The skeletal muscle Ca\(^{2+}\) release channel (RYR1), which plays a critical role in excitation-contraction coupling, is a homotetramer with a subunit molecular mass of 565 kDa. Oxidation of the channel increases its activity and produces intersubunit cross-links within the RYR1 tetramer (Aghdasi, B., Zhang, J., Wu, Y., Reid, M. B., and Hamilton, S. L. (1997) *J. Biol. Chem.* 272, 3739–3748). Alkylation of hyperreactive sulfhydryls on RYR1 with N-ethylmaleimide (NEM) inhibits channel function and blocks the intersubunit cross-linking. We used calpain and trypsin cleavage, two-dimensional SDS-polyacrylamide gel electrophoresis, N-terminal sequencing, sequence-specific antibody Western blotting, and [\(^{14}\)C]NEM labeling to identify the domains involved in these effects. Our data are consistent with a model in which 1) diamide, an oxidizing agent, simultaneously produces an intermolecular cross-link between adjacent subunits within the RYR1 tetramer and an intramolecular cross-link within a single subunit; 2) all of the cysteines involved in both cross-links are in either the region between amino acids 2100 and 2843 or the region between amino acids 4685 and 4882; 3) oxidation exposes a new calpain cleavage site in the central domain of the RYR1 (in the region around amino acid 2100); 4) sulfhydryls that react most rapidly with NEM are located in the N-terminal domain (between amino acids 426 and 1396); 5) alkylation of the N-terminal cysteines completely inhibits the formation of both inter- and intra-subunit cross-links. In summary, we present evidence for interactions between the N-terminal region and the putatively cytoplasmic central domains of RYR1 that appear to influence subunit-subunit interactions and channel activity.

In skeletal muscle, the Ca\(^{2+}\) release channel (RYR1) of the sarcoplasmic reticulum (SR)\(^1\) responds to T tubule depolarization by releasing luminal Ca\(^{2+}\) into the myoplasmic space (1), triggering the sequence of events that leads to muscle contraction. Single amino acid changes in RYR1 (2–8) are thought to produce the human diseases malignant hyperthermia and central core disease. Based on a hydrophathy analysis of the primary amino acid sequence of RYR1, the monomeric subunit is predicted to have a short cytoplasmic C terminus and between 4 and 10 membrane-spanning regions in the C-terminal one-fifth of the molecule (9, 10). The transmembrane regions of the monomers may combine to form the pore of the homotetrameric Ca\(^{2+}\) release channel. The large N-terminal part of the molecule is thought to extend into the cytoplasm as a “foot” structure (11), and this region of the protein plays an important role in regulation of the channel activity of RYR1. Most of the mutations in RYR1 that produce malignant hyperthermia and central core disease have been found in this region and cluster in two cytoplasmic locations (2–8). Also, most modulators of the channel are thought to interact with cytoplasmic domains of RYR1 (12–16).

Reactive oxygen intermediates are produced in resting muscle and their production increases with muscle activity (17–22). The reactive oxygen intermediates appear to enhance contractile function in muscle, and one mechanism for their effects of contractility could be a modulation of the activity of the Ca\(^{2+}\) release channel. Consistent with this, oxidation reduction reactions have been reported to modulate RYR1 activity (23–29). Previously, we have demonstrated that the sulfhydryl oxidizing agent, diamide (30), produces intersubunit cross-links between subunits of the RYR1 tetramer, enhances \(^{3}H\)ryanodine binding, and activates the Ca\(^{2+}\) release channel (31). In addition to redox modulation, RYR1 is also sensitive to reagents that react with free sulfhydryls but do not form disulfide bonds (31, 32). Treatment of RYR1 with the alkylating reagent N-ethylmaleimide (NEM) for very short periods of time alkylates a small percentage of the total sulfhydryls on the protein and inhibits both channel activity and \(^{3}H\)ryanodine binding (3). Alkylation of these hyperreactive sulfhydryls (representing less than 10% of the total sulfhydryls on RYR1) completely blocks the diamide-induced intersubunit cross-linking (3). The domain locations of the sulfhydryls on RYR1 involved in the NEM and oxidative effects are not known. In particular, it is not clear whether the same sulfhydryls are involved in both the alkylation and oxidation-reduction reactions or whether the alkylation of hyperreactive sulfhydryls has a long distance effect on the sulfhydryls involved in cross-linking. Formation of intersubunit disulfides activates the channel and the localization of the cysteine residues involved may help to identify a functionally important site of subunit-subunit contact.

Proteolysis has been used as a tool for probing the functional domains of RYR1 (16, 33, 34). For example, both high and low affinity ryanodine binding sites are found between the tryptic cleavage site Arg-4475 and the carboxyl terminus. If ryanodine is bound prior to digestion, it remains bound. If, however, ryanodine does not occupy the site, the ability of the channel to bind \(^{3}H\)ryanodine is rapidly lost upon tryptic digestion (33, 34). The tryptic complex composed only of RYR1 fragments from amino acid 4476 to the carboxyl terminus retains the ability to form channels in planar lipid bilayers (16). In contrast to trypsin, digestion of SR membranes with calpain II...
do not cause loss of ability of RYR1 to bind \(^{3}H\)ryanodine (35, 36). RYR1 is the major SR substrate for endogenous calpains (37), and calpain II digestion enhances the activity of the Ca\(^{2+}\) release channel (35). Calpain digestion does not appear to alter the sedimentation properties of RYR1, and the cleaved fragments appear to remain associated with the remainder of the complex (38, 39).

