THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 25, Issue of June 22, pp. 22579–22585, 2001
Printed in U.S.A.

Identification of Apocalmodulin and Ca^{2+}-Calmodulin Regulatory Domain in Skeletal Muscle Ca^{2+} Release Channel, Ryanodine Receptor*

Received for publication, March 27, 2001
Published, JBC Papers in Press, April 16, 2001, DOI 10.1074/jbc.M102729200

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Fusion proteins and full-length mutants were generated to identify the Ca^{2+}-free (apoCaM) and Ca^{2+}-bound (CaCaM) calmodulin binding sites of the skeletal muscle Ca^{2+} release channel/ryanodine receptor (RyR1). [^{35}S]Calmodulin (CaM) overlays of fusion proteins revealed one potential Ca^{2+}-dependent (aa 3553–3662) and one Ca^{2+}-independent (aa 4302–4430) CaM binding domain. W3620A or L3624D substitutions almost abolished completely, whereas V3619A or L3624A substitutions reduced [^{35}S]CaM binding to fusion protein (aa 3553–3662). Three full-length RyR1 single-site mutants (V3619A, W3620A,L3624D) and one deletion mutant (Δ4274–4535) were generated and expressed in human embryonic kidney 293 cells. L3624D exhibited greatly reduced [^{35}S]CaM binding affinity as indicated by a lack of noticeable binding of apoCaM and CaCaM (nanomolar) and the requirement of CaCaM (micromolar) for the inhibition of RyR1 activity. W3620A bound CaM (nanomolar) only in the absence of Ca^{2+} and did not show inhibition of RyR1 activity by 3 μM CaCaM. V3619A and the deletion mutant bound apoCaM and CaCaM at levels compared with wild type. V3619A activity was inhibited by CaM with IC_{50} ~200 nM, as compared with IC_{50} ~50 nM for wild type and the deletion mutant. [^{35}S]CaM binding experiments with sarcoplasmic reticulum vesicles suggested that apoCaM and CaCaM bind to the same region of the native RyR1 channel complex. These results indicate that the intact RyR1 has a single CaM binding domain that is shared by apoCaM and CaCaM.

Calcium release channels, also known as ryanodine receptors (RyRs),[1] control the release of Ca^{2+} from endoplasmic and sarcoplasmic reticulum in a wide range of tissues (1–3). Mammalian tissues express three structurally and functionally related RyR isoforms referred to as the skeletal muscle (RyR1), cardiac muscle (RyR2), and brain (RyR3) ryanodine receptors. All three isoforms have been purified as 30 S protein complexes composed of four 565-kDa RyR polypeptides in tight association with four 12-kDa FK506-binding proteins. They are cation-selective channels capable of multiple interactions with other molecules. These include small diffusible molecules, such as Ca^{2+}, Mg^{2+}, and ATP, and proteins, such as triadin and calmodulin (CaM) (1–4).

CaM is a ubiquitous cytosolic protein that has a critical role in regulating cellular functions by altering the activity of a large number of proteins. CaM regulates all three RyR isoforms. RyR1 and RyR3 are activated by Ca^{2+}-free CaM (apoCaM) and are inhibited by Ca^{2+}-bound CaM (CaCaM) (5–8), whereas RyR2 is not activated by apoCaM but is inhibited by CaCaM (8–10). Determination of the number of CaM binding sites and their location has been the focus of several studies. Early studies using [^{125}I]CaM (6, 11) or fluorescence-labeled CaM (12) showed a stoichiometry of 1 CaCaM and 2–6 apoCaM binding sites/RyR1 subunit. More recent studies using metabolically ^{35}S-labeled CaM showed one binding site/RyR1 monomer for both of apoCaM and CaCaM (8, 10, 13, 14). Binding site localization studies with fusion proteins and synthetic peptides revealed up to seven candidate CaM binding sites in RyR1 (15–18), clearly exceeding the number of 1 [^{35}S]apoCaM and 1 [^{35}S]CaCaM binding site/RyR polypeptide. To resolve this discrepancy, full-length RyR1 mutants were generated focusing on two CaM binding domains identified in [^{35}S]CaM overlays of fusion proteins spanning the full-length RyR1 (10). The RyR1 mutants were expressed in HEK293 cells, and their [^{35}S]CaM binding properties and regulation by CaM were determined. We found that two amino acid substitutions (W3620A,L3624D) resulted in a loss of high affinity CaCaM binding and inhibition of RyR1 by CaCaM (nanomolars). The L3624D substitution also resulted in a loss of apoCaM binding and activation of RyR1 by apoCaM. Portions of this study have been published previously in abstract form (19).

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]ryanodine was obtained from PerkinElmer Life Sciences, Tran^{35}S-label was from ICN Radiochemicals (Costa Mesa, CA), unlabeled ryanodine was from Calbiochem (La Jolla, CA), unlabeled CaM was from Sigma, and complete protease inhibitors were from Roche Molecular Biochemicals.

