Calmodulin Regulation and Identification of Calmodulin Binding Region of Type-3 Ryanodine Receptor Calcium Release Channel†

Naohiro Yamaguchi,‡ Le Xu,‡ Daniel A. Pasek,‡ Kelly E. Evans,‡ S. R. Wayne Chen,§ and Gerhard Meissner*,‡

Departments of Biochemistry and Biophysics, and Cell and Molecular Physiology, University of North Carolina, Chapel Hill, North Carolina 27599-7260, and Cardiovascular Research Group, Departments of Physiology and Biophysics, and Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta T2N 4N1, Canada

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ABSTRACT: Ryanodine receptors (RyRs) are a family of intracellular Ca^{2+} channels that are regulated by calmodulin (CaM). At low Ca^{2+} concentrations (<1 μM), CaM activates RyR1 and RyR3 and inhibits RyR2. At elevated Ca^{2+} concentrations (>1 μM), CaM inhibits all three RyR isoforms. Here we report that the regulation of recombinant RyR3 by CaM is sensitive to redox regulation. RyR3 in the presence of reduced glutathione binds CaM with 10−15-fold higher affinity, at low and high Ca^{2+} concentrations, compared to in the presence of oxidized glutathione. However, compared to RyR1 assayed at low Ca^{2+} concentrations under both reducing and oxidizing conditions, CaM binds RyR3 with reduced affinity but activates RyR3 to a greater extent. Under reducing conditions, RyR1 and RyR3 activities are inhibited with a similar affinity at $[Ca^{2+}] > 1$ μM. Mutagenesis studies demonstrate that RyR3 contains a single conserved CaM binding site. Corresponding amino acid substitutions in the CaM binding site differentially affect CaM binding and CaM regulation of RyR3 and those of the two other isoforms. The results support the suggestion that other isoform dependent regions have a major role in the regulation of RyRs by CaM [Yamaguchi et al. (2004) *J. Biol. Chem. 279,* 36433−36439].

Mammalian tissues express three closely related Ca^{2+} release channels/ryanodine receptors (RyRs)[1] encoded by separate genes (*1*−*3*). RyR1 is the dominant isoform in skeletal muscle. RyR2 is present in high levels in cardiac muscle. RyR3 was initially identified in brain but is expressed in many tissues including skeletal muscle (*4*). The RyR ion channels release Ca^{2+} ions from an intracellular membrane compartment, the endo/sarcoplasmic reticulum, to regulate muscle contraction and many other cellular activities. RyR ion channels are composed of four RyR 560 kDa peptide subunits, four small 12 kDa FK506 binding proteins (FKBP), and various associated proteins for a total molecular weight >2500 kDa. Numerous endogenous effectors ranging from divalent cations (Ca^{2+} and Mg^{2+}) to proteins such as calmodulin regulate RyR activities and cellular functions (*1*−*3*).

The ubiquitous cytoplasmic calcium binding protein calmodulin (CaM) regulates ion channels including the RyRs by direct binding (*5*, *6*). At Ca^{2+} concentrations < 1 μM, CaM activates RyR1 and inhibits RyR2, whereas CaM inhibits both isoforms at Ca^{2+} concentrations > 1 μM. CaM regulates RyR1 and RyR2 by binding to a single highly conserved CaM binding domain (*7*−*9*). CaM regulation of RyR3 is not as well understood because no tissue expresses RyR3 selectively in substantial amounts. Single channel measurements with the recombinant RyR3 (*10*) and studies with RyR1 knock-out myotubes (*11*) showed that RyR3 is activated by CaM at low Ca^{2+} concentrations and inhibited at elevated Ca^{2+} concentrations. However, neither the affinity of CaM binding nor the location of the CaM binding site(s) was determined.

The present study was undertaken to determine the site(s) and properties of CaM binding of RyR3 using recombinant channels transiently expressed in human embryonic kidney 293 (HEK 293) cells. The results show that RyR3 shares a single conserved CaM binding site with RyR1 and RyR2 but that its regulation by CaM differs in several respects from that of the two other isoforms.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Ryanodine was obtained from Perkin-Elmer Lifesciences (Boston, MA), Tran^{35}S-label was from MP Biomedicals (Irvine, CA), unlabeled ryanodine was from Calbiochem (La Jolla, CA), complete protease inhibitors and FuGENE6 were from Roche (Indianapolis, IN), and HEK 293 cells were from ATCC. Unlabeled CaM and [^{35}S]CaM were prepared as described (*12*).

Construction of RyR3 cDNAs. The full-length rabbit RyR3 cDNA (*10*) subcloned into expression vector pcDNA3 was used to construct RyR3 mutants. SmaI/SpeI (9266−10576) fragment of RyR3 subcloned into pBluescript KS vector

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* Address correspondence to Gerhard Meissner, Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599-7260. Tel: 919-966-5021. Fax: 919-966-2852. E-mail: meissner@med.unc.edu.

‡ University of North Carolina, Chapel Hill, North Carolina 27599-7260.

§ University of Calgary.

[1] Abbreviations: RyR, ryanodine receptor; RyR1, skeletal muscle RyR; RyR2, cardiac muscle RyR; RyR3, brain RyR; CaM, calmodulin; HEK, human embryonic kidney; CaMBD, CaM binding domain; SR, sarcoplasmic reticulum.

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served as template for mutagenesis. A SmaI site was created as described (*10*). Mutations were introduced by Pfu-turbo polymerase-based chain reaction, using mutagenic oligonucleotides and QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The complete mutated DNA fragments amplified by PCR were confirmed by DNA sequencing. Mutant SmaI/SpeI fragment was cloned back to the original position of a vector containing BamHI/PinAI (6954−11934) fragment of RyR3, and then to full-length RyR3 in pcDNA3, to construct the full-length RyR3 mutant expression plasmids. Nucleotide numbering is according to ref *13* with modifications as described (*10*).

