Multiple Classes of Sulfhydryls Modulate the Skeletal Muscle Ca$^{2+}$ Release Channel*

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Two sulfhydryl reagents, N-ethylmaleimide (NEM), an alkylating agent, and diamide, an oxidizing agent, were examined for effects on the skeletal muscle Ca$^{2+}$ release channel. NEM incubated with the channel for increasing periods of time displays three distinct phases in its functional effects on the channel reconstituted into planar lipid bilayers; first it inhibits, then it activates, and finally it again inhibits channel activity. NEM also shows a three-phase effect on the binding of $[^3H]$ryanodine by first decreasing binding (phase 1), followed by a recovery of the binding (phase 2), and then a final phase of inhibition (phase 3). In contrast, diamide 1) activates the channel, 2) enhances $[^3H]$ryanodine binding, 3) cross-links subunits within the Ca$^{2+}$ release channel tetramer, and 4) protects against phase 1 inhibition by NEM. All diamide effects can be reversed by the reducing agent, dithiothreitol. Diamide induces intersubunit dimer formation of both the full-length 565-kDa subunit of the channel and the 400-kDa generated by endogenous calpain digestion, suggesting that the cross-link does not involve sulfhydryls within the N-terminal 170-kDa fragment of the protein. NEM under phase 1 conditions blocks the formation of the intersubunit cross-links by diamide. In addition, single channels activated by diamide are further activated by the addition of NEM. Diamide either cross-links phase 1 sulfhydryls or causes a conformational change in the Ca$^{2+}$ release channel which leads to inaccessibility of phase 1 sulfhydryls to NEM alkylation. The data presented here lay the groundwork for mapping the location of one of the sites of subunit-subunit contact in the Ca$^{2+}$ release channel tetramer and for identifying the functionally important sulfhydryls of this protein.

The Ca$^{2+}$ release channel of skeletal muscle sarcoplasmic reticulum is a homotetramer with a subunit molecular mass of 565 kDa (1). The channel opens in response to a signal from the transverse tubules, which is triggered by membrane depolarization (2), and the resulting flux of Ca$^{2+}$ from the sarcoplasmic reticulum initiates the sequence of events that leads to muscle contraction. Several laboratories have clearly demonstrated that the activity of the Ca$^{2+}$ release channel is modulated by oxidation-reduction reactions (3–11). Oxidizing agents that stimulate Ca$^{2+}$ release from the sarcoplasmic reticulum include H$_2$O$_2$ (3), 2,2'-dithiodipyrindine, 4,4'-dithiodipyridine (4), Cu$^{2+}$ phthaloxyanine dyes (5), anthraquinone doxorubicin (6, 7), and thimerosal (8). Heavy metals such as Hg$^{2+}$, Ag$^+$, Cu$^{2+}$, Cd$^{2+}$, and Zn$^{2+}$ have also been reported to induce Ca$^{2+}$ release, either by directly interacting with a sulfhydryl or by causing oxidation (9). These studies led Abramson and Salama (10) to propose a model for redox modulation of the channel that involves three different sulfhydryl groups that exist in close proximity and that can form mixed disulfides to open or close the channel. The evidence that the channel can be altered by oxidation is conclusive (3–11); the question is whether this oxidation plays a physiological role in skeletal muscle. It is not yet known if disulfide interchange or oxidation-reduction of sulfhydryls on the Ca$^{2+}$ release channel contribute to normal excitation-contraction coupling, but an increasing body of evidence suggests that such a mechanism could be an important modulatory element. Under basal conditions, unfatigued skeletal muscle produces reactive oxygen species (12) and nitric oxide (NO) derivatives (13, 14) that have been shown to modulate excitation-contraction coupling (12, 13). Strentuous contractile activity increases reactive oxidant production (14–17), which contributes to fatigue of both isolated muscle preparations (15, 16, 18, 19) and human muscle in vivo (20). Redox modulation of the ryanodine-binding protein has been proposed as a common mechanism for these effects (21).

Proteins other than the Ca$^{2+}$ release channel may contribute to its modulation by oxidizing agents. Pessah and co-workers (22, 23) have suggested that oxidation may involve the crosslinking of triadin to the Ca$^{2+}$ release channel. The functional role of triadin in skeletal muscle and its relationship to the Ca$^{2+}$ release channel have remained elusive. Caswell and co-workers (24) have suggested that it is involved in coupling of the Ca$^{2+}$ release channel to the t-tubule voltage sensor. Others (25) on the basis of its putative arrangement in the membrane have argued against a role for triadin in connecting the voltage sensor to the Ca$^{2+}$ release channel. There is, however, general agreement that this protein interacts with the Ca$^{2+}$ release channel (24, 26). The functional significance of this interaction is not yet known.

