Apocalmodulin and Ca^{2+} Calmodulin Bind to the Same Region on the Skeletal Muscle Ca^{2+} Release Channel†

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ABSTRACT: The skeletal muscle Ca^{2+} release channel (RYR1) is regulated by calmodulin in both its Ca^{2+}-free (apocalmodulin) and Ca^{2+}-bound (Ca^{2+} calmodulin) states. Apocalmodulin is an activator of the channel, and Ca^{2+} calmodulin is an inhibitor of the channel. Both apocalmodulin and Ca^{2+} calmodulin binding sites on RYR1 are destroyed by a mild tryptic digestion of the sarcoplasmic reticulum membranes, but calmodulin (either form), bound to RYR1 prior to tryptic digestion, protects both the apocalmodulin and Ca^{2+} calmodulin sites from tryptic destruction. The protected sites are after arginines 3630 and 3637 on RYR1. These studies suggest that both Ca^{2+} calmodulin and apocalmodulin bind to the same or overlapping regions on RYR1 and block access of trypsin to sites at amino acids 3630 and 3637. This sequence is part of a predicted Ca^{2+} CaM binding site of amino acids 3614−3642 [Takeshima, H., et al. (1989) *Nature 339*, 439−445].

The Ca^{2+} release channel or ryanodine receptor (RYR1)[1] of the skeletal muscle sarcoplasmic reticulum releases Ca^{2+} from the SR lumen in response to depolarization of the t-tubule membrane. The voltage sensor in the t-tubule membrane is thought to be the dihydropyridine receptor, which is also a voltage-dependent Ca^{2+} channel (*1*). In addition to its regulation by the voltage sensor, the Ca^{2+} release channel is modulated by other intracellular proteins such as calmodulin (CaM) (*2*, *3*). CaM is a ubiquitously expressed dumbell-shaped protein with two Ca^{2+} binding sites at both the N- and C-termini. Upon binding Ca^{2+}, CaM undergoes a conformational change that exposes hydrophobic pockets, allowing physical interaction with many targets.

CaM is a bifunctional regulator of the skeletal muscle Ca^{2+} release channel; Ca^{2+}-bound CaM inhibits and Ca^{2+}-free CaM (apoCaM) activates RYR1 (*2*). Localization of modulator binding sites would greatly improve our understanding of the molecular mechanisms involved in CaM regulation of RYR1 channel activity. Several candidates for the Ca^{2+} CaM and apoCaM binding sites on RYR1 have been identified using sequence analysis (*4*, *5*) and [^{125}I]CaM binding to fusion proteins (*6*, *7*). However, binding sites for CaM on the native protein have not previously been identified.

Using limited tryptic digestion, we showed (*8*) that the binding site for [^{3}H]ryanodine is in the C-terminal region of the protein (after amino acid 4476). Using calpain digestion to generate large fragments, we demonstrated that a disulfide bond formed by oxidation is between two central cytoplasmic domains (*9*), the first from amino acid 1400 to 2843 and the second from amino acid 2844 to somewhere around 4685. Here we use the ability of CaM to protect its binding site from tryptic inactivation to localize the CaM binding site on RYR1.

EXPERIMENTAL PROCEDURES

Materials. For [^{125}I]CaM binding experiments, [^{125}I]CaM (∼8000 cpm/pmol) was prepared by derivatization with monoiodinated Bolton−Hunter reagent (2200 Ci/mmol) purchased from NEN-LifeSciences (Boston, MA). [^{35}S]-Methonine (>1000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Dithiothreitol (DTT), 3-(*N*-morpholino)propanesulfonic acid (MOPS), bovine serum albumin (BSA), nickel chloride, imidazole, soybean trypsin inhibitor, and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO). Trypsin (TPCK) was purchased from Pierce (Rockford, IL). Highly pure digitonin was purchased from CalBiochem (La Jolla, CA). Chelating Sepharose and Phenyl Sepharose resins were purchased from Pharmacia Biotech (Uppsala, Sweden). Unlabeled CaM (bovine brain) was obtained either from Sigma or from Upstate Biochemicals (Lake Placid, NY).