Expression of RyR3s in HEK 293 Cells. RyR cDNAs were transiently expressed in HEK 293 cells with FuGENE6 according to the manufacturer's instruction (*8*). Cells were harvested 48 h after transfection and crude membrane fractions were prepared as described (*8*).

Skeletal and Cardiac Muscle SR Vesicle Preparations. Heavy sarcoplasmic reticulum (SR) vesicles were isolated from rabbit hind limb and back muscle in the presence of protease inhibitors (*14*). Cardiac SR vesicles were isolated from canine heart in the presence of protease inhibitors (*15*). Endogenous CaM was removed by treating membranes with 1 μM of myosin light chain kinase derived calmodulin binding peptide (*12*).

[^{35}S]Calmodulin Binding. Membrane fractions of HEK 293 cells were incubated for 2.5 h at 24 °C with 5−200 nM [^{35}S]-CaM in 20 mM imidazole, pH 7.0, 0.15 M sucrose, 150 mM KCl, 0.125 mg/mL BSA, 5 mM glutathione (reduced), 20 μM leupeptin, 200 μM Pefabloc, and either 5 mM EGTA, 0.4 μM free Ca^{2+}, or 100 μM free Ca^{2+}. Aliquots were taken for determination of total radioactivity and centrifuged for 30 min at 90000*g* in a Beckman Airfuge to obtain bound [^{35}S]CaM. Radioactivities were determined by scintillation counting. Nonspecific binding of [^{35}S]CaM was determined by incubating equal amounts of protein derived from vector-transfected HEK 293 cells. In parallel experiments, B_{max} values of [^{3}H]ryanodine binding were determined by incubating membranes for 4 h at 24 °C with a saturating concentration of [^{3}H]ryanodine (40 nM) in 20 mM imidazole, pH 7.0, 0.6 M KCl, 0.15 M sucrose, 20 μM leupeptin, 200 μM Pefabloc, and 100 μM Ca^{2+}. Specific [^{3}H]ryanodine binding was determined as described below.

[^{3}H]Ryanodine Binding. Ryanodine binds with high specificity to RyRs and is widely used as a probe of channel activity because of its preferential binding to the open channel state (*2*, *16*). Unless otherwise indicated, membranes were incubated with 2.5 nM [^{3}H]ryanodine in 20 mM imidazole (pH 7.0), 0.15 M sucrose, 250 mM KCl, protease inhibitors, and indicated glutathione, Ca^{2+} and CaM concentrations. Nonspecific binding was determined using a 1000−2000-fold excess of unlabeled ryanodine. After 20 h at 24 °C, samples were diluted with 8 volumes of ice-cold water and placed on Whatman GF/B filters preincubated with 2% polyethyleneimine in water. Filters were washed with three 5-mL aliquots of ice-cold 100 mM KCl, 1 mM KPipes (pH 7.0) solution. The radioactivity remaining with the filters was determined by liquid scintillation counting to obtain bound [^{3}H]ryanodine.

Dose-dependent curves of CaM inhibition and activation of RyRs were fit by the following two Hill equations.

$$B = 100 + B_a(1/(1 + (K_a/\mathrm{CaM})^{n_a})) \quad (1)$$

$$B = 100 - B_i(1/(1 + (K_i/\mathrm{CaM})^{n_i})) \quad (2)$$

where B_i and B_a are maximal inhibition and activation values, K_i and K_a are inhibition and activation Hill constants, and n_a and n_i are the activation and inhibition Hill coefficients, respectively.

Single Channel Recordings. Single channel measurements were performed in planar lipid bilayers as described (*17*). Crude membrane fractions expressing WT and mutant RyRs were pretreated for 30 min with 1 μM myosin light chain kinase-derived CaM binding peptide to dissociate endogenous CaM. Final peptide concentration was 10 nM following the addition of membranes to the cis (cytosolic) chamber of the bilayer apparatus. A strong dependence of single channel activities on cis Ca^{2+} concentration indicated that the large cytosolic "foot" region faced the cis chamber of the bilayers. The trans (lumenal) side of the bilayer was defined as ground. Measurements were made in symmetrical 0.25 M KCl, 20 mM KHepes, pH 7.4, 0.5 mM EGTA and Ca^{2+} concentrations to yield the indicated concentrations of free Ca^{2+}. Exogenous CaM was added to the cis solution. Electrical signals were filtered at 2 kHz, digitized at 10 kHz, and analyzed as described (*17*). Channel open probabilities (P_o) in multichannel recordings were calculated using the equation $P_o = \sum iP_{o,i}/N$, where N is the total number of channels and $P_{o,i}$ is channel open probability of the ith channel.

Biochemical Assays and Data Analysis. Free Ca^{2+} concentrations were obtained by including in the solutions the appropriate amounts of Ca^{2+} and EGTA as determined using the stability constants and computer program of Shoenmakers et al. (*18*). Free Ca^{2+} concentrations $\geq$ 1 μM were verified using a Ca^{2+} selective electrode.

Results are given as means ± SE. Significances of differences in data ($p < 0.05$) were determined using Student's t test.

RESULTS

RyR3 is expressed in a variety of cells (*4*); however, no mammalian tissue expresses RyR3 as the dominant isoform. To investigate the mechanism of CaM regulation of RyR3, we expressed wild-type and mutant RyR3s in HEK 293 cells, which express endogenous RyRs at background levels. Wild-type and mutant RyR3s were expressed at a comparable level, with B_{max} values of [^{3}H]ryanodine binding ranging from 0.5 to 1.2 pmol/mg protein (not shown).

