Calmodulin Binding and Inhibition of Cardiac Muscle Calcium Release Channel (Ryanodine Receptor)*

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Metabolically 35S-labeled calmodulin (CaM) was used to determine the CaM binding properties of the cardiac ryanodine receptor (RyR2) and to identify potential channel domains for CaM binding. In addition, regulation of RyR2 by CaM was assessed in [3H]ryanodine channel domains for CaM binding. In addition, regulation by CaM of the skeletal and cardiac ryanodine receptors.

The ryanodine receptors (RyRs) are large, high conductance Ca2+ release channels found in a specialized subcompartment of the endoplasmic reticulum of many tissues (1). In muscle cells, this subcompartment is referred to as the sarcoplasmic reticulum (SR). There are three known mammalian RyR isoforms: RyR1, which is the dominant isoform in skeletal muscle; RyR2, which is found in cardiac muscle; and RyR3, which is expressed in many tissues at low levels but is mostly associated with diaphragm and brain. In both skeletal and cardiac muscle, Ca2+ release through the RyR in response to a signal received from the T-tubule membrane via the dihydropyridine receptor is a crucial step in excitation-contraction coupling (2). This event is highly regulated by small molecules such as Ca2+, Mg2+ and adenine nucleotides (3, 4), and through protein-protein interactions such as with triadin and calmodulin (CaM) (5–8).

CaM is a small (16.7 kDa) cytosolic protein, the structure of which has been determined by both x-ray crystallography and NMR (9, 10). The protein resembles a dumbbell, with two globular heads linked by a solvent-exposed α-helical stalk. Each of the N- and C-terminal domains contains two EF-hand Ca2+ binding motifs. Ca2+ binding domains I and II in the N domain have a lower Ca2+ affinity (10–15 μM) and are less Ca2+-selective than the corresponding domains III and IV (10–30 μM) in the C domain. CaM binds to and regulates a myriad of target proteins involved in almost every biological function in three distinct manners: 1) as CaCaM (CaM), 2) apoCaM (without Ca2+ bound), and 3) Ca2+-independent or constitutively bound (11, 12). Each of these binding events occurs through one of several poorly defined CaM binding motifs, the most common of which are composed of an amphipathic helix of ~20 amino acid residues that bind CaCaM or an IQ sequence motif that preferentially binds apoCaM.

CaM shows a biphasic regulation of RyR1, activating the channel at submicromolar cytosolic Ca2+ while inhibiting the channel at higher Ca2+ concentrations (7). RyR2, on the other hand, does not show activation by apoCaM but is inhibited by CaCaM in a manner similar to RyR1 (13, 14). Several studies have reported the stoichiometry of CaM binding to the RyR1. Early studies using 125I-CaM (7) or fluorescently labeled (15) CaM indicated a stoichiometry of 1 molecule of CaCaM and 4–6 molecules of apoCaM bound per subunit. Binding site localization studies with fusion proteins and synthetic peptides indicated three to six potential binding sites per subunit with a variable Ca2+ dependence (16, 17). More recent studies using metabolically 35S-labeled CaM indicate a stoichiometry of one binding site per RyR1 subunit for both apo- and CaCaM (8). A recent report suggests that cardiac SR vesicles bind five CaCaM molecules but only 1 apoCaM molecule per RyR2 tetramer (14).

This study presents a systematic analysis of the CaM binding properties of RyR2 and compares them to RyR1 assayed under identical conditions. Our data indicate that the purified RyR2 binds approximately one 35S-CaM molecule per subunit in both 100 μM Ca2+ and 5 mM EGTA. In native cardiac SR vesicles, 35S-CaM binds approximately two sites per RyR2 subunit in the presence of Ca2+ and a single site per subunit in the absence of Ca2+. Two possible explanations for this discrepancy are that a second CaCaM-binding protein in SR vesicles is lost on purification or that purification induces a conformational change masking the second site. We have also analyzed the effects of redox state on CaM binding to both RyR1 and RyR2. RyR1 and RyR2 are sensitive to redox regulation, showing a 2–3-fold reduction in CaCaM affinity in the presence of GSSG, which is accentuated in the absence of Ca2+ to a 4–9-

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fold reduction in affinity. CaCaM inhibition of native RyR2, unlike that of RyR1, is greatly diminished by the presence of two allosteric regulators of the ryanodine receptor, caffeine and AMPPCP. Unlike RyR1, apoCaM inhibited RyR2, as determined in [3H]ryanodine binding and single-channel measurements.

EXPERIMENTAL PROCEDURES

SR Vesicle Preparations and RyR Purification—Heavy SR vesicles were isolated from rabbit hind limb and canine cardiac muscle as previously described (18). In selected experiments, endogenous CaM was removed by incubating SR vesicles for 30 min at 24 °C with 1 μM myosin light chain kinase-derived calmodulin binding peptide (CaMBP) in the presence of 100 μM Ca2+ followed by centrifugation through a layer of 15% sucrose to remove complexed CaM and CaMBP. Where indicated, the centrifugation step was omitted. For purification, SR vesicles were solubilized in CHAPS, purified by centrifugation, and reconstituted into phosphatidylcholine liposomes (19). The endogenous SR-associated concentration of CaM was determined by the ability of CaM to stimulate phosphodiesterase hydrolysis of cAMP as described previously (20).

