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Nitric Oxide, NOC-12, and *S*-Nitrosoglutathione Modulate the Skeletal Muscle Calcium Release Channel/Ryanodine Receptor by Different Mechanisms

AN ALLOSTERIC FUNCTION FOR O_2 IN *S*-NITROSYLATION OF THE CHANNEL*

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The skeletal muscle Ca^{2+} release channel/ryanodine receptor (RyR1) contains ~50 thiols per subunit. These thiols have been grouped according to their reactivity/responsiveness toward NO, O_2, and glutathione, but the molecular mechanism enabling redox active molecules to modulate channel activity is poorly understood. In the case of NO, very low concentrations (submicromolar) activate RyR1 by *S*-nitrosylation of a single cysteine residue (Cys-3635), which resides within a calmodulin binding domain. *S*-Nitrosylation of Cys-3635 only takes place at physiological tissue O_2 tension (pO_2; *i.e.* ~10 mm Hg) but not at pO_2 ~150 mm Hg. Two explanations have been offered for the loss of RyR1 responsiveness to NO at ambient pO_2, *i.e.* Cys-3635 is oxidized by O_2 *versus* O_2 subserves an allosteric function (Eu, J. P., Sun, J. H., Xu, L., Stamler, J. S., and Meissner, G. (2000) *Cell* 102, 499–509). Here we report that the NO donors NOC-12 and *S*-nitrosoglutathione both activate RyR1 by release of NO but do so independently of pO_2. Moreover, NOC-12 activates the channel by *S*-nitrosylation of Cys-3635 and thereby reverses channel inhibition by calmodulin. In contrast, *S*-nitrosoglutathione activates RyR1 by oxidation and *S*-nitrosylation of thiols other than Cys-3635 (and calmodulin is not involved). Our results suggest that the effect of pO_2 on RyR1 *S*-nitrosylation is exerted through an allosteric mechanism.

The large, homotetrameric skeletal muscle Ca^{2+} release channel/ryanodine receptor (RyR1)[1] contains several classes of regulatory thiols. These classes are distinguished by reactivity or responsiveness to O_2 tension (pO_2) (1, 2), redox active molecules such as glutathione (3) and nitric oxide (NO) (1), transmembrane glutathione redox potential (4), and allosteric effector molecules (Ca^{2+}, Mg^{2+}) (5). It has recently been shown that cysteine 3635, which is localized to the calmodulin (CaM) binding domain of RyR1 (6–8), confers responsiveness to NO. In contrast, the identities of the remaining regulatory thiols are not known. NO forms a covalent bond with the thiol group of Cys-3635 (*i.e. S*-nitrosylation) *in vivo* and thereby reverses the inhibitory effect of CaM on the channel (6). Full-length RyR1 channels with an alanine residue substituted for Cys-3635 are not *S*-nitrosylated by physiological concentrations of NO, and channel activity is unaffected by NO. *S*-nitrosylation of Cys-3635 only occurs at low O_2 tension (pO_2 ~10 mm Hg, comparable with that found in skeletal muscle *in vivo*) (1, 6). At this pO_2, 6–8 (of ~50) thiols per RyR1 subunit are actively maintained in the reduced state (1). Thus, one explanation for the failure of NO to *S*-nitrosylate RyR1 at ambient pO_2 is that Cys-3635 is oxidized. An alternative possibility is that the oxidation of pO_2-sensitive thiols leads to a change in channel conformation; in this state *S*-nitrosylation of Cys-3635 is unfavorable. Alternatively stated, O_2 is either serving as an oxidant (of Cys-3635) or as an allosteric effector (of Cys-3635 reactivity).

NO donors, compounds capable of donating NO and redox active forms thereof, are widely used to mimic the effects of NO synthase (9). A number of these compounds are capable of modulating RyR1 activity (1, 10–15). RyR1 contains a large number of reactive thiols (1, 2), and the action of NO donors may differ widely depending on the mechanisms and rates of NO release, the chemistry of NO group transfer, the base structure of the NO donor compound, and the reactivity of substrate thiol. In particular, members of the *S*-nitrosothiol (SNO) class of NO donors can modulate protein function by transnitrosylation as well as NO release (16, 17). In contrast, the NONOate class of NO donors is thought to be less susceptible to transnitrosylation chemistry (18). It is important to note, however, that NONOate compounds may directly interact with proteins through polyamine recognition sites and/or through ionic interactions.

