Low N-Ethylmaleimide Concentrations Activate Ryanodine Receptors by a Reversible Interaction, Not an Alkylation of Critical Thiols

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Previous studies proposed that N-ethylmaleimide (NEM) alkylates 3 classes of thiols on skeletal muscle ryanodine receptors (RyRs) producing 3 phases of channel modification, as function of time and concentration. NEM (5 mM) decreased, increased, and then decreased the open probability (Pₒ) of the channel by thiol alkylation, a reaction not reversed by reducing agents. We now show that low NEM concentrations (20–200 μM) elicited Ca²⁺ release from sarcoplastic reticulum (SR) vesicles, but contrary to expectations, the effect was fully reversed by reducing agents or by washing SR vesicles. In bilayers, NEM (0.2 mM) increased Pₒ of RyRs within seconds when added to the cis (not trans) side, and dithiothreitol (DTT; 1 mM) decreased Pₒ in seconds. High (5 mM) NEM concentrations elicited SR Ca²⁺ release that was not reversed by DTT, as expected for an alkylation reaction. A non-sulfhydryl reagent structurally related to NEM, N-ethylsuccinimide (0.1–0.5 mM), also elicited SR Ca²⁺ release that was not reversed by DTT (1 mM). Other alkylating agents elicited SR Ca²⁺ release, which was fully (N-methylmaleimide) or partially (iodoacetic acid) reversed by DTT and inhibited by ruthenium red. Nitreric oxide (NO) donors at concentrations that did not activate RyRs inhibited NEM-induced Ca²⁺ release, most likely by an interaction of NO with NEM rather than an inactivation of RyRs by NO. Thus, at low concentrations, NEM does not act as a selective thiol reagent and activates RyRs without alkylating critical thiols indicating that the multiple phases of ryanodine binding are unrelated to RyR activity or to NEM alkylation of RyRs.

In striated muscle, the sarcoplastic reticulum (SR) is the major storage compartment of intracellular Ca²⁺ that controls cytosolic free Ca²⁺ and developed force by sequestering and releasing Ca²⁺ during each contraction. Studies of Ca²⁺ flux across the SR membrane and single channel recordings in planar lipid bilayers have suggested that Ca²⁺ release from skeletal muscle SR is mediated by a high conductance Ca²⁺ channel (ryanodine receptor; RyR) (1, 2). A wide range of intracellular signaling molecules and pharmacological agents modulated RyRs (3) in addition to the following oxidants have been shown to activate the channel: heavy metals, reactive disulfides, hydrogen peroxide, Fe²⁺/ascorbate, and Thimerosal (4–10). Sulfhydryl-reducing agents have been shown to reverse the effect of some oxidants to close the channel. Such studies demonstrated that skeletal and cardiac RyRs contain hyper-reactive or critical thiols of low pKₐ values that could be oxidized and reduced to reversibly open and close the Ca²⁺-release channel (7, 9). Heavy metals and sulfhydryl oxidants, much like Ca²⁺-induced Ca²⁺ release, exhibit biphasic effects on RyRs, where low concentrations activate and high concentrations inhibit the open probability of the channel (10–12).