[35S]Calmodulin Expression and Purification—Calmodulin was metabolically labeled with [35S] according to a protocol generously provided by Drs. Gerald Carlson and Kenneth Traxler (University of Missouri at Kansas City). The cDNA encoding CaM was the generous gift of Dr. Claude Klee (National Institutes of Health). Escherichia coli transformed with the plasmid DNA were grown in M63 minimal media, and expression was induced by heat shock at 42 °C followed by the addition of 1 mM/100 ml trypan blue (ICN Radiochemicals, Costa Mesa, CA). Expression was allowed to continue for 3 h before the bacteria were pelleted and resuspended in lysis buffer (50 mM MOPS, pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μg/ml lysozyme) and allowed to lyse overnight at 4 °C. After centrifugation at 30,000 × g for 30 min, CaM was purified from the cleared lysate on phenyl-Sepharose in the presence of 4 mM Ca2+ and eluted with 1 mM Ca2+. Peak elution fractions were dialyzed versus two changes of 0.15 M KCl, 20 mM K-Pipes, pH 7.0. CaM protein concentration was determined by absorption spectroscopy by the equation [CaM] = (A277 − A230)ε. For expressed CaM, ε was assumed to be 0.20 ml/mg cm (21).

[35S]Calmodulin Binding—Unless otherwise indicated, SR vesicles or purified RyR preparations were incubated with 1–300 nM [35S]CaM in 150 mM KCl, 20 mM K-Pipes, pH 7.0, 0.1 mg/ml bovine serum albumin (BSA, Sigma-A-9281), 0.2 mM Pefabloc, 20 μM leupeptin, and either 5 mM GSH or GSSG and 100 μM (200 μM CaCl2, 100 μM EGTA) or <10 nM (5 mM EGTA, no added CaCl2) free Ca2+. Equilibrium [35S]CaM binding was assayed after incubation at room temperature for 2 h by centrifugation in a Beckman Airfuge for 30 min at 90,000 × g (SR vesicles) or for 180 min at 225,000 × g in a Beckman Type 75 Ti rotor (soluble and reconstituted, purified RyR preparations). Centrifugation-based binding assays are ideal in situations of rapid ligand dissociation and low affinity since the receptor and ligand remain in equilibrium throughout the separation period. Nonspecific binding, including the trapped volume of [35S]CaM, was determined using a 100–1000-fold excess of unlabeled calmodulin (SR vesicles) or by determining [35S]CaM binding to CHAPS-solubilized phospholipid or liposomes that lacked RyR2. Bound [35S]CaM was determined by scintillation counting after solubilization of pellets in Tri-s-HCl buffer, pH 8.5, containing 2% sodium dodecylsulfate. The time course of [35S]CaM dissociation was determined at 23 °C with the use of a filter assay. To minimize nonspecific binding of [35S]CaM, Whatman GF/B filters were blocked for 1 h in 0.15 M KCl, 10 mM K-Pipes, pH 7.0, buffer containing 10 mg/ml BSA. Vesicles on the filters were washed with 3 × 5 ml of 0.15 M KCl, 10 mM K-Pipes, pH 7.0, buffer containing 0.1 mg/ml BSA.

[3H]Ryanodine Binding—Specific [3H]ryanodine binding was determined in the buffer system used for [35S]CaM binding after incubation with 1–2 nM [3H]ryanodine for 20 h at 23 °C as previously described (18). Bmax values of [3H]ryanodine binding were determined by Scatchard analysis or with 50 nM [3H]ryanodine in 0.6 M KCl buffer.

Fusion Protein Generation and Expression—Fusion proteins spanning the full-length coding sequences of rabbit RyR1 (fused to TrpE or glutathione S-transferase) and RyR2 (fused to glutathione S-transferase) were generated by using polymerase chain reaction to add unique restriction sites to the 5' and 3' ends of the region of interest (RyR2) or using existing restriction sites (RyR1) followed by cloning in-frame into pATH or pGEX-SX (RyR1) or pGEX-SX (Amersham Pharmacia Biotech, RyR2). The sequences expressed for each fusion protein are as follows: for RyR2, FP1 (1–333), FP2 (263–615), FP3 (561–908), FP4 (672–1207), FP5 (1157–1509), FP6 (1487–1817), FP7 (1791–2112), FP8 (2084–2401), FP9 (2385–2754), FP10 (2724–3016), FP11 (3003–3182), FP12 (3160–3352), FP13 (3298–3595), FP13short (3298–3577), FP14 (3545–3961), FP15 (3931–4299), FP16 (4205–4478), FP17 (4404–4563), FP18 (4548–4748), FP19 (4726–4968); for RyR1, FPA (1–282), FPB (282–
...lalysis by sonication. 15 min followed by resuspension in phosphate-buffered saline containing 50 nM [35S]CaM dissociation). The averaged time constants (in symbols) were blocked (2
...membranes were then exposed to 100 nM [35S]CaM in 150 mM KCl, 20 mM K-Pipes, pH 7.0, 0.04% Tween 20 with the appropriate Ca2+
...4430), FPN (4431–4771), FPO (4771–5037). Fusion proteins were ex-...799), FPC (799–1209), FPD (1209–1632), FPE (1632–2157), FPF (2156–2592), FPG (2502–2874), FPH (2804–3224), FPI (3225–3662), FPJ (3622–3880), FPK (3879–4222), FPL (4223–4302), FPM (4302–4430), FPN (4431–4771), FPO (4771–5037). Fusion proteins were expressed in the BL21 Gold strain of E. coli (Strategen) by induction (with 1 mM isopropyl-1-thio-β-D-galactopyranoside for GST and 10 μg/ml indocyanine green for TrpE fusion proteins) and a 2-h incubation at 37°C. Whole cell pellets were collected by centrifugation at 1500 g for 15 min followed by resuspension in phosphate-buffered saline containing Complete protease inhibitors (Roche Molecular Biochemicals) and lysis by sonication.