Construction of Wild Type and Mutant cDNA Plasmids—cDNAs for RyR1 fusion proteins tagged with trpE and GST were constructed using pATH and pGEX-5X vectors, respectively. The plasmids were transformed into BL21 *Escherichia coli* cells, and protein expression was induced by manufacturer's protocol (for GST) and as described previously (for trpE) (20). FPI (3225–3662), FPI-2 (3352–3392), FPI-3 (3391–3554), and FPI-4 (3553–3662) were expressed as trpE fusion proteins, and FPI-1 (3225–3353) and FPM (4302–4430) were expressed as GST fusion proteins (amino acid sequences are shown in parentheses). The full-length rabbit RyR1 cDNA (*Cla*I/*Xba*I) was constructed and cloned into expression vector pCMV5 as described previously (21). Single and multiple base changes were introduced by pfu polymerase-based chain

* The work was supported by National Institutes of Health Grant AR18687. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: RyR, ryanodine receptor; RyR1, skeletal muscle RyR; CaM, calmodulin; apoCaM, Ca^{2+}-free CaM; CaCaM, Ca^{2+}-bound CaM; HEK, human embryonic kidney; GST, glutathione *S*-transferase; SR, sarcoplasmic reticulum; KPipes, potassium 1,4-piperazinediethanesulfonic acid; BSA, bovine serum albumin; wt, wild type.

reaction using mutagenic oligonucleotides and the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). For the fusion proteins, FPI-4 was used as the template of mutagenesis. The construction of the full-length RyR1 mutants made use of mutated FPI-4 fragments, *Ecl*XI/*Ssp*BI(10872–11054). Alternatively, a partial fragment, *Pvu*I/*Nde*I(8600–11304), was used as the template of mutagenesis. Deletion of sequences encoding amino acids 4274–4535 was performed using two *Nar*I restriction enzyme sites (22). Mutated and deleted sequences were confirmed by sequencing. Mutated and deleted full-length expression plasmids were prepared by ligation of two fragments (*Cla*I/*Pvu*I and *Pvu*I/*Xba*I containing the mutated or deleted sequence) and expression vector pCMV5 (*Cla*I/*Xba*I).

Expression of Full-length RyR1 in HEK293 Cells—RyR1 cDNAs were transiently expressed in HEK293 cells with the LipofectAMINE Plus (Life Technologies, Inc.) or Fugene6 (Roche Molecular Biochemicals) methods according to the manufacturers' instructions. Cells were maintained at 37 °C and 5% CO_2 in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and plated the day before transfection. For each 10-cm tissue culture dish, 3–6 μg cDNA was used. Cells were harvested 42–48 h after transfection. Cells were washed twice with 3-ml ice-cold phosphate-buffered saline containing 1 mM EDTA and Complete protease inhibitors and harvested in the same solution by removal from the plates by scraping. Cells were collected by centrifugation, washed in the same buffer without EDTA, and stored at −80 °C. Sarcoplasmic reticulum (SR) vesicles were prepared from rabbit skeletal muscle as described previously (6).

[^{35}S]Calmodulin Overlay—CaM binding to RyR1 fusion proteins was assayed by [^{35}S]CaM overlays using whole cell preparations or inclusion bodies. [^{35}S]CaM was prepared using Tran^{35}S label as described previously (10). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked by treating membranes with a solution, 150 mM KCl, 20 mM KPipes, pH 7.0, containing 1 mg/ml bovine serum albumin (BSA) and 100 μM Ca^{2+} (blocking buffer) for 1 h. CaCaM binding was analyzed by incubating membranes with 100 nM [^{35}S]CaM in 150 mM KCl, 20 mM KPipes, pH 7.0, 0.04% Tween 20, and 100 μM Ca^{2+} for 1 h and washing with blocking buffer 4 times. Dried membranes were exposed to x-ray film, and radioactivity was determined by autoradiography. ApoCaM binding was analyzed in buffer solutions containing 5 mM EGTA instead of 100 μM Ca^{2+}.

[^{35}S]Calmodulin Binding—Crude membrane fractions prepared as described below were incubated for 2 h at room temperature with solutions, 5 or 15 nM [^{35}S]CaM in 10 mM KPipes, 10 mM imidazole, pH 7.0, containing 0.15 M sucrose, 150 mM KCl, 0.125 mg/ml BSA, 5 mM glutathione (reduced form), 20 μM leupeptin, 200 μM Pefabloc, and either 5 mM EGTA (apoCaM binding) or 200 μM Ca^{2+} (CaCaM binding). Aliquots were taken for determination of total radioactivity and centrifuged for 45 min at 30 p.s.i. 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 protein amounts of vector-transfected or non-transfected HEK293 cells. In parallel experiments, B_{max} values of [^{3}H]ryanodine binding were determined. Membranes were incubated for 5 h at room temperature with a saturating concentration of [^{3}H]ryanodine (30 nM) in 20 mM imidazole, pH 7.0, 0.6 M KCl, 0.15 M sucrose, 20 μM leupeptin, 200 μM Pefabloc, and 200 μM Ca^{2+}. Specific [^{3}H]ryanodine binding was determined as described above.

The time courses of [^{35}S]CaM binding to and dissociation from skeletal SR vesicles were determined by a filtration assay. To minimize nonspecific binding of [^{35}S]CaM, Whatman GF/B filters were blocked in buffer, 0.15 M KCl, 20 mM KPipes, pH 7.0, containing 10 mg/ml BSA. Vesicles on the filters were washed with three 5 ml of ice-cold buffer, 0.15 M KCl, 20 mM KPipes, pH 7.0, containing 0.1 mg/ml BSA and 100 μM Ca^{2+} ([^{35}S]CaCaM binding and [^{35}S]CaCaM dissociation) or 100 μM EGTA ([^{35}S]apoCaM dissociation).

[^{3}H]Ryanodine Binding—[^{3}H]Ryanodine binding experiments were performed with crude membrane fractions. HEK293 cell pellets were resuspended in 20 mM imidazole, pH 7.0, 0.3 M sucrose, 150 mM KCl, 1 mM glutathione (oxidized form), Complete protease inhibitors, and 0.1 mM EGTA and homogenized with a Tekmar Tissumizer for 5 s at a setting of 13,500 rpm. Homogenates were centrifuged for 45 min at 40,000 rpm in a Beckman Ti75 rotor, and pellets were resuspended in the above buffer without EGTA and glutathione. Unless otherwise indicated, membranes were incubated with 2.5 nM [^{3}H]ryanodine in 20 mM imidazole, pH 7.0, 0.3 M sucrose, 250 mM KCl, 0.5 mM glutathione (oxidized), 0.25 mg/ml BSA, protease inhibitors, and indicated Ca^{2+} concentrations. Nonspecific binding was determined using a 1000–2000-fold excess of unlabeled ryanodine. After 20 h, aliquots of the samples were diluted with 8.5 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 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.

