Calmodulin Activation and Inhibition of Skeletal Muscle Ca^{2+} Release Channel (Ryanodine Receptor)

Ashutosh Tripathy, Le Xu, Geoffrey Mann, and Gerhard Meissner
Departments of Biochemistry and Biophysics, and Physiology, University of North Carolina, Chapel Hill, North Carolina 27599 USA

ABSTRACT The calmodulin-binding properties of the rabbit skeletal muscle Ca^{2+} release channel (ryanodine receptor) and the channel's regulation by calmodulin were determined at ≤ 0.1 μM and micromolar to millimolar Ca^{2+} concentrations. [^{125}I]Calmodulin and [^{3}H]ryanodine binding to sarcoplasmic reticulum (SR) vesicles and purified Ca^{2+} release channel preparations indicated that the large (2200 kDa) Ca^{2+} release channel complex binds with high affinity (K_D = 5–25 nM) 16 calmodulins at ≤ 0.1 μM Ca^{2+} and 4 calmodulins at 100 μM Ca^{2+}. Calmodulin-binding affinity to the channel showed a broad maximum at pH 6.8 and was highest at 0.15 M KCl at both ≤ 0.1 μM and 100 μM Ca^{2+}. Under conditions closely related to those during muscle contraction and relaxation, the half-times of calmodulin dissociation and binding were 50 $\pm$ 20 s and 30 $\pm$ 10 min, respectively. SR vesicle-$^{45}Ca^{2+}$ flux, single-channel, and [^{3}H]ryanodine binding measurements showed that, at ≤ 0.2 μM Ca^{2+}, calmodulin activated the Ca^{2+} release channel severalfold. At micromolar to millimolar Ca^{2+} concentrations, calmodulin inhibited the Ca^{2+}-activated channel severalfold. Hill coefficients of $\sim$1.3 suggested no or only weak cooperative activation and inhibition of Ca^{2+} release channel activity by calmodulin. These results suggest a role for calmodulin in modulating SR Ca^{2+} release in skeletal muscle at both resting and elevated Ca^{2+} concentrations.

INTRODUCTION

In striated muscle, the release of Ca^{2+} from the SR is mediated by a Ca^{2+} release channel in response to an action potential by a mechanism referred to as E-C coupling (see reviews by Rios and Pizarro, 1991; Franzini-Armstrong and Jorgensen, 1994; Schneider, 1994). The SR Ca^{2+} release channels are also known as RyRs because of their ability to bind the plant alkaloid ryanodine with high affinity and specificity. Mammalian tissues express three structurally related RyRs encoded by three different genes: a skeletal muscle (RyR1), cardiac muscle (RyR2), and neuronal and smooth muscle (RyR3) isoform. The skeletal muscle and cardiac muscle RyRs have been purified as 30-S protein complexes composed of four polypeptides of $\sim$5000 amino acids each. The very large subunits presumably allow a complex pattern of regulation of channel activity, because functional studies have shown that the skeletal and cardiac muscle Ca^{2+} release channels may be regulated by various endogenous effector molecules including Ca^{2+}, Mg^{2+}, ATP, and CaM (see reviews by Coronado et al., 1994; Meissner, 1994).

Photoaffinity labeling studies with native SR vesicles indicated that CaM predominantly binds to high M_r skeletal and cardiac SR proteins now known to be the RyR polypeptides (Seiler et al., 1984). More recently, native skeletal muscle SR vesicles were used to study the channel's interaction with CaM by fluorescence anisotropy by using rhodamine maleimide-labeled wheat germ CaM (Yang et al., 1994). These studies indicated that CaM binds with high affinity to the skeletal muscle Ca^{2+} release channel protein in the presence and absence of Ca^{2+}. Correlation of CaM and [^{3}H]ryanodine binding data indicated the presence of multiple CaM-binding sites on each channel subunit with the number and affinities of the CaM-binding sites depending on Ca^{2+} and Mg^{2+} concentrations. Primary sequence predictions and [^{125}I]CaM overlay studies suggested the presence of up to nine CaM-binding sites on each of the four M_r 565,000 subunits of the skeletal muscle RyR (Takeshima et al., 1989; Zorzato et al., 1990; Chen and MacLennan, 1994; Menegazzi et al., 1994). Electron microscopic studies with purified RyRs and gold-cluster-labeled CaM indicated that one CaM-binding site is at the surface that faces the cytoplasm (Wagenknecht et al., 1994).

Single-channel measurements showed that CaM inhibits the SR Ca^{2+} release channel by reducing channel open time without having an effect on single-channel conductance (Smith et al., 1989). Channel inhibition was reversible and an only partial (two- to sixfold) rather than complete inhibition by CaM was observed. Calmodulin inhibited SR Ca^{2+} release and single-channel activities in the absence of ATP, which suggested a direct inhibitory action of CaM (Meissner, 1986; Meissner and Henderson, 1987; Plank et al., 1988; Fuentes et al., 1994).

In this study we compared the CaM-binding properties and regulation by CaM of the skeletal muscle Ca^{2+} release channel by using rabbit skeletal muscle SR vesicles and purified channel preparations. Our results indicated that the tetrameric channel complex binds with nanomolar affinity 4

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Address reprint requests to Dr. Gerhard Meissner, Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599–7260. Tel.: 919-966-5021; Fax: 919-966-2852.

Abbreviations used: SR, sarcoplasmic reticulum; E-C, excitation-contraction; RyR, ryanodine receptor; CaM, calmodulin; AMPPCP, β,γ-methyleneadenosine 5′-triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulphonic acid); EGTA, ethylene glycol-bis(β-aminoethyl ether)N,N,N′,*N*′-tetraacetic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethanetetraacetic acid; BSA, bovine serum albumin.

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CaMs in the presence of Ca^{2+} and 16 CaMs in the absence of Ca^{2+}. Measurements of channel activity showed that CaM activates the skeletal muscle Ca^{2+} release channel at submicromolar Ca^{2+} concentration whereas it inhibits the channel at micromolar to millimolar Ca^{2+}. Determination of binding kinetics suggested that CaM may exert its two opposing effects on channel activity without dissociating from the channel.

MATERIALS AND METHODS

Materials

[^{125}I]CaM and [^{3}H]ryanodine were purchased from DuPont NEN (Boston, MA) and $^{45}Ca^{2+}$ from ICN Biomedicals. Unlabeled CaM and the nonhydrolyzable ATP analogue AMPPCP were obtained from Sigma Chemical Co. (St. Louis, MO), unlabeled ryanodine from Calbiochem (La Jolla, CA), CHAPS and Pefabloc (a protease inhibitor) from Boehringer Mannheim (Indianapolis, IN), and phospholipids from Avanti Polar Lipids (Birmingham, AL). All other chemicals were of analytical grade.

Preparation of SR vesicles

Heavy SR vesicle fractions enriched in [^{3}H]ryanodine binding and Ca^{2+} release channel activities were prepared in the presence of protease inhibitors (100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride) as described (Meissner, 1984). Vesicles were purified by sucrose gradient equilibrium centrifugation in the presence of micromolar Ca^{2+} concentration and a high ionic strength buffer (0.6 M KCl) that favored dissociation of endogenous CaM from the SR Ca^{2+} release channel (see Fig. 2).

Purification and reconstitution of Ca^{2+} release channel

The Ca^{2+} release channel was purified as a 30-S protein complex by sucrose gradient rate centrifugation after solubilization of heavy SR vesicles in the presence of CHAPS (Lai et al., 1989). The purified 30-S protein complex was reconstituted into proteoliposomes by removal of CHAPS by dialysis (Lee et al., 1994a). Proteoliposomes were quick-frozen and stored at −75°C. Before use, samples were freeze-thawed and sonicated as described (Lee et al., 1994a).

[^{125}I]Calmodulin binding

Unless otherwise indicated, SR vesicles or purified Ca^{2+} release channel preparations were incubated with 2–200 nM [^{125}I]CaM at 23°C in 0.1 M KCl, 20 mM K-PIPES, pH 7.0, 0.2 mM Pefabloc, 20 μM leupeptin, and 2 mM EGTA or 100 μM Ca^{2+}. [^{125}I]CaM concentrations (10^5 cpm/ml) were obtained as admixtures of labeled (~100 μCi/μg protein) and unlabeled CaM. Nonspecific binding was determined with a 100- to 1000-fold excess of unlabeled CaM. After 60–120 min, aliquots of vesicle suspensions were placed into a glass tube to determine total radioactivity and centrifuged for 30 min at 90,000 × g in a Beckman Airfuge to determine free [^{125}I]CaM in the supernatant fractions. Soluble and reconstituted, purified Ca^{2+} release channel preparations were sedimented by centrifugation for 180 min at 225,000 × g in a Beckman Type 75 Ti rotor. Bound [^{125}I]CaM was determined as the difference between total and free [^{125}I]CaM. Radioactivity was determined by using a Packard auto-gamma counter.

The time course of [^{125}I]CaM binding and dissociation was determined at 23°C with the use of a filter assay. To minimize nonspecific binding of [^{125}I]CaM, Millipore filters (0.45 μm, type HA) were blocked for 1 h in 0.1 M KCl, 10 mM K-PIPES, pH 7.0 buffer containing 0.2 mM EGTA and 1 mg/ml BSA. Vesicles on the filters were washed three times with 5 ml of 0.1 M KCl, 10 mM K-PIPES, pH 6.8 buffer containing 0.2 mM EGTA and 0.1 mg/ml BSA.

$^{45}Ca^{2+}$ efflux measurements

SR vesicles (5–10 mg protein/ml) were passively loaded for 60 min at 23°C with 2 mM $^{45}Ca^{2+}$ in media containing 0.1 M KCl, 20 mM K-PIPES, pH 6.8, and the indicated concentrations of CaM as described (Meissner, 1986). $^{45}Ca^{2+}$ efflux was initiated by diluting vesicles 1:300 into isoosmolar efflux media and stopped by placing 0.4-ml aliquots at various times on a 0.45-μm filter (type HA, Millipore). Filters were washed with a quench solution containing 0.1 M KCl, 20 mM K-PIPES, pH 6.8, 10 mM Mg^{2+}, 20 μM ruthenium red, and 0.2 mM EGTA. Radioactivity remaining with the vesicles on the filters was determined by liquid scintillation counting. The time course of $^{45}Ca^{2+}$ efflux from the Ca^{2+}-permeable vesicle population was obtained by subtracting the amount not readily released (Meissner, 1986).

