The Carboxy-Terminal Calcium Binding Sites of Calmodulin Control Calmodulin's Switch from an Activator to an Inhibitor of RYR1†

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ABSTRACT: Calcium and calmodulin both regulate the skeletal muscle calcium release channel, also known as the ryanodine receptor, RYR1. Ca^{2+}-free calmodulin (apocalmodulin) activates and Ca^{2+}-calmodulin inhibits the ryanodine receptor. The conversion of calmodulin from an activator to an inhibitor is due to Ca^{2+} binding to calmodulin. We have previously shown that the binding sites for apocalmodulin and Ca^{2+}-calmodulin on RYR1 are overlapping with the Ca^{2+}-calmodulin site located slightly N-terminal to the apocalmodulin binding site. We now show that mutations of the calcium binding sites in either the N-terminal or the C-terminal lobes of calmodulin decrease the affinity of calmodulin for the ryanodine receptor, suggesting that both lobes interact with RYR1. Mutation of the two C-terminal Ca^{2+} binding sites of calmodulin destroys calmodulin's ability to inhibit ryanodine receptor activity at high calcium concentrations. The mutated calmodulin, however, can still bind to RYR1 at both nanomolar and micromolar Ca^{2+} concentrations. Mutating the two N-terminal calcium binding sites of calmodulin does not significantly alter calmodulin's ability to inhibit ryanodine receptor activity. These data suggest that calcium binding to the two C-terminal calcium binding sites within calmodulin is responsible for the switching of calmodulin from an activator to an inhibitor of the ryanodine receptor.

Electrical depolarization of the transverse tubule (t-tubule) results in a conformational change in the voltage sensor (DHPR) that is transmitted to the skeletal muscle calcium release channel (RYR1)[1] via a direct mechanical coupling (*1*). RYR1 controls the release of Ca^{2+} from the sarcoplasmic reticulum (SR) that is necessary for muscle contraction. RYR1 is a homotetramer containing four 565 kDa subunits; four-fifths of the molecule is cytoplasmic, and the remainder one-fifth is membrane-spanning or lumenal. Ca^{2+}, in addition to several endogenous proteins that bind within the large cytoplasmic domain, modulates RYR1 activity. In the muscle fiber, these endogenous modulators probably act together to affect RYR1 channel activity and ultimately Ca^{2+} release from the SR.

Calmodulin (CaM) is a ubiquitous Ca^{2+} binding protein that contains four EF-hand type Ca^{2+} binding motifs, two in the N-terminus and two in the C-terminus of the protein. CaM binds to RYR1 and affects its function in a Ca^{2+}-dependent fashion (*2*−*6*). At nanomolar Ca^{2+} concentrations, CaM is a partial agonist of RYR1, while at micromolar Ca^{2+} concentrations, CaM is an inhibitor of RYR1. Using a mutant CaM that cannot bind Ca^{2+}, we have recently shown that this switch from an agonist to an inhibitor is due to Ca^{2+} binding to CaM (*4*).

Behavioral mutants of *Paramecium* have been isolated in which naturally occurring mutations in the N-terminal lobe of CaM alter Ca^{2+}-activated Na^+ currents, while naturally occurring mutations in the C-terminal lobe reduce Ca^{2+}-activated K^+ currents (*7*). Because the phenotypes of these mutant *Paramecia* can be grouped according to the two lobes of CaM, these authors suggested that there is a "functional bipartition" of CaM that corresponds to its structural bipartition. More recently, Peterson et al. (*8*) demonstrated that the Ca^{2+}-dependent inactivation of the L-type calcium channel is due to Ca^{2+} binding to CaM. Mutating the C-terminal Ca^{2+} binding sites of CaM, to prevent Ca^{2+} binding, destroyed the Ca^{2+}-dependent inactivation of the cardiac L-type channel, demonstrating this "functional bipartition" of CaM in a mammalian ion channel. Mutating the N-terminal sites did not alter Ca^{2+}-dependent inactivation.

The goal of the current studies was to determine if the inhibition of RYR1 activity by Ca^{2+}-CaM can be attributed to a particular Ca^{2+} binding domain within CaM. We demonstrate that mutating the two C-terminal Ca^{2+} binding sites, but not the two N-terminal sites, of CaM prevents the Ca^{2+}-CaM inhibition of RYR1 activity. These results demonstrate that the functional regulation of RYR1 by Ca^{2+}-CaM also displays a bipartition that reflects CaM's structural bipartition.