Calmodulin Activation and Inhibition of RyR3. In agreement with previous single channel measurements (*10*), RyR3 was activated by CaM at [Ca^{2+}] < 1 μM and inhibited at [Ca^{2+}] > 1 μM. Figure 1A shows representative single channel recordings in which the effects of RyR3 were examined at 0.4 and 2 μM Ca^{2+} with K^+ ions as charge carrier in 250 mM KCl media on both sides of the lipid bilayer. To establish well-defined cytoplasmic Ca^{2+} concentrations, K^+ rather than Ca^{2+} was used as the current carrier (*19*). At 0.4 μM Ca^{2+}, addition of 50 nM and 1 μM CaM to the cis chamber of the bilayer apparatus increased averaged channel open probability (P_o) approximately 3- and 7-fold, respectively (Table 1). Kinetic analysis showed that CaM

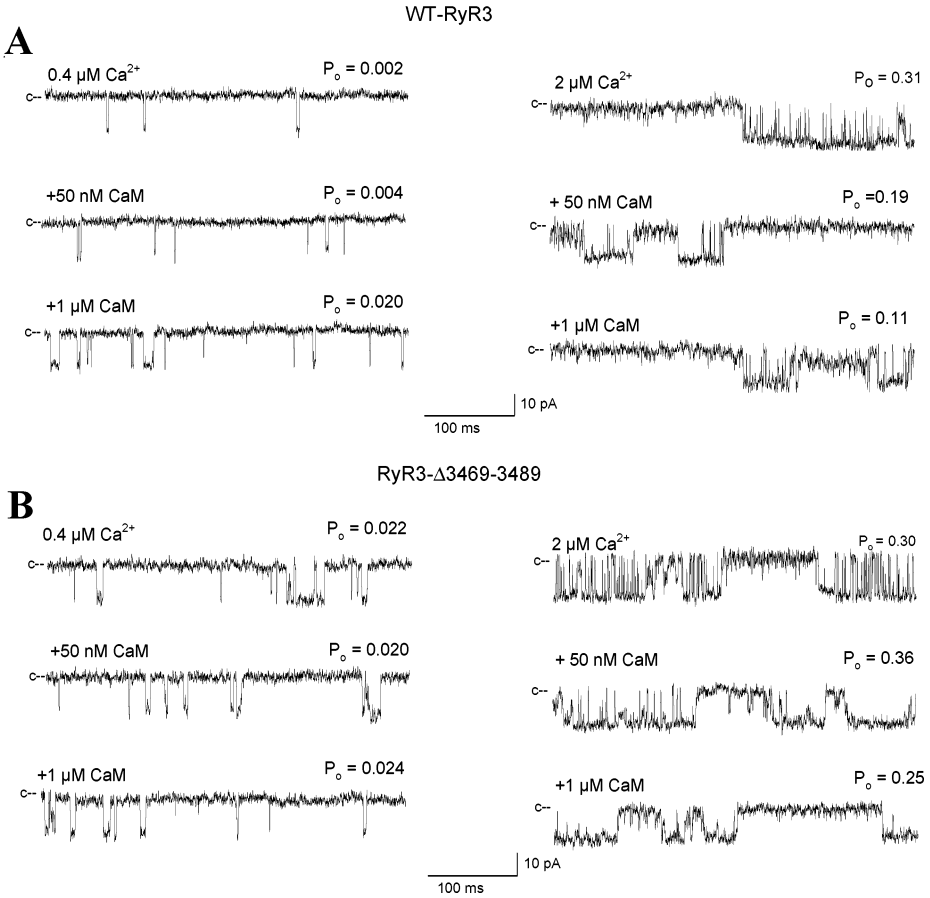

FIGURE 1: Effects of CaM on WT-RyR3 and RyR3-Δ3469−3489 single channel activities. Membrane fractions prepared from HEK293 cells expressing WT-RyR3 (A) or RyR3-Δ3469−3489 (B) were fused with a lipid bilayer. Representative single channel recordings are shown. Single channel currents were recorded at −20 mV (downward deflections from closed level, c, in symmetric 0.25 M KCl, 20 mM KHepes, pH 7.4 media with 0.4 μM Ca^{2+} (left panels) or 2 μM Ca^{2+} (right panels) before (top traces) and after the addition of 50 nM CaM (middle traces) and 1 μM CaM (bottom traces). Averaged control (−CaM) P_o values of WT-RyR3 and RyR3-Δ3469−3489 were 0.003 ± 0.001 (n = 4) and 0.013 ± 0.004 (n = 5) at 0.4 μM Ca^{2+}, and 0.36 ± 0.02 (n = 4) and 0.15 ± 0.05 (n = 8) at 2 μM Ca^{2+}, respectively. Kinetic parameters of single channel recordings of WT-RyR3 are summarized in Table 1.

Table 1: Effect of CaM on Single Channel Parameters of WT-RyR3

assay condition	P_o	events/min	T_o (ms)	T_c (ms)
0.4 μM Ca^{2+}	100	100	100	100
+50 nM CaM	323 ± 106	194 ± 48	142 ± 39	32 ± 1[a]
+1 μM CaM	681 ± 55[a]	630 ± 91[a]	123 ± 25	22± 10[a]
2 μM Ca^{2+}	100	100	100	100
+50 nM CaM	54 ± 9[a]	165 ± 68	41 ± 10[a]	93± 31
+1 μM CaM	44 ± 11[a]	149 ± 30	34 ± 10[a]	101± 33

Average control parameters (in the absence of CaM) at 0.4 μM Ca^{2+} where P_o = 0.0023 ± 0.0012, events/min = 148 ± 87, T_o = 0.83 ± 0.01 ms, T_c = 1431 ± 704 ms; at 2 μM Ca^{2+}: P_o = 0.36 ± 0.02, events/min = 4753 ± 619, T_o = 4.5 ± 0.8 ms, T_c = 9.9 ± 1.6 ms. Data are the mean ± SE of 3−4 experiments. [a] $p < 0.05$ compared to control.

