Molecular Basis of Calmodulin Binding to Cardiac Muscle Ca\(^{2+}\) Release Channel (Ryanodine Receptor)*

Calmodulin (CaM) is a ubiquitous Ca\(^{2+}\)-binding protein that regulates the ryanodine receptors (RyRs) by direct binding. CaM inhibits the skeletal muscle ryanodine receptor (RyR1) and cardiac muscle receptor (RyR2) at >1 \(\mu M\) Ca\(^{2+}\) but activates RyR1 and inhibits RyR2 at <1 \(\mu M\) Ca\(^{2+}\). Here we tested whether CaM regulates RyR2 by binding to a highly conserved site identified previously in RyR1. Deletion of RyR2 amino acid residues 3583–3603 resulted in background [\(^{35}\)S]CaM binding previously in RyR1. Deletion of RyR2 amino acid residues 3583–3603 resulted in background [\(^{35}\)S]CaM binding and inhibition of single channel activities by CaM depending on the Ca\(^{2+}\) concentration. An RyR2 mutant, which assessed the effects of 4 amino acid residues that differ between RyR1 and RyR2 in or flanking the CaM binding domain, bound [\(^{35}\)S]CaM and was inhibited by CaM, essentially identical to wild type (WT)-RyR2. Three RyR1 mutants (W3620A, L3624D, F3636A) showed responses to CaM that differed from corresponding mutations in RyR2. The results indicate that CaM regulates RyR1 and RyR2 by binding to a single, highly conserved CaM binding site and that other RyR type-specific sites are likely responsible for the differential functional regulation of RyR1 and RyR2 by CaM.

The ryanodine receptors (RyRs)\(^1\) are Ca\(^{2+}\)-channels that release Ca\(^{2+}\) from an intracellular Ca\(^{2+}\) store, membrane-bound compartment, the endo/sarcoplasmic reticulum (1–3). In mammalian cells, three structurally and functionally related RyR isoforms include RyR1, predominant in skeletal muscle, RyR2, predominant in cardiac muscle, and RyR3, which was initially isolated from brain but is found in many tissues. The three isoforms are comprised of four 560-kDa RyR subunits and three isoforms are comprised of four 560-kDa RyR subunits and three isoforms are comprised of four 560-kDa RyR subunits. The present study was undertaken to identify the CaM binding sites in RyR2. We generated eight RyR2 mutants focusing on the domain corresponding to the apo- and CaM regulatory domain that is highly conserved between RyR1 and RyR2. One mutant assessed the significance of a 1.5,10 CaM recognition motif (5) by substituting a corresponding amino acid residue in RyR1 and RyR2. The results of the study show that (i) like RyR1, RyR2 has a high affinity CaM binding domain that is shared by apoCaM and CaCaM; (ii) deletion of the CaM binding site eliminates inhibition of RyR2 by CaM at submicromolar and micromolar Ca\(^{2+}\); and (iii) corresponding mutations in the CaM binding site differentially alter the CaM binding properties and regulation by CaM of the skeletal and cardiac RyRs.

**EXPERIMENTAL PROCEDURES**

**Materials**—[\(^{3}H\)]Ryanodine was obtained from Perkin Elmer Life Sciences, Tran\(^{35}\)S label was obtained from ICN Radiochemicals (Costa Mesa, CA), unlabeled ryanodine was obtained from Calbiochem, unla- beled CaM was obtained from Sigma. Complete protease inhibitors were obtained from Roche Applied Science, and human embryonic kidney (HEK) 293 cells were obtained from ATCC. Full-length RyR2 cDNA was kindly provided by Dr. Junichi Nakai at National Institute of Physiological Sciences, Okazaki, Japan.

**Construction of Mutant cDNAs**—The full-length rabbit RyR2 cDNA (16) was subcloned into pCIneo. Single and multiple base changes and deletions were introduced by Pfu-turbo polymerase-based chain reaction by using mutagenic oligonucleotides and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The complete mutated sequences were confirmed by DNA sequencing. A 721-bp fragment (ClaI/ScaI II, 10483–11203) subcloned into pBlueScript vector (Stratagene) was used as template for mutagenesis of RyR2. The fragment with the mutation was subcloned back into the original position of RyR2 in two steps: to a vector containing a BbrI/SacI II (residues 5038–11203) fragment and to full-length RyR2 in pCIneo.