In the present study, we examined the activation of the skeletal muscle Ca^{2+} release channel by NOC-12 and GSNO, an endogenous S-nitrosothiol, and compared their effects to solutions of NO. We found that both NOC-12 and GSNO activated RyR1 independently of O_2 tension and that the NO scavenger, C-PTIO, blocked the effects of both. But whereas NOC-12 mediated its effects by *S*-nitrosylation of a single cysteine (Cys-3635), GSNO activation involved the *S*-nitrosylation and oxidation of multiple thiols. Moreover, Cys-3635 was not required for activation by GSNO. Thus, NO, NOC-12, and GSNO activate the prototypic redox-sensitive RyR1 channel by different mechanisms, and the effect of O_2 tension on *S*-nitrosylation by NO is best rationalized by an allosteric mechanism.

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[1] The abbreviations used are: RyR, ryanodine receptor; RyR1, skeletal muscle isoform of RyR; pO_2, O_2 tension; CaM, calmodulin; NO, nitric oxide; SNO, *S*-nitrosothiol; GSNO, *S*-nitrosoglutathione; SR, sarcoplasmic reticulum; NOC-12, *N*-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine; WT, wild type; HEK293, human embryonic kidney 293;

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Ryanodine was a product of PerkinElmer Life Sciences. CaM was obtained from Sigma. NO donors, monobromobimane, myosin light chain kinase-derived CaM binding peptide and anti-*S*-nitrosocysteine polyclonal antibody were from Calbiochem, and leupeptin and Pefabloc (protease inhibitors) were from Roche Molecular Biochemicals. An ECL detection reagent kit was from Amersham Biosciences. NO gas (purity >99%, National Welders) was scrubbed to remove O_2 and nitrite by passing through an argon-purged column filled with KOH pellets and then a solution of NaOH. The concentration of NO was determined by a hemoglobin titration assay and an NO electrode (WPI Instruments) as described (1). All other chemicals were of analytical grade.

Sample Preparations—Skeletal muscle sarcoplasmic reticulum (SR) vesicles enriched in RyR1 were prepared from rabbit skeletal muscle in the presence of protease inhibitors (19). The construction and expression of wild type (WT) and C3635A mutant RyR1s have been described (6). WT and C3635A RyR1s were expressed in HEK293 cells, and crude membrane fractions were prepared as described (6).

Quantification of RyR1 Free Thiols and S-Nitrosothio1s—RyR1 free thiol (SH) and SNO contents were determined by the monobromobimane fluorescence method and a photolysis/chemiluminescence-based NO detection assay, respectively (1).

Electrophoresis and Detection of S-Nitrosocysteine on Western Blots—All procedures were performed under non-reducing conditions (6). Membranes were incubated in 0.125 M KCl, 20 mM imidazole, pH7.0, and 8 μM free Ca^{2+} for 1 h at 24 °C in room air in the absence and presence of NOC-12 or GSNO. Protein samples were separated by 3–20% SDS-PAGE under non-reducing conditions and transferred to polyvinylidene difluoride membranes. The membranes were blotted with 5% nonfat milk in 0.05% Tween 20 phosphate-buffered 0.1 M saline solution at 24 °C for 2 h and probed with anti-*S*-nitrosocysteine polyclonal antibody (Calbiochem; 1:500) and secondary peroxidase-conjugated anti-rabbit IgG antibody (Calbiochem; 1:2000). Anti-*S*-nitrosocysteine signals were detected with an ECL kit (Amersham Biosciences). After that, the membranes were re-probed with anti-RyR1 monoclonal antibody D110 (1:10) and peroxidase-conjugated anti-mouse IgG (Calbiochem, 1:2000) using the ECL detection method.

[^{3}H]Ryanodine Binding—Functional effects of NO donors were determined in [^{3}H]ryanodine binding measurements as described (1). The assay conditions of [^{3}H]ryanodine binding are indicated in the legends to Figs. 1, 2, and 4.

Single Channel Recordings—Single channel measurements were performed at room air by fusing RyR1-containing membrane fractions with Mueller-Rudin-type bilayers containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio 5:3:2 (25 mg of total phospholipid per milliliter of *n*-decane) (1, 20). The side of the bilayer to which the RyR1-containing membrane fractions were added was defined as the *cis* (cytoplasmic) side. The *trans* (lumenal) side of the bilayer was defined as ground. Single channels were recorded in the buffer solutions given in the legends to Figs. 3 and 5. Measurement of the sensitivity of the channels to cytosolic Ca^{2+} indicated that in a majority of recordings (>98%) the cytosolic side of RyR1 faced the *cis* side and the lumenal side faced the *trans* side of the bilayer. Electrical signals were filtered at 2 kHz, digitized at 10 kHz, and analyzed with a commercially available software package (pClamp 8.2, Axon Instruments, Foster City, CA). P_o values in multichannel recordings were calculated using the equation $P_o = \Sigma\, iP_{o,i}/N$, where N is the total number of channels, and $P_{o,i}$ is channel open probability of the
i^{th} channel.

