Both apocalmodulin (Ca\(^{2+}\)-free calmodulin) and Ca\(^{2+}\)-calmodulin bind to and regulate the activity of skeletal muscle Ca\(^{2+}\) release channel (ryanodine receptor, RyR1). Both forms of calmodulin protect sites after amino acids 3630 and 3637 on RyR1 from trypsin cleavage. Only apocalmodulin protects sites after amino acids 1975 and 1999 from trypsin cleavage. Ca\(^{2+}\)-calmodulin and apocalmodulin both bind to two different synthetic peptides representing amino acids 3614–3643 and 1975–1999 of RyR1, but Ca\(^{2+}\)-calmodulin has a higher affinity than apocalmodulin for both peptides. Cysteine 3635, within the 3614–3643 sequence of RyR1, can form a disulfide bond with a cysteine on an adjacent subunit within the RyR1 tetramer. The second cysteine is now shown to be between amino acids 2000 and 2401. The close proximity of the cysteines forming the intersubunit disulfide to the two sites that bind calmodulin suggests that calmodulin is binding at a site of intersubunit contact, perhaps with one lobe bound between amino acids 3614 and 3643 on one subunit and the second lobe bound between amino acids 1975 and 1999 on an adjacent subunit. This model is consistent with the finding that Ca\(^{2+}\)-calmodulin and apocalmodulin each bind to a single site per RyR1 subunit (Rodney, G. G., Williams, B. Y., Strasburg, G. M., Beckingham, K., and Hamilton, S. L. (2000) Biochemistry 39, 7807–7812).

The skeletal muscle Ca\(^{2+}\) release channel (also known as the ryanodine receptor, RyR1) is a homotetramer containing four 565-kDa subunits; four-fifths of the molecule is cytoplasmic, and the remaining one-fifth is membrane-spanning and/or luminal to the sarcoplasmic reticulum (SR).\(^1\) Ca\(^{2+}\) and several endogenous proteins that bind within the large cytoplasmic domain modulate the activity of RyR1. In the muscle fiber, these endogenous modulators probably act together to regulate RyR1 channel activity and, ultimately, Ca\(^{2+}\) release from the SR. One of these modulators, calmodulin (CaM), binds to RyR1 at both nanomolar and micromolar Ca\(^{2+}\) concentrations (1). Apocalmodulin (apoCaM) is a weak partial agonist, whereas Ca\(^{2+}\)-calmodulin is an inhibitor of RyR1 (1). CaM in both its Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free forms binds to one site per RyR1 subunit (2).

ApoCaM and Ca\(^{2+}\)-CaM bound to RyR1 protect sites after amino acids 3630 and 3637 from trypptic digestion (3), suggesting that this sequence may form part of both the apoCaM and Ca\(^{2+}\)-CaM binding sites. Evidence that the Ca\(^{2+}\)-apoCaM and apoCaM binding sites are overlapping includes the following: (a) apoCaM and Ca\(^{2+}\)-CaM have the same number of binding sites (2); (b) the binding sites for both apoCaM and Ca\(^{2+}\)-CaM are destroyed by trypsin at the same rate (3); (c) the two forms of CaM protect each other's binding sites from trypsic destruction (3); (d) the protected cleavage sites within the 3614–3643 region are the same in high and low Ca\(^{2+}\); (e) the binding of [\(^{35}\)S]E1234Q (a mutant CaM that cannot bind Ca\(^{2+}\) at any of the four Ca\(^{2+}\) binding sites) is completely inhibited by Ca\(^{2+}\)-CaM (2); (f) no cooperativity in binding has been detected; (g) mutations W3620A or L3624A in RyR1 greatly decrease the affinity of both apoCaM and Ca\(^{2+}\)-CaM binding for RyR1 (4); (h) the 3614–3643 peptide binds the C-lobe but not the N-lobe of both apoCaM and Ca\(^{2+}\)-CaM (5); and (i) the interaction of 3614–3643 with CaM increases the Ca\(^{2+}\) affinity only of the C-lobe of CaM (5). These findings support a model in which the C-lobe of both apoCaM and Ca\(^{2+}\)-CaM binds to the sequence between amino acids 3614–3643. The C-lobe binding sites for apoCaM and Ca\(^{2+}\)-CaM are overlapping, nonidentical sites, and CaM shifts N-terminally within the site when Ca\(^{2+}\) binds to the C-lobe (2). The binding of Ca\(^{2+}\) to the C-lobe of CaM is responsible for its conversion from an activator to an inhibitor of RyR1 (2).

