Circulation Research

ORIGINAL RESEARCH

RyR2 Serine-2030 PKA Site Governs Ca^{2+} Release Termination and Ca^{2+} Alternans

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BACKGROUND: PKA (protein kinase A)–mediated phosphorylation of cardiac RyR2 (ryanodine receptor 2) has been extensively studied for decades, but the physiological significance of PKA phosphorylation of RyR2 remains poorly understood. Recent determination of high-resolution 3-dimensional structure of RyR2 in complex with CaM (calmodulin) reveals that the major PKA phosphorylation site in RyR2, serine-2030 (S2030), is located within a structural pathway of CaM-dependent inactivation of RyR2. This novel structural insight points to a possible role of PKA phosphorylation of RyR2 in CaM-dependent inactivation of RyR2, which underlies the termination of Ca^{2+} release and induction of cardiac Ca^{2+} alternans.

METHODS: We performed single-cell endoplasmic reticulum Ca^{2+} imaging to assess the impact of S2030 mutations on Ca^{2+} release termination in human embryonic kidney 293 cells. Here we determined the role of the PKA site RyR2-S2030 in a physiological setting, we generated a novel mouse model harboring the S2030L mutation and carried out confocal Ca^{2+} imaging.

RESULTS: We found that mutations, S2030D, S2030G, S2030L, S2030V, and S2030W reduced the endoplasmic reticulum luminal Ca^{2+} level at which Ca^{2+} release terminates (the termination threshold), whereas S2030P and S2030R increased the termination threshold. S2030A and S2030T had no significant impact on release termination. Furthermore, CaM–wild-type increased, whereas Ca^{2+} binding deficient CaM mutant (CaM-M [a loss-of-function CaM mutation with all 4 EF-hand motifs mutated]), PKA, and Ca^{2+}/CaMKII (CaM-dependent protein kinase II) reduced the termination threshold. The S2030L mutation abolished the actions of CaM–wild-type, CaM-M, and PKA, but not CaMKII, in Ca^{2+} release termination. Moreover, we showed that isoproterenol and CaM-M suppressed pacing-induced Ca^{2+} alternans and accelerated Ca^{2+} transient recovery in intact working hearts, whereas CaM–wild-type exerted an opposite effect. The impact of isoproterenol was partially and fully reversed by the PKA inhibitor N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide and the CaMKII inhibitor N-[2-[N-(4-chlorocinnamyl)-N-methylaminomethyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide individually and together, respectively. S2030L abolished the impact of CaM–wild-type, CaM-M, and N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinoline-sulfonamide–sensitive component, but not the N-[2-[N-(4-chlorocinnamyl)-N-methylaminomethyl] phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide–sensitive component, of isoproterenol.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: calcium ■ calmodulin ■ endoplasmic reticulum ■ mutation ■ phosphorylation ■ ryanodine receptor calcium release channel

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Cardiac RyR2 (ryanodine receptor 2) is an intracellular Ca^{2+} channel that governs the release of Ca^{2+} from the sarcoplasmic reticulum (SR). It is well established that SR Ca^{2+} release is initiated upon the activation of RyR2 via a mechanism known as Ca^{2+}-induced Ca^{2+} release that underlies excitation-contraction coupling in the heart.[1–3] It is also well known that activation of RyR2 is regulated by several ligands and proteins, and aberrant activation of RyR2 is associated with cardiac arrhythmias and sudden death.[4–16] Another important aspect of excitation-contraction coupling and muscle function is the inactivation of RyR2, which underlies the termination of SR Ca^{2+} release

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Novelty and Significance

What Is Known?

- Cardiac RyR2 (ryanodine receptor 2) S2030 is a major PKA (protein kinase A)-dependent phosphorylation site upon β-adrenergic stimulation; however, the physiological significance of this phosphorylation is poorly understood.
- Calcium/CaM (calmodulin) dependent inactivation of RyR2 plays an important role in calcium release termination and cardiac calcium alternans.

What New Information Does This Article Contribute?

- RyR2-S2030 is an important determinant of PKA-induced suppression of CaM-dependent calcium release termination.
- RyR2-S2030 mediates PKA-induced suppression of calcium alternans and calcium transient refractoriness in intact hearts upon β-adrenergic stimulation.
- PKA-dependent phosphorylation of RyR2-S2030 may protect against rapid heart rate–induced cardiac alternans during β-adrenergic stimulation or fight-or-flight response.

The physiological role of PKA-dependent phosphorylation of cardiac RyR2 has remained poorly understood. Biochemical and functional studies established that RyR2-S2030 is a major PKA phosphorylation site responding to PKA activation upon β-adrenergic stimulation. However, the physiological significance of PKA-dependent phosphorylation of RyR2-S2030 is unclear. Interestingly, recent high-resolution three-dimensional structural analyses of RyR2 revealed that RyR2-S2030 is located near the Ca^{2+}/CaM–binding domain that plays an important role in RyR2-mediated Ca^{2+} release termination and cardiac Ca^{2+} alternans. Here, we demonstrate, for the first time, that RyR2-S2030 mediates PKA-induced suppression of CaM-dependent Ca^{2+} release termination and PKA-induced suppression of rapid pacing-triggered Ca^{2+} alternans and acceleration of Ca^{2+} transient recovery in intact working hearts upon β-adrenergic stimulation. These findings suggest a novel physiological role of PKA-dependent phosphorylation of RyR2-S2030 in the regulation of CaM-dependent Ca^{2+} release termination and rapid heart rate–induced cardiac alternans during β-adrenergic stimulation or fight-or-flight response. Our work also reveals RyR2-S2030 as a promising target for controlling cardiac alternans.

Nonstandard Abbreviations and Acronyms

CaM	calmodulin
CaMKII	Ca^{2+}/CaM-dependent protein kinase II
CaM-M	a loss-of-function CaM mutation with all 4 EF-hand motifs mutated
CaM-WT	CaM-wild-type
ER	endoplasmic reticulum
GFP	green fluorescence protein
PKA	protein kinase A
RyR2	cardiac ryanodine receptor
SR	sarcoplasmic reticulum
WT	wild-type

and muscle relaxation.[1,17–19] Despite its fundamental importance, the molecular mechanism and regulation of SR Ca^{2+} release termination remains poorly understood.

We have recently shown that CaM (calmodulin) plays a critical role in the termination of RyR2-mediated Ca^{2+} release.[20] By monitoring the endoplasmic reticulum (ER) luminal Ca^{2+} dynamics in HEK293 (human embryonic kidney) cells expressing RyR2, we found that CaM wild-type (CaM-WT) increased the ER Ca^{2+} level at which Ca^{2+} release is terminated (the termination threshold) (i.e. promoted Ca^{2+} release termination), whereas Ca^{2+} binding–deficient CaM mutant (CaM-M [a loss-of-function CaM mutation with all 4 EF-hand motifs mutated]) decreased the termination threshold, resulting in suppressed/delayed Ca^{2+} release termination.[20] Importantly, impaired termination of Ca^{2+} release has been implicated in cardiac arrhythmias and cardiomyopathies.[21–23] We have also reported recently that CaM-dependent termination of RyR2-mediatd Ca^{2+} release underlies the induction of Ca^{2+} alternans,[24] which is a beat-to-beat alternation in the amplitude of the cytosolic Ca^{2+} transients. We showed that CaM-WT promoted Ca^{2+} alternans and prolonged Ca^{2+} transient recovery in intact working hearts, whereas CaM-M suppressed Ca^{2+} alternans and accelerated Ca^{2+} transient recovery.[24] Of note, Ca^{2+} alternans is thought to play a primary role in the genesis of cardiac alternans,[25–32] a well-recognized risk factor for ventricular fibrillation and sudden cardiac death.[33–39] These observations indicate that CaM plays an important role in RyR2-mediated Ca^{2+} release termination and cardiac Ca^{2+} alternans.

Recent determination of high-resolution 3-dimensional (3D) structures of RyR2 in complex with CaM using cryo-electron microscopy shed novel insights into the molecular basis of CaM-dependent inactivation of RyR2 and the termination of Ca^{2+} release.[40] These high-resolution 3D structures reveal that Ca^{2+}-CaM binds to

RyR2 and induces conformational changes in the central domain that is connected to the gate of the channel, resulting in pore closure (ie, Ca^{2+}-CaM–induced inactivation of RyR2).[40] Another exciting finding from these structural analyses is that the serine-2030 (S2030) residue is located near the CaM-binding domain in the 3D structure of RyR2[40] (Figure 1). We have shown previously that RyR2-S2030 is phosphorylated by PKA, but not by CaMKII (CaM-dependent protein kinase II).[41,42] Furthermore, RyR2-S2030 is the major PKA phosphorylation site in RyR2 responding to PKA activation upon β-adrenergic stimulation.[41–43] Inspired by these novel structural insights, we hypothesize that PKA-dependent phosphorylation of RyR2 at S2030 may affect the conformational changes induced by Ca^{2+}-CaM binding to RyR2 and thus modulate CaM-dependent Ca^{2+} release termination and Ca^{2+} alternans.

To test this hypothesis, in the present study, we assessed the impact of systematically mutating the PKA phosphorylation site RyR2-S2030 to various residues with different charges, sizes, and shapes on CaM-dependent Ca^{2+} release termination and on the actions of PKA and CaMKII in this process. To assess the role of the PKA site RyR-S2030 in cardiac Ca^{2+} alternans, we generated a mouse model expressing the RyR2-S2030L$^{+/+}$ mutation that markedly reduces the threshold for Ca^{2+} release termination. We found that the RyR2-S2030L$^{+/+}$ mutation suppressed Ca^{2+} alternans and diminished the effect of CaM on Ca^{2+} alternans. The RyR2-S2030L$^{+/+}$ mutation also abolished the effect of β-adrenergic stimulation–induced activation of PKA, but not CaMKII, on Ca^{2+} alternans. Our results suggest that PKA-dependent phosphorylation of RyR2 at S2030 regulates Ca^{2+} release termination and Ca^{2+} alternans.

METHODS

Data Availability

The authors declare that all supporting data are available within the article and its Supplemental Material. An expanded Materials and Methods is available in the Supplemental Material. All research materials are listed in the Materials and Methods are also included in the Major Resources Table.

The PKA phosphorylation site RyR2-S2030 was systematically mutated to amino acids with different charges, sizes, and shapes of side chains using site-directed mutagenesis. The impact of RyR2-S2030 mutations on Ca^{2+} release termination was assessed by performing single-cell ER luminal Ca^{2+} imaging using the ER luminal Ca^{2+} sensing protein D1ER. To assess the role of the PKA site RyR2-S2030 in CaM and PKA actions, we generated a knock-in mouse model harboring the RyR2-S2030L$^{+/+}$ mutation using the CRISPR-mediated gene editing technology. Confocal Ca^{2+} imaging was performed using adult RyR2-WT and RyR2-S2030L$^{+/+}$ mutant homozygous mice of both sexes (8–12 weeks) at the levels of intact hearts and isolated ventricular myocytes. Ca^{2+} alternans in the RyR2-WT and RyR2-S2030L$^{+/+}$ mutant hearts were induced by rapid electrical stimulation at increasing frequencies (6–16 Hz, 7 V). The recovery of voltage-induced Ca^{2+} transients was determined by using the S1S2 stimulation protocol. Expanded methods are provided in the Supplemental Material.