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[1] Abbreviations: RYR1, skeletal muscle calcium release channel; CaM, calmodulin; apoCaM, Ca^{2+}-free calmodulin; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; IPTG, isopropyl *β*-D-thiogalactopyranoside; BSA, bovine serum albumin; SR, sarcoplasmic reticulum.

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MATERIALS AND METHODS

Materials. Dithiothreitol (DTT), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 3-(*N*-morpholino)propanesulfonic acid (MOPS), 3-(cyclohexylamino)-l-propanesulfonic acid (CAPS), isopropyl β-D-thiogalactopyranoside (IPTG), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-Ryanodine (70−80 Ci/mmol) was purchased from Du Pont New England Nuclear (Boston, MA); unlabeled ryanodine was purchased from Calbiochem (La Jolla, CA). [^{35}S]-Methionine (>1000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Sarcoplasmic Reticulum (SR) Membrane Preparation. SR membranes were prepared as previously described (*9*) from rabbit hind leg and backstrap white skeletal muscle and purified using sucrose gradient centrifugation.

Expression of CaM. The CaMs used in this study include B12Q, in which the glutamic acid residues in the *z* position of the two E-F hands at the N-terminal lobe of CaM have been mutated to glutamines, and B34Q, where the corresponding mutations have been made at the C-terminal lobe. The *Drosophila* CaM (dCaM), wild-type, B12Q, and B34Q cDNAs (*10*, *11*) were subcloned into the *Nde*I and *Bpu*I *102* I sites of pET3a (Novagen, Madison, WI). Protein expression was induced with 0.3 mM IPTG. dCaM was purified by phenyl-Sepharose chromatography (*12*). B12Q and B34Q were purified by anion exchange chromatography as described previously for the full Ca^{2+} binding site mutant, B1234Q (*4*). [^{35}S]Methionine was used to metabolically label dCaM as previously described (*4*). Protein concentration of the CaMs was determined by the BioRad protein assay using bovine brain CaM as the standard.

Equilibrium [^{35}S]Calmodulin Binding. Calmodulin binding to SR membranes was assessed as previously described (*4*). Briefly, SR membranes (10 μg of protein per assay) were incubated with [^{35}S]dCaM (5.0 nM) and increasing concentrations of dCaM, B12Q, or B34Q (0.6−156 nM) for 2 h at room temperature in binding buffer [300 mM NaCl, 50 mM MOPS (pH 7.4), 100 μg/mL BSA, 0.1% CHAPS, 1 mM EGTA] in the absence (<10 nM free Ca^{2+}) or presence of 1.2 mM $CaCl_2$ (200 μM free Ca^{2+}). Bound radioligand was separated from free radioligand by filtration through Whatman GF/F filters presoaked in 0.3 mg/mL BSA/binding buffer. The filters were washed with 5 × 3 mL of ice-cold binding buffer, and [^{35}S]dCam binding was assessed by scintillation counting.

Equilibrium [^{3}H]Ryanodine Binding. SR membranes (20 μg/assay) were incubated with [^{3}H]ryanodine (5 nM) at room temperature (23 °C) for 16 h in binding buffer [300 mM NaCl, 50 mM MOPS (pH 7.4), 100 μg/mL BSA, 0.1% CHAPS, 100 μM EGTA] and increasing amounts of $CaCl_2$ to achieve free Ca^{2+} concentrations ranging from <5 nM to 1 mM. Nonspecific binding was defined in the presence of 10 μM unlabeled ryanodine. The bound [^{3}H]ryanodine was separated from free ligand by filtering through Whatman GF/F glass fiber filters. The filters were washed with 5 × 3 mL of ice-cold buffer [300 mM NaCl, 50 mM MOPS (pH 7.4), 100 μg/mL BSA, 100 μM $CaCl_2$]. The radioactivity bound to the filters was quantified by scintillation counting. Free Ca^{2+} concentrations were calculated as described by Fabiato (*13*). For the effect of increasing concentrations of B12Q and B34Q on [^{3}H]ryanodine binding, the assay conditions were essentially the same as described above except SR membranes (10 μg/assay) were incubated in 300 mM NaCl, 50 mM MOPS (pH 7.4), 100 μg/mL BSA, 0.1% CHAPS, 1 mM EGTA, 1.2 mM $CaCl_2$.

RYR1 Activity in Planar Lipid Bilayers. To assess the effects of B12Q and B34Q dCaM on the activity of RYR1, SR membranes were fused with planar lipid bilayers as previously described (*14*). Single-channel activity was recorded in 216 mM CH_3O_3SC, 50 mM MOPS (pH 7.4) and either 100 nM or 50 μM $CaCl_2$. The data were acquired at a holding potential of −40 mV, filtered at 2.5 kHz, and digitized at 10 kHz.