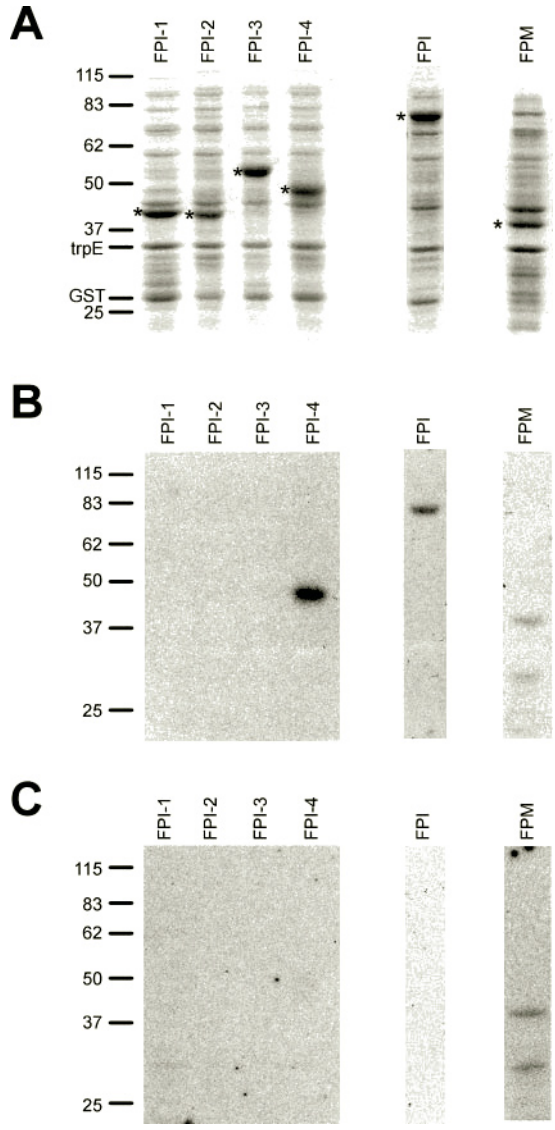

FIG. 1. **[^{35}S]CaM overlays to RyR1 fusion proteins.** *A*, SDS-polyacrylamide gel of whole cell fractions stained with Coomassie Brilliant Blue R250. The *asterisks* indicate fusion proteins as detected with trpE (FPI, FPI-2, FPI-3, and FPI-4) or GST (FPI-1 and FPM) antibodies. [^{35}S]CaM overlays in the presence of either 100 μM Ca^{2+} (*B*) or 5 mM EGTA (*C*). RyR1 amino acids included in fusion protein are 3225–3353 (FPI-1), 3352–3392 (FPI-2), 3391–3554 (FPI-3), and 3553–3662 (FPI-4), 3225–3662 (FPI), and 4302–4430 (FPM). Standard molecular masses are shown on the *left side* of *panels* in kDa. The positions of trpE and GST are also shown. Neither bound CaCaM nor ApoCaM (data not shown).

RESULTS

[^{35}S]Calmodulin Overlays of Wild Type and Mutant RyR1 Fusion Proteins—In a previous study, we used 15 fusion proteins spanning the full coding sequence of the RyR1 polypeptide to identify candidate CaM binding domains (10). We found that two fusion proteins including amino acids 3225–3662 of RyR1 (FPI) and amino acids 4302–4430 (FPM) specifically bound [^{35}S]CaM in a Ca^{2+}-dependent and independent manner, respectively (Fig. 1) (10). In this study, we further subdivided the larger of the two fusion proteins (FPI) into four

fragments (FPI-(1–4)) using specific restriction enzyme sites. The fragments were expressed as trpE fusion proteins. FPI-1 (3225–3353) was also expressed as a GST fusion protein because the expression level of the trpE fusion protein was very low. Since all fusion proteins were insoluble, [^{35}S]CaM overlays were done with whole cell fractions in Fig. 1. The amounts of proteins on the gels were adjusted to show similar Coomassie Blue staining for the fusion proteins (Fig. 1*A*). Fig. 1*B* shows that in the presence of 100 nM [^{35}S]CaM and 100 μM Ca^{2+}, three of the fusion proteins clearly showed detectable [^{35}S]CaM binding. The strongest binding was observed for FPI-4 followed by FPI and FPM. We also performed [^{35}S]CaM overlays in the presence of 5 mM EGTA instead of 100 μM Ca^{2+}. As previously found (10), FPM bound [^{35}S]apoCaM at a level comparable with CaCaM. FPI did not show apoCaM binding and, as expected, neither did the FPI-derived fragments. These results show that [^{35}S]CaM could bind to two fusion proteins derived from RyR1; binding to one fusion protein was Ca^{2+}-dependent, whereas binding to the other was Ca^{2+}-independent.