Other Biochemical Assays—Free Ca^{2+} concentrations were obtained by including in the solutions the appropriate amounts of Ca^{2+} and EGTA as determined using the stability constants and computer program published by Schoenmakers *et al.* (21). Free Ca^{2+} concentrations were verified with the use of a Ca^{2+} selective electrode. The protein concentrations were determined by the Amido Black method (22).

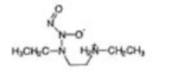
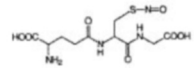
Data Analysis—Results are given as means ± S.D. unless otherwise indicated. Significance of differences of data was analyzed with Student's *t* test. Differences were regarded to be statistically significant at *, $p < 0.05$ and **, $p < 0.01$.

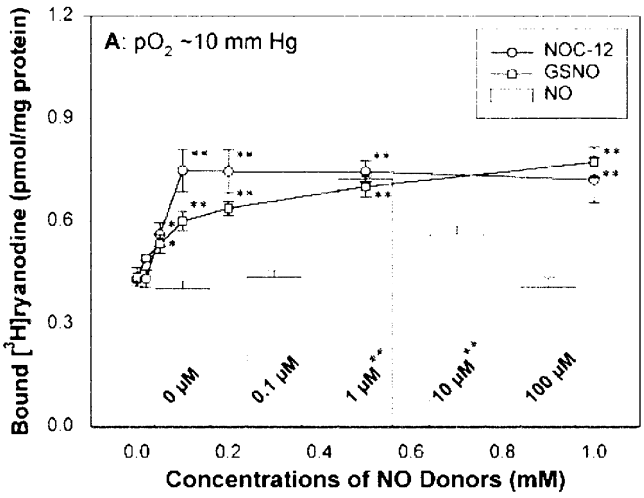
RESULTS

Release of NO by NOC-12 and GSNO—NOC-12 releases two NO molecules per donor (23), whereas GSNO releases only one (24). An NO electrode was used to characterize the peak concentrations and the durations of NO release under conditions employed in the [^{3}H]ryanodine binding measurements. NOC-12 (0.1 mM) and GSNO (0.1 mM) attained peak concentrations of 2.6 ± 0.4 μM and 1.4 ± 0.3 μM (n = 3 each) with

TABLE I
Peak concentrations and half lifetimes of NO released by NOC-12 and GSNO

The stock solutions of NO donors (10 mM) were prepared fresh, and NO release by 0.1 mM each NO donor was recorded with a NO electrode (WPI Instruments) in the buffer used for [^{3}H]ryanodine binding at 24 °C in room air (pO_2 ~ 150 mm Hg). The half-life time of 1 μM NO under this condition was ~10 min (1).

NO Donors (Molecular Formula)	Molecular Structure	Peak Concentration of Released NO	Half Lifetime of Released NO
NOC-12 ($C_6H_{16}N_4O_2$)		2.6 ± 0.4 μM	~ 6.5 hrs
GSNO ($C_{10}H_{16}N_4O_7S$)		1.4 ± 0.3 μM	~ 2.8 hrs