**[35S]Calmodulin Overlays**—Equivalent amounts of each fusion protein (as judged by Coomassie Brilliant Blue stain and Western analysis) were loaded onto 10% SDS-polyacrylamide gel electrophoresis, the proteins were transferred to nitrocellulose (as judged by Coomassie Brilliant Blue stain and Western analysis) and then were diluted 60-fold into media containing 50 nM nonradioactive CaM and either 100 μM free Ca2+ (filled symbols) or 5 mM EGTA (open symbols). The averaged time constants (in min) of [35S]CaM dissociation were determined as previously described (18). All data analyses were done using SigmaPlot version 5.

**RESULTS**

**[35S]Calmodulin Binding to RyR1 and RyR2**—Fig. 1A shows the results of Scatchard analysis of CaM binding to cardiac SR vesicles in reducing (5 mM reduced glutathione, GSH, circles) and oxidizing (5 mM oxidized glutathione, GSSG, triangles) conditions with 100 μM [35S]CaM (filled symbols) and <10 nM free (open symbols) Ca2+, respectively. The data are also summarized in Table I. In the presence of 100 μM Ca2+, cardiac SR vesicles bound 7.5 mol of [35S]CaM/mol of bound [3H]ryanodine. Since there is only one high affinity [3H]ryanodine binding site per tetramer (3, 4), this suggests that in the presence of Ca2+, RyR2 binds 2 CaM molecules per subunit or that other CaM-binding proteins are present in cardiac SR vesicles. One of the

**TABLE I**

|$^{[35S]}$CaM/[3H]Ryanodine $K_D$ $^{[35S]}$CaM/[3H]Ryanodine $K_D$ With 100 μM Ca2+ With <10 nM Ca2+
|---|---|---|---|---|
|Cardiac SR | +GSH | 7.4 ± 1.5 (12) | 3.5 ± 2.0 (12) | 3.8 ± 0.8 (5) | 2.6 ± 0.8 (5) |
| | +GSSG | 7.6 ± 1.3 (5) | 6.8 ± 2.3 (5)* | 2.6 ± 0.8 (5) | 261 ± 103 (5)* |
|Purified RyR2 | +GSH | 3.9 ± 1.0 (5) | 5.0 ± 2.2 (5) | 3.8 ± 1.5 (6) | 54 ± 34 (6) |
|Skeletal SR | +GSH | 4.9 ± 0.7 (16) | 4.7 ± 1.6 (16) | 4.2 ± 0.8 (8) | 12.9 ± 3.4 (8) |
| | +GSSG | 5.3 ± 0.9 (5) | 12.1 ± 1.8 (5)* | 4.4 ± 0.7 (5) | 108 ± 41 (5)* |
|Purified RyR1 | +GSH | 4.6 ± 0.9 (6) | 5.5 ± 2.6 (6) | 3.3 ± 1.3 (6) | 5.4 ± 2.5 (6) |
| | +GSH + Ca2+ | 4.6 ± 0.9 (6) | 5.5 ± 2.6 (6) | 3.3 ± 1.3 (6) | 5.4 ± 2.5 (6) |

* P < 0.01 when compared to $K_D$ in presence of GSH by unpaired Student’s t test.

**Fig. 2. Dissociation of bound [35S]-calmodulin.** Cardiac SR vesicles were prebound with 100 or 200 nM [35S]CaM in 100 μM free Ca2+ (filled symbols) or 5 mM EGTA (open symbols), respectively, in the presence of 5 mM GSH (circles) or 5 mM GSSG (triangles) and then were diluted 60-fold into media containing 50 mM nonradioactive CaM and either 100 μM free Ca2+ (filled symbols) or 5 mM EGTA (open symbols). The averaged time constants (in min) of [35S]CaM dissociation were determined as previously described (18). All data analyses were done using SigmaPlot version 5.

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binding sites appeared to be CaCaM-specific since, in the absence of free Ca\(^{2+}\), the stoichiometry approximates one CaM molecule per RyR2 subunit. Skeletal SR vesicles bound ~1 molecule of \(^{35}\text{S}\)CaM per RyR1 subunit both in the presence and absence of Ca\(^{2+}\), as has been previously reported (8). The results from these experiments have been corrected for the presence of endogenous CaM in the vesicle preparations \((1.0 \pm 0.6 (n = 4) and 0.13 \pm 0.05 (n = 5) \text{CaM} \text{per subunit for cardiac and skeletal SR vesicles, respectively), as determined by phosphodiesterase activation assay.}

After purification of RyR2 from cardiac SR vesicles (shown in Fig. 1B) in the presence of 5 mM GSH and after reconstitution into proteoliposomes, the CaCaM:\(^{16}\text{H\text{[ryanodine binding stoichiometry dropped from -2 to 1 molecule per subunit in the presence of 100 \text{mM Ca}^{2+}, suggesting that other CaM-binding proteins have been removed.}}\)

