Regulation of RYR1 Activity by Ca^{2+} and Calmodulin†

George G. Rodney,‡ Barbara Y. Williams,‡ Gale M. Strasburg,§ Kathy Beckingham,|| and Susan L. Hamilton*,‡

Department of Molecular Physiology and Biophysics, Baylor College of Medicine, 1 Baylor Plaza, Houston, Texas 77030, Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48824-1224, and Department of Biochemistry & Cell Biology, Rice University, 6100 Main Street, Houston, Texas 77005

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ABSTRACT: The skeletal muscle calcium release channel (RYR1) is a Ca^{2+}-binding protein that is regulated by another Ca^{2+}-binding protein, calmodulin. The functional consequences of calmodulin's interaction with RYR1 are dependent on Ca^{2+} concentration. At nanomolar Ca^{2+} concentrations, calmodulin is an activator, but at micromolar Ca^{2+} concentrations, calmodulin is an inhibitor of RYR1. This raises the question of whether the Ca^{2+}-dependent effects of calmodulin on RYR1 function are due to Ca^{2+} binding to calmodulin, RYR1, or both. To distinguish the effects of Ca^{2+} binding to calmodulin from those of Ca^{2+} binding to RYR1, a mutant calmodulin that cannot bind Ca^{2+} was used to evaluate the effects of Ca^{2+}-free calmodulin on Ca^{2+}-bound RYR1. We demonstrate that Ca^{2+}-free calmodulin enhances the affinity of RYR1 for Ca^{2+} while Ca^{2+} binding to calmodulin converts calmodulin from an activator to an inhibitor. Furthermore, Ca^{2+} binding to RYR1 enhances its affinity for both Ca^{2+}-free and Ca^{2+}-bound calmodulin.

The opening of the skeletal muscle Ca^{2+} release channel (RYR1)[1] in response to depolarization of the t-tubule membranes is thought to occur by direct mechanical coupling of the t-tubule voltage sensor to RYR1. Ca^{2+} binding to RYR1 alters both its activity in planar lipid bilayers and its ability to bind the plant alkaloid ryanodine (*1*). At micromolar concentrations, Ca^{2+} activates the channel and increases [^{3}H]-ryanodine binding. In the millimolar range, Ca^{2+} inhibits the channel and decreases [^{3}H]ryanodine binding.

RYR1 is also regulated by the binding of calmodulin (CaM), a ubiquitous Ca^{2+}-binding protein that plays a central role in Ca^{2+} signaling in eukaryotes (*2*). CaM contains four EF-hand Ca^{2+}-binding pockets, two in the N-terminal domain and two in the C-terminal domain of the molecule. CaM interacts with many target proteins as a result of increased hydrophobicity upon binding Ca^{2+}. However, some proteins, including RYR1, bind Ca^{2+}-free CaM (apoCaM) (*3*).

The role of CaM in excitation−contraction (E−C) coupling of intact skeletal muscle cells is not well understood. In vitro, CaM is an effective regulator of RYR1 (*4*). At nanomolar Ca^{2+} concentrations, RYR1 is not very active, but its activity is increased by the addition of CaM. At micromolar Ca^{2+} concentrations, RYR1 is much more active, but the addition of CaM is inhibitory. If CaM interacts with RYR1 in vivo, it is likely to alter the Ca^{2+} dependence of both activation and inactivation of the channel. In the presence of CaM, RYR1 should both activate and inactivate at lower Ca^{2+} concentrations than in the absence of CaM. These Ca^{2+}-dependent, bifunctional effects of CaM on RYR1 activity suggest that the functional consequences of CaM's interaction with RYR1 are likely to be different between the resting state and during the Ca^{2+} transient, and therefore, CaM is likely to modulate skeletal muscle E−C coupling in a complex fashion.

We have previously shown, using mammalian CaM (mCaM), that both [^{35}S]apoCaM and [^{35}S]Ca^{2+}CaM apparently bind to one site per RYR1 subunit (four per tetramer) (*5*). Moreover, both apoCaM and Ca^{2+}-CaM protect sites at amino acids 3630 and 3637 on RYR1 from cleavage by trypsin. Our data are consistent with a model in which apoCaM and Ca^{2+}-CaM bind to overlapping sites in this region of RYR1.