[^{35}S]Methionine Labeling of CaM. Mammalian CaM cDNA was generously provided by Z. Grabarek (Boston Biomedical Institute, Boston, MA) and subcloned into *Nde*I−*Sac*I sites of the pET28a vector (Novagen, Madison, WI) for bacterial expression. Expressed CaM was metabolically labeled as described by Lydan et al. (*10*) and purified by

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[1] Abbreviations: apoCaM, apocalmodulin; Ca^{2+} CaM, calcium calmodulin; CaM, calmodulin; RyR1, ryanodine receptor isoform 1; SR, sarcoplasmic reticulum; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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nickel affinity chromatography. The polyhistidine tag was cleaved as described previously (*11*), and CaM was further purified using Phenyl Sepharose chromatography (*12*, *13*).

SR Membrane Preparation. SR membranes were prepared from rabbit leg and backstrap white skeletal muscle and were purified using sucrose gradient centrifugation (*14*). Using an anti CaM antibody (Sigma) and known amounts of CaM to generate a standard curve, we have determined that the SR membranes isolated in this manner have less than 10% of the CaM binding sites occupied by endogenous CaM prior to the assay.

SDS−PAGE. Polyacrylamide (10%) gel electrophoresis was performed as described by Schagger and von Jagow (*15*).

Bolton−Hunter ^{125}I Labeling of CaM. Mammalian CaM (expressed) was iodinated by a modified Bolton−Hunter reaction (*16*). Briefly, CaM (2.0 mg/mL) was reacted with Bolton−Hunter reagent (NEN-LifeSciences) in 50 mM MOPS (pH 7.4) and 1 mM EGTA for 1 h on ice with frequent agitation. The reaction was quenched by the addition of 1 M glycine in 50 mM MOPS buffer (pH 7.4) and the mixture applied to a desalting column (Pierce D-Salt Dextran) equilibrated with 50 mM MOPS (pH 7.4) and 0.02% NaN_3. The column was eluted with 50 mM MOPS and 0.02% NaN_3 (pH 7.4), and 0.5 mL fractions were collected. Radioactivity in each fraction was monitored, and the two fractions with the highest counts were combined. The protein concentration was determined according to the method of Bradford, using CaM as a standard (*17*).

[^{125}I]CaM and [^{35}S]CaM Binding. SR membranes (10 μg per assay) were incubated with [^{125}I]CaM or [^{35}S]CaM (1.6−200 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 (low-Ca^{2+} buffer) and presence (high-Ca^{2+} buffer) of 1.2 mM $CaCl_2$. Nonspecific binding was defined in the presence of 5 μM unlabeled CaM. Bound radioligand was separated from free by filtration through Whatman GF/F filters presoaked in 0.3 mg/mL BSA/binding buffer, and the filters were washed five times with 3 mL of ice-cold binding buffer. The amount of [^{125}I]CaM bound to membranes was determined using a Beckman γ counter. [^{35}S]CaM binding was assessed by liquid scintillation counting. Free Ca^{2+} concentrations were calculated as described by Fabiato (*18*). The data shown are the mean ± the standard error of the mean (SEM). The concentration of CaM was determined by measuring its absorbance at 277 and 320 nm and calculating the concentration according to the following equation.

$$C\ (\text{mg/mL}) = (A_{277} - A_{320})/\epsilon$$

where the extinction coefficient, ϵ, is equal to 0.18 mL mg^{-1} cm^{-1} in high-Ca^{2+} buffer and 0.20 mL mg^{-1} cm^{-1} in low-Ca^{2+} buffer (*19*).

Purification of Trypsin-Digested Complexes of RYR1. Sarcoplasmic reticulum vesicles were digested with trypsin in 1 mM EGTA, 300 mM NaCl, and 50 mM MOPS (pH 7.4) for 10 min at 37 °C using a protease-to-protein ratio of 1:1000 (w/w). The cleavage reaction was terminated using a 10-fold molar excess of soybean trypsin inhibitor. The membranes were then solubulized using a 2% digitonin solution and sedimented for 18 h at 110000*g* on a 5 to 20% sucrose gradient containing 0.1% digitonin, 300 mM NaCl, and 50 mM MOPS (pH 7.4). The protein distribution on the gradient was determined by a Bradford (*17*) assay.

