Towards a Unified Theory of Calmodulin Regulation (Calmodulation) of Voltage-Gated Calcium and Sodium Channels

Article *in* Behaviour and Information Technology · September 2015

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Curr Mol Pharmacol. Author manuscript; available in PMC 2016 July 26.

Published in final edited form as:

Curr Mol Pharmacol. 2015 ; 8(2): 188–205.

Towards a unified theory of calmodulin regulation (calmodulation) of voltage-gated calcium and sodium channels

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Abstract

Voltage-gated Na and Ca^{2+} channels represent two major ion channel families that enable myriad biological functions including the generation of action potentials and the coupling of electrical and chemical signaling in cells. Calmodulin regulation (calmodulation) of these ion channels comprises a vital feedback mechanism with distinct physiological implications. Though long-sought, a shared understanding of the channel families remained elusive for two decades as the functional manifestations and the structural underpinnings of this modulation often appeared to diverge. Here, we review recent advancements in the understanding of calmodulation of Ca^{2+} and Na channels that suggest a remarkable similarity in their regulatory scheme. This interrelation between the two channel families now paves the way towards a unified mechanistic framework to understand vital calmodulin-dependent feedback and offers shared principles to approach related channelopathic diseases. An exciting era of synergistic study now looms.

Keywords

Sodium Channels; Calcium Channels; Calmodulation

Introduction

In recent years, the small 17 kDa bilobal Ca^{2+}-binding protein calmodulin (CaM) has emerged as a potent and pervasive modulator of ion channels, often serving as an obligatory channel subunit [1]. The tale of CaM interaction with ion channels is entwined with rich mechanistic insights, important biological ramifications, and promises of crucial therapeutic insights. In this context, an early result that highlighted the role of CaM in regulating ion channel function came about from the genetic dissection of motile behavior in *Paramecium*. Strikingly, mutations in the CaM gene resulted in organisms that were either ‘under-excitable’ or ‘over-excitable’ to certain stimuli, due to the selective loss of either a Ca^{2+}-dependent Na current or a Ca^{2+}-dependent K current [2]. This functional bipartition of CaM regulation foreshadowed a major theme of ion channel regulation, where the two lobes of

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*Dr. David T. Yue passed away on Dec. 23 2014. He was a remarkable scientist, mentor and friend. The world will miss this luminary scientist.

CaM impart distinct functions. Since then, the catalogue of ion channels identified to be under the charm of CaM has been ever-expanding [3], and the list now includes diverse channel families such as the small-conductance potassium (SK) channels [4], the KCNQ potassium channels [5–7], cyclic nucleotide-gated channels [8], N-methyl D-aspartate (NMDA) receptor [9, 10], transient receptor potential (TRP) channels [11], ryanodine receptors [12], voltage-gated Ca^{2+} channels [13–16], and voltage-gated Na channels [17]. Amongst these channel families, CaM regulation (calmodulation) of voltage-gated Ca^{2+} and Na channels is a prominent prototype for ion channel modulation with diverse functional effects, exquisite Ca^{2+} decoding capabilities, and profound biological implications for cellular excitability. Here, we review recent advancements in our understanding of calmodulation of Ca^{2+} and Na channels – two fields that have long-shared a parallel history that now converges with exciting synergy.

The rise of Ca^{2+} channel calmodulation

Calmodulin regulation of Ca^{2+} channels is a prominent feedback mechanism that tunes channel gating to adjust vital Ca^{2+} entry in accordance with cytosolic Ca^{2+} signals. The first signs of this modulation emerged in 1978 when Brehm and Eckert observed that increased cytosolic Ca^{2+} could accelerate the inactivation of Ca^{2+} currents in *Paramecium* [18]. Thus, unlike the canonical voltage-dependent inactivation (‘*h*-gate’) of Hodgkin and Huxley [19], inactivation of Ca^{2+} currents was highly sensitive to cytosolic Ca^{2+} concentrations [20]. Indeed, this modulation allows the cell to sculpt electrical activity based on the integration of cytosolic spatiotemporal Ca^{2+} dynamics.

Ca_V1 (L-type) channels serve as a prominent example of the regulatory principles and the molecular underpinnings of calmodulation. For many L-type channels, calmodulation manifests as a rapid Ca^{2+}-dependent inactivation (CDI) evolving typically over 50 – 100 milliseconds. A convenient experimental approach to characterize CDI with quantitative precision is illustrated in Figure 1A. Here, a step depolarization in membrane potential activates Ca^{2+} channels, furnishing Ca^{2+} entry that triggers CDI mediated by CaM. By contrast, calmodulation is absent when Ba^{2+} is the permeant ion through the channel, thus serving as a negative control. Figure 1B displays the typical electrophysiological profile of Ca^{2+}-dependent inactivation (CDI) as exemplified by $Ca_V1.3$, an archetypal channel with strong and unambiguous Ca^{2+}/CaM regulation. In this manner, the steady-state decrement in Ca^{2+} current (red) compared to Ba^{2+} (black) serves as a robust quantitative metric for Ca^{2+} regulation of L-type Ca^{2+} channels, enabling the systematic characterization of underlying biophysical mechanisms and their biological implications [21, 22].

At the single molecule level, Ca^{2+} dependent inactivation switches Ca^{2+} channels from a high open-probability (P_O) mode of gating (‘mode 1’) to a lower P_O gating mode (‘mode Ca’) in a quantized manner [23]. In ‘mode Ca’, channel openings are more sparse with a prolonged first latency [23]. The transition of channels from ‘mode 1’ to ‘mode Ca’ is governed by Ca^{2+}/CaM. This behavior is notably different from the canonical voltage-dependent inactivation of ion channels, where channels enter an absorbing non-conductive inactive state [24]. Indeed, systematic mapping of the state-dependence of calmodulation suggests that entry into ‘mode Ca’ proceeds equally from open and nearby closed states [21,

25]. By contrast voltage-dependent inactivation of Ca^{2+} channels appears to proceed preferentially from the open state [25]. Importantly, as Ca^{2+} influx through Ca^{2+} channels drives calmodulation, shifts in voltage-dependence of channel activation could indirectly alter the strength of channel regulation [21]. Of pathophysiological relevance, various channelopathic mutations that result in heritable disorders including Timothy syndrome [26, 27], Autism Spectral Disorders [28], and Familial Hemiplegic Migraine [29, 30] cause shifts in channel activation. These indirect effects of channel gating on CaM regulation are important considerations in the effort to develop effective therapeutic strategies.

The physiological phenomenon of Ca^{2+} regulation of L-type Ca^{2+} channels was well-established in many native systems including the heart, long before the definitive role of CaM in this process was confirmed [31–33]. We first consider key findings that led to the establishment of CaM as the Ca^{2+} sensor for Ca^{2+} channel regulation. The earliest proposals for the mechanism of Ca^{2+} channel CDI hypothesized both the direct binding of Ca^{2+} ions to the channel [34, 35] and Ca^{2+}-dependent phosphorylation and/or dephosphorylation of channels [36, 37]. Even then, the direct or indirect role of CaM in this regulatory process was suspected, but remained unclear for many years [20, 37]. The molecular cloning and functional expression of recombinant Ca^{2+} channels in heterologous systems, paved the way towards an in depth structure-function understanding of Ca^{2+} regulation [38]. The Ca^{2+} channel is a large protein complex composed of roughly 2000 amino acids in the main α-subunit. Identifying a molecular segment relevant for Ca^{2+} regulation on the channel complex thus seemed daunting. Some hope was garnered following the identification of a putative Ca^{2+}-binding ‘EF hand’ motif on the carboxy-terminus of both Ca^{2+} and Na channels through bioinformatic analysis (Figure 1C, EF) [39]. Classically, the EF hand motif is composed of a helix-loop-helix protein segment with acidic residues in the loop region spatially arranged to coordinate Ca^{2+} ions [40]. The channel EF hand, however, appeared to be vestigial in that it lacked key acidic residues essential for Ca^{2+} binding [39]. Further analysis of the channel carboxy-terminus revealed a second EF-hand motif analog [41, 42]. The field quickly focused on this channel locus and evaluated its role in CDI. To this end, it was fortuitous that the L-type ($Ca_V1.2$) channels exhibited strong CDI under elevated intracellular Ca^{2+} buffering, while the R-type ($Ca_V2.3$) channels demonstrated only weak CDI under the same conditions rendering them useful for chimeric channel analysis [43, 44]. Accordingly, segments of the carboxy-tail (Ca^{2+}-inactivating region; CI region) of $Ca_V1.2$ and $Ca_V2.3$ channels were swapped and Ca^{2+} regulation was quantified [44]. The proximal third of the channel carboxy terminus and, more specifically, the dual vestigial EF hand segment of $Ca_V1.2$ emerged as critical segments for strong Ca^{2+} regulation. It was then plausible that Ca^{2+} may directly interact with the channel to trigger CDI as postulated earlier [20, 34, 35]. However, point mutations of key residues in the Ca^{2+} binding loop of the EF1 segment spared CDI, arguing that Ca^{2+} does not directly interact with the channel itself [45, 46]. Overall, it was clear that the Ca^{2+} channel CI region, including the two vestigial EF hand regions, played a prominent role in calmodulation, though the precise function of these segments remained poorly defined.

Cognizant of these results, the search-space for a structural determinant of Ca^{2+} channel Ca^{2+} regulation soon expanded to include alternative segments of the channel CI region. Systematic deletions of overlapping segments of the $Ca_V1.2$ channel CI region identified an

IQ motif (Figure 1C) as yet another critical element in the CDI process [47]. The IQ domain, named for conserved isoleucine and glutamine residues, is a well-known target of Ca^{2+}-independent CaM binding [48]. Moreover, mutagenesis of the $Ca_V1.2$ IQ domain confirmed a critical role for this segment in CDI and renewed interest in the possibility that CaM could still be the Ca^{2+} sensor for Ca^{2+} channels [47, 49]. That said, prior experiments utilizing pharmacological inhibitors of CaM had failed to eliminate CDI of L-type Ca^{2+} channels [23, 47]. The resolution to this conundrum came from the development of a Ca^{2+}-insensitive mutant CaM, where point mutations were introduced within all four EF-hand Ca^{2+} binding sites (CaM_{1234}) [50]. If CaM were to associate with the Ca^{2+} channel complex in a Ca^{2+} independent manner, then this mutant CaM could act as a dominant negative to eliminate regulatory function. Indeed, CaM was identified as the Ca^{2+} sensor for the SK channels using such a maneuver [50]. Accordingly, when CaM_{1234} was coexpressed with $Ca_V1.2$ channels, CDI was eliminated, establishing CaM as the unambiguous Ca^{2+} sensor and gave birth to the concept of ‘calmodulation’ of Ca^{2+} channels [51, 52]. Importantly, Ca^{2+}-free CaM (apoCaM) was subsequently shown to bind the $Ca_V1.2$ CI region with a strong dependence upon the IQ domain [53–56]. Thus, CaM acts as a resident Ca^{2+} sensor molecule in the Ca^{2+} channel signaling complex. Since this discovery, nearly all Ca^{2+} channels have been shown to be regulated by CaM with diverse functional caricatures and elegant Ca^{2+} sensing properties [43, 57–63], as described in subsequent sections. More generally, the usage of a dominant negative CaM (CaM_{1234}) remains an indispensable tool for establishing CaM as a Ca^{2+}-sensor for various macromolecular targets where preassociation of apoCaM is a requisite.

