Regulation of the RYR1 and RYR2 Ca^{2+} Release Channel Isoforms by Ca^{2+}-Insensitive Mutants of Calmodulin†

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ABSTRACT: Calmodulin (CaM) may function as a regulatory subunit of ryanodine receptor (RYR) channels, modulating both channel activation and inhibition by Ca^{2+}; however, mechanisms underlying differences in CaM regulation of the RYR isoforms expressed in skeletal muscle (RYR1) and cardiac muscle (RYR2) are poorly understood. Here we use a series of CaM mutants deficient in Ca^{2+} binding to compare determinants of CaM regulation of the RYR1 and RYR2 isoforms. In submicromolar Ca^{2+}, activation of the RYR1 isoform by each of the single-point CaM mutants was similar to that by wild-type apoCaM, whereas in micromolar Ca^{2+}, RYR1 inhibition by Ca^{2+}CaM was abolished by mutations targeting CaM's C-terminal Ca^{2+} sites. In contrast to the RYR1, no activation of the cardiac RYR2 isoform by wild-type CaM was observed, but rather CaM inhibited the RYR2 at all Ca^{2+} concentrations (100 nM to 1 mM). Consequently, whereas the apparent Ca^{2+} sensitivity of the RYR1 isoform was enhanced in the presence of CaM, the RYR2 displayed the opposite response (RYR2 Ca^{2+} EC_{50} increased 7−10-fold in the presence of 5 μM wild-type CaM). CaM inhibition of the RYR2 was nonetheless abolished by each of four mutations targeting individual CaM Ca^{2+} sites. Furthermore, a mutant CaM deficient in Ca^{2+} binding at all four Ca^{2+} sites significantly activated the RYR2 and acted as a competitive inhibitor of RYR2 regulation by wild-type Ca^{2+}CaM. We conclude that Ca^{2+} binding to CaM determines the effect of CaM on both RYR1 and RYR2 channels and that isoform differences in CaM regulation reflect the differential tuning of Ca^{2+} binding sites on CaM when bound to the different RYRs. These results thus suggest a novel mechanism by which CaM may contribute to functional diversity among the RYR isoforms.

Muscle contraction is triggered by the rapid release of Ca^{2+} from the sarcoplasmic reticulum (SR)[1] through large (∼2300 kDa), high-conductance channels known as ryanodine receptors (RYRs). Different RYR isoforms, termed RYR1 and RYR2, are expressed in mammalian skeletal muscle and cardiac muscle, respectively (*1*, *2*). Both the RYR1 and RYR2 channel isoforms display a biphasic dependence on Ca^{2+} concentration, indicating that activating as well as inhibitory Ca^{2+} binding sites are present on the tetrameric RYR channel proteins (*3*). Ca^{2+} regulation of the RYRs, in turn, is modulated by various endogenous channel effectors, including ATP and Mg^{2+}. In addition, accumulating evidence suggests that calmodulin (CaM) may function as a Ca^{2+} binding regulatory subunit of the RYRs, potentially modulating both the activation and inhibition of these channels by Ca^{2+} (*4*, *5*).

CaM is a small (∼17 kDa), highly conserved intracellular Ca^{2+} sensor, comprised of four EF hand Ca^{2+} binding sites. Conformational rearrangements within CaM that result from Ca^{2+} binding provide a mechanism for transducing intracellular Ca^{2+} signals to diverse target proteins (*6*), including ion channels (*7*). When CaM is free in solution, the N-terminal Ca^{2+} binding sites (1 and 2) display a lower affinity for Ca^{2+} (K_d ∼10 μM) than the corresponding C-terminal sites (3 and 4, K_d ∼1 μM). However, important changes in the Ca^{2+} binding properties of CaM may result when CaM forms a multimolecular complex with a particular target, such that the affinity, selectivity, and kinetics of Ca^{2+} binding may be "tuned" to a particular biochemical pathway (*8*−*10*). Insights into the role of Ca^{2+} binding to CaM in the regulation of various targets have come from studies using site-directed CaM mutants in which the substitution of a critical glutamate residues within each EF hand selectively reduces Ca^{2+} binding at that site (*10*, *11*). These Ca^{2+}-insensitive CaM mutants thus help to define how the number and position of calcium ions bound determines the functional interactions of CaM with different targets.

The interactions of CaM with intracellular Ca^{2+} release channels are to date best described for the skeletal muscle RYR1 channel isoform, which binds CaM with high affinity

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[1] Abbreviations: RYR, ryanodine receptor; SR, sarcoplasmic reticulum; EC, excitation−contraction; CaM, calmodulin; AMPPCP, adenosine 5′-(β,γ-methylene)triphosphate; PIPES, 1,4-piperazinediethanesulfonic acid; GSH, reduced glutathione; BSA, bovine serum albumin.

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both in the absence and in the presence micromolar Ca^{2+} (*12*, *13*). [^{35}S]CaM binding determinations indicate a single high-affinity CaM binding site per RYR1 subunit (*14*−*16*), and 3D reconstructions show CaM bound within a cleft near the cytoplasmic face of the channel (*17*). The identification of residues critical for CaM binding to the RYR1 suggests that binding sites for apoCaM and Ca^{2+}CaM overlap within the primary sequence of the RYR1 (*14*, *18*, *19*), and this same region may participate in the physical coupling of RYR1 channels with voltage-sensing L-type Ca^{2+} channels on the transverse tubule membrane (*20*). Notably, however, the apoCaM and Ca^{2+}CaM species evoke opposing functional effects on RYR1 channel activity. Thus, apoCaM binding to the RYR1 is associated with channel activation, whereas Ca^{2+}CaM binding results in partial inhibition of the RYR1 when media contain >1 μM Ca^{2+} (*13*, *21*). This conversion of CaM from an activator to an inhibitor of the RYR1 has been attributed to Ca^{2+} binding within the C-terminal domain of CaM (*22*) and accounts for the pronounced sensitization of RYR1 channels to both activation and inhibition by Ca^{2+} in CaM-containing media (*4*, *5*).