Primary sequence predictions suggest the presence of several CaM binding sites in RyR1 (23, 24). One of these sites was predicted to be present in FPI-4 (3614–3637). Using nnPredict (University of California, San Francisco, CA), we identified a stretch of amino acids (aa 3617–3628) predicted to form an amphipathic α-helical structure but not in perfect agreement with reported CaM binding motifs. Therefore, we somewhat arbitrarily mutated three hydrophobic amino acid residues (Val 3619 to Ala, Trp 3620 to Ala, and Leu 3624 to Ala and Asp) lying on one face of the helix. We also substituted cysteine 3635 with an alanine because CaM blockage of *N*-ethylmaleimide alkylation of Cys 3635 suggested that this residue may be important for CaM binding (25). All of the mutant fusion proteins including wt were isolated as inclusion bodies and tested for [^{35}S]CaCaM binding using the overlay assay. Equivalent amounts of wt and mutated FPI-4s were used based on Coomassie Blue staining of SDS gels. The results of the overlay assay are shown in Fig. 2. The strongest binding was observed for wt and C3635A mutant proteins. Mutant proteins with V3619A or L3624A substitutions showed reduced binding, whereas mutant proteins with W3620A or L3624D substitutions barely showed detectable binding. The results identify two amino acid residues (Trp 3620, Leu 3624) that are critical for CaCaM binding to FPI-4. However, it was unclear whether the results with the fusion protein were directly applicable to the full-length RyR1. The information gained was limited because FPI-4 did not bind apoCaM and, therefore, could not be used to locate the apoCaM binding sites in RyR1. Also, [^{35}S]CaM overlays revealed two candidate CaCaM binding sites as opposed to one site/subunit in the native RyR1. Therefore, we extended our mutant studies to the intact RyR1.

[^{35}S]Calmodulin Binding to Wild Type and Mutant RyR1s—We introduced three site-specific mutations in the full-length RyR1 that led to nearly a complete loss (W3620A,L3624D) or a reduction (V3619A) of [^{35}S]CaM binding to FPI-4 (Fig. 2). We also generated a deletion mutant (RyR1Δ4274–4535) to address the significance of a Ca^{2+}-independent CaM binding site detected in the overlays in FPM (aa 4302–4430) (Fig. 1, *B* and *C*). The mutant RyR1s were expressed in HEK293 cells, and crude membrane fractions were prepared to determine their CaM binding properties. In parallel experiments, the RyR1 expression levels were quantified by a ligand binding assay using saturating [^{3}H]ryanodine concentrations as described under "Experimental Procedures." Expression of full-length wt and mutant RyR1s was confirmed by Western blot analysis using anti-RyR1 monoclonal antibody D110 (26) (data not shown). In Fig. 3, we used 5 and 15 nM [^{35}S]CaM, which are near and beyond the dissociation constants of apoCaM and CaCaM binding to the native and purified RyR1 under the assay conditions described under "Experimental Procedures" (10). Cells expressing wt-RyR1 bound 3–4 [^{35}S]apoCaM and 4–5 [^{35}S]CaCaM/[^{3}H]ryanodine binding site. As there is only one high affinity [^{3}H]ryanodine binding site/RyR1 tetramer, these ratios corresponded to ~1 apoCaM and 1 CaCaM binding site/RyR1 subunit. RyR1 mutant with a V3619A substitution and RyR1Δ4274–4535 bound [^{35}S]apoCaM and [^{35}S]CaCaM not significantly different from wt-RyR1. W3620A bound only [^{35}S]apoCaM, whereas L3624D showed a loss of high affinity [^{35}S]CaM binding both in the presence and absence of Ca^{2+}. These studies provided information beyond that obtained with the fusion proteins. The results of Fig. 3 indicate that in the intact RyR1 amino acid residues 4274–4535 are not important for high affinity apoCaM and CaCaM binding. Rather, they suggest that Leu 3624 constitutes a part of both the apoCaM and the CaCaM binding site in the intact RyR1, whereas Trp 3620 appeared to be only a part of the CaCaM binding site, results that could not be obtained with the mutant fusion proteins because FPI-4 did not show apoCaM binding.

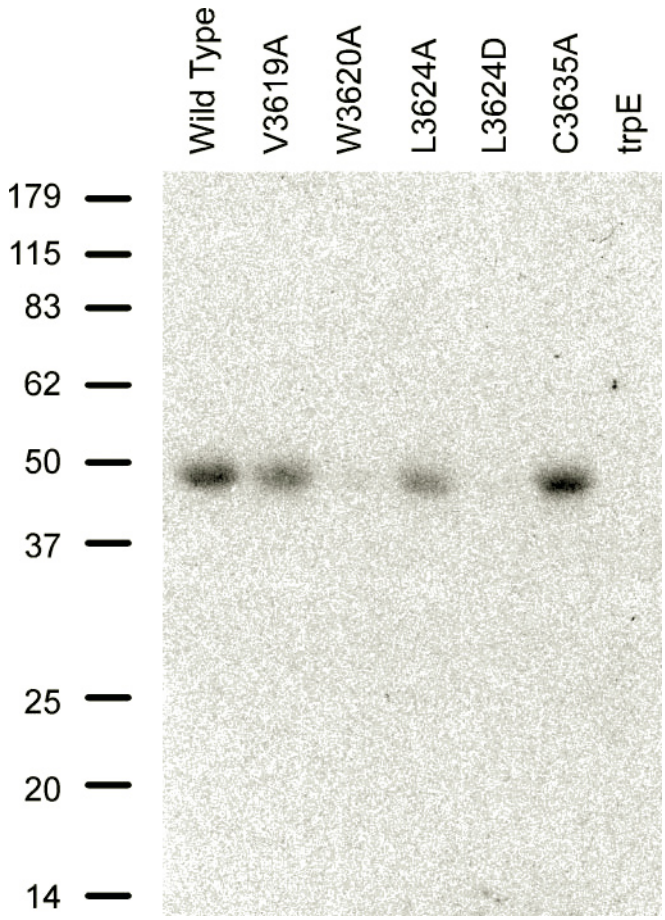

FIG. 2. **[^{35}S]CaM overlay to wild type and mutant FPI-4 fusion proteins.** [^{35}S]CaM overlays in the presence of 100 μM Ca^{2+} are shown. Standard molecular masses are shown on the *left side* of *panels* in kDa.