$^{45}Ca^{2+}$ influx measurements

SR vesicles (5–10 mg protein/ml) were incubated for 30 min at 23°C in media containing 0.1 M KCl, 20 mM K-PIPES, pH 7.0, 2 mM EGTA, 5 mM Mg^{2+}, 5 mM AMPPCP, 0.2 mM Pefabloc, 20 μM leupeptin, and the indicated CaM concentrations. $^{45}Ca^{2+}$ influx was started by diluting the vesicles with an equal volume of 0.1 M KCl, 20 mM K-PIPES, pH 7.0, and 4 mM $^{45}Ca^{2+}$. At the indicated times, 0.3 ml of a quench solution (0.1 M KCl, 20 mM K-PIPES, pH 7.0, 1.0 mM EGTA, 1.05 mM Ca^{2+}, 20 mM Mg^{2+}, and 20 μM ruthenium red) was added. The vesicle suspension was placed on a 0.45-μm Millipore filter and washed with the above quench solution (without EGTA). Radioactivity remaining with the vesicles on the filters was determined by liquid scintillation counting.

[^{3}H]Ryanodine binding

Unless otherwise indicated, samples were incubated at 12°C with 1–50 nM [^{3}H]ryanodine in 0.75 M NaCl, 20 mM K-PIPES, pH 7.0, 0.3 mM free Ca^{2+} (0.1 mM EGTA and 0.4 mM Ca^{2+}), 5 mM AMP, 0.2 mM Pefabloc, and 20 μM leupeptin. Nonspecific binding was determined with a 1000-fold excess of unlabeled ryanodine. After 44 h, aliquots of the samples were diluted with 20 volumes of ice-cold water and placed on Whatman GF/B filters soaked with 2% polyethyleneimine. Filters were washed with three 5-ml volumes of ice-cold 0.1 M KCl, 1 mM K-PIPES, pH 7.0 medium, and the radioactivity remaining with the filters was determined by liquid scintillation counting to obtain bound [^{3}H]ryanodine.

Single-channel recordings

Single-channel measurements were performed by fusing proteoliposomes containing the purified skeletal muscle Ca^{2+} release channel with Mueller-Rudin type bilayers containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio 5:3:2 (50 mg total phospholipid/ml n-decane) (Lee et al., 1994a). Single channels were recorded in a symmetric KCl buffer solution (0.25 M KCl, 20 mM K-PIPES, pH 7.1) containing the additions indicated in the text. Electrical signals were filtered at 2 or 4 kHz through an eight-pole low pass Bessel filter, digitized at 10 or 20 kHz, and analyzed. Data acquisition and analysis were performed with a commercially available software package (pClamp 6.0.1., Axon Instruments, Burlingame, CA) with an IBM-compatible 486 computer and 12-bit A/D to D/A converter (Digidata 1200, Axon Instruments). Data files were acquired with Clampex pulse protocol (100–200 episodes of 358 ms) or by continuous fetchex mode (file length 60–120 s). Some data were acquired by using both protocols without a significant difference.

Other biochemical assays

Protein concentrations were determined by the method of Kaplan and Pederson (1985) using Amido Black and 0.45-μm Millipore filters (type HA). BSA was used as the protein standard. Free Ca^{2+} concentrations of >1 μM were determined with a Ca^{2+}-selective electrode (World Precision Instruments, Sarasota, FL). Free Ca^{2+} concentrations of <1 μM were obtained by including in the solutions the appropriate amounts of EGTA (or BAPTA) and Ca^{2+} as determined by using the stability constants and computer program published by Shoenmakers et al. (1992).

Data analysis

Results are given as means ± SE with the number of experiments in parentheses. Significance of differences of data was analyzed with Student's *t*-test. Differences were regarded to be statistically significant at $P < 0.05$.

RESULTS

[^{125}I]Calmodulin binding to SR vesicles and purified Ca^{2+} release channel

Primary sequence predictions and [^{125}I]CaM overlay studies have suggested the presence of as many as 9 CaM-binding sites per skeletal muscle RyR polypeptide (or 36 sites per tetrameric RyR) (Takeshima et al., 1989; Zorzato et al., 1990; Chen and MacLennan, 1994; Menegazzi et al., 1994). In this study, the binding of the Ca^{2+}-free (at <0.1 μM free Ca^{2+}) and Ca^{2+}-bound (4 Ca^{2+}/CaM at ≥100 μM Ca^{2+}) forms of CaM was determined (George et al., 1990). CaM binding to the skeletal muscle Ca^{2+} release channel was quantitated by measuring the binding of [^{125}I]CaM to SR vesicles and purified RyR preparations. In parallel experiments, the RyR concentration was determined by radioligand analysis by using the neutral plant alkaloid [^{3}H]ryanodine as a high affinity probe (Lai et al., 1989). Fig. 1 shows that the extent of [^{125}I]CaM binding to the purified and reconstituted RyR increased severalfold when the free Ca^{2+} concentration was lowered from micromolar to submicromolar concentrations. Scatchard analysis (Fig. 1, inset) indicated the presence of high affinity CaM-binding sites with B_{max} values of 605 and 2560 pmol/mg protein in the presence of 100 μM free Ca^{2+} and 2 mM EGTA (~5 × 10^{-9} M free Ca^{2+}), respectively. These B_{max} values corresponded to a [^{125}I]CaM-to-[^{3}H]ryanodine binding ratio of 3.0 and 12.4, respectively.

Table 1 summarizes the K_D and B_{max} values of [^{125}I]CaM binding to SR vesicles, the purified CHAPS-solubilized RyR, and purified RyR reconstituted into proteoliposomes, along with the B_{max} values of high affinity [^{3}H]ryanodine binding. The averaged [^{125}I]CaM/[^{3}H]ryanodine binding ratios obtained for the three preparations ranged from 12.7 to 15.1 at <0.01 μM, and from 2.4 to 4.2 at 100 μM Ca^{2+}. The binding stoichiometries for the detergent-solubilized RyR and reconstituted RyR were obtained by using averaged values of 345 and 230 pmol bound [^{3}H]ryanodine/mg protein, respectively. These values were lower than the theoretical value of 440 pmol/mg protein for the purified

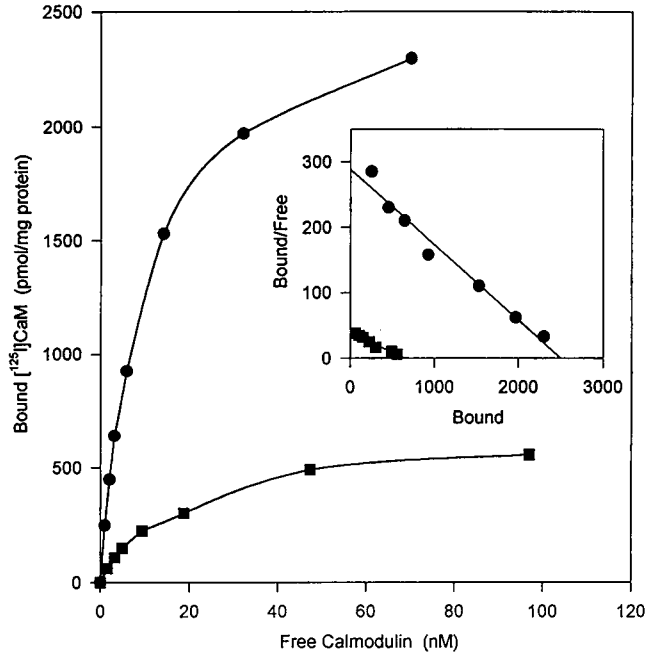

FIGURE 1 [^{125}I]CaM binding to proteoliposomes in the presence of submicromolar and micromolar Ca^{2+} concentrations. [^{125}I]CaM binding to proteoliposomes containing the purified Ca^{2+} release channel was determined in the presence of 2 mM EGTA with <0.01 μM free Ca^{2+} (●) or 100 μM free Ca^{2+} (■) as described in Materials and Methods. Scatchard analysis (*inset*) yielded K_D values of 9.5 and 14 nM and B_{max} values of 2560 and 605 pmol [^{125}I]CaM/mg protein, respectively. A B_{max} value of specific high affinity [^{3}H]ryanodine binding of 205 pmol/mg protein indicated [^{125}I]CaM/[^{3}H]ryanodine binding ratios of 12.4 and 3.0 at <0.01 μM and 100 μM Ca^{2+}, respectively. The B_{max} value of [^{3}H]ryanodine binding was determined by Scatchard analysis or by using a ligand concentration (50 nM [^{3}H]ryanodine) that resulted in occupancy of essentially all (>98%) high affinity binding sites (Lai et al., 1989).

receptor, indicating partial receptor inactivation during purification and reconstitution. It was therefore conceivable that the binding ratios for the two purified preparations may have been overestimated because of binding of [^{125}I]CaM to inactivated RyRs. However, against this possibility it can be argued that similar [^{125}I]CaM/[^{3}H]ryanodine binding ratios were obtained regardless of the [^{3}H]ryanodine binding activity of the purified RyR preparations. Furthermore, heating of SR vesicles and purified RyR (60°C for 30 min) resulted in loss of both [^{3}H]ryanodine- and [^{125}I]CaM-binding activities. The B_{max} values of [^{125}I]CaM binding to proteoliposomes are likely underestimated, as proteolysis studies have suggested that ~20% of the reconstituted RyRs have a disposition that renders them incapable of [^{125}I]CaM binding (Lee et al., 1994a). Taken together, the data of Table 1 suggest the presence of maximally 16 and 4 high affinity binding sites of CaM per RyR (1 and 4 CaM sites/RyR polypeptide) at <0.01 μM and 100 μM Ca^{2+}, respectively. The very similar binding ratios for the purified RyR preparations and SR vesicles are in agreement with photoaffinity labeling studies that have suggested that the RyR is the major CaM-binding protein of SR vesicles (Seiler et al., 1984; Yang et al., 1994).