The full-length rabbit RyR1 cDNA (ClaI/XhoI) was subcloned into pCMV5 (17). For construction of RyR1-F3638A, a 243-bp RyR1 cDNA fragment (EcoRI/BamHI, 10872–11114) subcloned into pBlueScript vector was used as template for mutagenesis. The mutated sequence was modulated through CaM-dependent protein kinases or by direct binding (5). CaM modulates the RyRs by direct binding since CaM affects channel function in the absence of ATP (6, 7). CaM inhibits all three RyRs at submicromolar Ca\(^{2+}\) concentrations above 1 \(\mu M\), however, differences in the regulation of the RyRs at submicromolar Ca\(^{2+}\) concentrations have been described. At free Ca\(^{2+}\) concentrations below 1 \(\mu M\), CaM has a stimulatory effect on RyR1 and RyR3 channel activities (8–10), whereas RyR2 is unaffected (11) or inhibited (12) by CaM.

Studies investigating the CaM binding properties of the RyRs have focused on RyR1. Trypsin digestion and peptide binding studies indicate that Ca\(^{2+}\)-free CaM (apoCaM) and Ca\(^{2+}\)-bound CaM (CaCaM) bind RyR1 amino acid residues 3614–3643 (13, 14). Mutations in this region resulted in loss of high affinity CaCaM and apoCaM binding and modulation of RyR1 channel activity (15).
confirmed by DNA sequencing and subcloned back into the original position of RyR1 in three steps: the sequence was subcloned back to a vector containing a Poul/Vel (residues 8600–11304) fragment and then back to a vector containing a Poul/Velb (residues 8600–15276) fragment, and finally, mutated RyR1 full-length plasmids were prepared by ligation of two fragments (Cla/I/Vel, Poulb/Vel containing the mutated sequence) and pCMV (Cla/I/Velb1). Nucleotide numbering is as described (16, 17).

Expression of Full-length RyRs in HEK293 Cells—RyR CDNAs were transiently expressed in HEK293 cells transfected with FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Cells were maintained at 37 °C and 5% CO2 in high glucose Dulbecco’s modified eagle medium containing 10% fetal bovine serum and plated the day before transfection. For each 10-cm tissue culture dish, 3.5 ml modified eagle medium containing 10% fetal bovine serum and plated

Ca2

ion concentrations to yield <10 nM, 0.4 μM, or 100 μM free Ca2+. Samples were centrifuged for 30 min at 30,000 × g in a Beckman Airfuge after aliquots were taken for determination of total radioactivity. Radioactivity in the pellet fractions was determined by scintillation counting to obtain bound [35S]CaM. Nonspecific binding of [35S]CaM was determined by incubating equal protein amounts of membranes obtained from vector-transfected HEK293 cells. In parallel experiments, Bmax values of [3H]ryanodine binding were determined by incubating membranes for 4 h at room temperature with a saturating concentration of [3H]ryanodine (40 nM) in 20 mM imidazole, pH 7.0, 0.6 M KCl, 0.15 M sucrose, 1 mM glutathione (oxidized), 20 μM leupeptin, 200 μM Pefabloc, and 1 mM EGTA plus Ca2+ concentrations to yield <10 nM, 0.4 μM, or 100 μM free Ca2+. Samples were centrifuged for 30 min at 30,000 × g in a Beckman Airfuge after aliquots were taken for determination of total radioactivity. Radioactivity in the pellet fractions was determined by scintillation counting to obtain bound [35S]CaM. Nonspecific binding of [35S]CaM was determined by incubating equal protein amounts of membranes obtained from vector-transfected HEK293 cells. In parallel experiments, Bmax values of [3H]ryanodine binding were determined by incubating membranes for 4 h at room temperature with a saturating concentration of [3H]ryanodine (40 nM) in 20 mM imidazole, pH 7.0, 0.6 M KCl, 0.15 M sucrose, 1 mM glutathione (oxidized), 20 μM leupeptin, 200 μM Pefabloc, and 1 mM Ca2+. Specific [3H]ryanodine binding was determined as described above.