[^{3}H]Ryanodine Binding to Wild Type and Mutant RyR1s—We next examined the functional effects of CaCaM on the four RyR1 mutants shown in Fig. 3 by determining their [^{3}H]ryanodine binding properties in the absence and presence of exogenously added CaM. The highly specific plant alkaloid ryanodine is widely used as a probe of channel activity because of its preferential binding to the open RyR channel states (1–3). The four mutants exhibited a specific [^{3}H]ryanodine binding affinity (determined by Scatchard analysis) and Ca^{2+} activation/

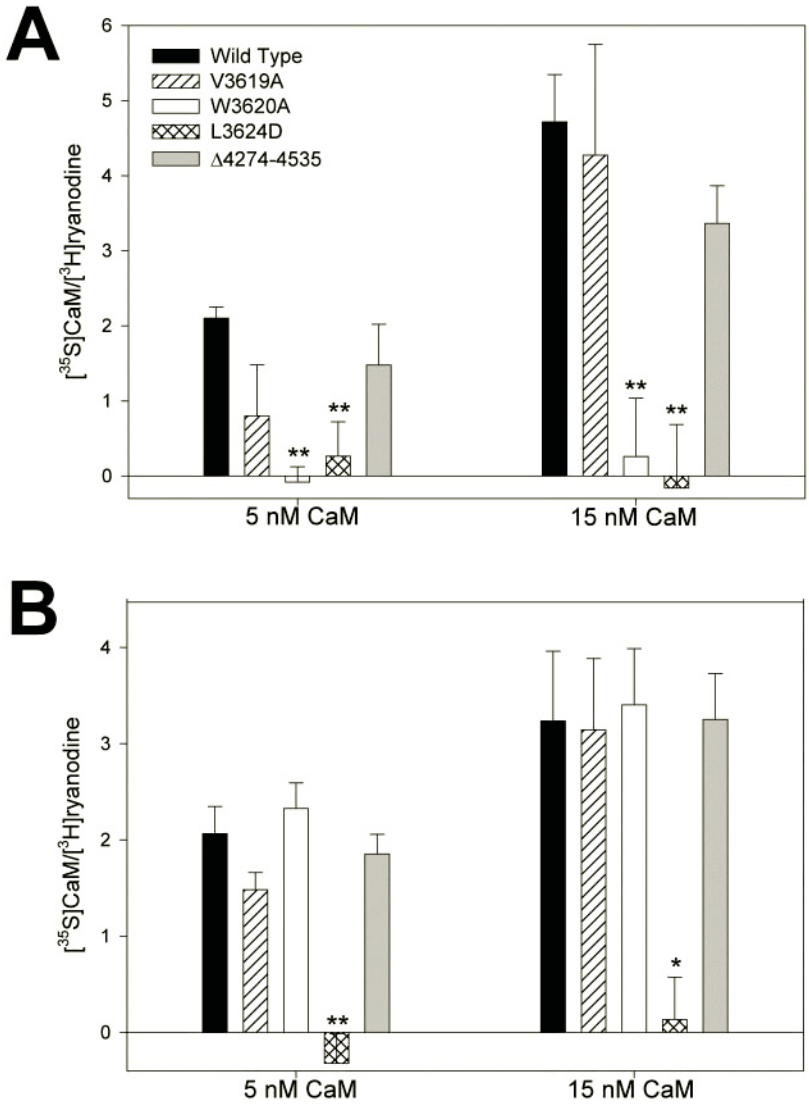

FIG. 3. **[^{35}S]CaM binding to wild type and mutant RyR1s.** Membrane fractions prepared from HEK293 cells expressing wt or mutant RyR1s were incubated for 2 h at room temperature with 5 or 15 nM [^{35}S]CaM and either 200 μM Ca^{2+} (*A*) or 5 mM EGTA (*B*). The ratios of [^{35}S]CaM binding values to maximal binding values of [^{3}H]ryanodine are shown. Maximal values of [^{3}H]ryanodine binding (in pmol/mg protein), determined as described under "Experimental Procedures", were 0.22 ± 0.03 (wt), 0.21 ± 0.02 (V3619A), 0.21 ± 0.03 (W3620A), 0.18 ± 0.02 (L3624D), and 0.69 ± 0.08 (Δ4274–4535). Data are the means ± S.E. of 3–6 experiments. *, $p < 0.05$; **, $p < 0.01$ as compared with wt at the same CaM concentration.

inactivation profile comparable with wt-RyR1 with the exception of RyR1Δ4274–4535, which showed an ~10-fold increased sensitivity to activating Ca^{2+} in agreement with a previous report (22) (data not shown). Fig. 4*A* shows that [^{3}H]ryanodine binding to wt-RyR1 was inhibited by CaCaM in a concentration-dependent manner with an IC_{50} ~50 nM. The maximal extent of inhibition (60% by ~1 μM CaM) was comparable with that observed for native RyR1s (6). The deletion mutant (RyR1Δ4274–4535) showed a response to CaCaM essentially identical to wt-RyR1. V3619A required a higher CaCaM concentration for the inhibition of [^{3}H]ryanodine binding (IC_{50} ~200 nM as compared with IC_{50} ~50 nM for wt-RyR1). L3624D exhibited a greatly reduced apparent affinity for CaCaM as indicated by the requirement of 3 μM CaCaM for partial inhibition of RyR1 activity, whereas W3620A did not show any inhibition at 3 μM CaCaM. Fig. 4*B* shows that, in agreement with the apoCaM binding data of Fig. 3*B*, 1 μM apoCaM significantly increased [^{3}H]ryanodine binding to wt and V3619A, W3620A, and Δ4274–4535 RyR1s but not L3624D. Taken together, the results of the [^{35}S]CaM (Fig. 3) and [^{3}H]ryanodine binding (Fig. 4) experiments suggest that Leu 3624 constitutes a part of the CaCaM inhibiting and apoCaM activating sites of RyR1, whereas Trp 3620 appears to be only essential for CaCaM inhibition.

