Oxidation of the skeletal muscle Ca$^{2+}$ release channel alters calmodulin binding

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Oxidation of the skeletal muscle Ca$^{2+}$ release channel alters calmodulin binding. Am. J. Physiol. 276 (Cell Physiol. 45): C46–C53, 1999.—This study presents evidence for a close relationship between the oxidation state of the skeletal muscle Ca$^{2+}$ release channel (RyR1) and its ability to bind calmodulin (CaM). CaM enhances the activity of RyR1 in low Ca$^{2+}$ and inhibits its activity in high Ca$^{2+}$. Oxidation, which activates the channel, blocks the binding of 125I-CaM at both micromolar and nanomolar Ca$^{2+}$ concentrations. Conversely, bound CaM slows oxidation-induced cross-linking between subunits of the RyR1 tetramer. Alkylation of reactive sulphydryls (<3% of the total sulphydryls) on RyR1 with N-ethylmaleimide completely blocks oxidant-induced intersubunit cross-linking and inhibits Ca$^{2+}$-free 125I-CaM but not Ca$^{2+}$-apoCaM binding. These studies suggest that 1) the sites on RyR1 for binding apocalmodulin have features distinct from those of the Ca$^{2+}$/CaM site, 2) oxidation may alter the activity of RyR1 in part by altering its interaction with CaM, and 3) CaM may protect RyR1 from oxidative modifications during periods of oxidative stress.

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**MUSCLE CONTRACTILE FUNCTION** is altered by both reactive oxygen intermediates (ROIs) and nitric oxide (NO) (20, 31). In unfatigued muscle, ROIs enhance contractile function, and NO opposes this effect (31). One of the targets of these agents is thought to be the skeletal muscle Ca$^{2+}$ release channel (1, 13, 41), also known as the ryanodine receptor (RyR). Oxidation-induced changes in RyR1 activity could arise either from direct modification of channel properties or from alterations in its interaction with endogenous modulators. There is now substantial evidence to suggest that oxidation of RyR1 modifies its intrinsic channel properties (1, 13, 40). We have previously shown that oxidation of RyR1 increases channel activity and produces intersubunit cross-links between neighboring subunits of the RyR1 tetramer (4). Consistent with the opposing effects of oxidation and NO on contractile function, both the oxidation-induced channel activation and the intersubunit cross-linking are blocked by NO (2). The effects of oxidation of RyR1 on its interaction with modulators have not been fully explored. Oxidation is, however, known to alter the interaction of the cardiac Ca$^{2+}$ release channel (RyR2) with calmodulin (CaM) (18, 24, 26, 40).

RyR1 regulates the release of Ca$^{2+}$ from the lumen of the sarcoplasmic reticulum (SR) into the muscle cytoplasm. RyR1, a homotetramer with a subunit molecular mass of 565 kDa (35), is regulated by numerous modulators that control the opening of the channel. Most of the binding sites for these modulators are in the amino-terminal four-fifths of the RyR1 molecule that form the cytoplasmic “foot” region extending from the surface of the SR to the transverse tubules. The channel-forming portion of the protein, which traverses the bilayer, consists of the carboxy-terminal one-fifth of the protein. Mutations in RyR1 are associated with human diseases such as malignant hyperthermia (MH) and central core disease. All of the currently identified mutations in RyR1 that produce MH or central core disease are also in the putative cytoplasmic domains (14, 28–30, 41).

CaM is a ubiquitous Ca$^{2+}$ binding protein. The crystal structure of CaM is that of a dumbbell-shaped molecule consisting of an amino-terminal lobe and a carboxy-terminal lobe separated by an eight-turn α-helix (5). The amino-terminal and carboxy-terminal lobes each contain two Ca$^{2+}$ binding sites that undergo Ca$^{2+}$-dependent exposure of their hydrophobic binding pockets to allow binding and activation of target proteins (for review, see Refs. 11, 17, 27, 37). Upon binding Ca$^{2+}$, both the amino-terminal and carboxy-terminal hydrophobic pockets of CaM bind to amphipathic α-helical domains within the target protein structure. Although CaM binding to most of its target proteins is Ca$^{2+}$ dependent, Ca$^{2+}$-free CaM (apoCaM) has also been shown to bind to some proteins (9, 10, 16, 23), including RyR1 (36, 39). The interactions of CaM with some of its targets, including RyR2, have been shown to be modified by oxidation (18, 24, 26).