RESULTS

Effect of PKA Phosphorylation Site RyR2-S2030 Mutations on the Termination of Ca^{2+} Release in HEK293 Cells

We have previously shown that CaM modulates the termination of RyR2-mediated Ca^{2+} release.[20,21] The close proximity of the PKA phosphorylation site RyR2-S2030 and the CaM-binding site in the 3D structure of RyR2[40] suggests that PKA phosphorylation of RyR2-S2030 may affect the action of CaM and CaM-dependent termination of Ca^{2+} release (Figure 1A and 1B). Structurally, phosphorylation of RyR2-S2030 would introduce changes to the charge, size, and shape of the S2030 side chain. Thus, we first wanted to determine whether altering the charge, size, and shape at the S2030 location affects the termination of RyR2-mediated Ca^{2+} release. To this end, we generated a number of S2030 mutations, including S2030A, S2030D, S2030G, S2030L, S2030P, S2030R, S2030T, S2030V, and S2030W with different charges, sizes, or shapes. We then produced stable, inducible HEK293 cell lines expressing each of these mutants and performed single-cell ER luminal Ca^{2+} imaging to determine their impact on Ca^{2+} release termination as described previously.[20] As shown in Figure 1C through 1I and Figure S1, the S2030D, S2030G, S2030L, S2030V, and S2030W mutations significantly reduced the termination threshold for store-overload induced Ca^{2+} release (SOICR) (ie, suppressed/delayed Ca^{2+} release termination). In contrast, the S2030P and S2030R mutations significantly increased the termination threshold (ie, promoted/accelerated Ca^{2+} release termination). The S2030A and S2030T mutations had no significant effect on the termination threshold (Figure 1C, 1D, and 1G; Figure S1D). Note that none of these S2030 mutations affected the activation threshold for SOICR (Figure 1H). All of these S2030 mutations had no significant impact on store capacity (Figure 1I). These data indicate that the PKA phosphorylation site RyR2-S2030 is an important determinant of Ca^{2+} release termination.

Effect of PKA Site RyR2-S2030 Mutations on the Action of CaM-WT and CaM-M in Ca^{2+} Release Termination in HEK293 Cells

We next determined whether the PKA site RyR2-S2030 mutations affect the action of CaM in Ca^{2+} release termination in HEK293 cells. We focused on the phosphorylation deficient mutation S2030A, the phosphomimetic mutation S2030D, and the S2030L

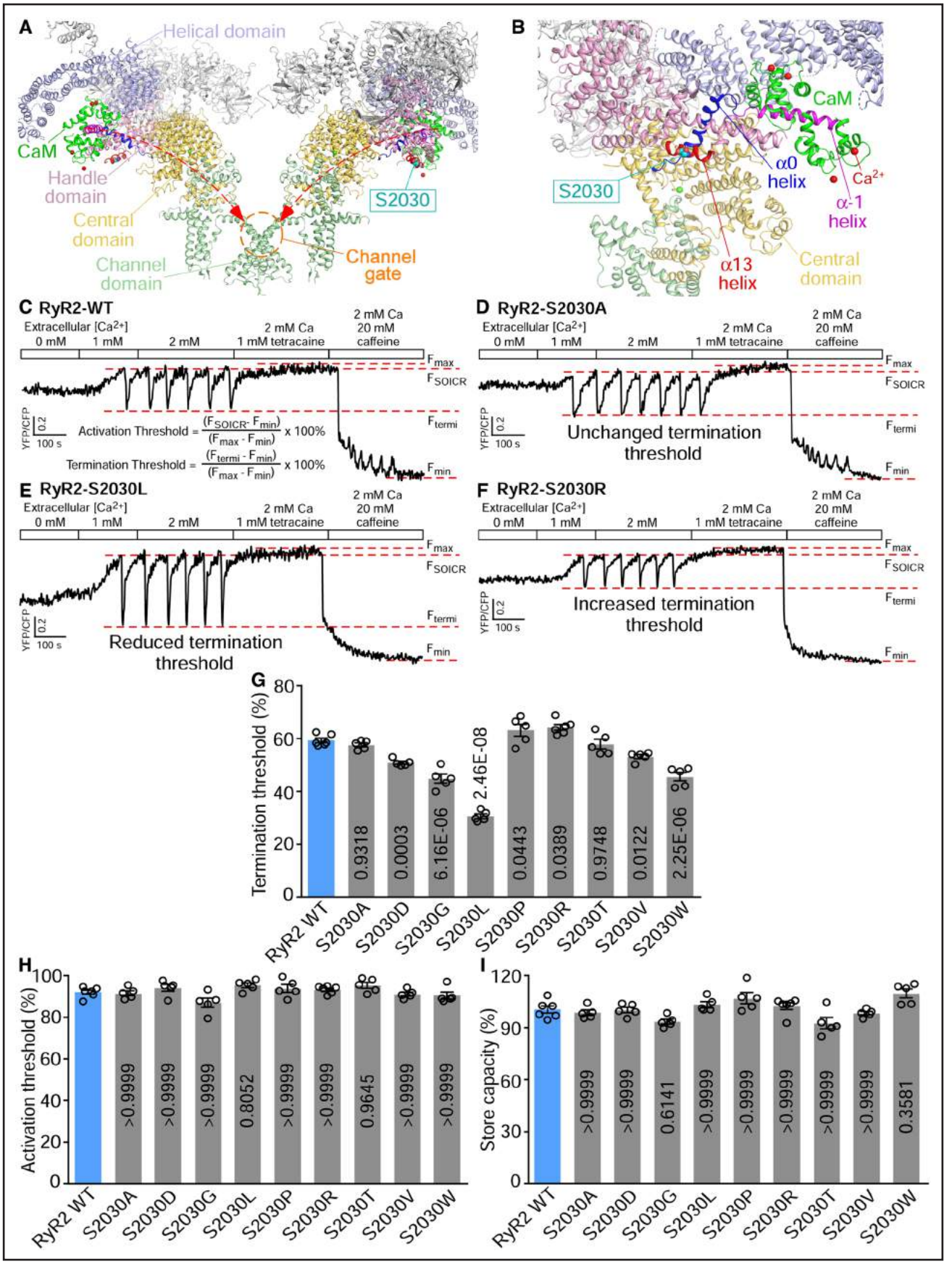

Figure 1. Impact of RyR2 (ryanodine receptor 2)–serine-2030 (S2030) mutations on Ca^{2+} release termination in HEK293 (human embryonic kidney) cells.(*Continued*)

mutation that exhibits the most reduced SOICR termination threshold. Consistent with our previous studies[20,21], CaM-WT increased the SOICR termination threshold, whereas the Ca^{2+} binding deficient CaM mutant (CaM-M), in which all of 4 EF-hand Ca^{2+} binding sites are disabled, reduced the termination threshold in RyR2-WT expressing HEK293 cells (Figure 2A, 2B, and 2E). The impact of CaM-WT and CaM-M on the termination threshold in RyR2-S2030A mutant-expressing cells was similar to that in RyR2-WT cells (Figure 2E, Figure S2A and S2B). CaM-WT and CaM-M also increased and decreased the termination threshold in the RyR2-S2030D mutant-expressing cells, respectively but to a smaller extent compared with that in RyR2-WT cells (Figure 2E, Figure S2C and S2D). However, CaM-WT and CaM-M had no effect on the termination threshold in RyR2-S2030L mutant cells (Figure 2C through 2E). Note that CaM-WT and CaM-M exerted no effect on the SOICR activation threshold or the store capacity in RyR2-WT and mutant-expressing cells (Figure 2F and 2G). Taken together, these data indicate that mutations of the PKA site RyR2-S2030 affect CaM regulation of Ca^{2+} release termination to different extents and that the S2030L mutation abolishes the action of CaM in Ca^{2+} release termination in HEK293 cells.

Effect of PKA Site RyR2-S2030 Mutations on the Action of PKA and CaMKII in Ca^{2+} Release Termination in HEK293 Cells

RyR2 is phosphorylated by PKA, but its role in Ca^{2+} release termination is unknown. To determine whether PKA modulates Ca^{2+} release termination and whether this action of PKA is mediated by the RyR2-S2030 site, we expressed PKA in HEK293 cells expressing RyR2-WT, RyR2-S2030A, RyR2-S2030D, and RyR2-S2030L and performed ER luminal Ca^{2+} imaging to monitor Ca^{2+} release termination. As shown in Figure 3, Figure S3 and Figure S4, PKA reduced the SOICR termination threshold in RyR2-WT, but not in S2030A, S2030D, or S2030L mutant-expressing HEK293 cells in the absence or presence of exogenously expressed CaM (Figure 3A, 3C, and 3E), compared with those in RyR2 WT cells. This indicates that S2030 is critical for PKA-dependent suppression of Ca^{2+} release termination. Notably, the phosphomimetic mutation, RyR2-S2030D, reduced the termination threshold to an extent similar to that induced by PKA in RyR2-WT expressing cells. This suggests that the phosphomimetic mutation S2030D indeed mimics the action of PKA in suppressing Ca^{2+} release termination. Note that PKA exerted little or no effect on the SOICR activation threshold or store capacity (Figure 3F, Figure S3, Figure S4).

RyR2 is also phosphorylated by Ca^{2+}/CaMKII. The role of CaMKII-dependent phosphorylation of RyR2 in Ca^{2+} release termination is also unknown. Hence, it is important to determine whether CaMKII affects Ca^{2+} release termination. Furthermore, we have previously shown that RyR2-S2030 is phosphorylated by PKA, but not by CaMKII.[41] To test the specificity of RyR2-S2030's role in PKA-dependent modulation of Ca^{2+} release termination, we expressed CaMKII in HEK293 cells expressing RyR2-WT, RyR2-S2030A, RyR2-S2030D, and RyR2-S2030L and performed ER luminal Ca^{2+} imaging. Interestingly, CaMKII reduced both the SOICR termination and activation thresholds in both RyR2-WT and mutant HEK293 cells in the absence or presence of exogenously expressed CaM (Figure 3B, 3D, 3E, and 3F), compared with those in RyR2 WT cells. However, CaMKII had no effect on the store capacity in RyR2-WT and mutant HEK293 cells (Figure S4). This indicates that S2030 is not essential for CaMKII-dependent regulation of Ca^{2+} release termination, consistent with our previous finding that S2030 is not a CaMKII phosphorylation site.[41] However, the specific CaMKII phosphorylation sites in RyR2 that mediate the action of CaMKII in Ca^{2+} release termination are unknown and have yet to be characterized. Collectively, these data demonstrate that the PKA phosphorylation site RyR2-S2030 is critical for PKA-dependent (but not CaMKII-dependent) modulation of Ca^{2+} release termination.