[^{35}S]Calmodulin Binding to Native RyR1—Dissociation and chase experiments were performed to determine whether CaCaM and apoCaM share a common binding domain in native RyR1s using a filtration assay. As shown in Fig. 5*A*, the dissociation of [^{35}S]CaM from skeletal muscle SR vesicles enriched in RyR1 is not largely dependent of whether CaM is bound in the presence or absence of Ca^{2+} but rather on whether Ca^{2+} is present in the dissociation buffer with apoCaM dissociating at a significantly greater rate than CaCaM. In a similar set of experiments, SR vesicles were preincubated with or without non-radioactive CaM in either the presence or absence of Ca^{2+} followed by the binding of radioactive CaCaM. The resulting rates of [^{35}S]CaM binding were dramatically slower to vesicles pretreated with non-radioactive CaM (Fig. 5*B*, *open symbols*) than the rates of binding to vesicles not pretreated with CaM (Fig. 5*B*, *closed symbols*). Furthermore, the rates were relatively independent of whether the preincubation had been performed in the absence or presence of Ca^{2+}. These experiments strongly support the mutant results that CaCaM and apoCaM bind to a common region of RyR1.

DISCUSSION

Calmodulin has a dual effect on skeletal muscle Ca^{2+} release channel activity. CaM activates the channel at Ca^{2+} concentrations below 1 μM, whereas at Ca^{2+} concentrations above 1 μM, the channel activity is inhibited by CaM. The data we have presented here indicate that these effects are mediated through

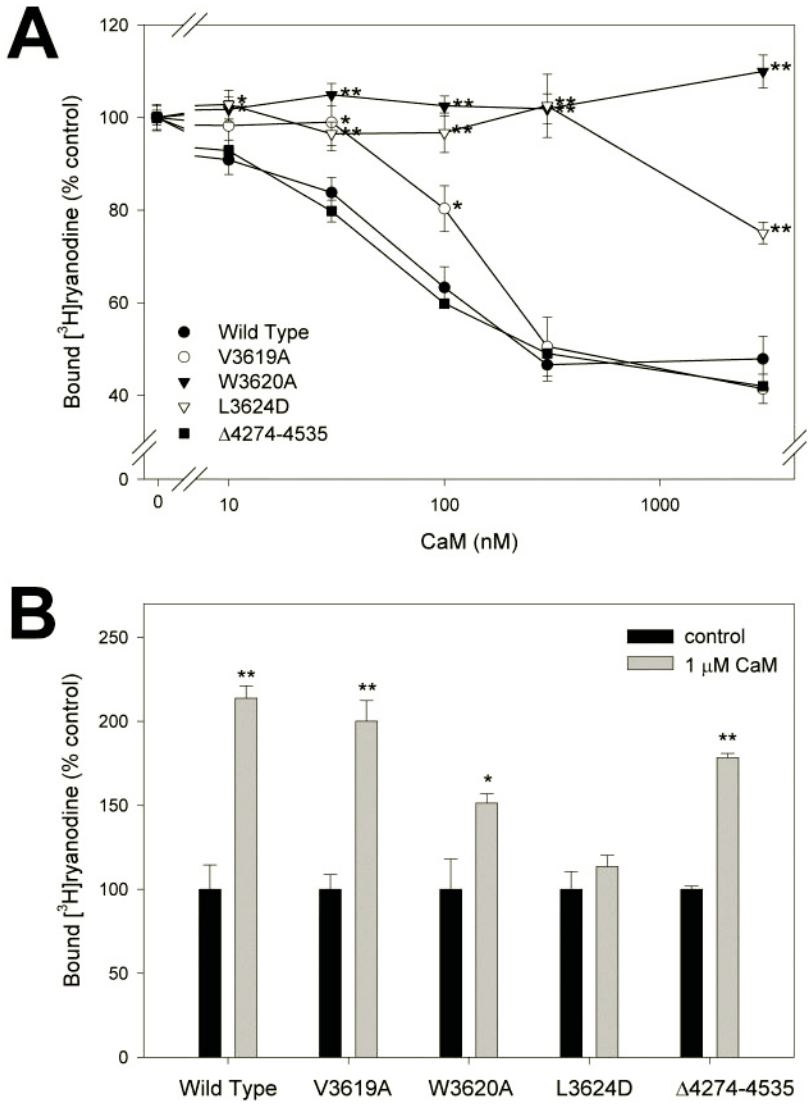

FIG. 4. **CaM inhibition and activation of [^{3}H]ryanodine binding to wild type and mutant RyR1s.** Specific [^{3}H]ryanodine binding to wt, V3619A, W3620A, L3624D, and Δ4274–4535 RyR1s was determined as described under "Experimental Procedures" in the presence of 100 μM Ca^{2+} (*A*) or 0.1 μM Ca^{2+} and 1 mM AMPPCP (a nonhydrolyzable ATP analog) (*B*) and the indicated concentrations of CaM. Normalized [^{3}H]ryanodine binding data are the means ± S.E. of four or more experiments. *, $p < 0.05$; ** $p < 0.01$ as compared with wt at the same CaM concentration (*A*) and wt and mutants in the absence of CaM (*B*).

a single CaM binding domain that is shared by apoCaM and CaCaM.

Several studies have reported the stoichiometry of CaM binding to RyR1 using SR vesicles (6, 8, 10–14) and purified RyR1 preparations (6, 10). The initial studies using either ^{125}I (6, 11) or fluorescently (12) labeled CaM revealed that the native RyR1 binds with nanomolar affinity 1 CaM/subunit in the presence of Ca^{2+}, and that there are as many as six high affinity binding sites for apoCaM on each of the four RyR1 subunits that comprise the functional channel. More recent studies using ^{35}S metabolically labeled CaM indicate that the tetrameric skeletal muscle channel complex binds 4 CaM molecules both in the absence and presence of Ca^{2+} or 1 CaM/subunit (8, 10, 13). These results imply that chemical modification of CaM increases the number of CaM binding sites of RyR1.