Alternatively, one of the two CaCaM binding sites in RyR2 may have been conformationally destroyed or buried during purification. We also considered the possibility that endogenous CaM remains associated with the purified RyR. However, if there is such a population of CaM, it would have to be very tightly bound to the receptor and, therefore, without effect on CaCaM binding measured in this study, which has a high rate of dissociation (see Fig. 2). Purification did not significantly alter the CaM:\(^{16}\text{H\text{[ryanodine binding stoichiometry for RyR2 in the absence of Ca}^{2+} or for RyR1 both in the presence and absence of Ca}^{2+} (Table I).}

Decrease in free Ca\(^{2+}\) concentration from 100 \text{mM to} \approx10 \text{mM lowered the affinity for CaM in cardiac SR vesicles 20-fold in the presence of reduced glutathione (GSH) (Fig. 1, A and B, circles) and} ~40-fold in the presence of oxidized glutathione (GSSG) (triangles). In either the presence or absence of Ca\(^{2+}\), oxidized glutathione decreased the affinity for CaM relative to reduced glutathione without changing \(B_{\text{max}}\). Likewise, the CaM binding affinity of RyR1 was reduced 2.5-fold by GSSG in the presence of Ca\(^{2+}\) and 9-fold in the absence, again without changes in \(B_{\text{max}}\) (Table I).

The Ca\(^{2+}\)-dependent changes in CaM binding to cardiac SR vesicles are shown in Fig. 1C. Increase in free Ca\(^{2+}\) from <10 to 100 nM was without significant effect on CaM binding affinity or CaM:\(^{16}\text{H\text{[ryanodine binding stoichiometry, implying that there is apoCaM binding at resting cytosolic Ca}^{2+} levels. The binding stoichiometry nearly doubled as Ca}^{2+} concentration was raised to 600 nM, to a value close to the one at 100 \text{mM Ca}^{2+}. Conversely, the increase in CaM binding affinity occurred over a broad Ca\(^{2+}\) concentration range, requiring Ca\(^{2+}\) concentrations between 1 and 10 \text{mM} for maximal affinity.

Dissociation experiments were performed to determine the effects of redox state on the stability of the apoCaM and CaCaM RyR complexes. As shown in Fig. 2, the rate of dissociation from RyR2 is largely independent of whether CaM is bound in the presence of 5 mM GSH or 5 mM GSSG. The rate of dissociation in EGTA containing media occurred with a \(\tau_{1/2}\) of \(\approx40\) s. The rate of dissociation was decreased by more than 10-fold in the presence of Ca\(^{2+}\), occurring with \(\tau_{1/2}\) of \(\approx9\) min. The results suggest that the rate of dissociation of CaM from RyR2 is largely independent of redox state but rather on whether Ca\(^{2+}\) is present in the dissociation buffer, with apoCaM dissociating at a significantly greater rate than CaCaM. Similar results were obtained for RyR1 (see the legend of Fig. 2), with less pronounced differences between the rates of dissociation of CaCaM and apoCaM.

Identification of Potential Calmodulin Binding Sites—To identify potential calmodulin binding sites within the linear sequence of RyR1 and RyR2, we have generated fusion proteins spanning the full coding sequence of the subunit. The design for the fusion proteins is indicated under “Experimental Procedures.” RyR1 sequences were fused to TrpE (with the exception of RyR1 FP E, L, M, and N, which are fused to GST to improve
expression), whereas the RyR2 fusion proteins were fused to GST. Since the vast majority of the fusion proteins were insoluble, [35S]CaM overlays were performed with whole cell fractions. Fig. 3A shows that, in the presence of 100 μM CaCl2 and 100 nM [35S]CaM, five of the RyR2 fusion proteins showed pronounced [35S]CaM binding. CaM binding was detected for RyR2 fusion proteins 2 (aa 263–615), 10 (2724–3016), 13 (3298–3662), 14 (4302–4430) bound CaM; in the presence of 5 mM EGTA, binding to FPM was not significantly altered, and binding to FP1 was abolished (not shown). These results suggest that there are multiple potential CaM binding sites within the linear RyR sequences, particularly RyR2. Most appear to be buried in the large intact RyR2 channel protein because their number exceeds the number of CaM binding sites in the intact receptor (Table I).

Functional Implications of Calmodulin Binding—It has been previously reported that CaCaM inhibits Ca2+ efflux from both skeletal and cardiac SR vesicles (13, 22). In addition, CaCaM inhibits [3H]ryanodine binding to RyR1 in skeletal SR vesicles (7, 14, 23) with little effect on [3H]ryanodine binding to RyR2 in cardiac SR vesicles (14). We have used a CaM binding peptide derived from the myosin light chain kinase (CaMBP) to determine and correct for the presence of endogenous calmodulin (see above) in studies of [3H]ryanodine binding, which has not been done in previous studies. Relatively low CaMBP concentrations (0.1 μM) were sufficient to reduce [35S]CaM binding in the presence of 100 μM free Ca2+ to skeletal SR vesicles to 4% of the control value (Fig. 4A). For cardiac SR vesicles, a higher CaMBP concentration (1 μM) was required to reduce [35S]CaM binding to a comparable, low level. It is crucial that relatively low concentrations of CaMBP be used if the experiments are done in the presence of the peptide since, as shown in Fig. 4B, vesicles assayed in the presence of high concentrations of CaMBP show a marked stimulation of [3H]ryanodine binding to skeletal SR in either GSH or GSSG by CaMBP at concentrations greater than 1 μM. A lower degree of stimulation was observed with cardiac SR. CaMBP was less effective in reducing [35S]CaM binding to SR vesicles at [Ca2+] < 1 μM. Where indicated, experiments were therefore done with SR vesicles pretreated with CaMBP, as described under “Experimental Procedures.”