The purpose of the study described here was to identify domains on RYR1 that contribute to the regulation of channel activity by oxidation and by alkylation. Our strategy to localize functional domains was to identify the calpain-derived fragments of the RYR1 cross-linked by diamide and/or alkylated by \(^{14}C\)NEM. We chose the proteolytic enzyme calpain for these studies because endogenous calpain is known to produce large fragments of RYR1 (35–39), and large fragments allow us to discriminate between inter- and intramolecular cross-links. We used three types of cross-linking experiments: 1) diamide cross-linking of purified RYR1 previously digested by calpain while in the SR membrane to eliminate the possibility of cross-linking between neighboring tetramers or with other proteins in the SR membrane; 2) calpain digestion of RYR1 followed by cross-linking in membranes to demonstrate that the cross-linking pattern was not altered by membrane solubilization, and 3) cross-linking of RYR1 followed by calpain digestion in SR membranes to assess whether cross-linking altered the sites of the calpain digestion or if calpain digestion altered the sites of the cross-linking.

**EXPERIMENTAL PROCEDURES**

**Materials**—\(^{3}H\)Ryanodine (81.5 Ci/mmol) and \(^{14}C\)NEM (0.05 Ci/mmol) were purchased from NEN Life Science Products. Ryanodine and calpain II were purchased from Calbiotech (La Jolla, CA). Diamide, dithiothreitol (DTT), NEM, CHAPS, MOPS, and CAPS were obtained from Sigma.

**SR Membrane Preparation**—SR membranes were prepared from rabbit leg white skeletal muscle and were purified using sucrose gradient centrifugation (40, 41).

**Preparation of Antibodies**—Antipeptide antibodies corresponding to 2727–2743 (Ab 2727), 4363–4373 (Ab 4363), and 4685–4697 (Ab 4685) were described previously (53). Ab 0901 is a polyclonal antibody against a fusion protein containing the first 280 amino acids from the N-terminal of RYR1. The GST fusion construct was prepared by ligation of the N-terminal RYR1 cDNA into to BamHI-digested pGEX. The GST fusion protein was expressed in *Escherichia coli* (Borers, AR) and the antibody was then affinity-purified with an Affi-Gel Protein A column (Bio-Rad). Ab 5029 was graciously provided by Dr. Andrew Marks (Mount Sinai School of Medicine, New York). The polyclonal RyR antibody against the intact RYR1 was prepared in collaboration with Dr. John Dedman (University of Cincinnati, OH).

**Proteolytic Digestion with Calpain and Purification**—Rabbit SR membranes (10 mg/ml) were labeled with \(^{3}H\)ryanodine (10 nM) in binding buffer containing 300 mM NaCl, 50 mM MOPS (pH 7.4), 100 mM Ca\(_{2+}\), and protease inhibitors for 1 h at 37 °C. Unlabeled ryanodine (130 μM) was then added, and the incubation was continued for an additional 10 min. This latter treatment slows dissociation of \(^{3}H\)ryanodine from the high affinity site (33). Subsequently, the membranes were centrifuged for 5 min at 30,000 g in a Beckman Airfuge to remove the protease inhibitors and unbound \(^{3}H\)ryanodine. The pellet was then resuspended in digestion buffer (50 mM NaCl, 20 mM MOPS, 3 mM CaCl\(_{2}\), 2 mM DTT, pH 7.4) and digested with endogenous and exogenous calpain (1.5–2.5 calpain:protein ratio) at 37 °C for 1.5 h. The reaction was terminated by the addition of leupeptin (10 μM), and the samples were centrifuged for 35 min at 190,000 × g. The resulting pellets were solubilized in a solution of 2% CHAPS in binding buffer with protease inhibitors for 30 min at 4 °C. The solubilized complex was layered onto 17 ml of 5–20% sucrose gradients containing 0.4% CHAPS, 200 mM NaCl, and 20 mM MOPS (pH 7.4) and centrifuged for 17 h at 110,000 × g. Fractions of 20 drops each were collected from the bottom of the sucrose gradient. Aliquots of 50 μl were taken from each fraction to determine radioactivity.

**SDS-PAGE**—5% polyacrylamide gel electrophoresis was performed as described by Laemmli (42). The molecular mass of the high molecular weight fragments of RYR1 was estimated using Rainbow molecular markers (46–220 kDa, Amersham Corp.), the full-length RYR1 as a 565-kDa marker, and the commonly observed second large fragment as a 410-kDa marker (36, 37).

**Western Blots**—Protein samples subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA) for 16–18 h at 25 V in 5% methanol, 10 mM CAPS (pH 11.0) at 4 °C. The blots were developed with primary antibodies and alkaline phosphatase-conjugated secondary antibodies as described previously (33).

**N-Terminal Sequencing of Proteolytic Fragment**—The calpain- and trypsin-digested RYR1 fragments were separated on 5% SDS-polyacrylamide gels and then prepared for sequencing as described previously (33). Sequencing was performed by Dr. Richard Cook at Baylor College of Medicine.

**Diamide Titration**—RYR1 proteolysed by endogenous calpain in the SR membrane was purified by sucrose gradient centrifugation and DEAE-Trisacryl ion exchange as described previously (41). The sample was stored in 300 mM NaCl, 10 mM MOPS (pH 7.4), 0.4% CHAPS, 5% sucrose, and 1 mM DTT. For the diamide titration, diamide at final concentrations of 0–2 mM in 200 mM NaCl, 20 mM MOPS (pH 7.4) was added to the purified sample before incubation at 4 °C for 30 min. Subsequently, the samples were incubated with 4 mM NEM at room temperature for 20 min, and then with sample buffer (0.06% Trit, 10% glycerol, 2% SDS, 0.001% bromphenol blue) for another 20 min prior to electrophoresis.

**Two-dimensional SDS-PAGE**—The purified proteolytic complex was cross-linked by diamide and then treated with 4 mM NEM for 20 min before solubilization in SDS sample buffer. Polyacrylamide gels (5%) were used for the first dimension electrophoresis. Electrophoresis was continued for a certain time period after the dye front ran off the gel. The time period varied according to the required resolution of the fragments. Subsequently, the lanes were excised, and the gel strips were incubated with 25–50 mM DTT in the sample buffer without SDS and bromphenol blue for 1 h at room temperature (23 °C). The gel strip was then loaded on top of another 5% polyacrylamide gel for the second dimension electrophoresis. The gap between the gel strip and the two-dimensional resolving gel was sealed with melted agarose (1% agarose, 2% SDS, 50 mM DTT), and the gels were electrophoresed for 1–2 h at 120–170 V at 4 °C. The two-dimensional gels were subsequently stained with Coomassie Blue or silver nitrate.