Figure 1 Continued. Potential role of RyR2-S2030 in Ca^{2+}-CaM (calmodulin) induced inactivation of RyR2. **A**, The 3-dimensional (3D) structure of RyR2 with the helical domain, handle domain, central domain, channel domain, channel gate, CaM, and the serine-2030 (S2030) residue highlighted. **B**, Enlarged view of the CaM-binding domain and its connecting/interacting helixes. High-resolution 3D structural analyses suggest that the binding of Ca^{2+}-CaM to the CaM-binding domain (α-1 helix) induces conformational changes in the central domain that controls the channel gate in the channel domain via the α0 helix, leading to pore closure (**A**). The major PKA phosphorylation site RyR2-S2030 is located in the α13 helix in the handle domain that interacts with the α0 helix (**B**). Stable, inducible HEK293 (human embryonic kidney) cell lines expressing RyR2-wild-type (WT) and S2030A, S2030D, S2030G, S2030L, S2030P, S2030R, S2030T, S2030V, and S2030W mutants were transfected with the fluorescence resonance energy transfer (FRET)-based endoplasmic reticulum (ER) luminal Ca^{2+} sensing protein D1ER, 48 hours before single-cell FRET imaging. The expression of RyR2 WT and mutants was induced 24 h before imaging. The cells were perfused with KRH buffer containing increasing levels of extracellular Ca^{2+} (0-2 mM) to induce store-overload-induced Ca^{2+} release (SOICR), followed by the addition of 1.0 mM tetracaine to inhibit SOICR, and then 20 mM caffeine to deplete the ER Ca^{2+} stores. **C** through **F**, Single-cell luminal Ca^{2+} dynamics (FRET recordings) from representative RyR2 WT and S2030A, S2030L, and S2030R mutants are shown, respectively. F_{SOICR} indicates the FRET level at which SOICR occurs, while F_{termi} represents the FRET level at which SOICR terminates. The maximum FRET signal F_{max} is defined as the FRET level after tetracaine treatment. The minimum FRET signal F_{min} is defined as the FRET level after caffeine treatment. Cyan and yellow fluorescent proteins (CFP and YFP, respectively). The termination threshold (**G**) and activation threshold (**H**) were determined as described in panel **C**. The store capacity (**I**) was calculated by subtracting F_{min} from F_{max} and was normalized to that of RyR2 WT. Data shown (**G**–**I**) are mean±SEM from RyR2 WT (n=6), S2030A (n=5), S2030D (n=5), S2030G (n=5), S2030L (n=5), S2030P (n=5), S2030R (n=6), S2030T (n=5), S2030V (n=5), S2030W (n=5, the number of independent experiments) with Kruskal-Wallis test followed by Dunn post hoc test for obtaining the adjusted *P* values shown in (**G**, **H**, **I**), all conditions vs RyR2 WT.

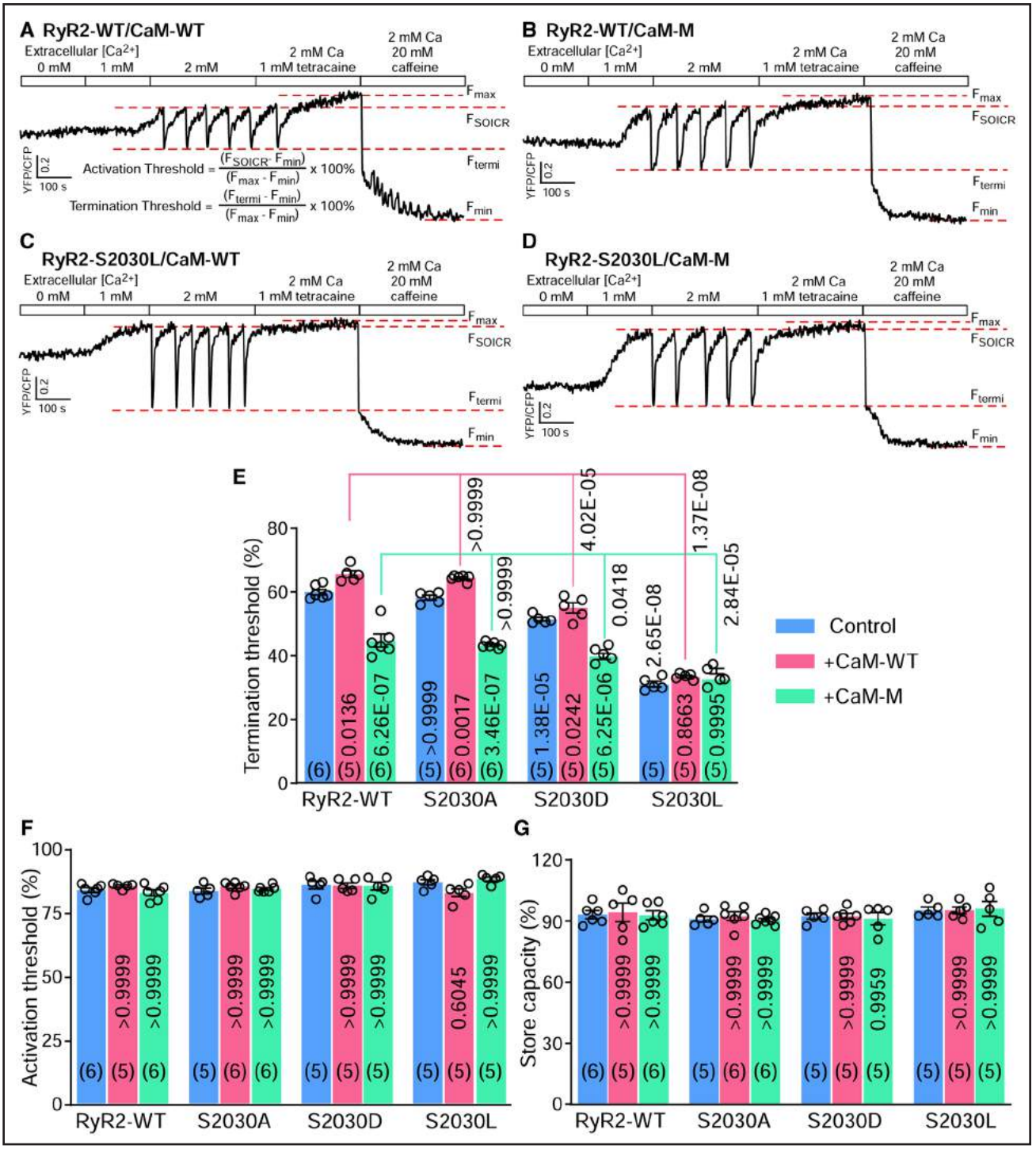

Figure 2. Impact of RyR2 (ryanodine receptor 2)–serine-2030 (S2030) mutations on the action of CaM (calmodulin) in Ca^{2+} release termination in HEK293 (human embryonic kidney) cells.

HEK293 cell lines expressing RyR2–wild-type (WT) and mutants were cotransfected with the fluorescence resonance energy transfer (FRET)-based endoplasmic reticulum (ER) luminal Ca^{2+} sensing protein D1ER and with (1) no CaM (control), (2) CaM-WT or (3) Ca^{2+} binding deficient CaM mutant, CaM-M (a loss-of-function CaM mutation with all 4 EF-hand motifs mutated). Representative single-cell luminal Ca^{2+} recordings of RyR2-WT cells transfected with CaM-WT (**A**), RyR2-WT cells transfected with CaM-M (**B**), RyR2-S2030L cells transfected with CaM-WT (**C**), and RyR2-S2030L cells transfected with CaM-M (**D**) are shown. Cyan and yellow fluorescent proteins (CFP and YFP, respectively). The termination threshold (**E**) and the activation threshold (**F**) were determined as shown in **A**. **G**, The store capacity was calculated by subtracting F_{min} from F_{max}. Data shown in (**E**–**G**) are mean±SEM with the number of independent experiments shown in brackets in each bar for each condition and analyzed by 2-way ANOVA with Bonferroni post hoc test for obtaining the adjusted *P* values shown (**E**, **F**, **G**). SOICR indicates store-overload-induced Ca^{2+} release.

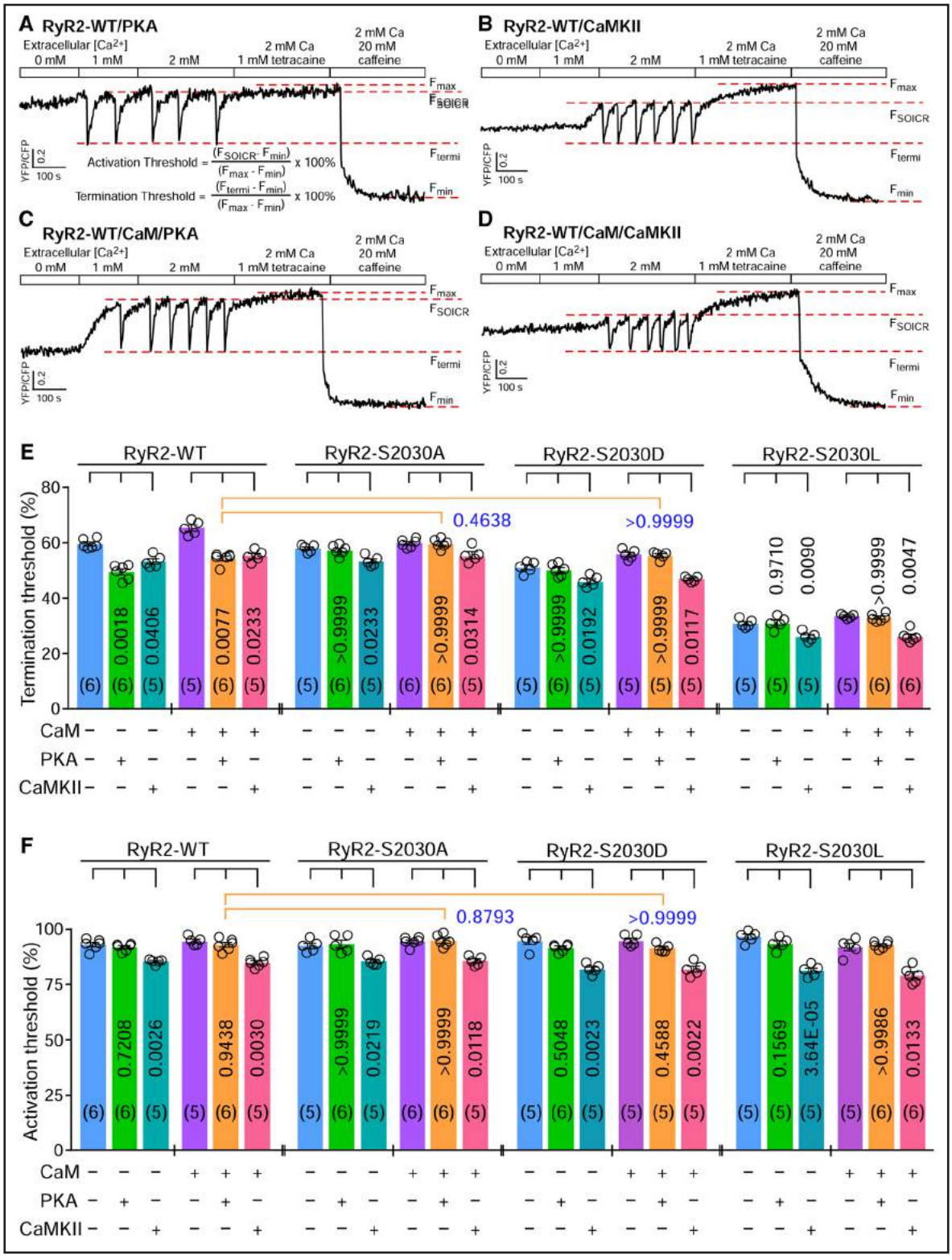

Figure 3. Impact of PKA (protein kinase A) and CaMKII (CaM-dependent protein kinase II) on Ca^{2+} release termination in RyR2 (ryanodine receptor 2)- wild-type (WT) and RyR2–serine-2030 (S2030) mutant HEK293 (human embryonic kidney) cells. HEK293 cell lines expressing RyR2-WT and mutants were cotransfected with the fluorescence resonance energy transfer (FRET)-based endoplasmic reticulum (ER) luminal Ca^{2+} sensing protein D1ER and with or without (1) CaM, (2) PKA, or (3) CaMKII. Representative single-cell luminal Ca^{2+} recordings of RyR2-WT cells transfected with PKA (**A**), RyR2-WT cells transfected with CaMKII (**B**), RyR2-WT cells transfected with CaM and PKA (**C**), and RyR2-WT cells transfected with CaM and CaMKII (**D**) are shown. Cyan and yellow fluorescent proteins (CFP and YFP, respectively). The termination threshold (**E**) and the activation threshold (**F**) were determined as shown in panel **A**. Data shown in (**E**, **F**) are mean±SEM with the number of independent experiments shown in brackets in each bar for each condition and analyzed by 2-way ANOVA with Bonferroni post hoc test for obtaining the adjusted *P* values. SOICR indicates store-overload-induced Ca^{2+} release.