Existing evidence suggests that the role of CaM in regulating the cardiac RYR2 isoform may be quite distinct from that suggested for the RYR1 (*15*, *16*), although mechanisms that may underlie these differences remain unclear. Early studies demonstrated Ca^{2+}CaM inhibition of the RYR2 in micromolar Ca^{2+} (*22*, *23*), and subsequent [^{35}S]-CaM binding studies have further indicated a single, high-affinity Ca^{2+}CaM site per RYR2 subunit (*15*, *16*). In submicromolar Ca^{2+}, RYR2 [^{125}I]CaM cross-linking and [^{35}S]-CaM binding were also demonstrated, suggesting that apoCaM might bind to and modulate RYR2 channels in cardiac muscle (*15*, *16*). However, in comparison to the RYR1, the affinity of RYR2 CaM binding was markedly reduced in submicromolar Ca^{2+}. Moreover, whereas apoCaM activated the RYR1, we originally reported that RYR2-mediated SR $^{45}Ca^{2+}$ release and [^{3}H]ryanodine binding were unaffected by CaM in equivalent 100 nM Ca^{2+}-containing media (*16*, *25*). Subsequently, Balshaw et al. (*15*) reported that the RYR2 was inhibited by CaM regardless of Ca^{2+} concentration and therefore concluded that the cardiac RYR2 isoform, in contrast to the RYR1, may be inhibited by both the apo- and Ca^{2+}-bound species. Taken together, these studies suggest that CaM binding may evoke isoform-specific effects on RYR1 and RYR2 channels. Notably, these isoform-specific effects may account for major differences in the Ca^{2+} sensitivities that characterize RYR1 and RYR2 channels in CaM-containing media (*4*, *15*, *16*).

To better understand the mechanisms underlying isoform differences in CaM's functional interaction with RYR1 and RYR2 channels, we examined the regulation of the two channel isoforms by a series of mutant CaMs deficient in Ca^{2+} binding at sites 1, 2, 3, and 4. Our results indicate that Ca^{2+} binding to CaM determines its functional interactions with both the RYR1 and RYR2 isoforms and suggest that isoform differences in channel regulation may be explained by the differential tuning of Ca^{2+} binding sites on CaM when bound to the different RYRs.

EXPERIMENTAL PROCEDURES

Materials. Pigs were obtained from the University of Minnesota Experimental Farm. Tran ^{35}S-label was from ICN Radiochemicals (Costa Mesa, CA). [^{3}H]Ryanodine and $^{45}Ca^{2+}$ were purchased from NEN Life Science Products (Boston, MA). Myosin light chain kinase-derived CaM binding peptide was from Peptide Technologies (Gaithersburg, MD). Adenosine 5′-(β,γ-methylene)triphosphate (AMP-PCP, an ATP analogue) and other reagents were from Sigma (St. Louis, MO).

Isolation of SR Vesicles. Skeletal muscle SR vesicles were isolated from pig longissimus dorsi muscle (*16*, *25*). Briefly, vesicles obtained by differential ultracentrifugation of a muscle homogenate were extracted with 0.6 M KCl and subsequently fractionated on discontinuous sucrose gradients. The terminal cisternae-derived (i.e., "heavy") SR vesicles that band at the 36−45% interface were collected and stored frozen at −70 °C. Cardiac muscle SR vesicles were isolated from pig ventricular tissue (*16*, *25*). Following homogenization in 10 mM $NaHCO_3$, the cardiac membranes were extracted in 0.6 M KCl and 20 mM Tris, pH 6.8, and then resuspended in 10% sucrose and stored frozen at −70 °C. Prior to ligand binding determinations, cardiac SR vesicles were incubated for 30 min in the presence of 1 μM CaM binding peptide and 100 μM Ca^{2+} and then centrifuged through 15% sucrose to ensure removal of endogenous CaM (*15*). All isolation buffers contained a mixture of protease inhibitors (100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin, 1 mM benzamidine, and 0.2 mM phenylmethanesulfonyl fluoride).

CaM Mutagenesis and Purification. Recombinant rat CaM was expressed in *Escherichia coli* using the pET-7 vector (*26*). Mutations targeting each of four EF hand Ca^{2+} binding sites in CaM were introduced using QuikChange mutagenesis kits (Stratagene, La Jolla, CA) and verified by DNA sequencing. Four single-point mutants (termed E1A, E2A, E3A, and E4A) were generated by mutating the conserved bidentate glutamate residue which coordinates Ca^{2+} at the $-Z$ position of each site to alanine. In addition, a CaM mutant termed E1234A was generated in which all four Ca^{2+} binding sites carried this E to A substitution. Following induction with isopropyl β-D-thiogalactopyranoside, wtCaM, E1A, E2A, E3A, and E4A were purified by phenyl-Sepharose chromatography (*26*). The E1234A mutant was subcloned into the pET-30 vector (Novagen), expressed as a His-tagged fusion protein, and purified by nickel affinity chromatography. Decreased Ca^{2+} affinities of mutant CaM N- and C-terminal domains were confirmed in fluorescent measurements using a Perkin-Elmer LS5, as previously described (*27*). Protein concentrations were determined by the bicinchoninic acid procedure (Pierce, Rockford, IL) using bovine brain CaM as the standard.

[^{35}S]CaM Binding. Wild-type CaM subcloned in the pET-7 vector was metabolically labeled with [^{35}S]methionine and purified by phenyl-Sepharose chromatography (*16*). Equilibrium binding of [^{35}S]CaM to SR vesicles (0.05 mg/mL) was determined in media containing 150 mM KCl, 20 mM K-PIPES (pH 7.0), 5 mM GSH, 1 μg/mL aprotinin/leupeptin, and 50 nM [^{35}S]CaM (*15*). Following 2 h incubations at room temperature, vesicles were pelleted at 80000*g*. Bound [^{35}S]-CaM was determined by scintillation counting after solubilization of the pellets in 2% sodium dodecyl sulfate. Nonspecific binding was measured in the presence of 10 μM unlabeled CaM. Data are normalized to maximal binding determinations, obtained in media containing 500 μM Ca^{2+}

and 50 nM [^{35}S]CaM (52.6 ± 3 pmol/mg for skeletal SR vesicles and 20.8 ± 3 pmol/mg for cardiac SR vesicles).

