Arrhythmogenic Calmodulin Mutations Affect the Activation and Termination of Cardiac Ryanodine Receptor-mediated Ca$^{2+}$ Release*

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Background: Mutations in the Ca$^{2+}$ sensing protein calmodulin (CaM) cause lethal cardiac arrhythmias.

Results: CaM mutations impair the activation and termination of store overload-induced Ca$^{2+}$ release via the cardiac ryanodine receptor (RyR2).

Conclusion: CaM mutations alter RyR2-CaM interaction, thereby affecting RyR2-mediated Ca$^{2+}$ release.

Significance: Aberrant regulation of RyR2 store Ca$^{2+}$ sensing is a potential component of calmodulin-mediated cardiac arrhythmias.

The intracellular Ca$^{2+}$ sensor calmodulin (CaM) regulates the cardiac Ca$^{2+}$ release channel/ryanodine receptor 2 (RyR2), and mutations in CaM cause arrhythmias such as catecholaminergic polymorphic ventricular tachycardia (CPVT) and long QT syndrome. Here, we investigated the effect of CaM mutations causing CPVT (N53I), long QT syndrome (D95V and D129G), or both (CaM N97S) on RyR2-mediated Ca$^{2+}$ release. All mutations increased Ca$^{2+}$ release and rendered RyR2 more susceptible to store overload-induced Ca$^{2+}$ release (SOICR) by lowering the threshold of store Ca$^{2+}$ content at which SOICR occurred and the threshold at which SOICR terminated. To obtain mechanistic insights, we investigated the Ca$^{2+}$ binding of the N- and C-terminal domains (N- and C-domain) of CaM in the presence of a peptide corresponding to the CaM-binding domain of RyR2. The N53I mutation decreased the affinity of Ca$^{2+}$ binding to the N-domain of CaM, relative to CaM WT, but did not affect the C-domain. Conversely, mutations N97S, D95V, and D129G had little or no effect on Ca$^{2+}$ binding to the N-domain but markedly decreased the affinity of the C-domain for Ca$^{2+}$. These results suggest that mutations D95V, N97S, and D129G alter the interaction between CaM and the CaMBD and thus RyR2 regulation. Because the N53I mutation minimally affected Ca$^{2+}$ binding to the C-domain, it must cause aberrant regulation via a different mechanism. These results support aberrant RyR2 regulation as the disease mechanism for CPVT associated with CaM mutations and shows that CaM mutations not associated with CPVT can also affect RyR2. A model for the CaM-RyR2 interaction, where the Ca$^{2+}$-saturated C-domain is constitutively bound to RyR2 and the N-domain senses increases in Ca$^{2+}$ concentration, is proposed.

During cardiac excitation, Ca$^{2+}$ entry into the cytoplasm of cardiomyocytes through sarcolemmal voltage-gated Ca$^{2+}$ channels (Ca$^{2+}$,1.2) activates RyR2$^2$ channels in the SR, giving rise to the so-called Ca$^{2+}$-induced Ca$^{2+}$ release (1–3). Ca$^{2+}$ released from the SR eventually leads to increases in cytosolic free Ca$^{2+}$ ([Ca$^{2+}$]$_{cyt}$) throughout the cardiomyocyte, where binding of Ca$^{2+}$ to myofilaments results in contraction (4). RyR2 channels are, however, not only sensitive to [Ca$^{2+}$]$_{cyt}$ but also the SR luminal free Ca$^{2+}$ ([Ca$^{2+}$]$_{SR}$), and both calcium concentrations modulate the activation and termination of Ca$^{2+}$ release (1, 5–9).

In the SR membrane, RyR2s arrange as homotetrameric channels extending into the cytosol, where the interaction with numerous proteins and ligands regulate the Ca$^{2+}$ release activity of the channel (4, 10). Among these RyR2 modulators, CaM is a cytosolic inhibitor of Ca$^{2+}$ release both at diastolic and systolic [Ca$^{2+}$]$_{cyt}$ and may also serve additional regulatory purposes (11–14). CaM is a ubiquitous Ca$^{2+}$ sensing protein in vertebrates and confers its sensing of intracellular Ca$^{2+}$ signals onto a multitude of protein targets, including ion channels and pumps responsible for excitation and contraction in cardiomyocytes (15). Although a cytosolic protein, CaM does...
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In the Ca\textsuperscript{2+} RyR2 around Trp3587 in the CaMBD, and the CaM N-domain RyR1(Lys3614–Pro3640) (PDB ID: 2BCX), differing in one residue from the mutant (CaM E31A/E67A/E104A/E140A, CaM1234) defective likely via the C-domain (18, 19). Also, an engineered CaM stoichiometry of four per channel, primarily via the CaM bind-
tion of RyR2 by the CaM WT (21). Hence, CaM binding to RyR2
demonstrate that both CPVT and LQTS-associated CaM
mutations alter RyR2-mediated Ca\textsuperscript{2+} release in permeabilized cardiomyocytes (15, 31). Conversely, LQTS is mainly associated with dysfunction of sar-colemmal voltage-gated Na\textsuperscript{+}, Ca\textsuperscript{2+}, or K\textsuperscript{+} channels in control of the action potential. Notably the activities of a majority of these channels are regulated by CaM, for example, CaM mutations N97S, D95V, F141L and D129G all confer reduced Ca\textsuperscript{2+}-dependent inhibition of the cardiac L-type voltage-gated Ca\textsuperscript{2+} channel (Ca\textsubscript{\textalpha},1.2) (15, 26, 32).