**Calpain Proteolysis and Diamide Cross-link in SR Membrane**—Rabbit SR membranes (2 mg/ml) were incubated either with calpain (calpain:protein ratio of 1:25 and 3 mM CaCl\(_{2}\)) at 37 °C for 40 min or with calpain at room temperature for 20 min in the presence of protease inhibitors in digestion buffer (50 mM NaCl, 20 mM MOPS, 2 mM DTT, pH 7.4). Subsequently, the membranes were centrifuged for 5 min at 30 p.s.i. in a Beckman Airfuge to remove the calpain, Ca\(_{2+}\), and DTT. The pellet was then resuspended and cross-linked with diamide (0.1–0.5 mM) in 200 mM NaCl and 20 mM MOPS (pH 7.4) at 4 °C for 20 min, during which the cross-linking, the preproteolysed sample was applied to two-dimensional electrophoresis. The nonproteolysed sample was centrifuged for 5 min at 30 p.s.i. to remove the diamide and then resuspended and digested with calpain at 37 °C for 40 min in the absence of DTT (50 mM NaCl, 20 mM MOPS, 3 mM CaCl\(_{2}\), pH 7.4). After the calpain was removed by centrifugation, the pellet was resuspended in buffer (200 mM NaCl and 20 mM MOPS, pH 7.4) and either applied to two-dimensional electrophoresis or reduced by 25 mM DTT for one-dimensional electrophoresis and Western blot analysis.

**Equilibrium \(^{3}H\)Ryanodine Binding**—SR membranes (10 μg assay) were incubated with \(^{3}H\)ryanodine (5 nM) at room temperature (23 °C) for 16 h in binding buffer (300 mM NaCl, 50 mM MOPS (pH 7.4), 100 μg/ml bovine serum albumin, 0.1% CHAPS, and 1 mM EGTA) in the absence and presence of 1.1 mM Ca\(_{2+}\). Non-specific binding was defined in the presence of 10 μM unlabeled ryanodine. The bound \(^{3}H\)ryanodine was separated from free ligand by filtering through Whatman GF/F glass fiber filters. The filters were washed with 5 × 3 ml of ice-cold buffer containing 300 mM NaCl, 100 mM CaCl\(_{2}\), and 20 mM MOPS (pH 7.4). The radioactivity bound to the filters was quantitated by liquid scintillation counting.

**\(^{14}C\)NEM Labeling in SR Membrane**—SR membrane was incubated with 0.3 mM \(^{14}C\)NEM for 5, 15, and 45 min. The reaction was stopped by the addition of 20 mM DTT, and samples were pelleted in an Airfuge to remove the free \(^{14}C\)NEM and DTT. Subsequently, the sample was resuspended in digestion buffer and digested by calpain (calpain:protein ratio of 1:25) and then electrophoresed on 5% gel. After stained with Coomassie Brilliant Blue, the fragment optical density (OD) was...
Functional Domains of RYR1

RESULTS

Identification of Calpain-derived Fragments of RYR1—Since the calpain cleavage sites on RYR1 were not known, it was necessary to identify the calpain-derived fragments of RYR1 prior to performing the cross-linking experiments. SR membranes were labeled with $[^3H]$ryanodine, proteolized with varying amounts of calpain II (0–1:25 calpain:protein weight ratio), and solubilized. The proteolyzed complexes were then purified on sucrose gradients. In contrast to tryptic digestion (33), neither the sedimentation behavior nor the ability to bind $[^3H]$ryanodine was significantly altered by calpain digestion (33), neither the sedimentation behavior nor the ability to bind $[^3H]$ryanodine was significantly altered by calpain digestion (33), neither the sedimentation behavior nor the ability to bind $[^3H]$ryanodine was significantly altered by calpain digestion (33), neither the sedimentation behavior nor the ability to bind $[^3H]$ryanodine was significantly altered by calpain digestion (33). To identify these fragments, we analyzed the proteolytic fragments of RYR1 by SDS-PAGE and Western blotting. Some of the smaller fragments were also analyzed by N-terminal sequencing. Fig. 1 shows proteolysis of RYR1 by calpain and the identification of these fragments with a polyclonal antibody against full-length RYR1 and an antibody against the last 9 amino acids of the RYR1 (Ab 5029). The protein fragments in all lanes are matched and alphabetically listed in Table I, along with their apparent molecular masses. In the control lane of Fig. 1A, RYR1 was already slightly proteolyzed, and several RYR1-derived fragments were detected: the full-length 565-kDa RYR1 (band a), the 410-kDa fragment (fragment b), and a doublet of 172 and 166 kDa (fragments h and i). This proteolysis detected in the membrane preparation may have occurred either in the intact muscle or during the isolation of membranes. Incubation of the membranes in the digestion buffer alone (calpain:protein ratio of 0) prior to the purification of RYR1 produced several new fragments, c (338 kDa), m (98 kDa), n (82 kDa), p (67 kDa), and r (61 kDa). The addition of calpain II increased the production of these bands and led to the production of several new bands (d, f, g, j, k, o, s, etc.) (Fig. 1), suggesting that they were calpain-digested fragments. The polyclonal RYR antibody recognized most of these fragments (Fig. 1B), demonstrating that they were derived from RYR1. One band (l) was not cleaved by calpain and was not recognized by the polyclonal antibody. Sequencing of tryptic fragments derived from this band revealed that this band is the Ca$^{2+}$-ATPase (data not shown). An antibody to the last 9 amino acids (Ab 5029) of RYR1 recognized several low molecular weight fragments (Fig. 1C) that were not stained sufficiently to be visualized in the Coomassie Brilliant Blue-stained gel, suggesting that they were derived from the C terminus of RYR1. These fragments had apparent molecular masses of 98 kDa (m), 82 kDa (n), 75 kDa (o), 67 kDa (p), 64 kDa (q), 61 kDa (r), 60 kDa (s), and 38 kDa (t). To further identify the fragments from the calpain digestion, we used other sequence-specific antibodies, as shown in Fig. 2. These data, including the N-terminal sequences of fragments g and f, are also summarized in Table I.