PKA Site Mutation RyR2-S2030L$^{+/+}$ Suppresses Ca^{2+} Alternans and Shortens Ca^{2+} Transient Refractoriness in Intact Hearts

We have previously shown that Ca^{2+} release termination is a major determinant of cardiac Ca^{2+} alternans.[24] Given that the PKA site RyR2-S2030 has an important role in Ca^{2+} release termination, S2030 may also play a crucial role in Ca^{2+} alternans. To test this, we generated a mouse model expressing the RyR2-S2030L$^{+/+}$ mutation (Figure S5) that markedly suppressed Ca^{2+} release termination and abolished the effect of PKA on Ca^{2+} release termination (Figs. 2, 3). Echocardiographic analysis revealed no significant differences in any of the echo parameters measured between RyR2-WT and RyR2-S2030L$^{+/+}$ mutant mice (Table S1). Hematoxylin/eosin and picrosirius red staining also revealed no major differences in cardiac morphology and fibrosis between RyR2-WT and RyR2-S2030L$^{+/+}$ hearts. There is also no significant difference in the heart weight/body weight between the RyR2-WT and RyR2-S2030L$^{+/+}$ mutant mice (Figure S6). To determine the impact of the RyR2-S2030L$^{+/+}$ mutation on Ca^{2+} alternans, we performed confocal Ca^{2+} imaging to monitor pacing-induced Ca^{2+} transients in epicardial ventricular myocytes of Langendorff-perfused intact RyR2-WT or RyR2-S2030L$^{+/+}$ hearts, as described previously.[24] As shown in Figure 4, substantial Ca^{2+} alternans was detected at a stimulation frequency of ≈11 Hz in RyR2-WT hearts (Figure 4A and 4B). Furthermore, the alternans ratio in RyR2-WT hearts increased with increasing stimulation frequency (from 12 to 16 Hz). Notably, the alternans ratio in the RyR2-S2030L$^{+/+}$ hearts was markedly reduced at each of the stimulation frequencies (12–16 Hz) compared with that in WT hearts (Figure 4C).

Alternations in the refractoriness of depolarization-induced Ca^{2+} transients are thought to contribute to the induction of Ca^{2+} transient alternans.[44–48] Thus, it is also important to determine whether RyR2-S2030L$^{+/+}$ affects Ca^{2+} transient refractoriness. To this end, we assessed the recovery of Ca^{2+} transients in ventricular myocytes in isolated Langendorff-perfused intact hearts using the S1S2 stimulation protocol.[47] We found that the recovery (50%) of the Ca^{2+} transient amplitude in ventricular myocytes in the RyR2-S2030L$^{+/+}$ mutant hearts was significantly accelerated compared to that of the WT hearts (Figure 4D through 4G). Collectively, these observations indicate that the PKA site RyR2-S2030L$^{+/+}$ mutation suppresses Ca^{2+} alternans and shortens the recovery time of Ca^{2+} transients in intact hearts.

RyR2-S2030L$^{+/+}$ Mutation Abolishes the Effects of CaM on Ca^{2+} Alternans and Ca^{2+} Transient Refractoriness in Intact Hearts

To assess whether the PKA site RyR2-S2030L$^{+/+}$ mutation affects the action of CaM in the setting of intact hearts, we infected mouse hearts in vivo by directly injecting adenoviruses harboring the CaM-WT/GFP (green fluorescence protein) or CaM-M/GFP (Figure S7) into the anterior wall of the left ventricle as we have done previously.[24,49] Here, GFP was used as a marker to directly visualize the confined expression of locally injected CaM-WT or CaM-M adenoviruses (Figure S7). We performed confocal Ca^{2+} imaging to monitor Ca^{2+} transients in ventricular myocytes near the adenovirus injection site in Langendorff-perfused intact hearts also as we have done previously.[24] As shown in Figure 5, Ca^{2+} alternans in intact RyR2-WT hearts without adenovirus injection (control) occurred at a stimulation frequency of ≈11 Hz (Figure 5A). This threshold frequency at which alternans occur was decreased to ≈9 Hz in ventricular myocytes near the CaM-WT/GFP adenovirus injection site in the anterior wall of the left ventricle of RyR2-WT hearts (Figure 5A). Furthermore, the recovery (50%) of the Ca^{2+} transient amplitude was significantly prolonged in ventricular myocytes near the CaM-WT/GFP adenovirus injection site in the anterior wall of the left ventricle of RyR2-WT hearts (Figure 5B and 5C). These effects of CaM-WT/GFP on RyR2-WT hearts are consistent with those reported previously.[24] In contrast, the Ca^{2+} alternans ratio at all stimulation frequencies (from 6 to 15 Hz) and the recovery (50%) of the Ca^{2+} transient amplitude in ventricular myocytes near the CaM-WT/GFP adenovirus injection site in the anterior wall of the left ventricle of the RyR2-S2030L$^{+/+}$ mutant intact hearts were similar to those of the control (without adenovirus injection) (Figure 5A through 5C).

We also determined the impact of CaM-M/GFP on Ca^{2+} alternans and Ca^{2+} transient refractoriness in RyR2-WT and S2030L$^{+/+}$ mutant hearts. Consistent with those described previously,[24] the threshold frequency for Ca^{2+} alternans in ventricular myocytes near the CaM-M injection site was markedly increased from ≈11 Hz to ≈13 Hz (Figure 5D). Furthermore, the recovery (50%) of the Ca^{2+} transient amplitude was significantly accelerated in ventricular myocytes near the CaM-M adenovirus injection site in the anterior wall of the left ventricle of RyR2-WT hearts (Figure 5E and 5F). In contrast, the Ca^{2+} alternans ratio at all stimulation frequencies (from 6 to 15Hz) and the recovery (50%) of the Ca^{2+} transient amplitude in ventricular myocytes near the CaM-M adenovirus injection site in the anterior wall of the left ventricle of the RyR2-S2030L$^{+/+}$ mutant intact hearts were similar to those of the control (without adenovirus injection) (Figure 5D through 5F). We also assessed the impact of RyR2-S2030L$^{+/+}$, CaM-WT, and CaM-M on the properties of Ca^{2+} transients in intact hearts. We found that RyR2-S2030L$^{+/+}$, CaM-WT, or CaM-M had little or no effect on the amplitude, time to peak, or decay time (at 50% or 90%) of Ca^{2+} transients in intact RyR2-WT or RyR-S2030L$^{+/+}$ mutant hearts (Figure S8). Taken together, these data indicate that the RyR2-S2030L$^{+/+}$

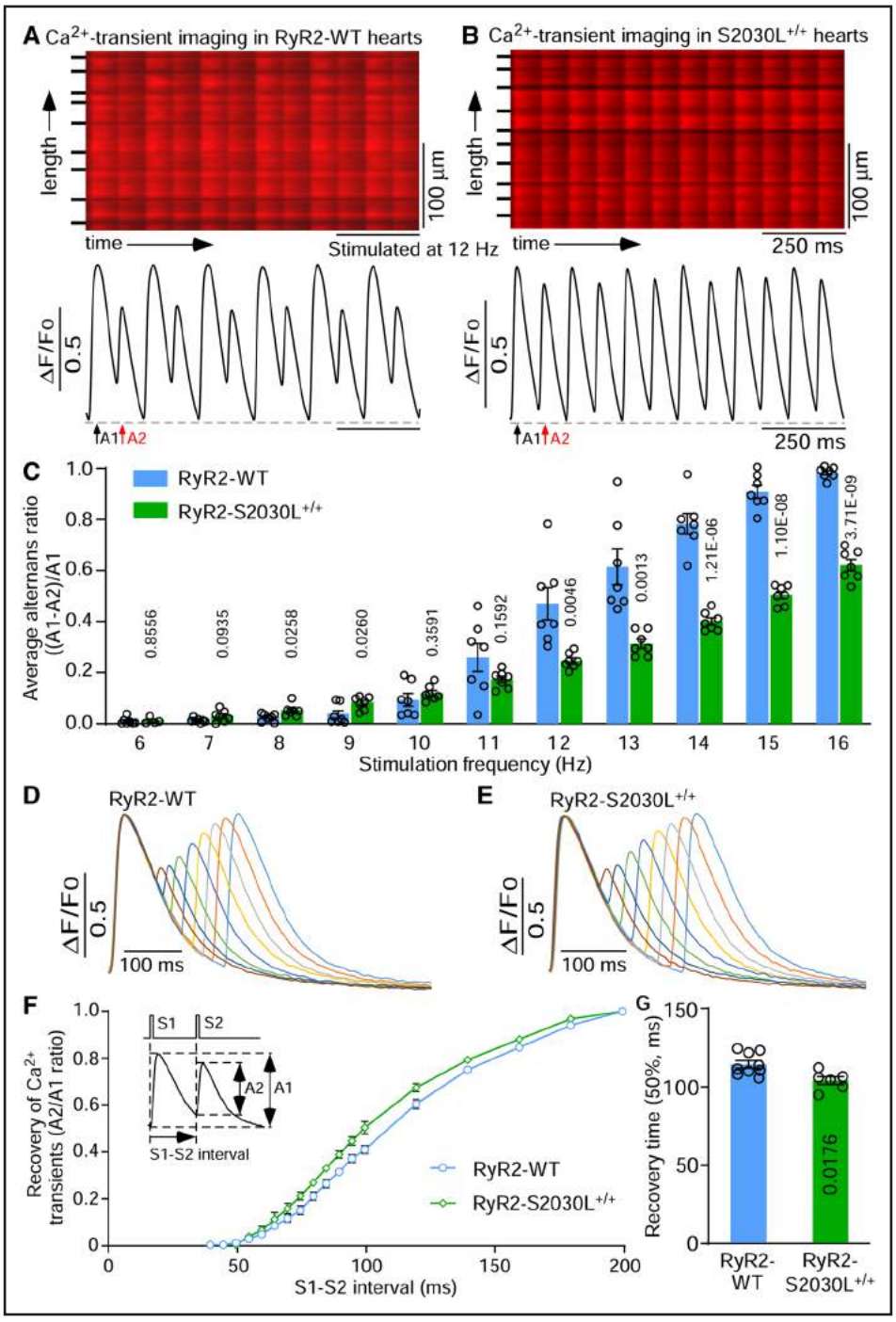

Figure 4. Effects of RyR2 (ryanodine receptor 2)–serine-2030L (S2030L)$^{+/+}$ mutation on Ca^{2+} alternans and Ca^{2+} transient recovery in intact hearts.

