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

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Metabolically ^{35}S-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 [^{3}H]ryanodine binding and single-channel measurements. Cardiac sarcoplasmic reticulum vesicles bound approximately four CaM molecules per RyR2 tetramer in the absence of Ca^{2+}; in the presence of 100 μM Ca^{2+}, the vesicles bound 7.5 CaM molecules per tetramer. Purified RyR2 bound approximately four [^{35}S]CaM molecules per RyR tetramer, both in the presence and absence of Ca^{2+}. At least four CaM binding domains were identified in [^{35}S]CaM overlays of fusion proteins spanning the full-length RyR2. The affinity (but not the stoichiometry) of CaM binding was altered by redox state as controlled by the presence of either GSH or GSSG. Inhibition of RyR2 activity by CaM was influenced by Ca^{2+} concentration, redox state, and other channel modulators. Parallel experiments with the skeletal muscle isoform showed major differences in the CaM binding properties and regulation by CaM of the skeletal and cardiac ryanodine receptors.

The ryanodine receptors (RyRs)[1] are large, high conductance Ca^{2+} 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, Ca^{2+} 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 Ca^{2+}, Mg^{2+} 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 Ca^{2+} binding motifs. Ca^{2+} binding domains I and II in the N domain have a lower Ca^{2+} affinity (10^{-5} M) and are less Ca^{2+}-selective than the corresponding domains III and IV (10^{-6} M) 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 2) as apoCaM (without Ca^{2+} bound), and 3) Ca^{2+} independent or constituently 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 Ca^{2+} while inhibiting the channel at higher Ca^{2+} 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 ^{125}I-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 Ca^{2+} dependence (16, 17). More recent studies using metabolically ^{35}S-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 [^{35}S]CaM molecule per subunit in both 100 μM Ca^{2+} and 5 mM EGTA. In native cardiac SR vesicles, [^{35}S]CaM binds approximately two sites per RyR2 subunit in the presence of Ca^{2+} and a single site per subunit in the absence of Ca^{2+}. 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 Ca^{2+} to a 4–9-

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[1] The abbreviations used are: RyR, ryanodine receptor; RyR1, skeletal muscle RyR; RyR2, cardiac muscle RyR; SR, sarcoplasmic reticulum; CaM, calmodulin; GSH, reduced glutathione; GSSG, oxidized glutathione; CaMBP, myosin light chain kinase-derived calmodulin binding peptide; BSA, bovine serum albumin; AMPPCP, adenosine 5′-(β,γ-methylene)triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; GST, glutathione *S*-transferase; aa, amino acids.

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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 [^{3}H]ryanodine binding and single-channel measurements.

EXPERIMENTAL PROCEDURES

SR Vesicle Preparations and RyR Purification—Heavy SR vesicles were isolated from rabbit hind limb and back muscle 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 Ca^{2+} 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 sucrose density gradient 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).

[^{35}S]Calmodulin Expression and Purification—Calmodulin was metabolically labeled with ^{35}S 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 mCi/100 ml Tran^{35}S-label (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 Ca^{2+} and eluted with 1 mM EDTA. Peak elution fractions were dialyzed *versus* two changes of 0.15 M KCl, 20 mM K^+-Pipes, 100 μM $CaCl_2$, pH 7.0. CaM protein concentration was determined by absorption spectroscopy by the equation [CaM] = $(A_{277} - A_{320})/\epsilon$. For expressed CaM, ϵ was assumed to be 0.20 ml/(mg cm) (21).

[^{35}S]Calmodulin Binding—Unless otherwise indicated, SR vesicles or purified RyR preparations were incubated with 1–300 nM [^{35}S]CaM in 150 mM KCl, 20 mM K-Pipes, pH 7.0, 0.1 mg/ml bovine serum albumin (BSA, Sigma A-0281), 0.2 mM Pefabloc, 20 μM leupeptin with either 5 mM GSH or GSSG and 100 μM (200 μM $CaCl_2$, 100 μM EGTA) or <10 nM (5 mM EGTA, no added $CaCl_2$) free Ca^{2+}. Equilibrium [^{35}S]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 [^{35}S]CaM, was determined using a 100–1000-fold excess of unlabeled calmodulin (SR vesicles) or by determining [^{35}S]CaM binding to CHAPS-solubilized phospholipid or liposomes that lacked RyR2. Bound [^{35}S]CaM was determined by scintillation counting after solubilization of pellets in Tris-HCl buffer, pH 8.5, containing 2% sodium dodecylsulfate. The time course of [^{35}S]CaM dissociation was determined at 23 °C with the use of a filter assay. To minimize nonspecific binding of [^{35}S]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 KCl, 10 mM K-Pipes, pH 7.0, buffer containing 0.1 mg/ml BSA.

[^{3}H]Ryanodine Binding—Specific [^{3}H]ryanodine binding was determined in the buffer system used for [^{35}S]CaM binding after incubation with 1–2 nM [^{3}H]ryanodine for 20 h at 23 °C as previously described (18). B_{max} values of [^{3}H]ryanodine binding were determined by Scatchard analysis or with 50 nM [^{3}H]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-5X (RyR1) or pGEX-5X (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 (872–1207), 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 (3543–3961), FP15 (3931–4229), FP16 (4205–4478), FP17 (4404–4563), FP18 (4548–4748), FP19 (4726–4968); for RyR1, FPA (1–282), FPB (282–