Data Analysis. In the competition studies with B12Q and B34Q, [^{35}S]CaM binding data were fit to eq 1:

$$B = B_o[(1 + K_d/L^*)(1 + x/K_i)] + C \quad (1)$$

where B is the amount of [^{35}S]dCaM bound at a given CaM concentration, B_o is the maximum amount bound, K_d is the equilibrium dissociation constant, L^* is the concentration of radiolabeled ligand, K_i is the equilibrium inhibition constant, and C is a constant.

The Ca^{2+} dependence of [^{3}H]ryanodine binding was fit to the product of an activation and an inactivation variable using the following equation as previously described (*4*):

$$B = \{B_o[[Ca^{2+}]/([Ca^{2+}] + K_{act})] \times [1 - [Ca^{2+}]/([Ca^{2+}] + K_{inh})]\} + C \quad (2)$$

where B is the amount of [^{3}H]ryanodine bound at a given $[Ca^{2+}]$, B_o is the maximum amount bound, K_{act} and K_{inh} are activation and inhibition constants, respectively, and C is a constant.

Steady-state single-channel open probabilities (P_o) were determined by the 50% threshold technique in 3 min of recording using FETCHAN and pSTAT software (Axon Instruments, Inc.).

The data shown are the mean ± the standard error of the mean (SEM) for at least three independent determinations. Unless stated otherwise, data were analyzed for differences using the Student's *t*-test for unpaired data. Differences were statistically significant at $p \leq 0.05$.

RESULTS

Relative Affinity of B12Q and B34Q for RYR1 at Nanomolar and Micromolar Ca^{2+} Concentrations. To determine the relative affinity of RYR1 for B12Q and B34Q, we examined the ability of these mutant CaMs to inhibit [^{35}S]-dCaM binding to SR membranes at nanomolar and micromolar Ca^{2+} concentrations. RYR1 is the major CaM binding protein in these membranes (*5*, *6*). Endogenous calmodulin is not detected in the SR membrane fractions with anti-calmodulin antibodies in Western blots (data not shown), presumably due to the extensive washing during the purification. At a free Ca^{2+} concentration of <10 nM, B12Q and B34Q inhibited [^{35}S]dCaM binding with K_i values of 83 ± 5 nM ($n = 3$) and 114 ± 8 nM ($n = 3$), respectively (Figure 1A). Under these conditions, dCaM had an apparent K_d of 48 ± 2 nM (*4*). At a free Ca^{2+} concentration of 200 μM, the relative affinities of B12Q and B34Q were 125 ± 2 nM

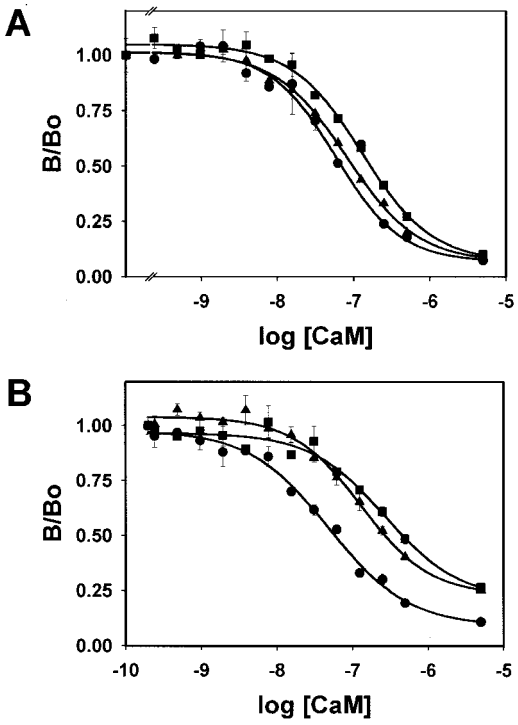

FIGURE 1: B12Q and B34Q have a lower affinity for RYR1. SR membranes (10 μg/200 μL) were incubated with 5 nM [^{35}S]dCaM and increasing concentrations (0.24 nM−5 μM) of dCaM (●), B12Q (▲), or B34Q (■) at <10 nM free Ca^{2+} (A) or 200 μM free Ca^{2+} (B) as described under Materials and Methods. The solid lines are derived from fitting the data to eq 1 under Materials and Methods. Data on the ordinate are plotted as the amount of radioligand bound normalized to the amount bound in the absence of competing cold ligand (B/B_o). No correction was made for nonspecific binding in this figure.