Samso and Wagenknecht (6) have localized the binding site for CaM in the three-dimensional structure of RyR1. They found that the two forms of CaM bind on the sides of the cytoplasmic domain of RyR1 to overlapping locations with centers separated by 33 Å. This large translocation, especially in light of a common C-lobe binding site (2, 3, 5), suggests that the binding sequence is moving a relatively large distance when the channel changes its conformation in response to CaM and Ca\(^{2+}\) and/or there is a major movement of one lobe of calmodulin when Ca\(^{2+}\) binds to both CaM and RyR1.

Additional information about the location of the CaM binding site in the RyR1 tetramer has come from oxidation studies. An intersubunit disulfide bond can be formed by treating RyR1 with oxidizing agents (7–9), and the formation of this cross-link is blocked by bound Ca\(^{2+}\)-CaM or apoCaM (7). These data suggest that the CaM binding site may be localized to a site of intersubunit contact. Consistent with this, one of the cysteines involved in this cross-link is found within the amino acid 3614–3643 binding site for the C-lobe of CaM (10).

\(^*\) This work is supported by grants from the Muscular Dystrophy Association and NIAMS, National Institutes of Health, Grants AR41802 and AR41729 (to S. L. H.). 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: SR, sarcoplasmic reticulum; CaM, calmodulin; apoCaM, apocalmodulin; Ca\(^{2+}\)-CaM, Ca\(^{2+}\)-calmodulin; DTT, dithiothreitol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MOPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; CAPS, 3-cyclohexylamino)propanesulfonic acid; Ab, antibody.
These findings raise the possibility that the CaM could be interacting simultaneously with two subunits in the RYR1 tetramer. Since the C-lobe of CaM appears to bind primarily within the 3614-3643 sequence, the site on the second subunit might represent an interaction site for the N-lobe of CaM. We have demonstrated the importance of the N-lobe in binding and regulation of RYR1 using N-terminal CaM mutants (5). The N-lobe of CaM appears to bind at an unidentified location distinct from that of the C-lobe (5). We now identify a new sequence that could be the binding site for the N-lobe of CaM on an adjacent subunit in the RYR1 tetramer.

EXPERIMENTAL PROCEDURES

Materials
Dithiothreitol (DTT), 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfoninate (CHAPS), 3-(N-morpholino)propanesulfonic acid (MOPS), 3-(cyclohexylaminol)-1-propane sulfonic acid (CAPS), bovine brain calmodulin, and bovine serum albumin were obtained from Sigma. Amplify and [3H]-ethymaleimide were obtained from Amer sham Biosciences. All antibodies except Ab 5029 were prepared in rabbit immunized with synthetic peptides coupled to keyhole limpet hemocyanin. Immunizations were performed either by our laboratory or by Pelfreeze Laboratories (Rogers, AR) using Freund’s complete adju vant, and the antibodies were purified as we have previously described (8). Ab 5029 was graciously provided by Dr. Andrew Marks (Columbia University, College of Physicians and Surgeons). E1234Q CaM was graciously provided by Dr. Kathleen Beckham (Rice University).

Methods
SR Membrane Preparation—SR membranes were prepared as previously described (11) from rabbit hind leg and back strap white skeletal muscle and purified using sucrose gradient centrifugation.

Expression of (N+3)CaM—(N+3)CaM was expressed and purified as described by Xiong et al. (5).

Generation of Trypsin-digested Complexes of RYR—SR membranes (10 mg of protein/ml) in 300 mM NaCl, 50 mM MOPS (pH 7.4), 100 mM CaCl2, were treated with trypsin for 1–5 min at 37 °C at a trypsin/protein ratio of 1:1000. Digestion was halted by the addition of a 10-fold excess of soybean trypsin inhibitor. After solubilization in 2% CHAPS, proteolysed complexes were purified over either our laboratory or by Pelfreeze Laboratories (Rogers, AR) using Freund’s complete adju vant, and the antibodies were purified as we have previously described (8). Ab 5029 was graciously provided by Dr. Andrew Marks (Columbia University, College of Physicians and Surgeons). E1234Q CaM was graciously provided by Dr. Kathleen Beckham (Rice University).

Protein Sequencing—Proteins were prepared for sequencing as described previously (12). N-terminal sequencing was performed in the Core Protein Facility by Dr. Richard Cook at Baylor College of Medicine.