increased P_o by raising the number of single channel events without significantly changing the mean open times (Table 1). At 2 μM Ca^{2+}, addition of 50 nM and 1 μM CaM reduced P_o about 2-fold (Figure 1A). CaM reduced P_o by decreasing the mean open time without affecting the number of events (Table 1). The results indicate that CaM activated the RyR3 ion channel at [Ca^{2+}] < 1 μM by increasing the transition rates from the closed to open states without significantly affecting the lifetimes of channel openings, whereas CaM inhibited the channel at elevated Ca^{2+} concentrations by shortening the lifetimes of channel openings. It should be noted that in previous single channel measurements, CaM-dependent changes in RyR3 activity were the result of concomitant changes in both the open and the closed times. The variations in the kinetics of channel opening and closing between the previous and the present study may have been due to performing the single channel measurements using detergent-solubilized RyR3 (*10*) and detergent-free membrane preparations (this study), respectively.

To further delineate the CaM dependence of RyR3 activity, [^{3}H]ryanodine binding experiments were performed. Since CaM regulation of RyR1 and RyR2 depends on glutathione redox potential (*6*), redox dependence of RyR3 regulation by CaM was determined in the presence of 5 mM reduced (GSH) or oxidized (GSSG) glutathione. In the absence of CaM, the level of [^{3}H]ryanodine binding to RyR3 was 1.5−2-fold greater under oxidizing than reducing conditions in the presence of both 0.4 μM Ca^{2+} and 25 μM Ca^{2+} (not shown). Figure 2 shows that CaM activated and inhibited RyR3 in a concentration-dependent manner. In the presence of 5 mM GSH, RyR3 was activated 6-fold by 1 μM CaM at 0.4 μM Ca^{2+} and inhibited 2-fold by 1 μM CaM at 25 μM Ca^{2+}, in good agreement with the single channel measurements.

The kinetic parameters of CaM activation and inhibition of RyR3 were determined by the two equations given in Experimental Procedures. These data are summarized in

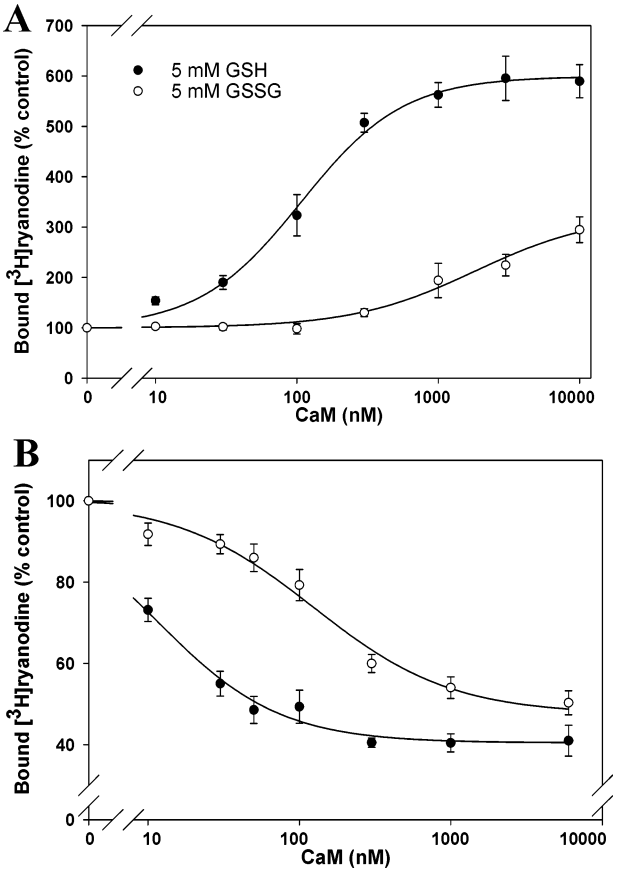

FIGURE 2: CaM activation and inhibition of [^{3}H]ryanodine binding to WT-RyR3. Specific [^{3}H]ryanodine binding to WT-RyR3 was determined as described under Experimental Procedures at 0.4 μM Ca^{2+} and 1.3 mM AMP−PCP (A) or 25 μM Ca^{2+} (B) and the indicated concentrations of CaM in the presence of 5 mM reduced glutathione (GSH, filled circles) or 5 mM oxidized glutathione (GSSG, open circles). Small amounts of endogenous CaM were removed by incubating membranes for 30 min at 24 °C with 1 μM myosin light chain kinase-derived calmodulin binding peptide in 100 μM Ca^{2+} followed by centrifugation through 15% sucrose to remove complexed and free peptide (*12*). Normalized [^{3}H]ryanodine binding data are the mean ± SE of 4−7 experiments. Data were fitted by eqs 1 and 2 in Experimental Procedures.

Table 2. Under reducing conditions, the Hill constants of CaM activation and inhibition were 107 ± 16 nM at 0.4 μM Ca^{2+} and 12 ± 2 nM at 25 μM Ca^{2+}, respectively. Hill coefficients of 1.2 and 1.1 indicated that CaM regulated the tetrameric RyR3 in a noncooperative manner. In the presence of 5 mM GSSG, the Hill constant and coefficient of CaM activation were 1828 ± 1218 nM and 1.0, and those of CaM inhibition were 125 ± 33 nM and 1.0 at 25 μM Ca^{2+}. The results show that CaM activates RyR3 with a lower affinity than it inhibits the receptor. They further indicate that CaM activates and inhibits RyR3 with an apparent higher affinity under reducing than oxidizing conditions.