(n = 3) and 266 ± 21 nM (n = 3), respectively (Figure 1B), compared to the wild-type affinity of 34 ± 1 nM (*4*). In addition, we were not able to demonstrate complete inhibition of [^{35}S]dCaM by either of these mutant CaMs at high [Ca^{2+}]. This may indicate that only one lobe of the mutant CaMs is binding at high [Ca^{2+}].

Analysis of B12Q and B34Q Binding to RYR1 Peptides. The binding of apoCaM and Ca^{2+}CaM to RYR1 protects sites at amino acids 3630 and 3637 on RYR1 from cleavage by trypsin (*3*). Using synthetic peptides of this region, we have previously shown (*15*) that amino acids 3614−3643 of RYR1 constitute the apoCaM and Ca^{2+}CaM binding sites and that upon binding Ca^{2+}, CaM shifts toward the N-terminus of this sequence and simultaneously becomes an inhibitor of the channel. B1234Q, which is a CaM that has all four Ca^{2+} binding sites mutated, does not undergo this shift. To further evaluate the Ca^{2+}-dependent binding of CaM to its RYR1 binding site, we assessed the ability of B12Q and B34Q to bind to a series of synthetic peptides (Table 1) within the CaM binding region of RYR1 at 200 μM Ca^{2+} concentration. B12Q bound peptides P3625−3644, P3614−3634, and P3614−3643 (Table 1, Figure 2). B34Q only bound peptides P3625−3644 and P3614−3643 (Table 1, Figure 3). (Note that peptide P3614−3634 is not able to bind all of the calmodulin on these gels. This is due to its limited solubility in aqueous solutions.) The pattern of B12Q binding is the same as we have previously reported for Ca^{2+}CaM, and that of B34Q is the same as apoCaM (*15*), suggesting

Table 1: Summary of the Interaction of Peptides Representing Sequences of RYR1 with the CaM Mutants B12Q and B34Q at 200 μM Ca^{2+}

peptide	Ca^{2+}CaM	B12Q	B34Q	B1234Q
P3636−3652: FRMTPLYNLPTHRACNM	−	−	−	−
P3614−3627: KSKKAVWHKLLSKQ	−	−	−	−
P3625−3644: SKQRRRAVVACFRMTP-LYNL	+	+	++	++
P3614−3634: KSKKAVWHKLLSKQRRR-AVVA	++	++	±	−
P3614−3643: KSKKAVWHKLLSKQRRR-AVVACFRMTPLYN	++	++	++	++

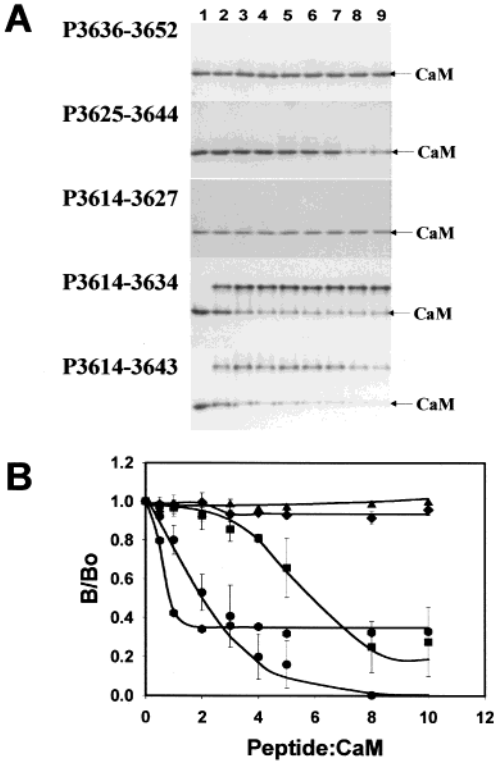

FIGURE 2: Ability of B12Q to bind to RYR1 peptides. Panel A shows a representative 20% nondenaturing polyacrylamide gel of B12Q (3.4 μM) with increasing molar ratios of the indicated RYR1 peptides in 200 μM $CaCl_2$. The peptides alone do not enter the gel. B12Q bound to the peptide diminishes the intensity of the B12Q band. Lane 1: B12Q alone. Beginning in lane 2 the ratios of peptide to B12Q are 0.5:1, 1:1, 2:1, 3:1, 4:1, 5:1, 8:1, and 10:1. In panel B, densitometric analysis of the B12Q (3.4 μM) band in the presence of increasing amounts of peptide is summarized from three independent nondenaturing gel shift assays under high Ca^{2+} conditions. P3636−3652 (▲), P3614−3627 (◆), P3625−3644 (■), P3614−3634 (●), P3614−3643 (●).

that B12Q can still undergo the N-terminal shift while B34Q cannot. The pattern of binding of both B12Q and B34Q at nanomolar Ca^{2+} concentrations is similar to that previously demonstrated for dCaM and B1234Q (*15*). If this shift in binding correlates with the functional effect of CaM on RYR1, then we predict that B12Q, like wild-type CaM, will inhibit the channel at high Ca^{2+} concentrations, while B34Q, like B1234Q, will be a channel activator at all Ca^{2+}-concentrations.