Langendorff-perfused intact RyR2-wild-type (WT) (**A**) and RyR2- S2030L$^{+/+}$ mutant (**B**) hearts were loaded with Rhod-2 AM. Ca^{2+} transients were elicited by pacing at 12 Hz and recorded using line-scanning confocal imaging. Cell boundaries were indicated by short bars to the left. The ΔF/Fo traces depict the average fluorescence signal of the scan area. **C**, Alternans ratios for each cell that displayed alternans in the scan area were determined and averaged per cell to yield the average alternans ratio. Alternans ratio is defined as the ratio of the difference in amplitude between the large and small Ca^{2+} transients over the amplitude of the large Ca^{2+} transient. Data are mean±SEM (n=7 hearts for RyR2-WT and 7 hearts for RyR2-S2030L$^{+/+}$ with their adjusted *P* values indicated for RyR2-S2030L$^{+/+}$ vs RyR2-WT, Two-way ANOVA with Bonferroni post hoc test (**C**). To determine the recovery of Ca^{2+} transients, RyR2-WT (**D**) and RyR2-S2030L$^{+/+}$ mutant (**E**) hearts were first stimulated at 5 Hz for 30 beats (S1), followed by a single S2 stimulation. A series of S1S2 stimulations were repeatedly applied with progressively reduced S1S2 intervals from 200 to 40 ms. Ca^{2+} transients were recorded using line-scanning confocal imaging. **F**, The relationship between A2/A1 ratio of the Ca^{2+} transient amplitude and the S1S2 interval. **G**, The 50% recovery time of the Ca^{2+} transient amplitude after pacing with the S1S2 protocol. Data are mean±SEM (n=9 hearts for RyR2-WT, and 7 hearts for RyR2-S2030L$^{+/+}$ with their *P* values indicated for RyR2-S2030L$^{+/+}$ vs RyR2-WT, Mann-Whitney test (**G**).

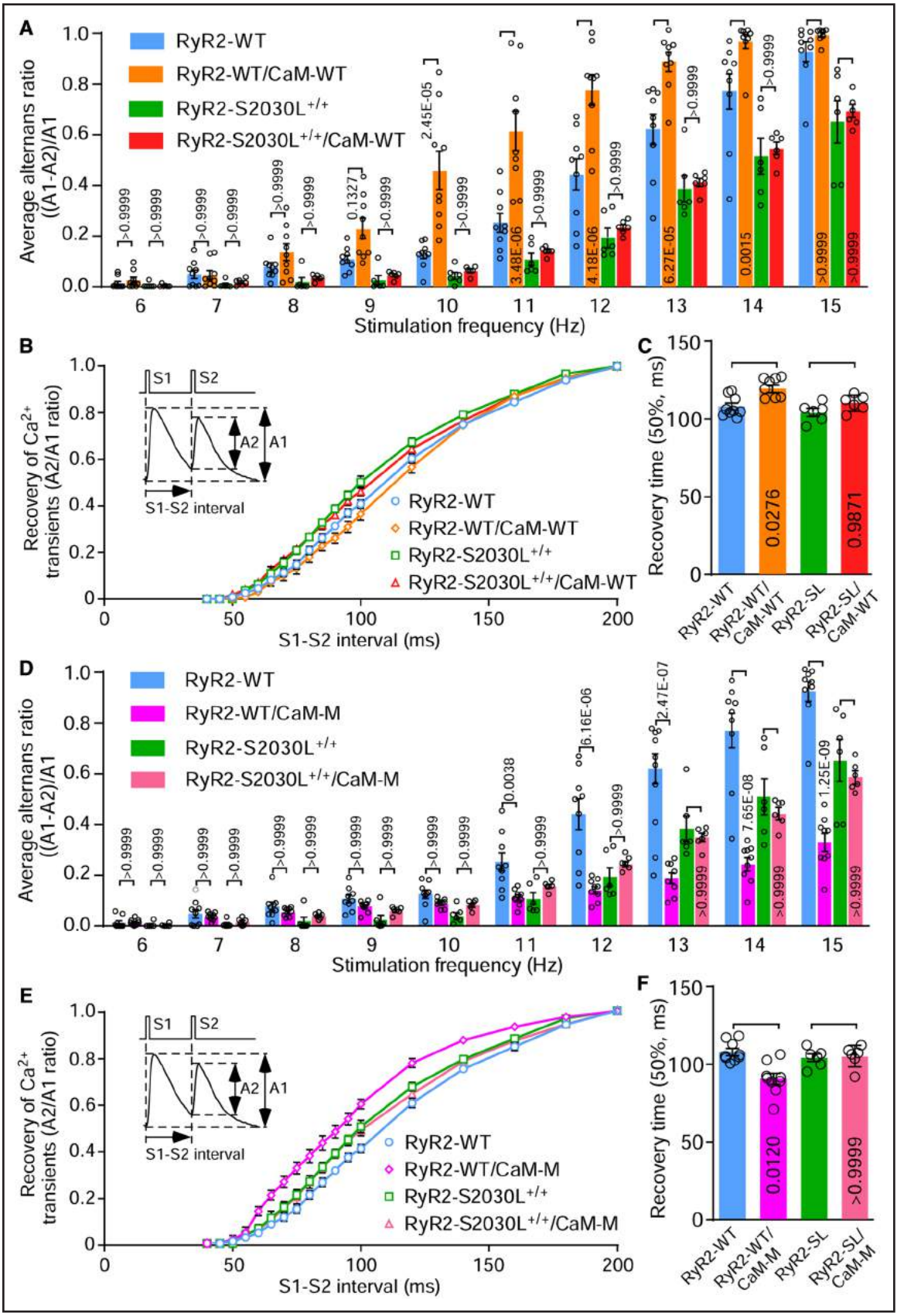

Figure 5. Effects of local injection of CaM (calmodulin)–wild-type (WT)/GFP (green fluorescence protein) or CaM-M (a loss-of-function CaM mutation with all 4 EF-hand motifs mutated)/GFP adenovirus on Ca^{2+} alternans and Ca^{2+} transient recovery in intact RyR2 (ryanodine receptor 2)-WT and RyR2–serine-2030L (S2030L)$^{+/+}$ mutant hearts. (*Continued*)

mutation abolishes the action of CaM in Ca^{2+} alternans and Ca^{2+} transient recovery but has little or no effect on Ca^{2+} transient properties in intact hearts.

RyR2-S2030L$^{+/+}$ Mutation Abolishes the Suppressive Effects of CaM on Ca^{2+} Sparks in Isolated Ventricular Myocytes

To determine the effects of the RyR2-S2030L$^{+/+}$ mutation and CaM on intracellular Ca^{2+} handling at the level of Ca^{2+} sparks, we performed confocal line-scanning Ca^{2+} imaging of isolated ventricular myocytes from RyR2-WT or RyR2-S2030L$^{+/+}$ mutant hearts infected with or without adenoviruses expressing CaM-WT/GFP. We found that RyR2-S2030L$^{+/+}$ mutant ventricular myocytes displayed increased frequency, amplitude, duration, and rate of rise of Ca^{2+} sparks, compared with RyR2-WT cells (Figure S9). We also found that CaM-WT decreased the frequency, amplitude, and rate of rise, but not the duration, of Ca^{2+} sparks in RyR2-WT ventricular myocytes but had no effect on Ca^{2+} spark properties in RyR2-S2030L$^{+/+}$ mutant ventricular myocytes (Figure S9). These data are consistent with the lack of effect of CaM on the luminal Ca^{2+} termination threshold, Ca^{2+} alternans, or Ca^{2+} transient recovery of the RyR2-S2030L$^{+/+}$ mutant.

RyR2-S2030L$^{+/+}$ Diminishes the Effects of β-Adrenergic Receptor Stimulation-Induced PKA Activation on Ca^{2+} Alternans and Ca^{2+} Transient Refractoriness in Intact Hearts

Activation of PKA upon β-adrenergic stimulation has been shown to suppress Ca^{2+} alternans in isolated atrial myocytes.[50] To determine whether β-adrenergic stimulation can suppress Ca^{2+} alternans in the setting of intact hearts and whether the PKA phosphorylation site RyR2-S2030 has any role in this process, we performed confocal Ca^{2+} imaging to monitor Ca^{2+} transients in ventricular myocytes in RyR2-WT and RyR2-S2030L$^{+/+}$ intact hearts paced at a wide range of frequencies (6–16 Hz) (Figure 6). Consistent with that observed in isolated atrial myocytes,[50] isoproterenol (1 μM) treatment significantly suppressed Ca^{2+} alternans, as evidenced by markedly reduced alternans ratio at stimulation frequencies of 11 to 16 Hz, at which substantial Ca^{2+} alternans occurred in control hearts (before isoproterenol treatment) (Figure 6A, 6B, and 6F). Also consistent with that seen in atrial myocytes, treatment with a PKA inhibitor H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) partially and significantly attenuated the suppressive effect of isoproterenol on Ca^{2+} alternans in intact RyR2 WT hearts at stimulation frequencies of 12 to 16 Hz (Figure 6A, 6C, and 6F). This suggests that activation of PKA contributes, in part, to the suppressive effect of isoproterenol on Ca^{2+} alternans in intact hearts. It has also been shown that activation of CaMKII also contributes, in part, to the suppressive effect of isoproterenol on Ca^{2+} alternans in atrial myocytes.[50] Consistent with this observation, treatment with a CaMKII inhibitor KN93 (N-[2-[N-(4-chlorocinnamyl)-N-methylaminomethyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide) also partially and significantly attenuated the suppressive effect of isoproterenol on Ca^{2+} alternans in intact RyR2-WT hearts at stimulation frequencies of 12 to 16 Hz (Figure 6A, 6D, and 6F).

The impact of isoproterenol, H89, and KN93 on Ca^{2+} alternans in intact RyR2-S2030L$^{+/+}$ mutant hearts is shown in Figure 6G through 6L. Isoproterenol treatment also significantly suppressed Ca^{2+} alternans in intact RyR2-S2030L$^{+/+}$ hearts, as evidenced by the reduced alternans ratios at stimulation frequencies of 12 to 16 Hz (Figure 6G, 6H, and 6L). However, treatment with KN93 (a CaMKII inhibitor), but not with H89 (a PKA inhibitor), abolished the suppressive effect of isoproterenol on Ca^{2+} alternans in intact RyR2-S2030L$^{+/+}$ hearts at stimulation frequencies of 12 to 16 Hz (Figs. 6G, 6I, 6J, and 6L). Thus, the PKA site mutation RyR2-S2030L$^{+/+}$ abolishes the PKA-associated, but not the CaMKII-associated, suppressive effect of isoproterenol on Ca2^{+} alternans. Together, these data suggest that the PKA site RyR2-S2030 is a critical determinant of PKA-dependent, but not CaMKII-dependent, suppression of Ca^{2+} alternans upon β-adrenergic stimulation in intact hearts.