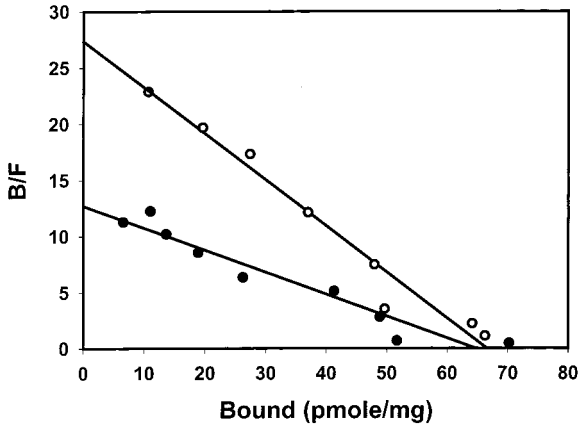


FIGURE 1: Ca^{2+} CaM and apoCaM bind to the same number of sites on RYR1. Scatchard analysis of [^{35}S]CaM binding to SR membranes. SR membranes (10 μg in 200 μL) were incubated with [^{35}S]CaM with increasing concentrations of unlabeled expressed mCaM (0.6−156 nM) for 2 h at room temperature in either 1 mM EGTA, 300 mM NaCl, and 50 mM MOPS (pH 7.4) (low-Ca^{2+} buffer) or 1.2 mM $CaCl_2$, 1 mM EGTA, 300 mM NaCl, and 50 mM MOPS (pH 7.4) (high-Ca^{2+} buffer): (●) [^{35}S]apoCaM binding and (○) Ca^{2+} [^{35}S]CaM binding.

Preparation of Samples for Sequencing. SDS−PAGE gels were cast 24 h before use. Sodium thioglycolate (0.1mM) was added to the upper cathode buffer to protect proteins from free radical oxidation. Following SDS−PAGE, samples were transferred to Immobilon-P-SQ (Millipore Corp.) for 16−18 h at 20 V and 4 °C. The bands were stained with Coomassie Brilliant Blue and excised for sequencing according to the method of LeGendre and Matsudaira (*20*).

N-Terminal Sequencing. Sequencing was performed in the laboratory of R. Cook at Baylor College of Medicine.

RESULTS

^{35}S-Labeled CaM Binds to One Site per Subunit of RYR1 at both High and Low Ca^{2+} Concentrations. Our objective in these experiments was to map the location of CaM binding sites on RYR1. To do this, it is crucial to obtain an accurate determination of the number of CaM binding sites per subunit of RYR1. For these studies, we examined the binding of metabolically labeled [^{35}S]CaM (expressed in bacteria) to SR membranes. We found approximately the same number of binding sites for [^{35}S]apoCaM and Ca^{2+} [^{35}S]CaM (Figure 1 and Table 1), but the affinity was slightly higher in the presence of Ca^{2+}. When compared to the number of ryanodine binding sites, there appeared to be approximately four CaM molecules binding per [^{3}H]ryanodine binding site. Since ryanodine binds to only one site per tetramer, our findings suggest that there are four CaM sites per RYR1 tetramer or one site per subunit.

We (*21*) and others (*2*) have reported that [^{125}I]CaM binds to more sites on RYR1 in nanomolar Ca^{2+} (low Ca^{2+}) than in micromolar Ca^{2+} (high Ca^{2+}). This is in contrast to the binding of the metabolically labeled [^{35}S]CaM that gave the same number of binding sites at high and low Ca^{2+} concentrations. These previous studies (*2*, *21*) were preformed with a CaM isolated from bovine brain and iodinated with Bolton−Hunter reagent. One possible cause for the discrepancy between the brain CaM and the expressed CaM

Table 1: Comparison of Binding Properties of Expressed [^{35}S]CaM and [^{125}I]CaM

CaM	K_D (nM) high Ca^{2+} (Ca^{2+} CaM)	B_{max} (pmol/mg) high Ca^{2+} (Ca^{2+} CaM)	K_D (nM) low Ca^{2+} (apoCaM)	B_{max} (pmol/mg) low Ca^{2+} (apoCaM)	ratio of Ca^{2+} CaM to ryanodine binding sites	ratio of apoCaM to ryanodine binding sites
[^{35}S]CaM ($n = 5$)	2.5 ± 0.5	65 ± 4	4.7 ± 0.4	57 ± 3	3.9 ± 0.2	3.4 ± 0.3
[^{125}I]CaM ($n = 3$)	5.2 ± 0.4	28 ± 6	8.2 ± 1.5	63 ± 4	1.6 ± 0.1	3.7 ± 0.5

