresses the M-clock, and so on, until the coupled-clock system attains a new steady-state.

Interestingly, model simulations show that the complex ivabradine effects extend further, beyond "biophysical" entrainment, and likely include an additional "biochemical" component. The aforementioned decrease in average Ca^{2+} influx produced by ivabradine not only decreases Ca^{2+} available for SR pumping, but also likely decreases protein phosphorylation signaling via Ca^{2+}-activated-CaMKII and Ca^{2+}-activated-ACs-cAMP/PKA pathways. This leads to further reductions in the average Ca^{2+} influx and, therefore, SR Ca^{2+} loading and AP firing rate. Simultaneously, reduction in cAMP shifts the I_f activation curve (effecting further M-clock slowing). If the "biochemical" crosstalk is lacking, model simulations (Yaniv et al., 2013a) predict only about 50% of the experimentally measured bradycardia produced by ivabradine. Thus, the entire ivabradine effect is explained by a crosstalk of equally important biophysical and biochemical mechanisms (including CaMKII signaling).

According to the coupled-clock theory (Maltsev and Lakatta, 2009) any selective perturbation of either clock will inevitably affect the function of the other and the entire coupled-clock system. In line with this postulate, the bradycardic effect is symmetric: it does not depend on which clock was initially perturbed. Both the LCR period and AP cycle length become prolonged by either perturbations of M-clock (e.g., using ivabradine) or Ca^{2+}-clock (e.g., using cyclopiazonic acid to selectively inhibit SERCA), with the LCR period reporting the resultant complex effect (Yaniv et al., 2013a).

Heart rate reductions produced by ivabradine or HCN4 mutations have been interpreted as a pure result of insufficient I_f function. However, based on the results discussed above, these effects are likely complex, involving the secondary changes in Ca^{2+}-clock and the entire coupled-clock system (that includes CaMKII signaling; Yaniv and Lakatta, 2013). Effects of mutations of Ca^{2+} cycling proteins on pacemaker function also likely include clocks coupling, i.e., secondary effect on I_f (via Ca^{2+}-activated-ACs and cAMP), rate-dependent effects on both clocks, ultimately resulting in mutual entrainment of the clocks (Yaniv and Lakatta, 2013; Yaniv et al., 2013a).

Luo et al. (2013) numerically modeled a further level of CaMKII effects related to cell death that is important to approach the mechanisms of insufficient pacemaker function in disease and aging. They developed a two-dimensional histologically reconstructed mathematical model that takes into account SAN cell death and fibrosis expressed in myocardial infarction by oxidizing CaMKII. Their simulations predict decreased conduction velocity and shift of the leading pacemaker site under these conditions. Thus, changes in CaMKII signaling can result in morphological changes of the SAN tissue which can affect cardiac impulse initiation.

LOCAL Ca^{2+} AND CaMKII SIGNALING IN PACEMAKER CELLS

The local Ca^{2+} control theory (Stern, 1992) remains a key in understanding the mechanisms of cardiac excitation-contraction coupling. This theory explained graded CICR phenomenon via statistics of success and failure of an initiating event (such as LCCh opening) to recruit stochastic Ca^{2+} release units (CRUs) to fire. While partially periodic LCRs (comprising Ca^{2+} clocks) in cardiac pacemaker cells are generated by the CRUs, they are, in fact, a product of complex local interactions of proteins residing in both cell membrane and the SR, i.e., RyR, SERCA, LCCh, and NCX. These interactions, in turn, are regulated by PKA and CaMKII signaling (**Figure 1**).

During the last decade mathematical models have been developed in ventricular myocytes to describe the CaMKII effects via regulation of ionic currents (Hund and Rudy, 2004; Grandi et al., 2007). More recent models describe CaMKII activity as a function of subspace Ca^{2+}, CaM, and phosphatase activity (Saucerman and Bers, 2008). These studies have demonstrated that the different affinities of CaM and CaMKII and calcineurin determine their sensitivity to local versus global Ca^{2+} signals that regulates excitation-contraction coupling. Hashambhoy et al. (2009) developed a stochastic model describing the dynamic interactions among CaMKII, LCCh, and phosphatases as a function of dyadic Ca^{2+} and CaM levels.

Local Ca^{2+} mechanisms have been recently modeled in pacemaker cells. The LCRs are generated via stochastic recruitment of the neighboring CRUs (Maltsev et al., 2011) regulated by local interactions of RyR, SERCA, and NCX (Maltsev et al., 2013). Some irregularity in RyR spatial distribution is not an imperfection,

but rather a functional modality of the pacemaker cells [abstract (Maltsev et al., 2014)]. The irregularity decreases nearest neighbor-to-neighbor distances among the CRUs and thereby facilitates local CICR forming wavelet-like LCRs. This new local control mechanism regulates the balance between robustness and flexibility of pacemaker cell function.

The most advanced SANC model (Stern et al., 2014) features stochastic propagated spontaneous diastolic Ca^{2+} release in three dimensions. This model describes explicit gating of individual Ca^{2+} channels (both RyR and LCCh), without assuming either a discrete sub-membrane compartment or an inactivated state of the RyR. The model succeeded in reproducing observed propagating local Ca^{2+} releases and realistic pacemaker rates only when RyR locations were assigned taking into account irregular, hierarchical distribution of RyR clusters (small and large) observed in 3D confocal scan sections of immunofluorescence staining. When the RyR sensitivity is very high or the NCX density is low, synchronization is lost, causing sympathetic stimulation to reduce (rather than increase) beating rate, often exhibiting arrhythmias (Maltsev et al., 2013; Stern et al., 2014). This regime may be important for rhythm abnormalities caused by heart failure, RyR mutations, or pharmacological NCX blockade.

Compared to previous models, lacking local Ca^{2+} dynamics (i.e., "common pool" models [Kurata et al., 2002; Maltsev and Lakatta, 2009)], the new models provide mechanistic insights into local crosstalk of the key molecules of the system: recruitment of RyRs (generating diastolic LCRs), RyR-LCCh and RyR-NCX crosstalk, and efficient SERCA operation (Maltsev et al., 2013). Indeed, Ca^{2+} signals within LCRs exhibit much higher amplitudes vs. those predicted by "common pool" models ($\sim$tens of μM vs. $\sim$1 μM). Thus, the "local" models, predicting the realistic scale of Ca^{2+} signals within the inhomogeneous signaling network of SANC, seem to be a better choice to explore CaMKII effects in future studies of pacemaker cells.

SUMMARY

In this review we have summarized the present state of experimental and numerical modeling studies on Ca^{2+} and CaMKII roles in cardiac pacemaker cells. Taking into account emerging importance of local Ca^{2+} control in cardiac pacemaker cells and also importance of local CaMKII signaling (reported in ventricular myocytes), accurate interpretation of experimental data on CaMKII effects in pacemaker cells will likely require integration of local (Saucerman and Bers, 2008) and molecular (Hashambhoy et al., 2009) mechanisms into new pacemaker cell models. Another important aspect that needs numerical integration is CaMKII involvement in SANC bioenergetics (Yaniv et al., 2013b). The new experimental studies combined with new model simulations will explore CaMKII interactions (**Figure 1**, red) with key regulatory molecules (e.g., ACs, PDEs, phosphatases, PKA, phospholamban), effector molecules (RyR, SERCA, NCX, LCCh, NCX, etc), and energy production of the system. This knowledge will contribute greatly to our understanding of cardiac impulse initiation and specific role of CaMKII signaling in the pacemaker regulation.

AUTHOR CONTRIBUTIONS

Both authors contributed to the conception of the work, drafted the paper, approved the version to be published, and are accountable for all aspects of the work.

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Calcium dysregulation in atrial fibrillation: the role of CaMKII

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Atrial fibrillation (AF) is the most frequently encountered clinical arrhythmia and is associated with increased morbidity and mortality. Ectopic activity and reentry are considered major arrhythmogenic mechanisms contributing to the initiation and maintenance of AF. In addition, AF is self-reinforcing through progressive electrical and structural remodeling which stabilize the arrhythmia and make it more difficult to treat. Recent research has suggested an important role for Ca^{2+}-dysregulation in AF. Ca^{2+}-handling abnormalities may promote ectopic activity, conduction abnormalities facilitating reentry, and AF-related remodeling. In this review article, we summarize the Ca^{2+}-handling derangements occurring in AF and discuss their impact on fundamental arrhythmogenic mechanisms. We focus in particular on the role of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase type-II (CaMKII), which acts as a major link between Ca^{2+}-dysregulation and arrhythmogenesis. CaMKII expression and activity are increased in AF and promote arrhythmogenesis through phosphorylation of various targets involved in cardiac electrophysiology and excitation-contraction coupling. We discuss the implications for potential novel therapeutic strategies for AF based on CaMKII and Ca^{2+}-handling abnormalities.

Keywords: atrial fibrillation, calcium, CaMKII, ectopic activity, reentry

INTRODUCTION

Atrial fibrillation (AF) is the most prevalent heart-rhythm disorder, estimated to affect more than 33 million people worldwide (Chugh et al., 2013). AF is associated with increased morbidity and mortality, notably as a risk factor for stroke and worsening of heart failure (Camm et al., 2012; Chugh et al., 2013). Current pharmacological treatments for rhythm-control of AF mainly include class-I and class-III antiarrhythmic drugs, which have modest efficacy, providing sinus-rhythm maintenance in only 30–70% of patients after >1 year of follow-up (Camm, 2012). In addition, these drugs are associated with substantial adverse side-effects including ventricular proarrhythmia and extra-cardiac toxicity (Zimetbaum, 2012; Heijman et al., 2013a). The AF incidence is expected to increase due to aging of the population, making the development of improved antiarrhythmic treatments of critical importance. A better understanding of AF pathophysiology is expected to foster this development (Dobrev et al., 2012). Accumulating evidence has highlighted a central role for abnormal Ca^{2+}-handling in AF-pathophysiology (Dobrev and Nattel, 2008; Heijman et al., 2012; Nattel and Dobrev, 2012). Here, we review recent studies detailing the proarrhythmic role of AF-related Ca^{2+}-handling abnormalities, with particular focus on the contributions of the Ca^{2+}/calmodulin-dependent protein kinase type-II (CaMKII).

ATRIAL CELLULAR ELECTROPHYSIOLOGY AND ARRHYTHMOGENIC MECHANISMS

NORMAL ATRIAL CELLULAR ELECTROPHYSIOLOGY AND Ca^{2+}-HANDLING

The atrial action potential (AP) is determined by depolarizing and repolarizing ionic currents (Dobrev and Ravens, 2003). Depolarizing currents include the cardiac voltage-gated Na^+-current (I_{Na}) and its persistent ("late") component ($I_{Na,late}$), the L-type Ca^{2+}-current ($I_{Ca,L}$) and the Na^+/Ca^{2+}-exchanger type-1 (NCX1) current (I_{NCX}), which, in its forward mode, extrudes one Ca^{2+}-ion in exchange for 3 Na^+-ions, resulting in a net depolarizing inward current. Repolarizing currents include the transient-outward K^+-current (I_{to}), delayed-rectifier K^+-currents with slow, rapid or ultra-rapid kinetics (I_{Ks}, I_{Kr}, and I_{Kur}, respectively), as well as the Na^+/K^+-ATPase current (I_{NaK}). In addition, AP duration (APD) and resting membrane potential are influenced by basal and acetylcholine-activated inward-rectifier K^+-currents (I_{K1} and $I_{K,ACh}$). The I_{Kur} and $I_{K,ACh}$ currents are predominantly expressed in the atria, thereby providing potential atrial-specific therapeutic targets.

Ca^{2+} entry through the L-type Ca^{2+}-channel activates Ca^{2+}-induced Ca^{2+}-release from the sarcoplasmic reticulum (SR) through type-2 ryanodine receptor channels (RyR2), producing the systolic Ca^{2+}-transient responsible for initiating contraction of atrial cardiomyocytes (Bers, 2002). In addition, inositol

1,4,5-triphosphate (IP_3)-receptor-mediated Ca^{2+}-release may contribute to Ca^{2+}-induced Ca^{2+}-release by activating neighboring RyR2, although direct IP_3-receptor-mediated activation of NCX1 has also been described recently (Dobrev and Nattel, 2008; Roderick and Knollmann, 2013).

Structural differences between atrial and ventricular cardiomyocytes may further contribute to a unique atrial Ca^{2+}-handling profile. Isolated atrial cardiomyocytes generally have a less well-developed T-tubular network than ventricular cardiomyocytes. However, cardiomyocytes of certain species including humans, sheep, goats, cows, and horses do have more T-tubules than those from rodents (Dibb et al., 2009; Lenaerts et al., 2009; Richards et al., 2011). At least in sheep, this T-tubular system contributes to a more uniform, ventricular-like, Ca^{2+}-induced Ca^{2+}-release (Dibb et al., 2009). Although a small T-tubular system is present in human atrial myocytes, it shows some variability depending on region and cardiomyocyte size (Trafford et al., 2013). Moreover, this T-tubular system can be remodeled by cardiac disease including AF (Lenaerts et al., 2009). In atrial cardiomyocytes with a less well-developed T-tubular structure, Ca^{2+}-induced Ca^{2+}-release starts at the plasma membrane and propagates slowly toward the cell-center (Dobrev et al., 2009; Bootman et al., 2011). Relaxation occurs when Ca^{2+} is extruded from the cell via NCX1 and the plasmalemmal Ca^{2+}-ATPase (PMCA), or is taken back up into the SR by the type-2a SR Ca^{2+}-ATPase (SERCA2a). The affinity of SERCA2a for intracellular Ca^{2+} is largely determined by the inhibitory proteins phospholamban (PLB) and sarcolipin. The expression of sarcolipin is atrial-specific, whereas PLB is more strongly expressed in the ventricles than in the atria (Dobrev et al., 2009).

ARRHYTHMOGENIC MECHANISMS IN AF

AF can occur as a result of abnormalities in electrical impulse formation or impulse conduction (Nattel et al., 2008; Wakili et al., 2011; Heijman et al., 2014). Electrical impulse generation outside of the sinoatrial node, termed ectopic activity, can sustain AF as a driver, and can trigger reentry in a vulnerable substrate characterized by a slow and inhomogeneous conduction and short effective refractory periods. This vulnerable substrate can arise from genetic conditions, normal aging, or co-morbidities such as heart failure or hypertension (Wakili et al., 2011). Reentry can occur around anatomical obstacles or can be functional (i.e., occurring in the absence of anatomical obstacles). Reentry is considered the predominant mechanism for AF maintenance. When AF is maintained, atrial tachycardia-related remodeling produces electrical and structural alterations that further promote AF maintenance and stabilization, contributing to the progression toward longer-lasting AF episodes that are more difficult to treat.