SDS-PAGE—Polyacrylamide gel electrophoresis was performed as described by Laemmli (13) or Scha fer and von Jagow (14). Two-dimensional SDS-PAGE—the purified proteolytic complex was compared by SDS-PAGE. The peptides with CaM by nondenaturing gel electrophoresis was performed in the presence of apoCaM (Fig. 1, A, lanes a and b, and B, lanes a and b). To aid in the visualiztion of the protected fragments, the purified proteolytic complexes were also labeled with [3H]-ethy lamide and visualized by fluorography (Fig. 1A, lanes c and d, and Fig. 2, lanes c and d). Protected bands that we have previously identified (3) are clearly seen in these gels, and, in addition, a new 71-kDa band is seen in the absence but not in the presence of apoCaM.

We routinely use two different one-dimensional SDS-PAGE systems to analyze the proteolytic fragments: (a) the gel system of Schagger and von Jagow (14) for separation of smaller fragments (5 and 6) and (b) 5% Laemmli gels (13) for separation of larger fragments. In the Schagger and von Jagow gels, large fragments are difficult to separate. The separation of these highly molecular weight fragments into multiple bands is more clearly achieved in the Laemmli gels (Fig. 1C). However, smaller fragments are best differentiated in the Schagger and von Jagow gels (Fig. 1A). Also, fragments with molecular masses less than ~40 kDa run with the dye front on the 7.5% Laemmli gels.

The identity of the tryptic fragments was determined by Western blotting with sequence-specific antibodies (Fig. 3 and Table I) and with N-terminal sequencing (Table II). The tentative assignment of the C-terminal amino acid for each of the fragments is based on the apparent size and our previously determined tryptic digestion sites (12). To assess precursor/product relationships, the intensities of the bands were analyzed by densitometry (Fig. 1C). Consistent with our previous findings, a cleavage at amino acid 3630 and/or 3637 converts fragment 1 (apparent molecular mass of 170 kDa, N-terminal amino acid 3120) to fragment 6 (~128 kDa, N-terminal amino acid 3120). Fragment 6 is found in variable amounts from preparation to preparation. In reduced membranes, fragment 6 is rapidly converted to fragment 9, but this cleavage is slowed if the membranes are oxidized (see below). Fragment 2 (~158 kDa, also beginning with amino acid 3120) is directly converted to fragment 9 (~110 kDa, beginning with amino acid 3631). Both CaM and apoCaM protect the sites after arginine 3630 and 3637 from trypsin cleavage (3) (Figs. 1 and 2).

A second site protected by apoCaM is also seen (Fig. 1A). When the digestion was performed at <10 μM Ca2+ concentrations, fragment 12 is found in samples digested in the absence but not in the presence of CaM (Fig. 1A, lane a versus lane b). N-terminal amino acid sequencing of this band identified it as beginning with amino acid 1509. To determine whether there is another site protected by apoCaM, we looked for both the precursor of this fragment at a higher molecular weight and the other part of a larger precursor fragment at a lower molecular weight. The fluorogram shows that the broad band around 110–125 kDa appears to be even broader when the digestion was performed in the presence of apoCaM (Fig. 1A, lanes b and d). We find a fragment with an apparent molecular mass of about 123 kDa (fragment 7) with an N-terminal sequence beginning with amino acid 1509. Furthermore, there is at least three times more of this fragment (determined by N-terminal sequencing) in the 110–120-kDa band if apoCaM is present during the proteolysis compared with that found in the absence
A final concentration of 5 μM H9262 aliquots of the RYR1 peak were incubated with 5 μM additional 30 min. Trypsin (10 μM) the same as in the experiment described in Fig. 1 was repeated using 5 μM CaM (lane b) or 5 μM (N+3) CaM (lane c) for protection. Lane a shows the proteolytic pattern obtained in the absence of CaM. Samples were electrophoresed on Schägger gels. Molecular mass markers are shown to the left and are the same as in Fig. 1A. Lanes d–f show the autoradiograms of samples a–c.

**FIG. 2.** Effect of (N+3)apoCaM on the digestion patterns. The experiment described in Fig. 1 was repeated using 5 μM CaM (lane b) or 5 μM (N+3) CaM (lane c) for protection. Lane a shows the proteolytic pattern obtained in the absence of CaM. Samples were electrophoresed on Schägger gels. Molecular mass markers are shown to the left and are the same as in Fig. 1A. Lanes d–f show the autoradiograms of samples a–c.