To distinguish between the effects of Ca^{2+} binding to RYR1 and Ca^{2+} binding to CaM, we made use of known mutations in the EF-hands of CaM that prevent Ca^{2+} binding to CaM. The Ca^{2+} binding site mutants of CaM were created by replacing the invariant glutamic acid in the Z-position of each EF-hand with a glutamine residue (*6*). Our findings are consistent with a model in which apoCaM enhances the affinity of the Ca^{2+} activation sites on RYR1, while CaM in its Ca^{2+}-bound state is an inhibitor of RYR1.

MATERIALS AND METHODS

Materials. [^{3}H]Ryanodine (70−80 Ci/mmol) was purchased from DuPont−New England Nuclear (Boston, MA), and unlabeled ryanodine was purchased from Calbiochem (La Jolla, CA). [^{35}S]Methionine (>1000 Ci/mmol) was

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* To whom correspondence should be addressed: e-mail susanh@bcm.tmc.edu; phone: (713) 798-3894; fax (713) 798-5441.

‡ Baylor College of Medicine.

§ Michigan State University.

|| Rice University.

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

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purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Dithiothreitol (DTT), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 3-(*N*-morpholino)-propanesulfonic acid (CAPS), 3-(cyclohexylamino)-1-propanesulfonic acid (MOPS), thrombin, isopropyl β-D-thiogalactopyranoside (IPTG), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). Unlabeled mammalian calmodulin (bovine brain) was obtained from Upstate Biochemicals (Lake Placid, NY).

Sarcoplasmic Reticulum Membrane Preparation. SR membranes were prepared from rabbit hind leg and backstrap white skeletal muscle and were purified by sucrose gradient centrifugation (*7*).

Expression of CaM. The *Drosophila* CaM (dCaM) wild-type and B1234Q cDNAs (*8*) were subcloned into the *Nde*I and *Bpu*1102I sites of pET3a (Novagen, Madison, WI). Protein expression was induced with 0.3 mM IPTG. Expressed CaMs (mCaM and dCaM) were purified by phenyl-Sepharose chromatography (*9*). B1234Q was purified by anion-exchange chromatography followed by HPLC as previously described (*6*). [^{35}S]Methionine was used to metabolically label the CaMs according to the procedure described by Lydan and O'Driscoll (*10*). Protein concentration of the CaMs was determined by measuring absorbance at 277 and 320 nm and calculating the concentration according to (*11*)

$$C = (A_{277} - A_{320})/\epsilon \quad (1)$$

where $\epsilon = 0.20$ mL/mg·cm for mCaM, 1874 L/M·cm for dCaM, and 1900 L/M·cm for B1234Q in 1 mM EGTA.

Equilibrium [^{35}S]Calmodulin Binding. SR membranes (7−10 μg of protein per assay) were incubated with [^{35}S]CaM (2.0−5.0 nM) and increasing concentrations of CaM (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, and 1 mM EGTA] in the absence or presence of 1.2 mM $CaCl_2$. For studies assessing the effect of Ca^{2+} on CaM binding, SR membranes (7−10 μg) were incubated with [^{35}S]CaM (5 nM) and increasing concentrations of Ca^{2+} (free Ca^{2+} concentration ranging from <5 nM to 1 mM). Free Ca^{2+} concentrations were calculated as described by Fabiato (*12*). Nonspecific binding was defined in the presence of 5 μM unlabeled CaM. Bound radioligand was separated from free radioligand by filtration through Whatman GF/F filters presoaked in 0.3 mg/mL BSA/binding buffer and the filters were washed with 5 × 3 mL of ice-cold binding buffer. For the experiments examining the concentration effect of CaM, the Ca^{2+} concentration in the filter soaking solution and the wash buffer matched the Ca^{2+} concentration of the binding buffer. In the experiments assessing the effect of Ca^{2+} on CaM binding, the soak and wash buffer had no added Ca^{2+}.

