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A Role for Cysteine 3635 of RYR1 in Redox Modulation and Calmodulin Binding

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Oxidation of the skeletal muscle Ca²⁺ release channel (RYR1) increases its activity, produces intersubunit disulfide bonds, and blocks its interaction with calmodulin. Conversely, bound calmodulin protects RYR1 from the effects of oxidants (Zhang, J.-Z., Wu, Y., Williams, B. Y., Rodney, G., Mandel, F., Strasburg, G. M., and Hamilton, S. L. (1999) Am. J. Physiol. 276, Cell Physiol. C46–C53). In addition, calmodulin protects RYR1 from tryptic cleavage at amino acids 3630 and 3637 (Moore, C. P., Rodney, G., Zhang, J.-Z., Santacruz-Toloza, L., Strasburg, G. M., and Hamilton, S. L. (1999) Biochemistry 38, 8532–8537). The sequence between these two tryptic sites is AVVACFR. Alkylation of RYR1 with N-ethylmaleimide (NEM) blocks both 35S-apocalmodulin binding and oxidation-induced intersubunit cross-linking. In the current work, we demonstrate that both cysteines needed for the oxidation-induced intersubunit cross-link are protected from alkylation with N-ethylmaleimide by bound calmodulin. We also show, using N-terminal amino acid sequencing together with analysis of the distribution of [3H]NEM labeling with each sequencing cycle, that cysteine 3635 of RYR1 is rapidly labeled by NEM and that this labeling is blocked by bound calmodulin. We propose that cysteine 3635 is located at an intersubunit contact site that is close to or within a calmodulin binding site. These findings suggest that calmodulin and oxidation modulate RYR1 activity by regulating intersubunit interactions in a mutually exclusive manner and that these interactions involve cysteine 3635.

Reactive oxygen species and nitric oxide (NO)¹ are produced by skeletal muscle even at rest, but their levels are dramatically increased by muscle activity (1–5). Both reactive oxygen species and NO alter muscle function (6), possibly by altering excitation-contraction coupling (7). One of the proteins involved in excitation-contraction coupling, the skeletal muscle Ca²⁺ release channel, is modulated by both oxidants (8–10) and NO (6, 11). This regulation appears to be controlled, at least in part, by the binding of calmodulin (CaM) to RYR1 (12, 13). Ca²⁺-free CaM is a partial agonist of RYR1, whereas Ca²⁺-CaM is an inhibitor (14). In addition to these direct functional effects, CaM bound to RYR1 protects the channel from oxidation-induced intersubunit cross-linking (12), and conversely, oxidation can block CaM binding to RYR1. In contrast to the effects of oxidation, alkylation of RYR1 with NEM rapidly destroys the ability of RYR1 to bind 35S-Ca²⁺-free CaM (apoCaM) but does not alter its ability to bind 35S-Ca²⁺–CaM. Alkylation also prevents oxidation-induced intersubunit cross-linking. Our studies suggest that there is only one CaM site per subunit of RYR1 at either high or low Ca²⁺ (13). Some indication of the location of the CaM binding site has been obtained from examination of sites on RYR1 protected from tryptic cleavage by CaM (13). Treatment of RYR1 with trypsin rapidly destroys its ability to bind CaM, but CaM bound to RYR1 can protect its binding site from tryptic digestion. The sites protected by Ca²⁺–CaM or apoCaM are at amino acids 3630 and 3637, suggesting that either the binding sites for CaM in both the Ca²⁺-free and Ca²⁺-bound forms are physically close to this region of RYR1 or that the binding of both forms of CaM produces a conformational change that buries this region of RYR1. The latter possibility seems unlikely because the functional effects of apoCaM are opposite those of Ca²⁺–CaM. The sequence between these two sites is AVVACFR, suggesting that cysteine 3635 may be one of the cysteines that, in the absence of CaM, can form the intersubunit disulfide bond. This intersubunit contact site is likely to represent an important site for regulating RYR1 activity. In the current study, we demonstrate that cysteine 3635 is one of the amino acids involved in redox modulation of RYR1.