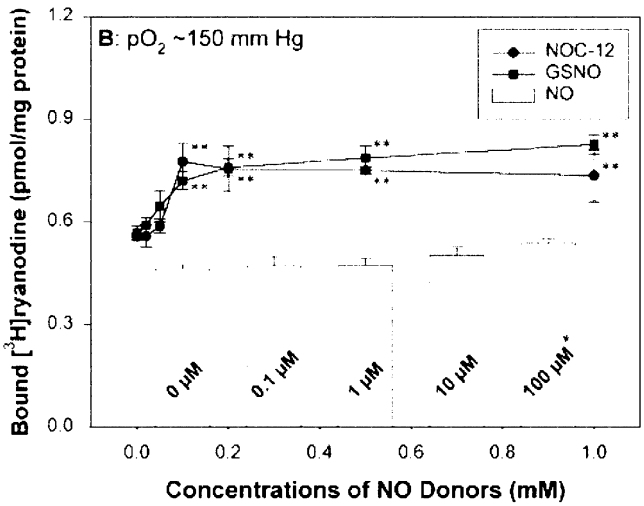


FIG. 1. **Effects of NO, NOC-12, and GSNO on [^{3}H]ryanodine binding to skeletal muscle SR vesicles in pO_2 of ~10 mm Hg (*A*) and ~150 mm Hg (*B*).** Specific [^{3}H]ryanodine binding to skeletal SR vesicles was determined in 0.125 M KCl, 20 mM imidazole, pH7.0, 8 μM free Ca^{2+}, the indicated concentrations of NO (*columns*) and NO donors (*lines* with *symbols*), and 5 nM [^{3}H]ryanodine at 24 °C for 5 h in pO_2 ~10 mm Hg (*A*) and pO_2 ~150 mm Hg (*B*), respectively. Data are the mean ± S.D. of four to six experiments. *, $p < 0.05$; **, $p < 0.01$ compared with respective control without NO or NO donor.

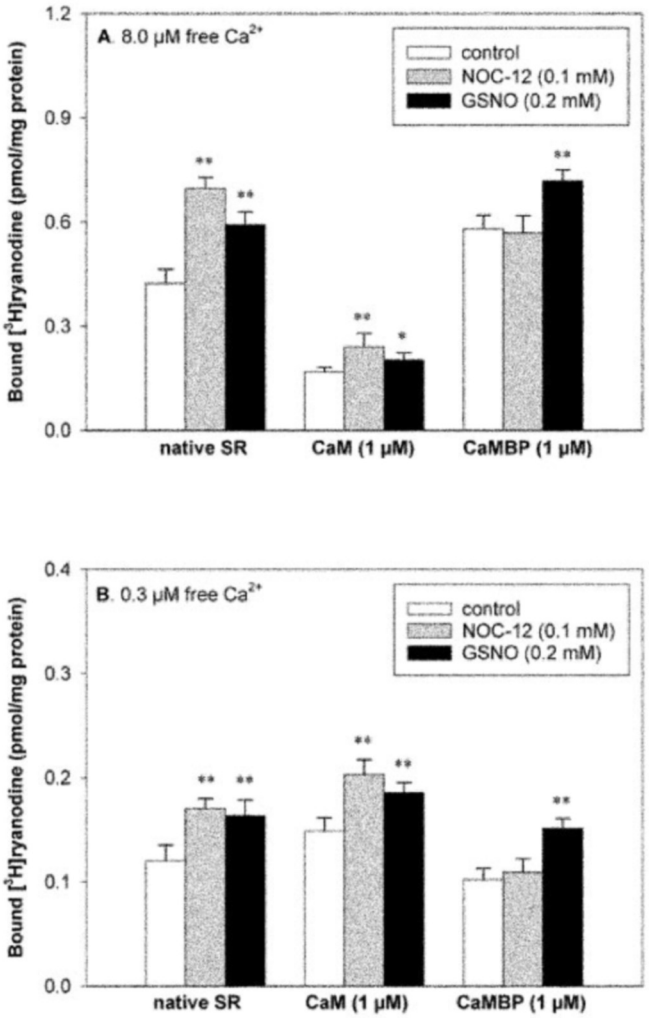


FIG. 2. **Effects of CaM, NOC-12, and GSNO on [^{3}H]ryanodine binding of skeletal muscle SR vesicles.** Skeletal muscle SR vesicles were pretreated in the presence of 100 μM Ca^{2+} without or with 1 μM CaM or 1 μM CaM binding peptide (*CaMBP*) at 24 °C for 30 min. Specific [^{3}H]ryanodine binding was assayed at 8 μM Ca^{2+} (*A*) or 0.3 μM Ca^{2+} (*B*) as described in Fig. 1 in the absence and presence of 0.1 mM NOC-12 or 0.2 mM GSNO in pO_2 ~150 mm Hg. Data are the mean ± S.D. of three to four experiments. *, $p < 0.05$; **, $p < 0.01$ compared with controls without NO donor in each group.