To correlate the binding of CaCaM to the inhibition of [3H]ryanodine binding, we measured [3H]ryanodine binding at increasing concentrations of CaM both in the presence of GSH and GSSG for cardiac (Fig. 5) and skeletal (not shown) SR vesicles. For cardiac SR, a higher extent of inhibition was observed in GSH than GSSG. The KIH for inhibition of ryanodine binding (0.6 ± 0.2 and 1.7 ± 0.7 nM for RyR2 in GSH and GSSG, respectively) was considerably lower than the KIH for [35S]CaM binding (see Table I). The Hill coefficients for CaM inhibition of ryanodine binding to RyR2 were 1.0 ± 0.3 both in GSH and GSSG. Skeletal SR also had KIH values (legend of Fig. 5) that were lower than the KD values and had Hill coefficients near unity. The results suggest that inhibition of ryanodine binding perhaps requires only a single CaCaM bound/tetramer.

CaM inhibition of [3H]ryanodine binding is also modulated by various regulators of the RyRs as indicated in Fig. 6 and Table II. At [Ca2+] > 1 μM, CaM (1 μM) inhibition of both cardiac and skeletal RyR is observed in both oxidizing and reducing conditions in the absence of MgAMPPCP (AMPFPCP is a nonhydrolyzable ATP analogue). CaM inhibits [3H]ryanodine binding to RyR2 by both rendering the receptor less sensitive to activation by Ca2+ and more sensitive to inhibition at high Ca2+ as well as by lowering the maximal level of [3H]ryanodine binding (Fig. 6, upper left panel). In the presence of MgAMPPCP and at [Ca2+] < 10 μM, CaM inhibits cardiac SR ryanodine binding in both reducing and oxidizing conditions (Fig. 6, upper right panel). However, at [Ca2+] > 10 μM, CaM inhibits RyR2 ryanodine binding only in reducing conditions. The lower left panel of Fig. 6 indicates that in agreement with a previous report (24), skeletal SR [3H]ryanodine binding is markedly
inhibited in the presence of GSH. [3H]Ryanodine binding to skeletal SR is activated by CaM at low Ca2+ concentrations both in the absence or presence of MgAMPPCP and the presence of GSH (see Table II) or GSSG and inhibited at higher Ca2+ concentrations in both oxidizing and reducing conditions (Fig. 6, lower two panels). In the presence of 5 mM MgAMPPCP, free Ca2+ concentrations in excess of 0.3 mM were not tested because of difficulties in keeping Ca2+ in solution.

In Table II, the effects of CaM on [3H]ryanodine binding to RyR2 and RyR1 are compared in 0.1 and 100 μM Ca2+ media that contained 10 mM caffeine, 5 mM AMPPCP, or 1 mM Mg2+. ApoCaM significantly inhibits RyR2 in reducing conditions in
either the presence or absence of AMPPCP. This apoCaM inhibition was attenuated in the presence of caffeine or in oxidizing conditions. CaCaM inhibition of RyR2 was significant in control (+GSH) and in the presence of 1 mM MgCl₂, with no significant effects in the presence of caffeine or AMPPCP. ApoCaM stimulation of RyR1 under both oxidizing and reducing conditions was maintained in the presence of 10 mM caffeine or 5 mM AMPPCP, compounds that further sensitize the RyR1 ion channel to low concentrations of Ca²⁺. CaCaM inhibition of ryanodine binding to RyR2 was observed under each condition, although the magnitude of the inhibition was decreased in the presence of AMPPCP. The inhibition by CaCaM in AMPPCP was lower in the presence of GSSG than GSH, whereas the inhibition in the presence of caffeine was unaffected by the redox state of glutathione.

**Calmodulin Interaction with Purified Ryanodine Receptors—** Purification of RyR1 by CHAPS solubilization and reconstitution into proteoliposomes had little effect on the equilibrium [³⁵S]CaM binding properties, as indicated in Table I; furthermore, CaCaM inhibition of [³⁵H]ryanodine binding to purified RyR1 was comparable with that of SR vesicles (not shown). Purification of RyR2, however, decreased the stoichiometry of CaM binding without affecting the Kᵢ. In addition, purified RyR2 (either solubilized or reconstituted) failed to show any inhibition of [³⁵H]ryanodine binding by CaCaM in equilibrium binding experiments.