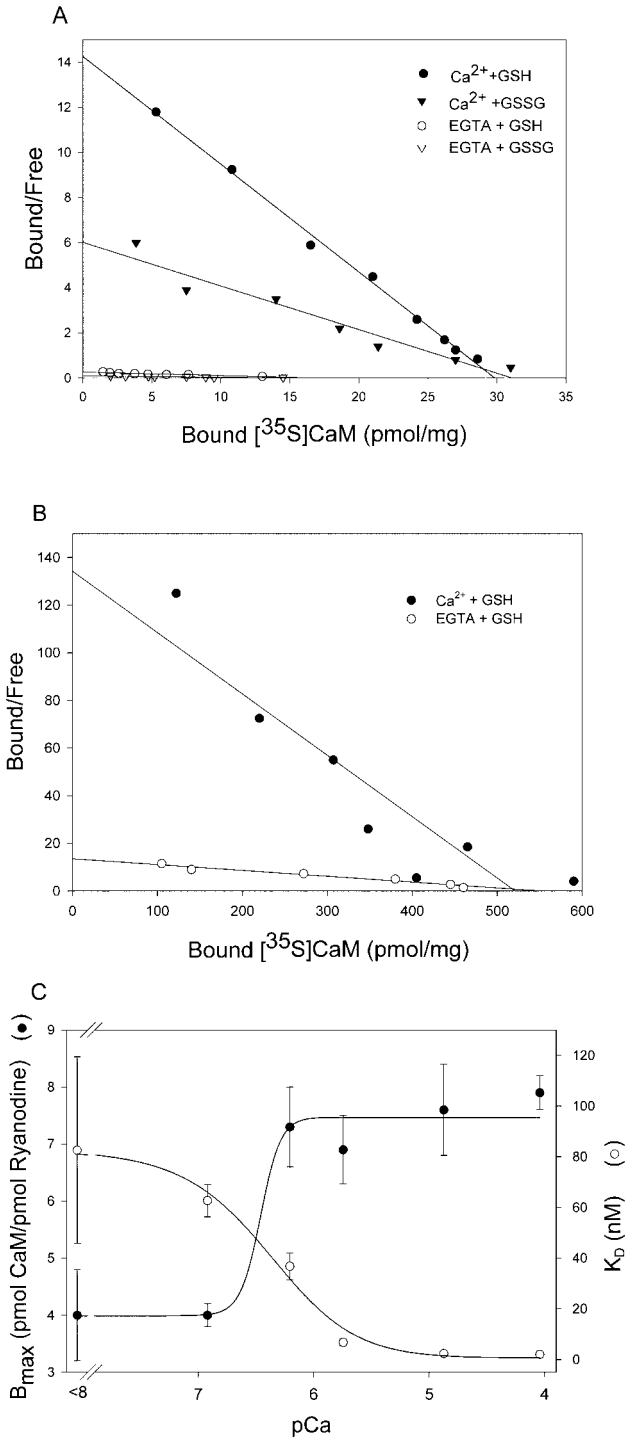

FIG. 1. **Analysis of ^{35}S-calmodulin binding to cardiac SR vesicles and purified RyR2.** Cardiac SR vesicles (*A*) and proteoliposomes containing the purified RyR2 (*B*) were incubated with increasing concentrations of [^{35}S]CaM in the presence of either 100 μM free Ca^{2+} (*filled symbols*) or 5 mM EGTA (*open symbols*) and reduced (GSH, *circles*) or oxidized (GSSG, *triangles*) glutathione. Averaged [^{35}S]CaM/[^{3}H]ryanodine binding ratios and K_D values from a total of between 5 and 16 experiments are shown in Table I. *C*, B_{max} (*left axis*, *filled circles*) and K_D (*right axis*, *open circles*) values for [^{35}S]CaM binding were determined as a function of free Ca^{2+} concentration. The data are mean ± S.D. for 8 experiments at <10 nM Ca^{2+}, 3 experiments at all other [Ca^{2+}].

TABLE I

[^{35}S]Calmodulin binding to cardiac and skeletal SR vesicles and purified RyR2 and RyR1

Values are the mean ± S.D. with the number of experiments in parenthesis. [^{35}S]CaM/[^{3}H]ryanodine is the ratio of B_{max} values in pmol/mg for [^{35}S]CaM and [^{3}H]ryanodine as determined by either Scatchard analysis or using saturating concentrations.

	with 100 μM Ca^{2+}		with <10 nM Ca^{2+}	
	[^{35}S]CaM/[^{3}H]Ryanodine	K_D	[^{35}S]CaM/[^{3}H]Ryanodine	K_D
		nM		nM
Cardiac SR				
+GSH	7.4 ± 1.5 (12)	3.5 ± 2.0 (12)	3.8 ± 0.8 (5)	72 ± 30 (5)
+GSSG	7.6 ± 1.3 (5)	6.8 ± 2.3 (5)[a]	2.6 ± 0.8 (5)	261 ± 103 (5)[a]
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)[a]	4.4 ± 0.7 (5)	108 ± 41 (5)[a]
Purified RyR1				
+GSH	4.6 ± 0.9 (6)	5.5 ± 2.6 (6)	3.3 ± 1.3 (6)	5.4 ± 2.5 (6)

[a] $P < 0.01$ when compared to K_D in presence of GSH by unpaired Student's t test.