EXPERIMENTAL PROCEDURES

Materials—Both [3H]NEM (47 Ci/mmol) and [14C]NEM (0.04 Ci/mmol) were purchased from NEN Life Science Products. Dithiothreitol (DTT), NEM, MOPS, CHAPS, EGTA, and bovine serum albumin were obtained from Sigma. Digitonin was purchased from CalBiochem (La Jolla, Ca). Unlabeled CaM (bovine brain) was obtained either from Sigma or from Upstate Biochemicals (Lake Placid, N.Y.).

SR Membrane Preparation—SR membranes were prepared from rabbit leg and backstrap white skeletal muscle and were purified using sucrose gradient centrifugation as described previously (15).

SDS-Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was performed as described by Laemml (16) or Schägger and von Jagow (17).

NeM Alkylation and Diamide Oxidation of SR Membranes—SR membranes were reduced with 0.1 mM DTT for 30 min at room temperature. Excess DTT was removed by pelleting in an Airfuge for 5 min at 30 p.s.i. The membranes (1 mg/ml) were then labeled with 10 μM [3H]NEM in the presence or absence of CaM for 10 min on ice. Reactions and CaM incubations were carried out in either high Ca²⁺ buffer (300 mM NaCl, 50 mM MOPS (pH 7.4), 1 mM EGTA, 1.2 mM CaCl₂) or low Ca²⁺ buffer (300 mM NaCl, 50 mM MOPS (pH 7.4), 1 mM EGTA). The reactions were stopped by the addition of DTT to 0.1 mM. Protein was centrifuged in a Beckman TL100.3 rotor for 45 min at 30,000 rpm and resuspended to remove CaM. Diamide was added to 100 μM and incubated for 5 min on ice to induce intersubunit cross-links of RYR1. NEM was added to 5 mM to stop the reaction with diamide. Equal amounts of protein were electrophoresed on a 5% Laemmli gel (16).

Generation of Trypsin-Digested Complexes of RYR1—SR membranes were trypsinized for 10 min at 37 °C at a protease to protein ratio of 1:100.

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RESULTS AND DISCUSSION

CaM Protects Both Cysteines Required for Oxidation-induced Intersubunit Cross-linking from NEM Alkylation—The activity of RYR1 is modulated by modification of its cysteine residues by oxidation (8–10), alkylation (11, 12, 19) or nitrosylation (6, 7, 11, 20). The cysteine residues involved in these reactions have not previously been identified. The identification of the cysteines involved in these modifications is difficult because RYR1 has 100 cysteines per subunit. However, the functionally important cysteines appear to be hyper-reactive (21–23). We have previously shown that the N terminus of RYR1 contains important cysteines that are very heavily labeled by [14C]NEM under these conditions, but their labeling is not altered by the presence of calmodulin. We have previously identified these bands as fragments of the N terminus (24). The bands obtained after labeling in low Ca2+ have also been excised and digested, and the radiolabel was quantified. We find a 65 ± 13% (n = 3) decrease in radioactivity in the 160-kDa band and a 41 ± 19% (n = 3) decrease in the 150-kDa when labeling is carried out in the presence of CaM.

Localization of the RYR1 Cysteine Protected by Bound CaM—An examination of the sequence between the two CaM protected cleavage sites shows a cysteine at position 3635. To test whether this cysteine modulates CaM binding, we incubated membranes in the presence and absence of CaM at μM Ca2+ and then reacted with [3H]NEM. In these experiments, CaM was then removed by successive washes, and the membranes were digested with trypsin. This treatment cleaves RYR1 after amino acids 3630 and 3637, producing two fragments in approximately equal amounts that differ by 7 amino acids. The N-terminal sequence of fragment 1 that begins at amino acid 3630 is AVVACFRMTPLYNLPTAC. In fragment 2 there are cysteines that will be detected at sequencing cycles 5 and 20, whereas fragment 2 has a cysteine that would be detected at cycle 13. Half of the sample from each round of sequencing was collected for scintillation counting. The 3H radiolabel was detected only in the fifth cycle of sequencing, corresponding to cysteine 3635 in fragment 1 (Fig. 3). Fragment 2 has a tyrosine at cycle 5. The incorporation was greatly decreased by the presence of CaM during the labeling (Fig. 3). No radiolabel was detected in cycle 13 or 20, the position of cysteine 3650 in fragments 2 and 1, respectively.