In this study, we investigated the impact of CaM mutations linked to CPVT (N53I), LQTS (D95V and D129G), or both (N97S) on the CaM-dependent regulation of RyR2 channels. To this end, we monitored the endoplasmic reticulum (ER) Ca\textsuperscript{2+} dynamics in RyR2-expressing HEK293 cells transfected with CaM WT or mutants. The RyR2-mediated Ca\textsuperscript{2+} release in this system is triggered by increasing the Ca\textsuperscript{2+} load in the ER, which mimics the store overload-induced Ca\textsuperscript{2+} release (SOICR) model for CPVT (14, 33). Furthermore, we investigated domain-specific interactions of CaM with Ca\textsuperscript{2+} in the presence of a peptide corresponding to the CaMBD of RyR2. Our results demonstrate that both CPVT and LQTS-associated CaM mutations alter RyR2-mediated Ca\textsuperscript{2+} release.

Experimental Procedures

Plasmid Constructs—Plasmid constructs based on the pMAL vector (New England Biolabs) for recombinant expression and purification of native, full-length CaM were prepared as described previously (1, 22). For expression of CaM variants in HEK293 cells, CaM coding sequences from the pMAL vectors were PCR-amplified and the products ligated into pcDNA3.1 vectors (Invitrogen). Chemically competent Escherichia coli DH5a cells (in-house stock) were transformed with pcDNA3.1 vectors, and overnight cultures were used to prepare purified plasmid preparations using the Qiagen plasmid maxi kit. pcDNA3.1 vectors were eluted in double-distilled water, and Sanger sequencing verified the sequence of CaM encoding inserts in all plasmids.

Model Fitting and Statistical Analysis—All fitting of data to mathematical models and statistical analyses was done using GraphPad Prism 6 for Mac (version 6.0f). Models and statistical method details are described below.
Endoplasmic Reticulum Luminal Ca\(^{2+}\) Imaging of HEK293 Cells Expressing RyR2 during Store Overload-induced Ca\(^{2+}\) Release—Stable expression of murine RyR2 in HEK293 cells co-transfected with plasmids encoding CaM, and the D1ER Ca\(^{2+}\) probe was done as previously described (14). Briefly, D1ER FRET signals reflecting ER luminal [Ca\(^{2+}\)]\(_{\text{free}}\) were monitored for individual cells in an epifluorescent microscope setting with perfusion (6, 14) (Fig. 2A). Each FRET signal trace was used to measure the Ca\(^{2+}\) release properties of the RyR2 channels relative to the ER Ca\(^{2+}\) content: the activation and termination thresholds (Fig. 2A) and their difference, the fractional Ca\(^{2+}\) release. The ER Ca\(^{2+}\) store capacities were calculated as the difference between maximum and minimum FRET signal (\(F_{\text{max}} - F_{\text{min}}\)). Experiments were also done with HEK293 cells

FIGURE 2. Store overload-induced Ca\(^{2+}\) release from RyR2-expressing cells transfected with CaM WT and CPVT-linked CaM mutations. A–C, the FRET signal from the ER luminal D1ER [Ca\(^{2+}\)]\(_{\text{free}}\) indicator oscillates as Ca\(^{2+}\) is released through RyR2. Rectangles and labels indicate the concentrations of Ca\(^{2+}\), tetracaine (RyR2 channel inhibitor), and caffeine ( activator) in the perfusion solution. Stepwise increase in Ca\(^{2+}\) concentration elicited RyR2 SOICR oscillations, tetracaine blocked Ca\(^{2+}\) release filling ER to maximum [Ca\(^{2+}\)]\(_{\text{free}}\) and finally caffeine opened RyR2 channels depleting ER Ca\(^{2+}\). Tetracaine and caffeine were used to establish maximum and minimum ER [Ca\(^{2+}\)]\(_{\text{free}}\) as measured using D1ER (\(F_{\text{max}}\) and \(F_{\text{min}}\)), respectively. The activation and termination thresholds were calculated relative to \(F_{\text{max}}\) and \(F_{\text{min}}\). Example traces for transfection with CaM WT (A), N53I (B), and N97S (C) are shown. D–G, the activation threshold (D), termination threshold (E), fractional release (F), and store capacity (G) averaged from multiple traces are shown as bar graphs. The error bars show S.D., and asterisks indicate significant changes compared with CaM WT (\(p < 0.01\)).
expressing a RyR2 mutant with the CaMBD deleted (RyR2 △CaMBD, murine RyR2 △Lys3583–Phe3663). Measured parameters were compared using one-way analysis of variance with Holm-Sidak multiple comparison test for all possible combinations and with \( p < 0.01 \) (against the CaM WT values) chosen as a conservative measure of significance.

**Protein Expression and Purification**—CaM was expressed from the pMAL (CaM N53I and N97S) or the pET15b (D95V and D129G) vector in *E. coli* Rosetta B cells (EMD Chemicals) or *E. coli* BL21 (DE3) cells (Novagen), respectively, and purified as previously described (1, 22, 23). The identity, purity, and integrity of each protein preparation were confirmed by SDS-PAGE and MALDI-TOF mass spectrometry of trypsin-digested proteins.

**Peptides Corresponding to the CaM Binding Domain of RyR2**—A peptide corresponding to the CaMBD of RyR2 (RyR2(Arg3581–Leu3611), human RyR23581RSKKAVWHKLKSQRKRAVVACFRMAPLYNL3611) was purchased from Peptide 2.0 Inc. (Chantilly, VA) at >98% purity.