At the cellular level, the effective refractory period is determined by APD and post-repolarization refractoriness. Conduction velocity is influenced by the depolarizing force through I_{Na}, and the electrical conduction between atrial cardiomyocytes is controlled by gap-junction channels as well as the structure of the atrial myocardium, notably the amount and composition of the extracellular matrix, particularly fibrosis. The cellular mechanisms of ectopic activity mainly involve early and delayed afterdepolarizations (EADs and DADs, respectively). EADs are caused primarily by recovery from inactivation of $I_{Ca,L}$ during excessive APD-prolongation, for example due to loss of repolarizing K^+-currents. DADs are likely the most common mechanism underlying ectopic (triggered) activity and result from intracellular Ca^{2+}-handling abnormalities. Spontaneous diastolic SR Ca^{2+}-release events resulting from SR Ca^{2+}-overload or intrinsic RyR2-dysfunction can activate NCX1, resulting in a transient-inward current that depolarizes the membrane potential as Ca^{2+} is extruded from the atrial cardiomyocyte (Dobrev and Wehrens, 2010). When the threshold for excitation is reached in a sufficient number of cardiomyocytes, an ectopic impulse is generated (Wakili et al., 2011).

STRUCTURE, ACTIVATION AND TARGETS OF CaMKII

CaMKII is a multifunctional serine/threonine protein kinase that is abundantly expressed in various tissues including the heart (Swaminathan et al., 2012). There are four CaMKII isoforms, with CaMKIIδ being the most abundant in heart. CaMKIIδ has a hypervariable region, giving rise to multiple splice variants, including a splice variant with a nuclear localization signal (NLS; $CaMKII\delta_B$) and one without such NLS sequence ($CaMKII\delta_C$). The latter was traditionally considered cytosolic (Swaminathan et al., 2012), although this localization is not absolute (Mishra et al., 2011). CaMKII is a holoenzyme consisting of two stacked hexameric rings of subunits. Each subunit has a catalytic domain that, under resting conditions, is inhibited by regulatory domains of neighboring subunits. When intracellular Ca^{2+}-levels periodically rise during the cellular Ca^{2+}-transient, Ca^{2+} binds to calmodulin and activates CaMKII by binding to the regulatory domain (Swaminathan et al., 2012). CaMKII subunits can auto-phosphorylate Thr287 on neighboring subunits, thereby hindering the re-association of the catalytic and regulatory domains, producing sustained Ca^{2+}-independent activation. This mechanism makes CaMKII activation strongly heart rate-dependent, with accumulating activity at faster rates. Furthermore, CaMKII can show Ca^{2+}-independent activation following oxidation of Met281/282 by reactive oxygen species (Erickson et al., 2008), via O-linked glycosylation of Ser280 by O-linked N-acetylglucosamine (Erickson et al., 2013), and via NO-dependent nitrosylation of Cys116, Cys273, or Cys290, the exact residue being at present unknown (Gutierrez et al., 2013). In contrast, phosphorylation of Thr306/307 promotes CaMKII inactivation by reducing the binding of Ca^{2+}/calmodulin complexes (Colbran, 1993).

CaMKII can phosphorylate multiple substrates in atrial cardiomyocytes (**Figure 1**). CaMKII-dependent phosphorylation of L-type Ca^{2+}-channels produces high-activity mode-2 gating resulting in increased open probability of $I_{Ca,L}$, thereby augmenting the amount of Ca^{2+} entering the atrial cardiomyocyte. CaMKII also contributes to the increase in $I_{Ca,L}$ following repeated depolarizing pulses (termed Ca^{2+}-dependent $I_{Ca,L}$-facilitation) (Swaminathan et al., 2012). CaMKII-dependent phosphorylation of Nav1.5 slows I_{Na} inactivation and augments the non-inactivating, "late" component of I_{Na} (Wagner et al., 2011). The Kv4.3 pore-forming subunit of I_{to} is also regulated by CaMKII-dependent phosphorylation through the accessory protein SAP97, resulting in increased I_{to} that would tend to

shorten APD (El-Haou et al., 2009; Wagner et al., 2009). Based on experiments involving CaMKII inhibition with an inhibitory peptide or the experimental drug KN-93, CaMKII also appears to acutely augment I_{K1} (Wagner et al., 2009) and I_{Kur} (Tessier et al., 1999), thereby offsetting the APD-prolonging effects of CaMKII-dependent $I_{Ca,L}$ and I_{Na} phosphorylation. In addition, both PLB and sarcolipin can undergo CaMKII-dependent phosphorylation, causing disinhibition of SERCA2a and increasing SR Ca^{2+}-reuptake (Dobrev and Wehrens, 2010). Finally, CaMKII-dependent hyperphosphorylation of Ser2814 on RyR2 increases channel open probability, augmenting SR Ca^{2+}-release. Taken together, CaMKII plays a nodal role in the modulation of atrial cellular Ca^{2+}-handling.

Ca^{2+}/ CaMKII DYSREGULATION IN AF

MECHANISMS PROMOTING CaMKII DYSREGULATION IN AF

CaMKIIδ protein expression and activity are increased in dogs with pacing-induced atrial tachycardia remodeling (Wakili et al., 2010), goats with long-standing AF (Greiser et al., 2009), and patients with chronic AF (cAF); (Tessier et al., 1999; Neef et al., 2010; Voigt et al., 2012), suggesting that increased CaMKII function can be a consequence of AF. Activation of CaMKII appears to be regulated locally within the myocyte, since autophosphorylation of Thr287 was increased for $CaMKII\delta_C$ but not $CaMKII\delta_B$ in patients with cAF (Voigt et al., 2012). Several AF-related conditions, including sympathetic hyperactivity, oxidative stress and atrial tachycardia *per se*, may promote CaMKII activation (**Figure 2**). High atrial-rates during AF can activate CaMKII via frequency-dependent mechanisms. In addition, neuronal autonomic dysbalance can contribute to AF initiation (Park et al., 2012) and atrial tachycardia, in turn, promotes neural remodeling including heterogeneous sympathetic hyperactivity (Jayachandran et al., 2000). Increased sympathetic activity can activate CaMKII through various pathways, including protein kinase-A (PKA)-dependent augmentation of cellular Ca^{2+}-cycling (Grimm and Brown, 2010). In addition, PKA-independent, exchange-protein activated by cAMP (Epac) can activate CaMKII following β-adrenoceptor stimulation (Mangmool et al., 2010; Pereira et al., 2013). Moreover, β1-adrenoceptor-activated Epac2 can promote SR Ca^{2+}-leak via phosphorylation of RyR2-Ser2814 (Pereira et al., 2013). It has also been suggested that the Epac-mediated CaMKII activation involves phosphorylation of CaMKII-Thr287 by protein kinase-C type-ε (PKCε) (Oestreich et al., 2009) and the upregulation of PKCε in cAF patients (Voigt et al., 2008) might contribute to increased CaMKII activity. Since PKCε translocation to the membrane is increased in atrial myocytes following *in vitro* tachypacing (Makary et al., 2011), this might promote local atrial tachycardia-dependent CaMKII stimulation, although this remains to be proven in future studies. AF is also associated with oxidative stress and oxidation of CaMKII is increased in AF patients (Purohit et al., 2013). Conversely, phosphorylation of the inhibitory Thr306/307 site is decreased in cAF patients, providing another pathway of CaMKII activation in AF (Voigt et al., 2012).

Atrial CaMKII activity is also increased in dogs with ventricular tachypacing-induced heart failure (Yeh et al., 2008), and in goats with atrial dilatation (Greiser et al., 2009), suggesting that CaMKII can be activated by AF-enabling cardiac pathologies, potentially contributing to the evolution of a vulnerable substrate for AF initiation. Similarly, increased body-mass index and diabetes are AF risk-factors (Dublin et al., 2006) that may further promote CaMKII activation via O-linked glycosylation in response to hyperglycaemia (Erickson et al., 2013). Thus, CaMKII activation is multifactorial, resulting from AF itself, as well as from AF-enabling risk factors and diseases (**Figure 2**).

ROLE OF CaMKII IN ECTOPIC ACTIVITY

CaMKII has been shown to promote EADs in ventricular cardiomyocytes (Qi et al., 2009), which can produce ectopic (triggered) activity. CaMKII-dependent phosphorylation of $I_{Ca,L}$ slows $I_{Ca,L}$ inactivation, increasing the $I_{Ca,L}$ window current that plays a major role in the generation of EADs (Qi et al., 2009). In addition, the APD-prolonging effects of CaMKII-dependent phosphorylation of I_{Na}, increasing $I_{Na,late}$, could further promote the occurrence of EADs and ectopic activity (Wagner et al., 2011). However, since most forms of AF are generally associated with abbreviated APD, the relevance of such EADs may be lower in atrial compared to ventricular arrhythmogenesis. On the other hand, EADs can also arise from Ca^{2+}-handling abnormalities that activate depolarizing NCX-current (late phase-3 EADs), which have been implicated in the initiation of AF in some animal models (Burashnikov and Antzelevitch, 2003; Patterson et al., 2006).

Ca^{2+}-handling abnormalities can also cause DADs and ectopic (triggered) activity, promoting AF initiation. Genetic mouse models have revealed that intrinsic RyR2-dysfunction is sufficient to increase the susceptibility to pacing-induced AF, as reviewed in (Dobrev et al., 2011). Mice with gain-of-function RyR2 mutations causing catecholaminergic polymorphic ventricular tachycardia (CPVT), and mice lacking the RyR2-stabilizing subunit FKBP12.6, develop Ca^{2+}-handling abnormalities including increased SR Ca^{2+}-leak and spontaneous SR Ca^{2+}-release events (i.e., sparks, waves). These mice also have an increased susceptibility to pacing-induced AF (Sood et al., 2008; Chelu et al., 2009; Shan et al., 2012). Rapid-pacing activates CaMKII and increases CaMKII-dependent RyR2 and PLB phosphorylation. Genetic and pharmacological CaMKII inhibition normalized the susceptibility to pacing-induced AF in mice with a CPVT mutation in RyR2 (Chelu et al., 2009). Of note, selective genetic inhibition of CaMKII-dependent RyR2-hyperphosphorylation (RyR2-Ser2814Ala) also reduced the incidence of rapid-pacing-induced AF in mice where a vulnerable substrate was created using stimulation with the muscarinic-receptor agonist carbachol, and pacing-induced AF in mice deficient of FKBP12.6 (Chelu et al., 2009; Li et al., 2012), strongly suggesting that CaMKII-dependent RyR2 hyperphosphorylation and associated Ca^{2+}-handling abnormalities are critical AF-promoting factors (Dobrev et al., 2011). In addition, recent work has identified calmodulin as a direct regulator of RyR2 that stabilizes SR Ca^{2+}-release (Yang et al., 2014). Although overall calmodulin levels are increased in cAF patients (Voigt et al., 2012), a reduced affinity between RyR2 and calmodulin, as observed in heart failure (Yang et al., 2014), could potentially contribute to RyR2 dysfunction in AF.

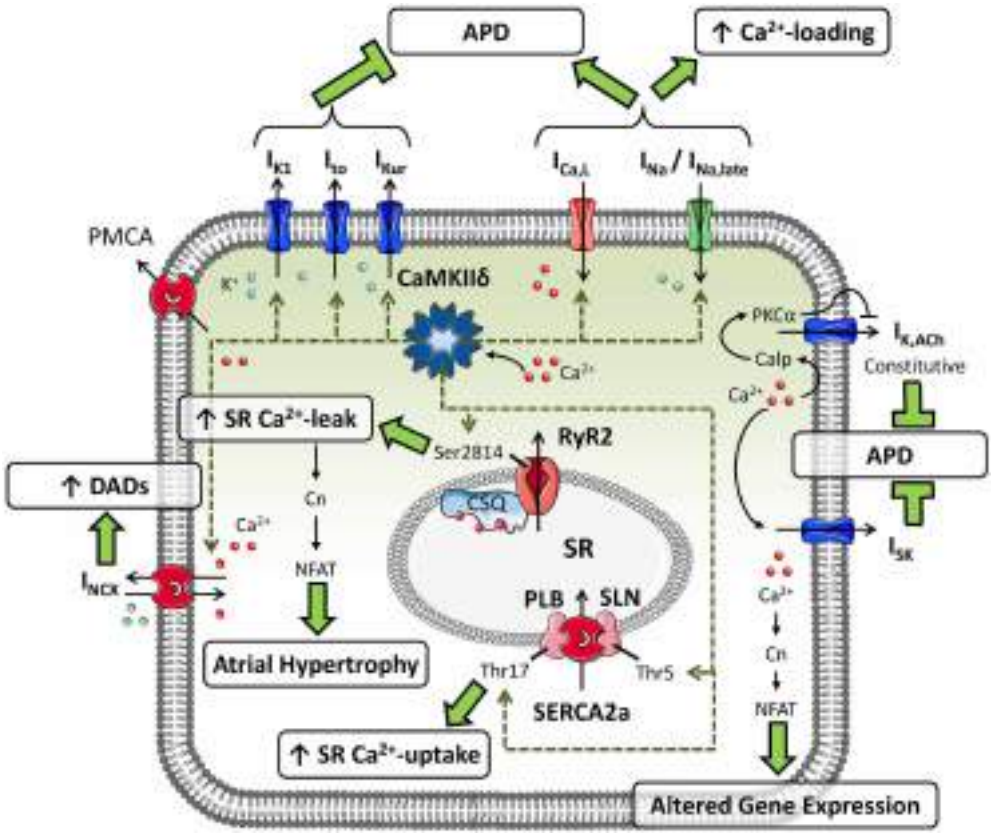

FIGURE 1 | Putative substrates for CaMKII-dependent phosphorylation in atrial cardiomyocytes and their consequences for atrial cellular electrophysiology and Ca^{2+}-handling. CaMKII can phosphorylate the transient-outward K^+-current (I_{to}), inward-rectifier K^+-current (I_{K1}) and ultra-rapid delayed-rectifier K^+-current (I_{Kur}), augmenting their functions and shortening action potential duration (APD). Phosphorylation of L-type Ca^{2+}-current ($I_{Ca,L}$) and Na^+-current (I_{Na}; resulting in an increased late component: $I_{Na,late}$) by CaMKII increases intracellular Ca^{2+} levels and prolongs APD. CaMKII-dependent phosphorylation of phospholamban (PLB) and sarcolipin (SLN) increases sarcoplasmic reticulum (SR) Ca^{2+}-uptake, whereas phosphorylation of type-2 ryanodine-receptor channels (RyR2) promotes diastolic SR Ca^{2+}-leak. CaMKII-dependent increases in expression of Na^+/Ca^{2+}-exchanger type-1 (NCX1) augment NCX-current (I_{NCX}), promoting the occurrence of delayed afterdepolarizations (DADs). In addition, Ca^{2+}-handling abnormalities can activate small-conductance Ca^{2+}-activated K^+-currents (I_{SK}) and agonist-independent "constitutive" $I_{K,ACh}$, shortening APD, and promote altered gene expression via the Ca^{2+}-dependent phosphatase calcineurin (Cn).