Single Channel Recordings—Single channel measurements were performed using the planar lipid bilayer method (18). Planar lipid bilayers contained phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio of 5:3:2 (25 mg of total phospholipid/ml of n-decane). Membrane fractions of HEK293 cells expressing wild type (WT) or mutant RyRs were pretreated for 30 min with 1 μM myosin light chain kinase-derived calmodulin binding peptide to remove endogenous CaM (12). Final peptide concentration was 10 nM following the addition of membranes to the cis (cytosolic) chamber of the bilayer apparatus. A strong dependence of single channel activities on cis Ca2+ concentration indicated that the large cytosolic “foot” region faced the cis chamber of the bilayers. The trans (luminal) side of the bilayer was defined as ground. Measurements were made with symmetrical 0.25 M KCl, 20 mM KPi, pH 7.4, with the indicated concentration of CaCl2. Exogenous CaCl2 was added to the cis solution. Electrical signals were filtered at 2 kHz, digitized at 10 kHz, and analyzed as described (18). Popen values in multichannel recordings were calculated using the equation Popen = Σ iPopen/N, where N is the total number of channels, and Popen is channel open probability of the ith channel.

Biochemical Assays and Data Analysis—Free Ca2+ concentrations were obtained by including in the solutions the appropriate amounts of Ca2+ and EGTA as determined using the stability constants and computer program published by Schoenmakers et al. (19). Free Ca2+ concentrations of ≳1 μM were verified with the use of a Ca2+ selective electrode.

Results are given as means ± S.E. Significances of differences in the data (p < 0.05) were determined using Student’s t test.

RESULTS

Identification of the CaM Binding Site in RyR2—Previous mutagenesis studies identified 2 residues in the RyR1 CaM binding domain that were required for high affinity CaCaM binding and inhibition of RyR1 channel activity. One mutation also resulted in loss of apoCaM binding and activation of RyR1.
Because the region of the RyR1 apoCaM and CaCaM binding site is highly conserved among the RyRs (Fig. 1), we introduced the corresponding mutations into RyR2. Membrane fractions prepared from HEK293 cells transiently expressing WT or mutant RyR2s were incubated with increasing [35S]CaM concentrations in the presence of 10 nM Ca2+ to study apoCaM binding, 0.4 μM Ca2+ (a Ca2+ concentration that results in activation of RyR1 but inhibition of RyR2 by CaM (12)), and 100 μM Ca2+ to study CaCaM binding. Bound [35S]CaM activities were measured using a centrifugation assay. The Bmax values of [3H]ryanodine binding were determined in parallel experiments.

The tetrameric WT-RyR2 bound [35S]CaM in a concentration-dependent manner with 1.9 ± 0.2 [3H]ryanodine binding site at 200 nM CaM and <10 nM Ca2+, which corresponds to 0.5 CaM/RyR2 subunit (Fig. 2), as there is only one high affinity [3H]ryanodine binding site/RyR2 tetramer. The use of 200 nM CaM likely did not result in saturation binding. However, higher CaM concentrations could not be used because these resulted in high background binding levels. The mean numbers of bound [35S]CaM/RyR2 subunit were 0.65 at 200 nM CaM and 0.4 μM Ca2+ and 0.73 at 75 nM CaM and 100 μM Ca2+ (Fig. 2). By comparison, WT-RyR1 bound/subunit ~1 apoCaM at <10 nM Ca2+ and ~1 CaCaM at 100 μM Ca2+ (15). Fig. 2 also shows the [3H]CaM binding properties of RyR2 mutants RyR2-W3587A and RyR2-L3591D that correspond to RyR1-W3620A and RyR1-L3624D. Each RyR1 mutation eliminated CaCaM binding at 100 μM Ca2+ with one of the mutations (RyR1-L3624D) resulting in loss of apoCaM binding at <10 nM Ca2+ (15). RyR2-W3587A retained and RyR2-L3591D lost CaM binding at <10 nM Ca2+ (Fig. 2), as observed previously for the two corresponding RyR1 mutants (15). However, CaM binding to the RyR2 mutants was not eliminated at 100 μM Ca2+ or 0.4 μM Ca2+. Thus, the corresponding RyR1 and RyR2 mutants have similar apoCaM but different CaCaM binding properties.