Previous studies performed to localize the CaM binding sites relied on the use of fusion proteins and synthetic peptides (Fig. 6). CaM overlays of RyR1 fusion proteins using ^{125}I (15) or digoxigenin-labeled (16) CaM revealed up to seven regions that bound CaM. With the exception of one site, CaM binding was abolished in the presence of EGTA, indicating that it was Ca^{2+}-dependent. Our protein overlays using [^{35}S]CaM identified potential binding domains in two fusion proteins; one of which, FPM (aa 4302–4430), bound CaM both in the absence and presence of Ca^{2+}. The other fusion protein, FPI-4 (aa 3553–3662), bound CaM only in the presence of Ca^{2+} in agreement with previous studies using fusion proteins (15, 16).

Studies with fusion proteins show that the fragmentation of the 565 kDa of RyR peptide into smaller pieces unmasks CaM binding sites not detected in the large channel complex. It is therefore necessary that full-length RyR1 mutants lacking putative CaM binding sites are constructed and that the functional consequences of these mutations are examined. Deletion of one of the potential CaM binding sites identified in the [^{35}S]CaM overlays, RyR1Δ4274–4535, was without effect on high affinity CaM binding and the inhibition and activation of [^{3}H]ryanodine binding by CaCaM and apoCaM, respectively (Figs. 3 and 4). In this study, we therefore focused on amino acid residues covered by FPI-4 (aa 3553–3662), which contained a CaM binding site implicated in all previous studies (Fig. 6). Three amino acid substitutions (V3619A,W3620A, L3624D) in FPI-4, leading to a reduction or nearly a complete loss of [^{35}S]CaCaM binding, were introduced in the full-length RyR1. One of the mutants (L3624D) showed a loss of both high affinity apoCaM and CaCaM binding, whereas a second mutant (W3620A) showed a specific loss of CaCaM binding as it maintained the ability to bind [^{35}S]CaM (nanomolar) in the absence of Ca^{2+}. These results suggest that Leu 3624 is critical for conferring both apoCaM and CaCaM binding, whereas Trp 3620 is critical only for CaCaM. The physiological relevance of these findings was supported by [^{35}S]CaM dissociation and chase experiments, which indicated that the native RyR1 has a