These experiments demonstrate that 1) calpain primarily cleaves the RYR1 first at the N terminus around residue 1400 and then at the C terminus between residues 4373 and 4685, generating four major fragments: b, c, h, and m; 2) fragment b is cleaved again after amino acid 2843 to produce fragments g and d; 3) fragment c is further proteolyzed after amino acid 2843 to generate fragments g and f; 4) the C-terminal fragment m, generated in the first C-terminal cleavage, is also further degraded into a number of small fragments (fragments n, o, p, q, r, s); and 5) other cleavages also appear to occur but at a slower rate under these conditions. These experiments were performed under reducing conditions to protect the calpain from inactivation by oxidation. The major calpain cleavage sites are summarized in the fragmentation map shown in

![Image](107x470 to 506x729)

**FIG. 1.** Degradation of RYR1 by calpain II. A, Coomassie Blue-stained SDS-PAGE gel of the purified RYR1 complex proteolyzed under four different proteolytic conditions. B, Western blot of the proteolyzed RYR1 using the polyclonal RYR antibody. C, Western blot of the proteolyzed RYR1 using the 5029 antibody. Excluding the molecular weight standards in panel A, the first lane in each panel is the control, where the RYR1 was directly purified from the SR membrane. The second lane is the RYR1 incubated under the digestion conditions but with no added calpain (calpain:protein (C/P) is 0). In the third and fourth lanes, calpain was added to a protein weight ratio of 1:50 and 1:25, respectively.
**Functional Domains of RYR1**

### Table I

| Labels | Apparent molecular mass (kDa) | Recognition or identification | Predicted proteolytic site | Predicted molecular mass (kDa) |
|--------|-------------------------------|------------------------------|---------------------------|-------------------------------|
| a      | 565                           | Full-length Ca\(^{2+}\) release channel, recognized by all antibodies. | 1401\(^a\)–5037         | 565                           |
| b      | 410                           | A major fragment, recognized by Ab 2727, Ab 4363, Ab 4685, and Ab 5029 antibodies but not Ab 0001. | 1401\(^a\)–4685\(^b\)   | 410                           |
| c      | 338                           | A major fragment recognized by Ab 2727 and Ab 4363 but not by Ab 0001, Ab 4685, or Ab 5029. | 2844\(^a\)–5037         | 368                           |
| d      | 307                           | A major fragment recognized by Ab 5029 but not by Ab 0001 or Ab 2727. | 2844\(^a\)–5037         | 246                           |
| d'     | 250                           | A minor fragment, recognized by Ab 2727 but not by Ab 0001 or Ab 5029. | 1401\(^a\)–2843\(^a\)   | 156                           |
| e      | 197                           | A fragment not recognized by Ab 0001, Ab 2727, or Ab 5029. N-terminal sequence is TAXXYXPXEXY, where X represents amino acids that cannot be conclusively identified. | 2844\(^a\)–4685\(^b\)   | 205                           |
| g      | 184                           | A major fragment recognized by Ab 2727. N-terminal sequence is QPPATPALPR. | 1401\(^a\)–5037         | 164                           |
| h      | 172                           | A major fragment, recognized by Ab 0001 antibody but not by Ab 2727 or Ab 5029. | 1–1400\(^a\)           | 156                           |
| i      | 168                           | Recognized by the same antibodies as h. |                            |                               |
| j      | 162                           | A minor fragment recognized by the same antibodies as h. |                            |                               |
| k      | 157                           | A fragment recognized by the same antibodies as h. |                            |                               |
| l      | 110                           | Full-length Ca\(^{2+}\)-ATPase. |                            |                               |
| m      | 98                            | Poorly staining fragment recognized by polyclonal RYR antibody and Ab 5029. | 4685\(^a\)–5037         | 41                            |
| n      | 82                            | Poorly staining fragment recognized by polyclonal RYR antibody and Ab 5029. |                            |                               |
| o      | 75                            | Same recognition as n. |                            |                               |
| p      | 67                            | Same recognition as n. |                            |                               |
| q      | 64                            | Same recognition as n. |                            |                               |
| r      | 61                            | Same recognition as n. |                            |                               |
| s      | 60                            | Same recognition as n. |                            |                               |
| t      | 38                            | Recognized by Ab 5029. |                            |                               |
| x      | 374                           | A fragment generated only by proteolysis of oxidized RYR1, recognized by Ab 5029 and Ab 2727 but not by Ab 0001. | 2100\(^a\)–5703         | 358                           |
| y      | 283                           | A fragment generated only by proteolysis of oxidized RYR1, recognized by Ab 2727 but not by Ab 0001 or Ab 5029. | 2100\(^a\)–4685\(^a\)   | 289                           |
| z      | 80                            | A fragment generated only by proteolysis of oxidized RYR1, recognized by Ab 2727 but not by Ab 0001 or Ab 5029. | 2100\(^a\)–2843\(^a\)   | 84                            |

\(^a\) The proteolytic sites determined by N-terminal sequencing of the fragment. N-terminal sequence on f and g was obtained twice.

\(^{b}\) Putative proteolytic sites according to antibody recognition.

Fig. 2C.