TABLE 1 [^{125}I]Calmodulin and [^{3}H]ryanodine binding to SR vesicles and purified Ca^{2+} release channels

	SR Vesicles	Chaps-solubilized RyR	Reconstituted RyR
[^{3}H]Ryanodine binding (pmol/mg protein)	15.6 ± 1.4 (8)	345 ± 35 (5)	230 ± 45 (4)
[^{125}I]CaM binding			
$<10^{-8}$ M Ca^{2+}			
B_{max} (pmol/mg protein)	235 ± 33 (8)	4600 ± 900 (5)	2920 ± 620 (4)
K_D (nM)	12.7 ± 1.5 (8)	5.3 ± 0.6 (5)	7.5 ± 2.1 (4)
10^{-4} M Ca^{2+}			
B_{max} (pmol/mg protein)	48.0 ± 6.0 (7)	1330 ± 90 (3)	480 ± 80 (3)
K_D (nM)	21.0 ± 3.7 (7)	22.0 ± 4.0 (3)	8.7 ± 2.7 (3)
[^{125}I]CaM/[^{3}H]ryanodine			
$<10^{-8}$ M Ca^{2+}	15.1 ± 0.8 (8)	12.7 ± 1.8 (5)	12.7 ± 1.5 (4)
10^{-4} M Ca^{2+}	3.1 ± 0.4 (7)	4.2 ± 0.4 (3)	2.4 ± 0.3 (3)

Binding parameters were determined as described in Fig. 1.

The skeletal muscle Ca^{2+} release channel is regulated by various effectors including Ca^{2+}, Mg^{2+}, and ATP, as well as pH and ionic strength (Meissner, 1994). Fig. 2 describes the dependence of [^{125}I]CaM binding on [Ca^{2+}], pH, and ionic strength. Fig. 2*A* shows that [^{125}I]CaM binding to SR vesicles decreased with increasing [Ca^{2+}]. Maximal levels of [^{125}I]CaM binding were observed at free [Ca^{2+}] < 0.1 μM. [^{125}I]CaM binding reached a half-maximal level at ~1 μM Ca^{2+}, and exhibited a constant, low level of binding at free [Ca^{2+}] ranging from ~10 μM to 1 mM Ca^{2+}. Scatchard analysis of binding data at <0.1 μM and 100 μM Ca^{2+} (Fig. 1, Table 1) suggested that the decrease of binding in Fig. 2*A* was mainly a result of a reduction in the number of high affinity sites. The pH dependence of [^{125}I]CaM binding was investigated in the pH range 6.1–8.0 (Fig. 2*B*). In <0.1 μM Ca^{2+} media, [^{125}I]CaM binding showed a broad maximum at pH 6.5–7.2. An approximately 50% reduction in binding was observed when the pH was decreased from pH 7.0 to pH 6.1 or inceased to pH 8.0. In the presence of 100 μM Ca^{2+}, a similar pH dependence of [^{125}I]CaM binding was obtained except that at pH 6.1 binding slightly increased rather than decreased. In Fig. 2*C*, the effect of ionic strength on [^{125}I]CaM binding was assessed in media containing 0–1 M KCl and either <0.01 μM or 100 μM free Ca^{2+}. At both Ca^{2+} concentrations, [^{125}I]CaM binding was maximal at 0.1–0.25 M KCl. Because the changes in [^{125}I]CaM binding in Fig. 2, *B* and *C*, might have been a result of a change in affinity and/or the number of high affinity sites, we determined the CaM-binding parameters by Scatchard analysis. These experiments were performed in media containing <0.1 μM Ca^{2+} because of the higher extent of binding obtained at low Ca^{2+} concentrations (Table 1, Fig. 2*A*). Scatchard analysis showed that a change in pH from 7.0 to 6.1 or 7.5 caused approximately a twofold decrease in binding affinity without appreciably changing the B_{max} value (not shown). Similarly, the decreased values at the lower and higher KCl concentrations were a result of a lower binding affinity and not a decrease in the number of binding sites (not shown). These results indicated that the pH- and ionic strength-induced decreases in CaM binding (Fig. 2, *B* and *C*) were mainly a result of a decrease in CaM-binding affinity. The number of CaM-binding sites (16 at low [Ca^{2+}]) was largely unaffected by pH or ionic strength. Similar experiments were also performed at 100 μM Ca^{2+}, but the binding levels were too close to background to calculate the binding parameters accurately. The K_D and B_{max} values of [^{125}I]CaM binding were not significantly affected by either the addition of 1 mM Mg^{2+} or 5 mM Mg-AMPPCP to 0.1 M KCl, 20 mM K-PIPES, pH 7 media containing <0.1 μM or 100 μM Ca^{2+}.

The time courses of CaM binding and dissociation were determined under conditions closely related to those occurring in muscle during SR Ca^{2+} release and Ca^{2+} uptake. SR vesicles were preincubated at low or high [Ca^{2+}] in media containing 5 mM Mg-AMPPCP and a relatively high CaM concentration of 1 μM. For comparison, the total CaM concentration in skeletal muscle has been estimated to be 2 μM (Klee and Vanaman, 1982). After the rapid addition of Ca^{2+} and EGTA to yield a final [Ca^{2+}] of 100 μM and <0.1 μM, respectively, [^{125}I]CaM binding was determined by a filter assay (see Materials and Methods). An increase in Ca^{2+} concentration from < 0.1 μM to 100 μM Ca^{2+} resulted in a decrease of high affinity CaM binding (from ~16 to 4 CaMs/RyR), whereas a decrease from the high to low Ca^{2+} medium caused a corresponding increase in high affinity CaM binding (from ~4 to 16 CaMs/RyR; Fig. 3). The half-times of dissociation and binding of ~12 CaMs/RyR were 50 ± 20 s and 30 ± 10 min (n = 4), respectively. Similar time courses of [^{125}I]CaM binding and dissociation were obtained when these experiments were carried out in the absence of Mg-AMPPCP. The results of Fig. 3 suggest that CaM binding and dissociation are two slow processes when compared with the very rapid changes in [Ca^{2+}] that occur in skeletal muscle during muscle contraction and relaxation.

We considered the possibility that the RyR polypeptide has four domains that bind CaM only at submicromolar Ca^{2+} and a fifth domain that binds CaM only at micro- to millimolar Ca^{2+} concentrations. The other possibility was that one of the four domains binds CaM with high affinity at both low and high [Ca^{2+}]. To distinguish between the

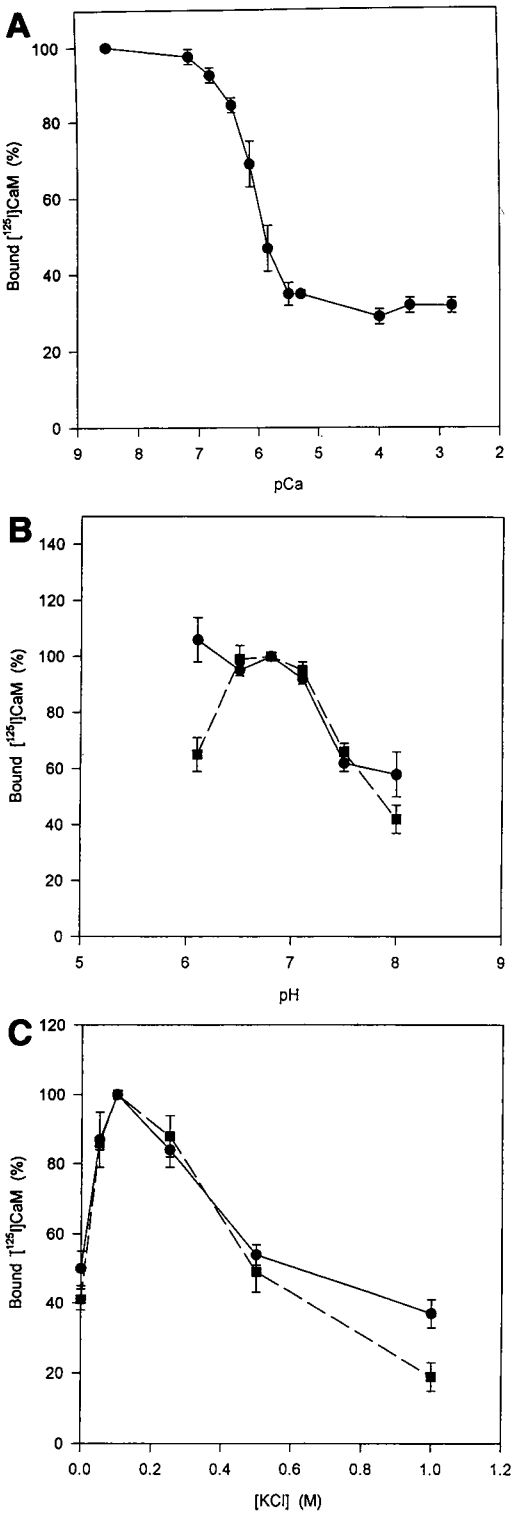

FIGURE 2 The effects of Ca^{2+}, pH, and ionic strength on [^{125}I]CaM binding to SR vesicles. [^{125}I]CaM binding to SR vesicles (0.1 mg protein/ml) was determined as described in Materials and Methods except that in *A* the free [Ca^{2+}] was varied and in *B* and *C* [^{125}I]CaM binding was determined at the indicated pH and [KCl], respectively. [^{125}I]CaM binding was determined with ligand concentrations that stressed changes in B_{max} (40 nM; *A*) and K_D (8 nM; *B* and *C*) values. In *B* and *C*, [Ca^{2+}] were <0.1 μM (■) and 100 μM (●). Values of [^{125}I] CaM binding are expressed as percent binding at <0.01 μM Ca^{2+} (*A*), at pH 6.8 (*B*), and 0.1 M KCl (*C*). Values are the means ± SE of three to five experiments.