A blueprint for Ca^{2+} channel regulation

The mechanism of CaM action on the channel and the functionally relevant CaM/channel interfaces has continued to be controversial. A prominent hypothesis has been an ‘IQ-centric’ model based on atomic structures of various Ca^{2+} channels in complex with Ca^{2+}/CaM [16, 64–67]. The merits and weaknesses of this model have been reviewed at length [15, 16]. Briefly, mutations in the IQ domain markedly alter Ca^{2+} regulation of many Ca^{2+} channels [41, 43, 47, 49, 52, 54, 61, 66–71]. ApoCaM preassociation has been shown to require the IQ domain [41, 53–55, 69] and Ca^{2+}/CaM also binds to the IQ domain as a peptide with high affinity [41, 43, 51, 52, 55, 56, 69, 70, 72, 73]. However, atomic structures of Ca^{2+}/CaM bound to wild-type and mutant IQ peptides of $Ca_V1.2$ show that the key isoleucine residue is deeply buried [64, 74], and that alanine substitution of this residue does not significantly alter CaM binding conformation [64]. It is unclear how a subtle change at this locus entombed within the hydrophobic Ca^{2+}/CaM interface could alter calmodulation [16, 64]. Further, several mutations in the channel IQ domain that disrupt CDI do not alter the binding affinity of Ca^{2+}/CaM for IQ peptides [41, 69]. As well, mutations that do weaken Ca^{2+}/CaM binding to IQ peptides do not alter the strength of CDI [41, 69]. Together, these results argue against a simple ‘IQ centric’ model leading to the possibility that alternative CaM interacting sites on the Ca^{2+} channel may bear critical functional relevance.

In part, the complexity in understanding the mutational effects on Ca^{2+} channel CDI stems from an incomplete consideration of the whole CaM regulatory system [41, 69]. In

particular, many studies have ascribed functional deficits in CDI resulting from mutations within Ca^{2+} channels to be a disruption of the Ca^{2+}/CaM interface on the channel. However, as calmodulation entails a system of configurational changes, mutations on the channel may affect the propensity for any number of channel states. A general conceptual framework for understanding the various states that underlie calmodulation is illustrated in Figure 1D [41]. In configuration *E*, channels lack apoCaM preassociation. As such, these channels may not undergo calmodulation [41, 58, 59, 69, 75]. Recently, this configuration has also been identified to represent a low P_O gating mode [76]. Upon apoCaM binding, channels traverse into configuration *A*. In this state, channel openings occur normally and the channels are primed to undergo calmodulation [41, 69]. As the two lobes of CaM often act semi-independently, we may further delineate the CaM regulatory process into multiple steps. When Ca^{2+} binds to the C-lobe of CaM, this lobe may interact with an appropriate channel interface yielding configuration I_C with a reduced P_O. Alternatively, once calcified, the N-lobe Ca^{2+}/CaM may interact with its effector site to yield configuration I_N, which also has a reduced P_O. Finally, Ca^{2+} binding to both lobes of CaM yields configuration I_{CN}, a fully inactivated channel with reduced P_O. Importantly, the entry into configuration I_{CN} may involve a positively cooperative step defined by a cooperativity-coefficient $\lambda>>1$.

Thus formulated, mutations of channel interfaces may alter either a single transition or multiple transitions in this regulatory scheme. Nonetheless, the effects on individual transitions may be evaluated using either wild-type or mutant CaM. For example, if a mutation were to alter the binding interaction of the C-lobe of Ca^{2+}/CaM to its effector interface, then the coexpression of mutant CaM_{12} (mutant CaM with Ca^{2+} binding enabled only to C-lobe) would isolate this component of CDI. Conversely, the N-lobe component of CDI could be isolated using CaM_{34} (a mutant CaM with Ca^{2+} binding enabled only to the N-lobe). In both cases, confounding effects of positive cooperativity are carefully avoided. If a mutation were to diminish apoCaM binding, then a fraction of channels may lack a preassociated apoCaM at baseline CaM levels in a cell. Under this condition, channels would preferentially reside in configuration *E*, incapable of undergoing calmodulation. However, overexpression of wild-type CaM would repopulate the channels with an apoCaM, thus redistributing them into configuration *A* by mass action. This maneuver would allow one to rescue a deficit in CDI resulting from a loss of apoCaM preassociation. This general framework allows one to dissect individual sub-configurations of calmodulation, thereby enabling the systematic characterization of relevant CaM/channel interfaces.

Towards a structural understanding of Ca^{2+} channel calmodulation

So far, the general framework presented here assumes a single CaM elicits CaM regulatory function on the channel. However, the stoichiometry of CaM to the Ca^{2+} channel complex remains controversial [77–81]. Recent atomic structures of Ca^{2+}/CaM complexed with subsegments of the $Ca_V1.2$ CI region suggest that multiple CaM molecules may be necessary for the full breadth of Ca^{2+} regulatory functions [77, 78]. Additionally, multiple peptides derived from Ca^{2+} channel cytosolic segments have been shown to interact with Ca^{2+}/CaM [73, 82–85]. From the perspective of channel function, the stoichiometry of the preassociated apoCaM may be more relevant, as the exchange of Ca^{2+}/CaM from the cytosol to the channel alcove is unlikely given the brevity of voltage-pulses used to probe

calmodulation (Figure 1A–1B) and the relative abundance of Ca^{2+}/CaM targets in the cell [86]. Live-cell FRET (Fluorescence Resonance Energy Transfer) binding experiments point to a 1:1 stoichiometry of apoCaM binding to the holo-$Ca_V1.2$ channel complex [81]. Functional experiments have also shown that the covalent fusion of a single CaM molecule to $Ca_V1.2$ and $Ca_V1.3$ channels is sufficient to elicit CDI [79, 80]. Overall, the 1:1 stoichiometry of CaM with Ca^{2+} channel complex simplifies the configurational states necessary to understand calmodulation, thus avoiding the complexity observed in systems that utilize multiple CaM molecules [87]. A possible implication of the singular stoichiometry is that the lobes of CaM may bridge various closely-juxtaposed Ca^{2+}/CaM binding sites on the channel. For example, a recent biochemical study highlights the preference of an amino-terminal Ca^{2+}/CaM binding segment (NSCaTE, N-terminal Spatial Ca^{2+} Transforming Element) to interact with a single lobe of CaM (N-lobe) [83, 88]. Furthermore, this NSCaTE element can interact with Ca^{2+}/CaM prebound to an IQ domain peptide, suggesting the possible bridging of the channel amino- and carboxy-termini [89].

Thus apprised, recent studies have formulated a new structure-function framework of CaM/channel configurations that underlie calmodulation of Ca^{2+} channels as illustrated by molecular models in Figure 1E–1F for $Ca_V1.3$ channels [41, 69]. This molecular framework was built based on functional characterization of extensive alanine scanning mutagenesis of the $Ca_V1.3$ CI region, and the subsequent correlation of functional deficits in calmodulation to underlying binding interactions [41, 69]. First, a single apoCaM preassociates to the Ca^{2+} channel carboxy terminus with the C-lobe of apoCaM bound to the channel IQ domain, and the N-lobe bound to the channel dual vestigial EF-hand segments [41, 54, 55, 69]. The *ab initio* structural model of the $Ca_V1.3$ carboxy-terminus in Figure 1E shows two vestigial EF hand segments (EF, green), a protruded helix representing the preIQ domain (green), and a helical IQ domain (blue). The structural models of the preIQ segment and the IQ domain correspond to known atomic structures of $Ca_V1.2$ channel peptides [64, 65, 77, 78]. Overall, the new consensus is that apoCaM preassociates with the Ca^{2+} channel CI region through a bipartite binding scheme that enhances CaM affinity through positive cooperativity.

Upon Ca^{2+} binding to CaM, Ca^{2+}/CaM is proposed to rearrange itself on the channel complex to elicit configuration I_{CN} (Figure 1F). In this model, the Ca^{2+}-bound N-lobe associates with the NSCaTE module on the channel amino terminus to elicit N-lobe CDI, as illustrated by the NMR structure depicted in Figure 1F [88]. The Ca^{2+} bound C-lobe of CaM has been argued to form a tripartite complex with the channel dual vestigial EF-hands and the IQ domain (Figure 1F) [41]. This ternary arrangement explains the importance of both the IQ domain and the dual vestigial EF-hand segments as determined in early characterization of Ca^{2+} channel CDI [44, 47, 51]. Interestingly, the C-lobe configuration likely corresponds to a traditional CaM/peptide complex, where the channel dual vestigial EF hands may serve as a surrogate lobe of CaM.

Lastly, apoCaM itself has been found to be a potent modulator of channel P_O [76]. In the past, it was believed that apoCaM affinity was sufficiently high that all channels must possess a preassociated CaM [53, 55]. The discovery of alternative splice variants of $Ca_V1.3$ [90, 91] and $Ca_V1.4$ channels [92] demonstrated the presence of a distal carboxy-terminal autoinhibitory domain (ICDI segment) that could compete with apoCaM, thus refining this

perspective [58, 59, 75, 93, 94]. The $Ca_V1.3$ channel IQ domain has also been found to be post-transcriptionally altered through RNA editing [95]. All these post-transcriptional modifications alter channel function by essentially weakening apoCaM preassociation [58, 59, 69, 75, 93]. As such, these $Ca_V1.3$ channel variants support variable strengths of CDI [59, 69, 90, 95, 96]. These channel variants have also been observed to possess a substantially diminished baseline P_O [76, 96]. However, it was not initially clear whether this enormous diversity in CDI and baseline P_O of $Ca_V1.3$ channel variants related to one and the same mechanistic process. Reassuringly, the overexpression of wild-type CaM rescued both CDI and baseline P_O of channel variants with weakened apoCaM preassociation [69, 76]. Accordingly, a simple scheme that unifies the mechanistic underpinnings of these diverse channel variants emerges – apoCaM binding itself can enhance the baseline P_O of L-type Ca^{2+} channels by nearly 7-fold and also prime them for calmodulation. Much like the switching of channels from 'mode 1' to 'mode Ca' during calmodulation, single-channel records of these channel variants depicted quantized behavior whereby channels switched from a high P_O gating mode to a low P_O gating mode [76]. Indeed, transient elevation of CaM near the Ca^{2+} channels using novel chemical biological tools strongly enhanced peak Ca^{2+} currents in channel variants lacking an apoCaM. The unified conceptual scheme is as follows – apoCaM itself enhances channel P_O, and Ca^{2+}-binding to CaM relieves this baseline enhancement [76]. The biological implications of these mechanisms are far-reaching. Of note, apoCaM affinities of many $Ca_V1.3$ channel variants are such that natural fluctuations of ambient CaM in native cells could redistribute channels between pools that lack CaM and ones charged with apoCaM [69, 75, 76]. This switch in channel distribution could profoundly alter both the maximal Ca^{2+} current as well as the shape of the Ca^{2+} waveform in response to electrical stimulation of a cell.