**Cross-linking of the Purified Ca\(^{2+}\) Release Channel Complex**—Our next step was to use the protease digestion to identify domains of RYR1 involved in diamide-induced intersubunit cross-links. Partially purified RYR1, proteolyzed by endogenous calpain, was cross-linked with diamide (0–2 mM) and analyzed by SDS-PAGE as shown in Fig. 3A. The full-length RYR1 monomer (band a) and all of its major proteolytic fragments (b, c, d, g, and h) disappeared with increasing concentrations of diamide. At fairly low diamide concentrations (0.3–2 mM), three new bands (bands A, B, and C) appeared and had trations of diamide. At fairly low diamide concentrations, other proteolytic sites, which were not recognized by any of the antibodies used, were cross-linked with diamide (0–2 mM) and analyzed by SDS-PAGE as shown in Fig. 3A. The full-length RYR1 monomer (band a) and all of its major proteolytic fragments (b, c, d, g, and h) disappeared with increasing concentrations of diamide. At fairly low diamide concentrations (0.3–2 mM), three new bands (bands A, B, and C) appeared and had a smaller molecular weight after reduction (the molecular weight of the reduced monomer) in the second dimension. They appear as off-diagonal spots on the left side of the diagonal line. Several off-diagonal spots can be seen on this two-dimensional gel. Spot Aa, aligned with band A in the first dimension and band a in the second dimension, is the monomer of RYR1 reduced from A. Thus, band A in the first dimension appears to be a dimer of full-length RYR1. Spot Bb aligned with band B in the 1st dimension and band b in the 2nd dimension, suggesting that band B is a dimer of fragment b. Similarly, band C is apparently a dimer of fragment c.

The data in Fig. 3 provide evidence that the high molecular weight bands A, B, and C are dimers of a full-length RYR1 subunit and its proteolytic fragments (a, b, and c), respectively. Therefore, the primary cross-link for the dimer formation is located within the smaller of these fragments (fragment c), which represents a central, putatively cytoplasmic domain of RYR1. The absence of mixed dimers (i.e. a + b, a + c, or b + c) suggests that all subunits within a tetramer have been digested by calpain at the same sites. This has been a consistent finding in all of our cross-linking experiments.

In these experiments (Fig. 3), the N-terminal fragment h was seen to be involved in the formation of higher oligomers. However, as will be described below, this fragment was not cross-linked when the cross-linking was performed with membrane-bound RYR1, suggesting that the conformation of the N-terminal domain in the soluble RYR1 may be different from...
that in the membrane-bound RYR1.

Cross-linking of the Proteolyzed Ca\textsuperscript{2+} Release Channel in SR Membrane—We next analyzed the cross-linking patterns of RYR1 in the SR membranes. In these experiments, we treated SR membranes with calpain and then cross-linked with diamide. Fig. 4 shows the two-dimensional gel from a sample generated in this way. The proteolysis in this sample was more extensive than seen in Fig. 3B. In addition to the dimers of b and c, we detected a cross-linked complex composed of fragments d and g and a complex of fragments f and g. The apparent size of these complexes prior to reduction was about 850 and 650 kDa, suggesting that these complexes may contain more than one copy of some of the components. This will be discussed below. The major cross-linked bands obtained with the membrane-bound RYR1 (Fig. 4) were similar to those obtained in the experiments with RYR1 proteolyzed in the membrane but purified prior to cross-linking (data not shown). The cross-links involving the N-terminal fragment h were, however, not detected when the cross-linking was performed with the membrane-bound RYR1, suggesting that this fragment was not involved in dimer formation in the membrane. The presence of bound ryanodine did not alter the cross-linking pattern (data not shown). In SR membranes, we also detected several additional off-diagonal spots, one of which appeared to arise from cross-linking of the Ca\textsuperscript{2+}-ATPase (the off-diagonal spot at the bottom of the two-dimensional gel in Fig. 4).

Similar to our results with purified RYR1, the experiment with membrane-bound RYR1 suggested that the primary cross-link was located in the middle domain of two adjacent subunits within the tetramer. Fragments g and f were the smallest fractions that were directly or indirectly (i.e., as part of b or c) involved in all of the cross-links, suggesting that at least one of the cysteines involved was located between 1401 and 2843 of RYR1 (g domain) and another cysteine was between residues 2844 and 4685 (f domain).

Calpain Digestion of Cross-linked RYR1 in the SR Membrane—It is possible that the conformation of the RYR1 is altered by the calpain digestion such that different sulfhydryls might be cross-linked in the native versus proteolyzed sample. There are considerable data from other laboratories suggesting that calpain digestion of the SR membrane does not alter the functional integrity of the RYR1 (35–37). Furthermore, diamide enhanced \textsuperscript{[3H]ryanodine binding to both control and calpain-digested membranes (data not shown), suggesting that the ability of diamide to modulate RYR1 is not altered by the digestion.

To determine whether diamide pretreatment altered the pattern of calpain digestion, membranes with or without diamide pretreatment were cleaved with calpain, and the cleavage pattern was analyzed by SDS-PAGE. A representative experiment (n = 3) is shown in Fig. 5. All samples were reduced prior to electrophoresis. Compared with the non-cross-linked sample, the pre-cross-linked proteolytic complex has increased amounts of three fragments with molecular masses of 374 kDa (fragment x), 283 kDa (fragment y) (Fig. 5A), and 80 kDa (fragment z, not shown on the 5% gel). Fragment x is recognized by Ab 5029 but not Ab 0001, indicating that this fragment has the C terminus of the RYR1 but not the N terminus. From its size, fragment x appears to arise from a new cleavage site on fragment b, which results in the removal of the N-terminal region. Neither fragment y and z was recognized by Ab 0001 or Ab 5029 (data not shown). They were, however, recognized by Ab 2727. Correlated with the appearances of fragments x, y, and z were the disappearances of some minor fragments with apparent molecular masses of 250 kDa (e) and 300 kDa (d') and decreases in fragments b and c.