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, and 100 μM EGTA] with 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, and 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 (*12*).

RYR1 Activity in Planar Lipid Bilayers. Analysis of the effect of CaM on the activity of RYR1 reconstituted into planar lipid bilayers was performed with cesium as the current carrier as previously described (*13*). 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, [^{35}S]CaM binding data were fit to

$$B = B_0/([1 + (K_d/L^*)][1 + (x/K_i)]) + C \quad (2)$$

where B is the amount of [^{35}S]CaM bound at a given CaM concentration, B_0 is the maximum amount bound, K_d is the equilibrium dissociation constant, L^* is the concentration of radiolabled ligand, K_i is the equilibrium inhibition constant, and C is a constant. Scatchard analysis of [^{35}S]CaM binding data was performed by linear regression with Sigma Plot (Jandel Scientific).

The Ca^{2+} dependence of [^{3}H]ryanodine binding was fitted by the product of an activation and an inactivation variable to the following equation modified from ref *1*:

$$B = (B_0\{[Ca^{2+}]/([Ca^{2+}] + K_{act})\} \times \{1 - [Ca^{2+}]/([Ca^{2+}] + K_{inh})\}) + C \quad (3)$$

where B is the amount of [^{3}H]ryanodine bound at a given [Ca^{2+}], B_0 is the maximum bound, K_{act} and K_{inh} are activation and inhibition constants, and C is a constant.

Single-channel recordings were analyzed with FETCHAN and pSTAT software (Axon Instruments, Inc.). Steady-state open probabilities (P_o) were determined by the 50% threshold technique in at least 2 min of recording.

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 by the Student's *t*-test for unpaired data. Differences were statistically significant at $p < 0.05$.

RESULTS

dCaM and B1234Q Bind to Approximately the Same Number of Sites on RYR1 at Nanomolar and Micromolar Ca^{2+} Concentrations. To define the interactions of wild-type *Drosophila* calmodulin (dCaM) and the B1234Q mutant with RYR1, the CaMs were metabolically labeled with [^{35}S]methionine and their binding to SR membranes was analyzed. dCaM differs from mCaM by only three amino acids. However, dCaM binds to RYR1 and has effects similar to those of mCaM (data not shown). Although in these assays the number of binding sites for [^{35}S]dCaM to SR membranes (Figure 1A) appeared to be somewhat greater at micromolar Ca^{2+} than at nanomolar Ca^{2+}, the number of binding sites for [^{35}S]B1234Q to SR membranes (Figure 1B) was not significantly different at nanomolar and micromolar Ca^{2+} concentrations (Table 1). The significance of the difference in the number of binding sites for [^{35}S]dCaM at nanomolar and micromolar Ca^{2+} concentrations is not yet known. The