**TABLE I**

| Sequence | Amino acids | Fragments recognized |
|----------|-------------|---------------------|
| 2391–2410 | ARDPGPGVRRRDRREHGFEEP | 5, 7, 10, 14, 16 |
| 2727–2742 | ATVDAGNFDPRPVET | 15 |
| 4196–4208 | SETNRAQWEMP | 1, 2, 3, 4, 6, 9 |
| 5029–5037 | RRQYEDQLS | 11 |

of CaM. Fragment 7 but not fragment 12 was recognized by an antibody to amino acid 2391 (Fig. 4). These findings suggest that apoCaM protects a site within fragment 7 from trypsin cleavage. The size of the piece removed from fragment 7 to form fragment 12 should be around 50 kDa, and, indeed, bands in the 50–kDa region were recognized by Ab 2391 (Fig. 3). We sequenced the broad 50-kDa band and found that it is composed of multiple fragments, including a sequence beginning with amino acid 1983 (fragment 14); a sequence beginning with either amino acid 2402, 2403, or 2404 (fragment 15, carboxy-terminal end with a jagged cut); and smaller and variable amounts of a fragment beginning with amino acid 2000 (fragment 16). In some preparations, there were also small amounts of a fragment of the sarcoplasmic reticulum Ca2+ ATPase, SERCA 1, in this band. When CaM was present during the proteolysis very little of either fragment 14 or 16 could be detected in the ~50-kDa band by N-terminal amino acid sequencing, suggesting that these two fragments are derived from cleavage of fragment 7. The appearance of the fragment 14 with an N-terminal sequence beginning after arginine 1982 appears to correlate most closely with the appearance of fragment 12 and the disappearance of fragment 7, suggesting that it represents the first cleavage. The cleavage at 1999 may represent a second protected site, or it may arise as a secondary cleavage after the primary cut at arginine 1982.

A Functionally Inactive ApoCaM (N+3)CaM Also Protects the 1982 Site from Tryptic Cleavage—ApoCaM is a weak partial agonist of the channel. We found that the addition of 3 amino acids to the N terminus of CaM produces a mutant CaM (designated (N+3)CaM) that binds with higher affinity than wild-type apoCaM but does not activate the channel (5). The cleavage of the fragment 7 to fragments 12 and 14 is also protected by (N+3)CaM (Fig. 2), suggesting that this represents a direct protection of this site from proteolysis rather than a protection arising allosterically.

Ca2+ CaM Does Not Protect the Arginine 1982 Site from Tryptic Cleavage—The presence of Ca2+ during the proteolysis with
Samples were electrophoresed on Schägger isolation, 100–300 mM NaCl, 50 mM MOPS (pH 7.4). The proteolytic complexes were bound RYR1.

Three concentrations were analyzed on both Schägger and Jaggow gels. Coomassie-stained gel was used. Lane a is again the digestion pattern obtained at high Ca\(^{2+}\) in the absence of CaM. C, densitometer analysis of the 58-kDa band from three independent experiments as performed as described for B, *p < 0.01.

trypsin does not greatly alter the tryptic digestion pattern. When we examined the effect of CaM on the proteolysis patterns at high Ca\(^{2+}\) concentrations (Fig. 3A), we found that fragment 12 is present in the proteolytic complexes prepared both with and without CaM (Fig. 3A, lane a versus lane b), indicating that Ca\(^{2+}\)-CaM did not prevent the cleavage after arginine 1982. Ca\(^{2+}\)-CaM did protect the previously described 158-kDa band (fragment 2) to 110-kDa (fragment 9) conversion (a cleavage after amino acid 3630/3637).

E1234QCaM, a Mutant CaM That Does Not Bind Ca\(^{2+}\), Protects the Site at Arginine 1982 from Tryptic Cleavage on the Ca\(^{2+}\)-bound RYR1—We have demonstrated a cleavage site that is protected by apoCaM on the Ca\(^{2+}\)-free RYR1, raising the question of whether the protection is due to the conformation of CaM or RYR1 or both. A mutant CaM that cannot bind Ca\(^{2+}\) at any of the four Ca\(^{2+}\) binding sites (E1234Q) was tested for the ability to protect the 1982 cleavage site on the Ca\(^{2+}\)-bound RYR1. Fig. 3B shows that the conversion of fragment 7 to fragments 12 and 14 occurs in high Ca\(^{2+}\) in the presence and absence of Ca\(^{2+}\)-CaM (Fig. 3B, lanes a and b). The presence of E1234Q, however, partially prevents this conversion (Fig. 3B, lane c, and C). We conclude that Ca\(^{2+}\)-free CaM can protect the 1982 cleavage site on the Ca\(^{2+}\)-bound RYR1, indicating that the protection has more to do with the conformation of CaM than any Ca\(^{2+}\)-driven changes in RYR1.