is that the brain CaM is binding to more sites than the expressed CaM. Iodination of the expressed CaM with Bolton−Hunter reagent, however, also produced a radioligand preparation that appeared to have fewer binding sites at high than at low Ca^{2+} concentrations (Table 1). These findings suggest that the binding of CaM to RYR1, especially at high Ca^{2+} concentrations, is altered by the iodination of lysines with the Bolton−Hunter reagent. In addition, the expressed CaM can completely inhibit the binding of [^{125}I]CaM from brain (data not shown), suggesting that the brain CaM is not binding to more sites than the expressed CaM.

Both the ApoCaM and Ca^{2+} CaM Sites on RYR1 Are Destroyed by Tryptic Digestion, but Bound CaM Protects the Sites from Tryptic Digestion. We have previously shown that RYR1 in SR membranes was rapidly cleaved by trypsin at six major sites (*8*). In the work described here, we tested the effect of tryptic digestion on the ability of SR membranes to bind [^{35}S]CaM. We found that tryptic digestion at both high and low Ca^{2+} concentrations rapidly destroyed the ability of the membranes to bind both [^{35}S]apoCaM and Ca^{2+} [^{35}S]CaM (Figure 2). The rate of the loss of Ca^{2+} [^{35}S]CaM binding sites with tryptic digestion was approximately the same as the rate of the loss of [^{35}S]apoCaM binding sites regardless of whether the digestion is carried out at high (Figure 2A) or low Ca^{2+} concentrations (Figure 2B). The $t_{1/2}$ for the loss of [^{35}S]apoCaM binding sites by tryptic digestion at low and high Ca^{2+} concentrations was 4.3 ± 0.2 ($n = 5$) and 4.7 ± 0.4 min ($n = 5$), respectively. The $t_{1/2}$ for the loss of Ca^{2+} [^{35}S]CaM sites at low and high Ca^{2+} concentrations was 5.0 ± 0.9 ($n = 4$) and 4.8 ± 0.7 min ($n = 5$), respectively.

Does the loss of the ability to bind [^{35}S]CaM reflect cleavage of the binding site itself or an overall change in the conformation of RYR1? To answer this question, we examined the ability of CaM to protect the sites from tryptic destruction. Either Ca^{2+} CaM or apoCaM, added prior to tryptic digestion, protected [^{35}S]apoCaM binding sites from tryptic destruction (Figure 3A). Likewise, either Ca^{2+} CaM or apoCaM, added prior to tryptic digestion, protected Ca^{2+} [^{35}S]CaM binding sites from tryptic destruction (Figure 3B). Although a 25-fold excess of CaM to CaM binding sites on RyR1 was used in Figure 3, complete protection was obtained with only a 5-fold excess of CaM relative to the number of sites (data not shown).

Identification of the Sites Protected from Tryptic Cleavage by CaM. As shown above, the protection of the CaM binding sites from tryptic destruction can be accomplished by either Ca^{2+} CaM or apoCaM. Since apoCaM activates the channel whereas Ca^{2+} CaM inhibits the channel, it seems unlikely that the protection of the site from cleavage represents an allosteric effect. Also, other studies with tryptic digestion in the presence of nonprotein modulators failed to detect any