Stoyanovsky et al. (13) first showed that NO donors oxidize free thiols on RyRs causing an increase in the open probability of the channel (Pₒ) and eliciting Ca²⁺ release from skeletal and cardiac SR vesicles. NO gas and NO donors interact with RyRs to promote channel opening by a nitrosylation of critical sulphydryl sites involved in the modulation of channel gating, and the effect is reversed by sulphydryl-reducing agents (e.g. reduced glutathione, GSH; dithiothreitol, DTT; and ω-cysteine) (10, 13). NO gas elicited SR Ca²⁺ but required high concentrations both in ambient oxygen levels (200–400 μM) and deoxygenated solutions (60–200 μM). Because SR Ca²⁺ transport is not altered by oxygen levels this indicated that direct nitrosylation of RyRs by NO gas is not biologically significant (13). Unlike authentic NO and non-thiol NO donors, nitrosothiols such as S-nitroso-cysteine (cys-SNO) and S-nitroso-N-acetyl-L-1-penicillamine (SNAP) were potent at activating RyRs even in the presence of ambient oxygen levels because of a robust transnitrosylation of NO from the donor to critical thiols on RyRs (13). As with other sulphydryl oxidants, the binding of radiolabeled [¹²⁵I]ryanodine to its receptor was inhibited by the NO donor cyst-SNO, whereas the activity of Ca²⁺-ATPases was not altered by NO (13). In contrast, Mészáros et al. (14) reported that SNAP inhibited SR Ca²⁺ release by partially reversing caffeine-induced Ca²⁺ release and decreased Pₒ of carboxyatractyloside; NEM, N-ethylmaleimide; NO, nitric oxide; cys-SNO, S-nitroso-cysteine; SNAP, S-nitroso-N-acetyl-L-1-penicillamine; DTT, dithiothreitol; GSH, reduced glutathione; Pipes, 1,4-piperazineethanesulfonic acid; AP, antipyrine; NMM, N-methylmaleimide; IAA, iodooacetic acid; NES, N-ethyl succinimide; NOC-9, MAHMA/NO, (z)-1-(N-methyl-N-[6-(N-methylammoniohexyl)amino]diaden-1-iium-1,2-diolate; NOC-15, PAPA-NONOate, PAPA/NO, (z)-1-(N-[3-ammoniopropyl]-N-(n-propyl)amino)diaden-1-iium-1,2-diolate.
The present study re-examines the triphasic effect of NEM on channel activity for the following three reasons: 1) The selectivity of NEM as a thiol-alkylating reagent cannot be relied upon. 2) The level of ryanodine binding is not equivalent to a measurement of channel activity, particularly for activation of the channel by sulfhydryl oxidants and nitrosylation of the channel. 3) Evidence that NEM alkylates free sulfhydryl on RyRs was lacking, leaving the possibility that NEM interacted with RyRs by other mechanisms that could be reversed by sulfhydryl-reducing agents, particularly at low NEM concentrations.
Low NEM Activates RyRs by Reversible Non-sulfhydryl Reaction

NEM-induced Ca\(^{2+}\) release from skeletal SR vesicles is reversible. Ca\(^{2+}\) transport across SR vesicles was determined by measuring extravesicular free [Ca\(^{2+}\)] through the differential absorption changes of AP III at 720–790 nm. After the SR vesicles (0.2 mg/ml) were actively loaded with Ca\(^{2+}\) using an ATP-regenerating system, NEM was added to the reaction mixture to elicit Ca\(^{2+}\) release. After release was completed, DTT was added to reverse the effects of the NEM. A23187 was added to measure the total intravesicular Ca\(^{2+}\) release was completed, DTT was added to reverse the effects of the NEM and then A23187 was added to measure the total intravesicular Ca\(^{2+}\). Traces were as follows: 1) 1 mM DTT; 2) 2 mM L-cysteine; 3) 1 mM GSH; 4) 1 mM mercaptoethanol. A prior addition of ruthenium red (5 \(\mu\)M) inhibited NEM-induced Ca\(^{2+}\) release (dotted line). B, NO donors inhibit Ca\(^{2+}\) release induced by low NEM concentrations. SR vesicles were actively loaded with Ca\(^{2+}\) using an ATP-regenerating system and then exposed to various NO donors for 5 min followed by NEM (25 \(\mu\)M) to test for the inhibition of NEM-induced Ca\(^{2+}\) release by NO donors. Traces were as follows: 1) 300 \(\mu\)M SNAP; 2) 300 \(\mu\)M NOC-15 (PAPA-NONOate); 3) 10 \(\mu\)M cys-SNO; 4) control.