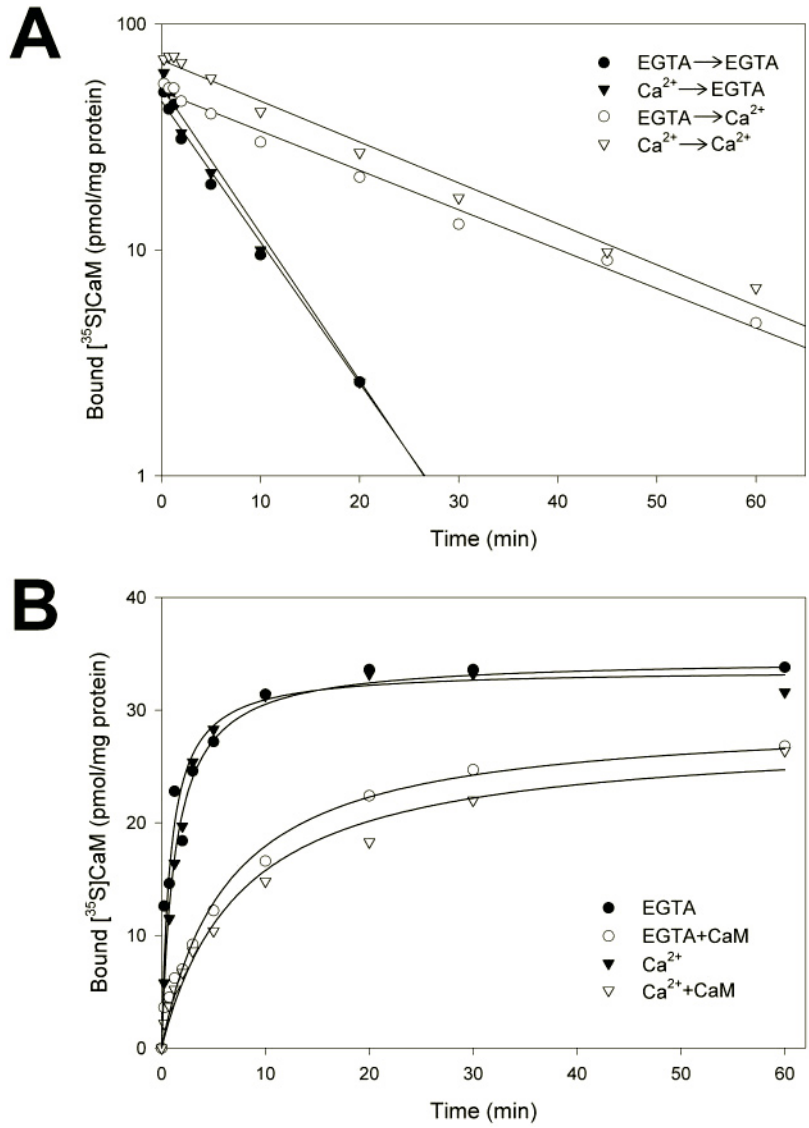

FIG. 5. **Time course of [^{35}S]CaM binding and dissociation.** *A,* skeletal SR vesicles were prebound with 100 nM [^{35}S]CaM in 150 mM KCl, 20 mM KPipes, pH 7.0, 5 mM glutathione (reduced form), 0.1 mg/ml BSA, 0.2 mM Pefabloc, 20 μM leupeptin with 5 mM EGTA (*circle*) or 100 μM Ca^{2+} (*triangle*) and then diluted 60-fold into media containing 50 nM non-radioactive CaM. Final EGTA-Ca^{2+} concentration was either 1 mM EGTA (*filled symbols*) or 100 μM Ca^{2+} (*open symbols*). Filter assays were performed as described under "Experimental Procedures." *B,* skeletal SR vesicles were preincubated for 1 h at room temperature in 0.15 M KCl, 20 mM KPipes, pH 7.0, buffer containing 5 mM glutathione (reduced form), 0.2 mM Pefabloc, 20 μM leupeptin, and 0.1 mg/ml BSA with (*open symbols*) or without (*filled symbols*) 100 nM non-radioactive CaM. Free Ca^{2+} concentration was <0.01 μM (*circle*) or 50 μM (*triangle*). Aliquots were then diluted 20-fold into media containing 2.5 nM [^{35}S]CaM and 0 (*open symbols*) or 5 nM (*closed symbols*) unlabeled CaM. Final CaM concentration was 7.5 nM, and final free Ca^{2+} concentration was 50 μM. The representative traces of three similar experiments are shown.

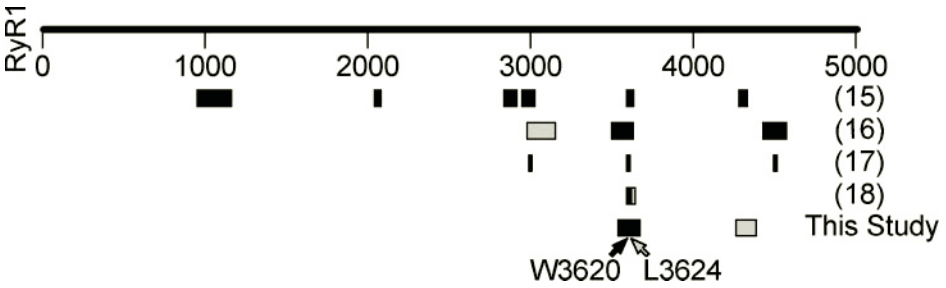

FIG. 6. **Diagram of calmodulin binding sites.** Sequence domains suggested by previous work and this study are shown. Potential CaCaM binding domains are *filled boxes*, whereas domains binding both apoCaM and CaCaM are *light gray* with a *black border*. *Arrows* indicate amino acids that were identified in this study to constitute part of the CaCaM (Trp 3620) and apoCaM and CaCaM (Leu 3624) binding sites.

site that interacts with both apoCaM and CaCaM. Using cryo-electron microscopy and three-dimensional reconstruction, Samso *et al.* (27) showed that apoCaM and CaCaM bind to two near but distinct cytoplasmic locations on each of the four subunits of the RyR1. This observation suggests that apoCaM and/or CaCaM binding induce major RyR1 protein conformational changes given that it is unlikely that a shift of CaM by several amino acids can be detected at the resolution achievable by electron microscopy.

Our data are in good agreement with a recent report by Moore *et al.* (13) who suggested that the region of the RyR1 identified in this study binds both apoCaM and CaCaM as both CaM forms were capable of protecting RyR1 from trypsin cleavage at arginines 3630 and 3637. Furthermore, these investigators showed that a synthetic peptide (aa 3614–3643), which included the two trypsin cleavage sites, bound both apoCaM and CaCaM (18). A shorter peptide (aa 3614–3635) bound CaCaM but showed a loss of apoCaM binding, whereas another peptide including neither Trp 3620 nor Leu 3624 (aa 3625–3644) bound apoCaM and with a reduced affinity CaCaM (18). Therefore, the results obtained with synthetic peptides (18) and the intact RyR1 in this study do not agree entirely.

The functional consequences of our mutations were assessed by determining their Ca^{2+} dependence and [^{3}H]ryanodine binding properties. The RyR1 mutants bound [^{3}H]ryanodine with an affinity and showed a Ca^{2+} dependence comparable with wt-RyR1 with the exception of RyR1Δ4274–4535, which showed an ~10-fold increased sensitivity to activating Ca^{2+}, as

previously reported (22). Therefore, the mutations did not introduce major global conformational changes, but rather they appeared to be mostly limited to the CaM binding sites. The functional studies also allowed tests of the effects of micromolar concentrations of CaM as opposed to the binding studies that are limited to nanomolar CaM concentrations due to experimental restraints. [^{3}H]Ryanodine binding to W3620A was not inhibited by 3 μM CaCaM, which suggests a complete loss or at least a very large reduction of CaCaM binding affinity. L3624D and V3619A were inhibited by CaM with IC_{50} ~3 μM and ~200 nM, respectively, as compared with IC_{50} ~50 nM for wt in agreement with the binding studies, which showed nearly a complete loss of CaCaM binding for L3624D but not for V3619A.

In addition to regulating the Ca^{2+} release channel, CaM probably also influences Ca^{2+} release through other proteins that interact with the release channel. Potential targets of CaM regulation are the transverse tubule Ca^{2+} channel, which via a direct interaction controls the SR Ca^{2+} release channel, calmodulin-dependent protein kinase, and calmodulin-stimulated protein phosphatase (calcineurin) (1–3, 28, 29). Our work provides information for future studies of distinguishing CaM regulation of the RyR1 from that of other proteins. We show that two single amino acid substitutions distinctly change the regulation of the skeletal muscle Ca^{2+} release channel by CaM; one of which (L3624D) results in a loss of activation by apoCaM and an inhibition by CaCaM, whereas the other (W3620A) specifically abolishes CaCaM inhibition.

Acknowledgments—We thank David Balshaw for suggestions and John Johnson and Daniel A. Pasek for technical assistance.

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