We also assessed the impact of isoproterenol, H89, and KN93 on Ca^{2+} transient refractoriness in intact

Figure 5 Continued. A, Alternans ratios in RyR2-WT or RyR2-S2030L$^{+/+}$ (SL) mutant hearts without injection and with local injection of CaM-WT/GFP adenovirus were stimulated at increasing frequencies (6–15 Hz). Data are mean±SEM (n=9 hearts for RyR2-WT without injection, 9 hearts for RyR2-WT with injection, n=6 hearts for RyR2-S2030L$^{+/+}$ without injection, and 6 hearts for RyR2-S2030L$^{+/+}$ with injection, their *P* values indicated, injection versus without injection for each genotype). **B**, The relationship between A2/A1 ratio of the Ca^{2+} transient amplitude and the S1S2 interval. **C**, The 50% recovery time of the Ca^{2+} transient amplitude after pacing with the S1S2 protocol. Data are mean±SEM (n=9 hearts for RyR2-WT without injection, 8 hearts for RyR2-WT with injection, n=6 hearts for RyR2-S2030L$^{+/+}$ without injection, and 6 hearts for RyR2-S2030L$^{+/+}$ with injection, their *P* values indicated, injection versus without injection for each genotype). **D**, Alternans ratios in RyR2-WT or RyR2-S2030L$^{+/+}$ mutant hearts without injection and with local injection of CaM-M/GFP adenovirus were stimulated at increasing frequencies (6-15 Hz). Data are mean±SEM (n=9 hearts for RyR2-WT without injection, 9 hearts for RyR2-WT with injection, n=6 hearts for RyR2-S2030L$^{+/+}$ without injection, and 6 hearts for RyR2-S2030L$^{+/+}$ with injection, their *P* values indicated injection versus without injection for each genotype). **E**, The relationship between A2/A1 ratio of the Ca^{2+} transient amplitude and the S1S2 interval. **F**, The 50% recovery time of the Ca^{2+} transient amplitude after pacing with the S1S2 protocol. Data are mean±SEM (n=8 hearts for RyR2-WT without injection, 9 hearts for RyR2-WT with injection, n=6 hearts for RyR2-S2030L$^{+/+}$ without injection, and 6 hearts for RyR2-S2030L$^{+/+}$ with injection, their *P* values indicated, injection versus without injection for each genotype). Two-way ANOVA with Bonferroni post hoc test for obtaining the adjusted *P* values shown (**A**, **C**, **D**, **F**).

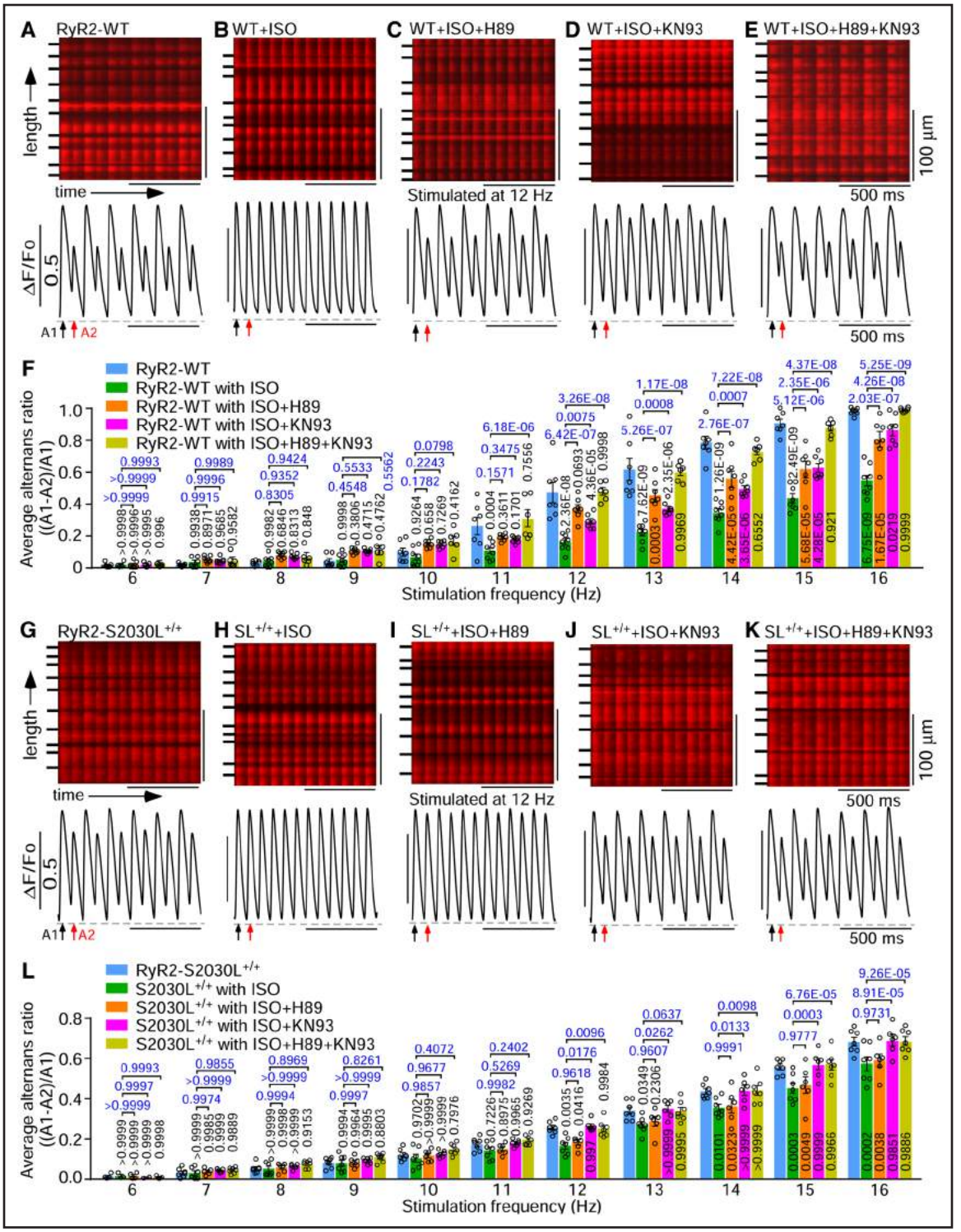

Figure 6. The RyR2 (ryanodine receptor 2)–serine-2030L (S2030L)$^{+/+}$ mutation diminishes the effects of β-adrenergic receptor stimulation-induced PKA (protein kinase A) activation on Ca^{2+} alternans in intact hearts.

RyR2-wild-type (WT) hearts without isoproterenol (ISO) (**A**), with ISO (**B**), with ISO+H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide) (**C**), with ISO+KN93 (N-[2-[N-(4-chlorocinnamyl)-N-methylaminomethyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide) (**D**), and with ISO+H89+KN93 (**E**) were stimulated at increasing frequencies (6-16 Hz). **F**, Alternans ratios. Data are mean±SEM (n=7 hearts for without ISO, 8 hearts for with ISO, 7 hearts for with ISO+H89, 7 hearts for with ISO+KN93, and 6 hearts for with ISO+H89+KN93, their *P* values indicated for each comparison). RyR2-S2030L$^{+/+}$ hearts without ISO (**G**), with ISO (**H**), with ISO+H89 (**I**), and with ISO+KN93 (**J**), and with ISO+H89+KN93 (**K**) were stimulated at increasing frequencies (6–16 Hz). **L**, Alternans ratios. Data are mean±SEM (n=7 hearts for without ISO, 7 hearts for with ISO, 6 hearts for with ISO+H89, 6 hearts for with ISO+KN93, and 7 hearts for with ISO+H89+KN93, their *P* values indicated for each comparison). Two-way ANOVA with Bonferroni post hoc test for obtaining the adjusted *P* values shown (**F** and **L**).

RyR2-WT and RyR2-S2030L$^{+/+}$ mutant hearts. As shown in Figure 7, isoproterenol treatment significantly accelerated the recovery (50%) of Ca^{2+} transients in RyR2-WT hearts compared to that of the control hearts (before isoproterenol treatment), as evidenced by a reduced recovery time (at 50%) of Ca^{2+} transients (Figure 7A, 7B, 7F, and 7G). Treatment with H89 or KN93 (1 μM) partially and significantly attenuated the isoproterenol-induced acceleration of Ca^{2+} transient recovery (50%) in RyR2-WT hearts (Figure 7C, 7D, 7F, and 7G). This suggests that the activation of PKA or CaMKII contributes, in part, to the isoproterenol-induced acceleration of Ca^{2+} transient recovery in RyR2-WT hearts.

Isoproterenol treatment also significantly accelerated the recovery (50%) of Ca^{2+} transients in RyR2-S2030L$^{+/+}$ mutant hearts, as evidenced by the reduced recovery time (at 50%) of Ca^{2+} transients (Figure 7H, 7I, 7M, and 7N). However, treatment with KN93, but not with H89, abolished the isoproterenol-induced acceleration of recovery (50%) of Ca^{2+} transients (Figure 7J, 7K, 7M, and 7N). Thus, the PKA phosphorylation site mutation S2030L$^{+/+}$ also abolishes the PKA-associated, but not the CaMKII-associated, acceleration of Ca^{2+} transient recovery upon β-adrenergic stimulation. Together, these data suggest that the PKA site RyR2-S2030 is also a critical determinant of PKA-dependent, but not CaMKII-dependent, acceleration of Ca^{2+} transient recovery upon β-adrenergic stimulation in intact hearts.

To determine the impact of inhibiting both PKA and CaMKII on isoproterenol-induced suppression of Ca^{2+} alternans and isoproterenol-induced acceleration of Ca^{2+} transient recovery, we treated intact RyR2-WT and RyR2-S2030L$^{+/+}$ hearts with isoproterenol in the presence of both inhibitors H89 and KN93. We found that inhibiting both PKA and CaMKII completely abolished isoproterenol-induced suppression of Ca^{2+} alternans and isoproterenol-induced acceleration of Ca^{2+} transient recovery (50%) in both intact RyR2-WT (Figure 6A, 6E, and 6F; Figure 7A, 7E, 7F, and 7G) and RyR2-S2030L$^{+/+}$ hearts (Figure 6G, 6K, 6L; Figure 7H, 7L, 7M, and 7N). These results suggest that isoproterenol-induced suppression of Ca^{2+} alternans and isoproterenol-induced acceleration of Ca^{2+} transient recovery in intact hearts is primarily mediated by PKA and CaMKII upon β-adrenergic stimulation.

DISCUSSION

The mechanism and regulation of RyR2 activation and Ca^{2+} release initiation in the heart are well understood, but those of RyR2 inactivation and Ca^{2+} release termination are not. Our recent functional and structural studies revealed that CaM is an important determinant of RyR2 inactivation and Ca^{2+} release termination and that the PKA-dependent phosphorylation site RyR2-S2030 may play a role in this CaM-dependent process. Consistent with this notion, we found, in the present study, that mutating the PKA phosphorylation site RyR2-S2030 altered Ca^{2+} release termination. Specifically, the RyR2-S2030L mutation reduced the threshold for Ca^{2+} release termination and abolished the effects of CaM and PKA on the termination of Ca^{2+} release. We have also shown recently that CaM-dependent RyR2 inactivation and Ca^{2+} release termination underlie rapid pacing–induced Ca^{2+} alternans in intact hearts.[24] To assess the role of the PKA site RyR2-S2030 in Ca^{2+} alternans and the actions of CaM and PKA in intact hearts, we generated a novel mouse model expressing the RyR2-S2030L$^{+/+}$ mutation. We found that the RyR2-S2030L$^{+/+}$ mutation suppressed rapid pacing–induced Ca^{2+} alternans and abolished the impact of CaM and β-adrenergic stimulation-induced PKA activation on Ca^{2+} alternans and Ca^{2+} transient recovery in intact hearts. These data demonstrate, for the first time, that the PKA phosphorylation site RyR-S2030 is a critical determinant of the actions of CaM and PKA in Ca^{2+} alternans and Ca^{2+} transient recovery. These findings also suggest that PKA-dependent phosphorylation of RyR2 at S2030 modulates CaM-dependent Ca^{2+} release termination and Ca^{2+} alternans.