Reversibility of NEM-induced Ca\(^{2+}\) Release—To determine whether the effect of NEM on SR Ca\(^{2+}\) release can be reversed by washing out the vesicles, skeletal SR vesicles were incubated in low concentration of NEM for 5 min and then washed of NEM by rapid centrifugation and resuspended in NEM-free reaction medium. SR vesicles pretreated with NEM were then tested for their capacity to actively load Ca\(^{2+}\) compared with control vesicles, which were treated in an identical manner but were resuspended in NEM in the reaction medium. Fig. 1B (dotted line, trace 1) illustrates that SR vesicles resuspended with NEM in the medium sequester Ca\(^{2+}\) upon the addition of ATP during an early phase of rapid Ca\(^{2+}\) transport and then release Ca\(^{2+}\) because of NEM-induced Ca\(^{2+}\) release. The rapid initial phase of Ca\(^{2+}\) uptake indicated that NEM acts primarily by activating Ca\(^{2+}\)-release channels rather than inhibiting Ca\(^{2+}\) uptake by Ca\(^{2+}\)-ATPase pumps. The addition of DTT reverses the effect of NEM resulting in complete reuptake of Ca\(^{2+}\) by the vesicles (trace 1). In contrast, SR vesicles that were exposed to NEM and then resuspended in NEM-free medium sequestered the same amount of Ca\(^{2+}\) as control vesicles, which were never exposed to NEM, and DTT had no further effect on enhancing SR Ca\(^{2+}\) uptake (solid line, trace 2). Hence, the pretreatment of vesicles with NEM (100 \(\mu\)M) was fully reversed by washing the vesicles. Fig. 2A shows that SR Ca\(^{2+}\) release induced by low NEM concentrations was reversed by all of the sulfhydryl-reducing agents that were tested. The order of relative potency was DTT > GSH > L-cysteine > mercaptoethanol. Ruthenium red was effective but did not fully inhibit NEM-induced Ca\(^{2+}\) release, supporting the view that release 

FIG. 1. NEM-induced Ca\(^{2+}\) release from skeletal SR vesicles is reversible. Ca\(^{2+}\) transport across SR vesicles was determined by measuring extravesicular free [Ca\(^{2+}\)] through the differential absorption changes of AP III at 720–790 nm. After the SR vesicles (0.2 mg/ml) were actively loaded with Ca\(^{2+}\) using an ATP-regenerating system, NEM was added to the reaction mixture to elicit Ca\(^{2+}\) release. After release was completed, DTT was added to reverse the effects of the NEM. A23187 was added to measure the total intravesicular Ca\(^{2+}\). A, NEM (20–5000 \(\mu\)M) elicited Ca\(^{2+}\) release from the SR vesicles, and DTT (1 mM) induced the reuptake of Ca\(^{2+}\) by SR, except for 5 mM NEM. B, SR vesicles were pretreated with 100 \(\mu\)M NEM at 4 °C for 5 min. The suspension of SR vesicles was diluted to 0.5 ml, centrifuged for 20 min at 45,000 \(\times\) g, and resuspended in 1 ml of medium with (dotted line, trace 1) or without (solid line, trace 2) NEM (0.1 mM). SR vesicles were actively loaded with Ca\(^{2+}\) and then DTT (1 mM) was added to reverse the effects of the NEM. SR with NEM exhibited a normal initial phase of Ca\(^{2+}\) uptake followed by a rapid release of Ca\(^{2+}\) (dotted line, trace 1). The phase of release was due to the presence of NEM, because DTT neutralized NEM resulting in rapid Ca\(^{2+}\) uptake. SR vesicles resuspended in NEM-free medium accumulated and retained Ca\(^{2+}\), and DTT had no effect (solid line, trace 2).