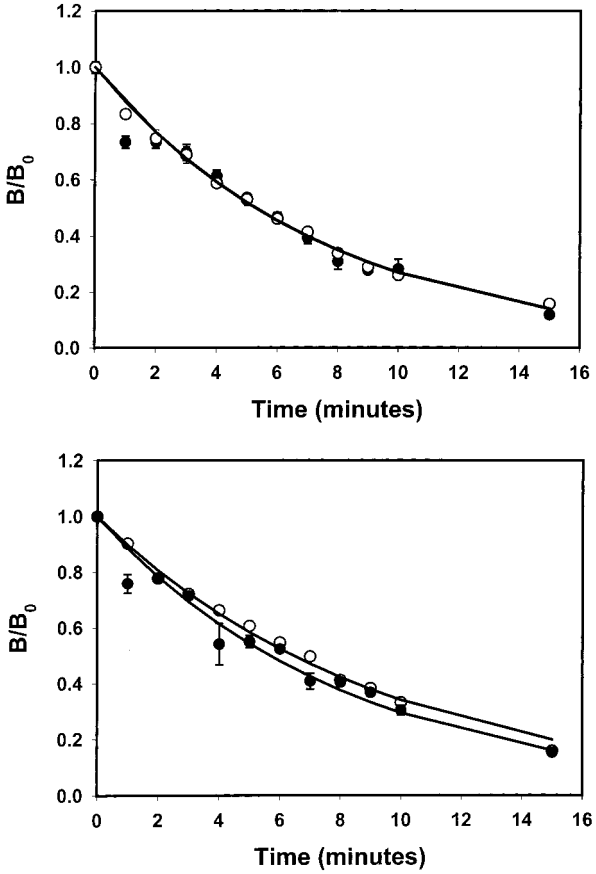


FIGURE 2: Both apoCaM and Ca^{2+} CaM binding sites on RYR1 are rapidly destroyed by trypsin. SR membranes (3.2 mg/sample) were reduced with 0.1 mM DTT for 30 min at room temperature and washed by pelleting in a Beckman airfuge. The samples were resuspended to a concentration of 1.5 mg/mL in either 1.2 mM $CaCl_2$, 1 mM EGTA, 300 mM NaCl, and 50 mM MOPS (pH 7.4) **(**A**)** or 1 mM EGTA, 300 mM NaCl, and 50 mM MOPS (pH 7.4) **(**B**)**. Trypsin (1:1000) was added, and the samples were incubated at 37 °C. At the indicated times, 75 μL aliquots were removed and placed into tubes containing 5 μL of 20 mg/mL soybean trypsin inhibitor and placed on ice. Samples digested at both high and low Ca^{2+} concentrations were then assayed for the ability to bind [^{35}S]CaM at high and low Ca^{2+} concentrations as described in Experimental Procedures: (A) digestion at high Ca^{2+} concentrations, and binding of (●) [^{35}S]apoCaM and (○) Ca^{2+} [^{35}S]CaM, and (B) digestion at low Ca^{2+} concentrations, and binding of (●) [^{35}S]apoCaM and (○) Ca^{2+} [^{35}S]CaM.

differences in the sites of tryptic digestion (unpublished observation). If it is assumed that Ca^{2+} CaM and apoCaM bind to the same region on RYR1, the site protected from tryptic cleavage is most likely close to or within both the apoCaM and Ca^{2+} CaM binding sites. To identify the cleavage site, we treated SR membranes with trypsin in the presence or absence of bound CaM (both apo- and Ca^{2+} CaM) and, after detergent solubilization, purified the trypsin-digested complexes of RYR1 by sucrose gradient centrifugation. The fragments of RYR1 generated by mild tryptic