In addition to modulation by oxidation-reduction, the Ca$^{2+}$ release channel is also sensitive to reagents that react with free sulfhydryls but do not form disulfide bonds. N-Ethylmaleimide (NEM), 1 a sulfhydryl alkylating agent, activates Ca$^{2+}$ release at low concentrations, while it inhibits release at higher concentrations (27). 2 NEM, however, induces Ca$^{2+}$ release with a slower onset than the heavy metals, a finding that has been interpreted to mean that the sulfhydryls responsible for the

*This work was supported by a grant from the Muscular Dystrophy Association and Grant AR41802 from the National Institutes of Health (both to S. L. H.) and Grant HL45721 from the National Institutes of Health (to M. B. R.). 1 The abbreviations used are: NEM, N-ethylmaleimide; DTT, dithiothreitol; SR, sarcoplasmic reticulum; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonic acid; EYR1, ryanodine receptor. 2 L. Mészáros, personal communication.

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observed effects on Ca\(^{2+}\) release are in a hydrophilic environment (9). Quinn and Ehrlich (28) reported that modification of cysteines on the Ca\(^{2+}\) release channel by methiosulfonate compounds reduces the conductance of the channel. The reaction occurs only when the channel is in the open state. These compounds reduce the conductance in multiple steps until complete closure of the channel is obtained, suggesting a reactive sulfhydryl in the ion conducting pathway. The multiple effects of sulfhydryl reagents on the activity of the Ca\(^{2+}\) release channel raise the question of whether all of these reagents are reacting with the same sulfhydryls or whether alteration of multiple classes of sulfhydryls can alter the function of the channel.

Each subunit of the Ca\(^{2+}\) release channel tetramer has 100 cysteines (1). In this report we attempt to distinguish between classes of functionally important sulfhydryls. We show that the alkylating reagent NEM has three distinct functional effects on the channel and the oxidizing agent, diamide (29), produces intersubunit cross-links within the tetrameric channel. Additional information about the location of the cross-links is obtained using a membrane preparation that has both the full-length 565-kDa subunit and the 400-kDa fragment generated by the action of endogenous calpain.

**EXPERIMENTAL PROCEDURES**

*Materials—*[^H]Ryanodine (61.5 Ci/mmol) was purchased from DuPont NEN. Ryanodine was obtained from Calbiochem. Diamide (15), NEM, and dithiothreitol (DTT) were obtained from Sigma. Phosphatidylethanolamine (bovine heart) and phosphatidylcholine (bovine brain) were obtained from Avanti Polar Lipids, Inc (Alabaster, AL). Ultima Gold scintillant was purchased from Packard (Meriden, CT).

*Sarcoplasmic Reticulum (SR) Membrane Preparation—*SR membranes were prepared from rabbit backstrapp and hindleg skeletal muscle and were purified by using sucrose gradient centrifugation as described elsewhere (30, 31). Protein was estimated by the method of Lowry et al. (32), using BSA as standard.

*Cross-linking—*For binding experiments, membranes in buffer I (300 mM NaCl, 100 mM Ca\(_{\text{Cl}_2}\), 50 mM MOPS (pH 7.4)) were incubated with 100 to 500 \(\mu\)M diamide for 30 min at 4°C or for 10 min at room temperature. Diamide was removed either by dilution (10-fold) or by pelleting and washing the membranes in a Beckman Airfuge by centrifuging 4 min at 30 p.s.i. For bilayer experiments the sample was incubated for 10 min at room temperature with 500 \(\mu\)M diamide in buffer II (225 mM CaSO\(_4\), 100 mM Ca\(_{\text{Cl}_2}\), 10 mM MOPS, pH 7.4). Membranes cross-linked in buffer I or II were washed and assayed for \[^{3}H\]ryanodine binding and for changes in gel patterns by SDS-PAGE. All \[^{3}H\]ryanodine binding assays were done in buffer I.

**SDS-PAGE Electrophoresis in One and Two Dimensions—**SR membranes were cross-linked with diamide for 10 min at room temperature (23°C) or 30 min on ice. The samples were treated with 5 mM NEM for 20 min at room temperature before solubilization in sample buffer. Electrophoresis on 5% SDS-PAGE was performed for the first dimension. Electrophoresis was continued for 15 min after the dye front ran off gel. The lanes were excised and used for second dimension electrophoresis. Each gel strip was treated with 50 mM DTT as sample buffer without SDS and bromophenol blue for 60 min at room temperature (23°C). The gel strips were loaded on the second dimension gel (5% SDS-PAGE). The gap was sealed with melted agarose (1% agarose). Gold scintillant was purchased from Packard (Meriden, CT).