[^{3}H]Ryanodine Binding. Ryanodine selectively binds to RYR channels in the open state, and [^{3}H]ryanodine binding measurements thus provide a useful index of RYR channel activity (*3*, *27*). Skeletal muscle SR vesicle [^{3}H]ryanodine binding was determined following 90 min incubations in 37 °C media containing 120 mM potassium proprionate, 10 mM K-PIPES (pH 7.0), 3 mM Na_2AMPPCP, and 100 nM [^{3}H]-ryanodine (*16*), except as otherwise indicated (Figures 3 and 6). Cardiac SR vesicle [^{3}H]ryanodine binding was determined as described by Balshaw et al. (*15*) in 22 °C media containing 150 mM KCl, 20 mM K-PIPES (pH 7.0), 5 mM GSH, 0.1 mg/mL BSA, 1 μg/mL aprotinin/leupeptin, and 7 nM [^{3}H]-ryanodine. Free Ca^{2+} concentrations were set using Ca^{2+}-EGTA buffers (*29*). Data are expressed as percentages of the maximal [^{3}H]ryanodine capacity of SR vesicle preparations, as estimated in media containing 600 mM KCl, 6 mM Na_2ATP, and 100 μM Ca^{2+}. Nonspecific binding was measured in the presence of 20 μM nonradioactive ryanodine. All assays were performed in duplicate and were repeated using at least three different SR vesicle preparations. EC_{50} values and Hill coefficients (n_H) for CaM activation and inhibition of [^{3}H]ryanodine binding are based on fits to the Hill equation (SigmaPlot Software, Chicago, IL). Analyses of the Ca^{2+} dependence of [^{3}H]ryanodine binding are based on fits to a biphasic Hill equation, assuming the presence of both a high-affinity Ca^{2+} activation site and a low-affinity inhibitory site (*30*).

$^{45}Ca^{2+}$ Efflux. SR vesicle $^{45}Ca^{2+}$ release was determined essentially as described (*16*). Vesicles passively loaded with 5 mM $^{45}Ca^{2+}$ (±5 μM CaM) were placed on the side of a polystyrene tube that contained 150 mM KCl, 20 mM K-PIPES (pH 7.0), and 300 nM ionized Ca^{2+} (buffered with EGTA), with or without 5 μM CaM, as indicated in Figure 5A. Ca^{2+} release was initiated by rapid mixing and stopped after 1−3 s by rapid dilution into a release inhibiting media (150 mM KCl, 20 mM K-PIPES, pH 7.0, 10 mM EGTA, 5 mM $MgCl_2$, and 20 μM ruthenium red) and then rapidly collected on 0.45 μm glass-fiber filters. Data are presented as percentages of the total loaded $^{45}Ca^{2+}$ that was released during 10 s incubations in a release medium that promotes maximal RYR activation (*16*).

RESULTS

Regulation of the Skeletal Muscle RYR1 Isoform by Single-Point Mutants of CaM. To investigate structural determinants of CaM activation and inhibition of the two RYR channel isoforms, single E to A substitutions were introduced into each of the four Ca^{2+} binding sites of mammalian CaM. Functional interactions of these mutant CaMs with the RYRs were then characterized through measurements of SR vesicle [^{3}H]ryanodine binding. Initial experiments characterized interactions of the mutant CaMs with the skeletal muscle RYR1 in 37 °C media which contained 100 nM Ca^{2+} and 3 mM AMPPCP, to favor apoCaM activation of the channel (*16*). In these media, wtCaM increased [^{3}H]ryanodine binding to skeletal muscle SR vesicles approximately 6-fold (Figure 1A; EC_{50} = 38.4 ± 6 nM). Each of the four mutant CaMs also activated skeletal muscle SR [^{3}H]ryanodine binding, and both the extent and concentration dependence of activation

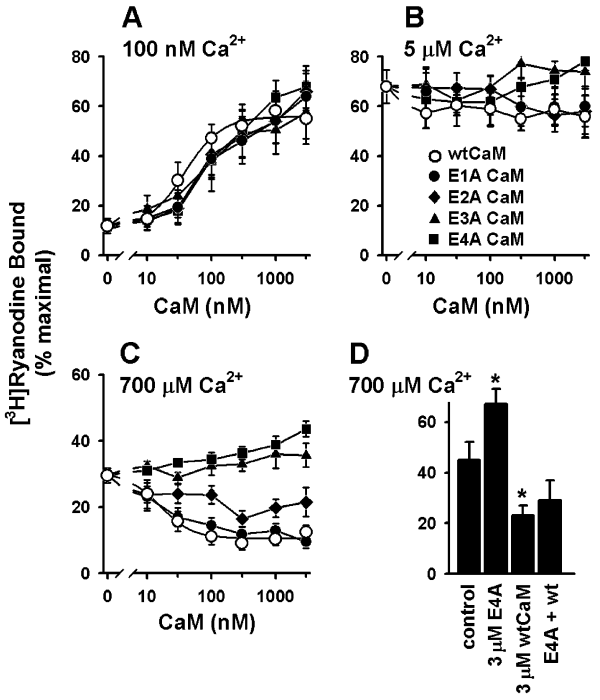

FIGURE 1: Activation and inhibition of the skeletal muscle RYR1 by CaM and CaM mutants. [^{3}H]Ryanodine binding to skeletal muscle SR vesicles was determined in 37 °C media containing 120 mM potassium propionate, 10 mM K-PIPES (pH 7.0), 3 mM Na_2-AMPPCP, and the indicated concentrations of Ca^{2+} (buffered with EGTA). Data are means ± SE from three to six experiments. (*) Significantly different than control value in the absence of CaM.

by the mutant CaMs were similar to wtCaM. Results in Figure 1A thus demonstrate that the single E to A mutations targeting each of CaM's four Ca^{2+} binding sites had little or no effect on RYR1 activation by apoCaM.