Atrial cardiomyocytes from cAF patients have unaltered RyR2 protein expression levels and SR Ca^{2+}-load (Voigt et al., 2012). However, they exhibit CaMKII-dependent RyR2-hyperphosphorylation that increases RyR2 open probability and augments SR Ca^{2+}-leak and spontaneous diastolic Ca^{2+}-release events. The enhanced SR Ca^{2+} leak results in enhanced DADs and cellular triggered activity and can be blocked using CaMKII inhibitors, thus supporting an important proarrhythmic role for these CaMKII-dependent Ca^{2+}-handling abnormalities in human AF (Voigt et al., 2012). In addition, cAF patients had significantly reduced levels of RyR2-stabilizing FKBP12.6 subunits (Vest et al., 2005) and larger transient-inward currents/depolarizations for a given SR Ca^{2+}-release. The latter is in part mediated by increased NCX1 mRNA (Gaborit et al., 2005) and protein expression levels (Schotten et al., 2002; El-Armouche et al., 2006; Voigt et al., 2012) in cAF patients. There is evidence that CaMKII can upregulate NCX1 transcription following β-adrenoceptor stimulation (Mani et al., 2010), suggesting that CaMKII could also be involved in the increased NCX1 expression in AF. Although atrial cardiomyocytes from paroxysmal AF (pAF) patients also have increased SR Ca^{2+}-leak, spontaneous SR Ca^{2+}-release events and DADs, these effects appear to be CaMKII-independent, since CaMKII expression and Thr287 autophosphorylation were not changed in pAF patients (Voigt et al., 2014). Similarly, CaMKII-dependent PLB and RyR2 phosphorylation, as well as NCX1 expression were also unaltered in pAF patients. However, RyR2 expression and RyR2 single-channel open-probability were increased and SR Ca^{2+}-load was larger in pAF, likely due to PKA-dependent PLB hyperphosphorylation (Voigt et al., 2014). Computational modeling showed that both increased SR Ca^{2+}-load and RyR2 dysregulation contribute to the spontaneous diastolic SR Ca^{2+}-release events in cardiomyocytes from pAF patients. Thus, although SR Ca^{2+}-handling abnormalities appear a central element in experimental and human AF, the underlying molecular mechanisms are complex. In addition, it is likely that the proarrhythmic consequences of Ca^{2+}-handling

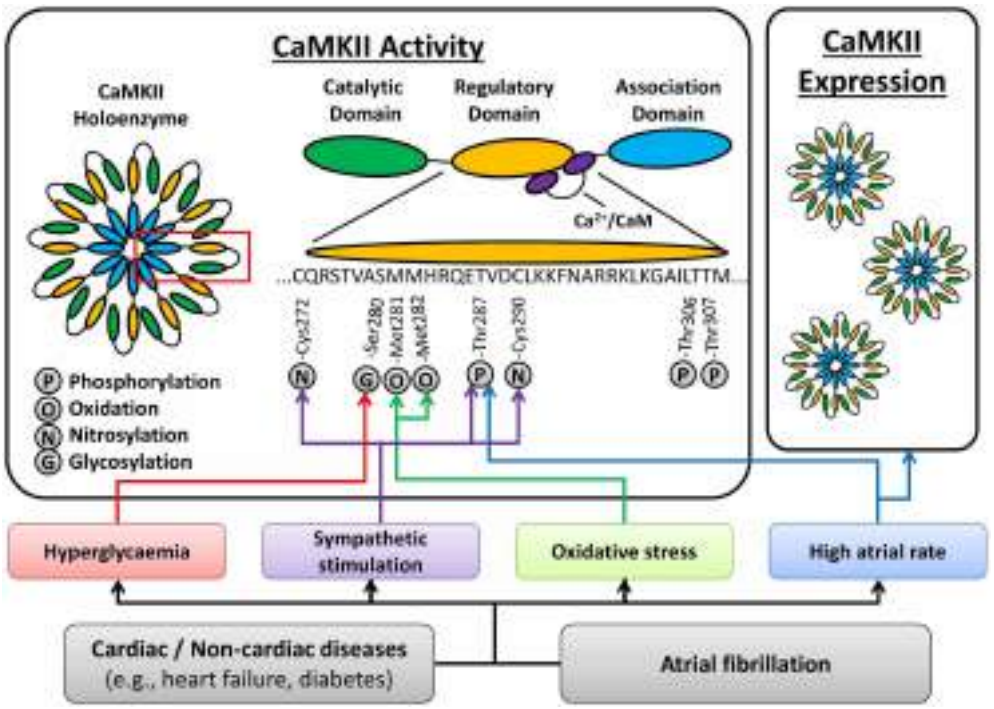

FIGURE 2 | Atrial fibrillation (AF)-related mechanisms promoting CaMKII activation. AF-promoting conditions and/or AF itself can activate CaMKII via high atrial rates, oxidative stress, sympathetic hyperactivity, and hyperglycaemia, resulting in post-translational modifications (phosphorylation, oxidation, nitrosylation and glycosylation) of various residues in the regulatory domain of CaMKII. In addition, AF is associated with an increased total expression of the CaMKII holoenzyme. See text for details.

abnormalities are distinct for different types of AF (**Figure 3**). Whereas Ca^{2+}-mediated triggered activity is a likely candidate for the re-initiation of AF episodes in pAF patients, its relevance for patients with long-standing persistent AF is incompletely understood. In persistent AF forms, Ca^{2+}-dependent evolution and progression of atrial remodeling may play a prominent role in arrhythmia maintenance and stabilization (as discussed below).

ROLE OF CaMKII IN REENTRY-PROMOTING REMODELING

Ca^{2+}-handling abnormalities also play a role in AF-promoting reentry. APD-shortening is a hallmark feature of AF-related remodeling that facilitates the maintenance of reentrant circuits. It is largely mediated by a reduction in depolarizing $I_{Ca,L}$ and an increase in several repolarizing K^+-currents. Various mechanisms contribute to reduced $I_{Ca,L}$ in AF (Dobrev et al., 2012). Cav1.2 expression is reduced in AF through a pathway involving the Ca^{2+}-dependent phosphatase calcineurin and nuclear factor of activated T-cells (NFAT) (Qi et al., 2008) and increased activation of the Ca^{2+}-dependent protease calpain promotes breakdown of $I_{Ca,L}$ channels (Brundel et al., 2004). $I_{Ca,L}$ phosphorylation is also reduced in AF, decreasing current amplitude, and could be due to either increased protein phosphatase activity or local reduction in CaMKII availability (Christ et al., 2004). I_{K1} is increased in cAF patients, and, together with an increase in the acetylcholine-independent "constitutive" activity of $I_{K,ACh}$, results in an overall increase in inward-rectifier K^+-current that contributes to APD shortening (Dobrev et al., 2005). A Ca^{2+}-dependent NFAT-mediated reduction in the inhibitory microRNA-26 in AF results in disinhibition of Kir2.1 expression, contributing to the increase in I_{K1} in cAF patients (Luo et al., 2013). Increased constitutive $I_{K,ACh}$ may also result from Ca^{2+}-dependent calpain-mediated reduction in inhibitory PKCα (Makary et al., 2011). Thus, the proarrhythmic increases in I_{K1} and constitutive $I_{K,ACh}$ are partially mediated by Ca^{2+}-dependent processes, althouth the potential involvement of CaMKII needs to be specifically addressed in future studies. Finally, the Ca^{2+}-dependent small-conductance (SK) K^+-current (I_{SK}) is upregulated in atria of cAF patients, which might contribute to APD shortening (Zhou et al., 2012), although others have reported reduced I_{SK} in AF (Yu et al., 2012). Acute Ca^{2+}-dependent regulation of currents such as I_{Na}, I_{SK} or $I_{Ca,L}$ can also contribute to beat-by-beat alterations in APD, including APD alternans and augmentation of dispersion of repolarization. These spatial and temporal repolarization heterogeneities favor unidirectional conduction block that can initiate reentry. In agreement, atrial APD alternans is emerging as a clinical index to assess the vulnerability to develop AF in patients (Lalani et al., 2013).

Ca^{2+}-entry into atrial fibroblasts via multiple ion channels contributes to fibroblast proliferation and differentiation into collagen-secreting myofibroblasts, which promote fibrosis-induced heterogeneous conduction slowing and reentry (Yue et al., 2011). Transient-receptor potential (TRP) melastatin-related-7 (TRPM7) and canonical-3 (TRPC3) channels are major sources of Ca^{2+}-entry into human atrial fibroblasts (Du et al.,

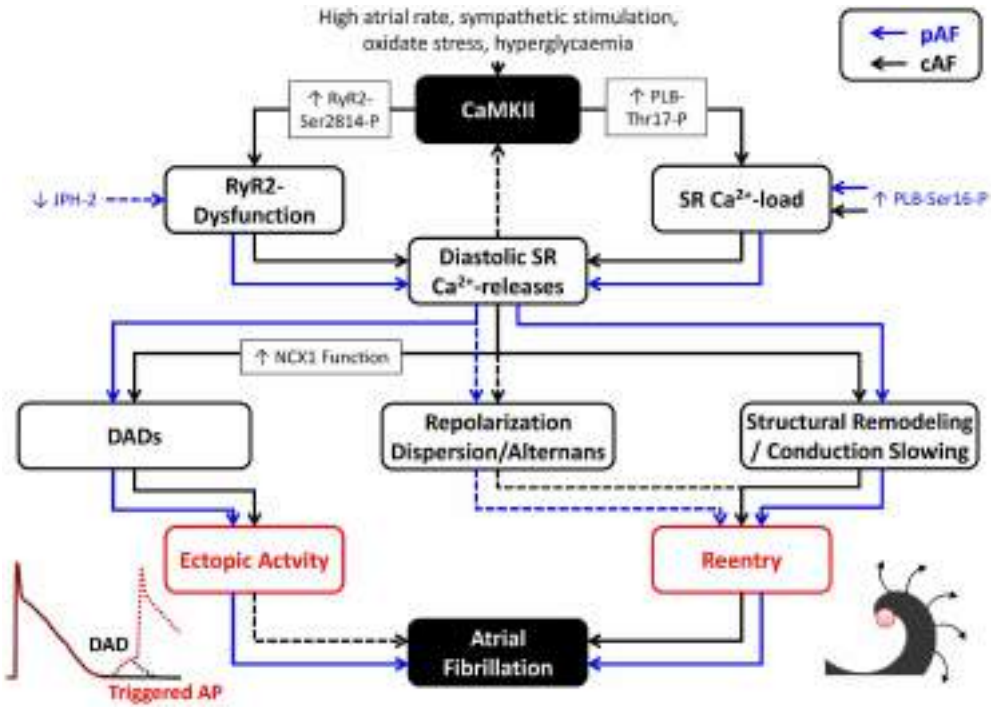

FIGURE 3 | Proarrhythmic consequences of Ca^{2+}/CaMKII dysregulation in atrial fibrillation (AF). CaMKII activation and CaMKII-dependent phosphorylation of type-2 ryanodine-receptor (RyR2) channels (RyR2-Ser2814-P) and phospholamban (PLB-Thr17-P), as well as other factors, promote spontaneous diastolic sarcoplasmic reticulum (SR) Ca^{2+}-release events through RyR2 dysfunction and modulation of SR Ca^{2+}-load in patients with paroxysmal AF (pAF; blue lines) and long-standing persistent, chronic AF (cAF; black lines). SR Ca^{2+}-leak and diastolic SR Ca^{2+}-release events can produce delayed afterdepolarizations (DADs) that contribute to ectopic activity. In addition, they can promote reentry through local repolarization abnormalities, as well as structural remodeling and conduction velocity (CV) slowing. Influences for which the proarrhythmic roles are more speculative have been indicated with dashed lines.

2010; Harada et al., 2012). Atrial fibroblasts from AF-patients have larger TRPM7 currents and increased TRPC3 expression, and are more prone to differentiate into myofibroblasts. Knockdown of TRPM7 expression reduces basal differentiation of fibroblasts from cAF patients (Du et al., 2010). Furthermore, pharmacological inhibition of TRPC3 channels reduces AF substrate development and AF duration in dogs with electrically maintained AF (Harada et al., 2012). TRPM7-like channels are inhibited by CaMKII in hepatocytes, which may support hepatocellular survival during proliferation (Mishra et al., 2009). Moreover, Ca^{2+}-influx through TRPC3 promotes CaMKII activation and NADPH-oxidase-mediated production of reactive oxygen species in a genetic mouse model (Kitajima et al., 2011). Thus, CaMKII could potentially act both upstream and downstream of TRP channels to alter fibroblast function in AF, although this requires confirmation in subsequent studies.

Ca^{2+}-handling abnormalities can also promote reentry by reducing atrial conduction velocity through a reduction in I_{Na} or direct inhibition of gap-junction channels in atrial cardiomyocytes (Heijman et al., 2013b; King et al., 2013b). The reduction in conduction velocity observed in mice with RyR2 mutations could be reproduced in wild-type mice with acute application of caffeine to increase SR Ca^{2+}-leak, and appears to be due to both acute Ca^{2+}-dependent inhibition of I_{Na}, as well as downregulation of Nav1.5 subunit expression under chronic conditions (King et al., 2013a). This Ca^{2+}-dependent reduction in I_{Na} is expected to promote reentry-mediated AF maintenance but may also reduce the likelihood of ectopic activity (Heijman et al., 2013b). At present the role of CaMKII in these reentry-promoting Ca^{2+}-handling abnormalities is largely unknown, although it has been suggested that CaMKII-dependent phosphorylation could also reduce peak I_{Na}, particularly at fast heart rates relevant for AF (Wagner et al., 2006), which could contribute to reentry by reducing atrial conduction velocity.