Other Tryptic Fragments of RYR1 Were Identified with N-terminal Sequencing and Western Blotting with Sequence-specific Antibodies—Other tryptic fragments of RYR1 were identified in these experiments and are listed in Table I. These were all consistent with the fragments that we had previously identified (12). Western blotting with four different antipeptide antibodies is shown in Fig. 4.

A Synthetic Peptide Binds Ca\(^{2+}\)-CaM—In an attempt to identify the amino acids involved in binding CaM, we made a series of synthetic peptides corresponding to the RYR1 sequence around the protected cleavage site (Table III). These peptides were screened for ability to bind to CaM at both high and low Ca\(^{2+}\) concentrations. In this gel system, the peptide alone does not enter the gel due to its positive charge. The complex of the peptide and CaM can be seen above the free CaM band, and the extent of interaction was assessed by the disappearance of the free CaM band. Representative gels using low and high Ca\(^{2+}\) concentrations are shown in Fig. 5, A and B, respectively. The summarized data (IUC, the ratio of intensity of the CaM band in the presence of the peptide compared with that of CaM alone) from the densitometric analysis of the CaM band at low and high Ca\(^{2+}\) with increasing peptide/CaM ratios are shown in Fig. 5C. By non-denaturing gel electrophoreses, only one peptide, 1975–1999, binds CaM. 1975–1999 binds Ca\(^{2+}\)-CaM with an affinity comparable with the 3614–3649 peptide (16). ApoCaM (Fig. 5) also binds to the 1975–1999 peptide but with lower apparent affinity. Other methods to detect the interaction (pull-down assays with bionylated 1975–1999 and streptavidin beads, and tryptophan fluorescence with a tryptophan substituted for the 1978 phenylalanine) also show a weak interaction of the peptide with apoCaM (data not shown). These findings suggest that although Ca\(^{2+}\)-CaM binds to the 1975–1999 sequence, its binding does not protect the 1982/1999 cleavage sites. In contrast, apoCaM binds more weakly to this sequence but does protect the cleavage site. There are a number of possible explanations for the different abilities of Ca\(^{2+}\)-CaM and apoCaM to protect the 1982/1999 cleavage sites: (a) both apoCaM and Ca\(^{2+}\)-CaM bind to this sequence, but Ca\(^{2+}\)-CaM does not protect the site (perhaps due to its conformation or to a slightly shifted interaction site); (b) apoCaM may bind at a different site on RYR1 and protect the cleavage site either sterically or allosterically; (c) the conformational change that occurs in RYR1 as a result of the inhibitory action of Ca\(^{2+}\)-CaM may increase the exposure of the 1983/1999 cleavage site; or (d) the binding affinities to the peptides do not accurately reflect the affinities of these regions for CaM in the native protein. Whether the 1975–1999 synthetic peptide binds only the N-lobe of CaM is not yet known.

Both CaM Binding Sites Are Close to a Site of Intersubunit Cross-linking—Cysteine 3635, located within the binding site for the C-lobe of CaM, can be cross-linked to a cysteine located on an adjacent subunit within the RYR1 tetramer (10). This cross-linking occurs in both 200 μM Ca\(^{2+}\) and at less than 10 nM Ca\(^{2+}\) concentrations (Fig. 6) and is blocked by apoCaM and Ca\(^{2+}\)-CaM (3, 7, 10). The close proximity of a neighboring subunit to the C-lobe binding site raises the question of whether the N-lobe of CaM is binding to a different subunit from the one that binds the C-lobe. To address this question, we cross-linked RYR1 with the disulfide-inducing agent diamide and treated the membranes with trypsin. After stopping the proteolysis with soybean trypsin inhibitor, the membranes were solubilized in CHAPS, and the RYR1 proteolytic complex was purified. The proteolytic patterns under both reducing and nonreducing conditions were analyzed on both Schägger and von An Intersubunit Binding Site for Calmodulin on RYR1