FIG. 2. Sulfhydryl reductants, ruthenium red, and NO inhibit NEM-induced Ca\(^{2+}\) release. Ca\(^{2+}\) transport across SR vesicles was measured with AP III at 720–790 nm. SR vesicles (0.2 mg/ml) were actively loaded with Ca\(^{2+}\) using an ATP-regenerating system and NEM (50 \(\mu\)M)-elicited Ca\(^{2+}\) release. A, after release was completed, various sulfhydryl-reducing agents were added to reverse the effects of the NEM and then A23187 was added to measure the total intravesicular Ca\(^{2+}\). Traces were as follows: 1) 1 mM DTT; 2) 2 mM L-cysteine; 3) 1 mM GSH; 4) 1 mM mercaptoethanol. A prior addition of ruthenium red (5 \(\mu\)M) inhibited NEM-induced Ca\(^{2+}\) release (dotted line). B, NO donors inhibit Ca\(^{2+}\) release induced by low NEM concentrations. SR vesicles were actively loaded with Ca\(^{2+}\) using an ATP-regenerating system and then exposed to various NO donors for 5 min followed by NEM (25 \(\mu\)M) to test for the inhibition of NEM-induced Ca\(^{2+}\) release by NO donors. Traces were as follows: 1) 300 \(\mu\)M SNAP; 2) 300 \(\mu\)M NOC-15 (PAPA-NONOate); 3) 10 \(\mu\)M cys-SNO; 4) control.
a wide range of concentrations and found that the inhibition of NEM-induced Ca\textsuperscript{2+} release by NO donors was dependent on the concentrations of the NO donor relative to that of NEM. The inhibition by NO was best studied at the lowest possible NEM (25 \mu M) concentration that caused release such that equally low concentrations of NO donors could be tested. This is important, because the higher concentrations of NO donor themselves elicit SR Ca\textsuperscript{2+} release. Fig. 2B illustrates that NEM (25 \mu M) induces Ca\textsuperscript{2+} release from SR vesicles (control, trace 4) and was inhibited by the prior addition of NO donors. The order of potency of NO donors as inhibitors of NEM was SNAP (trace 1) > NOC-15 (PAPA-NONOate) (trace 2) > cys-SNO (trace 3). At the concentration used here, these NO donors had no detectable effect on SR Ca\textsuperscript{2+} transport.

Effect of Low NEM Concentrations on Single Channel Properties of RyRs—The effect of low NEM concentrations on single channel fluctuations of skeletal muscle RyRs was investigated by reconstituting Ca\textsuperscript{2+}-release channels through the fusion of heavy SR vesicles with planar bilayers. Single channel fluctuations were measured in asymmetrical cesium solutions across the bilayer with cesium as the conducting ion (Fig. 3A). As illustrated in Fig. 3B, NEM (200 \mu M) added to the cis side (but not trans) produced a marked increase in \( P_o \) within 30 s. There was no detectable effect on single channel conductance at potentials ranging from ± 40 mV. In four separate channels, mean \( P_o \) increased from 0.28 ± 0.05 to 0.75 ± 0.1 in the presence of NEM. Continuous recordings showed that the presence of NEM (200 \mu M) on the cis side for up to 20 min did not cause further changes in \( P_o \) (\( n = 3 \)), and an initial inhibition of the channel was not observed. Lower concentrations (10, 25, and 50 \mu M) of NEM had no effect on single channel properties for 15 min, and 100 \mu M NEM also increased \( P_o \) with a slower onset of activation (~2 min). In three bilayers, the effect of NEM was tested on the trans side before testing it on the cis side. Single channel activation by NEM (200 \mu M) was effectively reversed by 1 mM DTT, resulting in a decrease in \( P_o \) to 0.05 in ~30 s (Fig. 3C).

Effects of Other Alkylating Agents and the Non-thiol Analog of NEM—N-methylmaleimide (NMM) had similar effects as NEM on SR Ca\textsuperscript{2+} release. As shown in Fig. 4A, NMM at 50–200 \mu M caused SR Ca\textsuperscript{2+} release, which was fully reversed by DTT. Higher NMM concentrations (1–5 mM) elicited release that was not reversed by DTT. Other alkylating agents like iodoacetate (IAA) (Fig. 4B) and iodoacetimide (not shown) also elicited SR Ca\textsuperscript{2+} release but with slower kinetics, and they were partially reversed by DTT. As for NEM, ruthenium red inhibited release by these alkylating reagents but did not fully block release (Fig. 4. A and B) consistent with a possible inhibition of SR Ca\textsuperscript{2+}-ATPase (26, 27). Interestingly, control experiments with a non-thiol-reactive compound, which is structurally related to NEM, N-ethyl succinimide (NES) also elicited SR Ca\textsuperscript{2+} release at 100–500 \mu M, and as shown for IAA, the rate of release was slower compared with NEM and NMM (Fig. 4C).
NEM-induced Ca\(^{2+}\) release was not reversed by DTT as expected for a non-sulphydryl reagent and was effectively blocked by ruthenium red, consistent with an activation of RyRs.