In the presence of 5 μM Ca^{2+}, basal levels of skeletal muscle SR [^{3}H]ryanodine binding were increased to approximately 70% of maximal (Figure 1B), and neither wtCaM nor the mutant CaMs significantly altered [^{3}H]-ryanodine binding in these media. However, when Ca^{2+} was further increased to 700 μM Ca^{2+} (Figure 1C), wtCaM inhibited skeletal muscle SR vesicle [^{3}H]ryanodine binding by one-third (IC_{50} = 16.5 ± 6 nM). The E1A CaM mutant also inhibited [^{3}H]ryanodine binding in 700 μM Ca^{2+}. Inhibition was reduced, however, by the single-point mutations that targeted Ca^{2+} sites 2, 3, or 4, with the C-terminal mutations showing greater effects. The rank order of the different CaMs in inhibiting the RYR1 was therefore wtCaM ≅ E1A > E2A > E3A > E4A. Subsequent experiments further documented that the E4A CaM significantly increased skeletal muscle SR vesicle [^{3}H]ryanodine binding in 700 μM Ca^{2+} (1.5-fold increase in the presence of 3 μM E4A CaM), whereas the same concentration of wtCaM reduced [^{3}H]-ryanodine binding by half (Figure 1D). Moreover, wtCaM (i.e., Ca^{2+}CaM) effectively blocked activation by the E4A CaM. This result is therefore consistent with the likelihood that wild-type Ca^{2+}CaM and the E4A mutant competed at the same or overlapping sites on the RYR1 to evoke opposing effects on channel activity. Taken together, the results in Figure 1 indicate that the mutations targeting single CaM Ca^{2+} sites selectively altered the conversion of CaM from an activator to an inhibitor and thereby reduced or abolished Ca^{2+}CaM inhibition of the RYR1 isoform.

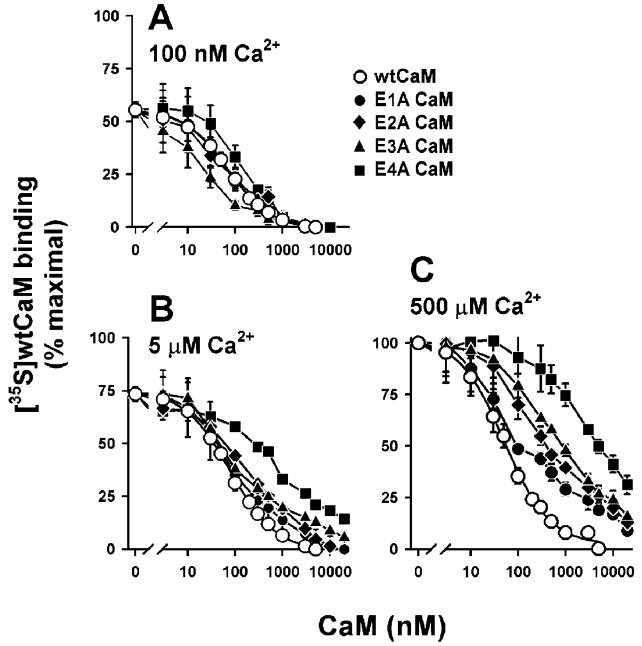

FIGURE 2: Inhibition of [^{35}S]CaM binding to the skeletal muscle RYR1 by CaM and CaM mutants. [^{35}S]CaM binding to skeletal muscle SR vesicles (0.05 mg/mL) was determined in media containing 150 mM KCl, 20 mM K-PIPES (pH 7.0), 5 mM GSH, and 50 nM [^{35}S]CaM. Data are means ± SE from three to five experiments.

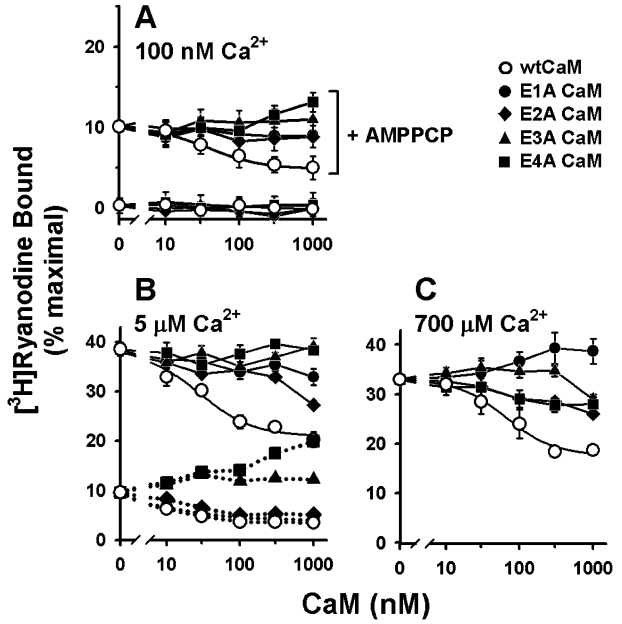

FIGURE 3: Regulation of the cardiac RYR2 isoform by CaM and CaM mutants. [^{3}H]Ryanodine binding to cardiac SR vesicles was determined at room temperature in media containing 150 mM KCl, 20 mM K-PIPES (pH 7.0), 5 mM GSH, and 0.1 mg/mL BSA. Na_2-AMPPCP (2 mM) was included as indicated (dotted lines) to boost the [^{3}H]ryanodine binding signal in the presence of 100 nM Ca^{2+}. Data are means ± SE from three to five experiments.

The RYR1 represents the major CaM binding protein in our skeletal muscle SR membranes (*12*, *16*), and determinations of [^{35}S]CaM binding to SR vesicles therefore provide a useful approach toward defining the relationship between binding at RYR1 CaM sites and the modulation of RYR1 activity. The relative affinities of the mutant CaMs for the RYR1 were investigated by characterizing the inhibition of [^{35}S]wtCaM binding to skeletal muscle SR vesicles. In media containing 100 nM Ca^{2+}, SR [^{35}S]wtCaM binding was fully inhibited by each of the four single-point mutant CaMs (Figure 2A), and the concentration dependence of inhibition was similar to that of the unlabeled wtCaM ($IC_{50} = 67 \pm 9$ nM for wtCaM). When the Ca^{2+} concentration was increased to either 5 μM (Figure 2B) or 500 μM (Figure 2C), the inhibition of [^{35}S]wtCaM binding by the mutant CaMs decreased in comparison to that by unlabeled wtCaM. None of the mutant CaMs completely inhibited SR vesicle [^{35}S]-wtCaM binding in 500 μM Ca^{2+}; however, the rank order effectiveness of the different CaMs suggested by these data was E1A > E2A ≥ E3A > E4A. These results thus indicate that reduced RYR1 inhibition by the mutant CaMs (Figure 1) was associated with a reduced affinity for RYR1 Ca^{2+}-CaM sites.