![Figure 3](image-url)
Table II

| Fragment | Mass (kDa) | N-terminal sequence | RYR1 sequence | Predicted mass | Ca\(^{2+}\) | Ca\(^{2+}\) + CaM | Ca\(^{2+}\) | Ca\(^{2+}\) + CaM |
|----------|------------|---------------------|---------------|---------------|------------|----------------|------------|----------------|
| 1        | 170        | TQVKGYGNQ          | 3120–4758     | 182           | –          | –              | –          | +              |
| 2        | 158        | TQVKGYGNQ          | 3120–4476     | 151           | –          | –              | –          | –              |
| 3        | 151        | ND                  | 3120–7        | 100           | +          | +              | +          | +              |
| 4        | 140        | ND                  | 1396–2401     | 113           | –          | –              | –          | –              |
| 5        | 133        | ND                   | Possibly      | 1396–2401     | 113        | –              | –          | –              |
| 6        | 128        | AVVAXFRM            | 3631–4758     | 124           | +          | +              | +          | +              |
| 7        | 123        | ISHTDLVIG           | 1509–2401     | 100           | –          | –              | –          | –              |
| 8        | 114        | GSGPPACPAL          | 426–1508      | 100           | +          | +              | +          | +              |
| 9        | 110        | AVVAXFRM            | 3631–4475     | 93            | +          | +              | +          | +              |
| 10       | 97         | ND                   | serca         |              |            |                |            |                |
| 11       | 88         | KLGVDGEEEE          | 4476–5037     | 64            | +          | +              | +          | +              |
| 12       | 71         | ISHTDLVIG           | 1509–1982     | 56            | +          | ±              | +          | +              |
| 13       | 61         | ND                   | serca         |              |            |                |            |                |
| 14       | 52         | APTMSAET            | 1982–2401     | 47            | +          | ±              | +          | +              |
| 15       | 50         | RGEPEEPE            | 2402–2840     | 50            | +          | +              | +          | +              |
| 16       | 49         | SPPQEQNML           | 2000–2401     | 45            | +          | ±              | +          | +              |
| 17       | 44         | TQVKGYGNQ           | 3120–3500     |              | ND         | ND             | ND         | ND             |

\(^{a}\) ND, not determined.

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Fig. 4. Identification of fragments using Western blotting. Samples prepared as described in the legends to Figs. 1 and 2 were electrophoresed on either Schägger and von Jagow or 7.5% Laemmli gels and transferred to Immobilon. Western blotting was performed as described under “Experimental Procedures.” A, on the left are the molecular weight standards, which are the same as those listed in Fig. 1. In all sections, lane a represents the sample obtained in the absence of CaM, and lane b represents the sample obtained in the presence of CaM. A, Schägger and von Jagow gel results; B, Laemmli gel results. Section 1, Coomassie-stained gel; section 2, Coomassie-stained transferred sample on Immobilon; section 3, Western blots with Ab 2391; section 4, Western blot with Ab 2727; section 5, Western blot with Ab 4198; section 6, Western blot with Ab 5029. The arrow in section 3 shows the position of band 10, which is a minor band that is also affected by the presence of CaM during the proteolysis. The identity of this band is not known.

Jaggow (Fig. 7A, lanes a and b) and Laemmli gels (Fig. 7B, lanes a and b). A number of minor bands at high molecular weight can be seen in the unreduced sample. Differences between the reduced and unreduced samples include the following. (a) There are several new bands in the 150–160-kDa region; (b) there is a decrease in the intensity of the 50-kDa band (containing fragments 14, 15, and 16); and (c) a band with an apparent molecular mass of 61 kDa (fragment 13), which is a fragment of SERCA 1 beginning with amino acid 208, undergoes a mobility shift as indicated by the diagonal arrow between lanes a and b in panels A and B. A Western blot of a 7.5% gel with antibodies 2391 and 2727 shows the fragment containing the “2391 sequence” but not the “2727 sequence” may be involved in formation of higher molecular weight complexes. The data obtained in this experiment suggest that the RYR1 fragments that are most likely to be involved in the cross-linking are fragments 1, 2, and 9 and a component the 50-kDa band.