**Titration Buffers and Verification of Free Ca\(^{2+}\) Concentrations**—Titration experiments were performed by mixing different volumes of pH- and Ca\(^{2+}\)-buffered solution (50 mM HEPES, 100 mM KCl, 0.5 mM EGTA, and 2 mM nitrilotriacetic acid at pH 7.2 (25 °C)) with the same buffer spiked to 3, 7, or 22 mM CaCl\(_2\) to reach precalculated [Ca\(^{2+}\)]\(_{\text{free}}\) levels (5). In practice, \( \times 1.5 \) concentrated buffer stocks were prepared and proteins, peptide, Ca\(^{2+}\) probes (final concentration, 0.75 \( \mu \)M), and reducing agent (tris(2-carboxyethyl)phosphine; final concentration, 16.5 \( \mu \)M) were added to the double distilled water used for diluting concentrated buffers. The calculated buffer ionic strength, which affects Ca\(^{2+}\) binding to CaM, was stable at 0.15 M. For all titration experiments, the [Ca\(^{2+}\)]\(_{\text{free}}\) was followed by including the Ca\(^{2+}\) probe Fura-2 or Fura-6F in solutions (Invitrogen) and indirectly via measuring of Ca\(^{2+}\) binding to CaM WT (1). Based on these measurements, a 15% error for the [Ca\(^{2+}\)]\(_{\text{free}}\) was included throughout data sets and fitting procedures.

**Titration of CaM/ RyR2(Arg3581–Leu3611) and Free CaM with Ca\(^{2+}\) from 15 to 15 \( \mu \)M Ca\(^{2+}\) in the presence of 16.5 \( \mu \)M RyR2(Arg3581–Leu3611) was titrated with Ca\(^{2+}\) as previously described (1, 5). Briefly, intrinsic protein fluorescence from the N- and C-domain of CaM were measured as partial Phe and Tyr emission spectra, respectively (Table 1), using a spectrofluorometer (HORIBA Jobin Yvon, FluoroMax®-4P) (1, 34, 35). In addition, the Trp fluorescence from the RyR2(Arg3581–Leu3611) peptide was also measured (Table 1). Titrations of free CaM WT, D95V, and D129G with Ca\(^{2+}\) were done by mixing discontinuous titration points in an automated liquid handler (Hamilton, Microlab STARlet) and serially transferring these to a 2-mm cuvette for emission spectra recording. Measurements were done in triplicate with 10 \( \mu \)M CaM. Each of the fractional saturations (\( \gamma \)) for the N- and C-domains of CaM were fitted to the raw fluorescence intensity (FI) signals from the partial Phe and Tyr spectra at 280 and 320 nm, respectively, according to the following,

\[
FI = Y \cdot a + b
\]

(\( \text{Eq. 1} \))

where the constants \( b \) and \( a \) indicate the initial FI and the span in FI from low to high [Ca\(^{2+}\)]\(_{\text{free}}\), respectively. \( \gamma \) is the fractional saturation of the monitored CaM domain binding to two Ca\(^{2+}\) as described by a two-site Adair model (36, 37),

\[
Y = \frac{K_1 \cdot [X] + 2 \cdot K_2 \cdot [X]^2}{2(1 + K_1 \cdot [X] + K_2 \cdot [X]^2)}
\]

(\( \text{Eq. 2} \))

where \( K_i \) is the sum of the microscopic equilibrium constants, and \( K_{2i} \) is the equilibrium constant for the particular domain binding to two Ca\(^{2+}\). The apparent dissociation constants for either domain in the free CaM or in the presence of RyR2(Arg3581–Leu3611) (\( K_{2i}^{\text{bound}} \) and \( K_{2i}^{\text{free}} \)) were calculated as the reciprocal square root of \( K_i \). When CaM binds to RyR2(Arg3581–Leu3611), the peptide Trp fluorescence shows a peak shift from ~350 to 340 nm, and furthermore the FI increases markedly upon Ca\(^{2+}\) binding to CaM (22). The raw FI for Trp fluorescence at 340 nm was also fitted to the model described above. Titration curves were normalized using the fitted \( a \) and \( b \) parameters for figure plotting purposes only. Statistical significances of differences in \( K_{2i} \) were evaluated via nonoverlapping 95% confidence intervals or one-way analysis of variance with Dunnett’s post hoc test against values measured for the CaM WT and \( p < 0.05 \) considered significant. Furthermore, the \( K_{2i} \) values were also used to calculate the mutation-induced change in Gibb’s free energy of Ca\(^{2+}\) binding to the domains of CaM (\( \Delta \Delta G^\circ_{\text{free}} \) and \( \Delta \Delta G^\circ_{\text{bound}} \) respectively) according to the following,

\[
\Delta \Delta G^\circ = -R \cdot T \cdot \ln \left( \frac{K_{2i}^{\text{mutant}}}{K_{2i}^{\text{WT}}} \right)
\]

(\( \text{Eq. 3} \))

using standard conditions of 1 M and 298.15 K (25 °C).