**DISCUSSION**

Several studies have shown that sulphydryl oxidation-reduction plays an important role in the pathophysiology of skeletal and cardiac muscle (10). It has been demonstrated that skeletal and cardiac RyRs contain critical thiols of low pK\(_a\), that can be oxidized and reduced to reversibly open and close the Ca\(^{2+}\) release channel. Free thiols on RyR can also be oxidized by NO donors resulting in an increase in the open probability of the channel and Ca\(^{2+}\) release from skeletal and cardiac SR vesicles (13, 16–18). Channel activation most likely occurs through the transnitrosylation of critical sulphydryl sites on RyRs resulting in the formation of stable S-nitrosothiol residues or transient S-nitrosothiol bonds followed by disulfide bond formation with vicinal thiols on RyR. The complex effects of NO and other sulphydryl oxidants highlight the multiple interactions that can occur and the need to identify the critical cysteine residues on RyRs to better understand the role of NO in the regulation of force in striated muscle (10).

**Low NEM Concentrations Do Not Act at Sulphydryl Sites on Skeletal RyRs**—The main findings of this study are that low and high NEM concentrations act by different mechanisms. Low concentrations of NEM (20–200 \(\mu\)M) activate RyRs but do not alkylate the protein, because the effect is fully reversed by washing out NEM or by adding sulphydryl-reducing agents. High concentrations of NEM (5 mM) also activate the channel but are not reversible with sulphydryl-reducing agents. It is important to note that low NEM concentrations activate RyRs and may partially inhibit Ca\(^{2+}\), Mg\(^{2+}\)-ATPases, because NEM effects were not fully reversed by ruthenium red. The Ca\(^{2+}\)-ATPase enzyme is known to be inhibited by sulphydryl reagents and up to three sulphydryl groups out of the 24 cysteines of the enzyme are considered essential for full enzymatic activity (26–29). High concentrations of alkylating reagents are typically required and used to inhibit Ca\(^{2+}\)-ATPase activity. Low concentrations of alkylating agents are ineffective, which is consistent with the fast initial phase of Ca\(^{2+}\) uptake seen after a brief exposure of the SR vesicles to NEM (Fig. 1B, trace 1). On the other hand, prolonged exposure of SR vesicles with low NEM concentrations may partially inhibit Ca\(^{2+}\)-ATPase activity, as shown in Fig. 1B.

The reversal of NEM-induced Ca\(^{2+}\) release by DTT indicates that the exogenously added DTT neutralizes the effect of NEM by the alkylaing of DTT by NEM and that NEM did not significantly inhibit Ca\(^{2+}\)-ATPase activity, because the vesicles take up the release Ca\(^{2+}\). These findings indicate that at low concentrations, NEM activates RyRs by a non-covalent interaction and at high concentrations by alkylating free thiols on RyRs. The NO donors SNAP, NOC-15 (PAPA-NONOate), and cys-SNO were tested at concentrations that had no effect on channel activity and were found to inhibit NEM-induced Ca\(^{2+}\) release from SR vesicles. NO donors most likely inhibit the effect of low NEM by interacting with NEM, because the inhibition is only seen at low NEM concentrations compared with NO donor concentrations and at NEM levels that do not alkylate the RyR. Thus, inhibition of NEM-induced Ca\(^{2+}\) release by NO cannot be explained by a poly-s-nitrosylation of essential cysteine residues on RyRs, because the action of NEM does not involve a sulphydryl-mediated reaction. These findings are supported by equivalent measurements with other alkylating agents and with a non-thiol-reactive compound, structurally related to NEM. In all cases, these reagents elicited SR Ca\(^{2+}\) release, and the effects of the thiol reagents were reversed by DTT, and as expected, the non-thiol reagent was not reversed by DTT. The finding that NEM, a non-thiol reagent structurally related to NEM, elicits SR Ca\(^{2+}\) release demonstrates that such release occurs without the possibility of a sulphydryl interaction with RyRs. The block of NEM-induced Ca\(^{2+}\) release by ruthenium red (Fig. 4C) confirms that NEM acts at RyRs.