*Recording solutions contained 225 mM CaSO\(_4\) in both the cis and trans chambers. All additions were made to the cis chamber. Agar/KCl bridges were used to connect the chambers to Ag/AgCl electrodes immersed in 2 M KCl. The holding potential was +400 mV. The data were filtered at 2.5 KHz and digitized at 10 KHz.*

**Data Analysis—**[^H]Ryanodine binding data were analyzed with nonlinear curve fitting using SigmaPlot (Jandel Scientific). Nonspecific binding was subtracted prior to analysis.

Single-channel recordings were analyzed using FETCHAN and pSTAT software (Axon Instruments, Inc). Steady-state open probabilities (\(P_o\)) were determined by measuring the fraction of time that the channel was open in at least 2 min of recording. Instantaneous open probabilities were determined as the fraction that the channel was open in 10 s of recording.

**RESULTS**

*NEM Has Multiple Effects on Both Channel Activity and[^H]Ryanodine Binding—*NEM is an alkylating reagent, which reacts primarily with cysteine residues (35). The effect of NEM alklylation on the activity of the Ca\(^{2+}\) release channel reconstituted into planar lipid bilayers is shown in Fig. 1A. Immediately after the addition of NEM, the channel was inhibited (phase 1). Continued exposure to the alkylating agent (3–10 min) produced a substantial activation (phase 2), but longer incubations led again to inhibition of channel activity (phase 3). This stepwise effect of NEM is shown in Fig. 1B in the plot of \(P_o\) values as a function of time after the addition of 5 mM NEM. To investigate the phase 1 inhibition, we examined the effect of 200 \(\mu\)M NEM on the activity of the channel in the bilayer (Fig. 1C). Only the phase 1 inhibition was seen with 200 \(\mu\)M NEM, but the subsequent addition of 5 mM NEM led to channel activation. These effects of stepwise addition of NEM on \(P_o\) values are summarized in Fig. 1D.

NEM also has time-dependent effects on \[^{3}H\]ryanodine binding. The effect of reaction of the membranes with 5 mM NEM is shown in Fig. 2A. For binding experiments, NEM was reacted in binding buffer at 4°C and the alkylated was stopped by the addition of 10 mM DTT. Similar to the effects on channel activity, there were three distinct phases of the alkylation that altered \[^{3}H\]ryanodine binding. The three phases are an initial and rapid inhibition (phase 1), a recovery or enhancement of binding (phase 2), and a second inhibitory step (phase 3). The phase 1 decrease with 5 mM NEM reaches 49.1 ± 4.4% (\(n = 3\)) inhibition prior to the onset of phase 2. The time course of these three phases was dependent on the concentration of NEM (Fig. 2B). Low concentrations of NEM (50 \(\mu\)M) slow the onset of phases 2 and 3, allowing phase 1 to plateau at 54.6 ± 5.7% (\(n = 4\)) inhibition. If the sulfhydryls that have reacted at 500 \(\mu\)M NEM are primarily phase 1 sulfhydryls, it should be possible to add 5 mM NEM to membranes at the plateau stage and recover the phase 2 enhancement of \[^{3}H\]ryanodine binding. This experiment is shown in Fig. 2C. The addition of 5 mM NEM 25
min after the 500 mM NEM addition gave rise to the recovery of binding (phase 2) followed by phase 3 inhibition. The reaction with NEM under bilayer conditions was faster than that obtained using the protocol employed for Fig. 2 (A–C). For comparison, the effect on [3H]ryanodine binding of the reaction of membranes with 500 μM and 5 mM NEM in bilayer buffer and at room temperature is shown in Fig. 2D. The three phases were still seen, and the time of onset of each phase corresponded to those observed in the bilayer; however, the extent of inhibition was always less under bilayer conditions, possibly reflecting the difference in the redox state of the channel under these conditions. All membrane preparations that we tested showed the three-phase effect of NEM. Some variation in the magnitude of the three phases was, however, observed. Some air oxidation of sulfhydryls did occur, and the difference in the three phases may reflect differences in the oxidation state of the membranes.

Diamide Treatment Increases Open Probability of the Channel, Enhances [3H]Ryanodine Binding, and Forms Intersubunit Cross-links—The sulfhydryl oxidizing agent, diamide, can also activate the channel reconstituted into planar lipid bilayers (Fig. 3). This activation was reversed by the addition of 5 mM DTT. The $P_o$ of the channel was increased from 0.035 ± 0.007 (n = 3) to 0.132 ± 0.030 (n = 3) in the presence of 250 μM diamide. Subsequent treatment with DTT decreases the $P_o$ to 0.022 ± 0.006 (n = 3).