Table 2: Summary of the Effects of B12Q and B34Q on Single-Channel Activity

		control	+CaM
I. B12Q	100 nM Ca^{2+}	0.007 ± 0.004	0.080 ± 0.020 ($n = 6$)[a]
	50 μM Ca^{2+}	0.150 ± 0.050	0.030 ± 0.020 ($n = 7$)[a]
II. B34Q	100 nM Ca^{2+}	0.001 ± 0.004	0.010 ± 0.002 ($n = 5$)[a]
	50 μM Ca^{2+}	0.080 ± 0.030	0.170 ± 0.050 ($n = 7$)[a]

[a] $P \leq 0.05$ versus control; Student's paired *t*-test was used to determine the significance of change.

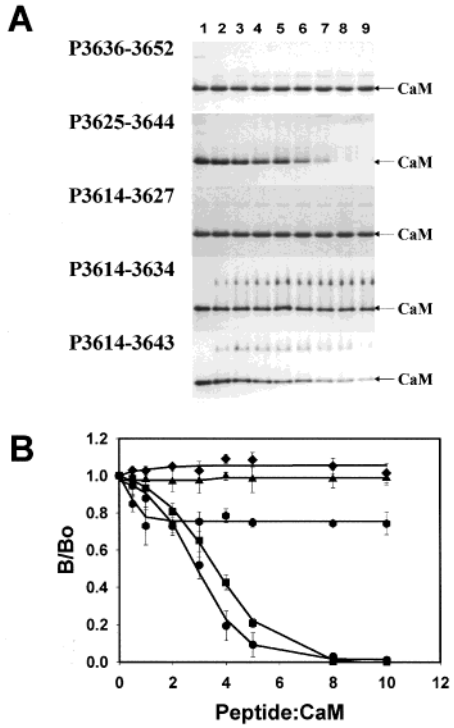

FIGURE 3: Ability of B34Q to bind to RYR1 peptides. Panel A shows a representative 20% nondenaturing polyacrylamide gel of B34Q (3 μM) with increasing molar ratios of the indicated RYR1 peptides in 200 μM free Ca^{2+}. The peptides alone do not enter the gel. The peptide binding to B34Q diminishes the intensity of the B34Q band. Lane 1: B34Q alone. Beginning in lane 2 the ratios of peptide to B34Q are 0.5:1, 1:1, 2:1, 3:1, 4:1, 5:1, 8:1, and 10:1. In panel B, densitometric analysis of the B34Q (3 μM) band in the presence of increasing amounts of peptide is summarized from three independent nondenaturing gel shift assays under high Ca^{2+} conditions. P3636−3652 (▲), P3614−3627 (◆), P3625−3644 (■), P3614−3634 (⬢), P3614−3643 (●).

Effect of B12Q and B34Q on Single-Channel Activity at High [Ca^{2+}]. We next tested the effects of B12Q and B34Q on the activity of RYR1 reconstituted into planar lipid bilayers. B12Q (1 μM) increased the steady-state mean open probability (P_o) of RYR1 from 0.007 ± 0.004 to 0.080 ± 0.020 ($p = 0.03$, $n = 6$) at a free Ca^{2+} concentration of <100 nM (Table 2). At a free Ca^{2+} concentration of 50 μM, B12Q (1 μM) decreased the P_o of the channel from 0.15 ± 0.05 to 0.03 ± 0.02 ($p = 0.04$, $n = 7$) (Figure 4A, Table 2). B34Q (1 μM), however, increased the P_o of RYR1 at both nanomolar and micromolar Ca^{2+} concentrations. At <100 nM free Ca^{2+}, B34Q increased the P_o from 0.001 ± 0.004 to 0.010 ± 0.002 ($p = 0.02$, $n = 5$, Table 2). At 50 μM free Ca^{2+}, B34Q increased the P_o from 0.08 ± 0.03 to 0.17 ± 0.05 ($p = 0.04$, $n = 7$) (Figure 4B, Table 2). These results suggest that either both or one of sites 3 and 4 in the C-terminus of CaM must bind Ca^{2+} to exert any inhibitory effect on RYR1.