Overall, the proposed structure-function framework in Figure 1E–1F reveals important channel interfaces with implications for the rational design of drugs that could alter Ca^{2+} channel function. For example, molecules that target apoCaM/CI region interface could serve as novel inhibitors of Ca^{2+} channels. Additionally, the switching of CaM/channel interactions upon Ca^{2+} binding implies drugs that target CaM effector interfaces could allosterically abolish Ca^{2+} channel inactivation without altering the peak current. In fact, natural molecules like CaBP (Ca^{2+}-binding protein) may exploit this mechanistic principle to switch off calmodulation [97]. Lastly, this framework will help guide future structural studies and is likely to serve as a model for understanding calmodulation of other channels including voltage-gated Na channels.

Shades of Calmodulation

Calmodulation underlies a pervasive mechanism across the entire $Ca_V1/2$ channel family. Beyond the tuning of channel function by apoCaM, Ca^{2+}/CaM regulation is capable of multiple modes of Ca^{2+} decoding and channel modulation. To a large extent, this customization of channel regulation is determined by the binding of Ca^{2+} to either the C- or N-lobe of CaM such that each lobe is capable of evoking distinct channel modulations [43, 61, 62, 70, 98–100] that allow for exquisite spatial and temporal decoding of Ca^{2+} signals. For example, $Ca_V2.1$ channels demonstrate functional bipartitioning of CaM in channel regulation. For these channels, Ca^{2+} regulation manifests as a rapid Ca^{2+}-dependent

facilitation (CDF) of Ca^{2+} current [101], followed by a slowly developing CDI. This dual regulation can be clearly observed in the response of Ca^{2+} currents to a train of action potentials (Figure 2A; CDF, circle; CDI, square). Moreover, these disparate regulatory effects can be triggered independently by the two lobes of CaM [70, 71]. Specifically, the C-lobe of CaM triggers CDF, whereas the N-lobe is responsible for CDI [70, 71]. Thus, each lobe of CaM can mediate signaling to a separate set of functions.

While the channel structural determinants of CDI versus CDF remain unknown, the dual regulation of Ca^{2+} channels by each lobe of CaM generalizes across channel subtypes. In most Ca_V1 channels, the C-lobe induces a kinetically distinct rapid phase of CDI, whereas the N-lobe yields a slower component [51, 61, 83]. In some studies, CDF has also been described in $Ca_V1.2$ channels, but the underlying mechanism remains mysterious. In Ca^{2+} channels of native cardiomyocytes, there is a weak manifestation of CDF which can be attenuated by blocking Ca^{2+} release from neighboring ryanodine receptors [102]. In addition, CDF of $Ca_V1.2$ channels in recombinant systems can only be viewed in the presence of channels bearing point mutations within the IQ domain, and only when expressed in frog oocytes [68]. Curiously, CDF is not observed in recombinant $Ca_V1.2$ channels expressed in mammalian cell lines [51]. By contrast, CDI of $Ca_V1.2$ channels is strong and universally observed across experimental platforms. Nonetheless, this latent CDF may provide shared mechanistic insight for Na channel regulation as described later.

Beyond simply splitting the Ca^{2+} signal, Ca^{2+}/CaM-dependent feedback exhibits remarkable spatial selectivity which can be revealed by probing channels in the presence of variable Ca^{2+} buffering. Some types of calmodulation are insensitive to strong intracellular Ca^{2+} buffering (e.g., $Ca_V1.2$ CDI), whereas others are not ($Ca_V2.3$ CDI). Strong Ca^{2+} buffering would spare the Ca^{2+} signals near the cytoplasmic face of channels where the large influx of Ca^{2+} would readily overwhelm the available buffers (Figure 2B, local Ca^{2+} signal) [103, 104]. This local signal is sufficient to evoke C-lobe CaM regulation. And the local Ca^{2+} selectivity allows for privileged signaling within a single CaM/channel complex without interference from neighboring Ca^{2+} sources. By contrast, the N-lobe of CaM generally imparts a regulatory effect only in the presence of low Ca^{2+} buffering, a condition that permits global elevation of cytosolic Ca^{2+} (Figure 2B, global Ca^{2+} signal). Thus, the N-lobe of CaM is able to ignore the intense local Ca^{2+} fluctuations and respond to the smaller global elevation in Ca^{2+} concentration [43, 70, 105, 106]. Such global selectivity of the N-lobe of CaM enables a resident Ca^{2+} sensor molecule to respond to remote Ca^{2+} signals, thus coordinating Ca^{2+} regulation across large portions of a cell [99, 100].

Further evidence of the local versus global selectivity of each lobe of CaM comes from single channel recordings of $Ca_V2.1$. In this mode, only a single channel is activated and therefore only a local Ca^{2+} signal exists. Probed thus, $Ca_V2.1$ channels exhibit CDF (driven by C-lobe), but not CDI (triggered by N-lobe) [105]. Similar results have also been shown for $Ca_V1.2$ channels [23]. These results confirm the proposed differential selectivity of each lobe of CaM.

By 2006, a general consensus appeared to converge for $Ca_V1/2$ channels. The N-lobe of CaM inevitably acted as a global Ca^{2+} sensor, while the C-lobe functioned as a local sensor.

This perspective was revised upon closer examination of $Ca_V1.2/1.3$ CDI [83]. These channels exhibited both N and C-lobe CDI even in the presence of strong intracellular Ca^{2+} buffering. This divergence in N-lobe selectivity is due to the NSCaTE channel segment conserved on the amino-terminus of $Ca_V1.2/1.3$ channels. NSCaTE is a Ca^{2+}/CaM binding site whose presence augments the overall affinity of the channel for Ca^{2+}/CaM over apoCaM [82, 83]. The existence of this element endows the N-lobe of CaM with local Ca^{2+} sensitivity in these channels. Elimination of the NSCaTE site then switches their N-lobe CDI to a global profile. Likewise, donation of the NSCaTE segment to Ca_V2 channels results in N-lobe CDI with local Ca^{2+} selectivity [83]. In this manner, the presence or absence of the NSCaTE module within the amino-terminus of a channel can tune the spatial Ca^{2+} selectivity of N-lobe mediated channel regulation.

The modularity of the NSCaTE element revealed that the local/global selectivity of each lobe of CaM is not immutable. The contrasting spatial Ca^{2+} selectivities can be explained as emergent properties of a system of channel transitions where a lobe of apoCaM transiently unbinds from a preassociation site before binding Ca^{2+} ions, and then reengages an effector interface upon Ca^{2+} binding to elicit channel regulation. The slow Ca^{2+} unbinding kinetics of the C-lobe of CaM invariably confers local Ca^{2+} selectivity. The rapid Ca^{2+} unbinding kinetics of the N-lobe of CaM, on the other hand, renders this lobe a global Ca^{2+} sensor. However, as the channel affinity for Ca^{2+}/CaM is enhanced, the system shifts to favor local Ca^{2+} sensitivity [107]. Thus, the N-lobe of CaM is a tunable Ca^{2+} sensor [107]. The full scheme of CaM regulatory function for Ca^{2+} channels is illustrated in Figure 2C. These remarkable Ca^{2+} decoding principles may be a general scheme that confers distinct spatial Ca^{2+} selectivities to a wide-range of biological molecules.

The rise of Na channel calmodulation

Developments in the understanding of Ca^{2+} regulation of Na channels closely paralleled the discoveries of Ca^{2+} channel calmodulation, though the functional outcome and underlying modulatory principles often appeared to diverge (Figure 3A). The first postulate that intracellular Ca^{2+} ions might regulate Na channels emerged with the early bioinformatic analysis that demonstrated the presence of a conserved vestigial EF hand segment in the carboxy-terminus of both Na and Ca^{2+} channels [39]. However, this possibility was nearly forgotten for almost a decade before pioneering biochemical studies explicitly demonstrated the robust binding of both apoCaM and Ca^{2+}/CaM to the Na channel IQ domain peptides [108]. This study also identified a second closely juxtaposed Ca^{2+}/CaM binding site, termed ‘Baa motif,’ immediately downstream of the Na channel IQ domain [108]. While these biochemical experiments demonstrated CaM interaction with Na channels, no functional outcome for this binding was yet ascribed [108].

A prominent feature of voltage-gated Na channels is its voltage-dependent inactivation [19, 109]. As such, early studies hypothesized that CaM and/or the channel vestigial EF hand segment might modulate particular attributes of Na channel voltage-dependent inactivation [110, 111]. Fitting with this idea, the Na channel carboxy-terminus was shown to alter fast inactivation properties through chimeric channel analysis of the neuronal $Na_V1.2$ and cardiac $Na_V1.5$ channels [112]. However, the effects of Ca^{2+} on Na channels often remained

controversial and isoform specific as reviewed in the past [65]. Typically, these effects were probed by characterizing Na current properties in two populations of cells dialyzed with either low or elevated Ca^{2+} (Figure 3B). For the cardiac $Na_V1.5$ channels, intracellular Ca^{2+} was shown to induce a subtle ~10 mV depolarizing or right shift in the voltage-dependence of steady-state inactivation or h_∞ curves (Figure 3C) – an effect that would contribute to an enhancement in Na channel availability during periods of high activity [42, 110, 113, 114]. However, this modulatory effect was not universally accepted with other studies reporting no significant effect of Ca^{2+} on $Na_V1.5$ steady-state inactivation curves [111, 115, 116]. For skeletal muscle $Na_V1.4$ channels, an initial report suggested a subtle Ca^{2+}-induced hyperpolarizing or left shift in voltage-dependence of steady-state inactivation curves [111], although later studies reported no significant shift of $Na_V1.4$ steady-state inactivation curves [115, 117]. The wild-type neuronal $Na_V1.2$ channel was reported to be insensitive to Ca^{2+}, however a channelopathic mutation within the IQ domain associated with autism (R1902C) was found to unveil a hyperpolarizing or left-shift in steady-state inactivation [118]. By contrast, the wild-type neuronal $Na_V1.1$ channels were argued to possess a subtle hyperpolarizing shift in voltage-dependence of steady-state inactivation in response to Ca^{2+} [119]. The neuronal $Na_V1.6$ channels showed no significant shift in steady-state inactivation curves [117]. Overall, the full spectrum of modulatory effects of Ca^{2+} on Na channels appeared to be complex and idiosyncratic, evading consensus for nearly a decade.