Regulation of the Cardiac RYR2 Isoform by Single-Point Mutants of CaM. Balshaw et al. (*15*) reported that the cardiac RYR2 channel was inhibited by CaM throughout the physiologic range of Ca^{2+} concentration and concluded that both the apo- and Ca^{2+}-bound species may act to inhibit this isoform. To further investigate the possibility that RYR2 inhibition by CaM may be independent of Ca^{2+} binding to CaM, we therefore examined the modulation of RYR2 [^{3}H]-ryanodine binding by the mutant CaMs, adopting the experimental conditions described in the Balshaw et al. study (Experimental Procedures). Under these conditions, RYR2 channel activation requires [Ca^{2+}] >100 nM. Accordingly, our initial determinations showed that [^{3}H]ryanodine binding to cardiac SR vesicles was similar to background levels in the presence of 100 nM Ca^{2+} (Figure 3A, solid lines). However, [^{3}H]ryanodine binding was increased to ~10% of maximal when the low Ca^{2+} media were supplemented with the RYR activator AMPPCP (2 mM; dotted lines in Figure 3A). In these media, cardiac SR vesicle [^{3}H]ryanodine binding was significantly inhibited by the addition of wtCaM ($p < 0.05$ in the presence of ≥300 nM wtCaM). Thus, whereas wtCaM activated the RYR1 in 100 nM Ca^{2+} (Figure 1A), the RYR2 displayed the opposite response. The wtCaM also inhibited RYR2 [^{3}H]ryanodine binding both in 5 μM Ca^{2+} (Figure 3B; $IC_{50} = 39 \pm 11$ nM) and in 700 μM Ca^{2+} (Figure 3C; $IC_{50} = 65 \pm 17$ nM). These results are therefore consistent with those of Balshaw et al. (*15*) in indicating an inhibitory action of CaM on the RYR2 at all physiologic Ca^{2+} concentrations.

The results in Figure 3 further demonstrate, however, that the inhibitory action of CaM on the RYR2 was abolished by the single-point mutations targeting Ca^{2+} binding to CaM. Thus, at each of the three different Ca^{2+} concentrations examined, inhibition of [^{3}H]ryanodine binding by each of the four single-point CaM mutants was reduced relative to that by wtCaM. By comparison, a reexamination of RYR1 regulation by the mutant CaMs in these same media (Figure 3B, dotted lines) confirmed that inhibition of this isoform was selectively abolished by the E3A and E4A mutations. Our results thus suggest that whereas CaM inhibition of the RYR1 may be critically dependent on Ca^{2+} binding at CaM's C-terminal, CaM inhibition of the RYR2 may depend on Ca^{2+} binding at both N- and C-terminal sites. Taken together, the results in Figure 3 indicate that although wtCaM inhibited the RYR2 in nanomolar as well as in micromolar Ca^{2+}, this inhibition may nonetheless be attributed to Ca^{2+}CaM and not to the apoCaM species.

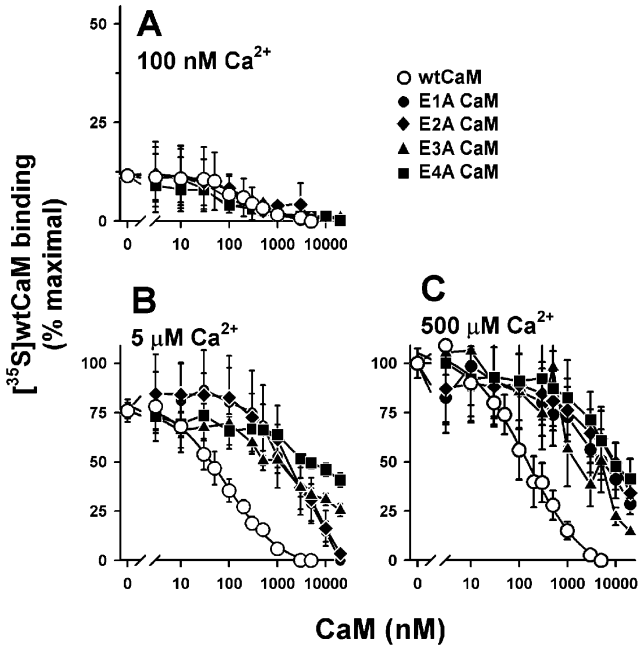

FIGURE 4: Inhibition of [^{35}S]CaM binding to the cardiac RYR2 by CaM and CaM mutants. [^{35}S]CaM binding to cardiac muscle SR vesicles (0.05 mg/mL) was determined in media containing 150 mM KCl, 20 mM K-Pipes, pH 7.0, 5 mM GSH, and 50 nM [^{35}S]-CaM. Data are means ± SE from four to five experiments.

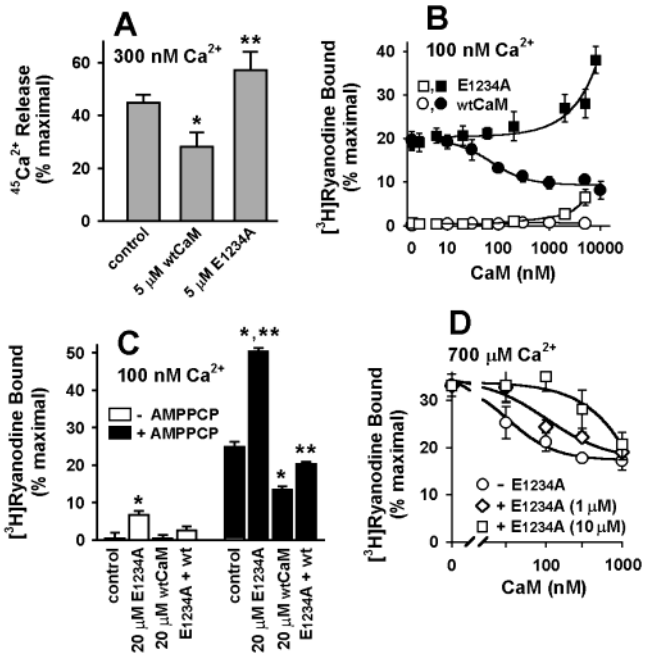

FIGURE 5: The E1234A CaM mutant activates the cardiac RYR2 isoform and competitively inhibits regulation by wild-type Ca^{2+}-CaM. Cardiac SR vesicle $^{45}Ca^{2+}$ release (panel A) and [^{3}H]-ryanodine binding (panels B−D) were determined as described in Experimental Procedures in media containing 150 mM KCl and 20 mM K-PIPES (pH 7.0). Na_2AMPPCP (2 mM) was included as indicated by filled symbols and bars in panels B and C. Data are means ± SE from three to five experiments. (*) Significantly different than the corresponding control value in the absence of CaM; (**) significantly different than the corresponding value in the presence of wtCaM.