Treatment of membranes with diamide caused an alteration in the electrophoretic mobility of the 565-kDa subunit of the Ca$^{2+}$ release channel. In diamide cross-linking experiments with either membranes (Fig. 4A) or purified Ca$^{2+}$ release channel, a new band was seen in the electrophoretic mobility consistent with dimer formation. Approximate molecular mass values of the oligomers were determined using a 200-kDa myosin standard and the full-length ryanodine-binding protein as a 565-kDa standard. Higher concentrations of diamide produced higher oligomers of the 565-kDa standard, which did not enter the gel. To demonstrate that the high molecular weight bands generated by diamide treatment are indeed dimers formed by intersubunit

3 Y. Wu, B. Aghdasi, S. J. Dou, and S. L. Hamilton, submitted for publication.
cross-linking and not by the cross-linking of the Ca\textsuperscript{2+} release channel to other proteins, we performed two-dimensional electrophoresis, reducing the disulfides between the first and second dimensions (37). The two-dimensional SDS-PAGE is shown in Fig. 4B. The membranes used in the experiment shown in Fig. 4B had a significant amount of a 400-kDa fragment of the Ca\textsuperscript{2+} release channel, which is derived from the 565-kDa band by calpain digestion (38, 39). Both bands underwent cross-linking to form higher molecular weight complexes. Upon reduction prior to the second dimension (Fig. 4B), the high molecular weight bands decreased to 565-kDa and 400-kDa bands. Surprisingly, very little cross-linking was detected between the 400-kDa and the 565-kDa proteins. Instead both the 565-kDa and the 400-kDa proteins appeared to form only homodimers, suggesting that, when the 565-kDa is digested to the 400-kDa, the other subunits within the tetramer are also digested. Although only high molecular weight bands are shown in this gel, we have extensively searched for lower molecular weight bands such as triadin and have found no evidence that any other protein is involved in the diamide-induced cross-linking to the Ca\textsuperscript{2+} release channel (data not shown).

To determine if the formation of intersubunit cross-links correlates with channel activation, membranes were treated with increasing concentrations of diamide. The effect of these treatments on \textsuperscript{3}H\textsubscript{ryanodine binding is shown in Fig. 5A. Diamide treatment enhanced \textsuperscript{3}H\textsubscript{ryanodine binding. To quantitate the formation of dimers, the Coomassie Brilliant Blue-stained gel was scanned with a densitometer and the optical

FIG. 2. Effect of NEM on \textsuperscript{3}H\textsubscript{ryanodine binding. A, the effect of 5 mM NEM on the binding of \textsuperscript{3}H\textsubscript{ryanodine to SR membranes. Membranes were incubated with NEM in binding buffer on ice for the indicated time periods. The alkylation was terminated with 10 mM DTT. \textsuperscript{3}H\textsubscript{ryanodine binding was performed on 20 \mu g of each sample as described under “Experimental Procedures.” The data points from \( t = 0 \) to \( t = 60 \) min are the average \pm S.E. of three independent experiments. Data points after 60 min are the average of two independent experiments. B, the effects of 500 \mu M, 1 mM, and 5 mM NEM on \textsuperscript{3}H\textsubscript{ryanodine binding. The alkylation at three different NEM concentrations were performed as described in A. ●, 500 \mu M NEM; ■, 1 mM NEM; ●, 5 mM NEM. C, phase 2 recovery with addition of 5 mM NEM. Membranes were incubated with 500 \mu M NEM, and aliquots were taken at the indicated times. The reaction was stopped with 10 mM DTT. In one sample, 5 mM NEM was added 30 min (arrow) after the first addition of 500 \mu M NEM. ●, control; ■, 5 mM NEM added at 30 min. D, the effect of 500 \mu M and 5 mM NEM on the binding of \textsuperscript{3}H\textsubscript{ryanodine to SR membranes under bilayer conditions. Reaction with NEM was done in 225 mM CsSO\textsubscript{3}CH\textsubscript{3}, 10 mM MOPS (pH 7.4), 10 \mu M CaCl\textsubscript{2} and room temperature (23°C). ●, 200 \mu M NEM; ●, 5 mM NEM.

FIG. 3. Effect on diamide and DTT on the activity of the Ca\textsuperscript{2+} release channel reconstituted into planar lipid bilayers. Single-channel recordings of heavy SR reconstituted into planar lipid bilayers. A, control traces of RYR at 40 mV. Traces were filtered at 2.5 kHz. B, addition of 250 \mu M diamide to the cis chamber. C, addition of 5 mM DTT to the cis chamber reversed the effect of diamide.