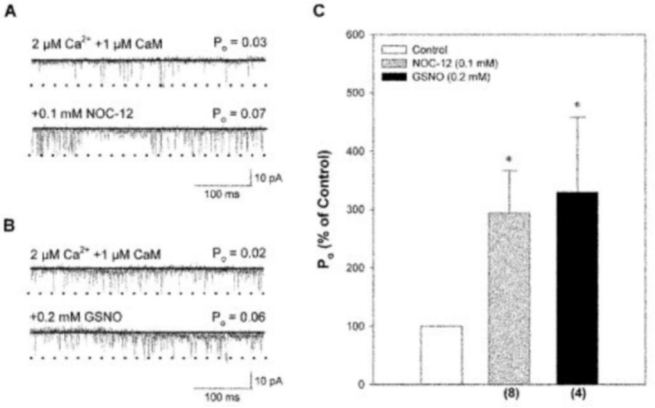


FIG. 3. **Effects of NOC-12 and GSNO on RyR1 activities.** SR vesicles were fused with lipid bilayers in pO_2 ~150 mm Hg. Single RyR1 channel currents, shown as downward deflections from closed levels (*solid lines*) to open levels (*dotted lines*), were recorded in symmetric 0.25 M $CsCH_3SO_3$, 10 mM cesium HEPES buffer, pH7.3, at a holding potential of −35 mV. *Top traces*, control with 2 μM free Ca^{2+} and 1 μM CaM; *bottom traces*, after the addition of 0.1 mM NOC-12 (*A*) or 0.2 mM GSNO (*B*) to the cytosolic side. *C*, normalized P_o before and after the addition of 0.1 mM NOC-12 or 0.2 mM GSNO. Data are the mean ± S.E. of the number of recordings indicated in parentheses. *, $p < 0.05$ compared with control (without NO donors).

half-life times of ~6.5 and 2.8 h, respectively (Table I). In a majority of the experiments, we matched the NO peak concentrations by comparing the groups treated with 0.1 mM NOC-12 with those treated with 0.2 mM GSNO. There was no difference in peak concentrations of NO released by either donor as a function of pO_2 (pO_2 ~10 mm Hg *versus* ~150 mm Hg) (data not shown). The half-life time of NO was ~10 min.

O_2 Tension-independent Modulation of RyR1 by NOC-12 and GSNO—Modulation of RyR1 by NO is O_2 tension dependent; only at a pO_2 comparable with that found in skeletal muscle *in vivo* (pO_2 ~10 mm Hg) can physiological amounts of NO (submicromolar) *S*-nitrosylate and activate RyR1 (1). In Fig. 1, SR vesicles were treated with increasing concentrations of NO, NOC-12, or GSNO, and RyR1 activities were determined by [^{3}H]ryanodine binding at pO_2 ~10 mm Hg (Fig. 1*A*) or at pO_2 ~150 mm Hg (Fig. 1*B*). Ryanodine is a highly specific plant alkaloid that is widely used as a probe of channel activity because of its preferential binding to the open channel states (25, 26). As shown previously (1), only at pO_2 ~10 mm Hg did NO (1–10 μM) cause a significant increase in [^{3}H]ryanodine binding (Fig. 1*A*). Elevated levels of NO were inhibitory at pO_2 ~10 mm Hg. In striking contrast, NOC-12 and GSNO activated RyR1 channel activity at either O_2 tension. Control experiments showed that NOC-12 and GSNO left to incubate for 48 h at room air (*i.e.* spent compounds) were without effect on RyR1 channel activity (data not shown). Under both O_2 tensions, NOC-12 concentrations higher than 0.1 mM caused a slight decrease in [^{3}H]ryanodine binding, whereas GSNO concentrations higher than 0.2 mM further increased [^{3}H]ryanodine binding. Thus, in contrast to NO, both NOC-12- and GSNO-activation of RyR1 is independent of pO_2 (over a wide range of NO donor concentrations).

Modulation of RyR1 by NOC-12 Is Dependent on CaM, whereas GSNO Modulation Is Not—The functional effects of *S*-nitrosylation of RyR1 Cys-3635 by NO are CaM-dependent (1, 6). At [Ca^{2+}] >1 μM, the Ca^{2+}-bound form of CaM (CaCaM) inhibits RyR1, whereas at [Ca^{2+}] <1 μM the Ca^{2+}-free form of CaM (apoCam) activates the receptor (27). We therefore assessed the effects of the NO donors on [^{3}H]ryanodine binding in the presence or absence of both the Ca^{2+}-bound form of CaM and the Ca^{2+}-free form of CaM. Sequestration of endogenous CaM with a CaM binding peptide (28) caused an increase in RyR1 channel activity over control at 8 μM free Ca^{2+} (Fig. 2*A*) and a decrease at 0.3 μM free Ca^{2+} (Fig. 2*B*). NOC-12 caused an increase in [^{3}H]ryanodine binding in the presence of CaM but not after CaM had been sequestered (Fig. 2, *A* and *B*). In contrast, GSNO caused an additional enhancement of RyR1 channel activity even after endogenous CaM sequestration. These results support the idea that NOC-12 controls RyR1 via the *S*-nitrosylation of Cys-3635, which is found in the CaM binding region of RyR1. On the other hand, redox modulation by GSNO does not appear to be dependent on S-nitrosylation or oxidation of Cys-3635. More definitive evidence for the role of Cys-3635 in the redox modulation of RyR1 is given below using a RyR1 construct with a Cys-3635 to Ala substitution.