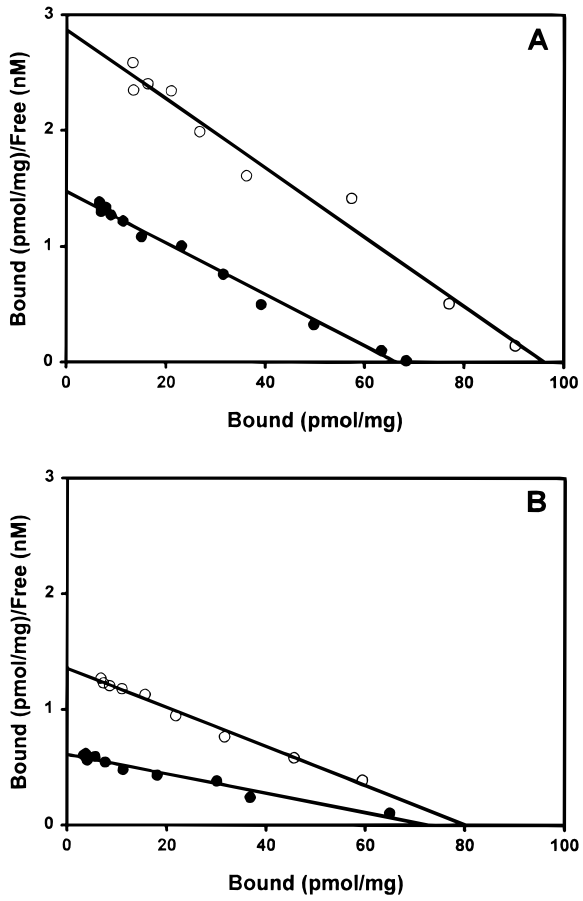

FIGURE 1: [^{35}S]dCaM and [^{35}S]B1234Q bind to 3−4 sites on RYR1 at low and high Ca^{2+} concentrations. Scatchard analysis of [^{35}S]dCaM and [^{35}S]B1234Q binding to SR membranes is shown. SR membranes (7 μg/200 μL) were incubated for 2 h at room temperature with 5 nM [^{35}S]dCaM (A) or 5 nM [^{35}S]B1234Q (B) and increasing concentrations (0.6−156 nM) of cold dCaM or B1234Q, respectively. (●) <10 nM Ca^{2+}; (○) 200 μM Ca^{2+}. Data were fit by linear regression.

Table 1: Summary of [^{35}S]dCaM[a] and [^{35}S]B1234Q Binding to RYR1 at Nanomolar and Micromolar [Ca^{2+}]

	calcium concentration	K_d[b] (nM)	B_{max}[c] (pmol/mg)	C/R[d]
[^{35}S]dCaM	<10 nM	48 ± 2	70 ± 2	3.1 ± 0.2
[^{35}S]dCaM	200 μM	34 ± 1[e]	97 ± 4[e]	4.3 ± 0.3
[^{35}S]B1234Q	<10 nM	129 ± 9	77 ± 3	3.4 ± 0.1
[^{35}S]B1234Q	200 μM	70 ± 14[e]	84 ± 9	3.8 ± 0.4

[a] dCaM = *Drosophila* CaM. [b] Equilibrium dissociation constant. [c] Maximum bound. [d] Calmodulin molecules bound per RYR1. Receptor concentration determined by [^{3}H]ryanodine binding was 22 ± 1 pmol/mg. [e] Significantly different ($p < 0.05$) from nanomolar Ca^{2+}.

receptor concentration in the SR membrane preparations was determined to be 22 ± 1 pmol/mg ($n = 4$) by [^{3}H]ryanodine binding. Since RYR1 binds one ryanodine molecule per tetramer, these data suggest that approximately 3−4 CaM molecules bind per RYR1 tetramer at nanomolar and micromolar Ca^{2+}.

To determine whether the B1234Q mutant binds to the same site as Ca^{2+}-bound dCaM, we examined the ability of dCaM to inhibit [^{35}S]B1234Q binding to SR membranes at micromolar Ca^{2+} concentrations. dCaM, at a free Ca^{2+} concentration of 200 μM, completely inhibited [^{35}S]B1234Q binding with a K_i of 43 ± 8 nM ($n = 3$) (Figure 2). This

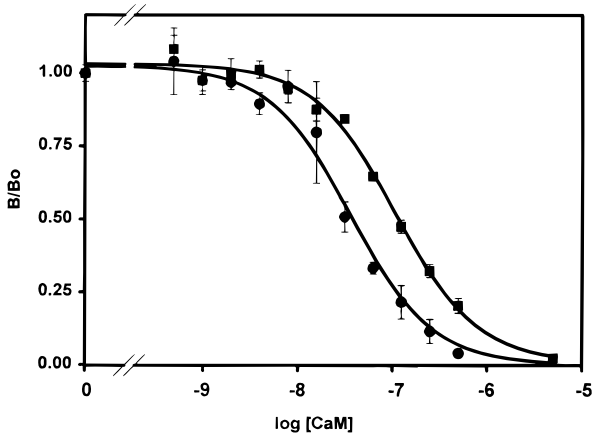

FIGURE 2: dCaM completely inhibits the binding of [^{35}S]B1234Q at micromolar Ca^{2+} concentration. SR membranes (10 μg/200 μL) were incubated with 10 nM [^{35}S]B1234Q and increasing concentrations (0.24 nM−5 μM) of dCaM (●) or B1234Q (■) at 200 μM Ca^{2+} as described under Materials and Methods. The solid lines are derived from fitting the data to eq 2 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).