The molecular mechanisms underlying these variegated functional effects of Ca^{2+} on Na channels were often contested. Of note, the identity of the Ca^{2+} sensor for Na channel regulation was debated [65], reflecting similar controversies in the Ca^{2+} channel field [16]. One possibility was the direct binding of Ca^{2+} ions to the channel dual vestigial EF hand segment. Early studies proposed putative Ca^{2+} coordinating residues in this segment based on sequence alignment [42]. Alanine substitution of these proposed critical acidic residues (termed ‘4X’, corresponding to E1788, D1790, D1792, and E1799 in the human $Na_V1.5$ channel) abolished Ca^{2+}-sensitive shifts in voltage-dependence of steady-state inactivation observed for $Na_V1.5$ channels [42, 120]. In this model, the IQ domain was suggested to alter Ca^{2+} sensitivity of the channel dual vestigial EF hand segment allosterically [121]. Fitting with this idea, the Na channel IQ domain has been argued to interact with the dual vestigial EF motifs also on the carboxy-terminus of the channel [121, 122]. Nonetheless, later structural studies demonstrated that these proposed critical residues (‘4X’) were arranged spatially in an orientation incompatible with Ca^{2+} coordination [118, 123–126]. Moreover, even in the presence of high Ca^{2+} concentrations, the atomic structures of both $Na_V1.5$ and $Na_V1.2$ failed to exhibit Ca^{2+} binding to the channel dual vestigial EF hand [118], suggesting that the functional effects observed with the ‘4X’ mutant may have been the result of indirect mechanisms.

The role of CaM in Ca^{2+} regulation of Na channels has been controversial with some studies proposing a prominent Ca^{2+} sensing role [115, 118, 127, 128], while others arguing for subtle secondary functions [120, 121, 125]. The direct role of CaM was envisioned based on biochemical studies that suggested strong interaction between the channel IQ domain and both apoCaM and Ca^{2+}/CaM [108]. Biochemically, CaM was shown to support a configurational change of the $Na_V1.2$ carboxy terminus in the context of a channelopathic mutation [127]. Functionally, some role for CaM in Ca^{2+} regulation of $Na_V1.5$ channels was

proposed based on the finding that Ca^{2+}/CaM could interact with the intracellular linker between domain III and domain IV (III-IV loop) of the $Na_V1.5$ channel [114, 128, 129]. In terms of overall Na channel function, the III-IV loop has been argued to serve a privileged role in eliciting the canonical fast-inactivation [130–132]. As such, it is conceivable that Ca^{2+}/CaM interaction with the Na channel III-IV loop might modulate fast inactivation. In this model, the C-lobe of CaM was proposed to migrate from the channel IQ domain to the III-IV loop upon calcification [114, 128].

In other studies, CaM was thought to play an indirect role in controlling channel steady-state inactivation that is revealed only upon disabling the dual vestigial EF hand Ca^{2+} sensing machinery [120].

Finally, a few methodological nuances utilized in these experiments merit attention. First, nearly all studies employed either EGTA (ethylene glycol tetraacetic acid) or BAPTA (1,2-bis(0-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid) to buffer Ca^{2+} in the 0.5 – 10 μM range, a concentration well above their dissociation constant (K_d ~ 100 nM) [133]. As such, contaminant Ca^{2+} ions may lead to drastic fluctuations in free Ca^{2+} concentration, rendering Ca^{2+} essentially unbuffered [115, 133]. Second, many of these studies also utilized F^- anions in internal solutions to facilitate patch clamp experiments. However, F^- ions have been shown to alter G-protein signaling status in cells[134], among other reported effects [65] and thus may regulate Na current through Ca^{2+}-independent pathways [135]. Third, prior to measurement, time-dependent voltage shifts in steady-state inactivation curves are typically allowed to equilibrate following the onset of cell pipet dialysis [120]. Variability in the actual equilibration process could obscure the precise quantification of Ca^{2+} dependent effects on Na current. Another limitation of Ca^{2+} delivery via pipet dialysis concerns the difficulty of detecting fast Ca^{2+}-induced changes in current amplitude without an overt change in the voltage-dependence of activation or inactivation of the channel. Changes in current amplitudes may relate to either disrupted channel gating or trafficking and could arise from numerous cellular factors. These challenges likely contributed to the conflicting reports, and point to the need for a robust and quantitative assay to probe Ca^{2+} effects of Na channels.

Rapid Ca^{2+} delivery reveals rapid Na channel modulation

A key methodological advance is the usage of simultaneous patch-fluorimetry and rapid Ca^{2+}-photouncaging. This approach had been used recently to quantitatively assess Ca^{2+}-regulation of $Ca_V1.3$ channels [136]. A schematic of this experimental method is illustrated in Figure 4A (left subpanel). Here, caged-Ca^{2+} molecules prepared from mixtures of a photolabile derivative of EDTA, DM-Nitrophen or 1-(2-Nitro-4,5-dimethoxyphenyl)-1,2-diaminoethane-N,N,N′,N′-tetraacetic acid, and Ca^{2+} ions are dialyzed into a cell along with a known ratio of the Ca^{2+} sensitive dye Fluo-4FF (K_d ~ 9.8 μM) and the red dye Alexa-568. Na currents were evaluated in response to a train of voltage step-depolarizations while quantitative Ca^{2+} measurements were obtained simultaneously using fluorescence measurements. Step increases in Ca^{2+} concentrations could be triggered at will using a brief UV light-pulse (Figure 4A, right subpanel). In this manner, Ca^{2+}-dependent alterations in Na

current could be evaluated and quantified with millisecond precision – each cell as its own control [115].

Surprisingly, when the cardiac Na channel, $Na_V1.5$, was studied in this manner, no significant Ca^{2+}-dependent effect was observed [115]. Figure 4B depicts this experimental outcome. Briefly, $Na_V1.5$ currents were evoked using a train of step depolarizations to 0 mV. The peak Na currents prior to Ca^{2+} uncaging (Figure 4B, gray dots) remained steady during the voltage-pulse protocol indicating negligible run-down of Na current. Moreover, Ca^{2+} measurements prior to Ca^{2+} uncaging showed resting Ca^{2+} to be <100 nM. Upon Ca^{2+} uncaging (blue line), the intracellular Ca^{2+} concentration rapidly elevated to ~ 10 μM. Nonetheless, the $Na_V1.5$ currents remained unperturbed, both in terms of the peak current amplitude as well as the kinetics of voltage-dependent inactivation. Additional experiments in this study also found no significant shift in voltage-dependence of steady-state inactivation of $Na_V1.5$ current upon elevation of cytosolic Ca^{2+}. Taken together, these results argue against the regulation of $Na_V1.5$ current by intracellular Ca^{2+} ions, and therefore stand in contrast to prior proposals of Ca^{2+} regulation of $Na_V1.5$ channels identified in similar recombinant systems. Yet, it is possible that these reduced recombinant systems may lack essential molecular components readily available in the native system [137]. To address this concern, Ca^{2+} regulation of native $Na_V1.5$ channels from freshly isolated guinea pig ventricular myocytes were also probed using Ca^{2+}-photouncaging (Figure 4C). Much as with the recombinant $Na_V1.5$ currents, Ca^{2+} steps ~ 10 μM in amplitude also failed to evoke any detectible Ca^{2+} modulation [115]. Importantly, as these Ca^{2+} steps exceeded the amplitude of Ca^{2+} transients typically observed in myocytes, it seems likely that Na currents in the heart are not subject to Ca^{2+}/CaM regulation.

Hope for a conserved Na channel calmodulation reemerged as $Na_V1.4$, the skeletal muscle channel, was shown to elaborate robust Ca^{2+} regulation [115]. Figure 4D reproduces these experimental results. Here, recombinant $Na_V1.4$ currents were subject to Ca^{2+} uncaging experiments. Peak Na currents prior to Ca^{2+} uncaging (gray dots) remained steady during a train of step depolarizations. Prior to photouncaging, the cytosolic Ca^{2+} concentration remained stable near ~100 – 200 nM as typical for cells during rest periods. Upon Ca^{2+} uncaging, the cytosolic Ca^{2+} concentration rapidly elevated to approach ~ 2 μM and the peak Na current declined without any apparent changes in the time-course of fast inactivation. The envelope of peak Na currents (red curve) illustrates the rapid millisecond time course of this modulation, reminiscent of L-type voltage-gated Ca^{2+} channel CDI (Figure 1B). Population data collected from Ca^{2+}-steps of differing amplitude from many cells define a steady-state CDI (fractional decrement in peak Na current following Ca^{2+} uncaging) with robust maximal CDI reaching ~ 0.35 with half-maximal Ca^{2+} sensitivity of ~ 1.5 μM. Indeed, the steady-state CDI – Ca^{2+} relation followed a Hill curve with a steepness coefficient of ~1.8 suggesting that the binding of two Ca^{2+} ions was required to elicit this modulation. Importantly, a similar effect of Ca^{2+} on native Na currents in mouse skeletal myotubes [138] was also demonstrated (Figure 4E) [115]. Reassuringly, Ca^{2+} transients in mouse skeletal muscle are often > 10 μM, a range well within the Ca^{2+} requirement for regulation of Na currents [139].

Mechanistically, Ca^{2+} regulation of $Na_V1.4$ currents appeared to be largely independent of voltage [115]. This regulation resulted in a uniform scaling-down of steady-state inactivation as opposed to a simple shift its voltage-dependence, suggesting that CDI may not be reversed by hyperpolarization of the cell [115]. Single molecule experiments of $Na_V1.4$ channels further established Ca^{2+} regulation of Na channels as a genuine molecular-level effect of Ca^{2+} on channel P_O [115]. In terms of channel gating, there are two possible molecular interpretations for $Na_V1.4$ CDI. First, Ca^{2+} might fractionally reduce channel availability independent of voltage. Thus, CDI might correspond to a silent state. Alternatively, Ca^{2+} might alter the transitions between closed and open states of $Na_V1.4$ akin to an alternate low P_O gating mode associated with Ca^{2+} channel calmodulation [23, 105]. These distinct possibilities will have to be resolved through single molecule measurements of $Na_V1.4$ channel gating. In all, these results firmly established the Ca^{2+} regulation of $Na_V1.4$ channels – a modulation with uncanny similarities to Ca^{2+} regulation of Ca^{2+} channels.