Cardiac myosin-binding protein-C (cMyBPC) is a critical regulator of myofilament function (Schlossarek et al., 2011). Ser282-phosphorylation of cMyBPC is decreased in dogs with pacing-induced atrial tachycardia remodeling (Wakili et al., 2010), in dogs with ventricular tachypacing-induced heart failure (Yeh et al., 2008), goats with long-standing AF or atrial dilatation (Greiser et al., 2009), and in cAF patients (Tessier et al., 1999; Neef et al., 2010; Voigt et al., 2012). Although there is indirect evidence that this could be due to increased local dephosphorylation by phosphatases, reduced local CaMKII-dependent phosphorylation of Ser282 could also be involved. In addition, contractile dysfunction is promoted by activation of Ca^{2+}-dependent proteases. Together, contractile dysfunction and

associated atrial dilatation result in a larger vulnerable substrate, promoting reentrant arrhythmias (De Jong et al., 2011).

Accumulating evidence suggests that CaMKII-dependent RyR2-hyperphosphorylation and the related SR Ca^{2+}-leak play an important role in AF-promoting structural remodeling. Mice with transgenic overexpression of the transcriptional repressor CREM-IbΔC-X in cardiomyocytes (CREM mice) develop age-dependent progression from spontaneous atrial ectopy to paroxysmal and long-lasting AF episodes (Li et al., 2014). The development of spontaneous AF episodes is preceded by Ca^{2+}-handling abnormalities and atrial enlargement. Genetic inhibition of CaMKII-dependent RyR2 phosphorylation (RyR2-Ser2814Ala) in CREM mice prevents Ca^{2+}-handling abnormalities and spontaneous AF, as well as atrial dilatation and conduction abnormalities (Li et al., 2014). Thus, CaMKII-dependent RyR2-dysregulation not only contributes to ectopic (triggered) activity, but also drives a progressive development of an AF substrate (**Figure 3**), promoting atrial hypertrophy and dilatation, and AF progression (Li et al., 2014). These studies suggest the interesting possibility that the progression of AF might be inhibited by targeted treatment of CaMKII or SR Ca^{2+}-leak via RyR2. Future studies in mice and large animal models are required to confirm this concept, since the pathophysiological mechanisms and the importance of CaMKII likely vary for different species and experimental AF models, as well as for different forms of clinical AF.

CaMKII DYSREGULATION AND Ca^{2+}-HANDLING ABNORMALITIES AS THERAPEUTIC TARGETS IN AF

The central role of Ca^{2+}-handling abnormalities in AF-pathophysiology suggests their potential as antiarrhythmic targets. Stabilization of RyR2 has emerged as a viable approach to normalize Ca^{2+}-handling abnormalities. Several currently-available antiarrhythmic drugs, including the class-Ic Na^+-channel blocker flecainide (Hilliard et al., 2010), the β-adrenoceptor blocker carvedilol (Zhou et al., 2011), and the antianginal drug ranolazine (Parikh et al., 2012), directly bind and inhibit RyR2 channels. Indeed, flecainide has been successfully employed in other Ca^{2+}-dependent arrhythmias such as CPVT (Van Der Werf et al., 2011). However, flecainide also inhibits atrial K^+-currents like $I_{K,ACh}$ (Voigt et al., 2010), which might contribute to its anti-AF efficacy. More specific RyR2 inhibitors are currently being evaluated in clinical studies (Dobrev et al., 2012).

Inhibition of CaMKII or elimination of CaMKII-dependent RyR2-phosphorylation has proven antiarrhythmic in mouse models of AF and has shown beneficial effects in atrial cardiomyocytes from cAF patients (Chelu et al., 2009; Li et al., 2012; Voigt et al., 2012). However, given the importance of CaMKII in various physiological processes, systemic CaMKII inhibition could have various undesirable side effects, including reduced fertility and impaired memory (Backs et al., 2010; Halt et al., 2012). Moreover, since CaMKII expression/autophosphorylation and CaMKII-dependent phosphorylation of RyR2 and PLB are not increased in pAF patients (Voigt et al., 2014), it is unclear whether CaMKII inhibition would be beneficial for this group of patients. Nonetheless, it appears likely that localized CaMKII inhibition could be a promising antiarrhythmic strategy for appropriately-selected AF patients. Future animal studies and clinical trials will be needed to determine which groups of AF patients are most likely to benefit from CaMKII inhibition. Local inhibition of CaMKII might be possible through inhibition of specific CaMKII-isoforms and splice variants, or by modulating different CaMKII-targeting proteins. Another potential avenue could be the modulation of microRNAs. Injection of complementary "antagomirs" to reduce the activity of certain microRNAs or overexpression of microRNAs has proven beneficial in a variety of experimental models, as reviewed in (Kumarswamy and Thum, 2013). Recent work has shown that CaMKIIδ expression is repressed by microRNA-145 (Cha et al., 2013) and microRNA-30b-5p (He et al., 2013). Increasing the levels of these microRNAs in the heart might, therefore, be an option to inhibit CaMKII.

CONCLUSIONS

Ca^{2+}-handling abnormalities promote both focal ectopic (triggered) activity and reentry that contribute to AF initiation and maintenance. The expected increase in the incidence of AF and the limited efficacy and safety of currently available antiarrhythmic drugs, make a better understanding of these AF-modulating processes critical for the development of improved therapeutic strategies. Ca^{2+}-handling abnormalities provide a novel set of potential antiarrhythmic targets for the treatment of AF. However, due to the multitude of etiologies and complexity of mechanisms underlying clinical AF, it is likely that tailored therapeutic strategies for specific groups of patients that target multiple pathophysiological processes will be necessary. Cardiac-specific inhibition of CaMKII could be a promising therapeutic strategy for certain groups of AF patients.

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CaMKII and stress mix it up in mitochondria

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CaMKII is a newly discovered resident of mitochondria in the heart. Mitochondrial CaMKII promotes poor outcomes after heart injury from a number of pathological conditions, including myocardial infarction (MI), ischemia reperfusion (IR), and stress from catecholamine stimulation. A study using the inhibitor of CaMKII, CaMKIIN, with expression delimited to myocardial mitochondria, indicates that an underlying cause of heart disease results from the opening of the mitochondrial permeability transition pore (mPTP). Evidence from electrophysiological and other experiments show that CaMKII inhibition likely suppresses mPTP opening by reducing Ca^{2+} entry into mitochondria. However, we expect other proteins involved in Ca^{2+} signaling in the mitochondria are affected with CaMKII inhibition. Several outstanding questions remain for CaMKII signaling in heart mitochondria. Most importantly, how does CaMKII, without the recognized N-terminal mitochondrial targeting sequence transfer to mitochondria?

Keywords: mitochondria, cell death, mitochondrial calcium uniporter, CaMKII, CaMKIIN

INTRODUCTION

CaMKII activity promotes heart failure by mediating pathological effects of ischemia reperfusion (IR) through induction of both apoptosis and necrosis (Salas et al., 2009). Cytosolic inhibition of CaMKII attenuates cell death in the heart that results from catecholamine stress, myocardial infarction (MI) or IR (Yang et al., 2006). The increase in cell death via CaMKII activity involves mitochondrial pro-death pathways (Salas et al., 2009; Joiner et al., 2012). Further, either membrane partitioned or mitochondrial matrix expression of a specific and potent inhibitor of CaMKII, CaMKIIN, reduces cell death from MI, catecholamine stress, and IR (Joiner et al., 2012). Therefore, inhibiting CaMKII either in the cytosol or in mitochondria can block CaMKII activity leading to cell death. CaMKII protein targets in cytosol are well studied and include Ca^{2+} entry pathways and proteins involved with Ca^{2+} handling at the ER (Salas et al., 2009; Koval et al., 2010; Ozcan and Tabas, 2010; Zhang et al., 2010). Mitochondrial-triggered cell death occurs from Ca^{2+} overload or excess reactive oxygen species (ROS) production in the mitochondria (Crompton and Costi, 1988; Gunter and Pfeiffer, 1990; Lemasters et al., 2009). Inhibiting or eliminating mitochondrial CaMKII activity reduced cell death in a number of cellular models of pathology (Timmins et al., 2009; Joiner et al., 2012; Yun et al., 2013). Reducing cell death by mitochondria Ca^{2+} overload may occur by either decreasing Ca^{2+} uptake or reducing mitochondrial permeability transition pore (mPTP) formation (Griffiths and Halestrap, 1993; Elrod et al., 2010; Pan et al., 2013) (**Figure 1**). The predominant mitochondrial Ca^{2+} uptake and efflux are via the mitochondrial calcium uniporter (MCU) and Na^+/Ca^{2+} antiporters (NCLX), respectively. However, a number of other channels have been described for these processes (**Figure 1** and described in recent reviews, including Ryu et al., 2010). Regulation of these ion channel complexes by post-translational modification and auxiliary proteins is best described for the MCU. In addition to Ca^{2+} exchange through channel proteins, Ca^{2+} can be sequestered in the matrix by forming phosphate complexes. These Ca^{2+}-phosphate complexes allow accumulation of Ca^{2+} in the matrix during periods of high levels cytosolic Ca^{2+} (Wei et al., 2012). In this review we focus on effects of CaMKII in mitochondrial Ca^{2+} uptake and permeability transition.

CaMKII EFFECTS ON MCU

Mitochondria take up Ca^{2+} primarily via the MCU (Kirichok et al., 2004; Baughman et al., 2011; De Stefani et al., 2011). The MCU pore-forming channel, a 350 amino acid protein, has two predicted transmembrane helices (**Figure 1**, from amino acids Lys233 to Trp255 and Thr266 to Met 283), each spanning the inner mitochondrial membrane with the N- and C-terminal ends extending into the matrix. The MCU channel is composed of two pore forming proteins, MCUa and MCUb, as well as at least three regulatory proteins, MICU1, MICU2, and EMRE (Perocchi et al., 2010; Sancak et al., 2013). Although a number of post-translational modifications were identified for MICU1 (Hornbeck et al., 2012), it is not known how these affect Ca^{2+} current or whether CaMKII phosphorylates any of the accessory proteins or MCUb.

A number of research groups use patch clamp onto mitoplasts (exposed inner membrane of mitochondrial) to measure ion currents, such as the MCU current, across the inner mitochondrial membrane. In order for the patch pipette to access the mitochondrial inner membrane, the mitochondria must be swollen to rupture the outer mitochondrial membrane. These manipulations raise the issue of whether, or how closely, the channel activity observed with patch-clamp corresponds to the *in vivo* state. Technical aspects of mitoplast patch-clamping remain highly non-standardized, as evidenced by inter-study variation in mitoplast capacitance (0.8–5 pF), (Kirichok et al., 2004; Fieni et al., 2012; Chaudhuri et al., 2013; Dolga et al., 2013; Hoffman et al., 2013) and patch-electrode resistance (4–40 MΩ) (Kirichok et al., 2004; Dolga et al., 2013). In combination

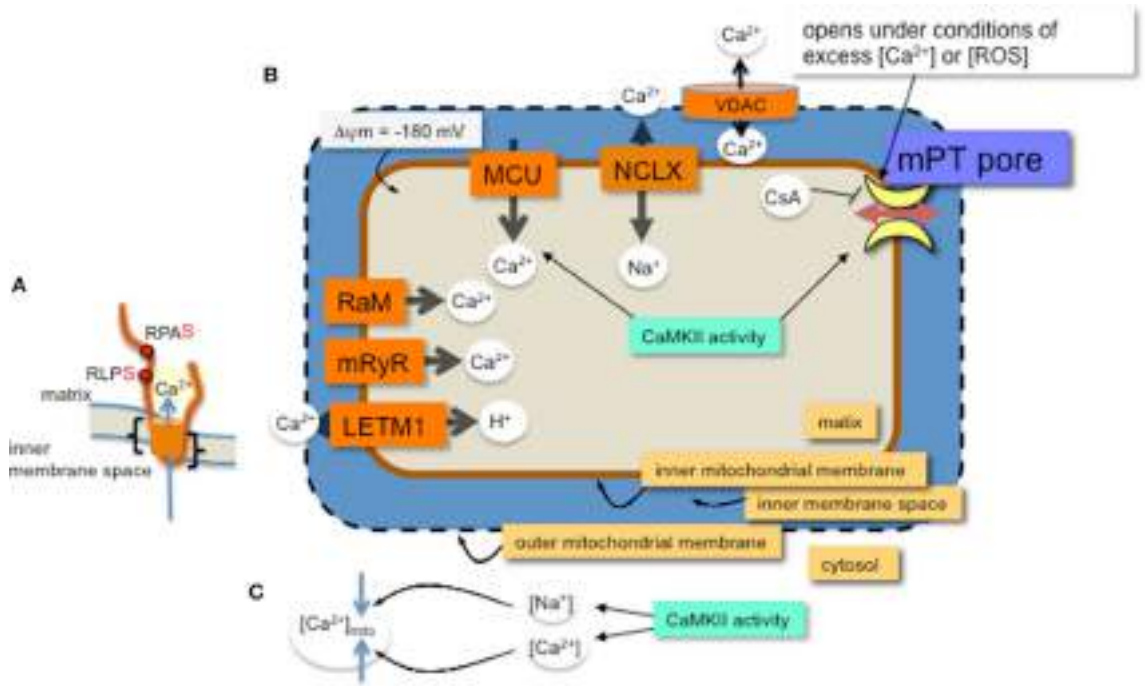

FIGURE 1 | MCU channel in the inner mitochondrial membrane. **(A)** A single monomer of MCU is shown (orange) with two phospho-serine residues (red dots, with consensus amino acids adjacent) on the N-terminal region in the matrix. The two transmembrane domains are indicated (black brackets). Layers of regulation include accessory proteins and a protein similar to MCU, MCUb (not shown). **(B)** Mitochondrial Ca^{2+} channels and exchangers on the inner membrane. Ca^{2+} predominantly enters the matrix through the MCU channel and efflux is via the Na^{+}-Ca^{2+} exchanger (NCLX). Other channels and an exchanger that were found to regulate Ca^{2+} across the inner membrane are the rapid mode of uptake (RaM), the ryanodine receptor (mRyR) and the Ca^{2+}-H^{+} exchanger (LETM1). Voltage dependent anion channels (VDAC) allow ions and metabolites across the outer membrane. The proton (H^{+}) gradient, a major component of the membrane potential ($\Delta\Psi$), is generated from the electron transport chain and drives the flow of H^{+} through ATP synthase in a reaction coupled to the generation of ATP from ADP and inorganic phosphate. The membrane potential produces a driving force for matrix Ca^{2+} accumulation. Excess Ca^{2+} or ROS will open the permeability transition pore, which can be inhibited with CsA or CaMKIIN. Mitochondrial CaMKII activity regulates 1. Ca^{2+} entry through the MCU and 2. transition pore opening. **(C)** CaMKII activity in the cytosol can increase both Ca^{2+} and Na^{+} ion levels in the cytosol with opposite effects on mitochondrial matrix Ca^{2+} accumulation.

with these technical aspects is the broader uncertainty in basic physiological characteristics of mitochondria, such as the ionic composition of the matrix. Reduced experimental conditions along with technical issues for patch-clamp studies of mitochondrial inner membrane ion currents can complicate data interpretation. For example, theoretical estimates of ionic currents are orders of magnitude lower than the currents through mitochondrial channels directly measured by patch-clamp (Kane and Pavlov, 2013). Accordingly, to understand channel modifications and regulation, studies should include a variety of techniques, not only patch clamp. Despite these acknowledged variations, overall effects of manipulating CaMKII function or its target sites in MCU have revealed effects of CaMKII activation on mitochondrial Ca^{2+} uptake. That is, phosphorylation of two serine sites on the N-terminus of MCU present a phenotype when mutated. Significantly, the CaMKII-induced larger current can be prevented by serine to alanine mutations of these two residues on MCU (Joiner et al., 2012) (**Figure 1**).