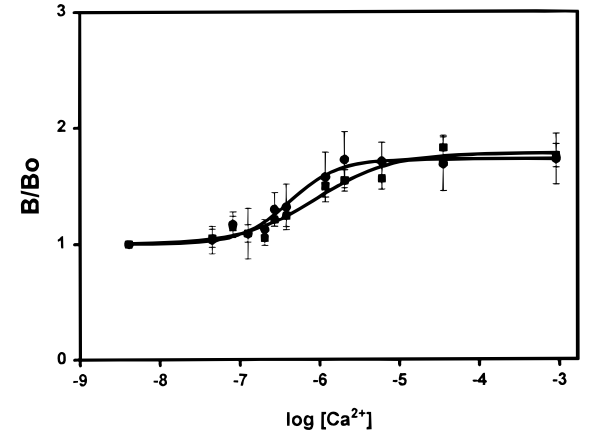

FIGURE 3: Ca^{2+} dependence of [^{35}S]dCaM and [^{35}S]B1234Q CaM binding. SR membranes (10 μg/200 μL) were incubated with 5 nM [^{35}S]dCaM (●) or [^{35}S]B1234Q (■) at the indicated free Ca^{2+} concentrations as described under Materials and Methods. The data were fit with a polynomial in Sigma Plot. Data on the ordinate are plotted as the amount of radioligand bound normalized to the amount bound at the lowest Ca^{2+} concentration (B/B_o).

value is in agreement with the K_D of dCaM obtained from direct binding (34 ± 1 nM, Table 1) and suggests competition for a common or overlapping binding site.

Ca^{2+} Binding to RYR1 Increases RYR1's Affinity for CaM. The affinity of both dCaM and B1234Q for RYR1 is greater under micromolar Ca^{2+} concentrations than at nanomolar Ca^{2+} concentration (Table 1). To further evaluate the binding of dCaM and B1234Q as a function of the Ca^{2+} concentration, we assessed the Ca^{2+} dependence of [^{35}S]dCaM and [^{35}S]B1234Q binding (Figure 3). The amount of both bound [^{35}S]dCaM and [^{35}S]B1234Q increased with increasing Ca^{2+} concentrations. The EC_{50} for the Ca^{2+} enhancement of dCaM binding (0.6 ± 0.2 μM, $n = 3$) appears to be lower than that for the Ca^{2+} enhancement of B1234Q binding (2.0 ± 0.8 μM, $n = 3$), but this did not reach statistical significance. The increase in binding of both [^{35}S]dCaM and [^{35}S]B1234Q at micromolar Ca^{2+} concentrations is due, in most part, to an increase in the affinity of RYR1 for CaM at micromolar

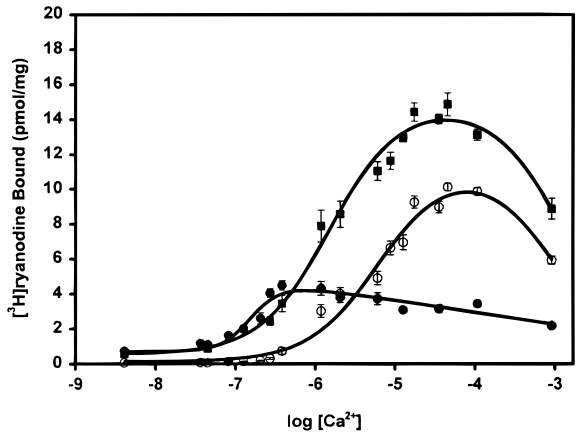

FIGURE 4: Effect of CaM on the Ca^{2+} dependence of [^{3}H]ryanodine binding to SR membranes. SR membranes (20 μg/200 μL) were incubated with [^{3}H]ryanodine (5 nM) at the indicated free Ca^{2+} concentrations in the absence (○) or presence of 1 μM dCaM (●) or 1 μM B1234Q (■) as described under Materials and Methods. The solid lines are derived from fitting the data to eq 3 under Materials and Methods.