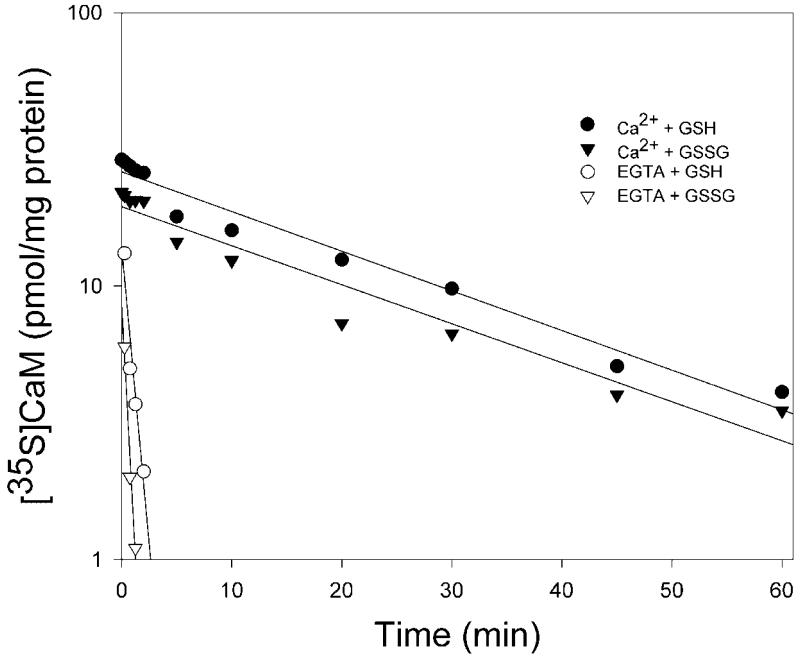

FIG. 2. **Dissociation of bound ^{35}S-calmodulin.** Cardiac SR vesicles were prebound with 100 or 200 nM [^{35}S]CaM in 100 μM free Ca^{2+} (*filled symbols*) or 5 mM EGTA (*open symbols*), respectively, in the presence of 5 mM GSH (*circles*) or 5 mM GSSG (*triangles*) and then were diluted 60-fold into media containing 50 nM non-radioactive CaM and either 100 μM free Ca^{2+} (*filled symbols*) or 5 mM EGTA (*open symbols*). The averaged time constants (in min) of [^{35}S]CaM dissociation ±S.D. of 3–5 experiments for RyR2 and RyR1, respectively, was 8.5 ± 2.9 and 12.4 ± 4.2 (Ca^{2+} + GSH), 9.0 ± 2.6 and 8.9 ± 3.4 (Ca^{2+} + GSSG), 0.7 ± 0.5 and 3.8 ± 1.0 (EGTA + GSH), and 0.7 ± 0.5 and 4.0 ± 0.9 (EGTA + GSSG).

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* (Stratagene) by induction (with 1 mM isopropyl-1-thio-β-D-galactopyranoside for GST and 10 μg/ml indoacrylic acid 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.

[^{35}S]Calmodulin Overlays—Equivalent amounts of each fusion protein (as judged by Coomassie Brilliant Blue stain and Western analysis) were loaded onto 10% SDS-polyacrylamide electrophoresis gels. After electrophoresis, the proteins were transferred to nitrocellulose (0.45-μm pore, Schleicher & Schuell) by semidry techniques. The membranes were blocked (2 × 30 min) in 150 mM KCl, 20 mM K-Pipes, pH 7.0, 1 mg/ml BSA with either 100 μM $CaCl_2$ or 5 mM EGTA. The membranes were then exposed to 100 nM [^{35}S]CaM in 150 mM KCl, 20 mM K-Pipes, pH 7.0, 0.04% Tween 20 with the appropriate Ca^{2+} concentration for 1 h at room temperature followed by four 10-min washes in blocking buffer at 4 °C. The membranes were dried overnight then exposed to Biomax MR x-ray film (Eastman Kodak Co.) for 1–10 days.

Single-channel Analysis—Single-channel measurements using purified RyR2 were carried out as previously described (18) in planar lipid bilayers containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio of 5:3:2 (25 mg of total phospholipid/ml of *n*-decane). The side of the bilayer to which the proteoliposomes containing the purified RyR2 were added was defined as the cis (cytoplasmic) side. The trans (SR lumenal) side of the bilayer was defined as ground. Measurements were made with symmetrical 0.25 M KCl, 20 mM K-Pipes, pH 7.0, with 50 μM free Ca^{2+} in the trans (lumenal) chamber. The cis (cytosolic) solution was varied according to experimental conditions. Data were acquired using test potentials of ±35 mV and were sampled at 10 kHz and filtered at 2 kHz. Channel open probabilities (P_o) were determined from at least 2 min of recordings for each condition.

Data Analysis—Free Ca^{2+} concentrations were determined as previously described (18). All data analyses were done using SigmaPlot version 5.

RESULTS

[^{35}S]Calmodulin Binding to RyR1 and RyR2—Fig. 1*A* 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 (*filled symbols*) and <10 nM free (*open symbols*) Ca^{2+}, respectively. The data are also summarized in Table I. In the presence of 100 μM Ca^{2+}, cardiac SR vesicles bound 7.5 mol of [^{35}S]CaM/mol of bound [^{3}H]ryanodine. Since there is only one high affinity [^{3}H]ryanodine binding site per tetramer (3, 4), this suggests that in the presence of Ca^{2+}, RyR2 binds 2 CaM molecules per subunit or that other CaM-binding proteins are present in cardiac SR vesicles. One of the