The PKA-dependent phosphorylation site RyR2-S2030 was discovered by 2-dimensional phosphopeptide mapping followed by Edman sequencing >15 years ago.[41] However, the impact of PKA phosphorylation of S2030 on RyR2 channel function and its physiological significance remain not well understood. Biochemical analyses revealed that RyR2-S2030 is stoichiometrically phosphorylated by PKA, but not by CaMKII. Treatment of cardiomyocytes with isoproterenol resulted in phosphorylation of S2030. This isoproterenol-induced phosphorylation of S2030 was markedly inhibited by PKI, a specific inhibitor of PKA.[41,42] These studies demonstrated that S2030 is a PKA phosphorylation site in RyR2 responding to PKA activation upon β-adrenergic stimulation. Single channel analyses revealed that PKA increases the response of single RyR2 channels to activation by luminal Ca^{2+}. Importantly, this effect of PKA on luminal Ca^{2+} activation of RyR2 is completely abolished by the S2030A mutation.[51] This indicates that the functional impact of PKA on the RyR2 channel is largely mediated by phosphorylation of S2030. To assess the functional role of the PKA phosphorylation site RyR2-S2030 in cardiomyocytes, Potenza et al[52] generated a mouse model expressing the RyR2-S2030A mutation and showed that ablation of the PKA phosphorylation site RyR2-S2030 blunted the response to β-adrenergic stimulation in isolated cardiomyocytes. In particular, it was found that the RyR2-S2030A mutation decreased isoproterenol-induced excitation-contraction–coupling gain and RyR2-mediated spontaneous SR Ca^{2+} release. It was suggested that PKA phosphorylation of RyR2 at S2030 may account for the enhanced RyR2 sensitivity,

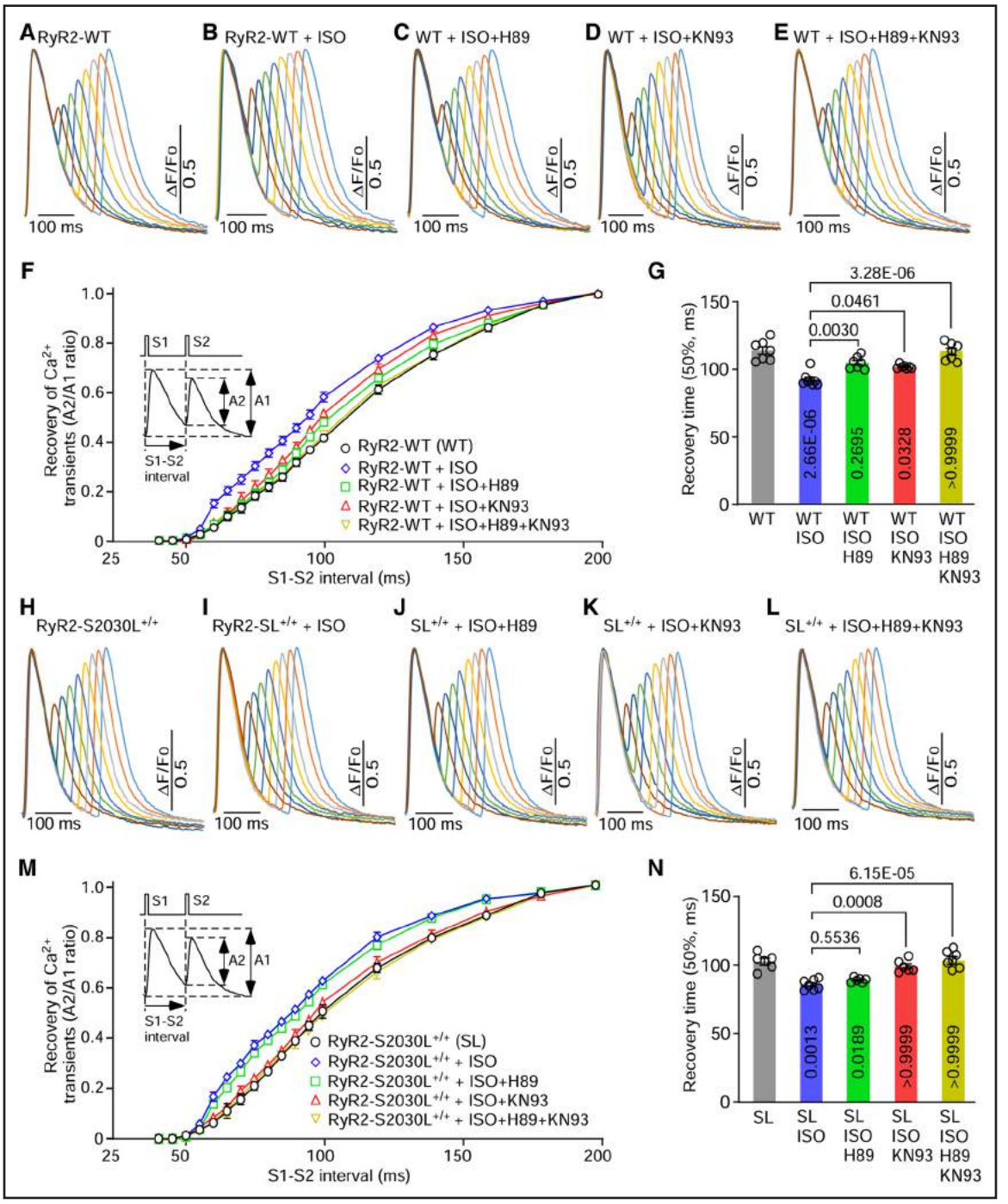

Figure 7. The RyR2 (ryanodine receptor 2)–serine-2030L (S2030L)$^{+/+}$ mutation diminishes the effects of β-adrenergic receptor stimulation-induced PKA (protein kinase A) activation on Ca^{2+} transient recovery in intact hearts.
Recovery of Ca^{2+} transients without isoproterenol (ISO) (**A**), with ISO (**B**), with ISO+H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide) (**C**), and with ISO+KN93 (N-[2-[N-(4-chlorocinnamyl)-N-methylaminomethyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide) (**D**), and with ISO+H89+KN93 (**E**) in RyR2-wild-type (WT) hearts. **F**, The relationship between A2/A1 ratio of the Ca^{2+} transient amplitude and the S1S2 interval. **G**, The 50% recovery time of the Ca^{2+} transient amplitude after pacing with the S1S2 protocol. Data are mean±SEM (n=7 hearts for without ISO, 8 hearts for with ISO, 7 hearts for with ISO+H89, 7 hearts for with ISO+KN93, and 7 hearts for with ISO+H89+KN93, their *P* values indicated for each comparison). Recovery of Ca^{2+} transients without ISO (**H**), with ISO (**I**), with ISO+H89 (**J**), with ISO+KN93 (**K**), and with ISO+H89+KN93 (**L**) in RyR2-S2030L$^{+/+}$ (SL) hearts. **M**, The relationship between A2/A1 ratio of the Ca^{2+} transient amplitude and the S1S2 interval. **N**, The 50% recovery time of the Ca^{2+} transient amplitude after pacing with the S1S2 protocol. Data are mean±SEM (n=6 hearts for without ISO, 7 hearts for with ISO, 6 hearts for with ISO+H89, 6 hearts for with ISO+KN93, and 7 hearts for with ISO+H89+KN93, their *P* values indicated for each comparison). Two-way ANOVA with Bonferroni post hoc test for obtaining the adjusted *P* values shown (**G**, **N**).

which may contribute to the positive inotropy during PKA activation upon β-adrenergic stimulation.[52] Interestingly, RyR2-S2030 phosphorylation is subjected to modulation by integrin β1D. Wang et al[53] showed that integrin β1D deficiency in arrhythmogenic right ventricular cardiomyopathy human patients and model mice resulted in hyperphosphorylation of RyR2 at S2030, but not S2808, leading to increased spontaneous SR Ca^{2+} release, triggered activities, and ventricular arrhythmias upon β-adrenergic stimulation.

In addition to providing a mechanistic understanding of the PKA site RyR2-S2030 as a mediator of PKA-dependent phosphorylation of RyR2, our present study also suggested new functional roles of RyR2-S2030. We showed that PKA suppressed CaM-dependent Ca^{2+} release termination and that mutations of RyR2-S2030 abolished the action of PKA on Ca^{2+} release termination. Furthermore, we found that β-adrenergic stimulation with isoproterenol suppressed rapid pacing-induced Ca^{2+} alternans and accelerated the recovery of pacing-induced Ca^{2+} transients in intact hearts. Importantly, the RyR2-S2030L$^{+/+}$ mutation abolished the PKA-associated suppressive effect of isoproterenol on Ca^{2+} alternans and Ca^{2+} transient refractoriness. Thus, our results reveal that the PKA phosphorylation site RyR2-S2030 also plays an important role in Ca^{2+} release termination and cardiac Ca^{2+} alternans. It is well known that β-adrenergic stimulation can substantially increase heart rate and that elevated heart rate can predispose the heart to cardiac alternans. Hence, the suppressive effect of PKA phosphorylation of RyR2-S2030 on Ca^{2+} alternans may serve as a protective mechanism against rapid heart rate–induced cardiac alternans during β-adrenergic stimulation or fight-or-flight response (Figure 8).

Apart from suppressing Ca^{2+} release termination, RyR2-S2030 phosphorylation has also been shown to enhance the activation of RyR2-mediated Ca^{2+} release.[51] We have previously shown that a catecholaminergic polymorphic ventricular tachycardia-associated RyR2 mutation R4496C enhances the activation of RyR2-mediated Ca^{2+} release and suppresses Ca^{2+} alternans.[24] Accordingly, RyR2-S2030 phosphorylation-induced activation of Ca^{2+} release could also suppress Ca^{2+} alternans. Thus, RyR2-S2030 phosphorylation not only affects the termination but also the activation of RyR2-mediated Ca^{2+} release, both of these effects would modulate the recovery of RyR2 from inactivation, Ca^{2+} release restitution, and thus Ca^{2+} alternans. Therefore, RyR2-S2030 phosphorylation-induced changes in both the activation and termination of Ca^{2+} release are likely responsible for RyR2-S2030 phosphorylation-induced reduction in Ca^{2+} alternans.