**Results**

**Arrhythmogenic CaM Mutations Decrease the Activation and Termination Thresholds for Spontaneous Ca\(^{2+}\) Release**—Spontaneous Ca\(^{2+}\) release can occur in cardiomyocytes during increased Ca\(^{2+}\) load in the SR lumen. This spontaneous SOICR is arrhythmogenic, because it may lead to delayed afterdepolarizations and triggered activity, a hallmark of CPVT (25, 38). To determine whether CaM mutations affect this arrhythmogenic SOICR, we transfected RyR2-expressing HEK293 cells with CaM and monitored the ER Ca\(^{2+}\) dynamics using a FRET-based ER luminal Ca\(^{2+}\) probe D1ER (14). Perfusion of these transfected cells with increasing extracellular Ca\(^{2+}\) concentrations induced SOICR in the form of ER Ca\(^{2+}\) oscillations (Fig. 2A), as reported previously (14, 39). The oscillating D1ER FRET signal was then used to calculate the ER Ca\(^{2+}\) level required for...
activating SOICR, the activation threshold, and the ER Ca\(^{2+}\) depletion required for terminating SOICR, the termination threshold (Fig. 2A and “Experimental Procedures”). Perfusion with the RyR2 inhibitor tetracaine and then the agonist caffeine established maximum \((F_{\text{max}})\) and minimum \((F_{\text{min}})\) ER Ca\(^{2+}\) content. The ER capacity for storing Ca\(^{2+}\), the store capacity, was calculated as \(F_{\text{max}} - F_{\text{min}}\).

In the SOICR experiments, the CPVT-linked CaM mutation, N53I, lowered the SOICR activation threshold (5%) relative to CaM WT, and so did the CPVT- and LQTS-linked mutation CaM N97S (4%) (Fig. 2, B–D, and Table 2). Similarly, the two LQTS-linked CaM mutations, D95V and D129G, also lowered the SOICR activation threshold by 6 and 4% (Fig. 3, B–D, and Table 2), respectively. The effects on the activation thresholds are modest, however, in cardiomyocytes SR Ca\(^{2+}\) release increases with increasing SR luminal Ca\(^{2+}\) concentrations in a steep and nonlinear fashion (40). Hence, even modest effects of CaM mutations on the response of RyR2 to SR luminal Ca\(^{2+}\) may have major impacts on SR Ca\(^{2+}\) release.

More strikingly, all of these CaM mutations markedly affected the ER Ca\(^{2+}\) level at which SOICR terminated. The CaM mutation N53I decreased the termination threshold by 21% relative to CaM WT (Fig. 2, B and E), and, likewise, the CaM mutations N97S, D95V and D129G also decreased the termination threshold by 30, 33, and 28% (Figs. 2, C and E, and 3, B, C, and E), respectively.

The amount of Ca\(^{2+}\) released from the ER during SOICR was calculated by subtracting the termination threshold from the activation threshold. As shown in Figs. 2 (B–E) and 3 (B–E), the disease-causing CaM mutations decreased the termination thresholds proportionally more than the activation thresholds and hence increased ER fractional Ca\(^{2+}\) release (Figs. 2F and 3F). CaM N53I increased the ER fractional Ca\(^{2+}\) release by 20% relative to the CaM WT, and CaM D95V, D129G, and N97S increased this release by 38, 38, and 34%, respectively (Table 2). Interestingly, the effect of CaM-N53I, located in the N-domain of CaM, on the ER fractional release (20%) was significantly less than that of the three CaM mutations located in the C-domain (average 33%), \((p < 0.001\) for each multiple comparison).

Notably, none of these CaM mutations affected the SOICR activation or termination thresholds in HEK293 cells expressing a RyR2 mutant with a deletion of the CaMBD (Fig. 4, A–D). This observation demonstrates that the CaMBD in RyR2, and not secondary effects of CaM overexpression (e.g. altered Ca\(^{2+}\) buffering), mediate the observed effects (Fig. 4, C–E, all individual data sets not shown). Furthermore, the deletion of the CaMBD in RyR2 increased the ER fractional Ca\(^{2+}\) release by 65% relative to that of WT RyR2 (Table 2), whereas the maximum increase in the ER fractional Ca\(^{2+}\) release caused by the CaM mutations was less than 40% (Figs. 2F and 3F and Table 2). It is noteworthy that this comparison clearly shows that the arrhythmogenic CaM mutations did not suppress CaM-mediated inhibition of Ca\(^{2+}\) release to the same extent as ablating the interaction between CaM and RyR2 by deleting the CaMBD in RyR2. In other words, the arrhythmogenic CaMs did interact with and inhibit RyR2, only not to the same extent as the CaM WT.

Ca\(^{2+}\) homeostasis in HEK293 cells differs from that in cardiomyocytes. The [Ca\(^{2+}\)]\(_{\text{cyt}}\) in HEK293 cells before RyR2 Ca\(^{2+}\) release (60 nM) is similar to that of diastolic cardiomyocytes (100 nM), but ER Ca\(^{2+}\) release is unlikely to increase [Ca\(^{2+}\)]\(_{\text{cyt}}\) to the same peak levels as within the cardiomyocyte dyadic clefts during systole (>100 μM) (41, 42). High Ca\(^{2+}\) concentrations mitigate the effects of mutations in the CaM C-domain (see below), and therefore their effect on RyR2 Ca\(^{2+}\) release termination may be less at peak systole [Ca\(^{2+}\)]\(_{\text{cyt}}\) in cardiomyocytes than in the HEK293 cells. However, the CaM mutations will on average lead to a suppressed inhibition of RyR2 by CaM and consequently increase the fractional Ca\(^{2+}\) release by RyR2 \textit{in vivo} (32). No significant differences in the HEK293 cell ER Ca\(^{2+}\) store capacities were observed (Figs. 2–4), and Ca\(^{2+}\) release in cardiomyocytes terminates at ~60% of diastole [Ca\(^{2+}\)]\(_{\text{3pS}}\), similar to the RyR2 termination thresholds in HEK293 cells (Table 2), which supports similar ER and SR luminal [Ca\(^{2+}\)]\(_{\text{free}}\) (7, 8).