Preliminary experiments indicated that CaM did not inhibit [3H]ryanodine binding to WT-RyR2 expressed in HEK293 cells (not shown) but was inhibitory in single channel measurements. Membrane fractions prepared from HEK293 cells transiently expressing WT- and mutant RyR2 cDNAs were incorporated into planar lipid bilayers. Single WT- and mutant RyR2 channel activities were recorded with K+ as current carrier in the absence and presence of exogenously added CaM. The use of K+ rather than Ca2+ as current carrier improved control of the cis Ca2+ concentration (20). The functional effects of 50 nM and 1 μM CaM were examined with 0.4 and 2 μM free Ca2+ in the cis (cytosolic) chamber, i.e., at two Ca2+ concentrations where CaM inhibits the native RyR2. Under these conditions, RyR1 is activated by CaM at 0.4 μM and inhibited at 2 μM free Ca2+ (see Fig. 6). A low micromolar Ca2+ concentration of 2 μM was used because CaM is less effective in inhibiting the RyR2 ion channel at elevated Ca2+ concentrations (12).

Figs. 3, A–C, and 4 compare the effects of CaM on single WT-RyR2, RyR2-W3587A, and RyR2-L3591D channels. The averaged channel open probability (Po) of WT-RyR2 in the
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Fig. 4. Channel open probabilities of WT- and mutant RyR2s and RyR1s. Data were obtained as described in legend for Fig. 3. RyR2-4M is RyR2-Q3580V/F3581K/K3596R/A3606T. Data show the relative mean channel open probability ($P_o$) at 0.4 μM Ca$^{2+}$ (top) and 2 μM Ca$^{2+}$ (bottom) of 4–8 single channel recordings for RyR2s, 4–6 single channel recordings for RyR1s.

presence of 0.4 μM free Ca$^{2+}$ was reduced to 35% of the control activity with 50 nM CaM and to 21% with 1 μM CaM in the cis chamber (Fig. 4). In the presence of 2 μM free Ca$^{2+}$, 50 nM and 1 μM CaM were less effective in inhibiting WT-RyR2, reducing $P_o$ to 75 and 54% of the control, respectively.

Single channel recordings showed that at 2 μM Ca$^{2+}$, CaM inhibited RyR2-W3587A (Figs. 3B and 4) and RyR2-L3591D (Figs. 3C and 4) to an extent comparable with WT-RyR2. These results are in agreement with a similar extent of [35S]CaM binding to wild type and the two mutant RyR2s in the presence of 100 μM Ca$^{2+}$. In contrast, CaM failed to inhibit RyR2-W3587A and RyR2-L3591D when the Ca$^{2+}$ concentration was lowered from 2 to 0.4 μM Ca$^{2+}$ despite the fact that both mutants bound CaM at 0.4 μM Ca$^{2+}$.

Both apoCaM and CaCaM binding to RyR2 was eliminated by deleting 21 amino acid residues (amino acids 3583–3603) (Fig. 2) corresponding to the CaM binding domain of RyR1 (Fig. 1). Loss of CaM binding resulted in loss of inhibition of RyR2-Δ3583–3603 activity by CaM in single channel measurements at 0.4 and 2 μM Ca$^{2+}$ (Figs. 3D and 4). The deletion of amino acid residues 3583–3603 did not introduce major global protein conformational changes because the mutant displayed a single channel open probability ($P_o = 0.33 \pm 0.12$ versus $0.39 \pm 0.05$ for WT-RyR2 at 2 μM Ca$^{2+}$), single channel conductance (Fig. 3D), and Ca$^{2+}$ activation/inactivation profile (Fig. 5) not significantly different from RyR2, as determined in single channel and [3H]ryanodine binding measurements, respectively. The results indicate that like RyR1, RyR2 has a single functional CaM binding site.