Modulation of RyR1 Single Channel Activities by NOC-12 and GSNO—The ability of the two NO donors to activate RyR1 under ambient oxygen tension was confirmed in single channel recordings. Skeletal SR vesicles were incorporated into planar lipid bilayers, and single RyR1 channels were recorded with Cs^+ as the current carrier. As shown in Fig. 3*A*, 0.1 mM NOC-12 significantly activated RyR1 channel in the presence of 2 μM free Ca^{2+} and 1 μM CaM. Similarly, 0.2 mM GSNO

TABLE II

Free thiol (SH) and S-nitrosothiol (SNO) contents of RyR1 and [^{3}H]ryanodine binding levels in the absence and presence of NOC-12 and GSNO

The amounts of free thiols (SH) and *S*-nitrosothiols (SNO) of RyR1 were assayed in $pO_2 \sim 150$ mm Hg by monobromobimane fluorescence and the photolysis/chemiluminescence NO detection method, respectively (1). For functional comparison, [^{3}H]ryanodine binding was carried out under the same conditions (with 8 μM free Ca^{2+}). Data are mean ± S.D. of number of experiments given in parentheses.

Preparations	Free thiol content (SH/RyR1 subunit)	*S*-nitrosothiol content (SNO/RyR1 subunit)	[^{3}H]ryanodine binding
			pmol/mg protein
Normal	29.3 ± 1.2 (7)	0.41 ± 0.02 (6)	0.56 ± 0.05 (6)
NOC-12			
0.1 mM	28.1 ± 1.0 (6)	1.43 ± 0.21 (6)	0.78 ± 0.05 (3)
1.0 mM	28.2 ± 0.2 (3)	1.45 ± 0.36 (3)	0.74 ± 0.08 (3)
GSNO			
0.2 mM	25.5 ± 0.8 (5)	2.05 ± 0.35 (4)	0.72 ± 0.03 (3)
1.0 mM	22.6 ± 1.2 (5)	3.02 ± 0.24 (4)	0.83 ± 0.06 (3)

activated the channel (Fig. 3*B*). Fig. 3*C* shows that the averaged channel open probability (P_o) of RyR1 tripled after the addition of 0.1 mM NOC-12 or 0.2 mM GSNO. Thus, both [^{3}H]ryanodine binding and single channel measurements show that under comparable conditions these two NO donors activate the RyR1 to the same extent.

Redox-related Basis of RyR1 Modulation by NOC-12 and GSNO—We next determined whether modulation of RyR1 by NO donors involved the formation of a single SNO per RyR1 subunit, as was shown previously for NO at $pO_2 \sim 10$ mm Hg (1). We thus determined both the free thiol and SNO content of RyR1s treated with NOC-12 or GSNO at $pO_2 \sim 150$ mm Hg. Exposure of SR vesicles to 0.1 or 1.0 mM NOC-12 increased [^{3}H]ryanodine binding to a similar extent and reduced the RyR1 thiol content by ~1 per RyR1 subunit, which was accounted for by the formation of ~1 SNO per RyR1 subunit (Table II). The stoichiometry of 1 SNO/RyR1 subunit agreed with that obtained by exposure to 0.75 μM NO at $pO_2 \sim 10$ mm Hg (1). 0.1 mM NOC-12 optimally activated RyR1 in single channel recordings in less than 1 min (Fig. 3*A*).