RyR1 is the major CaM binding protein of SR membranes (36, 39). Yang et al. (39) first demonstrated that there were more binding sites for CaM on RyR1 at nanomolar Ca$^{2+}$ than at micromolar Ca$^{2+}$. Tripathy et al. (36) subsequently showed that, at nanomolar Ca$^{2+}$ concentrations, CaM binds to four sites per subunit (16 per tetramer) and this binding activates the channel. They further showed that, at micromolar Ca$^{2+}$ concentrations, there appears to be only one site per subunit (4 per tetramer) and this binding inhibits channel activity.

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In the current study, we show that there is an interdependence of the regulation of RyR1 by oxidants and CaM. The formation of disulfide bonds within the RyR1 tetramer abolishes the binding of $^{125}$I-labeled CaM, suggesting the possibility that either CaM binds close to the site of intersubunit cross-linking or the formation of the intersubunit cross-link allosterically alters the CaM binding site(s). In addition, we show that alklylation of hyperreactive sulfhydryls on RyR1 blocks apoCaM binding but not Ca$^{2+}$/CaM binding, suggesting that there are distinct differences in the apoCaM and Ca$^{2+}$/CaM binding sites on RyR1. The ability of CaM to slow oxidative modification of RyR1 suggests that it may play a protective role during periods of oxidative stress.

**EXPERIMENTAL PROCEDURES**

Materials. $[^{3}H]$ryanodine (70–80 Ci/mmol) and N-$[^{14}C]$ethylmaleimide ($[^{14}C]$NEM; 0.05 Ci/mmol) were purchased from NEN (Life Science Products, Boston, MA). Monooxidinated Bolton-Hunter reagent (2,200 Ci/mmol) was prepared from Sigma (St. Louis, MO). Unlabeled CaM (bovine brain) was obtained either from Sigma or Upstate Biotechnology (Lake Placid, NY).

SR membrane preparation. SR membranes were prepared from rabbit leg and backstrap white skeletal muscle and were purified using sucrose gradient centrifugation (15).

Western blotting. Protein samples subjected to SDS-PAGE were transferred to Immobilon P membrane (Millipore, Bedford, MA) for 16–18 h at 25 V in 5% methanol-10 mM CAPS (pH 11.0). The blots were developed with primary antibodies and alkaline phosphatase-conjugated secondary antibodies as previously described (8).

Diamide cross-linking. SR membranes (1–5 mg/ml) with and without CaM (5 µM) were incubated with diamide (100 µM) for 5–30 min at 4°C in 300 mM NaCl, 50 mM MOPS (pH 7.4), and 1 mM EGTA, with or without 1.2 mM CaCl$_2$. Diamide was removed by pelleting the membranes for 4 min at 30 psi in a Beckman Airfuge. The membranes were then resuspended in 300 mM NaCl, 50 mM MOPS (pH 7.4), and 1 mM EGTA, with or without 1.2 mM CaCl$_2$. Alkylation of SR membranes with NEM. SR membranes (2.2 mg/ml) were treated with 1 mM NEM on ice. Alkylation was stopped at indicated time points by the addition of 20 mM DTT.

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 BSA, 0.1% CHAPS, and 1 mM EGTA, and 1.2 mM CaCl$_2$]. Nonspecific binding was defined in the presence of 5 µM unlabeled ryanodine. The bound $[^{3}H]$ryanodine was separated from free ligand by filtration through Whatman GF/F glass fiber filters. The filters were washed five times with 3 ml of ice-cold buffer containing 300 mM NaCl, 1 mM EGTA, 1.2 mM CaCl$_2$, 50 mM MOPS (pH 7.4), and 100 µg/ml BSA. The radioactivity bound to the filters was quantitated by scintillation counting.