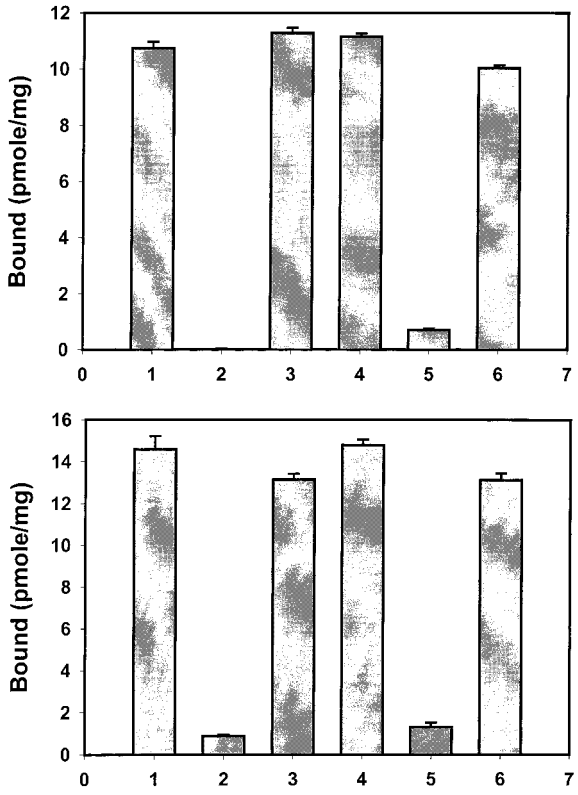


FIGURE 3: Both apoCaM and Ca^{2+} CaM protect the site from trypsin inactivation. SR membranes (1.1 mg/sample) were reduced with 0.1 mM DTT for 30 min at room temperature. Following removal of the DTT by pelleting in a Beckman airfuge, the samples were resuspended in either high-Ca^{2+} or low-Ca^{2+} buffer. CaM was added to the indicated samples to a final concentration of 1 μM. Trypsin (1:1000) was added to the indicated samples, and these samples were incubated for 10 min at 37 °C. The proteolysis was stopped by the addition of 5 μL of 20 mg/mL STI. Samples were washed twice by pelleting for 5 min at 30 psi in a Beckman airfuge. To remove bound CaM, the first pellet was resuspended in high-Ca^{2+} buffer while the second was resuspended in low-Ca^{2+} buffer. Samples were then assayed for [^{35}S]CaM (5 nM) binding at high and low Ca^{2+} concentrations. (A) Bars 1−3 are for [^{35}S]apoCaM binding to samples digested at low Ca^{2+} concentrations, and bars 4−6 are for [^{35}S]apoCaM binding to samples digested at high Ca^{2+} concentrations. Samples whose data are depicted in bars 1 and 4 were not treated with trypsin, and samples whose data are depicted in bars 2 and 5 were treated with trypsin; the sample whose data are depicted in bar 3 was preincubated with apoCaM prior to trypsin treatment, and the sample whose data are depicted in bar 6 was preincubated with Ca^{2+} CaM prior to trypsin digestion. (B) Lanes 1−3 show Ca^{2+} [^{35}S]CaM binding to samples digested at low Ca^{2+} concentrations, and lanes 4−6 show Ca^{2+} [^{35}S]CaM binding to samples digested at high Ca^{2+} concentrations. Samples whose data are depicted in bars 1 and 4 were not treated with trypsin, and samples whose data are depicted in bars 2 and 5 were treated with trypsin; the sample whose data are depicted in bar 3 was preincubated with apoCaM prior to trypsin treatment, and the sample whose data are depicted in bar 6 was preincubated with Ca^{2+} CaM prior to trypsin digestion.

digestion remain together in a rapidly sedimenting complex (*8*). The fragments obtained when the digestion was performed at high or low Ca^{2+} concentrations and in the presence or absence of bound CaM are shown in Figure 4. A major difference in the fragmentation pattern was seen with CaM bound to RYR1. In the absence of CaM, a 95 kDa band was detected that was not seen when the digestion was carried out in the presence of CaM. In contrast, a 160 kDa band was found only when CaM was bound at the time of tryptic digestion. The protection of this 160 kDa band

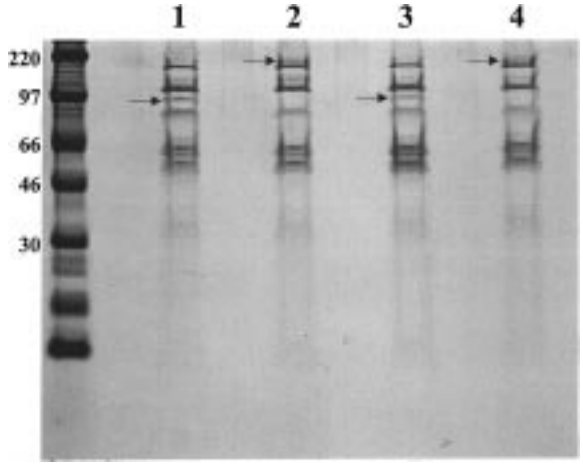


FIGURE 4: Protected cleavage sites are after amino acids 3630 and 3637. SR membranes (34 mg, reduced and washed) were resuspended in 11 mL of low-Ca^{2+} buffer. This sample was then divided into four aliquots of 2.5 mL. Ca^{2+} was added to a final concentration of 1.2 mM to samples 1 and 2. CaM was added to a final concentration of 20 μM to samples 2 and 4, and all samples were incubated for 30 min at room temperature. Digestion with trypsin was performed with a 1:1000 trypsin-to-protein ratio. Solubilization of the membranes and isolation of the proteolytic complexes were carried out as described in Experimental Procedures. Purified proteolytic complexes (20 μg) were electrophoresed on Schagger gels, and the gels were stained with Coomassie Brilliant Blue. The 160 and 95 Da fragments are identified with arrows: lane 1, RYR1 fragments isolated after tryptic digestion at high Ca^{2+} concentrations; lane 2, RYR1 fragments isolated after tryptic digestion of RYR1 with Ca^{2+} CaM bound prior to digestion; lane 3, RYR1 fragments after trypsin digestion at low Ca^{2+} concentrations; and lane 4, RYR1 fragments isolated after trypsin digestion of RYR1 with apoCaM bound prior to digestion.