Previous reports have indicated that the RYR2 comprises ≥50% of Ca^{2+}CaM binding sites in cardiac SR vesicle preparations and that the affinity of RYR2 [^{35}S]wtCaM binding is reduced 5−20-fold in nanomolar as compared to micromolar Ca^{2+} (*15*, *16*). Accordingly, in media containing 100 nM Ca^{2+}, cardiac SR vesicles displayed low levels of [^{35}S]wtCaM binding, and each of the mutant CaMs completely inhibited [^{35}S]wtCaM binding (Figure 4A). Cardiac SR vesicle [^{35}S]wtCaM binding was increased 7−9-fold in the presence of 5 and 500 μM Ca^{2+}, respectively (Figure 4B,C), and in these media the mutant CaMs were less effective than the unlabeled wtCaM in inhibiting [^{35}S]wtCaM binding. The C-terminal mutant CaM E4A was least effective in inhibiting cardiac SR [^{35}S]wtCaM binding in 5 μM Ca^{2+}, whereas in 500 μM Ca^{2+} the partial inhibition of [^{35}S]wtCaM binding by the four mutant CaMs was similar. Together, the results in Figure 4 demonstrate that each of the single-point mutant CaMs bound to cardiac SR vesicles and that the affinities of binding were reduced relative to wtCaM in the presence micromolar Ca^{2+}. Thus, the inability of the mutant CaMs to inhibit the RYR2 (Figure 3) may be associated with a decreased affinity for RYR2 Ca^{2+}CaM sites.

RYR Regulation by E1234A CaM. To further investigate the significance of apoCaM binding to the RYR2, we examined RYR2 regulation by a CaM mutant deficient in Ca^{2+} binding at all four Ca^{2+} sites (E1234A CaM). Figure 5A shows the results of experiments comparing the effects of E1234A and wtCaM on Ca^{2+} release from cardiac SR vesicles passively loaded with $^{45}Ca^{2+}$. In media containing 300 nM Ca^{2+}, $^{45}Ca^{2+}$ release from cardiac SR vesicles was significantly reduced in the presence of wtCaM (5 μM). In contrast, E1234A failed to inhibit $^{45}Ca^{2+}$ release, suggesting that RYR2 modulation by this Ca^{2+}-insensitive CaM mutant was distinct from that by wtCaM. The concentration dependence of RYR2 modulation by E1234A CaM was examined in [^{3}H]ryanodine binding studies. In media containing 100 nM Ca^{2+} and 2 mM AMPPCP (Figure 5B, closed symbols), cardiac SR vesicle [^{3}H]ryanodine binding was significantly increased by E1234A concentrations ≥2 μM (2-fold increase in the presence of 8 μM E1244A CaM). By comparison, wtCaM inhibited RYR2 [^{3}H]ryanodine binding, and this inhibition required ~10-fold lower concentrations (wtCaM $IC_{50} = 73 \pm 21$ nM; Figure 5B). In the absence of AMPPCP, a small but statistically significant activation of RYR2 [^{3}H]-ryanodine binding by E1234A CaM was also observed (Figure 5B, open symbols). These results thus demonstrate that the RYR2 isoform was activated by sufficiently high concentrations of the E1234A CaM. Results in Figure 5C further demonstrate that RYR2 activation by the E1234A CaM (20 μM) was blocked by wtCaM. Conversely, CaM inhibition of the RYR2 required higher concentrations of wtCaM in the presence of E1234A (Figure 5D). Furthermore, E1234A CaM inhibited cardiac SR binding of [^{35}S]wtCaM (50 nM) in media containing 5 μM Ca^{2+} (52% of control binding in the presence of 20 μM E1234A; data not shown). Together, these results thus suggest that RYR2 activation by the E1234A CaM and RYR2 inhibition by wild-type Ca^{2+}-CaM reflected competitive binding at the same or overlapping sites on the RYR2 isoform.

Subsequent experiments examined the effect of E1234A CaM on the Ca^{2+} dependence of the RYR1 and RYR2 in identical 22 °C media to allow for direct comparisons of the role of CaM Ca^{2+} binding on the different channel isoforms. Figure 6A shows that wtCaM (5 μM) activated RYR1 [^{3}H]ryanodine binding in Ca^{2+} <1 μM and inhibited [^{3}H]ryanodine binding in Ca^{2+} >1 μM, thus evoking a

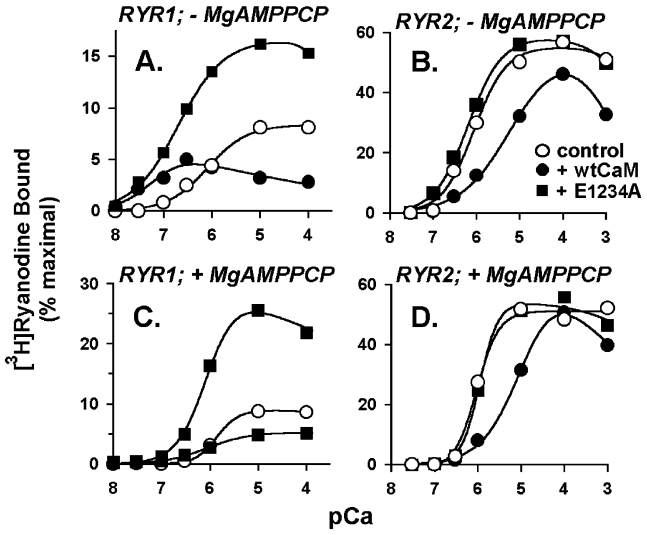

FIGURE 6: Effects of wild-type and E1234A CaM on the Ca^{2+} dependence of the RYR1 and RYR2 isoforms. [^{3}H]Ryanodine binding to skeletal muscle and cardiac muscle SR vesicles was determined as a function of free Ca^{2+} concentration in either the absence or presence of 3 mM MgAMPPCP, as indicated. Experiments were conducted at room temperature in 150 mM KCl, 20 mM K-PIPES (pH 7.0), 5 mM GSH, 0.1 mg/mL BSA, and 7 nM [^{3}H]ryanodine, with or without 5 μM CaM or 5 μM E1234A. Data are means from three to four experiments.