FIG. 4. Effect of NEM on the Ca\textsuperscript{2+} release channel.
densities of the dimer and monomer were plotted as a function of diamide concentration (Fig. 5B). The disappearance of the 565-kDa band correlated with the appearance of the dimer and with enhanced \(^{3}H\)ryanodine binding. At higher concentrations of diamide (1 mM and higher), the intensity of the dimer also decreased and higher oligomers appeared to accumulate at the top of the gel. Formation of higher oligomers was accompanied by a decrease in \(^{3}H\)ryanodine binding. Channels pretreated with diamide and washed prior to incorporation into planar lipid bilayers showed a substantial activation compared to controls (Fig. 5C). In the experiment shown, the \(P_o\) increased from 0.02 to 0.22 with 500 \(\mu M\) diamide.

**Fig. 5.** Activation of channel and enhancement of binding correlates with dimer formation. **A,** \(^{3}H\)ryanodine binding of samples treated with increasing concentrations of diamide. Binding was in 0.3 M NaCl, 50 mM MOPS, 0.1 mM CaCl\(_2\), 0.1% CHAPS, 100 \(\mu g/ml\) BSA with 5 nm \(^{3}H\)ryanodine. \(B_2\) is \(^{3}H\)ryanodine bound to membranes treated with different diamide concentrations, and \(B_1\) is \(^{3}H\)ryanodine bound to control membranes (not treated with diamide). \(B_2\) effect of diamide on the amount of monomer and dimer. The optical density obtained from a densitometer scan of the Coomassie-stained gels of monomer and dimer of RYR subunits is plotted as a function of diamide concentration. **C,** single-channel recordings of samples after removal of excess diamide. Top trace is control trace of RYR at 40 mV where no diamide was present. Bottom trace is from the diamide-treated sample, which was washed to remove the diamide by pelleting twice in an Airfuge. The data in this figure are representative of four independent experiments.

Diamide and NEM Can Be Used to Differentiate between Classes of Sulfhydryls on the \(Ca^{2+}\) Release Channel—To determine whether diamide and NEM react with the same sulphydryls, we examined the effect of NEM on the diamide-activated channel. The single-channel records are shown in Fig. 6. The first tracing is the control (\(P_o = 0.03 \pm 0.01, n = 3\)). Addition of 250 \(\mu M\) diamide activated the channel (\(P_o = 0.12 \pm 0.03, n = 3\)). The addition of 5 mM NEM to the diamide-modified channel led to a further increase in \(P_o\). This activation is not reversed by DTT (data not shown). NEM does not cause phase 1 inhibition in the diamide-pretreated membranes. Sulphydryls involved in channel activation by diamide, therefore, appear to be different than those involved in activation by NEM. Continued incubation with NEM leads to channel inhibition. Additional activation by NEM is also seen with channels pretreated with higher concentrations (500 \(\mu M\) to 1 mM) of diamide (data not shown).

To examine further the relationship between sulphydryls altered by diamide and those that react with NEM, we examined the effect of pretreatment of membranes with diamide on the ability of NEM to alter \(^{3}H\)ryanodine binding (Fig. 7A). In these experiments the membranes were reacted first with 250 \(\mu M\) diamide for 30 min and then with 5 mM NEM. At various times after NEM addition, aliquots were removed and the reaction stopped by the addition of DTT. The samples were then assayed for \(^{3}H\)ryanodine binding. As can be seen in Fig.
Diamide pretreatment on the isolated phase 1 reaction, we observed a reaction that is primarily phase 1. To examine the effect of NEM alkylation, the oxidation appeared to be protecting sulfhydryls during the second addition of 500 μM diamide to the cis chamber. Middle tracing, effects of addition of 250 μM diamide to the cis chamber. Bottom tracing, effect of subsequent addition of 5 mM NEM.

As shown in Fig. 2, lowering the NEM concentration greatly slows the onset of the phase 2 and 3 effects, allowing us to look at a reaction that is primarily phase 1. To examine the effect of diamide pretreatment on the isolated phase 1 reaction, we pretreated membranes with 250 μM diamide and then examined the effect of 500 μM NEM on [3H]ryanodine binding (Fig. 7B). No phase 1 inhibition was seen with the diamide-pretreated membranes. These findings suggest that diamide is interacting with and protecting the phase 1 sulfhydryls. Alternatively, diamide may be altering the conformation of the Ca2+ release channel, such that the phase 1 sulfhydryls no longer react as rapidly. To demonstrate this protection, we pretreated membranes with 250 μM diamide, diluted the diamide to less than 25 μM, reacted with 5 mM NEM for 30 min on ice, and then reduced with 10 mM DTT, washed, and tested the effect of a second addition of 500 μM NEM on the binding of [3H]ryanodine. The reaction was again stopped with 10 mM DTT prior to the binding assay. These data are shown in Fig. 7C. As can be seen in this figure, reduction after diamide and NEM treatment restored the ability of NEM to inhibit [3H]ryanodine binding in a phase 1-like reaction. The initial binding and plateau binding to the membranes treated with diamide and then NEM was increased, as would be expected from membranes that have been alkylated at the phase 2 sites (see Fig. 7A).