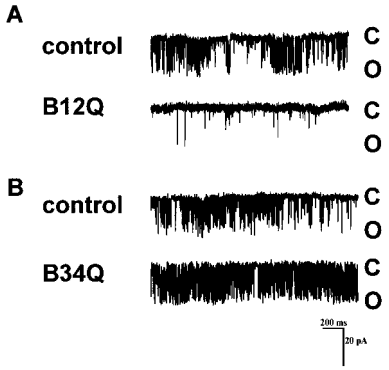

FIGURE 4: B12Q decreases while B34Q increases the single-channel activity of RYR1 reconstituted into planar lipid bilayers at 50 μM free Ca^{2+} concentration. Representative single-channel recordings of RYR1 reconstituted into planar lipid bilayers at 50 μM free Ca^{2+} concentration (O and C reflect the open and closed state of the channel, respectively). Panel A: Inhibition of RYR1 by 1 μM B12Q added to the cis chamber. Panel B: Activation of RYR1 by 1 μM B34Q added to the cis chamber. Data were analyzed for statistical significance using the paired *t*-test.

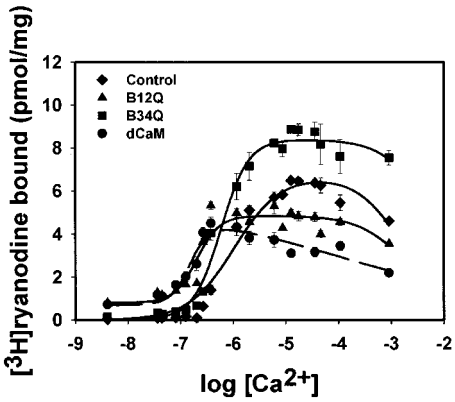

FIGURE 5: Effect of B12Q and B34Q on the [Ca^{2+}] dependence of [^{3}H]ryanodine binding to SR membranes. SR membranes (20 μg/200 μL) were incubated with 5 nM [^{3}H]ryanodine at the indicated free Ca^{2+} concentrations in the absence (◆) or presence of 1 μM B12Q (▲), 1 μM B34Q (■), or dCaM (●). The solid lines are derived from fitting the data to eq 2 under Materials and Methods. The solid circles represent the data for dCaM (1 μM), which we have reported previously (*4*) and are displayed here for reference.

Effect of B12Q and B34Q on the [Ca^{2+}] Dependence of [^{3}H]Ryanodine Binding. ApoCaM activates RYR1, in part, by increasing the affinity of RYR1 for Ca^{2+} (*15*). We assessed the effect of B12Q and B34Q on the Ca^{2+} dependence of [^{3}H]ryanodine binding to RYR1 (Figure 5). In the absence of CaM, the Ca^{2+} dependence of [^{3}H]-ryanodine binding showed the characteristic enhancement/inhibition curve, with apparent EC_{50}s for Ca^{2+} of 1.0 ± 0.2 μM ($n = 3$) and 2.1 ± 0.5 mM ($n = 3$), respectively. In the presence of B12Q (1 μM), the Ca^{2+} dependence of the enhancement of [^{3}H]ryanodine binding was shifted to lower Ca^{2+} concentrations compared to control ($EC_{50} = 0.20 \pm 0.01$ μM, Figure 5). At Ca^{2+} concentrations in which the two C-terminal Ca^{2+} binding sites are likely to be occupied by Ca^{2+}, B12Q also inhibited [^{3}H]ryanodine binding. In contrast, B34Q enhanced [^{3}H]ryanodine binding at all Ca^{2+} concentrations relative to control and increased the apparent affinity of the activating sites on RYR1 for Ca^{2+} ($EC_{50} =$

0.60 ± 0.06 μM). These data suggest that Ca^{2+} binding to the two C-terminal Ca^{2+} binding sites of CaM is absolutely required to convert CaM from an activator (apoCaM) to an inhibitor (Ca^{2+}CaM).