To correlate the new cleavage sites with the cross-linking event, we treated membranes with increasing amounts of diamide (Fig. 6A) and then cleaved them with calpain. Fig. 6B shows the sample electrophoresed without DTT, while in Fig. 6C, the samples were reduced. With increasing dimer formation, upon calpain digestion, there was a decrease in fragment c and an increase in fragments x and y in the proteolyzed fractions (Fig. 6C). This suggested that diamide treatment of SR membranes exposed a new calpain cleavage site on RYR1 found in both fragments b and c. Fragment y appears to arise from cleavage of fragment c at the new site, while fragment x is produced by a similar cleavage of fragment b. In addition, y may arise from x at the same carboxyl-terminal cleavage site that converts fragment b to fragment c (Fig. 5B). The other minor fragments, e and d', also appear to be derived from the central domain, probably generated by alternative cleavages that are a little different from y and c. They are, therefore, likely to be altered by the new cleavage.

We next examined the two-dimensional gels formed when RYR1 was cross-linked with diamide then cleaved with calpain. A representative experiment (n = 5) is shown in Fig. 7A. Again, the off-diagonal spots identified earlier were detected, as well as four additional spots of fragments x, y, d, and f. To determine if these new spots are cross-linked to smaller fragments, we used a 5% gel in the first dimension and an 8% gel in the second dimension (Fig. 7B). No off-diagonal fragments that align with the x and y spots were detected. One possible interpretation of these findings is that x and y also form dimers of themselves, but the shape and, hence, the mobility is anomalous. This is discussed below. The new off-diagonal spots of f and d both
align with a new 80-kDa spot (z). This fragment is recognized by Ab 2727 (data not shown). The 80-kDa off-diagonal spots can also be seen in Fig. 7A but are compressed at the bottom of the gel. Since fragment z was about half the size of fragment g, it is likely that fragment z is derived from fragment g from the new cleavage (Fig. 5B). Therefore, the appearance of the new off-diagonal spots f, d, and z appears to be due to a new cleavage of the previously identified higher molecular weight cross-linked complexes. This new cleavage site appears to occur somewhere within the sequence defined by fragment g. The cross-link involved in the formation of the off-diagonal spots

FIG. 3. Oxidation and reduction of endogenously proteolyzed ryanodine receptor on one- and two-dimensional SDS-PAGE. A, the RYR1 complex partially proteolyzed by endogenous calpain was purified in 1 mM DTT and then reacted with 0–2 mM of diamide as indicated at the top of each lane of the one-dimensional SDS-PAGE. All of the samples were treated with 4 mM NEM before they were dissolved in the sample buffer and electrophoresed on a 5% acrylamide gel. The gel was stained with Coomassie Brilliant Blue. The fragments originally generated in the proteolysis are labeled in lowercase letters as in Figs. 1 and 2 and Table I. The cross-linked products are labeled in uppercase letters. The sample in the first lane is fully reduced. B, two-dimensional SDS-PAGE of the cross-linked purified proteolytic complex (calpain:protein = 0). The purified RYR1 as in A was cross-linked by 0.65 mM diamide and then treated with 4 mM NEM before it was solubilized in sample buffer for the first dimension electrophoresis. Subsequently, the lanes were excised, and one of the lanes was incubated with 50 mM DTT for 1 h before loading on a 5% acrylamide for the second dimension electrophoresis. Another lane was stained with Coomassie Blue together with the two-dimensional gel, and then aligned on top of this two-dimensional gel to indicate the fragments in the first dimension gel. The molecular mass decreases from left to right in the first dimension and decreases from top to bottom in the second dimension. The fragments are labeled with letters as described in Figs. 1–3A and Table I.

FIG. 4. Two-dimensional SDS-PAGE of the cross-linked proteolytic RYR1 in SR (calpain:protein = 1:25). The membrane-bound RYR1 proteolyzed by calpain (calpain:protein = 1:25) was cross-linked by 0.5 mM diamide and then treated with 4 mM NEM before two-dimensional electrophoresis was performed as described in Fig. 3B. The two-dimensional gel was silver-stained, and the one-dimensional gel on the top was stained with Coomassie Blue.

FIG. 5. Comparison of the proteolytic fragments obtained from calpain digestion of reduced and oxidized RYR1. A, SR membranes pretreated with (lane 2) or without (lane 1) diamide (0.5 mM) were cleaved with calpain, resolved by 5% polyacrylamide gel electrophoresis, and then stained with Coomassie Brilliant Blue or Western blotted using N-terminal 0001 antibody, 2727 antibody, and C-terminal 5029 antibody. B, location map of fragments x, y, and z predicted according to the antibody recognition.
apparently involves the same cysteines discussed earlier, located in fragments g and f.

Hyperreactive Sulfhydryls and Diamide-induced Intersubunit Cross-linking—We have previously shown that a subset of cysteine residues, representing less than 10% of the total sulfhydryls on RYR1, reacted very rapidly with NEM to inhibit channel activity and [3H]ryanodine binding (31). Alkylation of these sulfhydryls completely blocks diamide-induced dimerization of the 565-kDa protein (31). To locate these sulfhydryls, we incubated SR membranes with [14C]NEM under conditions that primarily label the hyperreactive sulfhydryls. The alkylation was stopped by adding DTT at different time intervals. The samples were then cleaved with calpain, and the proteins were separated by 5% SDS-PAGE. N-terminal fragments of RYR1 were identified by Western blotting with Ab 0001. We also sliced both the SDS gel and the Western blot to quantitate the radioactivity in all of the fragments. As shown in Fig. 8A, when SR membranes were incubated with 300 μM [14C]NEM for 5–15 min, the radiolabel was found primarily in fragments h–k, the fragments detected by the N-terminal antibody (indicated by asterisks in Fig. 8). This is consistent in both the sliced gels (Fig. 8) and in the sliced Western blot (data not shown). Incubation times longer than 15 min did not significantly increase the radiolabeling in N terminus but began to label other fragments, such as b and c (Fig. 8B). Alkylation with NEM for 5 min (under the same conditions as used for the radiolabeling in Fig. 8A) prior to diamide treatment completely prevented the formation of off-diagonal RYR1 spots on the two-dimensional gels (data not shown). This treatment, however, did not block the cross-linking of the Ca2+-ATPase, demonstrating the specificity of the alkylation effect on RYR1. These findings suggest that the hyperreactive sulfhydryls are located in the N terminus (residues 1–1400) of RYR1 and that alkylation of these sulfhydryls blocks the cross-linking that occurs between amino acids 1401 and 4685.