Ca^{2+} compared to nanomolar Ca^{2+} (Table 1). Since B1234Q in not thought to bind Ca^{2+} under these conditions, the increase in the affinity of RYR1 for CaM (both apoCaM and Ca^{2+}-CaM) is a result of Ca^{2+} binding to RYR1. We cannot, however, eliminate the possibility that the binding of B1234Q to RYR1 enhances the affinity of one or more of B1234Q's binding sites for Ca^{2+}.

CaM binding to RYR1 increases RYR1's affinity for Ca^{2+}, while Ca^{2+} binding to CaM converts CaM from an activator to an inhibitor. Calmodulin enhances RYR1 activity at nanomolar Ca^{2+} concentrations and inhibits RYR1 activity at micromolar Ca^{2+} concentrations (*4*). [^{3}H]Ryanodine binding is frequently used to screen for effects of agents on RYR1 activity. Agents that increase [^{3}H]ryanodine binding generally activate RYR1, while those that inhibit [^{3}H]ryanodine binding generally inhibit channel activity (*14*−*16*). Because both CaM and RYR1 are Ca^{2+}-binding proteins, we first examined the effect of bound CaM on the Ca^{2+} dependence of [^{3}H]ryanodine binding to RYR1 (Figure 4). The control curve showed the characteristic bell-shaped Ca^{2+}-dependent activation/inhibition curve with constants of 4.9 ± 2.2 and 1130 ± 90.0 μM, respectively ($n = 3$). Bound dCaM shifts both Ca^{2+}-dependent enhancement and inhibition of [^{3}H]ryanodine binding to lower Ca^{2+} concentrations ($K_{act} = 0.21 \pm 0.05$, $K_{inh} = 600 \pm 140$ μM, $n = 3$). Similar results were obtained with mCaM (data not shown). This increase in the apparent affinity of the Ca^{2+}-activating sites of RYR1 for Ca^{2+} contributes to the enhancing effect of dCaM. Since Ca^{2+}-dependent inhibition occurs at much lower Ca^{2+} concentrations, this most likely reflects Ca^{2+} binding to CaM. B1234Q 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+} ($K_{act} = 2.5 \pm 1.2$ μM). These findings suggest that apoCaM (or CaM partially saturated with Ca^{2+}) enhances the Ca^{2+} sensitivity of RYR1 activation and that Ca^{2+} binding to CaM (CaM fully saturated with Ca^{2+}) converts CaM from a partial agonist to an inhibitor of RYR1.

Effect of dCaM and B1234Q on RYR1 Single-Channel Activity. CaM has been previously shown to decrease single-channel activity of RYR at micromolar Ca^{2+} concentrations (*4*). Consistent with these findings, dCaM (1 μM) decreased the steady-state mean open probability (P_o) of the channel from 0.19 ± 0.06 to 0.02 ± 0.01 ($p = 0.02$, $n = 7$) at 50 μM free Ca^{2+} concentration (Figure 5A). B1234Q (1 μM), however, increased the P_o of RYR1 from 0.10 ± 0.03 to 0.20 ± 0.04 ($p = 0.02$, $n = 6$) (Figure 5B). These results demonstrate that B1234Q is an activator of the Ca^{2+}-activated RYR1.