Taken together, each of the CaM mutations N53I, N97S, D95V, and D129G conferred a slightly increased propensity for SOICR and reduced the capability for terminating SOICR. The difference in termination threshold and ER fractional Ca\(^{2+}\) release between CaM N53I and the other CaM mutations may support a mechanistically distinct effect of the CaM N53I mutation.

**Arrhythmogenic Calmodulin Mutations Affect Binding of Ca\(^{2+}\) to Calmodulin in the Presence of the RyR2 CaM Binding Domain**—Regulation of RyR2 by CaM critically depends on (a) CaM binding to the CaMBD in RyR2 and (b) Ca\(^{2+}\) binding to CaM (43, 44). Furthermore, this regulation of RyR2 by CaM is dependent on the characteristics of binding of Ca\(^{2+}\) to each of the CaM domains (11, 14, 16, 21). Hence, we investigated whether the mutations would affect Ca\(^{2+}\) binding to CaM in the presence of a RyR2(Arg\(^{3581–3611}\)) peptide corresponding to part of the CaMBD in RyR2. Ca\(^{2+}\) binding was investigated by monitoring changes in CaM protein fluorescence (Phe for the N-domain and Tyr for the C-domain) as Ca\(^{2+}\) was titrated to the CaM-peptide complex (34). Although available from a previous study, Ca\(^{2+}\) binding to CaM in the absence of RyR2(Arg\(^{3581–3611}\)) was measured for D95V and D129G as reported for CaM N53I and N97S to ensure the reliability of comparisons made here (1, 23). Average [Ca\(^{2+}\)]\(_{\text{cyt}}\) varies between ~0.1 and 1 μM in cardiomyocytes during each heart beat, but there is a large spatial heterogeneity of systolic [Ca\(^{2+}\)]\(_{\text{cyt}}\). For example systolic [Ca\(^{2+}\)]\(_{\text{cyt}}\) can exceed 100 μM in the dyadic cleft in vicinity of RyR2 channels (41, 45). Thus, a wide range of Ca\(^{2+}\) concentrations (1 nM to 2 mM) was used in our Ca\(^{2+}\) titration experiments with RyR2(Arg\(^{3581–3611}\)).
The presence of the RyR2(Arg3581–Leu3611) increased the apparent Ca\(^{2+}\)/H11011 affinity of the CaM N-domain and CaM C-domain 20- and 80-fold, respectively (Fig. 5A and Table 3). These increases clearly demonstrate the thermodynamic coupling between the Ca\(^{2+}\)-CaM and the CaM-RyR2(Arg3581–Leu3611) binding events (Table 3) (46–48). Importantly, the presence of RyR2(Arg3581–Leu3611) increased the binding affinities of N-domain and C-domain for Ca\(^{2+}\) differentially, such that the Ca\(^{2+}\)-CaM interaction for both domains occurred in the range 0.001–6 \(\mu\)M \([\text{Ca}^{2+}]_{\text{free}}\) with an app\(K_D\)bound of 0.8 \(\mu\)M. This is 8-fold higher than the 0.1 \(\mu\)M \([\text{Ca}^{2+}]_{\text{cys}}\) at diastole in cardiomyocytes (Fig. 5A and Table 3) and within \([\text{Ca}^{2+}]_{\text{cyt}}\) range of the diastole to systole cycling. On the other hand, Ca\(^{2+}\) binding to the C-domain in the presence of RyR2(Arg3581–Leu3611) showed a much higher binding affinity with an app\(K_D\)bound of 0.03 \(\mu\)M, i.e. 3-fold lower than the diastolic \([\text{Ca}^{2+}]_{\text{cyt}}\). This suggests that the C-domain will be nearly Ca\(^{2+}\)-saturated (>90%) at 0.1 \(\mu\)M \([\text{Ca}^{2+}]_{\text{free}}\). In other words, these results imply that the...
C-domain of CaM in complex with RyR2 is inherently Ca\(^{2+}\)- loaded at diastolic levels of \([Ca^{2+}]_{\text{cyt}}\). Conversely, the N-domain of CaM in the same protein is poised for sensing increases in \([Ca^{2+}]_{\text{cyt}}\). Hence, the N-domain but not the C-domain will switch between apo and Ca\(^{2+}\)-loaded states during diastole to systole cycles and alter its interactions with RyR2.

An additional observation was made during the titrations of the CaM-peptide complex with Ca\(^{2+}\). During the titrations, we also monitored the change in RyR2(Arg3581–Leu3611) Trp fluorescence (data not shown), which coincided with the change in Tyr fluorescence from the CaM C-domain, but not with the change in Phe fluorescence from the CaM N-domain. This strongly supports that the C-domain of CaM WT binds around RyR2 Trp3587 and its Ca\(^{2+}\) binding induces a structural shift of the C-domain position on RyR2(Arg3581–Leu3611) as is also hinted by previous studies (18, 19, 22).