Convergence of Ca^{2+} and Na channel calmodulation

Several shared modulatory principles between Ca^{2+} and Na channel calmodulation have emerged in recent years (Figure 5A) [76, 115, 126]. First, Ca^{2+} regulation of Na channels now presents as a rapid and dynamic process that tunes Na channels to the fluctuations of cytosolic Ca^{2+}. Indeed, like Ca^{2+} channel calmodulation, Na channel regulation is attained in a matter of ~100 milliseconds and can be readily reversed as cytosolic Ca^{2+} wanes [115]. As with Ca^{2+} channels, the kinetics of Na channel calmodulation steeply depends upon the amplitude of cytosolic Ca^{2+} eliciting the modulation [115, 136].

Second, commonalities in the mechanism of Ca^{2+} sensing by both Na and Ca^{2+} channels have also been identified. A combination of structural [118, 123, 124, 126] and functional studies [115] now argue strongly that the channel dual vestigial EF hands are unlikely to bind Ca^{2+} ions and support functional manifestations of calmodulation. Functionally, alanine substitution of key acidic residues in the Ca^{2+} binding loop of the Na channel dual vestigial EF hands failed to alter the Ca^{2+} sensitivity of Ca^{2+} regulation for $Na_V1.4$ channels probed using Ca^{2+}-uncaging [115]. Parallel results have been observed for Ca^{2+} channel regulation as discussed previously [45, 46]. Nonetheless, it remains possible that the dual vestigial EF hands may support other critical roles for Na channel regulation, much as has been observed for Ca^{2+} channels [41, 45]. By contrast, growing structural and functional evidence points to a fundamental role for CaM as the Ca^{2+} sensor for Na channels [115, 118, 124, 126]. A key experimental gap preventing the establishment of CaM as the Ca^{2+} sensor of Na channels has been the difficulty in inhibiting Ca^{2+} regulatory effect upon disabling CaM function [65]. To this end, a greatly enabling tool that established CaM as the unequivocal Ca^{2+} sensor for Ca^{2+} channels has been the shunting of Ca^{2+} regulation in the presence of dominant negative CaM (CaM_{1234}) [43, 49, 51, 52, 61, 62, 70]. Likewise, the coexpression of CaM_{1234} with $Na_V1.4$ channels resulted in a complete disruption of Ca^{2+} regulation, thus establishing CaM as the Ca^{2+} sensor for these channels [115]. Moreover, the functional bipartition scheme of CaM function established for Ca^{2+} channels likely extends to CaM regulation of Na channels. Ca^{2+} binding to the N-lobe of CaM is both necessary and sufficient to recapitulate the full Ca^{2+} regulatory function of $Na_V1.4$ channels [115]. Fitting

with this functional role, a recent atomic structure of $Na_V1.2$ carboxy-tail in complex with Ca^{2+}/CaM has shown that only the N-lobe of CaM binds to Ca^{2+} ions [118].

Moreover, Na channel III–IV loop has been argued to be a functionally-relevant conserved interface for Ca^{2+}/CaM [114, 128]. Similarly, Ca^{2+}/CaM has been shown to interact with the $Ca_V1.3$ channel III-IV loop [115]. However, the binding of Ca^{2+}/CaM to the III-IV loop of both channel families now appears to be functionally irrelevant [115]. Instead, CaM interaction with the channel carboxy-terminus emerges as the primary structural determinant for both Na and Ca^{2+} channels [47, 49, 53, 55, 115, 118, 124, 126, 140, 141]. Furthermore, a striking similarity between $Na_V1.4$ and $Ca_V1.2$ channel CaM regulation is the curious phenomenon whereby substitution of isoleucine-glutamine residues in the IQ domain to dual alanines ($Na_V1.4$ IQ/AA and $Ca_V1.2$ IQ/AA) unveils latent Ca^{2+}-dependent facilitation for both channel families [52, 115].

Another burgeoning principle of calmodulation of Ca^{2+} channels is the effect of apoCaM binding to potently regulate channel P_O [76]. Remarkably, this principle appears to hold true for Na channels as well [76]. Like the $Ca_V1.3$ channels, the wild-type $Na_V1.4$ channels bind apoCaM with high affinity [76, 81, 117]. Disruption of CaM binding to the $Na_V1.4$ channels through mutation of the IQ domain ($Na_V1.4$ IQ/AA) results in channels with a very low P_O under conditions of low CaM. Overexpression of CaM fully rescues the P_O of these mutant Na channels, raising the possibility that CaM could tune Na channel peak P_O in a Ca^{2+}-independent manner [76]. Fitting with this resonance in channel function, a recent atomic structure of the $Na_V1.5$ carboxy-tail region in complex with apoCaM alone showed that the C-lobe preassociated with the IQ domain, and the N-lobe bound to the dual vestigial EF hand segment in a ‘lock-washer’ configuration [126]. This configuration is very reminiscent of the consensus apoCaM binding to the Ca^{2+} channel CI region as identified in recent functional studies [41, 69]. The apparent likeness in Na and Ca^{2+} channel regulatory function may correspond to a deep structural homology. It is worth noting that an alternate structure of a ternary complex of $Na_V1.5$ carboxy-tail bound to both apoCaM and fibroblast growth factor homologous factor (FHF) showed a subtly different apoCaM binding configuration (‘lollipop arrangement’) [124]. In this model, the FHF molecule is bound to the dual vestigial EF hand segment while the N-lobe of apoCaM is dislodged from the channel interface. Resolving these structural differences and possible functional implications remains an open question. Importantly, the full spectrum of biological implications of apoCaM binding to Na channels is yet to be fully explored and represents an exciting new avenue of research.

From the perspective of conservation, it is intriguing that nearly all high-voltage gated Ca^{2+} channels possess highly similar CI regions and elaborate robust and recognizably similar forms of CaM regulation (Figure 5B). The low voltage-activated (T-type) Ca^{2+} channels lack the CI region [142, 143] and are also insensitive to direct regulation by Ca^{2+}/CaM (Figure 5B) [144]. All members of the mammalian voltage-gated Na channel family also possess the CI region [65]. So far, the $Na_V1.4$ channel has been shown to be modulated by Ca^{2+}/CaM in a manner highly similar to Ca^{2+} channel calmodulation. Although rapid Ca^{2+} regulation appears to be absent in wild-type $Na_V1.5$ channels, molecular manipulations of this channel could confer latent Ca^{2+} regulation [115]. It remains to be seen how the static shifts in the

voltage-dependence of steady-state inactivation previously reported for this channel relate to Ca^{2+} channel calmodulation. Nonetheless, the extensive structural and functional similarity between calmodulation of Ca^{2+} and Na channels suggests that the CI region may be an evolutionarily conserved module [115, 126]. Sequence alignment of the CI region of both Ca^{2+} and Na channels demonstrates striking conservation across major eukaryotic phyla [115]. Notably, the CI region can be evolutionarily traced to the single cell organism *Paramecium*, which only possesses a voltage-gated Ca^{2+} channel but not a voltage-gated Na channel [115]. It was this Ca^{2+} channel that was first shown to regulated by Ca^{2+} ions by Brehm and Eckert [20]. This modulation now appears to be functionally preserved in mammalian orthologs of both voltage-gated Ca^{2+} and Na channels, suggesting that the CI region may be a modular protein element. An explicit demonstration of such modularity is illustrated in Figure 5C. Transplantation of the CI region from $Na_V1.4$ to $Ca_V1.3$ channels results in a chimeric channel that supports robust CDI reminiscent of wild-type $Na_V1.4$ channels [115]. Dominant negative CaM_{1234} abolishes CDI observed in these chimeric channels, thus validating the successful transfer of calmodulation across the Ca^{2+} and Na channel superfamilies [115]. Given the common heritage of these channels dating to early eukaryotes (~ 1 billion years ago), these results argue for a persistent link between modern CI elements of Ca^{2+} and Na channels to a primordial Ca^{2+} regulatory design. It is humbling, then, to realize that CaM has likely remained a modulatory partner for these two important ion channel families for nearly all of living history.

Calmodulinopathies – a calmodulation deficiency?

In recent years, genome sequencing of patients with long-QT syndrome (LQTS) [145–147] [148] and/or catecholaminergic polymorphic ventricular tachycardia (CPVT) [149], revealed a growing number of mutations in the three redundant genes of CaM [150] (Figure 6A). Clinically, the symptoms arising from LQTS-associated CaM mutations (LQT14/15 [151]) are resistant to conventional treatments [145–147] [148]. Could some of these disease manifestations, termed calmodulinopathies, arise from deficiencies in calmodulation of Ca^{2+} or Na channels? A tantalizing clue lies in prior experiments that showed how overexpression of mutant CaM with diminished Ca^{2+}-binding capacity resulted in severe action potential prolongation in ventricular myocytes [152, 153] – a cellular correlate of LQTS. This effect was argued to result from a loss of calmodulation of $Ca_V1.2$ channels [152, 153]. Therefore, one might postulate that the naturally occurring LQTS-associated CaM mutations could perturb the same process.

In general, many of these disease-causing mutations result in CaM molecules with substantially diminished Ca^{2+}-binding affinity [148, 149, 154] without significant alterations to other CaM interfaces [155]. However, given the redundancy in CaM genes, it is puzzling how mutations in a single gene could account for the severity of LQT14/15. The resolution to this paradox lies in the apoCaM preassociation to certain target molecules [48]. We first consider the case of L-type Ca^{2+} channels [53–55]. If apoCaM binding to these channels remains intact, then the fraction of channels occupied by a mutant CaM is proportional to the total fraction of mutant CaM available in cells. This proportion of channels bound to a mutant CaM is unable to undergo CDI, thus diminishing the overall inactivation of L-type currents. In terms of the net effect on the shape of the cardiac action potential, this reduced

inactivation of L-type currents would result in a substantial prolongation of the action potential duration due to enhanced inward currents during the plateau and repolarization phases [27, 152, 156, 157].