Parallel experiments with the skeletal muscle isoform using skeletal muscle SR membranes showed major differences in the regulation by CaM of RyR1 and RyR3. At 0.4 μM Ca^{2+}, CaM activated RyR1 with a more than 5-fold higher affinity both under reducing and oxidizing conditions, whereas the magnitude of activation was approximately 2-fold higher for RyR3 than RyR1 (Table 2). A similar difference in activation by CaM was also observed for the two recombinant channels in single channel measurements (*9*) (Table 1). At 25 μM Ca^{2+}, CaM inhibited RyR1 and RyR3 with a similar affinity and extent under reducing conditions, whereas a 5-fold difference in affinity was observed under oxidizing conditions. In agreement with the present study, Fruen et al. presented evidence for a more pronounced activation by CaM of RyR3 than RyR1 (*20*).

Regulation of RyR2 by CaM was examined using cardiac SR vesicles. At 0.4 μM Ca^{2+}, CaM inhibited RyR2 with Hill constants of 52 ± 7 nM and 161 ± 69 nM under reducing and oxidizing conditions, respectively (Table 2). The extent of inhibition was 66 ± 3% and 29 ± 4%, respectively. At 25 μM Ca^{2+}, CaM was less effective in inhibiting RyR2, resulting in approximately 15% inhibition of [^{3}H]ryanodine binding.

[^{35}S]Calmodulin binding to RyR3. The stoichiometry of CaM binding to the recombinant RyR3 was determined in the presence of 5 mM reduced glutathione at three Ca^{2+} concentrations, <10 nM Ca^{2+} that results in binding of Ca^{2+}-free CaM, 0.4 μM free Ca^{2+} that results in activation of RyR3 (Figure 1), and 100 μM Ca^{2+} that results in binding of Ca^{2+}-bound CaM and inhibition of RyR3. Wild-type RyR3 bound [^{35}S]CaM in a concentration-dependent manner (Figure 3). The receptor bound 2.7 ± 0.6 [^{35}S]CaM at <10 nM Ca^{2+} and 200 nM CaM per high-affinity [^{3}H]ryanodine binding site. This corresponds to 0.7 CaM bound per RyR subunit,

Table 2: Regulation by CaM of RyR Isoforms under Reducing and Oxidizing Conditions

	with 0.4 μM Ca^{2+}			with 25 μM Ca^{2+}		
	K_a (nM)	n_a	% activation	K_i (nM)	n_i	% inhibition
			RyR3			
+GSH	107 ± 16	1.2 ± 0.2	599 ± 20	12 ± 2	1.1 ± 0.2	60 ± 2
+GSSG	1828 ± 1218	1.0± 0.4	325 ± 54	125 ± 33	1.0 ± 0.2	52 ± 4
			RyR1			
+GSH	15 ± 2	1.7 ± 0.4	235 ± 5	11 ± 2	1.7 ± 0.4	78 ± 4
+GSSG	308 ± 240	1.1 ± 0.6	183 ± 23	25 ± 3	1.7 ± 0.3	64 ± 3
	K_i (nM)	n_i	% inhibition	K_i (nM)	n_i	% inhibition
			RyR2			
+GSH	52 ± 7	0.9 ± 0.1	66 ± 3	0.6 ± 0.6	0.8 ± 0.4	13 ± 1
+GSSG	161 ± 69	1.1 ± 0.3	29 ± 4	ND	ND	18 ± 13

CaM activation and inhibition of [^{3}H]ryanodine binding to RyRs was determined as in Figure 2. Data were obtained using eqs 1 and 2 in Experimental Procedures. K_a and K_i are activation and inhibition Hill constants, n_a and n_i are Hill coefficients, and % activation and inhibition are the maximal changes in [^{3}H]ryanodine binding compared to the controls (−CaM), respectively. ND, not determined. Data are the mean ± SE of 4−7 experiments.