In order to identify proteins in the mitochondrial CaMKII pathway, we deduced that MCU may be a target for CaMKII because well-established CaMKII targets in heart cells are located near Ca^{2+} sources (Koval et al., 2010; Purohit et al., 2013). Furthermore, recent publications that showed either CaMKII inhibition (Yang et al., 2006) or the MCU inhibitor, Ru360 (García-Rivas Gde et al., 2006) are protective from IR damage in heart. An immunoprecipitation assay indicated that mitochondrial CaMKII and the MCU interact in a complex (Joiner et al., 2012) and others showed that accumulation of mitochondrial Ca^{2+} is activated through CaMKII signaling (Timmins et al., 2009). Ca^{2+} current through the MCU is increased with CaMKII activation as shown with patch-clamp measurements onto prepared mitoplasts and expression of CaMKIIN in the matrix reduced mitochondrial Ca^{2+} uptake (Joiner et al., 2012). Others found that CaMKII inhibitors block A23187-stimulated arachidonic acid release, LDH release and the decrease in the subsequent mPTP formation is attributed to reduced MCU current (Yun et al., 2013). Taken together, these studies indicate that CaMKII activation in mitochondria is responsible for excess Ca^{2+} uptake under pathological conditions, which ultimately leads to increased levels of cell death. Conversely, in the absence of MCU, using a knock out mouse lacking the MCU channel, Pan et al showed no protective effect preventing necrosis in the heart after

IR (Pan et al., 2013). This study suggests that phosphorylation by CaMKII of a protein(s), other than on the MCU, underlies the transition to cell death by CaMKII activation.

CaMKII EFFECTS ON mPTP

Inhibiting mitochondrial CaMKII with CaMKIIN expression decreased cell death following MI, catecholamine stress and IR. As suggested above, phosphorylation of the MCU by CaMKII may promote cell death under stress conditions. However, reducing MCU current does not appear effective in reducing apoptosis (Pan et al., 2013), therefore, an alternative pathway for CaMKII inhibition may be to delay mPTP opening. The mitochondrial permeability transition allows flow of molecules of up to 1500 Daltons to pass across the inner mitochondrial membrane, leading to mitochondrial swelling, and cell death through apoptosis or necrosis. Opening of the transition pore on the inner membrane occurs under pathophysiological conditions and is triggered by either excess Ca^{2+} or ROS (Kim et al., 2006; Lemasters et al., 2009). The ATP synthase complex on the inner membrane is a leading contender for molecular identity of the transition pore (Giorgio et al., 2013). In addition, the phosphate carrier and auxiliary regulatory proteins are possible components (Halestrap, 2009). Blocking the opening of the mPTP with the inhibitor, cyclosporin A (CsA) (Nicolli et al., 1996; Halestrap and Brenner, 2003) can reduce cell death from stress and reduce infarct size in hearts after IR in patients (Piot et al., 2008). A number of mitochondrial kinases appear to regulate mPTP opening (Miura et al., 2010; Azarashvili et al., 2014). Furthermore, like CsA, CaMKII inhibition increases matrix Ca^{2+} capacity because expressing CaMKIIN in the mitochondrial matrix allowed as much or more Ca^{2+} retention as did CsA (Joiner et al., 2012), suggesting a level of regulation by CaMKII and other kinases in transition pore formation.

CaMKII EFFECTS ON METABOLISM

Protective effects of CaMKII inhibition may occur via auxiliary proteins to the transition pore. CaMKII regulates the interaction of carnitine palmitoyltransferase I with its inhibitor, malonyl CoA, to affect fatty acid metabolism in mitochondria (Sharma et al., 2010) with possible consequences for mPTP opening (Moon et al., 2012). A study using a knockdown approach to decrease a number of kinases, including CaMKII, showed decreases in ATP synthase activity correlated with a reduction in kinase activity (Sugawara et al., 2013). As mentioned above, components of the ATP synthase complex may form the transition pore under stress conditions (Giorgio et al., 2013). Taken together, excess CaMKII activation may promote mitochondrial cell death by its link to energy production and transition pore formation.

CaMKII TARGETS IDENTIFIED BY MITOCHONDRIAL PHOSPHOPROTEOMICS

Many post-translational modifications are being discovered in the mitochondrial proteome that are yet to be established as functionally significant. Using phosphoproteomics is one way to identify potential CaMKII targets in the mitochondria. However, it has been argued that few transient protein phosphorylation events are physiologically relevant (Clarke et al., 2008; Covian and Balaban, 2012), citing few phospho-sites revealed with a phospho-protein fluorescent dye on mitochondrial lysate before and after treatment to induce mPTP inhibition (Clarke et al., 2008), and also reasoning that phosphorylation can occur spontaneously, without a kinase. In contrast, numerous sensitive phosphoproteomic studies have identified hundreds of phospho-sites on mitochondrial proteins under different treatment regimes (Lee et al., 2007; Witze et al., 2007; Zhao et al., 2011; Koc and Koc, 2012), some of which are indeed functionally relevant as described in the previous two sections. The spontaneous reaction argument is reminiscent of the early days of assigning a role to superoxide dismutase. Arguments that an enzyme for the superoxide radical dismutation to H_2O_2 was not necessary, as the reaction could occur rapidly without an enzyme (Fridovich, 1983), were eventually overruled by findings that superoxide dismutase over expression or reduced expression can lead to drastic physiological changes *in vivo* (Antonarakis et al., 2004). Ultimately, uncovering functionally relevant phospho-sites, for CaMKII and other kinases, in the mitochondrial proteome will require extensive study of the individual sites under different phosphorylation conditions along with mutation analysis.

SPECIFICITY OF MITOCHONDRIAL CaMKII PHOSPHORYLATION SITES

Protein phosphorylation sites identified by phosphoproteomics described above may be attributed to kinases other than CaMKII. The consensus phosphorylation site, a serine or threonine, three amino acids down stream of an arginine (RxxS/T, x represents any amino acid) for CaMKII phosphorylation is shared by a number of other kinases, for example, PKCdelta (www.kinexus.ca). However, specificity of the inhibitor CaMKIIN occurs even in overexpression systems, where for example both CaMKII and PKC could phosphorylate the same consensus site. When each kinase was co-expressed with CaMKIIN, only phosphorylation by CaMKII was inhibited (Chang et al., 1998). Therefore, using the CaMKII inhibitor, CaMKIIN, endogenous to brain (Chang et al., 1998) raises confidence that CaMKII, rather than a different kinase, is responsible for promoting cell death under stress conditions in heart.

IMPACT OF NON-MITOCHONDRIAL CaMKII ON MITOCHONDRIAL FUNCTION IN DISEASE

CaMKII activity outside of mitochondria contributes to mitochondrial Ca^{2+} homeostasis. CaMKII activity elevates diastolic sarcoplasmic reticulum (SR) Ca^{2+} leak (Curran et al., 2007), which was later shown to contribute to mitochondrial Ca^{2+} overload (Zhang et al., 2010) specifically, under pathophysiological conditions such as rapid cardiomyocyte pacing (Sepúlveda et al., 2013) and diabetes (Luo et al., 2013), but also with the extreme physiological condition of endurance exercise (Rose et al., 2007). Disruption of cytosolic Ca^{2+} homeostasis promotes mitochondrial Ca^{2+} overload (Lemasters et al., 2009). Using genetic tools to overexpress CaMKII or the inhibitor of CaMKII, CaMKIIN, in different cell compartments will lead to a better understanding of where CaMKII activity is required for promoting disease with particular models of stress, including MI, IR, excess

catecholamine stimulation, and metabolic diseases. Conversely, CaMKII is a major contributor to myocyte Na^+ homeostasis in heart failure (Wagner et al., 2006) and Na^+ accumulation in heart failure was shown to influence mitochondrial Ca^{2+} load via enhanced NCLX -mediated Ca^{2+} removal (Maack et al., 2006). Thus, indirectly, cytosolic CaMKII can regulate mitochondrial Ca^{2+} levels.

SUMMARY

The role of CaMKII functioning in the mitochondria in physiology and disease is in the early stages of research and discovery. CaMKII may be central to regulating mitochondrial homeostasis as its activity is regulated by both Ca^{2+} (Miller and Kennedy, 1986) and ROS (Erickson et al., 2008) signaling pathways. CaMKII effects in the mitochondria are likely to be numerous and uncovering target sites promises to reveal regulation of mitochondrial signaling pathways that tune cellular responses for cardiac output.

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Toward a hierarchy of mechanisms in CaMKII-mediated arrhythmia

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Calcium/calmodulin-dependent protein kinase II (CaMKII) activity has been shown to contribute to arrhythmogenesis in a remarkably broad range of cardiac pathologies. Several of these involve significant structural and electrophysiologic remodeling, whereas others are due to specific channelopathies, and are not typically associated with arrhythmogenic changes to protein expression or cellular and tissue structure. The ability of CaMKII to contribute to arrhythmia across such a broad range of phenotypes suggests one of two interpretations regarding the role of CaMKII in cardiac arrhythmia: (1) some CaMKII-dependent mechanism is a common driver of arrhythmia irrespective of the specific etiology of the disease, or (2) these different etiologies expose different mechanisms by which CaMKII is capable of promoting arrhythmia. In this review, we dissect the available mechanistic evidence to explore these two possibilities and discuss how the various molecular actions of CaMKII promote arrhythmia in different pathophysiologic contexts.

Keywords: CaMKII, arrhythmias, afterdepolarizations, ryanodine receptor, cardiovascular diseases

INTRODUCTION

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a key regulator of excitation-contraction coupling in cardiac myocytes. As described in detail elsewhere in this special issue, CaMKII modulates the function and expression of numerous myocyte ion channels and calcium handling proteins (**Figure 1**). These include $Na_V1.5$, the L-type Ca^{2+} channel (LCC), several potassium channel subunits, the cardiac isoform of the ryanodine receptor (RyR2), and phospholamban (PLN; Bers and Grandi, 2009). Many of the effects at these targets are proarrhythmic (Swaminathan et al., 2012; Fischer et al., 2013b), although some are antiarrhythmic by conventional paradigms (Tessier et al., 1999; El-Haou et al., 2009; Cheng et al., 2012), and therefore need to be considered in combination. Integrating these pleiotropic effects is further complicated by the temporal, spatial, and biochemical complexities of CaMKII activation (see articles herein from Erickson, 2014 and Gray and Heller Brown, 2014) in the intact myocyte. Thus, deciphering how CaMKII activity contributes to arrhythmia in any disease context is complex and non-intuitive.

These complexities notwithstanding, the potential for CaMKII inhibition to reduce arrhythmogenic outcomes has been demonstrated in animal models of a remarkably broad range of human diseases: from genetic channelopathies, such as catecholaminergic polymorphic tachycardia (CPVT), to etiologically complex pathologies such as heart failure, atrial fibrillation (AF), and ischemia/reperfusion injury (I/R). For this reason, and in addition to its potential as a target for anti-hypertrophic therapy, CaMKII has become a leading candidate for anti-arrhythmic targeting in the heart. Here we use this diverse range of disease models to develop a conceptual hierarchy of mechanisms in CaMKII-mediated arrhythmia. Specifically, we interrogate whether current evidence suggests that CaMKII promotes arrhythmia via multiple mechanisms, each of which can be recruited by particular disease etiologies, or alternatively, that CaMKII drives the same terminal mechanism irrespective of the specific underlying pathology. We contend that, while CaMKII disrupts ionic homeostasis and impairs repolarization through its actions at many different targets, the available evidence suggests that these mechanisms eventually converge to result in spontaneous Ca^{2+} release (SCR) from the sarcoplasmic reticulum (SR) and delayed afterdepolarizations (DADs). Thus we suggest that this is a dominant terminal mechanism of CaMKII-mediated arrhythmia, and may explain why experimental manipulation of CaMKII phosphorylation at RyR2 has proven effective in many models of arrhythmogenic disease.