Role of a 1,5,10 CaM Recognition Motif—RyR2 has a 1,5,10 CaM recognition motif (Val-3599, Phe-3603, Leu-3608) (5) that is conserved in RyR1 (Fig. 1). We assessed the significance of this motif in the regulation of the RyRs by CaM by preparing RyR2-F3603A and corresponding RyR1-F3636A. For RyR2-F3603A, CaM binding was at background levels at <10 nM Ca$^{2+}$ but was present at 0.4 and 100 μM Ca$^{2+}$ (Fig. 2). The results suggest that RyR2-Phe-3603 is required for apoCaM but not for CaCaM binding. The expression level of RyR1-F3636A was too low to determine its CaM binding levels.

Single channel recordings showed that CaM inhibited RyR2-F3603A activity at 2 μM Ca$^{2+}$ but was without a significant effect at 0.4 μM Ca$^{2+}$ (Fig. 4). CaM inhibited WT-RyR1 and RyR1-F3636A single channel activities at >1 μM Ca$^{2+}$ (Fig. 6). A decrease in [3H]ryanodine binding by 1 μM CaM also indicated a decrease in WT and mutant RyR1 activities at >1 μM Ca$^{2+}$ (Fig. 7). At 0.3 μM Ca$^{2+}$ in the presence of 1 mM ATP to increase the otherwise very low channel activities, addition of CaM yielded the expected increase in activity of WT-RyR1 in both assays. In contrast, in the presence of 0.5 μM Ca$^{2+}$, a significant decrease in RyR1-F3636A [3H]ryanodine binding and single channel activities was observed after the addition of 1 μM CaM (although in single channel measurements not at 50 nM CaM). Thus, the loss of CaM modulation of RyR2-F3603A evident at <1 μM Ca$^{2+}$ is no longer present when the corresponding Phe in RyR1 is substituted with Ala. Remarkably, the RyR1 mutation led to inhibition by 1 μM CaM at submicromolar Ca$^{2+}$, as compared with activation of WT-RyR1.

Effects of CaM on Two RyR2 Double Mutations—None of the single site RyR2 mutants described above abolished CaM inhibition of RyR2 at 2 μM Ca$^{2+}$. The effects of two double mutations (W3587A/L3591D, L3591D/F3603A) were therefore determined. Both mutants had low [35S]CaM binding activities at <10 nM and 100 μM Ca$^{2+}$ (Fig. 2). Single channel recordings showed that, consistent with the binding data, addition of 50 nM or 1 μM CaM did not inhibit the single channel activity of either mutant at 0.4 μM Ca$^{2+}$ (Fig. 4). CaM also failed to inhibit RyR2-L3591D/F3603A at 2 μM Ca$^{2+}$ (Fig. 4). In contrast, at 2 μM Ca$^{2+}$, CaM inhibited W3587A/L3591D, notwithstanding that the mutant had low CaM binding levels ([35S]CaM)[3H]ryanodine = 0.25 ± 0.15 at 50 nM CaM, as compared with 1.6 ± 0.2 for WT-RyR2, n = 3).

Role of RyR2-specific 12-amino-acid Residues—RyR2 has a 12-amino-acid insert near the CaM binding site that is absent from RyR1 (Fig. 1). An RyR2 mutant with a deletion of this region (RyR2-Δ3564–3575) was tested as a possible explanation for the differential regulation of RyR2 and RyR1 by CaM at 0.4 μM Ca$^{2+}$. The deletion did not alter apoCaM and CaCaM
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**FIG. 6.** Effects of CaM on single WT-RyR1 and RyR1-F3636A ion channels. Single channel currents were recorded as described in the legend Fig. 3 at −20 mV (downward deflections from closed level, c) in symmetric 0.25 M KCl, 20 mM HEPES, pH 7.4, media with 0.3 μM Ca2+ and 1 mM ATP (left panels) or 2 μM Ca2+ (right panels) in the cis chamber before (top traces) and after the addition of 50 nM CaM (middle traces) and 1 μM CaM (bottom traces). Data of 4–6 single channel recordings are summarized in Fig. 4.

**FIG. 7.** CaM inhibition and activation of [3H]ryanodine binding to WT-RyR1 and RyR1-F3636A. Specific [3H]ryanodine binding to WT-RyR1 and RyR1-F3636A were determined as described under “Experimental Procedures” in presence of 0.3 μM Ca2+ and 1 mM AMP-PCP (a nonhydrolyzable ATP analog) (top) or 25 μM Ca2+ (bottom) in the absence (open bars) or presence (filled bars) of 1 μM CaM. Normalized [3H]ryanodine binding data are the means ± S.E. of 4–5 experiments. *, p < 0.05, as compared with control (−CaM).