Bolton-Hunter $^{125}$I labeling of CaM. Mammalian CaM (bovine brain, Upstate Biotechnology) was iodinated by a modified Bolton-Hunter reaction (6). Briefly, CaM (0.3 mg, 0.6 mg/ml) was reacted with monooxidinated Bolton-Hunter reagent (250 µCi) in 1 mM EGTA-50 mM MOPS (pH 7.4) for 1 h on ice with frequent agitation. The reaction was quenched by the addition of 1 M glycine (pH 7.4) and then applied to a desalting column (D-Salt dextran, Pierce) that had been preequilibrated with 5 vol of 50 mM MOPS (pH 7.4) followed by 5 vol of 50 mM MOPS-0.02% NaN$_3$ (pH 7.4). The column was eluted with 50 mM MOPS-0.02% NaN$_3$ (pH 7.4), and 0.5-ml fractions were collected. Radioactivity in each fraction was monitored, and the two fractions with the highest counts were combined. Protein content was determined according to the method of Bradford (7), using unlabeled CaM as a standard. The concentration of CaM was also determined by measuring its absorbance at 277 and 320 nm (A$_{277}$ and A$_{320}$, respectively) and calculating the concentration (C; in mg/ml) according to the equation (from Ref. 32) $C = (A_{277} - A_{320})/e$, where $e = 0.18$ ml·mg$^{-1}$·cm$^{-1}$ in high-Ca$^{2+}$ buffer and 0.20 ml·mg$^{-1}$·cm$^{-1}$ in low-Ca$^{2+}$ buffer. It should be noted that the iodination of CaM in the presence of micromolar Ca$^{2+}$ inactivated some of the CaM for binding to RyR1 when the binding was performed at micromolar Ca$^{2+}$ (unpublished observation). The iodination conditions did not alter CaM binding in low Ca$^{2+}$. All studies described were performed with $^{125}$I-CaM iodinated in the presence of EGTA.

$^{125}$I-CaM binding. SR membranes (10 µg/assay) were incubated with $^{125}$I-CaM (1.6–200 nM) for 2 h at room temperature in binding buffer [300 mM NaCl, 50 mM MOPS (pH 7.4), 100 µg/ml BSA, 0.1% CHAPS, and 1 mM EGTA] in the absence (low-Ca$^{2+}$ buffer) or presence (high-Ca$^{2+}$ buffer) of 1.2 mM CaCl$_2$. Nonspecific binding was defined in the presence of 5 µM unlabeled CaM. Bound radioligand was separated from free radioligand by filtration through Whatman GF/F or GF/C filters presoaked in 0.3 mg BSA/ml binding buffer, and the filters were washed five times with 3 ml of ice-cold binding buffer. For experiments on CaM inhibition of diamide-induced cross-linking of RyR1 (see Fig. 3), the wash buffer used was 1 mM EGTA, 300 mM NaCl, 50 mM MOPS (pH 7.4), 100 µg/ml BSA, and 0.1% CHAPS. Radioactivity was quantitated using a Beckman gamma counter. Free Ca$^{2+}$ concentrations were calculated as described by Fabiato (12). The data are presented as means ± SE.

**RESULTS**

CaM effects on $[^{3}H]$ryanodine binding to control and diamide-treated membranes. We have previously established conditions for $[^{3}H]$ryanodine binding that allow us not only to readily detect changes in the affinity of RyR1 for this ligand in the presence of channel modulators (15) but also to prevent the slow decline in $[^{3}H]$ryanodine binding often seen with SR membranes (22). Our assays are routinely performed in 300 mM NaCl, 50 mM MOPS (pH 7.4), 1 mM EGTA, 1.2 mM CaCl$_2$ (200 µM free Ca$^{2+}$), 100 µg/ml BSA, and 0.1% CHAPS. We examined the ability of CaM to alter $[^{3}H]$ryanodine binding under these conditions. In 200 µM free Ca$^{2+}$, CaM inhibited the binding of $[^{3}H]$ryanodine to SR membranes (Fig. 1A), as previously reported by Tripathy et al. (36). However, with 5 nM $[^{3}H]$ryanodine, the inhibition by CaM plateaued at ~50%, suggesting that the binding of CaM is not competitive with that of $[^{3}H]$ryanodine. The oxidation with diamide of RyR1 in SR membranes before incubation with CaM prevented the inhibition of $[^{3}H]$ryanodine binding by CaM (Fig. 1A). Using EGTA to decrease the free Ca$^{2+}$...
concentration to <10 nM, we found that CaM enhanced [3H]ryanodine binding (Fig. 1B), again consistent with the findings of Tripathy et al. (36). Treatment of membranes with diamide also enhanced [3H]ryanodine binding at low Ca2+ concentrations compared with control membranes, but subsequent addition of CaM produced no further increase (Fig. 1B).