CELLULAR MECHANISMS OF CaMKII-MEDIATED ARRHYTHMIA

A key feature of CaMKII signaling in arrhythmia is the presence of several positive feedback mechanisms, whereby CaMKII target phosphorylation results in changes to Ca^{2+} homeostasis (or metabolism) that further activate the kinase. These mechanisms are likely to contribute both during acute homeostatic challenge, such as β-adrenergic stimulation, and to the chronic disruption of ionic homeostasis in acquired disease. **Figure 2** shows two major forms of this feedback. Hyper-phosphorylation of LCCs, PLN, (**Figure 3A**, mechanism 1) and $Na_V1.5$ (**Figure 3A**, mechanism 2) increase whole-cell Ca^{2+} load either directly (LCC and PLN) or by impairing forward mode Na^+/Ca^{2+} exchange ($Na_V1.5$). This both promotes SCR in and of itself, and also further activates

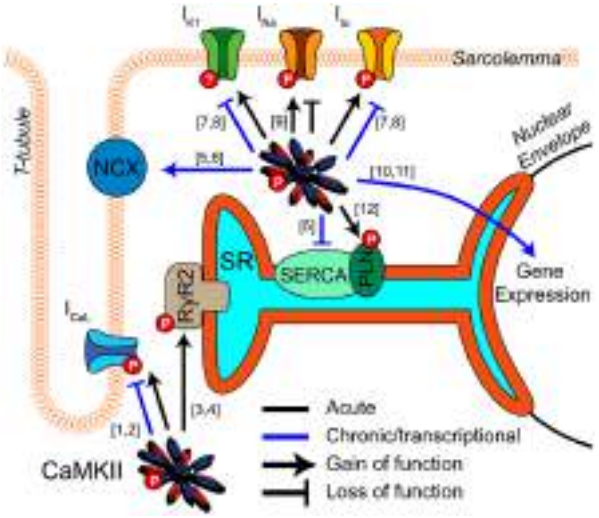

FIGURE 1 | CaMKII regulation of cardiomyocyte electrophysiology and Ca^{2+} handling. CaMKII exerts acute (black) and transcriptional (blue) regulation ([10]-Wu et al., 2006; [11]-Backs et al., 2006) of many key proteins in cardiac electrophysiology and calcium handling. Acute phosphorylation of LCCs by CaMKII potentiates I_{CaL} and slows inactivation ([1]-Yuan and Bers, 1994). Transcriptional effects are less established but likely downregulate channel expression ([2]-Ronkainen et al., 2011). Phosphorylation of RyR2 by CaMKII promotes Ca^{2+} release from the SR and is implicated in many proarrhythmic contexts ([3]-Witcher et al., 1991; [4]-Wehrens et al., 2004). Available data suggest CaMKII transcriptional regulation promotes Ca^{2+} extrusion from the cell by increasing NCX expression and decreasing SERCA ([6]-Mani et al., 2010; [5]-Lu et al., 2011). I_{to} and I_{K1} are enhanced by acute CaMKII phosphorylation ([7]-Li et al., 2006), and transcriptional downregulation of these currents is a well-established effect of long term CaMKII activity. This reduces repolarization reserve and may destabilize resting membrane potential ([8]-Wagner et al., 2009). Acute regulatory effects of CaMKII on $Na_V1.5$ enhance inactivation, decrease availability and potentiate I_{NaL}, but again any transcriptional regulation is unclear ([9]-Wagner et al., 2006). Acute phosphorylation of PLN disinhibits SERCA and enhances SR Ca^{2+} reuptake ([12]-Karczewski et al., 1997).

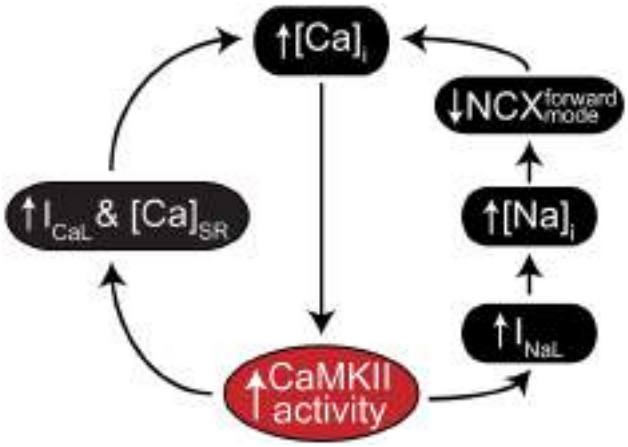

FIGURE 2 | CaMKII positive feedback mechanisms involved in cardiac disease. As a Ca^{2+} regulated kinase, CaMKII is sensitive to any physiological mechanism that alters intracellular Ca^{2+} cycling. Many of its own catalytic actions result in such alterations, and two gain-of-function effects that create positive feedback by enhancing Ca^{2+} cycling occur at I_{CaL} (left) and I_{Na} (right). CaMKII regulation of I_{Na} is thought to increase intracellular Na^{+} via enhanced late I_{Na}, which in turn reduces the thermodynamic potential for Ca^{2+} extrusion via NCX. CaMKII regulation of I_{CaL} more directly enhances Ca^{2+} cycling by increasing Ca^{2+} influx through slightly elevated peak current and slowed inactivation.

CaMKII, which independently drives SCR through phosphorylation of RyR2 (**Figure 3A**, mechanism 3; Wehrens et al., 2004). While it is not shown in **Figure 2**, the increased cytosolic Ca^{2+} also causes mitochondrial Ca^{2+} loading and probably invokes a third positive feedback loop involving ROS-dependent CaMKII activation (see Joiner and Koval, 2014 in this issue). Of course, the actions of CaMKII at LCCs, I_{Na}, and certain potassium currents also directly result in proarrhythmic changes to the action potential (AP), including prolonged repolarization and early afterdepolarizations (EADs, **Figure 3B**; Guo et al., 2006). Thus, even at the level of integrated physiological outcomes, there are a number of CaMKII-dependent mechanisms capable of disrupting cardiac ionic homeostasis and electrophysiology. Our focus here is to understand which of these predominates in experimental models of human disease.

ARRHYTHMOGENIC INFLUENCE OF CaMKII IN CONGENITAL HEART DISEASE

The last 20 years have witnessed rapid progress in understanding the molecular and genetic basis of congenital arrhythmogenic diseases (Curran et al., 1995; Cerrone et al., 2012; Napolitano et al., 2012). Somewhat surprisingly, CaMKII activity and hyperactivity have been shown to contribute in many experimental models of these diseases, even those with minimal or undetectable remodeling of myocardial structure. As such, these models offer a unique perspective of how CaMKII promotes arrhythmia in the absence of sequelae associated with structural disease. In this section, we review examples of CaMKII involvement in congenital arrhythmogenic disease, and identify key questions that remain for defining the roles, limits, and potential for therapeutic targeting of CaMKII in these contexts. Impressively, the available data suggest that CaMKII-dependent regulation of RyR2 is an important contributor to arrhythmogenesis in the majority of these congenital disease models. Here we focus specifically on how this regulation contributes to electrophysiologic instability, and suggest the review contributed by Camors and Valdivia (2014) for those interested in a more mechanistic description of CaMKII-dependent regulation of RyR2.

CATECHOLAMINERGIC POLYMORPHIC VENTRICULAR TACHYCARDIA

The autosomal dominant form of CPVT has become the prototypical disease of RyR2-mediated arrhythmia, and accounts for at least half of all positive CPVT diagnoses (Priori et al., 2002). This channelopathy results from a family of RyR2 mutations that cause channel hyperactivity due to increased receptor Ca^{2+} sensitivity. The outstanding physiological ramification of these mutations is increased myocyte susceptibility to SCR and DADs during catecholamine-induced Ca^{2+} overload. As such, β-blocker therapy is a front-line treatment for CPVT patients. However, in ~50% of cases, episodes of VT still present during β-blockade, and more aggressive treatment (including ICD implantation) is often required (Priori et al., 2002; Watanabe and Knollmann, 2011).

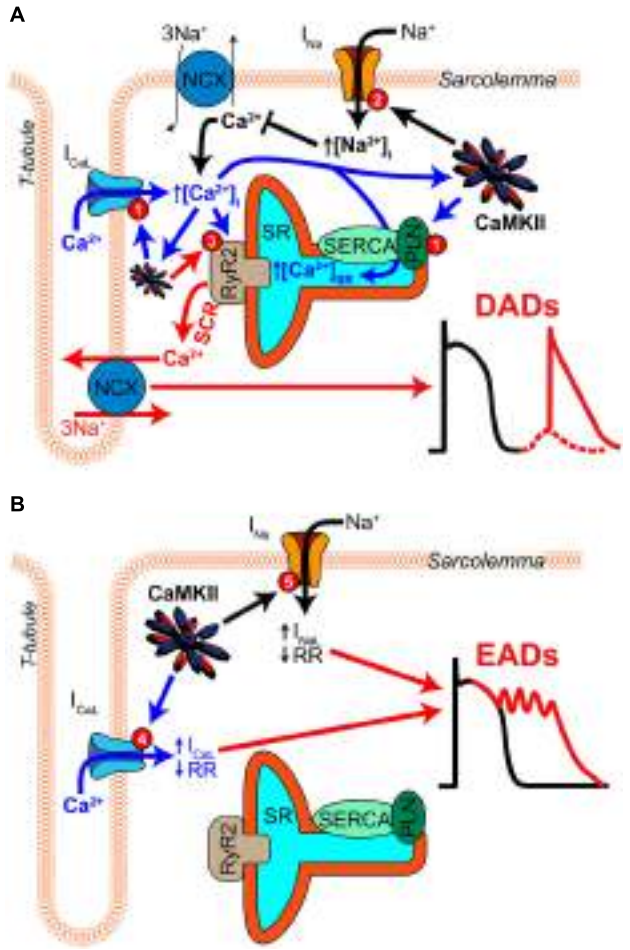

FIGURE 3 | Mechanisms of CaMKII-mediated afterdepolarizations. **(A)** Pathological CaMKII regulation can trigger diastolic SR Ca^{2+} release resulting in electrogenic Ca^{2+} extrusion by NCX and DADs (red arrows). This results from pro-arrhythmic CaMKII regulation at multiple target proteins, which together drive 3 mechanisms through which CaMKII promotes SCR. First, CaMKII elicits gain-of-function effects at I_{CaL} and SERCA (via PLN-mediated disinhibition), thereby increasing Ca^{2+} influx and resulting in Ca^{2+} overload (mechanism 1, blue arrows). Second, CaMKII hyper-phosphorylation of $Na_V1.5$ can elevate intracellular Na^+ resulting in Na^+-induced Ca^{2+}-overload by decreasing Ca^{2+} efflux through NCX (mechanism 2, black arrows). Third, CaMKII directly phosphorylates RyR2, which has been shown to promote SCR and has been strongly implicated in a number of different models of arrhythmogenic disease (mechanism 3, red arrow). **(B)** CaMKII phosphorylation of depolarizing currents reduces repolarization reserve and promotes EADs (red arrows) in certain disease contexts. Altered LCC gating due to CaMKII hyper-activity can elicit EADs by increasing and prolonging I_{CaL} (mechanism 4, blue arrows). $Na_V1.5$ phosphorylation increases I_{NaL} and may lead to non-equilibrium reactivation of I_{Na} (mechanism 5, black arrows), both of which can trigger EADs.

Thus, efforts to define additional effective pharmacotherapeutic approaches for this disease remain a high priority.

Conceptually, CaMKII presents an attractive target in CPVT because its established actions at various Ca^{2+} handling proteins would all be expected to exacerbate arrhythmia resulting from RyR2 hyperactivity. Indeed, transgenic overexpression of CaMKIIδ_C in the R4496C (+/−) mouse model of CPVT worsened the arrhythmia phenotype and increased mortality (Dybkova et al., 2011). However, few studies have assessed whether, and how, CaMKII activity contributes to arrhythmia in CPVT. Liu et al. (2011) showed that both chronic and acute administration of KN-93 dramatically reduced the incidence of arrhythmia in the R4496C (+/−) mouse. More specific CaMKII blockade by autocamtide-2 related inhibitory peptide (AIP) also markedly decreased the incidence of SCR, DADs, and triggered activity in isolated R4496C (+/−) ventricular myocytes (VMs). Interestingly, while AIP did remove signature effects of CaMKII on Ca^{2+} handling (e.g., frequency dependent acceleration of relaxation, FDAR), it did not achieve those effects by reducing SR Ca^{2+} load at any baseline pacing frequency or during isoproterenol (Iso) challenge. Thus, the antiarrhythmic actions of CaMKII blockade were unlikely to result from reduced SR or whole cell Ca^{2+} overload. Instead, they appeared to rely on reduction of CaMKII-induced RyR hyperactivity (**Figure 3A**, mechanism 3), as measured by triggered fractional release, and spark frequency. AIP reduced both of these measures in R4496C (+/−) myocytes, particularly during Iso challenge, and KN-93 reduced RyR2 phosphorylation at the established CaMKII site, Ser-2814. Thus, this study suggests that, even in myocytes already expressing hyperactive mutant RyR2, CaMKII phosphoregulation of this critical SR Ca^{2+} release channel may further contribute to *in vivo* arrhythmogenesis.

CONGENITAL LONG QT SYNDROME

Recent work has suggested that CaMKII is involved in several rare forms of congenital long QT (LQT) syndrome. Importantly, CaMKII mutations have not been established as the source of genetic susceptibility in these, or to date, any congenital arrhythmogenic disease. Rather, in all existing studies, CaMKII exerts its proarrhythmic influence by exacerbating the effects of disease-associated mutations occurring in other electrophysiologic or calcium handling proteins, many of which are CaMKII targets.

Ankyrin-B syndrome/LQT4

After remaining elusive for over 10 years, the molecular basis for type 4 LQT syndrome was identified in 2003 as a family of loss-of-function mutations in Ankyrin-B (Mohler et al., 2003). The electrophysiologic dysfunction associated with these mutations is broad, and often also involves sinus bradycardia and catecholamine-induced arrhythmia, but is not associated with gross structural defects. LQT is relatively mild in most cases, and absent for some mutations even though individuals harboring these mutations remain arrhythmia susceptible (Mohler et al., 2004). Owing to this spectrum phenotype and clear molecular source, LQT4 is now often referred to as Ankyrin B syndrome (Yong et al., 2003). Heterozygous deletion of AnkB in the mouse recapitulates many signatures of human LQT4 (Mohler et al., 2003), including inducible polymorphic VT, and this model has now been used extensively to study mechanisms of the disease.

In neonatal AnkB(+/−) myocytes, LQT4 mutations consistently reduce expression and disrupt localization of the myocardial Na^+/Ca^{2+} exchanger (NCX1), Na^+/K^+ ATPase (NKA), and inositol triphosphate (IP3) receptor (Mohler et al., 2003, 2004). In

adult myocytes, these changes promote signature arrhythmogenic changes including AP prolongation (DeGrande et al., 2012), a prominent increase in Ca^{2+} waves (Camors et al., 2012) and afterdepolarizations (DeGrande et al., 2012). The mechanistic link between altered AnkB molecular anchoring and these arrhythmogenic outcomes is not fully established, but recent studies summarized below have suggested an important role for altered Ca^{2+} homeostasis, and particularly for CaMKII-dependent regulation of RyR2.