In contrast to NOC-12, 0.2 mM GSNO activated RyR1 at ambient O_2 tension via the *S*-nitrosylation or oxidation of multiple thiols or a combination of both redox-based modifications. As shown in Table II, 0.2 mM GSNO *S*-nitrosylated approximately two RyR1 thiols in addition to oxidizing approximately two thiols per RyR1 subunit (loss of approximately four thiols per RyR1 subunit). At an elevated concentration (1.0 mM), GSNO further increased the level of [^{3}H]ryanodine binding and *S*-nitrosylated approximately three thiols and oxidized approximately four thiols (loss of approximately seven thiols/RyR1 subunit). Both oxidation and *S*-nitrosylation of RyR1 by GSNO (and *S*-nitrosylation by NOC-12) were prevented in the presence of 5 mM reduced glutathione (not shown).

We considered the possibility that GSNO *S*-nitrosylates RyR1 via transnitrosylation using C-PTIO, a NO scavenger, and NOC-12 as a control. NOC-12 (0.1 mM) no longer had any effect on RyR1 in the presence of 0.1 mM C-PTIO, neither S-nitrosylating nor activating RyR1 (not shown). Similarly, 0.1 mM C-PTIO eliminated RyR1 *S*-nitrosylation and activation by 0.2 mM GSNO (not shown). These results suggest that *S*-nitrosylation of RyR1 by GSNO is dependent on release of NO, as is the release of NO from NOC-12. We caution, nevertheless, that C-PTIO may have other effects, including scavenging and generating additional reactive radicals.

Cysteine 3635 Is Critical for RyR1 Modulation by NOC-12 but Not by GSNO—The aforementioned data using SR vesicles suggest that at ambient pO_2 NOC-12 S-nitrosylates Cys-3635 and activates RyR1 by antagonizing the inhibitory effect of CaM. In contrast, GSNO works by a different mechanism. We tested this hypothesis using a strategy that was previously employed to demonstrate selective modification of Cys-3635 by NO (6). Full-length WT or single-site C3635A RyR1 mutant

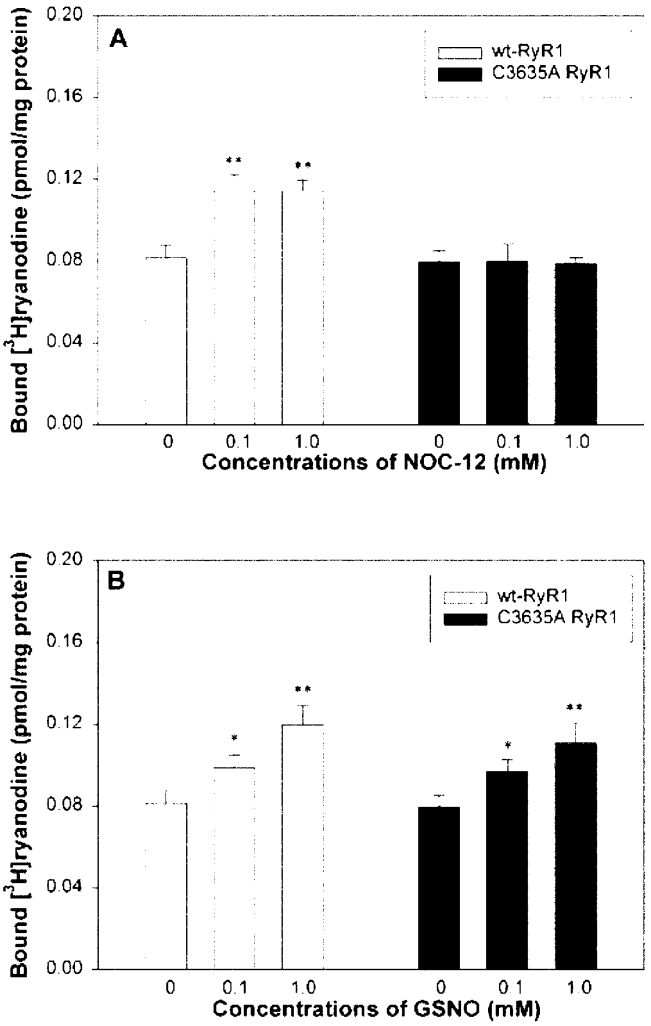


FIG. 4. **Effects of NOC-12 and GSNO on WT RyR1 and C3635A RyR1 activities.** Specific [^{3}H]ryanodine binding to membrane fractions prepared from HEK293 cells expressing WT or C3635A RyR1s was determined in 8 μM free Ca^{2+} medium as described in the Fig. 1 legend in the presence of indicated concentrations of NOC-12 (*A*) or GSNO (*B*) in $pO_2 \sim 150$ mm Hg. [^{3}H]Ryanodine binding data are the mean ± S.D. of three to five experiments. *, $p < 0.05$; **, $p < 0.01$, compared with each control (without NO donors).