[3H]-CaM binding to control and diamide-pretreated membranes. RyR1 is the major CaM binding protein in SR membranes (33, 39), thus enabling us to examine

Table 1. [3H]-CaM binding to control, oxidized, and alkylated sarcoplasmic reticulum membranes

<table>
<thead>
<tr>
<th>Membranes</th>
<th>Kd, nM</th>
<th>Bmax, pmol/mg</th>
<th>CaM/RyR1</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29 ± 2</td>
<td>113 ± 6</td>
<td>8 ± 1</td>
<td>8</td>
</tr>
<tr>
<td>nM Ca2+</td>
<td>39 ± 2</td>
<td>76 ± 4</td>
<td>5 ± 1</td>
<td>9</td>
</tr>
<tr>
<td>µM Ca2+</td>
<td>99 ± 22</td>
<td>13 ± 4</td>
<td>0.9 ± 0.3</td>
<td>3</td>
</tr>
<tr>
<td>Diamide pretreated</td>
<td>26 ± 7</td>
<td>21 ± 6</td>
<td>1.4 ± 0.4</td>
<td>3</td>
</tr>
<tr>
<td>nM Ca2+</td>
<td>27 ± 5</td>
<td>35 ± 5</td>
<td>2.4 ± 0.4</td>
<td>3</td>
</tr>
<tr>
<td>µM Ca2+</td>
<td>37 ± 7</td>
<td>64 ± 7</td>
<td>4.4 ± 0.5</td>
<td>3</td>
</tr>
</tbody>
</table>

Values are means ± SE for no. of membranes (n) indicated. Kd, dissociation constant; Bmax, maximum binding capacity; CaM/RyR1, ratio of calmodulin binding sites to ryanodine binding sites; NEM, N-ethylmaleimide.

The ratio in
induced decrease in Bmax for 125I-CaM binding was 87%.

CaM binding assay. Diamide pretreatment of SR membranes at either nanomolar or micromolar Ca2+ concentrations (data not shown).

Upon the addition of high concentrations of NEM (1 mM) to membranes, alkylation initially inhibits activity and [3H]ryanodine binding (phase 1); further reaction enhances channel activity and [3H]ryanodine binding (phase 2), and finally there is again inhibition of both channel activity and [3H]ryanodine binding (phase 3). A subset of sulfhydryls in the amino terminus of RyR1 between amino acids 1 and 1400 is alkylated at hyperreactive sulfhydryls on the ability of RyR1 to bind 125I-CaM. Set of 3 bars at left was obtained from studies performed at <10 nM Ca2+, and set of 3 bars at right was obtained from studies performed at 200 µM Ca2+. *Statistically significant difference vs. corresponding control (P < 0.05) in a paired t-test.

Fig. 3. CaM inhibits diamide-induced cross-linking of ryanodine receptor (RyR1). A: SDS-PAGE analysis. Lane 1, control membranes; lane 2, membranes treated with 100 µM diamide for 5 min at room temperature; lane 3, membranes preincubated with 5 µM CaM before cross-linking with diamide for 5 min at room temperature. The 410-kDa band represents a carboxy-terminal proteolytic fragment of 565-kDa, arising from calpain cleavage before or during membrane preparation. Proteins with apparent molecular masses of ≈100 kDa run at dye front in these gels. B: Western blot analysis. Western blot of gel shown in A, using an antibody specific for last 9 amino acids at carboxy terminus of RyR1 (8).

C: summary of effect of CaM on cross-linking of RyR1. Membranes were oxidized as for A, and RyR1 565-kDa Coomassie brilliant blue-stained bands in SDS gels were scanned with an Image Master densitometer. Optical densities (OD) of 565-kDa bands in cross-linked samples (ODx) were normalized to OD of 565-kDa band in parallel samples that were not cross-linked (ODc). Experiment was repeated with 3 different membrane preparations. Set of 3 bars at left was obtained from studies performed at <10 nM Ca2+, and set of 3 bars at right was obtained from studies performed at 200 µM Ca2+. *Statistically significant difference vs. corresponding control (P < 0.05) in a paired t-test.