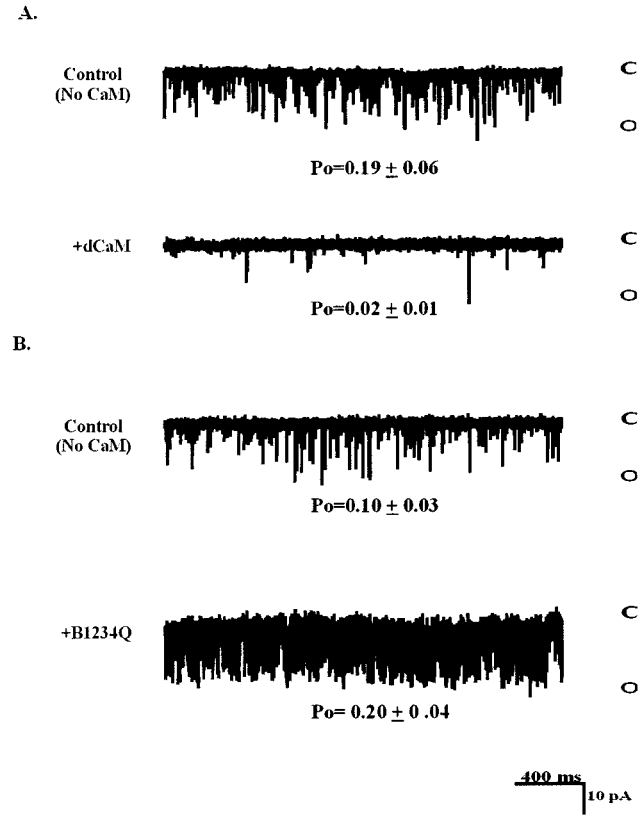

FIGURE 5: B1234Q increases single-channel activity of RYR1 reconstituted into planar lipid bilayers at 50 μM free Ca^{2+} concentration. Single-channel recordings of RYR1 reconstituted into planar lipid bilayers at 50 μM free Ca^{2+} concentration are shown (O and C reflect the open and closed state of the channel, respectively). (A) Inhibition of RYR1 by 1 μM dCaM added to the cis chamber. (B) Activation of RYR1 by 1 μM B1234Q added to the cis chamber. Data were analyzed for statistical significance by the paired *t*-test.

DISCUSSION

In vitro, CaM enhances RYR1 activity at nanomolar Ca^{2+} concentrations and inhibits RYR1 activity at micromolar Ca^{2+} concentrations (*4*). The Ca^{2+} dependence and bifunctional nature of the effects of CaM on RYR1 suggest that CaM may contribute to the regulation of both Ca^{2+}-dependent opening and closing of the channel. The binding of a Ca^{2+}-regulated modulator (CaM) to a channel that is itself regulated by Ca^{2+} raises the question of whether the Ca^{2+} dependence of CaM's functional effects on RYR1 are due to Ca^{2+} binding to CaM, Ca^{2+} binding to RYR1, or Ca^{2+} binding to both.

To answer this question we made use of a mutant *Drosophila* CaM that cannot bind Ca^{2+} under the conditions used in these studies (B1234Q). *Drosophila* CaM differs from mammalian CaM by three conservative amino acid

changes (Y99F, Q143T, and A147S, mCaM to dCaM). Despite these changes, dCaM binds to and exerts effects similar to those of mCaM on RYR1 activity at nanomolar and micromolar Ca^{2+} concentrations. Therefore, dCaM was used as a tool to assess the interactions between CaM and RYR1.

The binding of Ca^{2+} to RYR1 increased RYR1's affinity for both B1234Q CaM and Ca^{2+}-CaM (Table 1 and Figure 3). This increase in affinity at micromolar Ca^{2+} concentrations is greater for B1234Q than for dCaM. These differences could reflect the alteration in primary sequence (E → Q mutation) in B1234Q. Alternatively, the difference could arise from the ability of dCaM, when complexed to RYR1, to bind Ca^{2+} at one or more sites at these nanomolar Ca^{2+} concentrations. In other words, dCaM may not truly be in the apoCaM form at nanomolar Ca^{2+} concentrations while B1234Q is Ca^{2+}-free. We are currently investigating whether the Ca^{2+} affinity of CaM is enhanced when bound to its RYR1 binding site.

The presence of dCaM decreased the concentration of Ca^{2+} required for both activation and inhibition of RYR1 (Figure 4). B1234Q decreased the concentration of Ca^{2+} required for activation of RYR1 (Figure 4). Interpretation of the decrease in the Ca^{2+} concentration required to inhibit RYR1 in the presence of dCaM is complicated by the fact that CaM itself binds Ca^{2+}, and Ca^{2+}-bound CaM is also an inhibitor of RYR1. Since B1234Q remains an activator of both the Ca^{2+}-free and Ca^{2+}-bound RYR1, the inhibition of RYR1 by CaM is most likely due to Ca^{2+} binding to CaM, thereby overriding direct Ca^{2+} activation of RYR1.