One of the mutants (RyR2-W3587A/L3591D) exhibited low CaM inhibition and activation of [3H]ryanodine binding, as compared with control (−CaM).

**Method.** Deletion of amino acid residues 3583–3603 was sufficient to eliminate CaM binding and inhibition of RyR2 channel activity by CaM at submicromolar and micromolar Ca2+ concentrations. Mutagenesis generated four RyR2 single or double mutants in this region that eliminated or greatly reduced apoCaM binding with the double mutants also resulting in loss of or reduced CaM binding levels. Single channel recordings showed that at 0.4 μM Ca2+, RyR2-W3587A, -L3591D, and -F3603A bound CaM but were not inhibited by CaM concentrations as high as 1 μM. On the other hand, RyR2-W3587A/L3591D was inhibited by 50 nM CaM at 2 μM Ca2+ despite a low CaM binding level. Furthermore, an unexpected finding was that corresponding mutations in the CaM binding site affected the CaM binding properties and regulation by CaM of the skeletal and cardiac RyRs differently (Table I).

Functional characterization of WT and mutant RyR2s relied on single channel measurements because none showed CaM-dependent inhibition of [3H]ryanodine binding, an effect also observed for RyR2 purified from cardiac sarcoplasmic reticulum vesicles (12). In contrast, WT-RyR1 (this study) and the purified RyR1 (12) were regulated by CaM in the [3H]ryanodine binding assay. We previously showed that loss of CaM-dependent inhibition of [3H]ryanodine binding to the RyR2 was due to a conformational change in the purified receptor rather than to the removal of a necessary cofactor. Single channel measurements with purified RyR2 showed that CaM inhibition of channel activity was restored with application of a transmembrane potential.

One of the mutants (RyR2-W3587A/L3591D) exhibited low [35S]CaM binding levels but nevertheless was inhibited by 50 nM CaM at 2 μM Ca2+. Retention of CaM inhibition may have resulted from the application of an electrical potential that induced a conformational change associated with increased...
CaM binding. However, this could not be verified because CaM binding to single channels could not be measured.

Secondary structure predictions using PSSIPRED (21) and PSA (22) programs indicate a high α helix probability for the N-terminal and middle portions of RyR1 and RyR2 CaM binding sites. Mutating RyR2-Trp-3587 or RyR1-Trp-3620 to alanine does not change the secondary structure probabilities. Substitution of RyR2-Leu-3591 with aspartic acid and the corresponding substitution in RyR1 may similarly decrease helix probability in the N-terminal portion of the CaM binding domains, whereas the phenylalanine to alanine substitutions may similarly increase the helix probability of the C-terminal portion of RyR1 and RyR2 CaM binding domains (21, 22). It follows that the differential regulation of the three corresponding RyR1 and RyR2 mutants by CaM cannot be explained simply in terms of the CaM binding domain.

The CaM binding region identified in RyR2 is highly conserved in RyR1 with an amino acid identity of >90% as compared with 65% for the full-length RyRs, yet the two receptors are regulated differently by CaM. CaM activates RyR1 at Ca\(^{2+}\) concentrations below 1 μM, whereas channel activity is inhibited by CaM at >1 μM Ca\(^{2+}\). In contrast, RyR2 is inhibited by CaM at Ca\(^{2+}\) concentrations above and below 1 μM. One possibility we considered was that 4 nonidentical amino acid residues in or flanking the CaM binding domain were responsible for the differential regulation of RyR1 and RyR2 by CaM. However, the RyR2 quadruple mutant had [\(^{35}\)S]CaM binding and single channel activities essentially identical to WT-RyR2. A second possibility is that only the C-terminal half of CaM binds to the identified RyR1 CaM binding site (23), allowing the N-terminal half to interact with other receptor type-specific sites and thus to differentially regulate RyR1 and RyR2.