In fact, this effect of mutant CaM on L-type Ca^{2+} channels was recently confirmed in both recombinant and native systems [155, 158]. Firstly, live-cell FRET binding assays demonstrated no decrement in the binding affinity of mutant apoCaM to the carboxy-terminus of the L-type Ca^{2+} channel. Secondly, the expression of LQTS mutant CaM in either HEK293 cells or ventricular myocytes resulted in a significant attenuation of CDI (Figure 6B) [155, 158]. Thirdly, in terms of cardiac cellular function, this loss of Ca^{2+} channel inactivation contributed to a stark and often variable prolongation of the action potential duration (APD) (Figure 6C) [147, 155]. Lastly, the increased Ca^{2+} influx through L-type channels also enhanced Ca^{2+} transient magnitudes, elevated diastolic Ca^{2+} concentrations, and increased Ca^{2+} load in the sarcoplasmic reticulum (Figure 6D) [155, 158, 159]. Importantly, these experiments also showed hints of other proarrhythmic events including electrical alternans and after-depolarizations, recapitulating the disease phenotypes reported in patients [146, 148, 149]. At the tissue level, the immense cell-to-cell functional heterogeneity introduced by mutant CaM could be a major contributor to arrhythmia in the clinical setting [160]. Together, these functional effects rationalize the severe cardiac phenotype observed in LQT14/15 patients and shed light upon novel therapeutic strategies.

Voltage-gated Na channels also preassociate with apoCaM [81, 108, 124, 126]. As such, altered Na channel regulation may also augment the spectrum of clinical phenotypes associated with calmodulinopathies [158]. The effects of LQTS-associated CaM mutations on the cardiac $Na_V1.5$ channel were also evaluated [158]. However, no significant alteration of the Na channel inactivation properties or the late Na current were found with the overexpression of LQTS-associated mutant CaM in either recombinant systems or ventricular myocytes[158]. These results fit with the absence of Ca^{2+}-dependent regulation of $Na_V1.5$ currents previously discussed [115]. Nevertheless, calmodulinopathies may alter the Ca^{2+}-effects on $Na_V1.4$ channels [115]. As these channels are thought to be critical for normal action potential propagation in Purkinje fibers [161], deficiencies in Ca^{2+} regulation may contribute to arrhythmia in patients.

More generally, as CaM is a pervasive ion channel regulator, the pathophysiology associated with calmodulinopathies likely stems from misregulation of numerous target molecules, yet to be fully characterized. Of note, recent data suggests that CaM-ryanodine receptor interactions are also impacted by mutant CaM associated with various calmodulinopathies. These deficiencies may manifest clinically as CPVT as described elsewhere [154, 162]. The full implications of calmodulinopathies in human disease are yet to be determined; deducing further mechanistic principles and developing possible therapeutic intervention now looms on the horizon.

A shared framework of Ca^{2+} and Na channelopathies

Given that the constituents of Ca^{2+} and Na channel calmodulation are similar in both genetic sequence and underlying biophysical properties, mechanisms of related disease may also

accord to a unifying theme. A myriad of Ca^{2+} and Na channelopathies has been identified [163–171]. Timothy Syndrome (TS) represents one such disorder in which patients suffer from cardiac arrhythmias, immune deficiencies, and autism [172, 173]. This syndrome stems from a single *de novo* point mutation (either G406R or G402S) in the domain I S6 region of $Ca_V1.2$ channels [173]. The functional effects of the TS mutations on $Ca_V1.2$ is both a loss of voltage-dependent inactivation (VDI) [22, 26, 172, 173] and a reduction in CDI [26, 27]. Likewise, a similar mutation at a corresponding location of the homologous $Ca_V1.3$ channel (G407R) has been reported to be associated with autism spectral disorders [28]. Functional studies of this mutation argue for a similar slowing of Ca^{2+} current inactivation in both diseases [28].

In addition, mutations in $Ca_V1.4$ that result in missense, frame-shift, and premature termination of protein translation have also been correlated with incomplete congenital stationary night blindness (CSNB), which is inherited as an X-linked trait [174, 175]. $Ca_V1.4$ regulates calcium passage into retinal photoreceptors [176–178] and serves as a major proponent in triggering glutamate release [179]. Alternative splicing of $Ca_V1.4$ results in the inclusion of an autoinhibitory domain (ICDI segment) in the distal carboxy-terminus [92]. The presence of this ICDI segment competitively displaces CaM from the channel complex thus disabling CDI [58, 75]. The heritable disorder CSNB2 has been reported to result from a truncation of this ICDI segment (K1591X), thus re-enabling CDI [58, 59]. In this manner, calmodulation of Ca^{2+} channels may directly contribute to retinal disorders. Likewise, aberrations in alternative splicing [90, 91] and RNA editing [95] of $Ca_V1.3$ channels in substantia nigral cells may be relevant to Ca^{2+} overload, suspected to be a key determinant in neurodegeneration associated with Parkinson's disease [180–183].

In the brain, $Ca_V2.1$ plays a critical role in determining the release of neurotransmitters at synapses in the central nervous system and may be important for synaptic plasticity [184–186]. Furthermore, extensive genome-sequencing links genetic mutations in $Ca_V2.1$ to both familial hemiplegic migraine type 1 (FHM-1) and episodic ataxia [187, 188]. These findings provide a possible connection between FHM-1 and a mechanistic deficit for CDF [29] and channel P_O [189], which may allow for possible targeted pharmacological intervention [30].

Aberrant calmodulation of Na channels may also contribute to a wide-range of neuronal, cardiac, and muscular disorders, in accordance with the prominent role of these channels in cellular excitability and the brisk spatial propagation of action potentials [190]. The neuronal $Na_V1.1$ channel has been heavily studied in relation to a family of epilepsy disorders, including febrile seizures [191] and Dravet syndrome [192, 193]. Interestingly, several associated channelopathic mutations have been identified on the carboxy-terminus of these channels [192, 193]. Channelopathic mutations associated with familial autism have also been identified in the related neuronal channel $Na_V1.2$ channel [191, 194]. More specifically, an autism-associated channelopathic mutation in the CaM binding IQ domain of $Na_V1.2$ has been argued to unveil latent calmodulation [118]. For cardiac $Na_V1.5$ channels, various channelopathic mutations have been linked to both congenital long QT syndrome (LQTS) and Brugada syndrome [195]. The $Na_V1.5$ associated LQTS3 is thought to result from an enhancement of late Na current [195–198], while Brugada syndrome is argued to stem from a dramatic decrement of peak Na current syndrome [195, 199, 200]. The role of

CaM in these processes is yet to be fully elucidated. Nonetheless, many Brugada syndrome and LQTS3 associated mutations occur within the carboxyl-terminus of $Na_V1.5$ channels [201, 202]. Lastly, for the skeletal muscle $Na_V1.4$ channels, channelopathic mutations are associated with heritable forms of myotonia and periodic paralysis [171]. Moreover, channelopathic mutations in the carboxy-tail of $Na_V1.4$ associated with K-aggravated myotonia [203] and cold-aggravated myotonia [204] result in a decrement of CaM regulatory function [115]. It is, thus, plausible that alterations in calmodulation may contribute to heritable forms of myotonia. The full spectrum of CaM regulatory functions and its physiological implications remain to be established across Na channel families.

Until recently, Ca^{2+} and Na channels appeared to be divergent with respect to their regulatory mechanisms. An emerging theme, however, is the remarkable similarity in the conservation of CaM regulation across these channel families. This interrelation now paves the way towards a unified mechanistic framework to understand vital CaM-dependent feedback and offers shared principles to approach related channelopathic diseases.

Acknowledgments

The calcium signals lab would like to thank our advisor Dr. David Yue for unwavering support, boundless kindness, and superb mentorship across the years. His recent passing has left a void in the field of ion channels and in our hearts. His enthusiasm, passion and wonder will be greatly missed.

This work was supported by grants from the NHLBI (R37HL076795), NIMH (R01MH065531), NINDS (R01NS073874) and NINDS (R01NS08074)to DTY; AHA(13PRE16500029) to WL, NSF(DGE-1232825) to JN and MSTP fellowships to JBI and SRL.

Trainees of the Calcium Signals Laboratory

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*We are the calcium signals lab, and we are proud to have been trained by David Yue.

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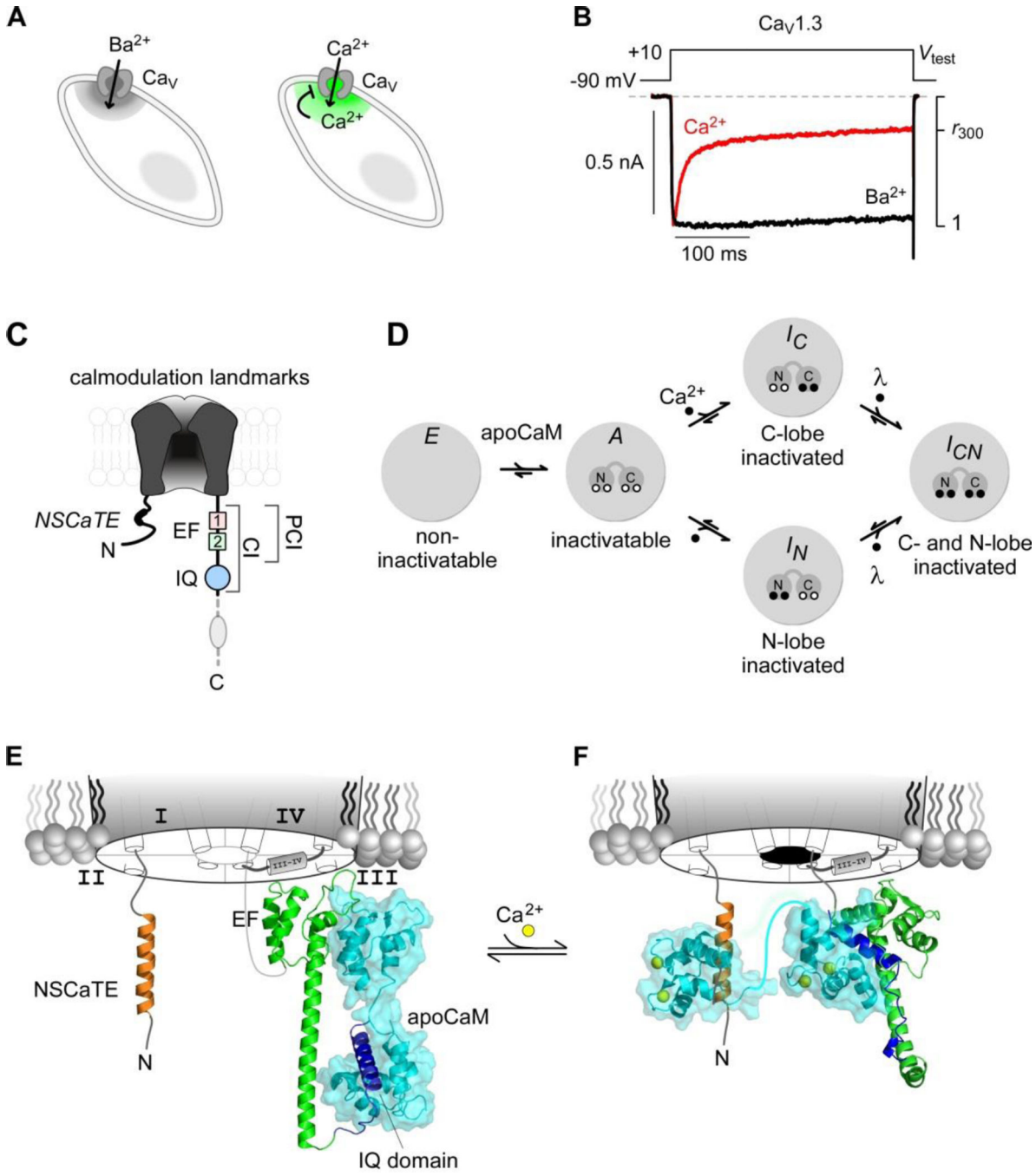
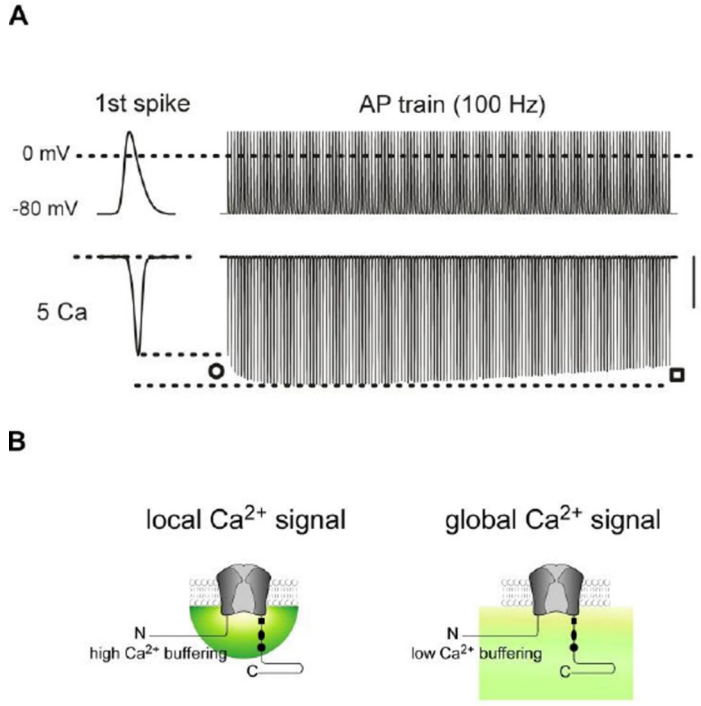