To confirm the location of the hyperreactive sulfhydryls, we also used tryptic digested RYR1. SR membranes labeled with [14C]NEM under conditions that primarily label the hyperreactive sulfhydryls (31), were digested with trypsin for 5 min and isolated as a 28 S complex. The major band labeled was the 100-kDa band (data not shown), which has an N-terminal sequence of PRGSGPAPGP, indicating that it arises from a cleavage after amino acid 425. Formed simultaneously with the 100-kDa band was another fragment that begins at amino acid 1397 (data not shown). Therefore, the 100-kDa fragment most likely represents the sequence from 426 to 1396. These data further narrow the location of the hyperreactive sulfhydryls to amino residues 426–1396.

**DISCUSSION**

Reactive oxygen intermediates may enhance contractile function by increasing the activity of the Ca2+-release channel (23–29). We have attempted to define domains of RYR1 that are involved in this redox modulation using diamide as the agent to induce disulfide formation and calpain as the proteolytic enzyme to generate large identifiable fragments. Aghdasi et al. (31) have shown that diamide activates the Ca2+-release channel reconstituted into planar lipid bilayers and enhances [3H]ryanodine binding by increasing the apparent affinity of RYR1 for this ligand. Concomitant with the change in channel activity and [3H]ryanodine binding in the presence of diamide is the formation of intersubunit cross-links within the RYR1 tetramer (31). In contrast to the effects of diamide, alkylation of RYR1 for very short periods of time with NEM inhibits both channel activity and [3H]ryanodine binding (31). Under the reaction conditions used in these experiments, only a small...
fraction of the total cysteine residues on RYR1 were modified (<10%); however, this reaction completely blocked dimer formation and channel activation by diamide. Our goal in the current studies was to identify domains of RYR1 involved in both the intersubunit cross-linking and in the rapid alkylation.

Calpain has three advantages for these experiments. First, it is selective. As shown in Fig. 1, the Ca\(^{2+}\)-ATPase, which is the major contaminating protein in these preparations, is not proteolysed by calpain II. This selectivity simplifies the analysis of the cross-linking patterns. Second, calpain degradation appears to occur in an ordered sequence of susceptible sites. Therefore, only a few large fragments are produced in the early proteolysis steps. This helped us to initially locate the cross-linking sites within large domains and to discriminate between intra- and intermolecular disulfide bonds. Third, RYR1 proteolyzed by calpain retains channel activity and the ability to bind ryanodine (35–37), and the sedimentation behavior is unaltered, indicating that the functional integrity of the channel is preserved after calpain proteolysis. Another intriguing aspect of calpain cleavage, suggested by the cross-linking patterns, is that calpain digests all of the subunits within the tetramer essentially simultaneously, indicating that either the cleavage

![Fig. 7. Two-dimensional SDS-PAGE of the cross-linked RYR1 in SR followed by proteolysis by calpain (calpain:protein = 1:25). SR membranes were reduced with 2 mM DTT, washed, and cross-linked in 0.5 mM diamide. Subsequently, the membranes were proteolyzed with calpain (calpain:protein = 1:25) in the absence of DTT and resolved by two-dimensional electrophoresis as described in the legends of Figs. 3B and 4. Both one and two-dimensional gels were silver-stained. The samples were electrophoresed in a 5% acrylamide gel for one dimension, and then the excised gel strip was loaded on either a 5% gel (A) or an 8% acrylamide gel (B) followed by the second dimension electrophoresis. The gels were silver-stained. The gels in A and B were generated in independent experiments.](image7)

![Fig. 8. RYR1 N-terminal alkylation by ^14^C-NEM. SR membranes (24 pmol of [\(^3^H\)]ryanodine binding sites/mg of protein) were incubated with 0.3 mM [\(^14^C\)]NEM for 5, 15 (A), or 45 min (B). The reaction was stopped by the addition of 20 mM DTT. The sample was resuspended in digestion buffer and digested with calpain (calpain:protein = 1:25) and then electrophoresed on 5% gels in triplicate. 50 μg of SR protein (1.2 pmol of [\(^3^H\)]ryanodine binding sites) was applied per well. 480 pmol of cysteines (1.2 pmol of RYR1 \(\times\) 4 subunits \(\times\) 100 cysteines per subunit) were theoretically available for alkylation. After staining with Coomassie Brilliant Blue, the fragment OD on the gels was analyzed by densitometry. Thereafter, each lane of the gel was sliced into 1-mm slices, the slices were digested, and the radioactivity of the gel slices was quantitated by liquid scintillation counting. Each point on the radioactivity curves represents the average of triplicates ± S.D. (The points without error bars represent averages of duplicates.) Since the proteolytic pattern is not altered by NEM within a 15-min incubation, the fragment OD in A is the average from six lanes (triplicates of each 5- and 15-min sample). The fragment OD in B is the average of triplicates ± S.D. The background OD of the gels is also averaged and labeled as bkg. The asterisks show the fragments recognized by the N-terminal antibody.](image8)
is cooperative (i.e., cleavage of one subunit is rapidly accompanied by cleavages of the other subunits at the same sites within the tetramer) or that we have reached a limit digest and a population of the RYR1 tetramers is resistant to calpain cleavage at some sites. This phenomenon is currently being investigated.