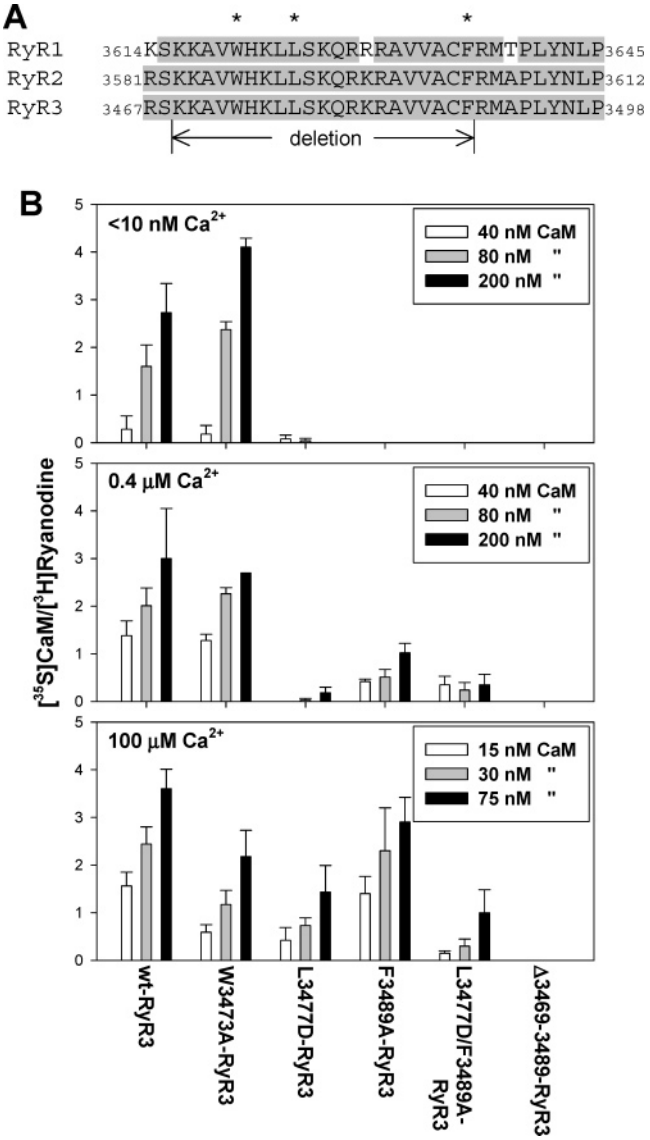

FIGURE 3: Sequence alignment of CaM binding domains and [^{35}S]-CaM binding to WT and mutant RyR3s. (A) Rabbit sequences of CaM binding domains of RyR1, RyR2, and RyR3. Indicated are amino acid segments that were deleted in RyR2 (*9*) and RyR3 (this study) and amino acid residues that were mutated in two previous (*8*, *9*) and this study. Sequences are from refs *13*, *37*, and *38*. (B) Membrane fractions prepared from HEK293 cells expressing WT and mutant RyR3s were incubated for 2.5 h at room temperature with indicated concentrations of [^{35}S]CaM in the presence of <10 nM Ca^{2+}, 0.4 μM Ca^{2+}, and 100 μM free Ca^{2+}. The ratios of [^{35}S]-CaM binding to maximal [^{3}H]ryanodine binding were obtained, taking into account that there is one high-affinity [^{3}H]ryanodine binding site per RyR3 tetramer. Data are the mean ± SE of 3−5 experiments.

as the tetrameric RyRs have only one high-affinity [^{3}H]-ryanodine binding site. The average number of bound [^{35}S]-CaM per RyR3 subunit was 0.75 at 0.4 μM Ca^{2+} and 200 nM CaM, and 0.9 at 100 μM Ca^{2+} and 75 nM CaM. The results indicate that each RyR3 subunit bound with nanomolar affinity one CaM at Ca^{2+} concentrations that activate and inhibit the receptor.

Identification of RyR3 CaM Binding Site. RyR1 and RyR2 have a single high-affinity CaM binding site that is shared by the Ca^{2+}-bound and Ca^{2+}-free forms of CaM (*8*, *9*). Figure 3A shows that RyR3 contains a 32 amino acid segment identical to RyR2 and similar to RyR1. Deletion of 21 amino acid residues from RyR3 (Δ3469−3489) reduced [^{35}S]CaM binding to background levels both at Ca^{2+} concentrations below and above 1 μM (Figure 3B). RyR3-Δ3469−3489 bound [^{3}H]ryanodine and showed a Ca^{2+}-dependence of [^{3}H]-ryanodine binding comparable to wild-type (not shown), which suggests that the deletion did not induce major changes in protein conformation. The results indicate that RyR3 has a single CaM binding domain that is conserved among the mammalian RyRs and is shared by the Ca^{2+}-free and Ca^{2+}-bound forms of CaM.

Next, we tested the effects of single and double amino acid substitutions in the CaM binding domain of RyR3, focusing on three residues that eliminated high affinity CaM binding and/or CaM regulation in RyR1 and RyR2 (*8*, *9*). We constructed three single-site RyR3 mutants (RyR3-W3473A, -L3477D, -F3489A) and one mutant with two amino acid substitutions (RyR3-L3477D/F3489A) (Figure 3A, asterisks). RyR3-W3473A bound [^{35}S]CaM at all three Ca^{2+} concentrations. RyR3-L3477D and -F3489A retained CaM binding at 100 μM Ca^{2+} but lost or showed a reduced ability to bind CaM at <10 nM and 0.4 μM Ca^{2+}. CaM binding to RyR3-L3477D/F3489A was greatly decreased at 100 μM Ca^{2+} and was reduced to background levels at <10 nM and 0.4 μM. The four mutants showed a Ca^{2+}-dependence of [^{3}H]ryanodine binding essentially identical to WT-RyR3 (not shown).

Calmodulin Activation and Inhibition of Mutant RyR3s. Single channel traces of Figure 1B show that deletion of 21 amino acid residues in RyR3 (RyR3-Δ3469−3489) resulted in loss of CaM modulation of RyR3 activity at [Ca^{2+}] below and above 1 μM. At 0.4 μM Ca^{2+}, the average open probabilities were 130 ± 54% and 146 ± 50% of control (−CaM) in the presence of 50 nM and 1 μM CaM, respectively ($n = 5$). At 2 μM Ca^{2+}, averaged P_o values were 171 ± 40% (50 nM CaM) and 143 ± 26% (1 μM CaM) of control ($n = 8$). None of the data were significantly different from the control.

CaM regulation of RyR3 mutant activities was assessed at 0.4 μM Ca^{2+} in the presence of 1.3 mM AMPPCP, a non-hydrolyzing ATP analogue, and at 25 μM Ca^{2+} in [^{3}H]-ryanodine binding measurements (Figure 4). In agreement with single channel measurements (Figure 1B), RyR3-Δ3469−3489 was neither activated by 1 μM CaM at 0.4 μM Ca^{2+} nor inhibited by 1 μM CaM at 25 μM Ca^{2+}. CaM activated WT-RyR3 and RyR3-W3473A at 0.4 μM Ca^{2+} but was without effect on the remaining four mutants at a CaM concentration as high as 1 μM. At 25 μM Ca^{2+}, RyR3-W3473A, and -L3477D, and -F3489A were inhibited by 1 μM but only modestly by 50 nM CaM. The results suggest a reduced ability of the mutants to transduce CaM binding into a functional effect. A small but significant inhibition by 1 μM CaM was observed for RyR3-L3477D/F3489A.