To obtain additional evidence that diamide has not altered the ability of NEM to enhance binding in a phase 2 reaction, membranes were pretreated with diamide and with low concentrations of NEM under conditions that in the absence of diamide would have produced phase 1 inhibition. NEM was then added under conditions determined to produce phase 2 enhancement (Fig. 7D). Phase 2 enhancement is not blocked by diamide pretreatment.

To demonstrate that the phase 1 reaction with NEM protects the sulfhydryls involved in the diamide cross-link, we assessed the ability of NEM in the different phases to block dimer formation. The steps in this experiment were: 1) incubation of membranes with 5 mM NEM for different periods of time, 2) reduction with DTT, 3) removal of DTT, and 4) cross-linking with 250 μM diamide. NEM at the earliest incubation times blocked dimer formation (Fig. 8D). If dimer formation is truly protecting sulfhydryls from the phase 1 reaction, it should be possible to reduce diamide-formed disulfides after the phase 2 NEM reaction and then reform dimers with diamide. The experiment involved the following steps: 1) incubation of membranes with 250 μM diamide, 2) treatment with NEM for different periods of time, 3) reduction with DTT, 4) removal of DTT, and 5) cross-linking with diamide. Dimers could reform if, prior to NEM and subsequent DTT treatment, the sulfhydryls were protected by diamide cross-linking (Fig. 8C).

The phase 1 effects on [3H]ryanodine binding correlated well with the rate of incorporation of [14C]NEM into the 565-kDa band of the RYR1. This is demonstrated in Fig. 9, where membranes with and without diamide pretreatment were incubated with 1 mM [14C]NEM for increasing periods of time. The 565-kDa band from a Coomassie Brilliant Blue-stained gel was excised, digested as described under “Experimental Procedures,” and the radioactivity in the bands was quantitated by liquid scintillation counting. The data were fit as the sum of two exponentials, with the rapid labeling component having a kobs of 0.26 min⁻¹ and the slower component having a kobs of 0.067 min⁻¹. From the fits it was determined that diamide pretreatment reduced the fast phase by 81% and the slow phase 13%. In these experiments the incorporation of [14C]NEM into the 565-kDa band under phase 1 conditions (the fast component of labeling) reached a maximum of about 13.5 pmol/sample. The quantity of [3H]ryanodine binding sites applied to each well of this gel was 0.5 pmol, and, since 4 subunits produce a single binding site, this would correspond to 2 pmol of the RYR1 subunits (assuming that all of the RYR1 can bind ryanodine). However, in this membrane preparation 32% of the RYR1 was in the 400-kDa calpain-derived fragment and, therefore, each well should have about 1.4 pmol of the 565-kDa band. Since there are 100 cysteines/subunit, the [14C]NEM labeled about 10% of the RYR1 sulfhydryls under phase 1 conditions or less than 10 cysteines/subunit. This is in reasonable agreement with direct labeling experiments, where the incorporation of radiolabeled NEM under phase 1 conditions is 10% (n = 2) of that which is incorporated into the 565-kDa band with 1 mM [14C]NEM in SDS sample buffer. For comparison the effect of unlabeled NEM under these same conditions on [3H]ryanodine binding is also shown in this figure. The rapid phase of [14C]NEM labeling appears to correlate with the phase 1 inhibition of [3H]ryanodine binding, and this labeling is partially blocked by diamide pretreatment.

DISCUSSION

Redox modulation of excitation-contraction coupling is important physiologically. In the native state, intact skeletal muscle fibers produce detectable levels of both reactive oxygen species (12) and NO derivatives (13, 14). Oxidant depletion alters contractile function. In unfatigued muscle, selective scavenging of reactive oxygen species depresses force generation (12); inhibition of NO synthesis has the opposite effect, increasing force (13). Strenuous contractile activity accelerates the rate at which myocytes produce free radicals, and other reactive oxidants, e.g. superoxide anion radicals (16), hydroxyl radicals (17), and NO derivatives (14). Oxidants accumulate in the active muscle and contribute directly to the loss of contractile function that occurs in muscular fatigue (15). Oxidative effects on both unfatigued and fatigued muscle are consistent with an increase in cytoplasmic Ca2+ concentrations due to
activation of the sarcoplasmic reticulum Ca\textsuperscript{2+} release channel (21). An important role of sulfhydryl groups in the modulation of the activity of the skeletal muscle Ca\textsuperscript{2+} release channel has been demonstrated by several laboratories (3–9, 27, 28). Both oxidizing compounds (3–9) and reagents that modify free sulfhydryls (27, 28) alter the activity of Ca\textsuperscript{2+} release channel. To explore these phenomena we choose NEM, a reagent that alkylates cysteine residues, and diamide, a sulfhydryl oxidizing agent that cross-links near neighbor cysteine residues. NEM shows a three-step effect on the activity of the Ca\textsuperscript{2+} release channel; first it inhibits, then it activates, and finally it again inhibits the channel reconstituted into planar lipid bilayers. In general, agents that activate the Ca\textsuperscript{2+} release channel increase the apparent affinity of the protein for \textsuperscript{3}H\textsuperscript{]ryanodine}, while those that inhibit channel activity decrease the apparent affinity (36). Consistent with this, NEM produces a similar three-phase effect on \textsuperscript{3}H\textsuperscript{]ryanodine} binding: phase 1 inhibition, phase 2 enhancement, and phase 3 inhibition. As shown by the effects on \textsuperscript{3}H\textsuperscript{]ryanodine} binding and channel activity, and by direct \textsuperscript{3}H\textsuperscript{]NEM} labeling, sulfhydrs on the Ca\textsuperscript{2+} release channel are reacting sequentially with NEM to alter the structure and function of the protein.