Figure (1).

A) Convenient experimental setup to examine CDI of Ca^{2+} channels. Left, no feedback regulation triggered upon Ba^{2+} influx through channel. Right, Ca^{2+} influx triggers Ca^{2+} regulation. **B)** Typical Ca^{2+} and Ba^{2+} current profiles through $Ca_V1.3$ channel. In response to a step depolarization, the Ca^{2+} current (red trace) activates and then sharply inactivates. Ba^{2+} current (black trace), does not inactivate. Adapted with permission [115]. **C)** Diagram depicting regions of Ca^{2+} channels critical for calmodulation, including dual vestigial EF hands (pink and green), IQ region (blue) on the C-terminus and NSCaTE (black) on the N-

terminus of the channel. **D)** Mechanist scheme for CaM regulation of Ca^{2+} channels. In the *E* state, the channel is devoid of CaM and incapable of undergoing CDI. Following apoCaM binding (*A* state), the channel can undergo N- or C-lobe-driven CDI, state I_N and I_C, respectively. Configuration I_{CN} corresponds to a fully inactivated state where N-and C-lobe of CaM engages respective effector interfaces. **E)** Molecular model of apoCaM preassociation to $Ca_V1.3$. The N-lobe of apoCaM preassociates with the dual vestigial EF hand segments, and the C-lobe with the channel IQ domain. **F)** Proposed model of Ca^{2+}/CaM interaction with channel interfaces. Upon Ca^{2+} binding, the N-lobe of Ca^{2+}/CaM interacts with the NSCaTE segment. The C-lobe of Ca^{2+}/CaM forms a tripartite complex with the channel dual vestigial EF hands and the IQ domain. Adapted with permission [41].



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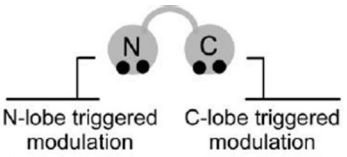

$Ca_V1.2$ L-type channel	CDI[1] (local*)	CDI[2-3] (local)
$Ca_V1.3$ L-type channel	CDI[4] (local)	CDI[4] (local)
$Ca_V1.4$ L-type channel	CDI[5,6] (global)	CDI[5,6] (local)
$Ca_V2.1$ P/Q-type channel	CDI[7,8] (global)	CDF[7,8] (local)
$Ca_V2.2$ N-type channel	CDI[9] (global)	(not found)[9]
$Ca_V2.3$ R-type channel	CDI[9] (global)	(not found)[9]

* intermediate local / global (Tadross *et al*, 2008)

[1] (Dick *et al*, 2008)
[2] (Peterson *et al*, 1999)
[3] (Zuhlke *et al*, 1999)
[4] (Yang *et al*, 2006)
[5] (Singh *et al*, 2006)
[6] (Wahl-Schott *et al*, 2006)
[7] (DeMaria *et al*, 2001)
[8] (Lee *et al*, 2003)
[9] (Liang *et al*, 2003)

Figure (2).

A) Functional bipartition of CaM in $Ca_V2.1$ channel. Top, $Ca_V2.1$ currents evoked by a train of action potentials. Rapid Ca^{2+} dependent facilation of Ca^{2+} current (circle, bottom subpanel) is followed by Ca^{2+}-dependent inactivation (square, bottom subpanel). Adapted with permission [70]. **B)** Distinct modes of spatial Ca^{2+} signaling. Under high Ca^{2+} buffering, Ca^{2+} elevations are restricted to the channel nanodomain (left). Low Ca^{2+} buffering permits global elevation of cytoplasmic Ca^{2+} (right). The resident CaM molecule can decode these distinct Ca^{2+} signals Adapted with permission [83]. **C)** Table summarizes dual regulation of Ca_V channels by the two lobes of CaM. Adapted with permission [16].

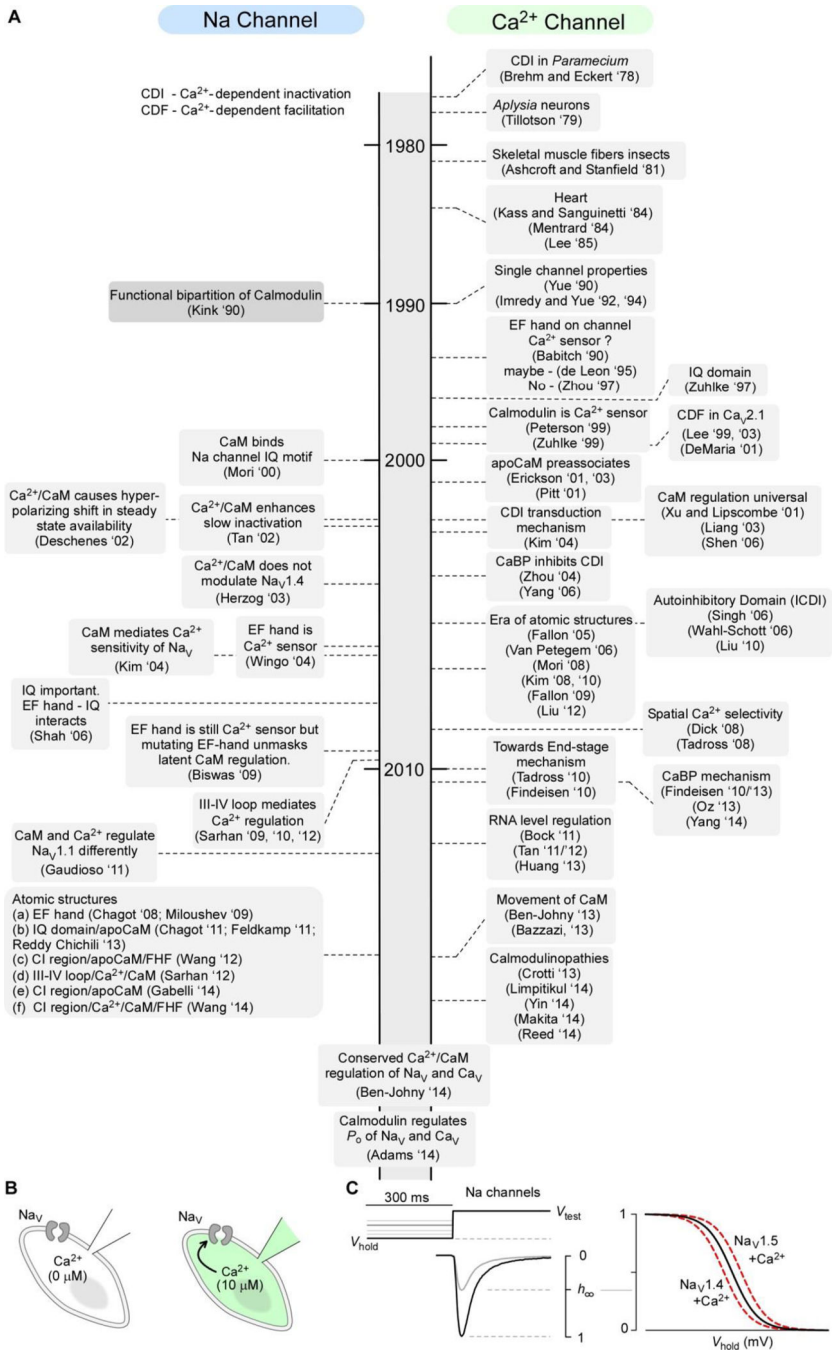

Figure (3).

A) Milestones in Na channel (left) and Ca^{2+} channel (right) calmodulation. **B)** Experimental scheme to study Ca^{2+} regulation of Na channels. Na current properties are evaluated from two population of cells dialyzed internal solutions containing either low and high Ca^{2+} concentrations **C)**. Protocol used to determine steady-state inactivation of Na channels (left). Expected Ca^{2+} dependent effects for two well-studied Na channel families. Adapted with permission [115].