DISCUSSION

The present study provides new insights into the regulation by calmodulin of the type-3 ryanodine receptor (Ca^{2+} release channel) (RyR3). Our work shows that regulation of RyR3 by calmodulin differs in several respects from that of type-1 and type-2 RyR isoforms and thus provides cells with regulatory mechanisms to differentially control intracellular Ca^{2+} fluxes. While all three isoforms are inhibited by CaM

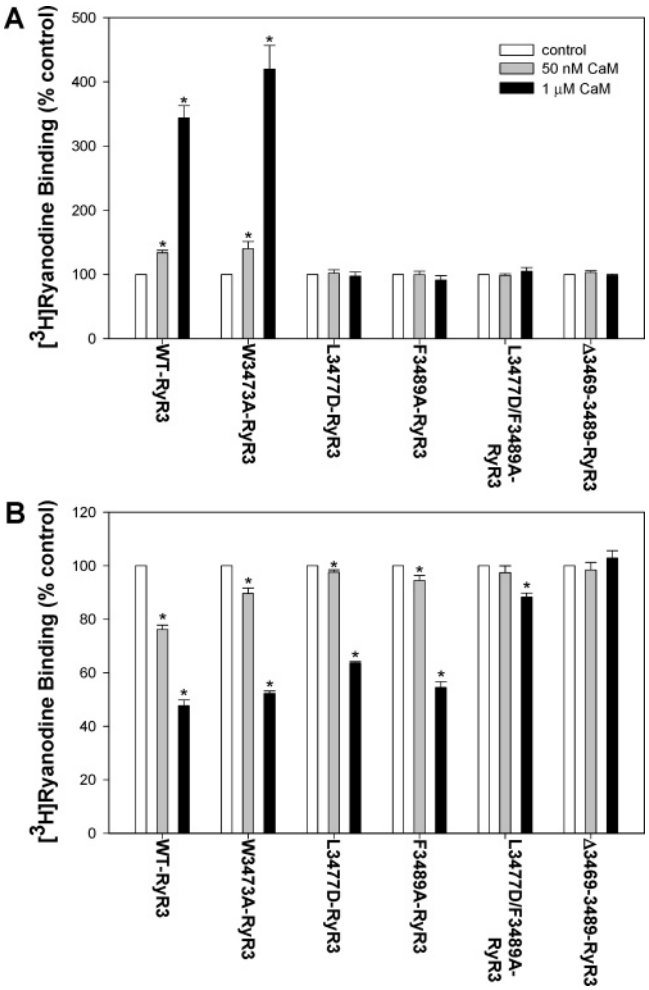

FIGURE 4: CaM activation and inhibition of WT and mutant RyR3s. Specific [^{3}H]ryanodine binding was determined in the presence of 0.2 mM GSSG at 0.4 μM Ca^{2+} and 1.3 mM AMP−PCP (A) or 25 μM Ca^{2+} (B) and the indicated concentrations of CaM. Normalized [^{3}H]ryanodine binding data are the means ± SE of 4−13 experiments. *p < 0.05 as compared to control (−CaM).

at elevated Ca^{2+} concentrations, they differ in their response to CaM at low Ca^{2+} concentrations. RyR1 and RyR3 are activated with a different affinity and to a different extent by CaM, whereas RyR2 is inhibited by CaM at Ca^{2+} concentrations less than 1 μM. Our mutagenesis studies show that despite these differences a single conserved binding site is responsible for sensitizing RyR3 to calmodulin. Corresponding amino acid substitutions in the CaM binding site differentially affected the three RyR isoforms, thus providing a mechanistic basis for the differential regulation of RyRs by calmodulin.

We found that the sequence of the CaM binding site of RyR3 is identical to that of RyR2, yet CaM activates RyR3, whereas it inhibits RyR2 at [Ca^{2+}] < 1 μM. Differences in the regulation by CaM were also observed when corresponding amino acid residues were mutated in the three mammalian RyRs. Table 3 compares the effects of three corresponding mutations on CaM binding and regulation of RyRs by CaM. Substitution of Trp with Ala had no or only a moderate effect on RyR3 but eliminated CaM binding to and CaM inhibition of RyR1 at [Ca^{2+}] > 1 μM. RyR2−W3587A bound [^{35}S]CaM but was not able to transduce CaM binding into a functional effect at [Ca^{2+}] < 1 μM. Substitution of

Table 3: [^{35}S]CaM Binding and CaM Regulation of WT and Mutant RyRs

	CaM Binding			CaM Regulation	
	10 nM Ca^{2+}	0.4 μM Ca^{2+}	>1 μM Ca^{2+}	0.4 μM Ca^{2+}	>1 μM Ca^{2+}
WT-RyR1	+++	ND	+++	↑↑↑	↓↓↓
-W3620A	+++	ND	−	↑↑↑	−
-L3624D	−	ND	−	−	−
-F3636A	ND	ND	ND	↓	↓↓↓
WT-RyR2	+++	+++	+++	↓↓↓	↓↓↓
-W3587A	+++	++	+++	−	↓↓↓
-L3591D	−	++	+++	−	↓↓↓
-F3603A	−	++	+++	−	↓↓↓
WT-RyR3	+++	+++	+++	↑↑↑	↓↓↓
-W3473A	+++	++	++	↑↑↑	↓↓↓
-L3477D	−	−	+	−	↓↓
-F3489A	−	+	+++	−	↓↓↓

Data for RyR1 and RyR2 are from refs 8 and 9, and RyR3 from this study. Upward and downward arrows denote CaM activation and inhibition of RyRs, respectively. Number of symbols shows relative affinity of CaM binding and regulation as compared with wild type. +++, ↑↑↑ and ↓↓↓ not significantly different from WT; −, denotes absence of [^{35}S]CaM binding, and regulation by 1 μM CaM; ND, not determined.