The ability of Ca^{2+} to convert CaM from an activator to an inhibitor can be explained in at least two ways. Ca^{2+}-CaM and apoCaM could bind to separate sites on RYR1, and the resulting functional effect would be dependent on which site CaM occupies. Alternatively, CaM could be bound to the same region on RYR1 at all Ca^{2+} concentrations, and the conformational change in CaM upon binding Ca^{2+} and the subsequent altered interactions with RYR1 could induce a conformational change in RYR1. Our data support the later model. Scatchard plots of [^{35}S]CaM binding at nanomolar and micromolar [Ca^{2+}] are linear (Figure 1), indicating a single class of binding sites. Both unlabeled dCaM and B1234Q completely inhibit the binding of [^{35}S]B1234Q at micromolar Ca^{2+} concentrations, suggesting competition for a common binding region on RYR1. Direct binding studies with [^{35}S]dCaM and [^{35}S]B1234Q show that they bind to the same number of sites at nanomolar and micromolar Ca^{2+} concentrations. In addition, both apoCaM and Ca^{2+}-CaM can protect sites at amino acids 3630 and 3637 from trypsin cleavage (*5*), suggesting that the binding sites for both apoCaM and Ca^{2+}-CaM overlap these amino acids. However, alkylation of cysteines of RYR1 with *N*-ethylmaleimide selectively destroys apoCaM binding but not Ca^{2+}-CaM binding (*17*), demonstrating that some features of the apoCaM binding site are different than those of the Ca^{2+}-CaM binding site. Our data suggest that the bifunctional effects of CaM on RYR1 (activation under nanomolar Ca^{2+} concentrations and inhibition under micromolar Ca^{2+} concentrations) are due to both effects on the affinity of the activating sites on RYR1 for Ca^{2+} and effects on the activity of the channel due to Ca^{2+} binding to CaM. The binding of Ca^{2+} to RYR1 increases the affinity of RYR1 for CaM.

Although our data suggest that the apoCaM and Ca^{2+}-CaM binding sites on RYR1 are overlapping in the primary sequence, structural studies by Wagenknecht and colleagues (*18*) have indicated that the apoCaM and Ca^{2+}-CaM binding sites are separated by about 30 Å in the 3D structure of RYR1. However, the low resolution of this study (>30 Å) makes precise localization of CaM binding sites very difficult. In addition, the binding of both Ca^{2+} and CaM to RYR1 alter its functional state and are, therefore, likely to change the 3D structure. In fact, Serysheva et al. (*19*) have shown changes in the conformation of RYR1 upon binding of Ca^{2+}. One explanation of this apparent discrepancy is that the binding of both Ca^{2+} and CaM cause the movement of the CaM-binding sites due to global conformational transitions in the channel structure. This results in a difference in the 3D localization of bound apoCaM versus Ca^{2+}-CaM, but the actual binding sites would represent the same region of the primary sequence.

Our ability to evaluate the role of CaM in E−C coupling is limited by the lack of knowledge of the in vivo situation. Thus far, all of the CaM interaction studies have been performed on RYR1 in vitro, in the absence of the DHPR. It is not known whether coupling of RYR1 to the DHPR alters RYR1's ability to bind CaM. The cardiac DHPR is also regulated by CaM in vitro (*20*−*22*) and our preliminary data suggest that the skeletal DHPR also binds CaM (data not shown). It is not known whether coupling to RYR1 alters the interaction of CaM with the DHPR. It is conceivable that only uncoupled RYR1s and DHPRs are regulated by CaM. The interaction of CaM with the coupled and uncoupled channels must be evaluated before we can adequately assess the role of CaM in E−C coupling.

In summary, our data are consistent with a model in which at nanomolar Ca^{2+} concentrations, Ca^{2+}-free CaM sensitizes RYR1 to Ca^{2+}. Upon an increase in cytosolic Ca^{2+}, CaM, bound to RYR1, binds Ca^{2+} and undergoes a conformational change that inhibits the activity of RYR1.

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