To confirm the identity of the fragments involved in cross-linking, the cross-linked proteolytic complexes were analyzed using the two-dimensional gel electrophoresis approach first described by Wang and Richards (17). In a previous study using this approach with a calpain-digested and purified RYR1 complex, we localized the second cysteine to a region between amino acids 1400 and 2100 (8). We have also found that all off-diagonal spots can be eliminated by preincubation of the membranes with CaM prior to cross-linking. Our data (3, 7–10, 12) are consistent with a single major inter-subunit cross-linking event.

The two-dimensional gel of RYR1 that was cross-linked with diamide to produce dimers (also designated oxidized RYR1), proteolyzed with trypsin in nanomolar Ca\(^{2+}\), and then purified, is shown in Fig. 5A. It should be noted that there are some subtle differences in the tryptic digest pattern obtained with oxidized RYR1, including (as discussed below) larger quantities of fragment 6, and the cleavage at 1999 predominates over cleavage at 1983. Western blots of the two-dimensional gels using Ab 2391 and 2727 are shown in Fig. 8, B and C, respectively. Four major off-diagonal spots (a–d) are clearly detected and appear to represent complexes prior to reduction of a–c and b–d. Using N-terminal Edman sequencing of the two upper bands, we found that both spots a and b begin with the N-terminal amino acid sequence AVVAXFRM, indicating that

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\(^{a}\) Y. Wu and S. L. Hamilton, unpublished observation.
their N-terminal amino acid is 3631 and corresponds to fragments 6 and 9, respectively. Spots c and d both begin with amino acid 2000, and both correspond to fragment 16. We did not detect the sequence beginning at 1983, presumably because both cleavages occur in these experiments using oxidized RYR1. In all of our oxidized preparations, fragment 16 was present in higher amounts than fragment 14, suggesting that oxidized RYR1 protein is more readily digested at this site than reduced RYR1. This is consistent with our findings with calpain fragments, where calpain cleaved a new site around amino acid 2100 in the oxidized RYR1 (8).

From the size of these fragments and our previous identification of tryptic fragments, we propose that the fragments containing cysteine 3635 (spot a, corresponding to fragment 6, and spot b, corresponding to fragment 9) are cross-linked by diamide treatment to a cysteine located in the fragment 16, found in both spots c and d.

**DISCUSSION**

How CaM can function as both an activator and an inhibitor of RYR1 is unknown. In addition, there appears to be a very large translocation of CaM within the three-dimensional structure of RYR1 when Ca\(^{2+}\) binds to both CaM and RYR1. Clues to the molecular mechanisms contributing to these phenomena can be obtained from an analysis of the binding sites for apoCaM and Ca\(^{2+}\)CaM on RYR1.

A binding site for CaM on the native RYR1 was identified using the ability of bound CaM to protect sites on RYR1 from tryptic cleavage (10). A synthetic peptide composed of amino
acids 3614–3643 was then shown to bind both apoCaM and Ca\(^{2+}\)/CaM, and cysteine 3635 was identified as one of the amino acids involved in the intersubunit disulfide bond (2, 10). ApoCaM requires additional amino acids between 3634 and 3643, whereas Ca\(^{2+}\)/CaM needs only amino acids 3614–3634. We recently found, however, that only the C-lobe of CaM binds to this sequence (5). This raised questions about the binding site for the N-lobe.

Deletion of amino acids 2–8 of calmodulin greatly decreases the affinity of calmodulin for RYR1 at both nanomolar and micromolar Ca\(^{2+}\)/CaM concentrations (5). These amino acids are also required for maximal inhibition of the channel at micromolar Ca\(^{2+}\)/CaM concentrations. In contrast, the addition of 3 amino acids to the N terminus of calmodulin increases its affinity for RYR1 at both nanomolar and micromolar Ca\(^{2+}\)/CaM concentrations but destroys its functional effects on RYR1 in nanomolar Ca\(^{2+}\)/CaM (5). These studies suggest that the N-lobe of CaM is binding to RYR1, but its binding site is likely to be noncontiguous with the C-lobe site. In the current study, we have attempted to identify the location of the binding site for the N-lobe.