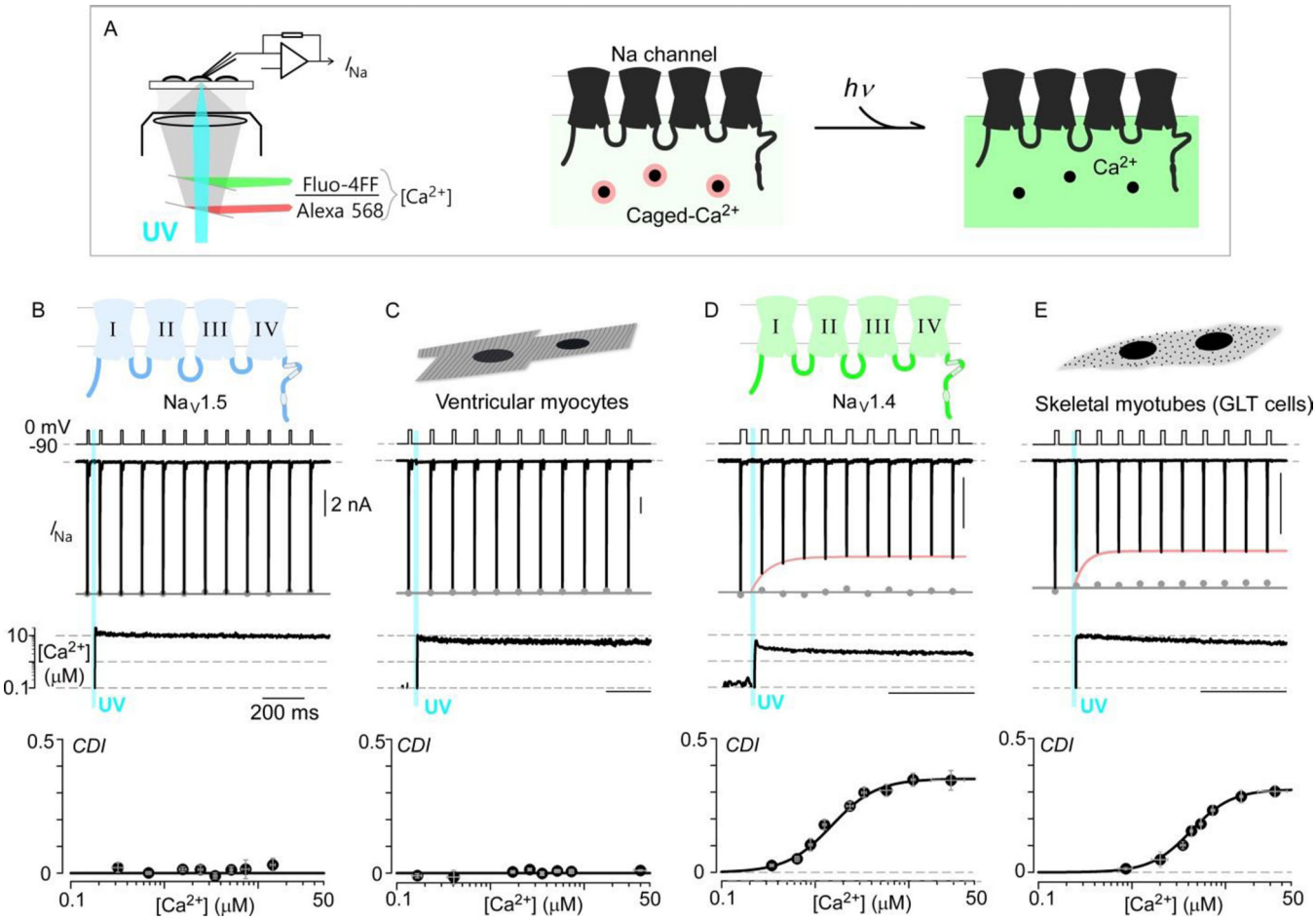

Figure (4).

A) Na channel regulation probed using rapid delivery of Ca^{2+} by photouncaging. Schematic shows simultaneous patch-fluorimetry and Ca^{2+} photouncaging. Ca^{2+} measured quantitatively using ratiometric fluorescence measurement. Ca^{2+} elevations are triggered using a brief UV pulse. **B)** Absence of Ca^{2+} regulation of cardiac Na channels ($Na_V1.5$) in a heterologous (HEK293) system. **C)** Ca^{2+} regulation of native $Na_V1.5$ current from ventricular myocytes. **D)** Robust Ca^{2+}-dependent inactivation in heterologously expressed NaV1.4 channels. Here, intracellular Ca^{2+} elevation decreases peak Na current (second row). Population data shows a dose-dependent response of CDI to intracellular Ca^{2+} (bottom). **E)** Robust Ca^{2+}-dependent inactivation of native NaV1.4 currents from skeletal myotubes (GLT cells). Adapted with permission [115].

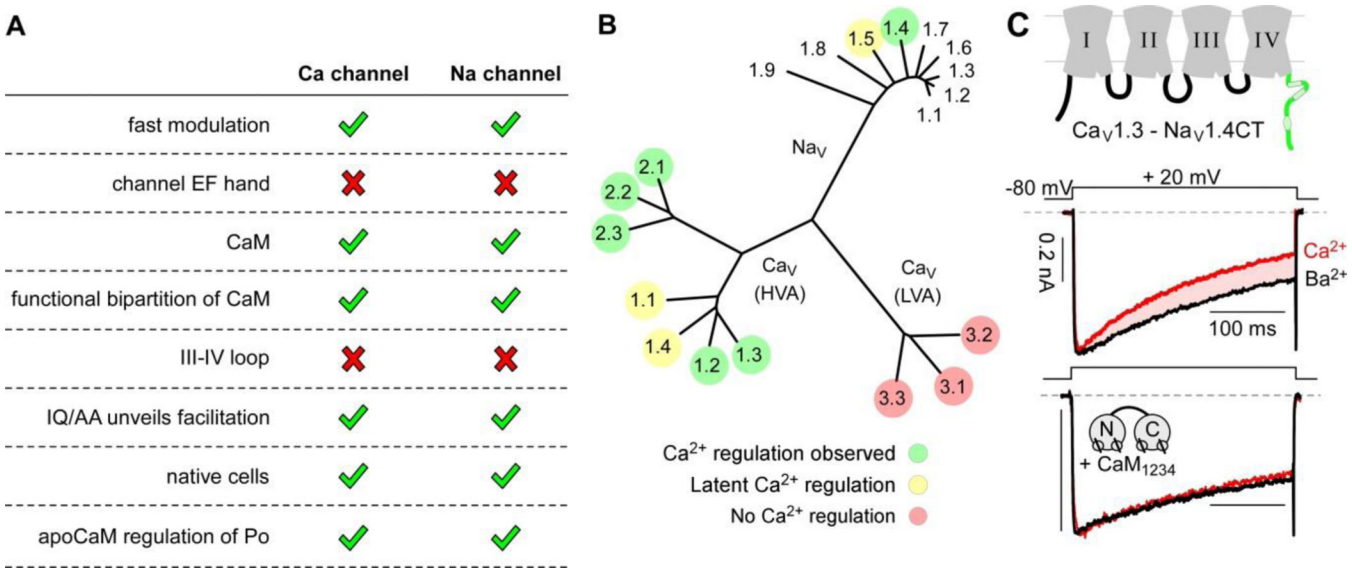

Figure (5).

A) Table shows conservation of Ca^{2+}/CaM regulation of Ca^{2+} and Na channels. **B)** Phylogenetic tree of the Ca^{2+} and Na channel superfamilies. HVA, high-voltage activated Ca^{2+} channels. LVA, low-voltage activated Ca^{2+} channels. **C)** Modularity of CI region. Transplanting CI region of $Na_V1.4$ to $Ca_V1.3$ supports CDI. Coexpression of mutant CaM_{1234} abolishes Ca^{2+} regulation of these chimeric channels. Adapted with permission [115].

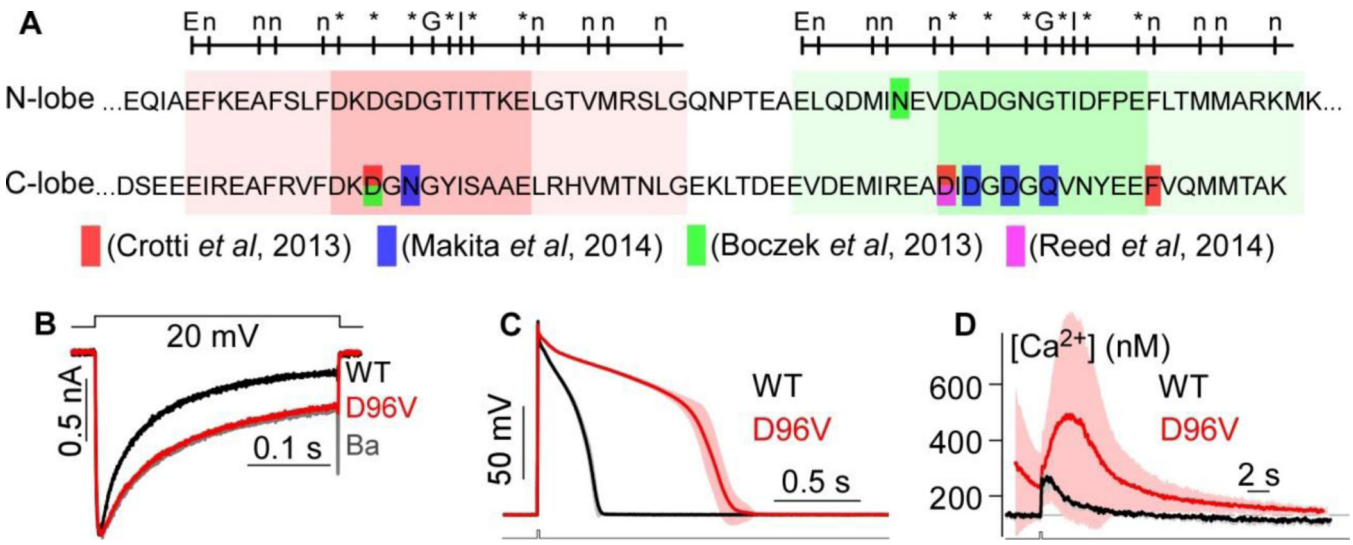

Fig. (6).

A) Sequence alignment of the two lobes of CaM. The EF hand segments are shaded rose and green. LQTS-associated CaM mutations are highlighted. **B)** Reduction of CDI of Ca^{2+} currents in adult guinea pig ventricular myocytes (aGPVMs) expressing CaM_{D96V} (red), compared to CaM_{WT} (black). Ba^{2+} current through the same channels (gray) shows the extent of VDI in these cells. **C)** Averaged action potentials from aGPVMs expressing CaM_{WT} (black) and CaM_{D96V} (red). Shaded regions represent standard deviation. CaM_{D96V} significantly prolongs the APD. **D)** Averaged Ca^{2+} transient waveforms from aGPVMs expressing CaM_{WT} (black) and CaM_{D96V} (red). Shaded regions represent standard deviation. CaM_{D96V} significantly increases the Ca^{2+} transient magnitude. Adapted with permission [155].