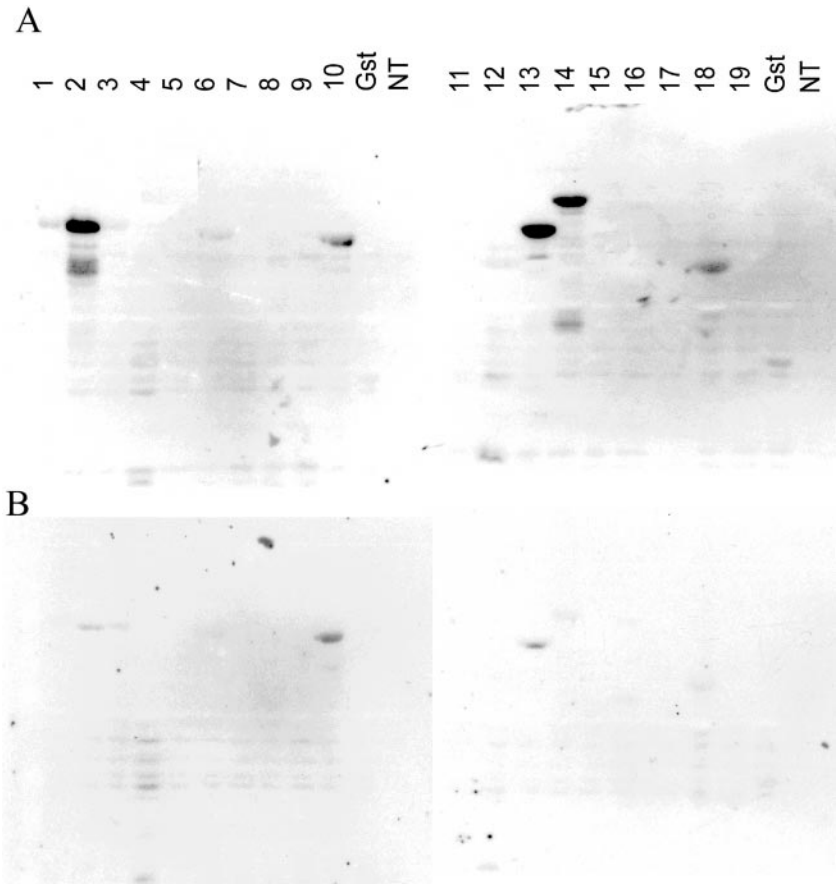

FIG. 3. **Identification of potential calmodulin binding sites.** Fusion proteins 1–19 derived from the full-length RyR2 sequence (fused to GST) as well as GST without an insert were expressed in *E. coli* and equivalent amounts of expressed fusion protein as well as nontransformed (*NT*) *E. coli*, as judged by Coomassie-stained SDS-polyacrylamide electrophoresis gels, were transferred to nitrocellulose. After blocking with 1 mg/ml BSA, 100 nM [^{35}S]CaM was overlaid for 1 h in 0.15 M KCl, 20 mM K-Pipes, pH 7, 0.04% Tween 20 with 100 μM $CaCl_2$ (*A*) or 5 mM EGTA (*B*). After four washes in blocking buffer and overnight drying, the membranes were exposed to x-ray film.

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}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 ± 0.6 (n = 4) and 0.13 ± 0.05 (n = 5) CaM 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. 1*B*) in the presence of 5 mM GSH and after reconstitution into proteoliposomes, the CaCaM:[^{3}H]ryanodine binding stoichiometry dropped from ~2 to 1 molecule per subunit in the presence of 100 μM 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:[^{3}H]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 μM to <10 nM 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_{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_{max} (Table I).

The Ca^{2+}-dependent changes in CaM binding to cardiac SR vesicles are shown in Fig. 1*C*. Increase in free Ca^{2+} from <10 to 100 nM was without significant effect on CaM binding affinity or CaM:[^{3}H]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 μM 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 μM 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 ~40 s. The rate of dissociation was decreased by more than 10-fold in the presence of Ca^{2+}, occurring with $\tau_{1/2}$ of ~9 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, [^{35}S]CaM overlays were performed with whole cell fractions. Fig. 3*A* shows that, in the presence of 100 μM $CaCl_2$ and 100 nM [^{35}S]CaM, five of the RyR2 fusion proteins showed pronounced [^{35}S]CaM binding. CaM binding was detected for RyR2 fusion proteins 2 (aa 263–615), 10 (2724–3016), 13 (3298–3595), 14 (3543–3961), and 18 (4548–4748), whereas inconsistent binding was observed for fusion proteins 1 (1–333), 3 (561–908), 6 (1487–1817), and 16 (4205–4478). Fig. 3*B* shows that in 5 mM EGTA, the binding to FP10 is only slightly decreased, whereas the binding to the remaining fusion proteins is greatly reduced, indicative of a Ca^{2+} dependence of binding to FPs 2, 13, 14, and 18.

The CaM binding to FP13 is localized to the C-terminal portion of the fusion protein since a truncated form, FP13short (3298–3577), does not bind either CaCaM or apoCaM in overlay experiments (not shown). Hence, it is likely that FPs 13 and 14 contain the same CaM binding domain. RyR1 fusion proteins I (aa 3225–3662) and M (4302–4430) bound CaCaM; in the presence of 5 mM EGTA, binding to FPM was not significantly altered, and binding to FPI 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 Ca^{2+} efflux from both skeletal and cardiac SR vesicles (13, 22). In addition, CaCaM inhibits [^{3}H]ryanodine binding to RyR1 in skeletal SR vesicles (7, 14, 23) with little effect on [^{3}H]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 [^{3}H]ryanodine binding, which has not been done in previous studies. Relatively low CaMBP concentrations (0.1 μM) were sufficient to reduce [^{35}S]CaM binding in the presence of 100 μM free Ca^{2+} to skeletal SR vesicles to 4% of the control value (Fig. 4*A*). For cardiac SR vesicles, a higher CaMBP concentration (1 μM) was required to reduce [^{35}S]CaM binding to comparable, low levels. 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. 4*B*, vesicles assayed in the presence of high concentrations of CaMBP show a marked stimulation of [^{3}H]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 [^{35}S]CaM binding to SR vesicles at $[Ca^{2+}] < 1$ 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 [^{3}H]ryanodine binding, we measured [^{3}H]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 K_{Hi} 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 K_D for [^{35}S]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 K_{Hi} values (legend of Fig. 5) that were lower than the K_D values and had Hill coefficients near unity. The results suggest that inhibition of ryanodine binding perhaps requires only a single CaCaM bound/tetramer.