Using diamide cross-linking, calpain digestion, and one- and two-dimensional electrophoresis, we showed that the central domains of RYR1 on near neighbor subunits within the tetramer are cross-linked by diamide (model shown in Fig. 9). In addition to the major cross-links observed between like large fragments (b + b, c + c), we also observed some cross-links that appear to involve three or more smaller fragments. The size of the complex that involves fragments d and g is most consistent with two copies of the larger fragment d and one copy of g in the complex. The size of the complex of fragments f and g suggests that it may also be a three-fragment complex, perhaps two copies of f and one copy of g. The occurrence of these complexes raises the question of why the multiple cross-links are more predominant than single cross-links such as f + g, f + f, g + d, or d + d. Since these simpler cross-linked species were not detected even with lower concentrations of diamide, it seems unlikely that they are due to the higher oligomer formation. Instead, they appear to arise from two simultaneous cross-links, one intermolecular and one intramolecular (Fig. 9). Since the simpler complexes are not detected, the two events would need to be cooperatively coupled, i.e., the intrasubunit cross-linking event occurs whenever the intermolecular cross-link is formed or vice versa.

When RYR1 is oxidized with diamide prior to calpain digestion, there is a decrease in fragments b, c, g, and e and an increase in two previously minor fragments, which we designated as x and y. Based on their size and recognition by sequence-specific antibodies, we suggest that x and y arise from a new cleavage of b and c, respectively, both at a sequence somewhere around amino acid 2100. On two-dimensional gels, the cross-linking patterns obtained in these experiments are very similar to those obtained with diamide cross-linking of the calpain-digested fragments except for the four new off-diagonal spots (Fig. 7A). The fragments in the four new spots are x, y, d, and f. The cross-linked products of the fragments x and y migrate much faster than one would predict if they were dimers. The apparent size of the oxidized complex of x is 580 kDa, while the apparent size of the complex of y is 380 kDa. This would represent an increase in mass of 200 kDa for x and 100 kDa for y when cross-linked. This mobility change suggests that the oxidized complex is larger than a monomer but smaller than a dimer. No off-diagonal spots with apparent molecular mass between 100 and 200 kDa align with the off-diagonal x and y, respectively. These off-diagonal spots are, therefore, either monomers of x and y that migrate more slowly in the first dimension gel than in the second dimension when fully reduced, or they are from dimers that migrate much faster in the first dimension gel than would be predicted. The altered mobility could arise from a more compact shape that allows them to move through the gel more rapidly. Our data are most consistent with these x and y complexes being dimers that migrate as though they are in a more compact conformation than the b and c dimers.

There are two other off-diagonal spots, involving bands f and d, which increase in intensity if the calpain digestion comes after the oxidation. The increase in the x, y, f, and d off-diagonal spots is accompanied by a corresponding decrease in the off-diagonal spots that arise from the c and b dimers.

There are four of the new off-diagonal spots are due to the additional cleavage of the b and c dimers. The new off-diagonal spots

![Fig. 9. Model for RYR1 domains cross-linked by diamide and cleaved by calpain. Fragments generated by calpain cleavage are indicated by lowercase letters. The scissors indicate the sites of cleavage by calpain, and the short lines connecting either gz with f or gz with the f on the next subunit represent the putative positions of the intra- and intermolecular cross-links. The scissors N-terminal to the fragment labeled gz represent the new site of calpain cleavage detected in the oxidized RYR1.](image-url)
involving fragments d and f both align with an 80-kDa fragment that is recognized by Ab 2727. This fragment (fragment z) appears to arise from the new cleavage (at about amino acid 2100) and extends to 2843. Both the original cleavage after 2843 and the new cleavage at about 2100 occur in dimers of c or b to generate z + f or z + d. The cross-linked complex of z (80 kDa) with f (197 kDa) has an apparent molecular mass of about 410 kDa, while the complex of z with d (307 kDa) has an apparent mass of slightly smaller than the dimer of c (about 680 kDa), suggesting that they are also multiple fragment cross-links. In the samples that were not oxidized prior to calpain cleavage, fragment z is a part of fragment g. We have previously suggested that g forms both intra- and intersubunit cross-links with either fragments f or d. This suggests that the cysteines involved in both the inter- and intramolecular cross-links are all on either the z (80 kDa, from residue ~2100 to 2843) or the f (190 kDa, from residue 2844 to somewhere before amino acid 4685) fragments. For example, the intramolecular cross-link could occur between z on subunit 1 and f on subunit 2, and another cysteine on z of subunit 1 would form an intramolecular cross-link with f on subunit 1 (Fig. 9). There are 100 cysteines in an intact RYR1 subunit, ~18 in fragment z, and ~22 in fragment f.

Since diamide cross-linking activates the channel and this correlates well with the formation of the observed cross-links, our findings suggest the possibility that subunit-subunit interactions within this central domain are involved in channel activation. The hyperreactive sulfhydryls modified in the reaction with NEM, however, appear to be located in the N terminus. This domain of RYR1 must, in some way, interact with the central domain, since alkylation of the N-terminal sulfhydryls completely blocks the cross-linking between the central domains. We do not know whether this is a long distance (i.e., allosteric) effect or if the N terminus physically interacts with the domains of RYR1 involved in the intersubunit interactions.

Recently, Zorzato et al. (44) suggested that there is an intramolecular interaction of the sequence around Gly-341 with a calmodulin binding domain at amino acids 3010–3225 and perhaps with a domain at amino acids 799–1172. These findings are completely consistent with our proposed interaction between the N-terminal domain and the central domain. The sequence of the first fusion protein (amino acids 335–500) used between the N-terminal domain and the central domain. The tramolecular interaction of the sequence around Gly-341 with a cysteine on z of subunit 1 would form an intramolecular cross-link with either fragments f or d. This suggests that the previously suggested that g forms both intra- and intersubunit interactions.

In summary, we provide evidence that the N terminus of RYR1 (amino acids 426–1396) can modulate the interaction between adjacent subunits within the RYR1 tetramer located at the central domain of the subunits. The central domain interactions involve the cooperative formation of both an inter- and an intramolecular disulfide bond, and all cysteines involved in these cross-links are either between amino acids 2100 and 2843 or between amino acids 2844 and 4685.

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