Single-channel measurements using purified RyR2 indicate that in an applied electrical field CaM inhibition of RyR2 is maintained after purification and reconstitution into proteoliposomes (Fig. 7, Table III). In Fig. 7A, a single channel was recorded in the presence of 100 μM cis (cytoplasmic) Ca²⁺. The addition of 1 μM cis CaM reduced channel open probability (Pₒ) from 0.62 to 0.27. The Hill inhibition constant and coefficient of 7 experiments were 42 ± 1 and 1.4 ± 0.1, respectively (Fig. 7B). In 100 μM free cis Ca²⁺ and symmetrical 0.25 mM KCl, the addition of 1 μM CaM to the cis chamber reduced Pₒ from 0.73 ± 0.09 to 0.32 ± 0.08 (n = 7) (Table III). The inhibition of purified RyR2 was attributed to a decrease in mean open time from 9.83 ± 6.57 to 1.45 ± 0.47 ms (n = 5) (statistically significant normalized decrease in open time of 44 ± 12%) without other significant effects on channel gating parameters. The inhibitory effect was magnified in the presence of 2 mM Mg²⁺ and 100 μM free Ca²⁺ with a decrease in Pₒ from 0.51 ± 0.15 to 0.05 ± 0.03 (n = 4). As in the [³⁵H]ryanodine binding studies using SR membranes, MgATP reversed the inhibition by CaM at 100 μM free Ca²⁺. At 100–200 nM free Ca²⁺ concentrations, the addition of 1 μM CaM had a weak inhibitory effect alone as well as in the presence of caffeine; this inhibition was statistically significant only when the data were normalized (PₒCaM/ Pₒ(−CaM) = 0.22 ± 0.05 for control and 0.66 ± 0.10 in the presence of caffeine). Thus, RyR2 was not activated by apoCaM, as is the case for RyR1 (7). It therefore appears that purification of RyR2 alters the conformation of the channel sufficiently to mask in [³⁵H]ryanodine binding measurements the CaCaM binding site that is responsible for inhibition of the channel, but that this site, mediating inhibition by CaCaM and presumably apoCaM (Fig. 1C), can be recovered in single-channel measurements by the application of an electrical field.

**DISCUSSION**

It has been known for more than 10 years that the Ca²⁺-binding protein calmodulin is capable of inhibiting Ca²⁺ release from isolated SR membranes both from cardiac and skeletal muscle (13, 22). Since that time, extensive work has attempted to characterize the nature of CaM binding to the ryanodine receptor. Initial studies using either [³⁵S] (7) or fluoro-labeled (15) labeled CaM found that there are as many as six binding sites for apoCaM on each of the four RyR1 subunits that compose the functional channel. This number was supported in part by studies using fragments of the full-length subunit, which indicated that there were three regions that strongly bound CaM as well as at least three other regions that had weaker CaM binding (16, 17). With the exception of one site, CaM binding was Ca²⁺-dependent, which indicated differences in the Ca²⁺ dependence of CaM binding to the intact receptor and isolated fragments. More recent studies using SR membranes and [³⁵S] metabolically labeled CaM indicate that the tetrameric skeletal muscle channel complex binds a total of four CaM molecules both for apo- and CaCaM on an average of one CaM per subunit (8). The implication of these studies is that chemical modification induces changes in calmodulin leading to nonphysiological binding. The data that we have presented here show that, in agreement with previous studies using [³⁵S]CaM, the skeletal muscle isoform binds ~4 molecules of CaM per tetramer both in the presence and absence of Ca²⁺. Furthermore, the stoichiometry of CaM binding to RyR1

| Assay medium | Bound [³⁵H]ryanodine | RYR2 | +CaM | −CaM | Bound [³⁵H]ryanodine | RyR1 | +CaM | −CaM |
|--------------|---------------------|------|------|------|---------------------|------|------|------|
| In 5 mM GSH  |                     |      |      |      |                     |      |      |      |
| 0.1 μM Ca²⁺  | 0.013 ± 0.003       | 0.005 ± 0.002* | 0.002 ± 0.002* | 0.002 ± 0.002* |
| +10 mM caffeine | 0.059 ± 0.04       | 0.050 ± 0.07    | 0.08 ± 0.01     | 0.14 ± 0.02*    |
| +5 mM AMPPCP | 0.06 ± 0.02         | 0.025 ± 0.005* | 0.025 ± 0.005* | 0.09 ± 0.03*    |
| 100 μM CaCl₂ | 1.2 ± 0.1           | 1.2 ± 0.2      | 0.17 ± 0.01     | 0.04 ± 0.01*    |
| +10 mM caffeine | 1.2 ± 0.1         | 1.2 ± 0.2      | 0.19 ± 0.01     | 0.04 ± 0.01*    |
| +5 mM AMPPCP | 1.4 ± 0.1           | 1.3 ± 0.1      | 3.3 ± 0.3       | 2.1 ± 0.2*      |
| +1 mM MgCl₂  | 0.90 ± 0.05         | 0.30 ± 0.04*   | 0.03 ± 0.01     | 0.01 ± 0.01     |
| In 5 mM GSSG |                     |      |      |      |                     |      |      |      |
| 0.1 μM Ca²⁺  | 0.025 ± 0.006       | 0.17 ± 0.003   | 0.01 ± 0.01     | 0.06 ± 0.01*    |
| +10 mM caffeine | 0.55 ± 0.05        | 0.55 ± 0.05    | 0.40 ± 0.1      | 0.7 ± 0.1*      |
| +5 mM AMPPCP | 0.17 ± 0.02         | 0.11 ± 0.04    | 0.30 ± 0.05     | 0.85 ± 0.20*    |
| 100 μM Ca²⁺  | 1.3 ± 0.1           | 1.1 ± 0.1      | 1.2 ± 0.1       | 0.2 ± 0.1*      |
| +10 mM caffeine | 1.3 ± 0.1          | 1.1 ± 0.1      | 1.3 ± 0.1       | 0.2 ± 0.1*      |
| +5 mM AMPPCP | 1.4 ± 0.1           | 1.4 ± 0.1      | 3.5 ± 0.3       | 3.5 ± 0.3       |
| +1 μM MgCl₂  | 1.2 ± 0.1           | 0.8 ± 0.1*     | 0.20 ± 0.05     | 0.05 ± 0.01*    |

* P < 0.05 when compared to bound [³⁵H]ryanodine in the absence of CaM by Student’s unpaired t test.
is not influenced by the redox state, whereas previous studies using sulfhydryl-reacting agents and [125I]CaM binding have indicated a decrease in the number of apoCaM binding sites (23).