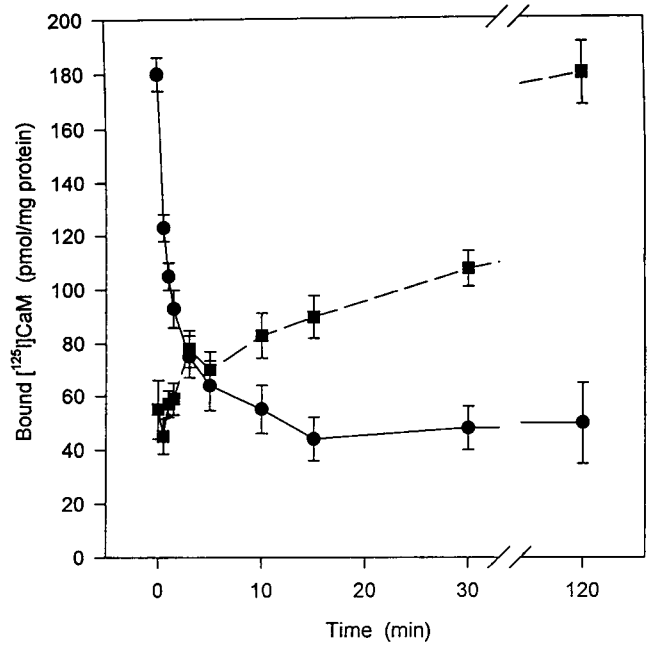

FIGURE 3 Time courses of [^{125}I]CaM binding and dissociation after a change in [Ca^{2+}]. SR vesicles (0.4 mg protein/ml) were incubated for 90 min at 23°C in 0.1 M KCl, 20 mM K-PIPES, pH 7.0 media containing 5 mM Mg-AMPPCP, 1 μM [^{125}I]CaM, and either 1 mM EGTA (●) or 400 μM Ca^{2+} (■). Rates of [^{125}I]CaM dissociation and binding were determined with the use of a filter assay (see Materials and Methods) after the addition of a small aliquot of 100 mM Ca^{2+} or EGTA to yield final free Ca^{2+} concentrations of 400 μM (●) and <0.1 μM (■), respectively. Values are the means ± SE of three to four experiments.

four- and five-domain models, SR vesicles were preincubated in <0.1 μM Ca^{2+} media in the presence or absence of 300 nM unlabeled CaM. The vesicles were then placed into 400 μM Ca^{2+} media containing 300 nM [^{125}I]CaM. Fig. 4 shows that preincubation of the vesicles with unlabeled CaM resulted in a considerably slower rate of [^{125}I]CaM binding. We conclude from these observations that one CaM per RyR polypeptide bound to the same domain at low and high [Ca^{2+}]. Accordingly, the data of Fig. 4 strongly favor the four-CaM-binding-domain model.

$^{45}Ca^{2+}$ flux measurements

The effects of CaM on SR Ca^{2+} release channel activity were assessed in SR vesicle-$^{45}Ca^{2+}$ flux, [^{3}H]ryanodine binding, and single-channel measurements. Fig. 5 illustrates the $^{45}Ca^{2+}$ efflux behavior of vesicles in the presence and absence of exogenously added CaM. Vesicles were passively loaded with 2 mM $^{45}Ca^{2+}$ in the presence and absence of 10 μM CaM and diluted 300-fold into either <0.1 μM or 200 μM Ca^{2+} efflux media containing or lacking 250 nM CaM, respectively. As previously observed (Meissner, 1986), $^{45}Ca^{2+}$ efflux from SR vesicles containing the Ca^{2+} release channel was relatively slow in media containing <0.1 μM or 200 μM Ca^{2+}. In the presence of CaM, we observed an increase or decrease in the $^{45}Ca^{2+}$ efflux rate depending on the Ca^{2+} concentration of efflux media. In

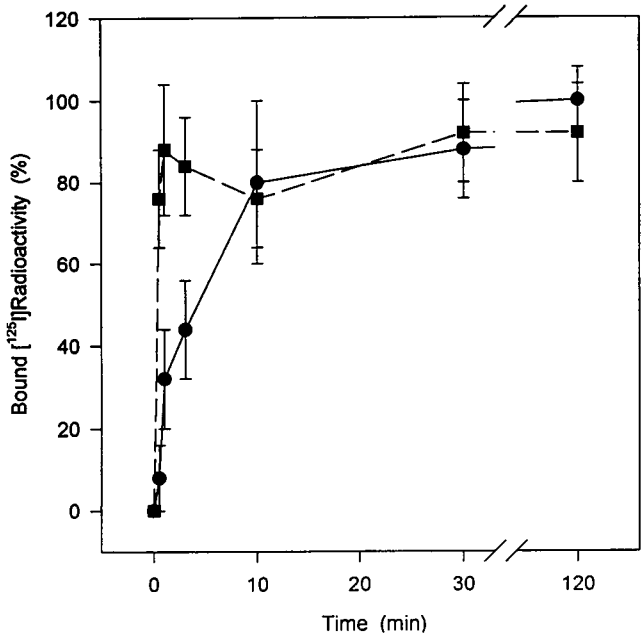

FIGURE 4 Time courses of [^{125}I]CaM binding to vesicles preincubated with and without unlabeled CaM. SR vesicles (0.5 mg protein/ml) were preincubated for 90 min at 23°C in 0.1 M KCl, 20 mM K-PIPES, pH 7.0 media containing 5 mM Mg-AMPPCP, 1 mM EGTA, and 0 (■) or 300 (●) nM unlabeled CaM. Rates of [^{125}I]CaM binding were determined with a filter assay (see Materials and Methods) after the addition of Ca^{2+} and [^{125}I]CaM to yield a final free Ca^{2+} concentration of 400 μM and CaM concentration of 300 nM. Maximal value of binding (100%) corresponded to the binding of four CaMs/RyR. Values are the means ± SE of four experiments.

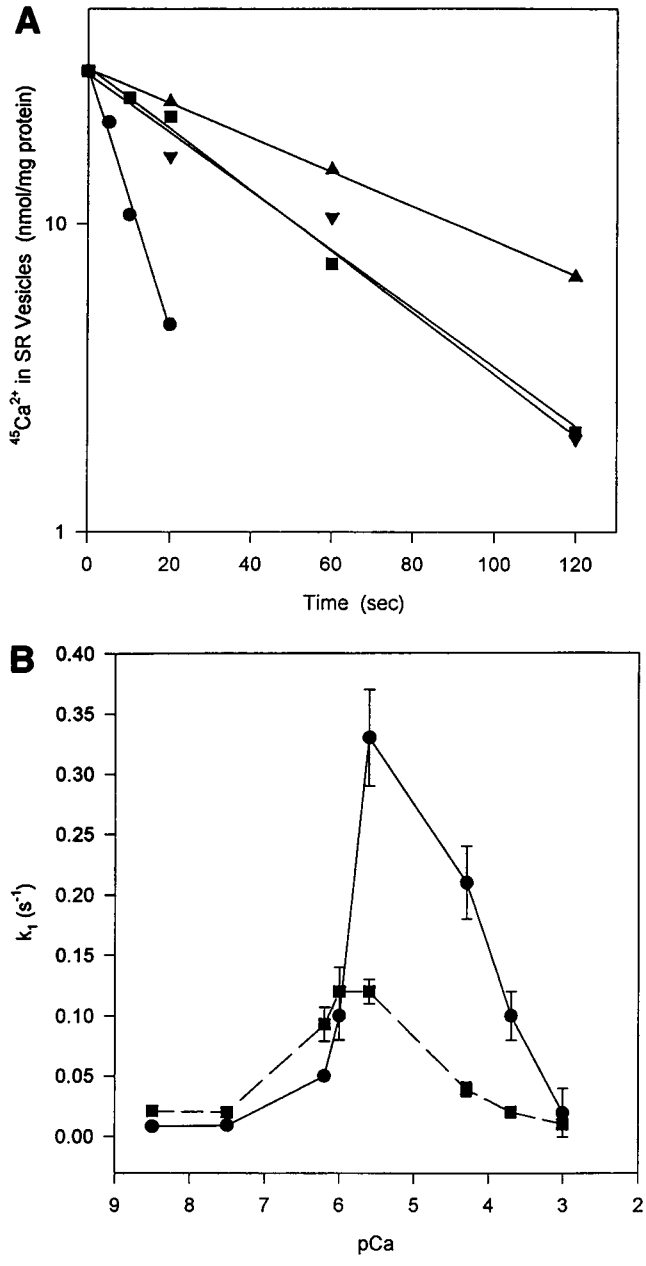

FIGURE 5 Effect of CaM on $^{45}Ca^{2+}$ efflux from SR vesicles. (*A*) SR vesicles (5 mg protein/ml) were incubated for 60 min at 23°C with 2 mM $^{45}Ca^{2+}$ in the absence (●,▲) or presence (▼,■) of 10 μM CaM. $^{45}Ca^{2+}$ efflux was initiated by diluting vesicles 300-fold into efflux media containing either 1 mM BAPTA (<0.1 μM free Ca^{2+}) and 0 (▲) or 250 (▼) nM CaM or 200 μM Ca^{2+} and 0 (●) or 250 (■) nM CaM. Amounts of $^{45}Ca^{2+}$ remaining with Ca^{2+}-permeable vesicles at the indicated times were determined by filtration as described in Materials and Methods. (*B*) The first order rate constants of $^{45}Ca^{2+}$ efflux from SR vesicles were determined in the absence (●) or presence (■) of CaM at the indicated concentrations of free Ca^{2+} in the efflux media as described in *A*. Values are the means ± SE of three to six experiments. Differences were significant ($P < 0.05$) with the exception at pCa 6.

media of low [Ca^{2+}], CaM binding increased the first order rate constant of $^{45}Ca^{2+}$ efflux by a factor of 2–3 (Fig. 5, *A* and *B*). In contrast, CaM slowed $^{45}Ca^{2+}$ efflux from the vesicles when these were placed in media containing 10 μM to 1 mM Ca^{2+} (Fig. 5, *A* and *B*). The finding of an activation of the Ca^{2+} release channel by CaM at low [Ca^{2+}] was unexpected. Whereas previous studies, including those from this laboratory, showed inhibition of channel activity by CaM (Meissner, 1986; Plank et al., 1988; Smith et al., 1989; Fuentes et al., 1994), activation of the skeletal muscle Ca^{2+} release channel by CaM has not been reported previously to our knowledge.