Table 1: Ca^{2+} Dependence of [^{3}H]Ryanodine Binding to RYR1 and RYR2: Effect of wtCaM and Mutant CaM E1234A[a]

	skeletal SR (RYR1)		cardiac SR (RYR2)	
	Ca^{2+} EC_{50} (nM)	n_H	Ca^{2+} EC_{50} (nM)	n_H
−MgAMPPCP				
control	800 ± 160	1.1	870 ± 160	1.2
5 μM wtCaM	65 ± 20[b]	1.5	6400 ± 2400[b]	0.8
5 μM E1234A	200 ± 54[b]	1.0	640 ± 50[c]	1.1
+MgAMPPCP				
control	1400 ± 24	1.9	940 ± 56	1.2
5 μM wtCaM	860 ± 160[b]	1.0	9800 ± 1700[b]	1.0
5 μM E1234A	890 ± 20[b]	1.4	1100 ± 140[c]	1.1

[a] Ca^{2+} EC_{50} values and Hill coefficients (n_H) are based on fits of the data presented in Figure 6 to the Hill equation (see Experimental Procedures). [b]Significantly different than the corresponding control value in the absence of CaM. [c]Significantly different than the corresponding value in the presence of wtCaM ($p < 0.05$, Student's *t*-test).

leftward shift in the biphasic Ca^{2+} dependence of the skeletal muscle isoform (*4*, *5*, *15*). A significant increase in RYR1 Ca^{2+} sensitivity was also observed when media contained 3 mM MgAMPPCP (Figure 6C, Table 1). The E1234A CaM, by comparison, activated the RYR1 at all Ca^{2+} concentrations and evoked both an increase in the maximal extent of Ca^{2+} activation (Figure 6A,C) and a leftward shift in EC_{50} for Ca^{2+} activation (Table 1). These data are thus consistent with previous results (Figure 1; ref *31*) in indicating that Ca^{2+}-insensitive mutants selectively abolish Ca^{2+}CaM inhibition of the RYR1 isoform.

Figure 6B shows that wtCaM inhibited [^{3}H]ryanodine binding to the RYR2 isoform at all Ca^{2+} concentrations and evoked a rightward shift in the Ca^{2+} dependence of the cardiac channel isoform (7-fold increase in Ca^{2+} EC_{50} relative to control; Table 1). A comparable (10-fold) increase in RYR2 Ca^{2+} EC_{50} in the presence of wtCaM was also apparent in media containing 3 mM MgAMPPCP (Figure 6D, Table 1). Thus, whereas wtCaM enhanced the Ca^{2+} sensitivity of the RYR1 isoform, the RYR2 isoform exhibited the opposite response. In contrast to wtCaM, E1234A (5 μM) did not significantly alter the Ca^{2+} sensitivity of the RYR2 relative to control values in the absence of CaM. Rather, the Ca^{2+} EC_{50} in the presence of E1234A CaM was reduced ~10-fold in comparison to that in the presence of wtCaM. These results therefore demonstrate that RYR2 Ca^{2+} sensitivity was significantly decreased in the presence of wtCaM and further indicate that this effect was entirely dependent on the binding of Ca^{2+} to CaM.

DISCUSSION

In this study, we used a series of Ca^{2+}-insensitive CaM mutants to define and compare the structural determinants of CaM activation and inhibition of the RYR1 and RYR2 channel isoforms. Our results suggest new insights into the mechanism by which CaM may function as an isoform-specific modulator of RYR channel Ca^{2+} sensitivity.

For the RYR1 isoform, the action of CaM is dependent on Ca^{2+} concentration (*4*, *5*), and accordingly our results show that the conversion of wtCaM from an activator (apoCaM) to an inhibitor (Ca^{2+}CaM) required [Ca^{2+}] ≥1 μM (Figure 6A). RYR1 inhibition by Ca^{2+}CaM was reduced or abolished by single E to A substitutions within the Ca^{2+} binding sites of mammalian CaM, with mutations targeting CaM C-terminal sites displaying progressively greater effects. Similarly, Rodney et al. reported that Ca^{2+}CaM inhibition of the RYR1 was abolished by a two-site mutant of *Drosophila* CaM with E to Q substitutions in Ca^{2+} sites 3 and 4 (22). The present study and that of Rodney et al. are thus in agreement in indicating that (i) Ca^{2+} binding at CaM C-terminal sites chiefly determines whether CaM activates or inhibits the RYR1 isoform and (ii) mutations that target these Ca^{2+} sites selectively disrupt Ca^{2+}CaM, but not apoCaM, regulation of the RYR1 isoform.

In contrast to the RYR1, the cardiac RYR2 isoform was inhibited by wtCaM at all physiologically relevant Ca^{2+} concentrations (100 nM to 1 mM; Figures 3 and 6). This result is thus consistent with the view that both apoCaM and Ca^{2+}CaM may act to inhibit the RYR2 (*5*, *15*). Arguing against this view, however, we further demonstrate that Ca^{2+}-insensitive mutants of CaM failed to inhibit the RYR2 isoform. Accordingly, each of the four single E to A substitutions targeting the individual Ca^{2+} sites of CaM effectively abolished RYR2 inhibition by CaM (Figure 3). These results therefore indicate that Ca^{2+} binding to CaM is necessary for the inhibition of the cardiac RYR2 as well as the skeletal muscle RYR1 isoform. Remarkably, however, RYR2 inhibition by Ca^{2+}CaM was observed in ~10-fold lower Ca^{2+} concentration than was Ca^{2+}CaM inhibition of the RYR1 isoform (Figures 3, 5, and 6).