Leu with Asp eliminated CaM binding to all three isoforms at [Ca^{2+}] < 0.01 μM and regulation at [Ca^{2+}] < 1 μM, whereas differential effects were observed at [Ca^{2+}] >1 μM. The third mutation (Phe to Ala) resulted in CaM inhibition of RyR1 and eliminated RyR2 and RyR3 regulation by CaM at [Ca^{2+}] < 1 μM but had no or only a moderate effect on CaM regulation at [Ca^{2+}] > 1 μM. Taken together, the results of the present study support the suggestion that CaM transduces its differential functional effects via other isoform-dependent regions of the RyRs, as discussed below. They further suggest that an interaction with the isoform-dependent regions in the RyRs affects the structure of the CaM binding domain (CaMBD) and thereby their CaM binding affinities.

Several studies have attempted to identify the regions in the RyRs that are responsible for their differential regulation by CaM. We previously showed that five nonconserved amino acids (RyR2−3647, 3649−3652) in the C-terminus region flanking the CaMBD have a specific role in CaM inhibition of RyR2 (*21*). Substitution of the five amino acid residues in RyR2 with those of RyR1 decreased the efficacy of CaM inhibition, whereas substitution of the corresponding RyR2 amino acid residues in RyR1 was without effect on CaM activation and CaM inhibition of RyR1. Trypsin protection and CaM peptide binding experiments suggested that the carboxyl-terminal half of CaM binds to the RyR1 CaMBD, whereas the N-terminal half interacts with RyR1 amino acid residues 1975−1999 (*22*). However, substitution of the nonidentical amino acid residues in RyR1 with those of RyR2 indicated that this region is not responsible for the differential regulation of RyR1 and RyR2 by CaM at [Ca^{2+}] < 1 μM (*21*). Synthetic peptides corresponding to the CaMBD affected RyR1 channel function (*23*). In permeabilized frog skeletal muscle, which contains both RyR1 and RyR3, the RyR1 CaMBD peptide increased the number of Ca^{2+} release events (sparks) (*24*). These studies suggested that peptides can be used to probe for regions that interact with the CaMBD. Xiong et al. (*25*) tentatively identified such a region by showing that a peptide corresponding to CaMBD bound to calmodulin-like peptide of RyR1 (amino acid

residues 4064−4210). Gangopadhyay and Ikemoto (*26*) labeled SR membranes with CaMBD peptide and identified a tryptic peptide encompassing amino acid residues 2900−4400 as the site of binding.

All three RyR isoforms are widely expressed in excitable and nonexcitable cells (*4*, *27*−*29*). In a recent study, it was shown that in dorsal root ganglion neurons, Ca^{2+} release from subsurface cisternae by RyR3 plays a role in mediating and modulating excitation-secretion coupling (*30*). In neuronally differentiated PC12 cells, RyR2 and RyR3 are coexpressed (*31*). RyR2 appeared to be primarily localized in the soma, with RyR3 showing a wide distribution throughout the cells. Coexpression of RyR1 and RyR3 in smooth (*32*) and skeletal (*4*, *29*) muscle has been described. In airway smooth muscle cells, RyR1 is localized near the cell membrane, whereas RyR3 is more centrally localized (*32*). In neonatal skeletal muscle and diaphragm, RyR1 and RyR3 are segregated in the junctional SR membrane and in a parajunctional position adjacent to the junctional SR, respectively (*33*). The different intracellular distribution of RyRs provides neuronal and muscle cells with spatial control of cytoplasmic Ca^{2+} levels. Differences in regulation of the three RyRs by CaM indicate additional regulatory mechanisms to control intracellular Ca^{2+} fluxes.

CaM-dependent regulation of RyR1 and RyR3 has been studied in skeletal muscle fibers. Ikemoto et al. (*11*) distinguished between the two isoforms, using chemically skinned skeletal muscle fibers isolated from mutant mice expressing either RyR1 or RyR3. In RyR3 expressing fibers, CaM increased the rate of Ca^{2+} release at pCa 6 and inhibited Ca^{2+} release at pCa 5, indicating activation and inhibition of RyR3, respectively, in reasonable agreement with the results of the present study. In RyR1 expressing fibers, CaM also potentiated SR Ca^{2+} release at pCa 6. At pCa 5, the effect of CaM depended on the presence of Mg^{2+}. While a small inhibitory effect was observed in the presence of Mg^{2+} in RyR1 expressing fibers, CaM increased the rate of Ca^{2+} release in the absence of Mg^{2+}. In permeabilized frog skeletal muscle fibers, addition of CaM increased the frequency of Ca^{2+} release events (sparks) (*34*). An EC_{50} value of 1.1 μM suggests that the increase in spark frequency was due to the activation of the RyR3 isoform, although a clear distinction between the two isoforms is not possible because RyR1 and RyR3 are expressed in equal amounts in amphibian muscle.

RyR1 and RyR3 isoforms are activated by CaM at Ca^{2+} levels of ~0.1 μM found in unexcited muscle cells and inhibited at Ca^{2+} levels > 1 μM of contracting muscle. Under physiological reducing conditions, CaM activated RyR1 at low Ca^{2+} and inhibited RyR1 at high Ca^{2+} with similar affinities (K_D = 10−15 nM, Table 2). An intracellular free CaM concentration of ~50 nM (*35*, *36*) suggests high intracellular CaM occupancy of RyR1. Activation of RyR3 by CaM (K_D = 100 nM) and inhibition of RyR3 by CaM (K_D = 10 nM) suggests CaM occupancy of RyR3 depends on intracellular Ca^{2+} levels. CaM binds and dissociates from RyR1 on a time scale of seconds to minutes (*8*). Thus, we envision that more sustained changes in intracellular Ca^{2+} resulting from hormonal and excitatory mechanisms influence CaM occupancy and regulation of RyR3 by CaM.

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