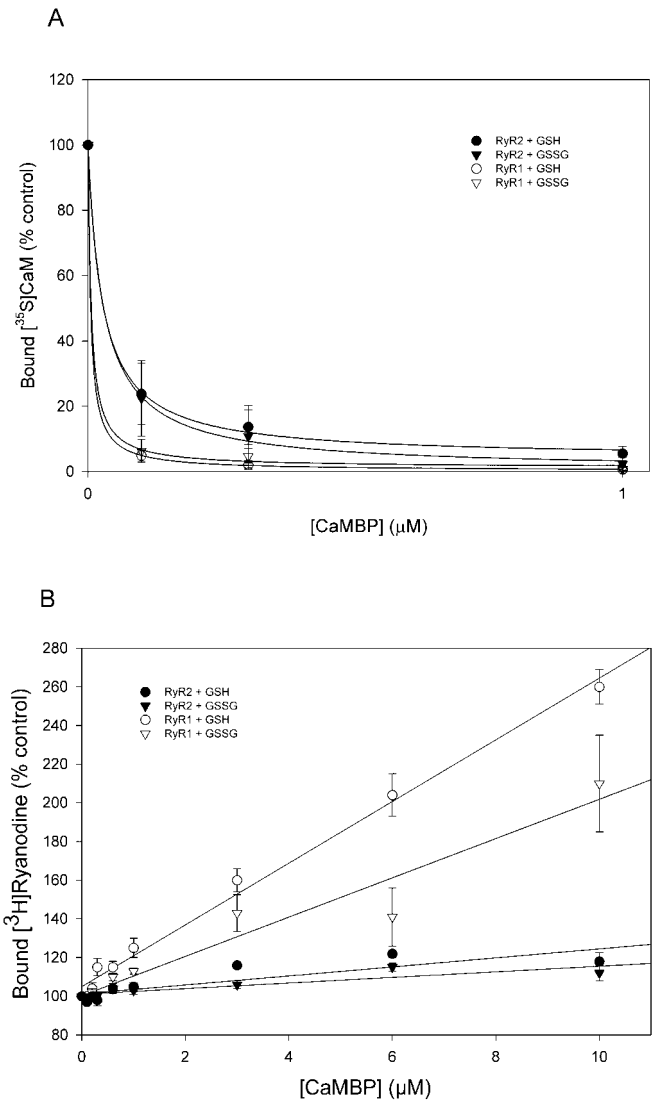

FIG. 4. **CaMBP effects on [^{35}S]calmodulin and [^{3}H]ryanodine binding.** *A*, [^{35}S]CaM binding in 10 nM [^{35}S]CaM and 100 μM free Ca^{2+} was determined in either 5 mM GSH (*circles*) or GSSG (*triangles*) as a function of increasing CaMBP concentration to SR vesicles from either cardiac (*filled symbols*) or skeletal (*open symbols*) muscle. *B*, cardiac or skeletal SR vesicles were preincubated with 1 μM CaMBP and centrifuged to remove endogenous CaM and added CaMBP, then [^{3}H]ryanodine binding was determined in 0.15 M KCl, 20 mM K-Pipes, pH 7.0, 100 μM Ca^{2+}, 0.1 mg/ml BSA in either 5 mM GSH or GSSG with increasing concentrations of CaMBP. Data are the mean ± S.D. of 3–4 experiments.

CaM inhibition of [^{3}H]ryanodine binding is also modulated by various regulators of the RyRs as indicated in Fig. 6 and Table II. At $[Ca^{2+}] > 1$ μM, CaM (1 μM) inhibition of both cardiac and skeletal RyR is observed in both oxidizing and reducing conditions in the absence of MgAMPPCP (AMPPCP is a nonhydrolyzable ATP analogue). CaM inhibits [^{3}H]ryanodine binding to RyR2 by both rendering the receptor less sensitive to activation by Ca^{2+} and more sensitive to inhibition at high Ca^{2+} as well as by lowering the maximal level of [^{3}H]ryanodine binding (Fig. 6, *upper left panel*). In the presence of MgAMPPCP and at $[Ca^{2+}] < 10$ μM, CaM inhibits cardiac SR ryanodine binding in both reducing and oxidizing conditions (Fig. 6, *upper right panel*). However, at $[Ca^{2+}] > 10$ 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 [^{3}H]ryanodine binding is markedly