Alkylation of RyR1 with NEM blocks 125I-apoCaM binding but not 125I-Ca2+ CaM binding. We have previously demonstrated that the NEM alkylation of hyperreactive sulfhydryls on RyR1 blocks intersubunit cross-linking (4). The extent of the NEM reaction with RyR1 occurs (38). We examined the effect of alkylation of the hyperreactive sulfhydryls on the ability of RyR1 to bind 125I-CaM. For comparison, we also assayed the same membranes for ability to bind [3H]ryanodine. Alkylation of hyperreactive sulfhydryls on RyR1 with 1 mM

nanomolar Ca2+ is lower than those reported by Tripathy et al. (36) and Yang et al. (39) and may reflect the different binding conditions used in our assay.

We next examined the effect of 100 µM diamide pretreatment of SR membranes on 125I-CaM binding (Fig. 2). Diamide was removed from the membranes by centrifugation in a Beckman Airfuge before the 125I-CaM binding assay. Diamide pretreatment of SR membranes greatly reduced the number of 125I-CaM sites at both nanomolar and micromolar Ca2+ concentrations. The Kd for 125I-CaM binding to diamide-pretreated membranes in low-Ca2+ buffer was 99 ± 22 nM, and the Bmax was 13 ± 4 pmol/mg (n = 3). The oxidation-induced decrease in Bmax for 125I-CaM binding was 87 ± 3% (n = 3, P < 0.01, paired t-test) in low-Ca2+ buffer.

The Kd for 125I-CaM binding to diamide-pretreated membranes in high-Ca2+ buffer was 26 ± 7 nM, and the Bmax was 21 ± 6 pmol/mg (n = 3). In high-Ca2+ buffer, diamide caused a 76 ± 3% (n = 3, P < 0.01, paired t-test) decrease in the Bmax. Pretreatment of 125I-CaM itself with diamide had no effect on its ability to bind to SR membranes at either nanomolar or micromolar Ca2+ concentrations (data not shown).

CaM slows diamide-induced intersubunit cross-linking. We have previously shown that diamide activates RyR1 and, concomitantly, produces cross-links between nearest neighboring subunits within the RyR1 tetramer (4). Here we investigated the effect of CaM on RyR1 intersubunit cross-linking in SR membranes. Diamide pretreatment of SR membranes resulted in the formation of new bands with an electrophoretic mobility consistent with that of RyR1 dimers. When diamide cross-linking was performed in the presence of 5 µM CaM at either high or low Ca2+ concentration, the formation of cross-linked products was reduced, as indicated by the reduction in dimers in the Coomassie-stained gels and Western blots shown in Fig. 3, A and B, respectively. The extent of inhibition of cross-linking by CaM was determined by densitometric scanning of the 565-kDa band under each condition for three different membrane preparations (Fig. 3C). Paired t-test analysis indicated that the inhibitory effect of CaM on the cross-link formation was statistically significant (P < 0.05).
NEM completely blocked 125I-CaM binding in low-Ca2+ buffer, enhanced 125I-CaM binding in high-Ca2+ buffer, and, as expected, inhibited [3H]ryanodine binding (Fig. 4A). Further alkylation to phases 2 and 3 caused no further changes in either 125I-apoCaM or 125I-Ca2+/CaM binding (data not shown). Further examination of the data in Fig. 4A showed that the loss of 125I-apoCaM binding appeared to occur more rapidly than either the enhancement of 125I-Ca2+/CaM binding or the loss of [3H]ryanodine binding. In fact, the loss of 125I-apoCaM binding can still be detected at 50-fold lower NEM concentrations (Fig. 4B). Reaction of SR membranes with 20 µM NEM produced a rapid loss in 125I-apoCaM binding with little or no effect on [3H]ryanodine binding or 125I-Ca2+/CaM binding. It should be noted that, although there are apparently two binding sites for 125I-apoCaM per subunit, both the alkylation with NEM and the oxidation with diamide blocked CaM binding to both sites. As we have previously reported for the higher concentrations of NEM, the reaction of SR membranes with 20 µM NEM also blocked the cross-linking of the 565-kDa band by diamide (Fig. 5).