Fig. 6 shows that the effects of CaM on the first order rate constants of $^{45}Ca^{2+}$ efflux are dependent on the extent of [^{125}I]CaM binding. Both parameters were determined in parallel by incubating SR vesicles under identical conditions in media containing either radioactively labeled Ca^{2+} or CaM. $^{45}Ca^{2+}$ efflux rates were measured by placing the vesicles in <0.1 μM or 200 μM Ca^{2+} media. A similar level of CaM binding was maintained by using the same free CaM concentrations in $^{45}Ca^{2+}$ loading and efflux media. Fig. 6 shows that CaM decreased in a dose-dependent manner the first order rate constant (k_1) of $^{45}Ca^{2+}$ efflux at 200 μM Ca^{2+}. The binding of two CaMs/RyR polypeptide (50% binding in Fig. 6) decreased k_1 approximately twofold (from 0.11 to ~0.05 s^{-1}) whereas, at a saturating concentration of 1 μM CaM (~4 CaMs/RyR at 1.5 mM Ca^{2+}), k_1 was decreased approximately fourfold (from 0.11 to 0.025 s^{-1}). Calmodulin increased in a dose-dependent manner the first order rate constant of $^{45}Ca^{2+}$ efflux (k_1) when the

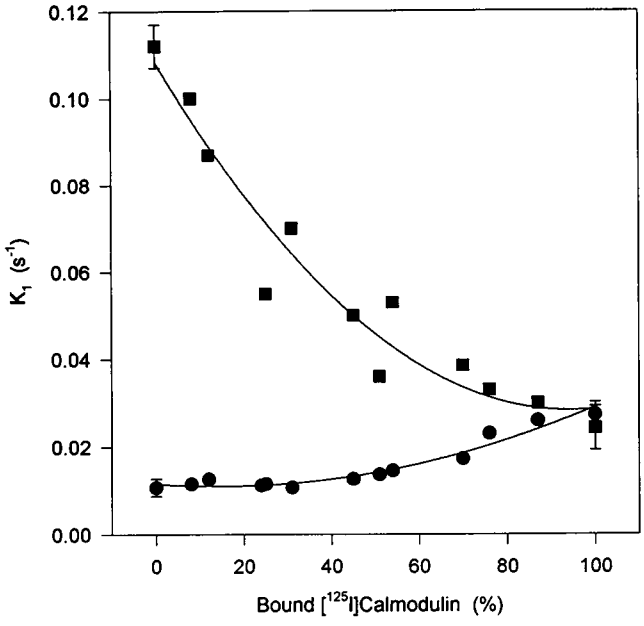

FIGURE 6 Dependence of first order rate constants of $^{45}Ca^{2+}$ efflux on the extent of CaM binding. [^{125}I]CaM binding and $^{45}Ca^{2+}$ efflux rates were determined in parallel with SR vesicles (1 mg protein/ml) that were incubated for 90 min at 23°C in media containing 1.5 mM Ca^{2+} and 30 nM to 1 μM [^{125}I]CaM or 1.5 mM $^{45}Ca^{2+}$ and 30 nM to 1 μM CaM. Maximal value (100%) of [^{125}I]CaM binding was determined by Scatchard analysis. First order rate constants (k_1) were determined by diluting SR vesicles into efflux media containing 1 mM BAPTA (●) or 200 μM Ca^{2+} (■). Efflux media contained free CaM concentrations corresponding to those in the vesicle media after incubation for 90 min.

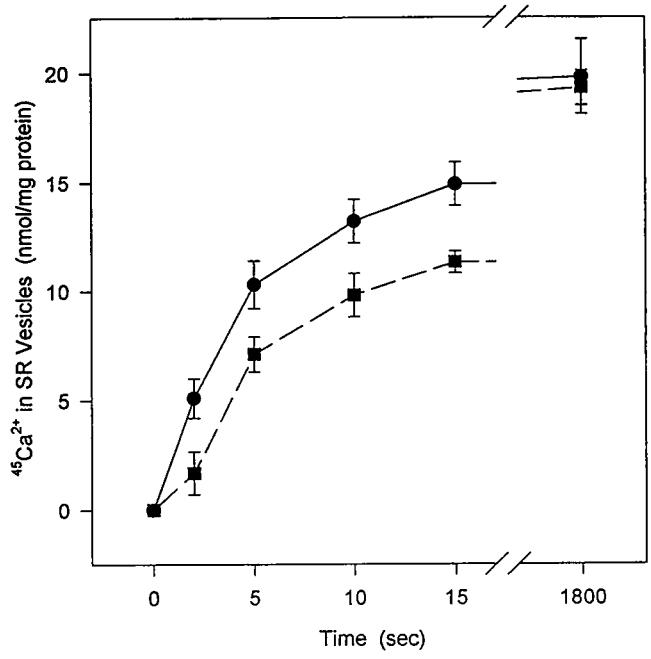

FIGURE 7 Effect of CaM on $^{45}Ca^{2+}$ influx into SR vesicles. SR vesicles (8 mg protein/ml) were incubated for 30 min at 23°C in 0.1 M KCl, 20 mM K-PIPES, pH 7.0 media containing 2 mM EGTA, 5 mM Mg-AMPPCP, 0.2 mM Pefabloc, 20 μM leupeptin, and 0 (●) or 10 (■) μM CaM. A relatively high CaM concentration was used to assure that all CaM-binding sites were occupied. $^{45}Ca^{2+}$ influx was initiated by adding an equal volume of 0.1 M KCl, 20 mM K-PIPES, pH 7.0 medium containing 4 mM $^{45}Ca^{2+}$, and determined as described in Materials and Methods. Values are the means ± SE of three to four experiments. The differences for the 2- to 15-s data points were significant ($P < 0.05$).

vesicles were diluted into <0.1 μM Ca^{2+} efflux media (Fig. 6). It was likely that the maximal (~2.5-fold) increase observed in Fig. 6 was a result of the binding of 4 and not 16 CaMs/RyR. In support of the lower number are the facts that the vesicles bound 4 CaMs/RyR in the $^{45}Ca^{2+}$ incubation medium and that the extent of CaM binding increased only slowly on transfer from a high Ca^{2+} to low Ca^{2+} medium (Fig. 3). Furthermore, a similar increase in k_1 was observed when the vesicles were diluted into <0.1 μM Ca^{2+} media containing or lacking 1 μM CaM.

In studies with sea urchin egg microsomes, CaM potentiated the activation of the RyR by cyclic ADP ribose (Lee et al., 1994b). The effects of CaM were observed at low [Ca^{2+}]. We were unsuccessful in demonstrating a significant potentiating effect of cyclic ADP ribose on $^{45}Ca^{2+}$ release at submicromolar Ca^{2+} concentrations in the presence or absence of CaM (not shown).

The effects of binding of more than 4 CaMs to the RyR were examined in $^{45}Ca^{2+}$ influx experiments. Vesicles were incubated in <0.1 μM Ca^{2+} media in the presence and absence of 10 μM CaM, and $^{45}Ca^{2+}$ influx was initiated by the addition of an equal volume of a $^{45}Ca^{2+}$ solution to yield a final free Ca^{2+} concentration of 1 mM. Fig. 7 shows that the vesicles initially sequestered smaller amounts of $^{45}Ca^{2+}$ when preincubated with CaM. Essentially identical amounts of $^{45}Ca^{2+}$ were sequestered when the vesicles were incubated with 1 mM $^{45}Ca^{2+}$ for 30 min. Calmodulin dissociated relatively slowly from the RyR on transfer from a low to high Ca^{2+} medium (Fig. 3). It was therefore likely that the intial time points (2 and 5 s) in Fig. 7 were obtained with SR vesicles that bound close to 16 CaMs/RyR

[^{3}H]Ryanodine binding

The neutral plant alkaloid ryanodine has been shown to bind with higher affinity to the open than the closed channel states of the skeletal muscle Ca^{2+} release channel (Coronado et al., 1994; Meissner, 1994). Therefore, [^{3}H]ryanodine binding was also used to study the effects of CaM on Ca^{2+} release channel activity. An intermediate ionic strength buffer (0.25 M KCl) was used to increase the extent of [^{3}H]ryanodine binding by SR vesicles in the control condition (without CaM). We also chose to use 5 mM AMPPCP at <0.01 μM Ca^{2+} because the ATP analogue afforded a higher level of channel activation. Similarly, as observed in $^{45}Ca^{2+}$ flux experiments (Figs. 5–7), CaM either inhibited or activated [^{3}H]ryanodine binding, depending on the Ca^{2+} concentration of the binding media. At 100 μM Ca^{2+}, a maximally fourfold decrease in specific [^{3}H]ryanodine binding was observed at CaM concentrations of 300 nM and greater (Fig. 8). [^{3}H]Ryanodine binding decreased half-maximally at 28 ± 1 nM free CaM, in good agreement with [^{125}I]CaM-binding studies that yielded

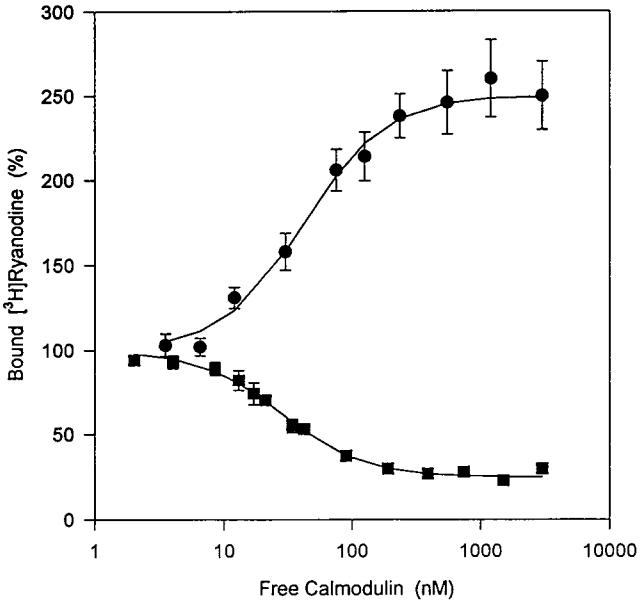

FIGURE 8 Effect of CaM on [^{3}H]ryanodine binding. Specific [^{3}H]ryanodine binding to SR vesicles was determined as described in Materials and Methods in a 0.25 M KCl, 20 mM K-PIPES, pH 7.0 buffer containing 0.2 mM Pefabloc, 20 μM leupeptin, the indicated concentrations of free CaM, and either 5 nM [^{3}H]ryanodine, 200 μM Ca^{2+}, and 100 μM EGTA (100 μM free Ca^{2+}) (■) or 20 nM [^{3}H]ryanodine, 5 mM EGTA, and 5 mM AMPPCP (●). Control values (100%, without added CaM) corresponded to 0.75 and 0.4 pmol [^{3}H]ryanodine/mg protein in the presence of 200 μM Ca^{2+} and 5 mM EGTA (<0.01 μM free Ca^{2+}), respectively. Values are the means ± SE of three to four experiments. Solid lines were obtained according to the equation $B = B_0\,(1 + ([CaM]/K_H)^{nH})^{-1}$, where B and B_0 are the binding of [^{3}H]ryanodine in the presence and absence of the indicated concentrations of CaM, respectively. The Hill dissociation constants (K_H) were 28 (■) and 42 (●) nM, and the Hill coefficients (n_H) were both 1.3.