Summary and Conclusions—Our data suggest that one potential site of redox modulation of RYR1 is cysteine 3635 and that the reactivity of this residue is regulated by the binding of CaM to RYR1. CaM protects RYR1 from oxidation-induced intersubunit cross-linking and alkylation-induced inactivation of the apoCaM-binding site. Our findings support the assignment of cysteine 3635 as the site of NEM alkylation that blocks apoCaM binding. The question is whether this cysteine is also one of the two cysteines involved in the intersubunit cross-link. NEM can prevent the formation of the diamide-induced disul-
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Fig. 2. Effect of CaM on the incorporation of [14C]NEM into the 160-kDa tryptic fragment. SR membranes (34 mg, reduced and washed) were resuspended in 10 ml of low Ca2+ binding buffer and divided into four aliquots of 2.5 ml. Ca2+ (1.2 mM) was added to aliquots 3 and 4. CaM (20 μM) was added to aliquots 2 and 4, and all samples were incubated for 30 min at room temperature. [14C]NEM was added to all samples to a final concentration of 10 μM. After 5 min on ice, alkylation was stopped by the addition of 1 mM DTT. All samples were then digested with trypsin (1:1000) in high Ca2+ buffer and in the presence of 20 μM CaM to prevent the trypsin cleavage at amino acids 3630 and 3637. Digestion with trypsin, solubilization of the membranes, and isolation of the proteolytic complexes was carried out as described under “Experimental Procedures.” Purified proteolyzed RYR1 (20 μg) from each treatment was electrophoresed on Schagger gels, and the gels were stained with Coomassie Brilliant Blue. The gel was then treated with Amplify (Amersham Pharmacia Biotech) and exposed to x-ray film. The arrows indicate the position of the 160- and 150-kDa band (previously shown to begin with amino acid 3119; Ref. 13). Lane 1, alkylated in low calcium conditions, no CaM present during alkylation; lane 2, low calcium alkylation, CaM present; lane 3, high calcium, no CaM; and lane 4, high calcium, CaM present during alkylation. The small letters indicate previously determined (Ref. 24) RYR1 fragment identities and the number of cysteines in each: a, aa 1320–4475 (150 kDa), 19 cysteines; b, aa 1320–4475 (150 kDa), 19 cysteines; c, aa 1397–2401 (113 kDa), 24 cysteines; and d, aa 1397–2401 (113 kDa), 24 cysteines. The two lower bands are calsequestrin and the T1 fraction of the Ca2+-ATPase (25). Identification of these bands was based on the basis of N-terminal sequence and Western blotting with sequence-specific antibodies. The relative mobility of the fragments on this gel system does not accurately reflect their mass.

Fig. 3. Alkylation of cysteine 3635 is blocked by CaM. As in Figs. 1 and 2, SR membranes were reduced, washed by centrifugation, and resuspended at 1 mg/ml in high Ca2+ binding buffer. Samples were incubated for 30 min at room temperature in the presence and absence of 10 μM CaM. [3H]NEM was added to both samples to 1 mM. After incubation for 10 min on ice, the alkylation was terminated with 5 mM DTT. Trypsin treatment and isolation of tryptic complexes was performed as described previously (24). Samples were electrophoresed on Schagger gels (17) and transferred to Immobilon-S for sequencing. Half of each sample at each cycle of sequencing was collected for scintillation counting. The figure shows the [3H]NEM cpm per cycle of sequencing for both samples. Open symbols (○) represent samples obtained from the alkylation in the presence of CaM, closed symbols (■) indicate those alkylated in the absence of CaM. This experiment was performed twice with similar results.

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