DISCUSSION

The skeletal muscle Ca^{2+} release channel of the SR is regulated by a number of endogenous modulators, including Ca^{2+} and CaM. RYR1 shows a biphasic response to Ca^{2+}: Ca^{2+} enhances channel activity at micromolar concentrations and inhibits channel activity at millimolar concentrations. CaM also exerts a bifunctional effect on the activity of RYR1, enhancing channel activity in its Ca^{2+}-free state (apoCaM) and inhibiting channel activity in its Ca^{2+}-bound state (Ca^{2+}CaM). The ability of apoCaM to enhance RYR1 activity is due, in part, to increasing the affinity of the Ca^{2+} activation sites on RYR1 (*15*). Using a mutant CaM that cannot bind Ca^{2+} at any of the four Ca^{2+} binding sites, we have shown that the inhibitory effect of CaM at high [Ca^{2+}] is due to Ca^{2+} binding to CaM (*15*). The inhibition of channel activity by Ca^{2+}CaM occurs at Ca^{2+} concentrations which, in the absence of CaM, enhance RYR1 activity.

In the current study, we demonstrate that the inhibition of RYR1 activity by CaM is due to Ca^{2+} binding to the two C-terminal Ca^{2+} binding sites of CaM. Mutation of sites in only one lobe of the CaM molecule reduces its affinity in high [Ca^{2+}] relative to both the wild-type CaM and CaM mutated at all four Ca^{2+} sites (B1234Q). The ability of B1234Q to bind to RYR1 in the presence of micromolar concentrations of Ca^{2+} suggests that both the Ca^{2+}-free and the Ca^{2+}-bound RYR1 has binding sites for apoCaM. The lower affinities of B12Q and B34Q compared to both B1234Q and wild-type CaM and the lack of complete inhibition of [^{35}S]wild-type CaM binding at high [Ca^{2+}] suggest that (1) one lobe in the hybrid molecule is not binding to RYR1, (2) one lobe is binding to an apoCaM site while the other is binding at a Ca^{2+}CaM site, or (3) the mutations are interfering with allosteric interactions between the two lobes of CaM. Since B12Q is able to inhibit the channel at high [Ca^{2+}] but B34Q is not, it would appear that Ca^{2+} binding to sites 3 and 4 of the CaM molecule and the interaction of this lobe of CaM with the Ca^{2+}CaM site on RYR1 are adequate for inhibition of channel activity. In contrast, the Ca^{2+} bound to the N-terminal lobe is not sufficient to inhibit channel activity. Both of the hybrid molecules can, however, activate the channel at low [Ca^{2+}], and B34Q can activate at high [Ca^{2+}].

Both dCaM and B1234Q have a higher affinity at 200 μM Ca^{2+} than at <10 nM Ca^{2+} (*4*), indicating that Ca^{2+} binding to RYR1 increases its affinity for CaM. In contrast, both hybrid molecules, B12Q and B34Q, have a higher affinity for RYR1 at nanomolar Ca^{2+} concentrations compared to micromolar Ca^{2+} concentrations. These data suggest that there is a complex interaction between RYR1 and CaM, in particular when CaM is partially saturated with Ca^{2+}, as is the case with B12Q and B34Q. Both B12Q and B34Q bind P3614−3643, a synthetic peptide representing the CaM binding site within RYR1, supporting the idea that these mutants are binding to the RYR1 CaM binding site. Furthermore, at high Ca^{2+} concentrations, B12Q binds to the same RYR1 peptides as does Ca^{2+}CaM and B34Q binds to the same RYR1 peptides as does apoCaM. These data suggest that the determinants within the CaM binding site on RYR1 for B12Q and B34Q parallel those we have previously shown for Ca^{2+}CaM and apoCaM binding, respectively.

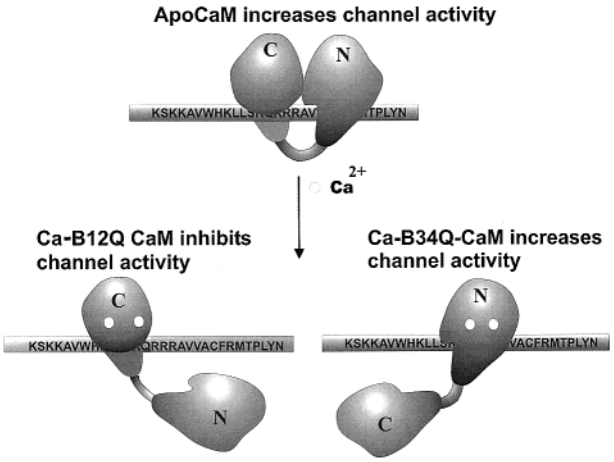

FIGURE 6: Model for the interaction between CaM and RYR1. ApoCaM binds to RYR1 between amino acids 3625 and 3643. Upon binding Ca^{2+}, CaM's binding site is shifted toward the N-terminus of RYR1 with the C-terminal domain of CaM regulating the functional state of RYR1. The binding orientation between CaM and P3614−3643 is head-to-tail, with the N-terminal domain of CaM bound to the C-terminus of P3614−3643. With B12Q and B34Q, we speculate that the movement of the Ca^{2+}-bound lobe caused the Ca^{2+}-free lobe to be released, accounting for the reduced affinity detected with these mutants. B12Q inhibits the channel at high [Ca^{2+}] while B34Q is an activator, indicating that it is the C-terminal lobe in its Ca^{2+}-bound form that is responsible for inhibition of channel activity.