was seen with both Ca^{2+} CaM and apoCaM. The 160 kDa band was composed of two fragments with N-terminal sequences of TQVKGVGQNL and GVGQNLTY, indicating two cleavages, four amino acids apart, after amino acids 3119 and 3123. From the apparent size of these bands, these fragments probably extend to tryptic cleavage sites around residue 4475 (*8*). The 95 kDa band found in the absence of CaM during the cleavage also had two N-terminal sequences, AVVACFRMTPL and MTPLYNLP, representing cleavages at residues 3630 and 3637 and are likely to represent the C-terminal region of the 160 kDa fragment. The cleavage sites protected by CaM are, therefore, at amino acids 3630 and 3637, and the 95 kDa fragment is the C-terminal fragment arising from cleavage of the 160 kDa fragment at these sites.

DISCUSSION

The Ca^{2+} release channel in skeletal muscle is regulated by the transverse tubular voltage sensor, the binding of Ca^{2+}, and interactions with a number of modulators. Ca^{2+} CaM is an inhibitor of the skeletal muscle Ca^{2+} release channel, while apoCaM is a partial agonist. These findings suggest that both the response to the voltage sensor and the Ca^{2+} dependence of the channel could be regulated by CaM. The opposing functional effects of CaM in its Ca^{2+}-bound and Ca^{2+}-free states could occur because (1) two forms of CaM bind to different sites on RYR1 to regulate its function in different ways or (2) CaM binds to a site on RYR1 over a range of Ca^{2+} concentrations with Ca^{2+} binding to the bound CaM, altering its conformation and its effect on RYR1 activity.

Knowledge of the location of the apoCaM and Ca^{2+} CaM binding sites on RYR1 would help in the discrimination of these two possibilities.

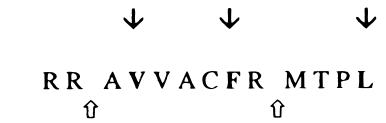
Although previous reports from our laboratory (*21*) and others (*2*) have suggested the existence of multiple binding sites for apoCaM per subunit of RYR1 and one site per subunit for Ca^{2+} CaM, the studies presented here in which metabolically labeled [^{35}S]CaM was used are consistent with one apoCaM and one Ca^{2+} CaM binding site per subunit. The previously identified difference in the number of binding sites for apoCaM and Ca^{2+} CaM appears to be due to an artifact arising from the use of Bolton-Hunter ^{125}I-labeled CaM. This reagent reacts with lysines, potentially producing a mixture of CaMs (CaMs iodinated at different lysines, and unmodified CaM). CaMs modified at different lysines could have different affinities for RYR. The modification of lysines on CaM has been previously suggested to alter its interactions with some targets (*22*−*24*). In our situation, the modifications appear to differentially alter the ability of CaM to bind to RYR1 at high Ca^{2+} concentrations. Since the expressed CaM can completely displace the [^{125}I]CaM, it is unlikely that [^{125}I]CaM is binding to more sites on RYR1 than [^{35}S]CaM. We suggest that Bolton−Hunter ^{125}I-labeled CaM be used for qualitative analysis of the interaction of CaM with RYR1 but not for quantifying the number of binding sites.

A possible difference between expressed CaM and CaM isolated from brain, which could produce changes in affinity or specificity, is the absence of post-translational modifications in the expressed CaM. We are currently assessing the possibility that these changes [N-terminal acetylation (*25*) or trimethylation of lysine 115 (*26*)] alter the interaction of CaM with RyR1.

We propose that Ca^{2+} CaM and apoCaM bind to the same or overlapping regions on RYR1. Evidence for this is as follows: (1) Ca^{2+} [^{35}S]CaM and [^{35}S]apoCaM have the same number of binding sites on RYR1, (2) trypsin destroys both binding sites at the same rate, (3) both apoCaM and Ca^{2+} CaM bound to RYR1 can protect the Ca^{2+} CaM site from tryptic inactivation, (4) both apoCaM and Ca^{2+} CaM bound to RYR1 can protect the apoCaM site from tryptic inactivation, and (5) both Ca^{2+} CaM and apoCaM bound to RYR1 prevent tryptic cleavage at the same sites.