Aghdasi et al. (19) reported three distinct time-dependent phases of channel modification primarily from measurements of ryanodine binding with the tacit assumption that ryanodine binding is a direct measurement of single channel activity. The greater the binding, the more active the channel. In general, it is known that agents that increase and decrease channel activity typically increase and decrease ryanodine binding (\(B_{\text{max}}\)), respectively. However, that is not always the case, because sulphydryl reagents that activate RyRs have the opposite effect on ryanodine binding. The heavy metal Ag\(^{+}\) is a robust activator of RyRs (4) and dissociates ryanodine from its high affinity binding site in seconds instead of the normally very slow rate of dissociation (>45 min) (30). Similarly, reactive disulfide compounds and NO activate RyRs and inhibit ryanodine binding (7, 13). Aghdasi et al. (19) found that low NEM concentrations alkylate thiols involved in phase 1 channel inhibition without progressing to phases 2 and 3. This was shown as an inhibition of RyR activity with 0.2 mM NEM in bilayers and a decrease in ryanodine binding with 0.5 and 1.0 mM NEM (19). However, no evidence was given to indicate that low NEM concentrations alkylate thiols or that 0.5 or 1 mM NEM inhibited activity in single channel recordings before progressing to phases 2 and 3 of channel modification.

In contrast, we found that low concentrations of NEM (0.2 mM) activated RyRs in bilayer experiments, and we found no signs of channel inhibition in either bilayer or SR vesicle measurements. In bilayer experiments, the activation of the channel by low concentrations of NEM was reversed by DTT (Fig. 4C) consistent with the reversal of NEM-induced Ca\(^{2+}\) release in vesicle experiments by washing out NEM or adding DTT. The most likely explanation is that DTT interacts directly with NEM to neutralize NEM and NEM-induced Ca\(^{2+}\) release. At high NEM concentrations (5 mM), Ca\(^{2+}\) release from SR was not reversed by DTT as expected for an alklylation of thiol groups.

Several studies have shown that NO donors activated (13, 16, 17) or inhibited (14, 15) skeletal and cardiac RyR. Aghdasi et al. (19) proposed that low levels of NO inactivate and high levels activate the receptor. Their conclusions were based on the findings that NEM could interact with multiple classes of sulphydryls resulting in inactivation or activation of RyR as a function of time and NEM concentration (19). NO and H\(_2\)O\(_2\) blocked the phase 1 inhibitory effect of 5 mM NEM. NO donors, at low concentrations that have no detectable effect on channel activity, blocked intersubunit cross-linking, whereas higher NO levels activate the channel (17). The authors suggested that the two effects of NO resulted from interaction with distinct sulphydryls. Our data show that NO donors can partially reverse the effects of low concentration of NEM, but the reversal of this reaction can also be accomplished by washing the SR vesicles or by adding a sulphydryl-reducing agent. When SR vesicles were preincubated with NO donors at subactivating concentrations of NO donors, the subsequent NEM-induced Ca\(^{2+}\) release was inhibited but not fully blocked. In this case, NO appears to interact directly with NEM, decreasing its effective concentration rather than causing an indirect inhibition of NEM-induced Ca\(^{2+}\) release through an inactivation of RyRs.

The present findings indicate that 5 mM NEM alkylates thiols on RyRs, but low concentrations (20–200 \(\mu\)M) act by a reversible non-covalent interaction indicating that phase 1 effects measured as a decrease in ryanodine binding do not
correspond to the inhibition of the channel with low NEM concentrations. The latter interpretation of the data rests heavily on ryanodine binding measurements and the questionable assumption that ryanodine binding is a measure of channel activity under all conditions.

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