K_D = 25 ± 6 nM. A Hill coefficient of 1.3 ± 0.1 suggested that CaM inhibited [^{3}H]ryanodine binding by a weak cooperative interaction. In control experiments, mastoparan was used to assess possible inhibition of channel activity by endogenous CaM. Mastoparan is a CaM inhibitor that has been shown to reverse inhibition of Ca^{2+} release activity by CaM in single-channel experiments (Smith et al., 1989). At concentrations ranging from 10 to 50 nM, mastoparan increased [^{3}H]ryanodine binding by less than 10% over the control value. This result suggested, in agreement with previous studies (Meissner, 1986), minimal inhibition of Ca^{2+} release channel activity by endogenous CaM. Heavy SR vesicles were prepared in the presence of a high ionic strength buffer (0.6 M KCl), which may have resulted in dissociation of endogenously bound CaM.

At <0.01 μM Ca^{2+}, CaM increased [^{3}H]ryanodine binding maximally 2.5-fold (Fig. 8). Similarly, as at 100 μM Ca^{2+}, a nearly linear relationship was obtained between CaM binding and the change in [^{3}H]ryanodine binding, except that the binding of 16 and not 4 CaMs was required to observe maximal activation. The K_H and n_H values of activation were 42 ± 3 nM and 1.3 ± 0.1, respectively. Scatchard analysis of [^{125}I]CaM binding yielded K_D = 45 ± 10 nM (n = 3).

Single-channel recordings

The effects of CaM on the activity of single purified Ca^{2+} release channels were studied at <0.2 μM and 50–100 μM Ca^{2+} in the presence and absence of ATP or Mg-ATP. Single channels were recorded in symmetric 250 mM KCl media, pH 7.1. With K^+ as the current carrier, single-channel conductance was ~770 pS (Xu et al., 1993). Calmodulin was added to the cis chamber of the bilayer apparatus. This side corresponded to the SR cytoplasmic side, as shown by the ability of cis Ca^{2+} and ATP to activate the channel.

In the two upper recordings of Fig. 9, the effects of CaM on the Ca^{2+} release channel were studied at a low cis [Ca^{2+}]. Because, at this level of Ca^{2+}, channel open probability (P_o) is close to zero, it would be difficult to discern any low level activation or inhibition of channel activity by CaM. We adapted therefore the following two strategies. In one, we activated the channel by 2 mM ATP (Smith et al., 1986) in the presence of 15 nM Ca^{2+}. In the second one, we adjusted the cis free Ca^{2+} to 150 nM (the resting level normally found in the cytoplasm) to achieve a small level of channel activity. In this case, the cis chamber also contained 2 mM Mg-ATP to further simulate the myoplasmic composition.

We observed that addition of 2 mM ATP to the 15 nM Ca^{2+} medium on the cis side of the bilayer chamber increased channel open probability from close to zero to either a lower level (P_o increased from ~0 to 0.006 ± 0.002, n = 4) or higher level (P_o increased from 0.010 ± 0.003 to 0.06 ± 0.01, n = 4) (not shown). We have no explanation for the differential effects of ATP at this time. The addition of 1 μM CaM further activated the ATP-activated channels (right top trace of Fig. 9). In all eight experiments, a two- to sixfold increase in P_o was recorded without a noticeable effect on single-channel conductance. The effects of CaM on the activity of the more active channels were analyzed by carrying out a detailed time analysis of the single-channel events (Table 2). The open and closed time histograms could each be fitted by the sum of two exponentials (not shown). The addition of 1 μM CaM significantly increased (approximately twofold) the number of events and the open time constant of the short open state. Calmodulin significantly decreased the two closed time constants. The long open time constant and the probability of events having short and long durations were not significantly changed by the addition of 1 μM CaM. Accordingly, the increase in channel activity by CaM could be largely accounted for by an increase in the number of events and the duration of the short open state.

At 150 nM Ca^{2+} cis in the presence of 2 mM Mg-ATP cis, the level of channel activity was low (P_o = 0.0010 ± 0.0003, n = 9; second recording of Fig. 9, Table 2). Addition of 1 μM CaM cis increased P_o 1.5- to 5-fold ($P < 0.03$, n = 9). For time analysis, we selected four recordings in which channel activity was continuously recorded for 2 min before and after the addition of CaM. The mean number of

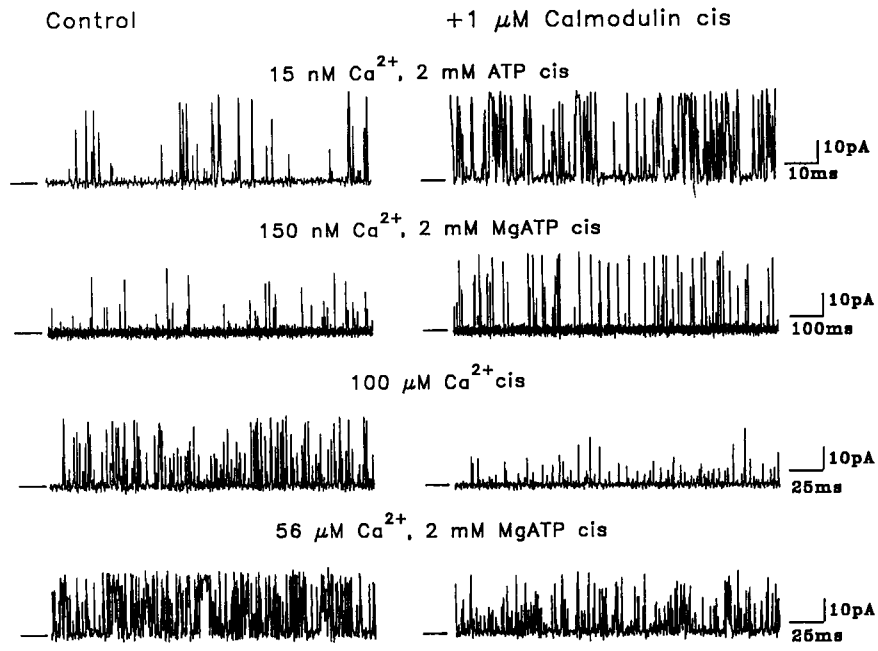

FIGURE 9 Effect of CaM on single-channel activity of purified and reconstituted RyR complex. Shown are four separate single-channel recordings before (*left panels*) and after (*right panels*) the addition of 1 μM CaM cis. Single-channel currents, shown as upward deflections, were recorded in symmetrical 0.25 M KCl, 20 mM K-PIPES, pH 7.1 media. The trans chamber solution contained 0.15 mM $CaCl_2$, 0.1 mM EGTA. The cis chamber solution contained the following: Top recording: 0.15 mM $CaCl_2$, 2 mM EGTA, and 2 mM ATP (15 nM free Ca^{2+}). *Left panel*, control, P_0 = 0.036; *right panel*, P_0 = 0.195. Holding potential = 40 mV. Second recording: 0.15 mM $CaCl_2$, 0.5 mM EGTA, and 2 mM Mg-ATP (150 nM free Ca^{2+}). *Left panel*, P_0 = 0.003; *right panel*, P_0 = 0.011. Holding potential = 40 mV. Third recording: 5.08 mM $CaCl_2$, 5 mM EGTA (100 μM free Ca^{2+}). *Left panel*, P_0 = 0.081, *right panel*, P_0 = 0.011. Holding potential = 35 mV. Bottom recording: 5.08 mM $CaCl_2$, 5 mM EGTA, and 2 mM Mg-ATP (56 μM free Ca^{2+}). *Left panel*, P_0 = 0.33; *right panel*, P_0 = 0.14. Holding potential = 35 mV.

events increased significantly from a control value of 2823 ± 479 to 6693 ± 1448. In the presence and absence of CaM, the open time histograms could be fitted by one exponential and the closed time histograms by the sum of two exponentials. Addition of 1 μM CaM increased the duration of open events and decreased the duration of the closed events. However, of these, only the decrease in the duration of the long closed events was significant (Table 2). Accordingly, in the presence of 150 nM Ca^{2+} and 2 mM Mg-ATP, two significant effects of CaM were an increase in the number of events and a decrease in the duration of the long closed state.

In control experiments, we tested the specificity of the activating effects of CaM by adding to the cis chamber BSA instead of CaM. Addition of 1 μM BSA was without any noticeable effect, whereas the subsequent addition of 1 μM CaM increased P_o threefold ($P < 0.05$, $n = 10$). This suggests that, at low [Ca^{2+}], CaM activated the Ca^{2+} release channel by a specific interaction.

The two lower recordings of Fig. 9 illustrate the effects of CaM on Ca^{2+} release channel activity in the presence of micromolar (50–100 μM) concentrations of free Ca^{2+} in the presence and absence of 2 mM Mg-ATP. In both recordings, the addition of 1 μM CaM cis caused a severalfold

TABLE 2 Effect of calmodulin on channel parameters at <0.2 μM Ca^{2+} cis

Channel parameter	Additions to cis bilayer chamber			
	15 nM Ca^{2+} and 2 mM ATP		150 nM Ca^{2+} and 2 mM Mg-ATP	
	−CaM	+CaM	−CaM	+CaM
No. of events	52,643 ± 13,643	101,080 ± 14,221*	2,823 ± 479	6,693 ± 1,448*
P_o	0.06 ± 0.01	0.18 ± 0.01*	0.0010 ± 0.0003	0.003 ± 0.001*
A_{o1}	0.97 ± 0.02	0.84 ± 0.06	1	1
A_{o2}	0.03 ± 0.02	0.16 ± 0.06		
τ_{o1} (ms)	0.17 ± 0.02	0.24 ± 0.02*	0.11 ± 0.01	0.17 ± 0.03
τ_{o2} (ms)	1.28 ± 0.24	0.98 ± 0.11		
A_{c1}	0.44 ± 0.12	0.54 ± 0.13	0.34 ± 0.12	0.45 ± 0.14
A_{c2}	0.56 ± 0.12	0.46 ± 0.13	0.66 ± 0.12	0.55 ± 0.14
τ_{c1} (ms)	1.70 ± 0.14	0.83 ± 0.25*	30.0 ± 8.1	17.2 ± 1.12
τ_{c2} (ms)	8.10 ± 1.30	4.20 ± 2.00*	107.7 ± 15.3	47.3 ± 7.1*

Channel parameters were obtained from 2-min continuous recordings as described in Materials and Methods. P_o refers to open time probability. Dwell-time data were fitted by the maximum likelihood method to the probability density function: $f(t) = \Sigma A_i\,(1/\tau_i)\exp(-t/\tau_i)$, where A_i and τ_i are the relative areas of the distributions and time constants of the ith state, respectively (Colquhoun and Sigworth, 1983). In 15 nM Ca^{2+}, 2 mM ATP cis, both the open and closed time histograms could be fitted by the sum of two exponentials, and in 150 nM Ca^{2+}, 2 mM Mg-ATP cis, the open time histogram could be fitted by a single exponential and the closed time histogram by the sum of two exponentials.