The CPVT- and/or LQTS-linked CaM mutations differentially perturbed this domain-specific interaction of Ca\(^{2+}\) with CaM. The CPVT-linked CaM-N53I mutation slightly decreased (app\(K_D^{\text{bound}}\) 1.2 \(\mu M\)) the Ca\(^{2+}\) affinity of the N-domain in the presence of RyR2(Arg3581–Leu3611) and showed no measurable effect on Ca\(^{2+}\) binding to the C-domain (Fig. 5B and Table 3). Conversely, the CaM-N97S mutation linked to both CPVT- and LQTS, had no measurable effect on Ca\(^{2+}\) binding to the N-domain, but markedly decreased (app\(K_D^{\text{bound}}\) 0.15 \(\mu M\)) the Ca\(^{2+}\) affinity of the C-domain (Fig. 5C and Table 3).

The LQTS-linked CaM mutations, D95V and D129G, both increased (app\(K_D^{\text{bound}}\) 0.48 and 0.22 \(\mu M\)) the Ca\(^{2+}\) affinity of the N-domain in the presence of RyR2(Arg3581–Leu3611) and both also markedly decreased the Ca\(^{2+}\) affinity of the C-domain (app\(K_D^{\text{bound}}\) 0.14 and 4 \(\mu M\)) (Fig. 4, D and E, and Table 3). The effect of the D129G mutation was so marked that the Ca\(^{2+}\) affinity of the C-domain was weaker than that of the N-domain. This suggests that the N-domain of CaM D129G may bind the CaMBD in RyR2 at a lower Ca\(^{2+}\) concentration than the C-domain. Our data support the notion that mutations in the CaM C-domain (D95V, N97S, and D129G) all markedly perturb the interaction between Ca\(^{2+}\) and CaM bound to the CaMBD in RyR2. Because CaM will bind to the CaMBD even in the absence of Ca\(^{2+}\), the reduction in C-domain Ca\(^{2+}\) affinity for the mutants may result in binding to the CaMBD in RyR2 in a partially saturated or even Ca\(^{2+}\)-unbound form (18). The small...
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FIGURE 5. Domain-specific titration curves for Ca$^{2+}$ binding to CaM in the presence of RyR2(Arg$^{3581}–$Leu$^{3611}$). A–E, CaM/RyR2(Arg$^{3581}–$Leu$^{3611}$) (black) Ca$^{2+}$ titration points for CaM N-domain (circles) and C-domain (squares) were fitted with a two-site Adair model (dashed and solid, respectively), and normalized $Y$ is plotted as a function of $[$Ca$^{2+}]_{\text{free}}$. The dotted vertical line indicates the cardiomyocyte diastolic equivalent 0.1 mM [Ca$^{2+}]_{\text{free}}$. Also in A, Ca$^{2+}$ titration of free CaM WT is shown in gray (1), illustrating the thermodynamic coupling of CaM binding Ca$^{2+}$ and RyR2(Arg$^{3581}–$Leu$^{3611}$). In B–E, gray lines represent the Adair fits for CaM WT/RyR2(Arg$^{3581}–$Leu$^{3611}$) binding Ca$^{2+}$ to illustrate the effects of CaM mutations. $F$ shows the quantified mutation effect ($\Delta G^o_{\text{bound}}$) on Ca$^{2+}$ binding to CaM in the presence of RyR2(Arg$^{3581}–$Leu$^{3611}$). Error bars indicate S.D., and asterisks indicate statistical significant differences ($p < 0.05$).

TABLE 3
Measured thermodynamic parameters for Ca$^{2+}$ binding to CaM

|          | appK$^b_{\text{bound}}$ | appK$^f_{\text{free}}$ | $\Delta G^o_{\text{bound}}$ | $\Delta G^o_{\text{free}}$ |
|----------|-------------------------|-------------------------|-----------------------------|-----------------------------|
| N-domain |                         |                         |                             |                             |
| WT       | 0.78 16                 |                         |                             |                             |
| N53I     | 1.21 19                 |                         | 2.1                         | 1.1                         |
| N97S     | 0.71 16                 |                         | −0.5                        | 0.1                         |
| D95V     | 0.48 3.2                |                         | −2.5                        | −6.4                        |
| D129G    | 0.22 13                 |                         | −6.4                        | −1.9                        |
| C-domain |                         |                         |                             |                             |
| WT       | 0.03 2.5                |                         |                             |                             |
| N53I     | 0.03 2.3                |                         | −0.3                        | −0.3                        |
| N97S     | 0.15 10.4               |                         | 8.4                         | 7.2                         |
| D95V     | 0.14 31                 |                         | 8.3                         | 13.2                        |
| D129G    | 4.0 84                  |                         | 25.3                        | 18.2                        |

increase in the Ca$^{2+}$ affinity of the N-domain in the presence of RyR2(Arg$^{3581}–$Leu$^{3611}$), as conferred by the LQTS-linked CaM mutations D95V and D129G, suggests that the N-domain CaM mutations will still respond to an increase in [Ca$^{2+}]_{\text{cyt}}$ from diastole to systole. In contrast to the marked perturbations of Ca$^{2+}$ binding by the CaM C-domain mutations, the CPVT-linked CaM N53I mutation showed only a small decrease in the CaM N-domain affinity for Ca$^{2+}$ in the presence of RyR2(Arg$^{3581}–$Leu$^{3611}$), yet a substantial effect on the interaction with RyR2 is expected because there is a substantial effect on SOICR.