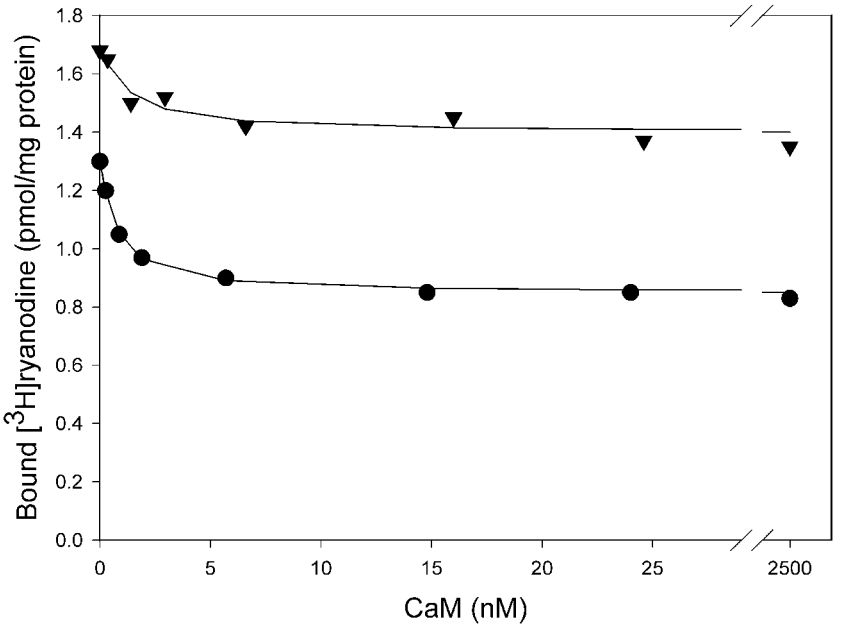

FIG. 5. **Calmodulin inhibition of [^{3}H]ryanodine binding to cardiac SR vesicles.** Cardiac SR vesicles were preincubated with 1 μM CaMBP and centrifuged to remove endogenous CaM and added CaMBP. [^{3}H]Ryanodine binding was determined in 0.15 M KCl, 20 mM K-Pipes, pH 7.0, 100 μM Ca^{2+}, 0.1 mg/ml BSA, 0–2500 nM CaM, and either 5 mM GSH (*circles*) or GSSG (*triangles*). Free CaM concentrations were measured on paired samples using [^{35}S]CaM. *Solid lines* were obtained according to the equation $B = B_o\,(1 + (([CaM]/K_{Hi})^{n_{Hi}})^{-1}$, where B and B_o are bound [^{3}H]ryanodine in the presence and absence of CaM. The averaged Hill inhibition constants (K_{Hi}) ± S.D. of 3–4 experiments in the presence of GSH and GSSG were 0.6 ± 0.2 nM and 1.7 ± 0.7 nM, respectively. Corresponding Hill coefficients (n_{Hi}) were 1.0 ± 0.3 and 1.0 ± 0.3. Corresponding K_{Hi} for RyR1 were 1.6 ± 0.2 nM and 4.7 ± 0.4 nM, and n_{Hi} were 1.2 ± 0.4 and 1.1 ± 0.3.

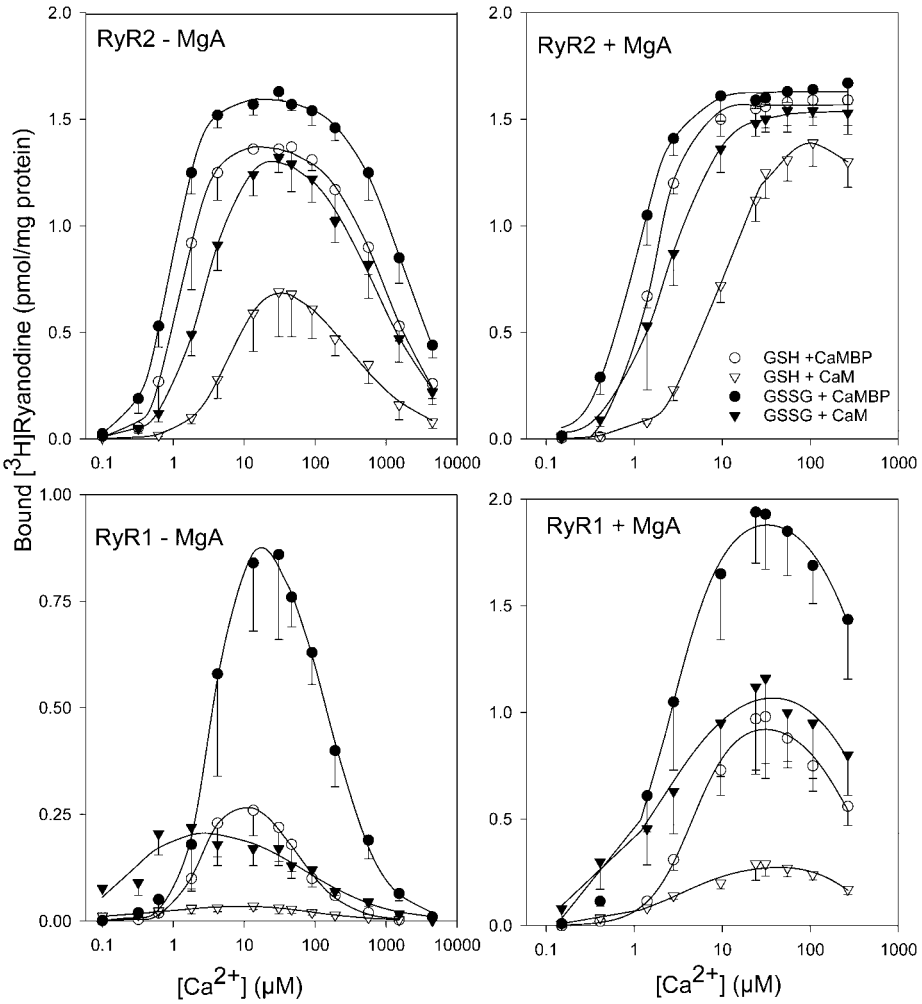

FIG. 6. **Ca^{2+} dependence of calmodulin inhibition of [^{3}H]ryanodine binding.** [^{3}H]Ryanodine binding was determined as a function of free Ca^{2+} concentration for both cardiac (RyR2) and skeletal (RyR1) SR vesicles either in the absence (*−MgA*) or presence (*+MgA*) of 5 mM MgAMPPCP and either in the absence (*circles*) or presence (*triangles*) of 1 μM added CaM and either with 5 mM GSH (*open symbols*) or 5 mM GSSG (*filled symbols*). Endogenous CaM was removed by pretreating vesicles with 1 μM CaMBP (experiments at <1 μM Ca^{2+}) followed by centrifugation or dissociated from the receptor by carrying out the binding assay in the presence of 0.5 μM CaMBP (experiments at > 1 μM Ca^{2+}). The averaged data ± S.D. of 3–4 experiments are fit to a two-site (one activation, one inhibition) logistic function (34).

inhibited in the presence of GSH. [^{3}H]Ryanodine binding to skeletal SR is activated by CaM at low Ca^{2+} concentrations both in the absence or presence of MgAMPPCP and the presence of GSH (see Table II) or GSSG and inhibited at higher Ca^{2+} concentrations in both oxidizing and reducing conditions (Fig. 6, *lower two panels*). In the presence of 5 mM MgAMPPCP, free Ca^{2+} concentrations in excess of 0.3 mM were not tested because of difficulties in keeping Ca^{2+} in solution.