*Values significantly different from the control ($P < 0.05$; $n = 4$).

decrease in channel activity. Ca^{2+}-activated channel activities were inhibited by CaM in a dose-dependent manner (Fig. 10). An only partial, approximately fourfold inhibition of channel activity was observed at 100–1000 nM CaM. Single channels were half-maximally inhibited at 16 nM CaM. These values were in agreement with those obtained from $^{45}Ca^{2+}$ vesicle flux and [^{3}H]ryanodine binding experiments that also suggested that there exists a good correlation between the extent of CaM binding and channel inhibition.

At 100 μM Ca^{2+} cis in the absence of Mg-ATP, the open time histograms could be fitted by the sum of two exponentials and the closed time histograms by the sum of three exponentials (Table 3). In the absence of Mg-ATP, addition of 1 μM CaM decreased the number of open and closed events approximately threefold, and P_o approximately sixfold. The decrease in the number of events resulted in a significant increase in the probability of the longest closed state at the cost of the shortest closed state (Table 3). None of the other channel parameters were significantly altered by CaM. Comparison of the second and fourth columns of Table 3 shows that addition of 2 mM Mg-ATP to the 100 μM Ca^{2+} medium increased the number of events and increased channel open probability by increasing the duration of open states and decreasing those of the closed states. Another effect of Mg-ATP was to render the channel less sensitive to inhibition by 1 μM CaM. In the presence of Mg-ATP, CaM lowered P_o by decreasing significantly the open time constants and increasing the probability of the intermediate closed state. In addition, an approximately twofold (not significant) increase in the three closed time constants was observed. Taken together, the data of Tables 2 and 3 suggest that other channel effectors such as Ca^{2+} and Mg-ATP play a significant role in determining the way in which SR Ca^{2+} release channel activity is modulated by CaM.

DISCUSSION

Previous vesicle-ion flux, single-channel, and CaM-binding studies with SR vesicles and fusion peptides have indicated that CaM inhibits SR Ca^{2+} release channel activity by a direct interaction. In this study, the CaM-binding properties and regulation of the SR Ca^{2+} release channel by CaM were compared by using isolated membrane and purified channel preparations. These studies led to two new observations that may have important implications for the mechanism of SR Ca^{2+} release in skeletal muscle. First, the data reported here provide the first evidence for an activation of the SR Ca^{2+} release channel by CaM. Second, our results suggest that CaM likely regulates skeletal muscle Ca^{2+} release activity without dissociating from the release channel.

Calmodulin binding to Ca^{2+} release channel

Recent fluorescence anisotropic measurements with rhodamine-CaM and skeletal muscle SR vesicles showed that, at low Ca^{2+} concentrations, the tetrameric RyR binds 20–24 molecules of CaM with high affinity (K_D = 8.6 nM) (Yang et al., 1994). In the presence of 100 μM Ca^{2+}, approximately 4 high affinity and 16 low affinity sites per RyR were measured. In the presence of 100 μM Ca^{2+} and 1 mM Mg^{2+}, a single binding site with a very high affinity (K_D = 0.1 nM) and approximately 7 sites with a lower affinity (K_D = 17 nM) were obtained. In reasonable agreement with the results of Yang et al. (1994), we find that the skeletal muscle RyR binds with high affinity 16 and 4 CaMs at submicromolar and micromolar to millimolar Ca^{2+} concentrations, respectively. One of the sites (4 CaMs/RyR) appeared to be able to bind CaM in both its Ca^{2+}-free and Ca^{2+}-bound forms (Fig. 4). Binding to low affinity sites in the presence of micromolar to millimolar Ca^{2+} or the appearance of a very high affinity binding site in the presence

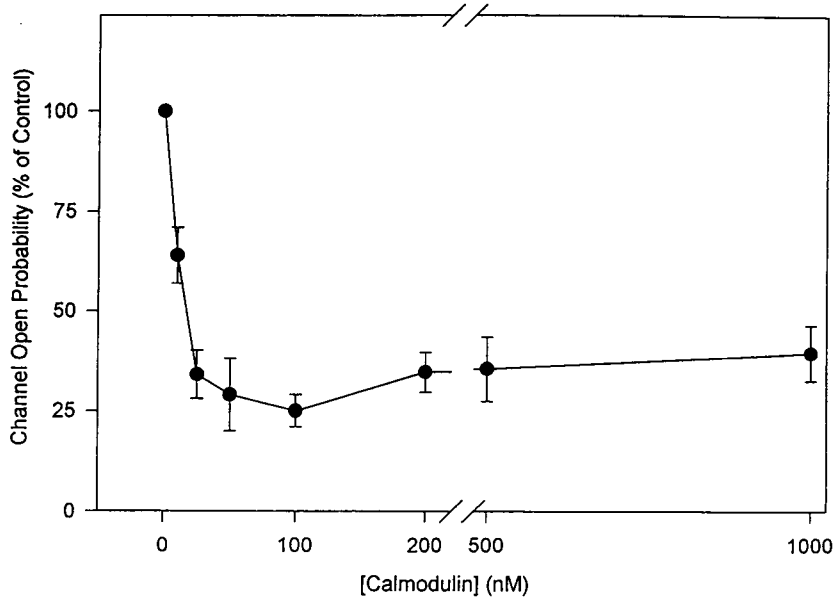

FIGURE 10 Dose-dependent inhibition of channel activity by CaM. Experiments were carried out in symmetrical 250 mM KCl, 20 mM K-PIPES, pH 7.1 medium. The free Ca^{2+} in the cis and trans sides was 50 μM. Holding potential = 40 mV. Control P_o value (without CaM) corresponded to 0.22 ± 0.04. Data points are the means ± SE of eight experiments.

TABLE 3 Effect of calmodulin on channel parameters at 50–100 μM Ca^{2+}

Channel parameter	Additions to cis bilayer chamber			
	100 μM Ca^{2+}		56 μM Ca^{2+} and 2 mM Mg-ATP	
	−CaM	+CaM	−CaM	+CaM
No. of events	36,190 ± 10,145	10,071 ± 3,124*	111,681 ± 17,016	94,062 ± 19,001
P_o	0.06 ± 0.01	0.01 ± 0.00*	0.34 ± 0.09	0.15 ± 0.04*
A_{o1}	0.97 ± 0.01	0.97 ± 0.01	0.91 ± 0.03	0.96 ± 0.00
A_{o2}	0.03 ± 0.01	0.03 ± 0.01	0.09 ± 0.03	0.05 ± 0.01
τ_{o1} (ms)	0.18 ± 0.01	0.15 ± 0.01	0.37 ± 0.06	0.23 ± 0.02*
τ_{o2} (ms)	1.03 ± 0.16	1.19 ± 0.21	1.84 ± 0.22	1.06 ± 0.15*
A_{c1}	0.45 ± 0.10	0.17 ± 0.05*	0.64 ± 0.12	0.47 ± 0.06
A_{c2}	0.53 ± 0.10	0.45 ± 0.08	0.29 ± 0.09	0.49 ± 0.04*
A_{c3}	0.02 ± 0.02	0.39 ± 0.13*	0.12 ± 0.01	0.07 ± 0.01
τ_{c1} (ms)	2.04 ± 0.23	2.56 ± 1.44	0.57 ± 0.09	1.03 ± 0.22
τ_{c2} (ms)	11.68 ± 3.59	13.61 ± 2.80	2.10 ± 0.37	3.17 ± 0.70
τ_{c3} (ms)	345.70 ± 118.46	437.80 ± 373.20	6.89 ± 2.19	13.06 ± 3.93

Channel parameters were determined as described in Table 2.
*Values significantly different from control ($P < 0.05$; $n = 4$).

of Mg^{2+} and Ca^{2+} were not observed in our binding studies. We found that the addition of Mg^{2+} or Mg-AMPPCP to <0.01 μM or 100 μM Ca^{2+}-binding media was without a significant effect on the K_D and B_{max} values of CaM binding. CaM binding showed a broad peak at pH 6.8 and was maximal in the presence of 50–250 mM KCl, in good agreement with the results of Yang et al. (1994), who found that CaM binding was maximal at ~0.3 M KCl. Taking into account a total CaM concentration of 2 μM in skeletal muscle (Klee and Vanaman, 1982), the binding data obtained in the present study suggest that, at a resting myoplasmic [Ca^{2+}] of 100–150 nM, each RyR tetramer may bind close to 16 CaMs.

Ligand overlay experiments with skeletal muscle RyR fusion peptides have indicated the presence of up to seven different CaM-binding regions (Chen and MacLennan, 1994; Menegazzi et al., 1994). With the exception of one binding region, CaM binding was dependent on the presence of micromolar to millimolar Ca^{2+} concentrations. The reasons for the differences in the number of binding sites and Ca^{2+} dependence of CaM binding to the native RyR and RyR fusion peptides are not clear at present. The CaM overlay method might not result in the full renaturation of the CaM-binding sites on the nitrocellulose membranes. Sites that are recognized in the native RyR might therefore not have been detected by the Ca^{2+}-free form of CaM. On the other hand, the overlays might have detected Ca^{2+}-dependent sites not accessible in the native RyR complex. Third, the apparent binding affinities of CaM to the RyR fusion peptides were estimated to be as high as 25 μM. Therefore, the overlays might have visualized low affinity binding sites that are not readily detected by more conventional binding assays.