Discussion
The domain-specific affinities of CaM for Ca$^{2+}$ in the presence of the RyR2(Arg$^{3581}–$Leu$^{3611}$) offer novel insight into the distinct roles of the two domains of CaM in regulating the Ca$^{2+}$ release from RyR2 (Fig. 6, A–C). Our results support an interaction between CaM and the CaMBD in RyR2 where the Ca$^{2+}$-saturated C-domain of CaM is constitutively tethered around RyR2 Trp$^{3587}$ even at the low diastole level of 0.1 mM Ca$^{2+}_{\text{free}}$ (Fig. 6B) and thus not releasing Ca$^{2+}$ during cardiac contraction cycles (Fig. 6, B and C). This implies a critical role for Ca$^{2+}$ binding to the C-domain in inhibiting Ca$^{2+}$ release from RyR2. In line with this view, Tian et al. (14) showed that a CaM double mutant (D93A/D129A) with loss of C-domain Ca$^{2+}$ binding is defective in regulating the termination of RyR2 SOICR. Both the work of Tian et al. and this study also demonstrate that the interaction between the Ca$^{2+}$-unbound CaM C-domain with the CaMBD in RyR2 inhibits Ca$^{2+}$ release (Fig. 6A), but not to nearly the same extent as the Ca$^{2+}$-saturated C-domain. Thus, the novel observation in this study is that the CaM C-domain under physiologically relevant conditions binds to the RyR2 CaMBD in a Ca$^{2+}$-saturated form.

The pivotal role of the tethered CaM C-domain for inhibiting RyR2 Ca$^{2+}$ release is consistent with studies showing that >70% of RyR2 channels in intact cardiomyocytes have CaM bound and that dissociation of CaM leads to excessive Ca$^{2+}$ release through RyR2 channels (13, 43, 49, 50). In addition, several studies show that single mutations in the binding site for the CaM C-domain in the CaMBD of RyR2 (W3587A or L3591D) decrease the affinity for CaM to RyR2 at 0.4 and 1 nM [Ca$^{2+}]_{\text{free}}$ and that this decrease also leads to impaired inhibition of RyR2-mediated Ca$^{2+}$ release (14, 16, 44, 51).

With the region of the RyR2 CaMBD around Trp$^{3587}$ occupied by the C-domain of CaM, the binding site for the N-domain would be adjacent and likely covering RyR2 Phe$^{3603}$, similar to the structure of CaM binding to the CaMBD in RyR1
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In contrast to the C-domain, our results suggest that the N-domain is Ca\(^{2+}\)-unbound at the diastolic level of [Ca\(^{2+}\)]\(_{\text{cyt}}\), but binds Ca\(^{2+}\) upon increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) above the diastolic level. Hence, the N-domain appears to cycle between the apo and Ca\(^{2+}\) saturated states during a heartbeat, thereby alternating between low and high affinity for binding to the RyR2 CaMBD. Accordingly, our data suggest that the CaM N-domain is a sensor of [Ca\(^{2+}\)]\(_{\text{cyt}}\) for RyR2 channels, albeit with a so far unknown function. Conversely, the CaM C-domain may not be a Ca\(^{2+}\) sensor because of its Ca\(^{2+}\)-saturated state at physiological [Ca\(^{2+}\)]\(_{\text{cyt}}\), when bound to the RyR2 CaMBD. Noteworthy, several Ca\(^{2+}\) binding proteins that interact with RyR2, as well as the endogenous RyR2 sensor of [Ca\(^{2+}\)]\(_{\text{cyt}}\), all contribute to the response of the RyR2 macro complex to [Ca\(^{2+}\)]\(_{\text{cyt}}\) in vivo (52, 53). We further propose that the Ca\(^{2+}\)-unbound N-domain interacts with regions of RyR2 outside the canonical CaMBD and then binds in the vicinity of Phe\(^{3603}\) upon cardiomyocyte excitation (Fig. 6, B and C) (18, 20). This proposed interaction would be consistent with the CaMBD in RyR2 not being the only RyR2 region reported to interact with CaM (11, 18, 54, 55). Moreover, at low [Ca\(^{2+}\)]\(_{\text{free}}\), the N-domain may interact with a region in the skeletal muscle SR Ca\(^{2+}\) release channel, ryanodine receptor (RyR1) that is noncontiguous with the RyR1 CaMBD (18–20, 56). On the other hand, cryo-electron microscopy models of RyR2 channels indicate that CaM does not change position on RyR2 when comparing samples prepared in low and high Ca\(^{2+}\) (55, 57). However, because activation of the RyR2 channel involves substantial movements in the channel structure, it cannot be ruled out that the N-domain may bind different RyR2 regions in the open and closed channel, respectively, without CaM markedly shifting position.

In summary, we propose that the interaction between CaM and RyR2 involves constitutive tethering of the Ca\(^{2+}\)-saturated CaM C-domain to the CaMBD in RyR2, which serves to inhibit Ca\(^{2+}\) release by stabilizing the RyR2 channel closed state throughout Ca\(^{2+}\) release (14, 21, 43, 58). In our model, N-domain binding to RyR2 is a Ca\(^{2+}\) sensing step that responds to increased [Ca\(^{2+}\)]\(_{\text{cyt}}\) upon cardiomyocyte excitation. However, determining the exact function of Ca\(^{2+}\) binding to CaM N-domain in regulating RyR2 Ca\(^{2+}\) release will require further investigation.