The effects of NEM on the binding of 125I-CaM in both high- and low-Ca2+ buffers were also assessed by Scatchard analysis (Fig. 6). In low-Ca2+ buffer, the Kd for 125I-CaM binding to membranes pretreated with 20 µM NEM was 27 ± 5 nM, and the Bmax was 35 ± 5 pmol/mg (n = 3). This represents a 70 ± 3% (n = 3, P < 0.01) decrease in the number of binding sites in low Ca2+, with no statistically significant change in the apparent affinity of the membranes for 125I-CaM. In high-Ca2+ buffer, the Kd for 125I-CaM binding to membranes pretreated with 20 µM NEM was 37 ± 7 nM, and the Bmax was 64 ± 7 pmol/mg (n = 3). In high-Ca2+ buffer...
buffer, therefore, 20 µM NEM pretreatment produced no statistically significant change in either the number of binding sites for [125I]-CaM or the affinity.

To determine whether the effects of pretreatment of RyR1 with 20 µM NEM on [125I]-CaM binding correlated with alkylation of RyR1, we analyzed the incorporation of [14C]NEM into the 565-kDa RyR1 protein band on SDS gels. The incorporation of radiolabel into the 565-kDa RyR1 band in nanomolar Ca2+ with 20 µM [14C]NEM is shown in Fig. 7A. In four independent experiments, the reaction with 20 µM [14C]NEM for 50 s labeled 2.5 ± 0.2% of the total sulfhydryls on RyR1. The labeling of the 565-kDa band was reduced in the presence of 5 µM CaM by 32 ± 7% (n = 4, with gels for each experiment run in triplicate). We also examined the ability of [125I]-CaM to bind to the membranes used for the SDS gel analysis. The presence of CaM almost completely protected the apoCaM binding site from inactivation by NEM (Fig. 7B) when the incubation was <1 min in duration. These findings suggest that CaM blocks the alkylation of a specific subset of the phase 1 hyperreactive sulfhydryls, approximately equal to one cysteine per apoCaM binding site. Furthermore, the labeling of the 565-kDa band occurs with approximately the same time course as the inhibition of [125I]-CaM binding when assayed in nanomolar Ca2+.

DISCUSSION

Skeletal muscle function is altered by oxidants in the form of ROI's (20), and these are formed at significant levels, especially during periods of exercise. There is now substantial evidence to support the concept that RyR1 of the SR is one of the targets of this reaction (34). Altered release of Ca2+ from the SR is likely to contribute to altered contractile function. In addition to a direct effect on the activity of the channel, the oxidation of RyR1 may alter its interactions with important cellular modulators of Ca2+ release, and, conversely, these modulators may protect RyR1 from oxidative damage. In this study, we demonstrate that the formation of disulfide bonds within the RyR1 tetramer has a dramatic effect on its interaction with CaM, a bifunctional modulator of channel activity, and, conversely, that CaM binding to RyR1 can protect it from oxidative modification.

CaM is present in micromolar concentrations in skeletal muscle (19), and Ca2+ binding to CaM initiates its interaction with most target proteins. However, several proteins bind CaM in the absence of Ca2+ or bind it in both nanomolar and micromolar Ca2+ (9, 10, 16, 23). RyR1 is apparently one of the proteins that is capable of binding both apoCaM and Ca2+/CaM (36, 39). Binding of CaM to RyR1 in nanomolar Ca2+ enhances channel activity, whereas the binding in micromolar Ca2+ is inhibitory (36). Although the mechanism by which CaM modulates RyR1 is poorly understood, its importance to excitation-contraction coupling is supported by the observation that CaM-dependent activation of porcine MH-susceptible channel is altered compared with the normal channel (25).

Our data support a ratio of four to five CaM binding sites per ryanodine site (or one CaM binding site per subunit) at nanomolar Ca2+ and eight CaM binding sites per ryanodine site (two per subunit) at micromolar Ca2+. These workers, however, found four to five sites per subunit at nanomolar Ca2+ concentrations. The difference in the number of binding sites at low Ca2+ may reflect the different binding conditions used. However, both the inhibiting and
activating effects of CaM on the activity of RyR1 can be detected under our buffer conditions.

Oxidants produce intersubunit cross-links and channel activation of RyR1 (4), and these effects are reversed by the addition of the reducing agent DTT. The present study provides evidence that redox-sensitive sulfhydryl groups on RyR1 modulate channel activity in part by altering the ability of RyR1 to bind CaM. We showed that CaM slows the formation of intersubunit cross-links in RyR1 and, conversely, that oxidation of the channel blocks the binding of \(^{125}\)I-CaM by decreasing the apparent number of binding sites. Thus CaM appears to alter subunit-subunit interactions (as assessed by ability to form intersubunit cross-links), and the binding of CaM is dependent on the oxidation state of the channel. CaM, therefore, either slows the access of diamide to the site of cross-linking or causes a conformational change in the protein such that the cysteine residues are no longer oriented to form the intersubunit cross-link.