In Table II, the effects of CaM on [^{3}H]ryanodine binding to RyR2 and RyR1 are compared in 0.1 and 100 μM Ca^{2+} media that contained 10 mM caffeine, 5 mM AMPPCP, or 1 mM Mg^{2+}. ApoCaM significantly inhibits RyR2 in reducing conditions in

TABLE II

Regulation of native RyR1 and RyR2 by calmodulin in the presence of other ligands

Bound [^{3}H]ryanodine (pmol/mg) was determined under the indicated conditions with vesicles pretreated with CaMBP (−CaM) to complex endogenous CaM or in the presence of 1 μM CaM (+CaM). Data are mean ± S.D. of 3–4 experiments.

Assay medium	Bound [^{3}H]ryanodine			
	RyR2		RyR1	
	−CaM	+CaM	−CaM	+CaM
	pmol/mg of protein			
In 5 mM GSH				
0.1 μM Ca^{2+}	0.013 ± 0.003	0.005 ± 0.002[a]	0.002 ± 0.002	0.012 ± 0.002[a]
+10 mM caffeine	0.59 ± 0.04	0.50 ± 0.07	0.08 ± 0.01	0.14 ± 0.02[a]
+5 mM AMPPCP	0.06 ± 0.02	0.025 ± 0.005[a]	0.025 ± 0.005	0.09 ± 0.03[a]
100 μM Ca^{2+}	1.2 ± 0.1	0.8 ± 0.1[a]	0.17 ± 0.01	0.04 ± 0.01[a]
+10 mM caffeine	1.2 ± 0.1	1.2 ± 0.2	0.19 ± 0.01	0.04 ± 0.01[a]
+5 mM AMPPCP	1.4 ± 0.1	1.3 ± 0.1	3.3 ± 0.3	2.1 ± 0.2[a]
+1 mM Mg^{2+}	0.90 ± 0.05	0.30 ± 0.04[a]	0.03 ± 0.01	0.01 ± 0.01
In 5 mM GSSG				
0.1 μM Ca^{2+}	0.025 ± 0.006	0.017 ± 0.003	0.01 ± 0.01	0.06 ± 0.01[a]
+10 mM caffeine	0.58 ± 0.05	0.55 ± 0.05	0.4 ± 0.1	0.7 ± 0.1[a]
+5 mM AMPPCP	0.17 ± 0.02	0.11 ± 0.04	0.30 ± 0.05	0.85 ± 0.20[a]
100 μM Ca^{2+}	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	0.2 ± 0.1[a]
+10 μM caffeine	1.3 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	0.2 ± 0.1[a]
+5 mM AMPPCP	1.4 ± 0.1	1.4 ± 0.1	3.8 ± 0.3	3.5 ± 0.3
+1 mM Mg^{2+}	1.2 ± 0.1	0.8 ± 0.1[a]	0.20 ± 0.05	0.03 ± 0.01[a]

[a] $P < 0.05$ when compared to bound [^{3}H]ryanodine in the absence of CaM by Student's unpaired t test.

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 $MgCl_2$, 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 Ca^{2+}. CaCaM inhibition of ryanodine binding to RyR1 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 [^{35}S]CaM binding properties, as indicated in Table I; furthermore, CaCaM inhibition of [^{3}H]ryanodine binding to purified RyR1 was comparable with that of SR vesicles (not shown). Purification of RyR2, however, decreased the stoichiometry of CaCaM binding without affecting the K_D. In addition, purified RyR2 (either solubilized or reconstituted) failed to show any inhibition of [^{3}H]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. 7*A*, a single channel was recorded in the presence of 100 μM cis (cytoplasmic) Ca^{2+}. The addition of 1 μM cis CaM reduced channel open probability (P_o) from 0.62 to 0.27. The Hill inhibition constant and coefficient of 7 experiments were 42 ± 1 nM and 1.4 ± 0.1, respectively (Fig. 7*B*). In 100 μM free cis Ca^{2+} and symmetrical 0.25 M KCl, the addition of 1 μM CaM to the cis chamber reduced P_o 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 Mg^{2+} and 100 μM free Ca^{2+} with a decrease in P_o from 0.51 ± 0.15 to 0.05 ± 0.03 ($n = 4$). As in the [^{3}H]ryanodine binding studies using SR membranes, MgATP reversed the inhibition by CaM at 100 μM free Ca^{2+}. At 100–200 nM free Ca^{2+} 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 ($P_{o(+CaM)}/P_{o(-CaM)} = 0.22 \pm 0.05$ 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 [^{3}H]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. 1*C*), 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 Ca^{2+}-binding protein calmodulin is capable of inhibiting Ca^{2+} 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 ^{125}I (7) or fluorescently (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 Ca^{2+}-dependent, which indicated differences in the Ca^{2+} dependence of CaM binding to the intact receptor and isolated fragments. More recent studies using SR membranes and ^{35}S metabolically labeled CaM indicate that the tetrameric skeletal muscle channel complex binds a total of four CaM molecules both for apo- and CaCaM or 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 [^{35}S]CaM, the skeletal muscle isoform binds ~4 molecules of CaM per tetramer both in the presence and absence of Ca^{2+}. Furthermore, the stoichiometry of CaM binding to RyR1