Very little is known about the CaM binding properties of the cardiac isoform of the ryanodine receptor. A recent report by Fruen et al. (14) suggests that cardiac SR membranes bind a single molecule of CaCaM per subunit. Their results also indicate that the binding of apoCaM is greatly reduced, with a stoichiometry of ~1 molecule per tetramer. The most likely cause of the discrepancy between their report and ours (7.5 molecules of CaCaM per tetramer and 4 molecules apoCaM per tetramer) is due to their use of a filtration-based assay given the rapid rate of dissociation of CaM from the cardiac receptor, particularly in the presence of EGTA, where the $\tau_{1/2}$ for dissociation was about 40 s. For both skeletal and cardiac SR vesicles, the CaM binding affinity was decreased both by the removal of Ca$^{2+}$ and by the presence of oxidizing GSSG. This agrees with previous reports suggesting that superoxide anion decreases cardiac SR CaM content (25) and sulfhydryl-reacting reagents diminish CaM binding and inhibition of RyR1 (23, 26). The Ca$^{2+}$-dependent change in stoichiometry appears to be the result of Ca$^{2+}$ binding to CaM given the highly cooperative nature of the increase, whereas the increase in affinity may be due to Ca$^{2+}$ binding to the cardiac ryanodine receptor, as previously suggested for the skeletal receptor (27).

Recent data suggest there is a single CaM binding domain that binds both apo- and CaCaM at distinct but closely apposed sequences (8, 29). Attempts to localize the CaM binding domain in RyR2 illustrated additional differences between skeletal and cardiac ryanodine receptors. Only two potential CaM binding sites were identified in fusion proteins derived from RyR1; one of which, fusion protein I (aa 3225–3662), bound only CaCaM, whereas the other, fusion protein M (aa 4302–4430), bound CaM both in 100 mM Ca$^{2+}$ as well as 5 mM EGTA. The fusion proteins derived from the RyR2 sequence, however, revealed many more potential CaM binding sites. Two overlapping sites (FP13, aa 3298–3595, and FP14, aa 3543–3961) displayed pronounced CaM binding. Fusion proteins I (RyR1) and 13 and 14 (RyR2) contain a sequence implicated in each study, localizing CaM binding sites in the RyR1 sequence (8, 16, 17, 28). C-terminal truncation of 18 amino acids from FP13 (FP13Short, aa 3298–3577) removed both Ca$^{2+}$- and apoCaM binding in overlay experiments, suggesting that the sequence (RyR2 aa 3578–3595) HPQRSKAVWHKLLSKQR is crucial for conferring CaM binding. Furthermore, our data imply that the affinity of CaM binding to this site is Ca$^{2+}$-dependent. A portion of this sequence with additional C-terminal residues (fusion protein PC26 RyR1, aa 3552–3661, and peptide PM2 RyR1, aa 3617–3634 and RyR2 aa 3583–3601) was found to bind calmodulin in 10 mM Ca$^{2+}$ but not in 10 mM EGTA (17); the corresponding peptide derived from RyR2 was also found to have similar Ca$^{2+}$ dependence (28). Interestingly, this single domain has been implicated in both CaCaM and apoCaM binding, as both forms are capable of protecting RyR1 from trypsin cleavage at arginines 3630 and 3637 (8). A recent publication has used peptides derived from this RyR1 CaM binding domain to further refine the putative apo and CaCaM binding sites as an N-terminal CaCaM-specific domain from 3614 to 3634 and an overlapping domain also capable of binding apoCaM from 3625 to 3644 (29). In RyR2, however, the results do not entirely agree, as FP13 bound both apo- and CaCaM (albeit to different extents) but does not contain this apoCaM domain. Furthermore, in the Rodney et al. (29) study, a peptide (3614-KSKKAVWHKLLSKQR-3627), which agrees with the critical sequence for CaM binding to RyR2 FP13, was unable to bind either Ca$^{2+}$ or apoCaM. The results from our localization of potential CaM binding sites as well as those reported by others are summarized in Fig. 8.