Our results may be explained by postulating that the affinity of Ca^{2+} binding to CaM is increased ~10-fold when CaM is bound to the RYR2 as compared to the RYR1 isoform. Consistent with this interpretation, thermodynamic coupling predicts that Ca^{2+}-dependent changes in the affinity of CaM for a particular target will be balanced by proportionate changes in the Ca^{2+} binding affinity of CaM when bound to that target (*32*). Accordingly, in micromolar Ca^{2+} concentrations the affinity of [^{35}S]CaM binding to the RYR2

is increased 5−20-fold relative to that in the presence of EGTA (*15*, *16*). We therefore postulate that the increased affinity of the RYR2 for Ca^{2+}CaM relative to apoCaM acts to drive Ca^{2+} binding to the CaM-RYR2 complex. In comparison to the RYR2, Ca^{2+}-dependent changes in the affinity of [^{35}S]CaM binding to the RYR1 isoform are less pronounced [≤2-fold increase in affinity in Ca^{2+} relative to EGTA (*14*, *15*)]. Thus, in comparison to the RYR2, the RYR1 would be expected to exert a lesser influence on the affinity of Ca^{2+} binding to CaM within the multimolecular channel complex.

The modulation of CaM Ca^{2+} binding parameters mediated through CaM's interactions with different targets has been termed *intermolecular tuning* and reflects an important mechanism through which the Ca^{2+} binding properties of CaM may be optimized for the regulation of different biochemical pathways (*8*−*10*). Intermolecular tuning of CaM thus allows various targets to respond to Ca^{2+} signals within the appropriate range of Ca^{2+} concentrations and with the appropriate kinetics. In regard to RYR channels, a CaM binding domain defined by RYR1 residues 3614−3643 is highly conserved among the different channel isoforms (*4*, *5*). Despite homology within this defined region, our results nonetheless confirm important differences in CaM's functional interactions with the intact RYR1 and RYR2 isoforms. Specifically, the dual functional effects of apoCaM and Ca^{2+}-CaM act to shift the biphasic Ca^{2+} dependence of the RYR1 isoform leftward (Figure 6) and decrease the RYR1 Ca^{2+} EC_{50} (Table 1). In contrast, CaM evoked a rightward shift in the Ca^{2+} dependence of the RYR2 isoform, increasing the RYR2 Ca^{2+} EC_{50} by ∼7−10-fold (Figure 6, Table 1). We propose that these distinctly different actions of CaM may be largely explained by the differential tuning of Ca^{2+} binding sites on CaM in the presence of the RYR1 and RYR2 isoforms, coupled with the characteristic Ca^{2+} dependence of channel activation. Thus, we postulate that the RYR2 isoform effectively reduces the Ca^{2+} concentration required for formation of the inhibitory Ca^{2+}CaM species, such that CaM inhibits the channel at all physiologic Ca^{2+} concentrations. In this view, CaM inhibition of the RYR2 in submicromolar Ca^{2+} concentrations is explained not by the distinct response of this isoform to apoCaM but rather by properties of the RYR2 that increase the affinity of Ca^{2+} binding to CaM within the multimolecular channel complex.

That the apoCaM species may also bind the RYR2 isoform (albeit with low affinity) was suggested by previous results that demonstrated RYR2 CaM binding in media containing ≤10 nM Ca^{2+} (*15*, *16*). However, the functional consequences of RYR2 apoCaM binding have remained unclear, with previous reports suggesting either no effect (*16*), inhibition (*15*), or slight activation (*16*) in different experimental media. These discrepancies may be explained in part by difficulties in determining whether CaM effects observed in various low-Ca^{2+} media may accurately be ascribed to apoCaM, Ca^{2+}CaM, or a mixture of species. Here we demonstrate that the Ca^{2+}-insensitive CaM mutant E1234A significantly activated the RYR2 in 100 nM Ca^{2+} (Figure 5A,B). Furthermore, RYR2 activation by E1234A CaM was blocked by wtCaM, indicating that activation was not a nonspecific effect of the mutant CaM but rather reflected binding at the RYR2 Ca^{2+}CaM site. We therefore conclude that apoCaM and Ca^{2+}CaM may bind at the same or overlapping sites on the RYR2, as has been previously proposed for the RYR1 isoform (*14*, *18*, *19*). In contrast to the E1234A CaM, however, wtCaM inhibited the RYR2 in 100 nM Ca^{2+} (Figure 3A), suggesting that Ca^{2+}CaM rather than apoCaM was the predominant species of wtCaM bound to the RYR2 in these media.

The physiological significance of RYR channel regulation by CaM is not yet clear, and additional studies are required to resolve the molecular details of CaM's physical and functional interactions with the different RYR isoforms. A recent report has indicated that high-affinity CaM binding to region 3614−3643 of the RYR1 was not essential for RYR1 activation via voltage-sensing L-type Ca^{2+} channels in skeletal muscle-derived myotubes (*34*). In cardiac muscle, the possibility that CaM may function as a Ca^{2+}-sensing subunit of the RYR2 channel isoform suggests that Ca^{2+} binding to CaM may change in response to changes in the amplitude or frequency of intracellular Ca^{2+} signals near the channel and thereby modulate SR Ca^{2+} release during excitation−contraction coupling. Alternatively, the present results suggest that the inhibitory Ca^{2+}CaM species may remain constitutively bound to the RYR2 at all physiologic Ca^{2+} concentrations. In cardiac muscle, CaM might therefore function not as a Ca^{2+}-sensing subunit of the RYR2 but as a constitutive inhibitor of the channel. In this view, RYR2 Ca^{2+} sensitivity might then be modulated by physiologic/pathophysiologic conditions that modify RYR2 CaM binding, for example, via oxidation, nitrosylation, or phosphorylation of the channel complex (*4*, *5*, *33*).

In summary, our results indicate that CaM regulation of both the RYR1 and the RYR2 isoforms is determined by Ca^{2+} binding to CaM and suggest that isoform differences in these channels' response to CaM reflect the differential tuning of CaM Ca^{2+} sites when bound to the different RYRs. These results thus support the possibility that CaM may serve distinct roles in regulating RYR channels in cardiac and skeletal muscle and point to a novel mechanism by which functional differences among the RYR isoforms may be mediated through CaM.

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