To identify the N-lobe binding site, we used trypsin digestion of the membrane-bound RYR1. Tryptic fragments were identified by N-terminal sequencing and Western blotting. Fig. 9 illustrates the tryptic digest patterns detected in the presence (yellow lines) and absence of apoCaM (red lines). In low Ca\(^{2+}\), we found that, in addition to the protected sites after arginines 3630 and 3637, there are sites after amino acid 1982 (and, possibly 1999) protected by apoCaM. Since apoCaM is an activator of RYR1, the protection could be arising from a burying of the site due to the conformational change in RYR1. However, an inactive CaM with 3 additional N-terminal amino acids, (N+3)CaM also protects the 1982/1999 sites, suggesting that this site is not protected as the result of a change in the conformation of the binding site arising from the ability of CaM to bind to RYR1.
apoCaM to activate RYR1. (N+3)CaM is a high affinity antagonist of the Ca\(^{2+}\)-free RYR1.

The sites after amino acids 1982 and 1999 are not protected by Ca\(^{2+}\)CaM, although the 1975–1999 sequence binds Ca\(^{2+}\)CaM. It is possible that Ca\(^{2+}\) binding to CaM bound to this sequence produces a conformational change in CaM itself or a movement of CaM within the binding site that uncovers this site. Another possible explanation of the lack of protection of this site by Ca\(^{2+}\)CaM is that there is an increased exposure of the site arising from the Ca\(^{2+}\)-CaM-driven inhibition of RYR1 activity. This possibility is currently being investigated. The lack of protection by Ca\(^{2+}\)CaM does not, however, appear to arise from a conformational change driven solely by Ca\(^{2+}\) binding to RYR1, since a Ca\(^{2+}\) partial agonist of RYR1 at all Ca\(^{2+}\) sites can protect the 1983/1999 cleavage sites. E1234Q is an increased exposure of this site by Ca\(^{2+}\)provides some protection of the 1982 and 1999 cleavage sites on the apoCaM-protected tryptic site. Another possible explanation of the lack of protection of the site arising from the Ca\(^{2+}\) binding to CaM is that there is an increased exposure of the site arising from the Ca\(^{2+}\)-CaM-driven inhibition of RYR1 activity. This possibility is currently being investigated. The lack of protection by Ca\(^{2+}\)CaM does not, however, appear to arise from a conformational change driven solely by Ca\(^{2+}\) binding to RYR1, since a Ca\(^{2+}\) binding site mutant of CaM (E1234Q) can protect the 1983/1999 cleavage sites. E1234Q is a partial agonist of RYR1 at all Ca\(^{2+}\) concentrations (2) and provides some protection of the 1982 and 1999 cleavage sites on the Ca\(^{2+}\)-bound RYR1, suggesting that the apoCaM binding site is similar in the Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free RYR1.

The site identified in the protection and peptide studies (amino acids 1975–1999) is clearly not close to the C-lobe binding to CaM bound to this sequence in the primary sequence of RYR1, but is it on the same subunit? Cysteine 3635 is one of the cysteines involved in the formation of an intersubunit disulfide bond within the RYR1 tetramer (10), raising the possibility that the N-lobe binding site is also on a second subunit. One approach to assessing this is to identify the location of the second cysteine and determine whether it is close to the site protected by apoCaM. Consistent with the idea that the N-lobe binding site is on a second subunit, we previously demonstrated that the second cysteine was located somewhere between amino acids 1400 and 2100 (8), a sequence that contains the apoCaM-protected tryptic site. To narrow the location of the cross-linking cysteine, we used diamide as a disulfide-inducing cross-linker and tryptic digestion to generate fragments. We found that fragments containing cysteine 3635 could be cross-linked to a 50-kDa fragment that had an N-terminal sequence corresponding to amino acids 2000 and from its size was predicted to extend to amino acid 2401. These studies place the cysteine involved in the intersubunit cross-link with cysteine 3635 in close vicinity of the apoCaM-protected cleavage site at amino acid 1982, suggesting that the N-lobe of CaM is binding to the subunit that is adjacent to the binding site for the C-lobe.

In summary, we propose that CaM binds to RYR1 with its C-lobe bound between amino acids 3614 and 3643 and its N-lobe bound to the adjacent subunit between amino acids 1975 and 1999, and Ca\(^{2+}\) binding to CaM allows its movement within these two sequences to inhibit channel activity.

Acknowledgments—We thank Dr. Liang-Wen Xiong, Dr. Ivan Delgado, Dr. Sergy Lemeshko, Oluwatoyin Thomas, D. Brent Halling, Dr. Mihail Chelu, Dr. Serap Sencer, Dr. Rao Papineni, and Shawn Robison for helpful comments on the manuscript.

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*J. Biol. Chem. 2003, 278:8348-8355.*
doi: 10.1074/jbc.M209565200 originally published online December 31, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209565200

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