The sulfhydryl alkylating agent NEM can modify a rapidly reacting class of sulfhydryls on RyR1 (phase 1 alkylation), which results in inhibition of channel activity, \(^{3}H\)ryanodine binding, and intersubunit cross-linking (3). We also previously showed that NO blocks the inhibitory phase of the NEM alkylation of RyR1 (3). The loss of \(^{3}H\)ryanodine binding appears to correlate with the alkylation of a class of sulfhydryls (<10% of the total RyR1 sulfhydryls) between the amino terminus and amino acid 1400 (38). Oxidation blocks the alkylation of this class of cysteines and forms an intersubunit cross-link and, conversely, alkylation of hyperreactive sulfhydryls blocks intersubunit cross-linking. One of the cysteines that forms the disulfide bond between subunits is located between amino acids 1400 and 2843, and the second is located between amino acids 2844 and 4683 (38). The ability of modified cysteines in the amino-terminal domain to block cross-linking in a central domain of RyR1 suggests an interaction (either direct or allosteric) between these two domains.

A surprising finding of this study was that NEM treatment of RyR1 rapidly decreases \(^{125}\)I-apoCaM binding, but not \(^{125}\)I-Ca\(^{2+}\)/CaM binding. In contrast, both apoCaM and Ca\(^{2+}\)/CaM prebound to RyR1 can protect the apoCaM binding sites from both NEM and diamide inactivation. These findings suggest that some of the structural features of the Ca\(^{2+}\)/CaM site are distinct from those of the apoCaM sites. An examination of the ability of NEM to block \(^{125}\)I-apoCaM binding has shown that the sulfhydryls involved react even faster than the phase one alkylation, suggesting a distinct subset of reactive cysteines that alter apoCaM binding. The rapid alkylation is still sufficient to block intersubunit cross-linking by diamide, suggesting that the subset of sulfhydryls is not the same as the sulfhydryls that inhibit channel activity and \(^{3}H\)ryanodine binding. We therefore refer to the sulfhydryls that alter \(^{125}\)I-CaM binding and intersubunit cross-linking as "pre-phase 1 sulfhydryls." The alkylation of these pre-phase 1 sulfhydryls appears to be silent with respect to \(^{3}H\)ryanodine binding and channel activity (both assessed at high Ca\(^{2+}\)). Our labeling studies have shown that about three to five cysteines per subunit are alkylated within 1 min of treatment with 20 µM \(^{14}C\)NEM. CaM blocks the labeling of one or two of these cysteines but completely protects the binding site from inactivation, suggesting that not all of the hyperreactive sulfhydryls are important for apoCaM binding. We have previously shown that most of the hyperreactive sulfhydryls are in the amino-terminal domain of RyR1 (38). We will now be able to use CaM binding to identify the specific cysteines that influence apoCaM. In summary, our data suggest that CaM may play a role in protecting RyR1 from oxidative modification. Studies with intact muscle fibers have shown that ROIs enhance contractile function in resting muscle but that strenuous activity leads to the accumulation of oxidants and the loss of contractile function (31). Oxidants, therefore, appear to have bifunctional effects on RyR1. Our current studies suggest that CaM may play a role in regulating the susceptibility of RyR1 to oxidative modification. One possible consequence of this interaction is that CaM protects RyR1 from the detrimental effects of oxidants. Both oxidation and CaM binding alter the activity of RyR1. At resting intracellular Ca\(^{2+}\) concentrations, either oxidation or CaM binding would be expected to enhance channel activity. However, as Ca\(^{2+}\) rises, CaM binding would become inhibitory, whereas oxidation would still be activating. CaM may, therefore, serve to protect the channel from this effect of oxidants and facilitate channel closing after a Ca\(^{2+}\) transient. Strenuous muscle activity generates higher concentrations of ROIs that could be sufficient to override the CaM protection by blocking the CaM interaction with RyR1, producing conditions that lead to muscle damage.

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