Identification of naturally occurring mutations in CaM that result in altered phenotypes of *Paramecium* led to the idea of a "functional bipartition" of CaM that reflects CaM's structural bipartition (*7*). These authors found that mutations in the N-terminal lobe of CaM altered Ca^{2+}-dependent Na^+ currents while mutations in the C-terminal lobe altered Ca^{2+}-dependent K^+ currents. This bipartition phenomenon has also been shown for the Ca^{2+}-dependent inactivation of the L-type Ca^{2+} channel (*8*). In our studies, we found that the B34Q mutant enhanced [^{3}H]ryanodine binding and increased single-channel activity at 50 μM Ca^{2+} concentrations while the BI2Q mutant showed inhibitory effects, similar to those of native CaM. These data indicate that the Ca^{2+}-dependent conversion of CaM from an activator to an inhibitor of RYR1 is due to Ca^{2+} binding to the two C-terminal Ca^{2+} binding sites within CaM, but strongly suggest that the N-terminal domain is contributing to the affinity of CaM for its binding site.

Our current model of the regulation of RYR1 by CaM (*15*) is that upon binding Ca^{2+}, CaM undergoes a conformational change and shifts toward the N-terminus of its RYR1 binding site. Ca^{2+} binding to the C-terminal domain of CaM regulates the functional effects of CaM on RYR1 (Figure 6). The N-terminal domain of CaM may be interacting with the C-terminus of the region between amino acids 3614 and 3643. However, we cannot exclude the possibility that the N-terminal domain of CaM binds another region within RYR1. Our current results suggest that RYR1 is another channel in which CaM regulates function according to its structural bipartition. We propose that there are multiple

mechanisms by which CaM uniquely transduces the Ca^{2+} signal for different ion channels, allowing for an increased diversity in CaM's role in Ca^{2+} signal transduction.

REFERENCES

1. Rios, E., Ma, J. J., and Gonzalez, A. (1991) *J. Muscle Res. Cell Motil. 12*, 127−135.
2. Buratti, R., Prestipino, G., Menegazzi, P., Treves, S., and Zorzato, F. (1995) *Biochem. Biophys. Res. Commun. 213*, 1082−1090.
3. Moore, C. P., Rodney, G., Zhang, J. Z., Santacruz-Toloza, L., Strasburg, G., and Hamilton, S. L. (1999) *Biochemistry 38*, 8532−8537.
4. Rodney, G. G., Williams, B. Y., Strasburg, G. M., Beckingham, K., and Hamilton, S. L. (2000) *Biochemistry 39*, 7807−7812.
5. Tripathy, A., Xu, L., Mann, G., and Meissner, G. (1995) *Biophys. J. 69*, 106−119.
6. Yang, H., Reedy, M. M., Burke, C. L., and Strasburg, G. M. (1994) *Biochemistry 33*, 518−525.
7. Saimi, Y., and Kung, C. (1994) *FEBS Lett. 350*, 155−158.
8. Peterson, B. Z., DeMaria, C. D., Adelman, J. P., and Yue, D. T. (1999) *Neuron 22*, 549−558.
9. Hawkes, M. J., Diaz-Muñoz, M., and Hamilton, S. L. (1989) *Membr. Biochem. 8*, 133−145.
10. Maune, J. F., Klee, C. B., and Beckingham, K. (1992) *J. Biol. Chem. 267*, 5286−5295.
11. Mukherjea, P., and Beckingham, K. (1993) *Biochem. Mol. Biol. Int. 29*, 555−563.
12. Gopalakrishna, R., and Anderson, W. B. (1982) *Biochem. Biophys. Res. Commun. 104*, 830−836.
13. Fabiato, A. (1988) *Methods Enzymol. 157*, 378−417.
14. Aghdasi, B., Reid, M. B., and Hamilton, S. L. (1997) *J. Biol. Chem. 272*, 25462−25467.
15. Rodney, G. G., Moore, C. P., Williams, B. Y., Zhang, J. Z., Krol, J., Pedersen, S. E., and Hamilton, S. L. (2001) *J. Biol. Chem. 276*, 2069−2074.

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