Given the observed changes to expression and distribution of major Na^+ transporters, Camors et al. (2012) investigated whether altered Na^+ handling could explain arrhythmogenicity in AnkB(+/−) myocytes. They found that, while maximal NKA function was depressed, this had little impact on measures of basal or challenged Na^+ homeostasis, and did not increase diastolic $[Ca^{2+}]_i$. However, AnkB(+/−) myocytes did exhibit slightly increased SR Ca^{2+} load, enhanced Ca^{2+} transient amplitude and fractional release, and a marked increase in spark frequency and Ca^{2+} waves. Soon after, the same group was able to show that these effects are associated with RyR2 hyperactivity and increased phosphorylation of RyR2 at the primary CaMKII site, S2814 (DeGrande et al., 2012). These effects could be normalized by crossing the AnkB(+/−) mouse with one expressing the CaMKII inhibitory peptide, AC3-I. These mice were also resistant to both cellular afterdepolarizations and organ level arrhythmia present in the AnkB(+/−) mice during adrenergic challenge (DeGrande et al., 2012).

While the authors acknowledged that these RyR2 effects are one component of what are probably many alterations associated with AnkB loss-of function, the observed changes in Ca^{2+} handling are consistent with a mechanism involving RyR2 hyperactivity (Camors et al., 2012). Because AnkB is not known to anchor CaMKII itself, the authors instead suggested that loss of AnkB may have disrupted local phosphatase activity because protein phosphatase 2A is a known binding partner of AnkB and regulator of RyR2 phosphorylation.

Timothy syndrome/LQT8

The extremely rare LQT8, more frequently referred to as Timothy syndrome (TS), results from substitution mutations in the alpha subunit ($Ca_V1.2$) of the cardiac L-type calcium current (I_{CaL}). Only two mutations have been described to date, and both replace glycine residues with either arginine (pos. 406) or serine (pos. 402; Splawski et al., 2004, 2005). These mutations profoundly impair voltage-dependent inactivation of I_{CaL} (VDI), and this is generally accepted to be the proximal molecular dysfunction associated with the disease (Barrett and Tsien, 2008). The cardiac signatures of TS include a range of cardiac structural defects and markedly prolonged ventricular repolarization (Splawski et al., 2004, 2005). Supraventricular arrhythmia has also been noted, particularly AV block (Marks et al., 1995), but ventricular arrhythmogenesis is the most serious presentation of the disease, and most TS patients experience life-threatening events in the first years of life (Splawski et al., 2004). The existence and severity of the TS phenotype has forced clinicians and scientists to broadly reconsider the importance of VDI in normal and pathologic cardiac electrophysiology.

Since the original mechanistic descriptions from Splawski et al. (2004, 2005), a number of experimental models have been developed to investigate how these point mutations destabilize cardiac electrophysiology. In general, those models have provided compelling (albeit not entirely consistent) evidence for the involvement of CaMKII. Erxleben et al. (2006) were the first to suggest a role for CaMKII, and observed that heterologous expression of TS-mutated (G406R) rabbit $Ca_V1.2$ yielded LCCs exhibiting more frequent prolonged openings (mode-2 gating). In noticing that this substitution also created a consensus site for CaMKII at nearby rabbit Ser-439 (homologous to human Ser-409), they hypothesized that CaMKII activity may be required for both mode-2 gating and slowed macroscopic inactivation of I_{CaL} in TS. CaMKII inhibition by KN-62, and genetic ablation of the putative CaMKII phosphoacceptor (S439A) indeed eliminated the increased intrinsic mode-2 openings of the mutant channels in that study. Thiel et al. (2008) extended this work to adult VMs by double-mutating $Ca_V1.2$ to include both G406R and resistance to dihydropiridine inhibition via T1066Y. This permitted the authors to modulate the contribution of endogenous LCCs to macroscopic I_{CaL} via nifedipine, and thereby study the effects of the loss of I_{CaL} control, independent of differences in peak I_{CaL}. Unlike Erxleben et al. (2006), they were unable to observe a requirement for CaMKII in the loss of VDI accompanying the G406R mutation. However, they did observe an interaction between VDI loss and CaMKII, whereby TS myocytes exhibited greater I_{Ca} facilitation than WT, and this could be removed through peptide blockade of CaMKII via AC3-I. To explain this they suggested that Ca^{2+} loading due to impaired VDI caused enhanced secondary activation of CaMKII, which in turn exaggerated I_{Ca} facilitation. They also observed a pronounced increase in SCR and DADs, both of which could be prevented by AC3-I. As a result they reasoned that DADs occurring secondary to Ca^{2+} overload may reflect the dominant arrhythmogenic role of CaMKII in TS, and mathematical modeling supported this contention. Thus, in VMs, CaMKII activation may compound the effects of TS mutations by enhancing I_{CaL} facilitation in response to impaired VDI and increased Ca^{2+} influx.

Further cardiac-specific models of TS have now been developed, most notably the G436R transgenic mouse developed by Cheng et al. (2011). This model exhibits increased $Ca_V1.2$ expression, and the entirety of this increase (∼40%) is due to expression of the G436R transgene. Somewhat surprisingly this additional $Ca_V1.2$ does not increase peak I_{CaL} in these mice but does slow VDI in the expected manner, and intriguingly, this effect required the anchoring protein AKAP150. To explain this result, the authors suggest that AKAP150 provides a key structural link among LCCs within the dyadic ensemble, and they provide evidence that, in the presence of G436R-mutated channels, AKAP150 promotes coupled gating of LCCs and increases mode-2 behavior. Similarly to Thiel et al. (2008), Yarotskyy et al. (2009), and Cheng et al. (2011) observed that slowed inactivation in this model is not impacted by CaMKII inhibition, and therefore suggest that the gating effects underlying TS are probably not reliant upon G436R-mediated creation of a CaMKII phosphorylation site at Ser-439/409. They did, however, observe markedly enhanced Ca^{2+} cycling, prolonged AP duration, and exaggerated susceptibility to cellular

afterdepolarizations. These effects were confirmed in a recent and more detailed study of Ca^{2+} handling in these mice (Drum et al., 2014), and again like Thiel et al. (2008) the authors conclude that Ca^{2+} overload and SCR are the likely terminal mechanisms of arrhythmogenesis in this model.

In summary, these investigations suggest that CaMKII plays an important role in TS arrhythmogenesis, but at least in VMs it seems probable that this accompanies Ca^{2+} overload secondary to the intrinsic effects of the TS mutations on LCC gating. An important qualifier here is that, to date, all experiments suggesting this role for CaMKII have been conducted in primary cardiomyocytes from rodents. The rapid repolarization of these cells probably biases arrhythmogenic mechanisms away from disrupted repolarization and toward SCR and arrhythmia arising during diastole. Thus, similarly detailed investigations in large mammalian myocytes remain desirable for clarifying if changes in I_{CaL} gating are capable of playing a more direct role in human TS arrhythmia, and if CaMKII still contributes in a similar manner and to a similar degree.

Phosphomimetic mutation of $Na_V1.5$

CaMKII is an established regulator of the myocardial Na^+ current (I_{Na}), and simultaneously potentiates late I_{Na} (I_{NaL}) while decreasing channel availability (Herren et al., 2013). While the specific phosphorylations required for these effects remain debated, available evidence suggests that either or both Ser-571 and Ser-516 may be key sites (Hund et al., 2010; Ashpole et al., 2012; Koval et al., 2012; Herren et al., 2013), and it is generally agreed that the I-II intracellular linker is the critical phosphoregulatory domain.

An interesting line of investigation has suggested that mutation of residues immediately adjacent to Ser-571 are associated with congenital disease, and may result in phosphomimetic effects at I_{Na}. Two mutations, A572D and Q573E, were originally uncovered by genetic screening of Romano-Ward syndrome probands, and therefore thought to be novel forms of LQT3 (Paulussen et al., 2003; Tester et al., 2005). The A572D mutation has since been dismissed as an independent source of LQT3 susceptibility, but may still be associated with arrhythmogenic cardiac diseases because it cosegregates with the established arrhythmogenic mutation H558R (Tester et al., 2010). Having previously identified Ser-571 as a potential site for CaMKII regulation, Koval et al. (2012) recently investigated whether the functional effects of these mutations exhibited any dependence on CaMKII activity. They showed that heterologous expression of either A572D or Q573E recapitulated CaMKII-dependent effects at I_{Na}. Neither constitutively active CaMKII nor blockade of endogenous CaMKII altered these effects, suggesting that they are autonomous to the mutant $Na_V1.5$ and independent of additional CaMKII phosphoregulation. Further, the mutant gating defects could be reconstituted in primary mouse VMs transfected with channels sensitive to low dose tetrodotoxin to allow silencing of the exogenous $Na_V1.5$. While conflicting results from other groups (Tester et al., 2010; Ashpole et al., 2012) suggest that these findings require further interrogation, they provide the first evidence that CaMKII regulation of I_{Na} may be sufficient to induce an arrhythmogenic phenotype in the absence of structural or ischemic disease.

ARRHYTHMOGENIC INFLUENCE OF CaMKII IN ACQUIRED HEART DISEASE

An extensive literature describes the arrhythmogenic mechanisms attributable to CaMKII hyperactivity in acquired heart disease. A large portion of this is related to heart failure, but significant effort has also been invested in AF and ischemia/reperfusion challenge (I/R). The CaMKII-dependent mechanisms involved in AF and I/R are detailed separately in this issue by Heijman et al. (2014) and Bell et al. (2014) and we direct the interested reader to those comprehensive treatments. For the purposes of this review it suffices to note that recent studies of AF and I/R mechanisms in mice and humans have again implicated RyR2 dysregulation, and SR Ca^{2+} handling in general (Said et al., 2008, 2011; Voigt et al., 2012; Purohit et al., 2013). These aspects are consistent among the various acquired disease even though other important pathological conditions differ. For example, some signature electrophysiologic alterations apparent in AF (Nattel et al., 2008), such as shortened APD and more negative resting potential, are precisely the opposite of the alterations present in failing VMs (Tomaselli and Marbán, 1999).

As might be expected of the pathologic complexity in acquired disease, the range of CaMKII-dependent mechanisms thought to contribute to arrhythmia is also broader than for congenital disease. Specifically, CaMKII has been shown to contribute to Na^+ overload in heart failure (**Figure 3A**, mechanism 2), and several studies have suggested that CaMKII effects at I_{CaL} may be sufficient to directly destabilize electrophysiology in the failing ventricle (**Figures 3A,B**). An additional feature of CaMKII-dependent arrhythmia in heart failure and AF, and probably in acquired disease in general, is the importance of CaMKII-oxidation as a source of kinase hyperactivity (Luczak and Anderson, 2014; see also the review by Erickson, 2014 in this special issue). This mode of CaMKII activation appears to be particularly important in sinus node (SN) dysfunction accompanying heart failure (Swaminathan et al., 2011), in AF (Purohit et al., 2013), and diabetic cardiomyopathy (Luo et al., 2013). Finally, one of the most important roles that CaMKII plays in acquired disease is as a controller of the expression of several key ion channels and transporters, and many disease-associated changes in expression of these proteins appear to require CaMKII. The best-described of these involve K^+ currents, namely the transient outward current (I_{to}) and inward rectifier current (I_{K1}; Wagner et al., 2009; reviewed elsewhere in this issue by Mustroph et al., 2014), but this transcriptional regulation may also extend to I_{CaL} (Ronkainen et al., 2011), NCX1 (Mani et al., 2010), and SERCA2 (Lu et al., 2011).

HEART FAILURE

The role of CaMKII in heart failure pathophysiology has been the subject of intense investigation since elevated CaMKII levels were first found in the myocardial tissue of heart failure patients (Hoch et al., 1999; Kirchhefer et al., 1999). Increased CaMKII expression and activity have since been mechanistically linked to structural and electrophysiological dysfunction in numerous experimental models of severe cardiomyopathy and heart failure. However, deciphering precisely how CaMKII is driving electrophysiologic dysfunction is made all the

more difficult by the extensive structural and electrophysiologic remodeling that accompanies CaMKII hyperactivity in the failing heart.

Target-specific roles of CaMKII in heart failure

Murine models of heart failure, CaMKII hyperactivity (overexpression), and CaMKII regulation of RyR2 have provided a wealth of information describing how CaMKII contributes to both arrhythmogenesis and disease progression in cardiomyopathy. Cardiac restricted overexpression of CaMKIIδ_C in the mouse leads to heart failure, inducible arrhythmia, and premature death (Zhang et al., 2003; Wagner et al., 2006). In these mice, acute inhibition of CaMKII (via KN-93) prevents catecholaminergic arrhythmia *in vivo*, and RyR2 dysfunction was implicated in this arrhymogenic mechanism (**Figure 3**, mechanism 3) by a substantial and CaMKII-dependent increase in SR Ca^{2+} leak, elevated diastolic Ca^{2+}, and DADs during Iso challenge (Sag et al., 2009). SR-targeted inhibition of CaMKII with SR-AIP restored calcium handling but worsened the heart failure phenotype suggesting that other CaMKII mechanisms are integral to pathological remodeling (Huke et al., 2011). The phosphomimetic (S2814D) and non-phosphorylatable (S2814A) RyR2 mutants developed by Wehrens et al. (2004), have provided a powerful model for investigating the role of CaMKII-dependent RyR2 regulation in a large number of acute and chronic diseases. Most recently, the knock-in mice expressing these engineered RyR2 variants have provided compelling evidence that CaMKII regulation of this protein contributes to arrhythmogenesis in several models of acquired disease. The S2814D mouse slowly develops a heart failure phenotype and is susceptible to sustained VT during epinephrine/caffeine challenge, and pressure-overload initiated prior to overt heart failure also increased mortality due to arrhythmias in these mice (van Oort et al., 2010). Genetic ablation of CaMKII-dependent RyR2 phosphorylation in the S2814A mouse provides protection from pacing-induced arrhythmias after pressure-overload, slows the development of contractile dysfunction, and reduces ventricular remodeling and cellular SCR (van Oort et al., 2010; Respress et al., 2012).