Previous reports show that the activity of the skeletal muscle ryanodine receptor is stimulated by CaM at low free Ca$^{2+}$ concentrations (7, 14). This effect appears to be lacking in the few studies performed to date using cardiac SR vesicles (14). Recently it was also shown that cardiac SR vesicle ryanodine binding was not inhibited by CaCaM in the presence of adenine nucleotides, although SR Ca$^{2+}$ efflux was inhibited by CaM in
Calmodulin Binding and Inhibition of Ryanodine Receptors

TABLE III

| Assay medium | Single-channel activities ($P_o$) | Number of experiments |
|--------------|----------------------------------|-----------------------|
|              | −CaM  | +CaM                |                        |
| 100 µM Ca$^{2+}$ | 0.73 ± 0.09 | 0.32 ± 0.08*       | 7                     |
| 100 µM Ca$^{2+}$ + 2 mM Mg$^{2+}$ | 0.51 ± 0.15 | 0.05 ± 0.03*       | 4                     |
| 100 µM Ca$^{2+}$ + 2 mM MgATP | 0.74 ± 0.16 | 0.67 ± 0.16       | 5                     |
| 10 µM Ca$^{2+}$ + 2 mM MgATP | 0.034 ± 0.014 | 0.016 ± 0.012 | 5                     |
| 0.1–0.2 µM Ca$^{2+}$ | 0.0011 ± 0.0005 | 0.0002 ± 0.0001b | 7                     |
| 150 nM Ca$^{2+}$ + 3–40 mM caffeine | 0.10 ± 0.05 | 0.07 ± 0.04b       | 8                     |
| 0.1–0.4 µM Ca$^{2+}$ + 2 mM MgATP | <0.001 | <0.001       | 3                     |

* $P < 0.05$ when compared to $P_o$ in the absence of CaM by paired Student’s $t$ test, b $P < 0.05$ when compared as normalized $P_o$ ($P_o$ + CaM)/($P_o$ − CaM).

**FIG. 8. Schematic summary of potential calmodulin binding sites.** Sequence domains suggested by previous works (16, 17, 28, 29) and this report are aligned with cardiac binding domains above and skeletal binding domains below a linear representation of the full-length amino acid sequence of the ryanodine receptor. CaCaM-specific binding domains are dark gray, whereas domains binding both apo- and CaCaM are light gray with a black border.

a manner similar to previous Ca$^{2+}$ efflux measurements (14). We show here that inhibition of RyR2 by CaM is dependent on the assay conditions. [3H]Ryanodine binding to cardiac SR vesicles was inhibited by CaM in the absence and presence of Mg$^{2+}$ and adenine nucleotide, with the effect being more pronounced at [Ca$^{2+}$] < 10 µM in the presence of GSH. CaCaM was without a significant effect at 100 µM Ca$^{2+}$ in the presence of GSSG and adenine nucleotide or caffeine. ApoCaM inhibited [3H]ryanodine binding to cardiac SR in the absence and presence of AMPPCP. In single-channel measurements, apoCaM had a small inhibitory effect in the absence and presence of caffeine.

We have also found that, although the use of the CaMBP is necessary to eliminate the effects of endogenous SR calmodulin, which is associated with both the skeletal and cardiac SR vesicles, it is imperative that it be used at low concentrations since it had a pronounced stimulatory effect on skeletal [3H]ryanodine binding after preincubation of the vesicles to remove the endogenous CaM. Since CaMBP is derived from a CaCaM-specific binding protein, it is ineffective at removing endogenous CaM in the absence of Ca$^{2+}$. To correct for the effect of endogenous apoCaM, we first removed endogenous CaM by incubation with CaMBP in the presence of Ca$^{2+}$ followed by centrifugation.

Purification of RyR2 decreases the stoichiometry of CaCaM binding, eliminating one site for $[^{35}S]$CaM per subunit without affecting apoCaM binding, an effect not observed with RyR1. In addition, purification of RyR2 also eliminated CaCaM-dependent inhibition of [3H]ryanodine binding. This effect appears to be due to a conformational change in the purified receptor rather than to the removal of a necessary cofactor since single-channel measurements using purified RyR2 show that upon application of a transmembrane potential, CaCaM inhibition of the channel is restored. However, the $K_{NH}$ for inhibition of channel open probability was considerably higher than the $K_{NH}$ for [3H]CaM binding. Whether CaM binding affinity is voltage-dependent or whether restoration of function correlates with unmasking of a second CaM binding site in RyR2 is not known because CaM binding to single channels cannot be measured. To resolve these issues, RyR2 mutants lacking putative CaM binding sites need to be constructed and examined.

Our data suggest that CaM is a major regulator of Ca$^{2+}$ release from intracellular stores during excitation-contraction coupling. Under conditions of oxidative stress, such as exercise-induced fatigue or ischemia in which levels of GSSG are increased relative to GSH, the affinity for both apo- and CaCaM is decreased. In skeletal muscle, the oxidizing effects can result both in an increase (oxidation of RyR1) and decrease (decrease in apoCaM affinity) of basal Ca$^{2+}$ release. One possible effect consistent with our data is that oxidation will result in a pronounced increase in peak Ca$^{2+}$ release, resulting from RyR1 oxidation and diminished CaCaM binding affinity. In cardiac muscle, in the presence of Mg$^{2+}$ and adenine nucleotide, a shift from reducing to oxidizing conditions will attenuate the inhibition of RyR2 by saturating CaM at low Ca$^{2+}$ while eliminating inhibition by CaM at high Ca$^{2+}$. In addition, the CaM binding affinity is reduced in the presence of oxidizing conditions, further sensitizing the channel to activation by cytosolic Ca$^{2+}$. This could lead to an increase in the sensitivity of RyR2 to trigger Ca$^{2+}$ release provided by the dihydropryridine receptor. Furthermore, CaM is a key mediator of Ca$^{2+}$-dependent inactivation and facilitation of cardiac L-type Ca$^{2+}$ channels (30–33) and is, therefore, a regulator of two of the most tightly regulated steps in excitation-contraction coupling.

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