Our present work demonstrates that the PKA site RyR2-S2030 has an important role in modulating the termination of Ca^{2+} release, but the underlying molecular mechanism is unclear. It appears that introducing a negative charge to the S2030 location (ie, adding a phosphate group PO_4 as a result of PKA phosphorylation or mutation to aspartate S2030D) reduced the termination threshold (ie, suppressed/delayed Ca^{2+} release termination). Reducing or increasing the size of the side chain at the S2030 location (S2030G, S2030L, S2030V, and S2030W) also reduced the termination threshold, while mutations S2030A and S2030T with similar sizes had no significant effect on the termination threshold. However, introducing a positive charge (S2030R) into the S2030 location increased the termination threshold (ie, promoted/accelerated Ca^{2+} release termination). Interestingly, mutating S2030 to proline (S2030P), a well-known helix breaker, also increased the termination threshold. Among the 9 RyR2-S2030 mutations characterized, the RyR2-S2030L mutation caused the most dramatic impact on Ca^{2+} release termination. The exact mechanism underlying the action of RyR2-S2030L on Ca^{2+} release termination is unknown. Interestingly, S2030 is located in the middle of a stretch of leucine-rich sequence, LTIRGRLLS(2030)LVEKVTYL. Mutating reside S2030 to leucine may turn this sequence into a leucine-rich domain, which is well-known for protein-protein interactions. Thus, it could be speculated that the RyR2-S2030L mutation, by introducing a leucine-rich domain, may strengthen some protein-protein interactions that suppress the action of CaM on Ca^{2+} release termination. Taken together, these observations suggest that the charge, size, and shape of the S2030 side chain are important determinants of Ca^{2+} release termination.

The structural basis by which mutations or phosphorylation of RyR2-S2030 affect the threshold for Ca^{2+} release termination has yet to be resolved. High-resolution 3D structural analysis reveals that Ca^{2+}-CaM binding to the CaM-binding domain (α-1 helix) causes channel pore closure by inducing conformational changes in the central domain that is connected to the pore domain (Figure 1A and 1B). This is consistent with our functional studies showing that Ca^{2+}-CaM inhibits single RyR2 channel and induces the termination of Ca^{2+} release.[40] Notably, the CaM-binding domain (α-1 helix) is connected to the central domain via the α0 helix. Interestingly, the PKA phosphorylation site RyR2-S2030 is located in the α13 helix in the handle domain that interacts with the α0 helix (Figure 1B).[40] Based on these structural insights, we propose that changing the charge, size, and shape of the side chain of residue S2030 (as in the case of S2030 mutations or adding a phosphate group to S2030 upon its phosphorylation) is likely to exert steric effects on the conformation of the α13 helix and the α13 helix-interacting α0 helix, which in turn affects the CaM-induced conformational changes in the central domain. In other words, PKA phosphorylation of RyR2 at S2030 or S2030 mutations may affect the CaM-dependent inactivation of RyR2 and thus the termination of RyR2-mediated Ca^{2+} release by altering

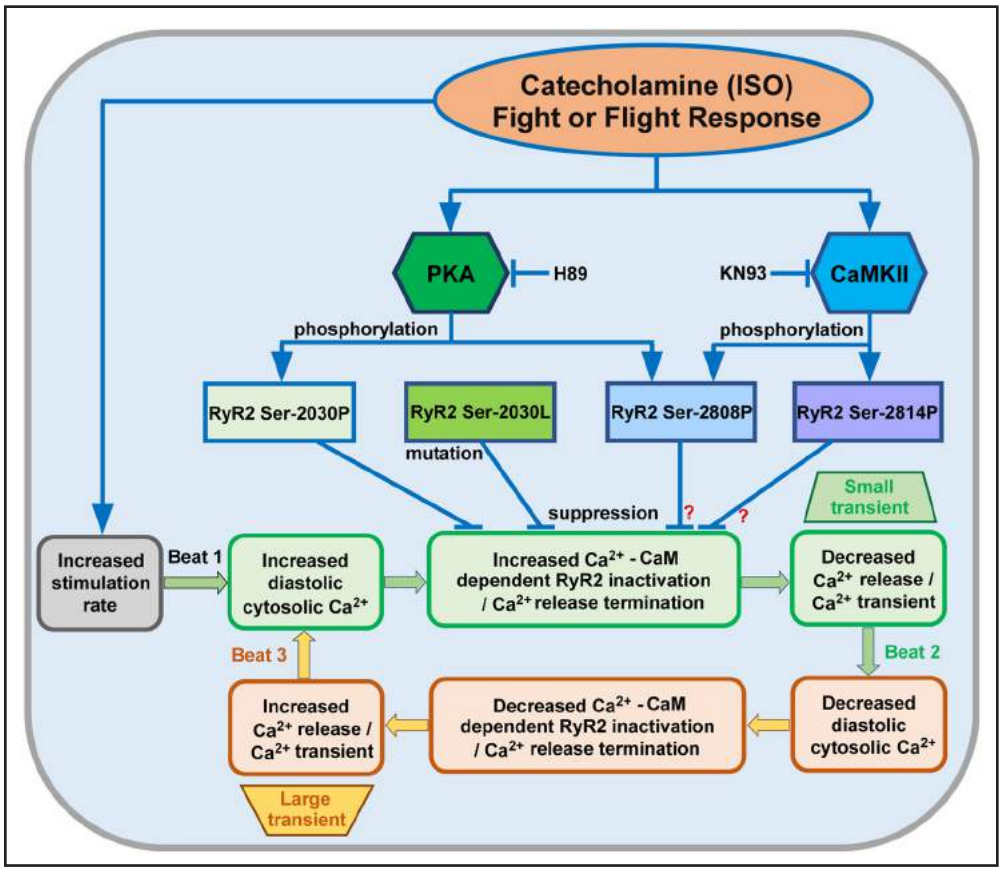

Figure 8. A schematic representation for the proposed role of PKA (protein kinase A)-dependent RyR2 (ryanodine receptor 2) phosphorylation in the modulation of Ca^{2+} release and Ca^{2+} alternans.

Our previous numerical modeling analyses suggested that diastolic cytosolic Ca^{2+} elevation as a result of rapid pacing is an important trigger of Ca^{2+}-CaM (calmodulin) dependent inactivation of RyR2 and Ca^{2+} release termination.[24] Since Ca^{2+}-CaM-dependent inactivation of RyR2 requires the elevation of diastolic cytosolic Ca^{2+} level, and the diastolic cytosolic Ca^{2+} level undergoes beat-to-beat oscillation during rapid pacing, the extent of Ca^{2+}-CaM–dependent inactivation of RyR2 and Ca^{2+} release termination would also undergo beat-to-beat oscillation, thereby resulting in Ca^{2+} alternans. We propose that PKA-dependent RyR2–serine-2030 (S2030) phosphorylation upon β-adrenergic stimulation (catecholamine or flight-or-fight response) or RyR2-S2030 mutations suppress the Ca^{2+}-CaM dependent inactivation of RyR2 and Ca^{2+} release termination, and thus Ca^{2+} alternans. We also propose that CaMKII (CaM-dependent protein kinase II)-dependent phosphorylation of RyR2 also contributes to the suppressive effect of catecholamine on Ca^{2+} alternans, but the exact phosphorylation sites in RyR2 mediated the action of CaMKII in Ca^{2+} alternans have yet to be determined. ISO indicates isoproterenol.

CaM-induced conformational changes. Further structural studies will be required to determine the role of PKA phosphorylation of RyR2-S2030 or the impact of RyR2-S2030 mutations on CaM-induced conformational changes in the 3D structure of the RyR2.

We have previously shown that RyR2 is phosphorylated by PKA at 2 major sites S2030 and S2808.[41] The properties and functional significance of the RyR2-S2808 site are markedly different from those of the RyR2-S2030 site. RyR2-S2030 was phosphorylated in cardiomyocytes upon β-adrenergic stimulation with isoproterenol in a concentration- and time-dependent manner. However, RyR2-S2808 was already substantially phosphorylated (>50%) before β-adrenergic stimulation, and the extent of isoproterenol-induced increase in S2808 phosphorylation was much less than that for S2030.[41,43,53–55] Unlike the S2030 site, the basal or isoproterenol-induced phosphorylation of the RyR2-S2808 site was not affected by the PKA inhibitor PKI.[42] RyR2-S2808, but not RyR2-S2030, could also be phosphorylated by CaMKII and PKG in addition to PKA.[42] Also different from the RyR2-S2030A mutation, the RyR2-S2808A mutation had no effect on PKA-induced luminal Ca^{2+} activation of single RyR2 channels.[51] Consistent with this in vitro functional study, the RyR2-S2030A mutation, but not the RyR2-S2808A mutation, markedly reduced the isoproterenol-induced increase in the frequency of spontaneous Ca^{2+} sparks and Ca^{2+} waves in cardiomyocytes.[52] These observations are consistent with the notion that RyR2-S2030 is a major PKA-dependent phosphorylation site that mediates the action of PKA in increasing RyR2 activity upon β-adrenergic stimulation.

The suppressive effect of isoproterenol on Ca^{2+} alternans and Ca^{2+} transient refractoriness was partially attenuated by the PKA inhibitor, H89, or by the CaMKII inhibitor, KN93, but was completely abolished by the

treatment of both H89 and KN93. These observations indicate that the suppressive effect of isoproterenol is largely mediated by the activation of PKA and CaMKII. Our data reveal that RyR2-S2030 mediates the PKA-associated suppressive effect of isoproterenol. However, the exact sites in RyR2 (eg, S2808 and/or S2814) that mediate the CaMKII-associated suppressive effect of isoproterenol are unknown. We have previously shown that PKG can also phosphorylate RyR2 at S2030 and S2808.[42] It has also been shown recently that RyR2-S2030 or S2808 is required for PKG-dependent modulation of RyR2-mediated spontaneous Ca^{2+} release.[56] These observations raise a possibility that PKG may also modulate Ca^{2+} release termination and Ca^{2+} alternans. Further systematic and detailed studies will be required to determine whether RyR2-S2808 and/or -S2814 or PKG plays a role in Ca^{2+} release termination and cardiac Ca^{2+} alternans (Figure 8).

We have previously shown that the 50% recovery time of the Ca^{2+} transients (≈140–270 ms) in the control, CaM-WT, or CaM-M expressing ventricular myocytes is substantially longer than the 50% recovery time from inactivation of the L-type Ca^{2+} current (≈40–80 ms).[24] Thus, although the recovery from inactivation of the L-type Ca^{2+} current can influence the recovery of Ca^{2+} transients, CaM-dependent modulation of the recovery from inactivation of the L-type Ca^{2+} current is unlikely to be the rate-limiting determinant of the action of CaM in the recovery of Ca^{2+} transients and Ca^{2+} alternans.[24] Instead, our finding that the RyR2-S2030L$^{+/+}$ mutation abolishes the action of CaM in Ca^{2+} alternans and Ca^{2+} transient recovery suggests that the effect of CaM-WT or CaM-M on Ca^{2+} alternans and Ca^{2+} transient refractoriness is primarily mediated by RyR2.

In summary, our present study demonstrates that the PKA phosphorylation site RyR2-S2030 plays an important role in CaM- and PKA-dependent modulation of Ca^{2+} release termination and rapid pacing–induced cardiac Ca^{2+} alternans. Based on our data, we propose a novel functional role of PKA phosphorylation of RyR2-S2030 in the regulation of Ca^{2+} release termination and Ca^{2+} alternans.

ARTICLE INFORMATION

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Author Contributions

J. Wei, R. Wang, R. Benitez, L. Hove-Madsen, S.R.W. Chen designed the research; J. Wei, W. Guo, R. Wang, J.P. Estillore, D. Belke, Y.X. Chen performed the research; J. Wei, D. Belke, Y.X. Chen, A. Vallmitjana, R. Benitez, LHM, S.R.W. Chen analyzed data; and J. Wei, R. Wang, A. Vallmitjana, R. Benitez, L. Hove-Madsen, S.R.W. Chen wrote the article.

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Supplemental Material

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