Ca\(^{2+}\) binding to the CaM N-domain does not appear to affect the SOICR activation and termination thresholds of RyR2 Ca\(^{2+}\) release, because no effect was detected in experiments using a CaM mutant with dramatically decreased N-domain Ca\(^{2+}\) affinity (CaM D20A/D56A) (14). Also, in the current study only minor effects of the CaM N53I mutation on the binding of Ca\(^{2+}\) to the N-domain in the presence of RyR2(Arg\(^{3581}\)-Leu\(^{3611}\)) were observed (see below). The binding of Ca\(^{2+}\) to the N-domain of CaM, when CaM is tethered to RyR2, may instead be part of inhibiting Ca\(^{2+}\)-induced Ca\(^{2+}\) release during the high systolic [Ca\(^{2+}\)]\(_{\text{cyt}}\). Alternatively, Ca\(^{2+}\) binding to the N-domain could be part of the triggering mechanism for the activation of RyR2 channel Ca\(^{2+}\) release during Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Both of these functions would be in combination with endogenous cytosolic and SR luminal Ca\(^{2+}\) sensors in RyR2 (52, 59). An inhibiting or facilitating function of CaM on Ca\(^{2+}\)-induced Ca\(^{2+}\) release would not be detected in the SOICR experiments performed in this study (6, 14).

Our results support the notion that arrhythmogenic CaM mutations adversely affect the native interaction between CaM and RyR2. We show that the C-domain mutations (D95V, N97S, and D129G) perturb the interactions between CaM, Ca\(^{2+}\) and the CaMBD in RyR2. Notably, this perturbation occurs within physiological relevant Ca\(^{2+}\) concentrations. We suggest that at diastole [Ca\(^{2+}\)]\(_{\text{cyt}}\), CaM with either of these C-domain mutations will bind to the RyR2 CaMBD with the CaM C-domain in a partially saturated or Ca\(^{2+}\)-unbound form as opposed to the native Ca\(^{2+}\)-saturated state (transition from Figs. 6B to 6A). This in turn leads to an insufficient inhibition of the RyR2 Ca\(^{2+}\) release during SOICR in intact cells, similar to the aberrant regulation of RyR2 by CaM(D1234) and CaM D93A/D129G mutants (14, 21). In vivo CaM D95V, N97S, and D129G are likely to still tether to RyR2 because each
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mutated CaM retains a significant affinity for intact RyR2 channels, despite reduced affinity for Ca\(^{2+}\) (31).

Interestingly, the N53I mutation in the N-domain of CaM adversely affected the regulation of RyR2-mediated Ca\(^{2+}\) release during SOICR, without any changes to the affinity of the CaM N53I C-domain for binding Ca\(^{2+}\) in the presence of RyR2(Arg\(^{3581}\)–Leu\(^{3611}\)). This strongly suggests that the integrity of the CaM N-domain is important in inhibiting RyR2 Ca\(^{2+}\) release but not dependent on Ca\(^{2+}\) binding to the N-domain, as discussed above (14). In strong support of this hypothesis is that CaM D20A/D56A, with markedly decreased N-domain Ca\(^{2+}\) affinity, does not affect activation or termination thresholds for RyR2-mediated SOICR, whereas CaM N53I does. A likely explanation is that the N53I mutation affects an interaction of the Ca\(^{2+}\)-unbound N-domain of CaM with a region of RyR2 that is outside the CaMBD. The significantly different impact of the CaM N53I mutation on RyR2 Ca\(^{2+}\) release, compared with the C-domain mutations, also supports this notion. Furthermore, we previously found divergent effects of the N53I and N97S mutations on protein properties and the binding of Ca\(^{2+}\) to CaM, which supports different molecular disease mechanisms for the two mutations (1).

Overexpression of CaM in HEK293 cells could theoretically lead to buffering of [Ca\(^{2+}\)]\(_{\text{cyt}}\), which in turn may affect RyR2 Ca\(^{2+}\) release through mechanisms independent of the interaction between CaM and RyR2, and lead to differences in the CaM buffering capacities caused by differences in Ca\(^{2+}\) buffering capacities. This was, however, not the case in the SOICR experiments as judged from three observations: (a) CaM N53I had marked effects on RyR2-mediated Ca\(^{2+}\) release, although it displayed Ca\(^{2+}\) binding properties highly similar to those of CaM WT both in the RyR2-bound and free form (Fig. 2, A and B, and Table 3); (b) CaM WT and CaM D129G had no effect on Ca\(^{2+}\) release from the RyR2 ΔCaMBD mutant, although they exhibited widely different Ca\(^{2+}\) binding properties (Fig. 4, A and B (1, 14); and (c) because the Ca\(^{2+}\) affinity (appK\textsubscript{D,free}) of the CaM C-domain not bound to a protein target is ~25 μM, the buffering capacity of free CaM is highly limited at the ~60 nM [Ca\(^{2+}\)]\(_{\text{cyt}}\) in HEK293 cells.

The combined results of this study show that both CPVT- and LQTS-linked CaM mutations can lead to excessive Ca\(^{2+}\) release from RyR2 channels, primarily from insufficient termination of RyR2-mediated Ca\(^{2+}\) release. Also, the same CaM mutations lower the activation thresholds for SOICR, which would increase the propensity for SOICR in vivo during conditions with increased SR Ca\(^{2+}\) load. Excessive Ca\(^{2+}\) release and leaky RyR2 channels are a well documented hallmark of CPVT-linked RyR2 mutations. It follows that dysregulation of RyR2 likely underlies the disease mechanisms of CaM N53I and N97S mutation-associated CPVT (38). Taken together, we propose that the regulation of RyR2 Ca\(^{2+}\) release is highly sensitive to CaM and that aberrant regulation of RyR2 may be a common component of both CPVT and LQTS arrhythmias caused by CaM mutations.

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