Regulation of Ca^{2+} release channel activity by CaM

In the present study, the functional interactions of CaM with the Ca^{2+} release channel were examined by carrying out vesicle-$^{45}Ca^{2+}$ flux, [^{3}H]ryanodine binding, and single-channel measurements. The results of these experiments can be described by the following scheme:

$$
\begin{array}{ccccc}
{}^{0}\mathrm{RyR} + 16\,{}^{0}\mathrm{CaM} & & & & \\
\downarrow\uparrow\ \text{(Activation)} & & & & \\
{}^{0}\mathrm{RyR_A}\,{}^{0}\mathrm{CaM_{16}} & \overset{Ca^{2+}}{\rightleftharpoons} & [{}^{Ca}\mathrm{RyR_A}\,{}^{Ca}\mathrm{CaM_{16}}] & \rightleftharpoons & {}^{Ca}\mathrm{RyR_I}\,{}^{Ca}\mathrm{CaM_{16}} \\
\downarrow\uparrow\ 12\,{}^{0}\mathrm{CaM} & & & & \downarrow\uparrow\ -12\,{}^{Ca}\mathrm{CaM} \\
{}^{0}\mathrm{RyR_A}\,{}^{0}\mathrm{CaM_4} & \rightleftharpoons & [{}^{0}\mathrm{RyR_I}\,{}^{0}\mathrm{CaM_4}] & \overset{-Ca^{2+}}{\rightleftharpoons} & {}^{Ca}\mathrm{RyR_I}\,{}^{Ca}\mathrm{CaM_4} \\
 & & & & \downarrow\uparrow\ \text{(Inhibition)} \\
 & & & & {}^{Ca}\mathrm{RyR} + 4\,{}^{Ca}\mathrm{CaM}
\end{array}
$$

In the above scheme, the Ca^{2+} release channel (RyR) and CaM are present in their Ca^{2+}-free forms, designated ^{0}RyR and ^{0}CaM at ≤0.2 μM Ca^{2+}, and in their Ca^{2+}-bound forms ^{Ca}RyR and ^{Ca}CaM (4 Ca^{2+}/CaM) (George et al., 1990) at ≥100 μM Ca^{2+}. A CaM-activated ($^0\mathrm{RyR_A}\,{}^0\mathrm{CaM_{16}}$) and CaM-inhibited ($^{Ca}\mathrm{RyR_I}\,{}^{Ca}\mathrm{CaM_4}$) channel state were identified in media containing a saturating concentration of CaM (0.3–1 μM) and a low and high [Ca^{2+}], respectively. The scheme further proposes that additional transitional CaM-activated and CaM-inhibited channel states may be formed after a change from submicromolar to micromolar to millimolar Ca^{2+} concentrations and vice versa. Of the four proposed transitional channel states, two were identified with reasonable certainty, whereas the two other ones (shown in brackets) are hypothetical. Below we shall first describe the scheme before discussing the physiological implications of our findings.

Binding of ^{0}CaM to ^{0}RyR

Our [^{3}H]ryanodine binding (Fig. 8), and single-channel (Figs. 9 and 10) measurements showed that CaM keeps the skeletal muscle SR Ca^{2+} release channel in an activated form at <0.2 μM Ca^{2+}. CaM activated the channel with a Hill coefficient of 1.3 suggesting a weak cooperative inter-

action. A two- to threefold stimulation of channel activity from a low baseline level was observed under conditions that resulted in the binding of 16 CaMs/RyR (Tables 1 and 2, Figs. 8–10). Failure of previous studies to observe channel activation may have been a result of the low levels of channel activity seen at low Ca^{2+} concentrations.

Binding of ^{Ca}CaM to ^{Ca}RyR

Inhibition of the Ca^{2+} release channel by CaM was analyzed in the presence of 50 μM to 1 mM Ca^{2+}. Vesicle-$^{45}Ca^{2+}$ efflux and [^{3}H]ryanodine binding measurements showed a three- to fourfold decrease in channel activity (Figs. 5, 6, and 8). Single-channel measurements confirmed that CaM inhibited the channel at 50–100 μM Ca^{2+}, i.e., under conditions that were found to result in the high affinity binding of four CaMs/RyR. A Hill coefficient of 1.3 showed that CaM inhibited the channel by a weak cooperative interaction.

Effects of raising the Ca^{2+} concentration

An increase from submicromolar to millimolar Ca^{2+} concentration led to a rapid (<2 s) inhibition of $^{45}Ca^{2+}$ influx into SR vesicles (Fig. 7), whereas dissociation of 12 of the 16 bound CaMs took place with an appreciably slower time course ($\tau_{1/2} \approx 1$ min; Fig. 3). In the above scheme, these observations are taken into account by proposing that an increase in [Ca^2] mediates the transition from a Ca^{2+}-free, CaM-activated channel form ($^0RyR_A{}^0CaM_{16}$) to a Ca^{2+}-bound, CaM-inactivated channel form ($^{Ca}RyR_I{}^{Ca}CaM_{16}$). Then, 12 of the 16 CaMs dissociate slowly with the 4 remaining CaMs being sufficient to maintain the channel in an inactivated state. The scheme indicates that, in addition, a Ca^{2+}-bound, CaM-activated channel intermediate ([$^{Ca}RyR_A{}^{Ca}CaM_{16}$]) may be formed before the complex converts to its CaM-inhibited forms. In our $^{45}Ca^{2+}$ influx experiments, we could not ascertain whether a Ca^{2+}-bound, CaM-activated channel is formed or how fast these transitions could take place, but we could show that a channel-inhibited form that bound close to 16 CaMs appeared in <2 s.

Effects of lowering the Ca^{2+} concentration

The question of how fast the channel switches from a CaM-inhibited to a CaM-activated form was addressed in $^{45}Ca^{2+}$ efflux experiments. Preincubation of passively loaded vesicles with CaM resulted in increased $^{45}Ca^{2+}$ efflux rates without an apparent lag period when these were transferred from a millimolar to a submicromolar Ca^{2+} medium. The transient formation of a Ca^{2+}-free, CaM-inhibited channel form ([$^0RyR_I{}^0CaM_4$]), if it occurs, may be therefore of a short duration. The binding of 4 CaMs/RyR appeared to be sufficient to observe significant channel activation (Figs. 5 and 6), because vesicles incubated with 1 μM CaM bound only slowly additional CaMs when transferred from a high to low Ca^{2+} medium (Fig. 3). However, it should be noted that our [^{3}H]ryanodine binding (Fig. 8) and single-channel (Fig. 9) measurements indicated that optimal activation required the binding of 16 CaMs/RyR (Figs. 8 and 9).

The effects of cyclic ADP ribose on Ca^{2+} release channel activity were tested because CaM has been recently reported to potentiate the activity of the cyclic ADP ribose-liganded sea urchin egg RyR (Lee et al., 1994b). However, we were unable to observe a significant stimulating effect of cyclic ADP ribose on the CaM-activated skeletal muscle Ca^{2+} release channel.

Single-channel measurements

Single-channel measurements have provided the most detailed information on the effects of CaM on SR Ca^{2+} release channel function. Only an inhibition and not activation was observed in previous studies. Smith et al. (1989) found that CaM inhibited Ca^{2+}- and ATP-activated Ca^{2+} release channel activities without affecting unitary channel conductance. Calmodulin decreased channel open probability by reducing the mean duration of single open events of skeletal and cardiac muscle channels. A second effect of CaM was an increase of the closed-time constants of the channels. In a more recent study, Fuentes et al. (1994) reported that CaM blocked skeletal muscle Ca^{2+} release channels in a Ca^{2+}-dependent manner by decreasing the number of open events without affecting the mean open time. Data reported here show that CaM affects Ca^{2+} release channel activity in a complex manner by changing both the mean duration of the open and closed events as well as the frequency of channel opening and closing. The effects of CaM depended on Ca^{2+} concentration and were affected by Mg-ATP, suggesting that other effectors besides Ca^{2+} modulate the functional interaction of CaM with the Ca^{2+} release channel. Additional studies will be required to more fully characterize the functional interaction of CaM with the Ca^{2+} release channel at the single-channel level.

Physiological implications

Previous $^{45}Ca^{2+}$ release measurements with SR vesicles have suggested that CaM exerts its inhibitory effects within time on the order of milliseconds (Fuentes et al., 1994) to seconds (Meissner, 1986). Simon et al. (1991) proposed a model in which inactivation of Ca^{2+} release in frog skeletal muscle fibers was brought about by the formation of an inactivating Ca^{2+}-CaM complex, thus providing a negative feedback for SR Ca^{2+} release. The underlying assumption in these studies was that CaM exerts its inhibitory action by rapidly binding to and dissociating from the release channel.

The results of the present study challenge the idea that CaM regulates the Ca^{2+} release channel by rapid binding

and dissociation. We found that CaM bound to and dissociated relatively slowly from the release channel when the Ca^{2+} concentration was changed (Fig. 3). Therefore, it is likely that the extent of CaM binding remains largely constant during an E-C coupling cycle in fast twitch muscle, unless other mechanisms contribute to CaM binding. One possible mechanism is channel phosphorylation, which has been reported to reverse CaM inhibition of the cardiac Ca^{2+} release channel (Witcher et al., 1991). Another possibility would be that other endogenous effectors or proteins removed during membrane isolation affect the kinetics of CaM binding.

Although the results of this study suggest that during an E-C coupling cycle the extent of CaM binding is not substantially changed, they do not answer the question of how many CaMs are bound to the Ca^{2+} release channel in skeletal muscle. We would expect that the number lies between 4 and 16 and that extended periods of muscle inactivity increase the extent of CaM binding, whereas increased muscle activity lowers the number of bound CaMs. Other unknown mechanisms such as channel phosphorylation may affect the level of CaM binding, perhaps lowering the extent of binding to less than 4 CaMs/RyR.

In conclusion, we envisage that CaM modulates SR Ca^{2+} release in a muscle cell during E-C coupling as follows. At rest, at a myoplasmic $[Ca^{2+}]$ of 100–150 nM, up to 16 CaMs bind to each RyR tetramer, keeping the SR release channel in a slightly activated form. During E-C coupling, as Ca^{2+} is released from the SR, the RyR is further activated by the released Ca^{2+} (Schneider, 1994) and the $[Ca^{2+}]$ near the release sites rises rapidly. Another consequence of a rise in $[Ca^{2+}]$ is that Ca^{2+} binds to CaM and the Ca^{2+}-bound form of CaM inactivates the channel. We suggest that CaM-mediated channel inactivation may be sufficiently slow to maintain the release channel in an activated form during the initial release phase. Calmodulin then contributes to channel closure by inactivating the channel and maintaining the channel in an inactivated form until Ca^{2+} is pumped back into the SR and the cytoplasmic $[Ca^{2+}]$ has fallen to the resting level. Again, the return to the activated form may be sufficiently slow to cause a time delay during which cytoplasmic $[Ca^{2+}]$ falls sufficiently low before the channel returns to its resting level activity. Thus, CaM may play a role in E-C coupling by modulating both Ca^{2+} release channel activation and inactivation.

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