Diamide activates the channel reconstituted into planar lipid bilayers and enhances \textsuperscript{3}H\textsuperscript{]ryanodine} binding by increasing apparent affinity. The observation that either an oxidizing or an alkylating reagent can activate the channel raised the question of whether the activation was due to the loss of a free sulfhydryl. If this were true, we would expect the activation by the two reagents to involve the same cysteine residues. This is not the situation for diamide and NEM; instead, to activate the channel, NEM and diamide appear to react with different sulfhydryls. This conclusion is based on a number of observations: 1) NEM can further stimulate the activity of the diamide-activated single channel, 2) diamide blocks the phase 1 inhibitory effects of NEM on \textsuperscript{3}H\textsuperscript{]ryanodine} binding, 3) phase 1 alkylation by NEM blocks dimer formation by diamide, and 4) diamide pretreatment enhances the activating effects of NEM on \textsuperscript{3}H\textsuperscript{]ryanodine} binding. When phase 1 sulfhydrs are protected by diamide cross-linking, NEM alkylation markedly enhances \textsuperscript{3}H\textsuperscript{]ryanodine} binding. Our data are most consistent with a model in which the activation by diamide is due to cross-linking of phase 1 sulfhydrs while the NEM activation is associated with alkylation of phase 2 sulfhydrs. Without actually identifying the cysteine residues involved in phase 1 alkylation and those involved in diamide cross-linking, it is impossible to totally eliminate the possibility that diamide is having a long distance effect on phase 1 sulfhydrs. However, extensive reaction with NEM after diamide cross-linking fails
Fig. 8. Correlation of sulfhydryls alkylated in phase 1 with NEM and those involved in dimer formation by diamide. A, SDS-PAGE of proteins of SR membranes used in binding experiments shown in Fig. 7. The unlabeled lane to the left contains Rainbow molecular size markers (Bio-Rad). The only markers that can be seen on these gels are the 220-kDa marker and, at the very top of the gel, an oligomer of BSA. Lane 1, control membranes; lane 2, control membranes with 10 mM DTT; lane 3, diamide cross-linked membranes; lane 4, diamide-treated membranes with 10 mM DTT. Bands A (2 × 565 kDa) and B (2 × 400 kDa) are dimers of full-length and proteolyzed RYR (36). Bands a and b are monomers of A and B, respectively. B, Coomassie Brilliant Blue-stained 5% SDS-PAGE of SR membrane proteins treated with 500 μM NEM for the indicated times (phase 1) and then incubated with 250 μM diamide. C, Coomassie Brilliant Blue-stained 5% SDS-PAGE of samples first treated with 250 μM diamide, reacted with 500 μM NEM for the indicated periods of time, reduced with DTT, washed by pelleting in an Airfuge, and then cross-linked with 250 μM diamide. Bands A (2 × 565 kDa) and B (2 × 400 kDa) are dimers of full-length and proteolyzed RYR (36). Bands a and b are monomers of A and B, respectively.

Fig. 9. Labeling of sulfhydryls on the Ca2+ release channel with [14C]NEM. Membranes (11 mg/ml, 20 pmol of [3H]ryanodine binding sites/mg) were incubated with 500 μM diamide for 30 min on ice. The diamide was removed by pelleting the membranes in a Beckman Airfuge (4 min at 30 p.s.i.) and resuspending the membranes in binding buffer (6.2 mg/ml). The membranes were then incubated with 1 mM [14C]NEM for the indicated periods of time. The reaction was stopped with the addition of 20 mM DTT. Aliquots (22 μg) were then electrophoresed on 5% SDS gels, and, after Coomassie Brilliant Blue staining, the 565-kDa RYR1 band was excised, digested, and counted as described under “Experimental Procedures.” All samples were electrophoresed three times. ●, control; ■, diamide-pretreated. Also shown in this figure (○) is the effect of a similar treatment of the same membranes at 6 mg/ml with unlabeled 1 mM NEM on the binding of [3H]ryanodine. This was performed as described in Fig. 2A.