The ability of CaM bound to RYR1 to protect its binding site from tryptic inactivation provides us with the tool for mapping the location of the CaM binding site and for comparing this site to CaM binding sites on other proteins. CaM regulates most of its targets in its Ca^{2+}-bound form. These target proteins recognize hydrophobic regions exposed on Ca^{2+} CaM via a basic amphiphilic α-helix, or BAA motif (*27*, *28*). These motifs have a large number of positively charged and hydrophobic residues, but few Asp or Glu residues. The BAA Ca^{2+} CaM binding motifs are further divided into two classes known as the 1-8-14 and the 1-5-10 motifs, where the numbers indicate the position of a hydrophobic residue [for a review, see Rhoads (*29*)]. ApoCaM can also interact and regulate target proteins such as iNOS (*30*, *31*), syntrophin (*32*), and neuromodulins (*33*). Many targets for apoCaM have a common motif, known as an IQ or modified IQ motif. This motif is represented by a primary structure of IQXXXRGXXXR that assumes α-helical structure. There are a number of possible binding sites for CaM on RYR1. Sequence analysis has suggested that there are potential Ca^{2+} CaM binding sites on RYR1 at amino acids 2807−2840, 2909−2930, 3031−3049, 3614−3637, and 4295−4325 (*4*, *5*). In addition, an apparent IQ domain similar to that found in the IQ domain in connexin (*29*) is found beginning at amino acids 1613−1624 on RyR1.

Using cryoelectron microscopy and three-dimensional image reconstruction, Wagneknecht et al. (*34*) have shown that CaM appears to bind in the large cytoplasmic domain of RYR1. Chen and MacLennan (*6*), using RYR1 fusion proteins, [^{125}I]CaM, and protein overlays, suggested that Ca^{2+} CaM binding sites are located at amino acids 2063−2091, 3611−3642, and 4303−4328. Buratti et al. (*35*) localized one of the sites that binds CaM at low Ca^{2+} concentrations to a fusion protein containing the sequence of residues 3010−3225. Also using fusion proteins, Menegazzi et al. (*36*) suggested that the Ca^{2+} CaM site is located between residues 3546 and 3655. Our data show that both the Ca^{2+} CaM and apoCaM can protect sites at amino acids 3630 and 3637 on RYR1 from digestion by trypsin. Protection of the site from trypsin digestion could arise from CaM bound at or near this site, or it could arise from a CaM-induced conformational change that buries this site. Although we cannot totally eliminate the possibility of an allosteric effect, this seems unlikely given the ability of both a channel activator (apoCaM) and a channel inhibitor (Ca^{2+} CaM), which presumably produce completely different conformational changes in RYR1, to protect the same sites from trypsin cleavage. These protected sites are within a potential Ca^{2+} CaM site identified by Takeshima et al. (*35*), using sequence analysis, and by both Chen and MacLennan (*6*) and Menegazzi et al. (*37*), using fusion proteins matching RYR1 sequences and [^{125}I]CaM overlays. The sequence is within the following predicted Ca^{2+} CaM binding site (amino acids 3630−3649):



The open arrows denote the trypsin cleavage sites protected by CaM, and the closed arrows denote hydrophobic residues, identifying this sequences as a 1-5-10 Ca^{2+} CaM binding motif. Although this could be a Ca^{2+} CaM binding site, it is less likely to be an apoCaM binding site. One possibility is that apoCaM protects this site sterically rather than by direct binding. The actual binding site for apoCaM could involve different amino acids in close proximity to this site. The ability of *N*-ethylmaleimide to block apoCaM binding but not Ca^{2+} CaM binding supports differences in the binding sites (*21*). Since both apoCaM and Ca^{2+} CaM can protect RYR1 from oxidation-induced intersubunit cross-linking (*21*), the protected cleavage sites could be located at a site of contact between two adjacent subunits. In this situation, the apoCaM binding site could even be located on the adjacent subunit, possibly at the IQ domain beginning at amino acid 1613.

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