CaMKII has also been shown to contribute to heart failure arrhythmias in large mammals. A series of studies in a rabbit model of non-ischemic heart failure provides further mechanistic evidence of the importance of CaMKII regulation of RyR2 in heart failure arrhythmogenesis. In this model, total CaMKII expression was elevated and the amount and activity of CaMKII localized to RyR2 was increased in failing ventricular tissue, thus leading to enhanced CaMKII-dependent phosphorylation of RyR2 (Ai et al., 2005). Elevated SR Ca^{2+} leak was significantly reduced with inhibition of CaMKII but not PKA (Ai et al., 2005), and Iso-induced SCR events (Ca^{2+} waves) were CaMKII-dependent (Curran et al., 2010). Belevych et al. (2011) also described progressive pathological RyR2 dysfunction in a tachycardia-induced canine model of heart failure. Mirroring changes in SR Ca^{2+} leak observed in the rabbit, phosphorylation of RyR2 by CaMKII but not PKA was increased after 1- and 16-months of tachycardic pacing. CaMKII inhibition with KN-93 abolished proarrhythmic diastolic calcium waves after 1 month but not 16-months. Instead, the increased wave frequency at 16 months was attributed to increased ROS production, which highlights the progressive nature of arrhythmia mechanisms in heart failure, and suggests that CaMKII hyperactivity may contribute more greatly to arrhythmogenesis early in heart failure progression. Significantly, the clinical importance of CaMKII modulation of RyR2 was reinforced recently when Fischer et al. (2013a) demonstrated that CaMKII inhibition with KN-93 or AIP reduced SR Ca^{2+} leak in myocytes isolated from failing human hearts.

CaMKII regulation of cardiac Na^+ currents is another potential source of arrhythmogenic regulation during heart failure. The mechanisms of this regulation are presented briefly in **Figures 2** and **3** and in detail elsewhere in this issue (see Grandi and Herren, 2014), thus we only briefly mention them in the context of their importance in heart failure. I_{NaL} is increased in human heart failure (Valdivia et al., 2005), and evidence from numerous animal models suggests CaMKII inhibition normalizes I_{NaL}. In a murine TAC-induced heart failure model, CaMKII inhibition by AIP reversed increases in I_{NaL}, APD_{90}, Iso-induced DAD frequency, and SR Ca^{2+} leak (Toischer et al., 2013). In that study, CaMKII phosphorylation of $Na_V1.5$ was increased during decompensated heart failure and application of ranolazine had a similar result to CaMKII inhibition suggesting an important role for CaMKII regulation of Na^+ handling. Interestingly, myocytes from pressure-overloaded hearts did not exhibit EADs in that study, suggesting that Na^+ does not carry the arrhythmogenic current. Instead, the authors suggested that I_{NaL} was indirectly responsible for DADs by causing Na^+-induced Ca^{2+}-overload (**Figure 2**). This idea is consistent with reports from several other studies. Most recently Morotti et al. (2014) have suggested that Na^+-induced Ca^{2+}-overload is a component of a positive feedback loop that is quantitatively capable of eliciting CaMKII hyperactivity, which in turn promotes arrhythmogenic outcomes and, probably, further Na^+ loading (**Figure 2**). Indeed, experiments involving Na^+ overload induced by anemone toxin (ATX-II, a potent agonist of I_{NaL}) and an LQT3 mutation (N1325S), have shown these maneuvers to be capable of inducing CaMKII activation and arrhythmia (Yao et al., 2011). Similar effects have been observed during glycoside-induced Na^+ overload (Gonano et al., 2011; Ho et al., 2013), and arrhythmias resulting from glycoside treatment were susceptible to both SR-targeted peptide inhibition of CaMKII (Gonano et al., 2011) and ablation of CaMKII phosphorylation at RyR2 via S2814A mutagenesis (Ho et al., 2013). Thus, it may again be that these Na^+-dependent forms of arrhythmogenesis converge at CaMKII-dependent regulation of RyR2. It should be noted that some evidence in large mammals also supports a more direct arrhythmogenic role for CaMKII-dependent regulation of Na^+ currents (**Figure 3B**). In a canine model of dyssynchronous heart failure with extensive electrophysiological remodeling (Aiba et al., 2009), CaMKII expression, activity and autophosphorylation were all increased, particularly in the late-activated lateral LV wall (Chakir et al., 2008). Myocytes isolated from these dogs exhibited shifted voltage-dependent availability, enhanced intermediate inactivation, increased I_{NaL} and ranolazine sensitive EADs (Aiba et al., 2013). Although inhibition studies were not performed in these dogs, Maltsev et al. (2008) had previously identified CaMKII as an important regulator of I_{NaL} in the failing canine heart.

Regulation of LCCs by CaMKII presents a third potential mechanism for proarrhythmic function during heart failure. However, as for I_{Na} the sites of CaMKII phosphorylation of LCCs remain somewhat controversial, and this has caused mutagenic approaches to investigating LCC-dependent mechanisms to be more challenging. Similarly to TS, CaMKII regulation of I_{CaL} in heart failure is thought to elicit arrhythmia either by disrupting repolarization and driving EADs (**Figure 3B**), or by promoting cellular Ca^{2+} overload (**Figures 2** and **3A**). Again, a complete treatment of CaMKII effects at I_{Ca} is provided elsewhere in this issue (see the review from Bers), but briefly, heart failure-associated changes to LCC gating are quite similar to those accompanying CaMKII regulation (Schröder et al., 1998; Dzhura et al., 2000). Specifically, CaMKII elicits mode-2 gating in single channels, and slows inactivation while hastening recovery from inactivation of the macroscopic current (Guo et al., 2006). All of these effects would be expected to promote EADs by reducing repolarization reserve, independently promoting I_{CaL} reactivation, or both (Anderson et al., 1994; Hashambhoy et al., 2010; Koval et al., 2010). The most direct evidence for the ability of CaMKII to elicit EADs via I_{CaL} (in large mammal VMs), was presented by Koval et al. (2010). In that study, the $Ca_V1.2$ β_{2a} regulatory subunit was expressed in rabbit VMs, which increased I_{Ca} facilitation and promoted EADs. Those effects were reversed by peptide (CaMKIIN) or shRNA inhibition of CaMKII, and ablation of the proposed CaMKII phosphorylation or binding sites (Koval et al., 2010). Two caveats when extending these findings to CaMKII hyperactivity in normal or failing myocytes are: (1) the β_{2a} subunit is probably a lesser component of LCCs in non-diseased hearts, although its expression may be increased in heart failure (Hullin et al., 2007), and (2) the slowed inactivation in this study also increased total Ca^{2+} influx without destabilizing the AP. *In vivo*, the effect of this on whole-cell and SR Ca^{2+} overload could easily be as or more important than effects upon repolarization.

Together these studies suggest that CaMKII-mediated ventricular DADs and EADs exert a proarrhythmic influence in the failing heart, and that the specific mode of arrhythmia initiation and maintenance is likely dictated by the underlying etiology and stage of disease development. The genetic tools available for studying the role of CaMKII regulation of RyR2 in mice have provided compelling evidence that this target is central to the acute arrhythmogenic outcomes of CaMKII in the failing ventricle, and this contention is largely supported by studies in large mammals. Given the extensive structural and electrophysiological remodeling accompanying heart failure, and relatively poor specificity of small molecule inhibitors available for *in vivo* CaMKII blockade, it is still not entirely clear how effective acute CaMKII inhibition may be as an anti-arrhythmic strategy in heart failure. However, evidence published to date support an optimistic outlook for potential therapeutic applications in this and other acquired cardiac diseases.

Sinus node dysfunction in heart failure

Another series of recent studies, reviewed in this issue by Wu and Anderson (2014), has suggested that oxidized-CaMKII (ox-CaMKII) plays a critical role in SN dysfunction accompanying heart failure, and that this involves pronounced structural remodeling of the SN. Swaminathan et al. (2011) first observed that, relative to non-diseased controls and heart failure patients without SN dysfunction, heart failure patients with SN dysfunction exhibit increased ox-CaMKII in right atrial tissue. This comprehensive study observed an analogous effect in a canine model of pacing-induced heart failure, and utilized their previously developed model of Ang II-dependent oxidation/activation of CaMKII to elicit and study the mechanisms of SN dysfunction in mice. With this model ∼70% of mice develop sinus pause or exit block after 3 weeks of Ang II infusion, and heart rate at rest and during activity is blunted compared to untreated controls. SN dysfunction was absent in AC3-I (cardiac specific) mice treated with Ang II even though the pressor response to Ang II was similar to WT. The observed dysfunction was associated with increased apoptosis and fibrosis within the SN, and structurally based computational analysis suggests that the cell loss observed experimentally may be sufficient to explain both reduced SN firing frequency, and episodes of exit block. One particularly elegant component of this study showed that local adenoviral gene transfer of the CaMKIIN inhibitory peptide (via painting of the SN) was capable of preventing Ang II-driven SN dysfunction in WT mice. Thus providing proof-of-principle that gene transfer of CaMKII-inhibitors may be a viable therapeutic avenue for this specific heart failure phenotype. A follow-up study from Luo et al. (2013) investigating SN dysfunction in a mouse model of combined insulin deficiency and myocardial infarction, suggests that the mechanistic cascade described by Swaminathan et al. (2011), may extend to other cardiac conditions exhibiting pronounced oxidative stress.

The role of oxidized CaMKII in driving structural remodeling and dysfunction of the SN in response to oxidative stress highlights the ability of CaMKII to impact arrhythmogenic outcomes via structural as well as functional changes. To date, the impact of these tissue-level effects in CaMKII-mediated arrhythmia has not been studied to the same extent as the effects on cellular electrophysiology and Ca^{2+} handling. This aspect of CaMKII-driven arrhythmia warrants further work, as some evidence suggests that effects at this level may be important. For example, CaMKII activity may exhibit regional heterogeneity in the heart (Chakir et al., 2008). Even without considering this heterogeneity of kinase action, transmural dispersion of repolarization is a predicted result of CaMKII hyperactivity (Bers and Grandi, 2009) due exclusively to its effects at I_{to}. Thus, putative roles for CaMKII in reentrant or alternans-driven arrhythmias remain largely untested.

SUMMARY AND CONCLUSION

The ability of CaMKII to contribute to arrhythmia in models of cardiac disease that result from widely varying etiologies is testament to the importance of this kinase in the control of cardiac electrophysiology and calcium handling. It also suggests that CaMKII exerts its proarrhythmic influence either by regulating some convergent mechanism that is active in all of these diseases, or by regulating a divergent range of proarrhythmic mechanisms, which contribute to differing degrees in each disease. To date, the available mechanistic evidence suggests that CaMKII-dependent regulation of diastolic SR Ca^{2+} release is

the dominant cellular mechanism by which the kinase promotes arrhythmia (**Figure 3A**). Of the various CaMKII targets capable of driving this mechanism, RyR2 is the most clearly implicated. This may in part be due to the quality of the tools available for genetically manipulating CaMKII phosphorylation and regulation of RyR2, but it also suggests that developing exogenous compounds capable of impairing the ability of CaMKII to phosphorylate RyR2 could constitute a broadly applicable therapeutic strategy. Additional effects of CaMKII upon I_{Na} and I_{CaL} may also directly contribute to arrhythmia in some contexts (**Figure 3B**), but in several of the examples described above it appears likely that these effects again converge to elicit arrhythmia via SCR and DADs (**Figure 3A**).

Both SCR and DADs are generally induced by some form of acute Ca^{2+} overload, and it is probable that this partially explains why CaMKII-dependent arrhythmia most often requires conditions of tachycardia and, notably, β-adrenergic challenge. The involvement of this additional signaling cascade brings in further complexity that is almost certainly important in defining the transition from stable to unstable electrophysiology. As suggested by studies involving the Ankyrin B (+/−) mouse (DeGrande et al., 2012), this may depend on less well-understood aspects of the signaling network, such as local phosphatase balance. As such, strong conclusions regarding any one CaMKII target as being crucial for broad arrhythmia phenotypes should remain contentious until these finer details have been described with some clarity.

Tissue-level effects of CaMKII are also undeniably important, as highlighted by SN dysfunction in heart failure, but beyond these investigations, little direct evidence is available to describe how CaMKII disrupts normal propagation of the cardiac AP. By incorporating regional heterogeneities in cellular electrophysiology, computational modeling may aide in understanding these tissue level effects provided sufficient experimental data for parameterization and validation are available. The reviews by Onal et al. (2014), Greenstein et al. (2014), and Yaniv and Maltsev (2014) offer a comprehensive survey of previous work modeling CaMKII on the cellular and tissue levels.

CAVEATS AND IMPORTANT FUTURE DIRECTIONS

Even with the wealth of information that has amassed to describe the role of CaMKII in cardiac arrhythmia over the last 20 years, several key aspects either remain challenging or are otherwise conspicuously absent from the existing literature. First, no study has been capable of identifying a gain-of-function mutation in CaMKII that is an independent risk for arrhythmogenic cardiac disease, or indeed, any form of cardiac disease. This is surprising given the data available from studies involving the various CaMKII inhibitors. Second, a key unrealized objective of the CaMKII field is to have more specific CaMKII inhibitors that can be applied acutely and *in vivo* (see the review from Pellicena and Schulman, 2014 in this special issue). This is particularly true for studies of arrhythmia, where such inhibitors would allow more straightforward translation between cell-based experiments and arrhythmia outcomes in intact animals, and perhaps eventually humans. Indeed, as mentioned above, the tissue-level effects of CaMKII activity and hyperactivity are poorly understood compared to the cellular effects, and one reason for this is the lack of suitably specific small molecule inhibitors. Third, as mentioned with respect to TS, the use of murine myocytes for cell-based arrhythmia assays probably biases the active mechanisms away from those occurring during repolarization (EADs), and toward those that are active during diastole (DADs). This is because the rapidly repolarizing murine myocyte has markedly increased repolarization reserve, and current dynamics during repolarization that are at least very different to those in large mammals and humans (Nerbonne, 2004). As techniques for differentiating and reprogramming h-IPSCs improve, models based on this approach may offer a useful new tool for studying the role of CaMKII in congenital arrhythmogenic diseases at least. Examples of early studies in this direction have recently appeared for CPVT (Di Pasquale et al., 2013) and TS (Yazawa et al., 2011). However, concerns surrounding how well these cells recapitulate adult cardiomyocyte Ca^{2+} handling are particularly poignant in these and other Ca^{2+}-related arrhythmogenic diseases. As such, the evidence supporting myocyte-specific differentiation should be considered carefully and specifically for any such future model.

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