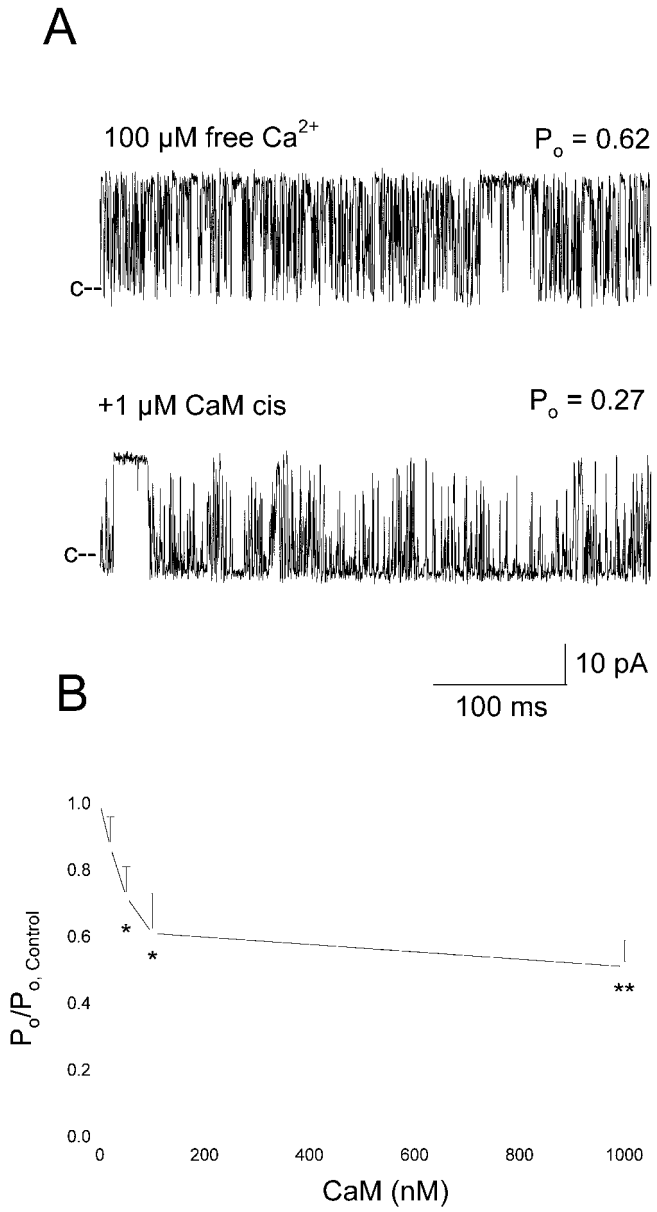

FIG. 7. **Effect of cytosolic calmodulin on single purified RyR2 ion channels.** Proteoliposomes containing purified RyR2 were fused with lipid bilayers. *A*, single-channel currents were recorded at 35 mV (upward deflection from closed levels, *c*-) in symmetric 0.25 M KCl, 20 mM K^+-Pipes, pH 7.0, media containing 5.08 mM $CaCl_2$ and 5 mM EGTA (free Ca^{2+} of 100 μM). *Top trace*, control, P_o = 0.62. *Bottom trace*, after the addition of 1 μM CaM, P_o = 0.27. *B*, dependence of cardiac Ca^{2+} release channel activity on CaM concentration. Relative channel open probability ($P_o/P_{o,\text{control}}$) was obtained from 7 single-channel recordings similar to those shown in *A*. *Solid lines* were obtained according to the equation $P_o = P_{o,\text{control}}(1 + (([\text{CaM}]/K_{\text{Hi}})^{n_{\text{Hi}}})^{-1}$ where P_o and $P_{o,\text{control}}$ are single-channel activities in the presence and absence of CaM. The Hill inhibition constant and coefficient ± S.D. of seven experiments were 42 ± 1 nM and 1.4 ± 0.1, respectively. * and ** indicate the means of data were significantly different from control at $p < 0.05$ and $p < 0.001$, respectively.

is not influenced by the redox state, whereas previous studies using sulfhydryl-reacting agents and [^{125}I]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 μM 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) HPQRSKKAVWHKLLSKQR 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 μM 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-KSKKAVWHKLLSKQ-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

TABLE III
Calmodulin inhibition of purified RyR2 single channel activities

Data are the means ± S.E. of number of indicated experiments.

Assay medium	Single-channel activities (P_o)		Number of experiments
	−CaM	+CaM	
100 μM Ca^{2+}	0.73 ± 0.09	0.32 ± 0.08[a]	7
100 μM Ca^{2+} + 2 mM Mg^{2+}	0.51 ± 0.15	0.05 ± 0.03[a]	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 ± 0.0001[b]	7
150 nM Ca^{2+} + 3–40 mM caffeine	0.10 ± 0.05	0.07 ± 0.04[b]	8
0.1–0.4 μM Ca^{2+} + 2 mM MgATP	<0.001	<0.001	3

[a] $P < 0.05$ 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 ($P_{o(+CaM)}/P_{o(-CaM)}$).

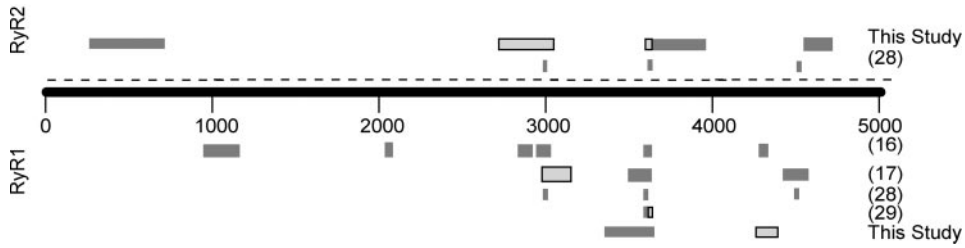

FIG. 8. **Schematic summary of potential calmodulin binding sites.** Sequence domains suggested by previous works (16, 17, 28, 29) and this report are aligned with cardiac binding domains above and skeletal binding domains below a linear representation of the full-length amino acid sequence of the ryanodine 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. [^{3}H]Ryanodine binding to cardiac SR vesicles was inhibited by CaCaM 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 [^{3}H]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 [^{3}H]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]CaCaM per subunit without affecting apoCaM binding, an effect not observed with RyR1. In addition, purification of RyR2 also eliminated CaCaM-dependent inhibition of [^{3}H]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_{Hi} for inhibition of channel open probability was considerably higher than the K_D for [^{35}S]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+} provided by the dihydropyridine 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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