Another possible explanation for the differential regulation of RyR1 and RyR2 by CaM is that the CaM binding domains interact with regions that are specific to RyR1 and RyR2. Such an interdomain interaction could alter the structure of the CaM binding domains. Conformational constraints imposed on the CaM binding domain seem to vary with receptor type and are dependent on Ca\(^{2+}\) concentration, as indicated by the different responses of RyR1 and RyR2 on binding CaM. The differential regulation at <1 μM Ca\(^{2+}\) may be due to CaM binding domain conformations that increase the Ca\(^{2+}\) binding affinity of CaM on binding to RyR2 as compared with RyR1. CaM would inhibit RyR2 below 1 μM Ca\(^{2+}\) because it is present in a Ca\(^{2+}\)-bound form, whereas CaM would activate RyR1 because it remains in its Ca\(^{2+}\)-free form. Ca\(^{2+}\)-dependent changes (between 0.1 and 1 μM Ca\(^{2+}\)) in CaM binding to cardiac sarcoplasmic reticulum vesicles (12) and a change in the Ca\(^{2+}\) affinity of CaM on binding to a peptide from RyR1 (amino acids 3609–3643) (23) have been described.

We previously showed that RyR1-W3620A and RyR1-L3624D eliminated high affinity CaCaM binding and inhibition with one of the mutations (RyR1-L3624D) causing loss of apoCaM binding and loss of activation of RyR1 by CaM (15) (Table I). The corresponding RyR2-W3587A and RyR2-L3591D mutants had comparable retention and loss of apoCaM binding, respectively; however, both mutants failed to transduce CaM binding into a functional effect at 0.4 μM Ca\(^{2+}\), and CaCaM binding to and inhibition of the two RyR2 mutants were not abolished. These results can be best rationalized by the aforementioned interaction of the CaM binding domains with other receptor regions that are specific to RyR1 and RyR2. One mechanism is that a mutation causes a different change in interdomain conformation, resulting in loss of CaM binding in only one of the receptor types (RyR1-W3620A and -L3624D but not RyR2-W3587A and -L3591D at micromolar Ca\(^{2+}\)). A second possible mechanism is that a mutation alters the interaction of the CaM binding domain with its interacting site(s) such that the mutant binds CaM but is not able to transduce the binding step into a functional effect (RyR2-W3587A and -L3591D at 0.4 μM Ca\(^{2+}\)). In contrast, a conformation comparable with WT may be preserved at a different Ca\(^{2+}\) concentration, thus transducing CaM binding into a functional effect (RyR2-W3587A and -L3591D at 2 μM Ca\(^{2+}\)). A Ca\(^{2+}\)-dependent and receptor type-specific functional interaction is also supported by the finding that RyR1-F3636A was inhibited by CaM at 0.4 μM Ca\(^{2+}\) and not activated as WT-RyR1, whereas the corresponding RyR2-F3603A mutation resulted in loss of CaM inhibition of channel activity at 0.4 μM Ca\(^{2+}\).

In summary, RyR1 and RyR2 have a single highly conserved high affinity CaM binding domain that is shared by apoCaM and CaCaM. Studies with the native receptors and site-directed mutagenesis nevertheless reveal major differences in the CaM binding properties and regulation by CaM of the skeletal and cardiac RyRs. We propose that the conserved CaM binding domain of RyRs interacts with RyR1- and RyR2-specific sites in the large channel protein complexes and that, in turn, these interactions have a critical role in transducing the functional effects of CaM. Future experiments are needed to identify the amino acid residues that interact with the CaM binding domains of the massive RyR1 and RyR2 channel complexes.

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**Table I.**

[| CaM Binding | CaM Regulation |
|---|---|
| WT-RyR1 | ++ | ++ | ++ |
| RyR1-W3620A | ++ | ++ | ++ |
| -L3624D | - | - | - |
| -F3636A | ND | ND | ND |
| WT-RyR2 | ++ | ++ | ++ |
| RyR2-W3587A | ++ | ++ | ++ |
| -L3591D | - | - | - |
| -F3603A | - | - | - |
| -W3587A/L3591D | - | - | - |
| -L3591D/F3603A | - | - | - |
| -Δ3583–3603 | ++ | ++ | ++ |
| -Δ3564–3575 | ++ | ++ | ++ |
| RyR2-4M | ++ | ++ | ++ |

* Data are from Ref. 15.
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