channel activity are summarized in Table I. Intersubunit cross-links are likely to involve different cysteines on the two adjacent subunits. We have not yet determined whether both of these sulfhydryls are alkylated by NEM in the phase 1 reaction. The second sulfhydryl may be alkylated at a later stage, or it may not react with NEM. A completely different sulfhydryl could be alkylated in phase 2. The sequentially reacting cysteine residues could be at different locations in the primary sequence of each subunit or could be the same residues on different subunits, reacting at different rates as a result of conformational changes in the protein. These issues remain to be resolved.

Diamide-induced cross-links are detected in intact membranes, in detergent-solubilized membranes, and in purified ryanodine-binding proteins, suggesting that the cross-links are occurring between subunits of a tetramer. Extended incubations with diamide lead to the formation of higher molecular weight oligomers, which could be trimers or tetramers. Formation of higher oligomers suggests either that the cross-link involves different sulfhydryls on adjacent subunits or that there is more than one type of cross-link formed. The partners in the formation of the cross-linked complexes were identified by two-dimensional electrophoresis. The membrane preparations used for these studies contained a significant amount of a 400-kDa band, which has been identified previously as a proteolytic fragment of the Ca2+ release channel (38, 39). Experiments have been performed with membrane preparations that have very little proteolysis and membranes that were proteolyzed by endogenous calpain. The effects of NEM and diamide on the binding of [3H]ryanodine was unaffected by this proteolytic event. Both the 565-kDa full-length ryanodine-binding protein subunit and the 400-kDa fragment appear to be cross-linked by diamide to form dimers, suggesting that the site of the intersubunit cross-link is within the 400-kDa fragment. Very little cross-linking, however, occurs between the 400-kDa and the 565-kDa bands. This surprising finding suggests that the proteolytic events that produce the 400-kDa fragment are

to prevent the recovery of the phase 1 effects of NEM after reduction of the cross-linked sulfhydryls, a finding that strongly supports our model of phase 1 sulfhydryls being at subunit-subunit contact domains. The effects of oxidation, reduction, and alkylation on the binding of [3H]ryanodine and on
Nonrandom. The most frequent neighbor of a 400-kDa proteolyzed subunit within a tetramer is another 400-kDa subunit, while the most frequent neighbor of the full-length 565-kDa protein is another 565-kDa protein. The proteolytic event is presumably due to the action of endogenous calpains (38, 39). One interpretation of these findings is that some of the Ca\(^{2+}\) release channel tetramers are by some means targeted for proteolysis such that all of the subunits in the protein are proteolyzed simultaneously. Other tetramers contain all intact subunits. This would suggest that some modification of the Ca\(^{2+}\) release channel allows it to be recognized by endogenous calpains. A second possibility is that once calpain binds to the subunits, it proteolyzes simultaneously. Other tetramers contain all intact subunits, and the most frequent neighbor of the full-length 565-kDa protein is another 565-kDa protein. The proteolytic event is presumably due to the action of endogenous calpains (38, 39).

Cross-linking of sulfhydryls by diamide activates the channel, indicating that sulfhydryls within this class may be the targets of redox modulation of the Ca\(^{2+}\) release channel and that these redox-sensitive sulfhydryls are located in domains where subunits contact one another. There is no indication of the involvement of any other protein in the disulfide bond formation induced by diamide. This is in contrast to the results of Liu et al. (22, 23), who suggest that triadin is cross-linked to the Ca\(^{2+}\) release channel by oxidizing agents. We were unable to find evidence of such a cross-link.

In summary, we demonstrate the existence of at least three classes of functionally important sulfhydryls on the Ca\(^{2+}\) release channel, modification of which alter channel activity and \(^{3}H\)ryanodine binding (Table I). Both NEM and diamide can activate the Ca\(^{2+}\) release channel, but the reactions involve different sulfhydryls. Diamide cross-links subunits within the tetramer. This cross-linking appears to correlate with channel activation and with oxidation of a class of sulfhydryls, which, in the absence of diamide, react rapidly with NEM to inhibit the channel. Alkylation of these phase 1 sulfhydryls prevents dimer formation. The phase 1 sulfhydryls, therefore, may be located at contact domains between subunits. These studies will be useful in designing strategies to differentially label phase 1 and phase 2 sulfhydryls to map their location in the primary sequence of the Ca\(^{2+}\) release channel. This may also enable us to define some of the parts of the protein that are in regions of subunit-subunit contact in the three-dimensional structure of the protein.

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