channels were expressed in HEK293 cells. Membranous fractions containing WT and C3635A mutant RyR1s were isolated from the HEK293 cells, and the effects of the two NO donors were assessed at $pO_2 \sim 150$ mm Hg in [^{3}H]ryanodine binding (Fig. 4) and in single channel measurements (Fig. 5). NOC-12 had no effect on the mutant RyR1 (Figs. 4*A* and 5, *A* and *C*), whereas the GSNO effect was preserved (Figs. 4*B* and 5, *B* and

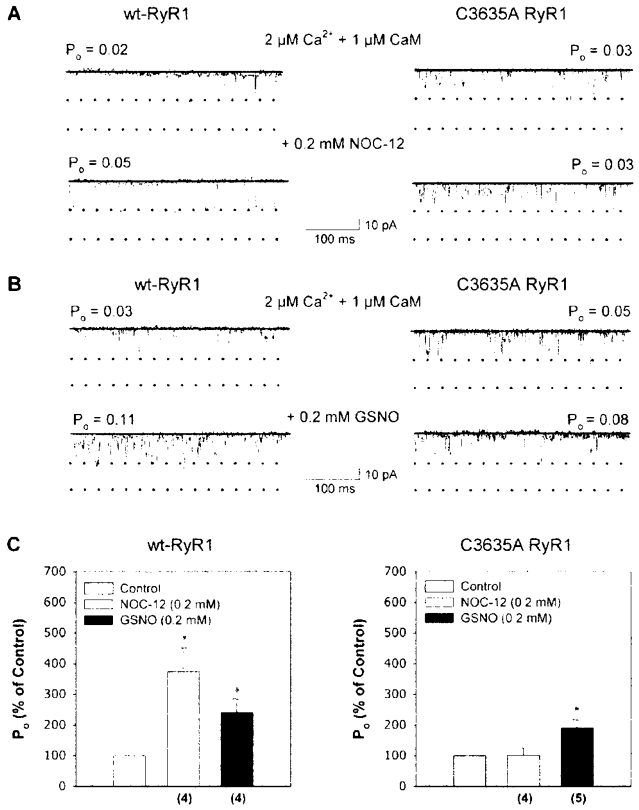


FIG. 5. **Effects of NOC-12 on single channel activities of WT RyR1 and C3635A RyR1.** *A* and *B*, membrane fractions containing WT and C3635A RyR1s were fused with lipid bilayers in pO_2 ~150 mm Hg. *Left side*, single channel currents of two WT RyR1 channels, shown as downward deflections from closed levels (*solid lines*) to open levels (*dotted lines;* the two dotted lines in each trace indicate the amplitude of two channels), were recorded in symmetric 250 mM KCl, 20 mM potassium Hepes buffer, pH 7.4, at holding potential of −35 mV. *Top traces*, control with 2 μM free Ca^{2+} and 1 μM CaM; *bottom traces*, after the addition of 0.2 mM NOC-12 (*A*) or 0.2 mM GSNO (*B*) to the cytosolic side. *Right side,* single channel currents of two C3635A RyR1 channels recorded in same condition as WT RyR1. *Top traces*, control with 2 μM free Ca^{2+} and 1 μM CaM; *bottom traces*, after the addition of 0.2 mM NOC-12 (*A*) or 0.2 mM GSNO (*B*) to the cytosolic side. *C*, normalized P_o before and after the addition of 0.2 mM NOC-12 or 0.2 mM GSNO. Data are the mean ± S.E. of the number of experiments indicated in parentheses. *, $p < 0.05$, compared with control (without NO donors).

C). The failure of NOC-12 to activate RyR1 C3635A was not due to a lack of CaM binding, because the C3635A mutation does not eliminate modulation of RyR1 activity by CaM (6, 8).

We used an anti-nitrosocysteine polyclonal antibody to determine whether NOC-12 and GSNO *S*-nitrosylated the RyR1 C3635A mutant channel. We first confirmed that NO increased the immunoreactivity of the native and WT RyR1s in pO_2 ~10 mm Hg but not pO_2 ~150 mm Hg (6) (not shown). NO did not, however, increase immunoreactivity of the C3635A mutant RyR1 at either oxygen tension. A weak signal was detected by the antibody in the control samples (without NO donor) in a region of the immunoblots containing the RyR1 (Fig. 6, *left panel*), as determined by an anti-RyR1 antibody (Fig. 6, *right panel*). NOC-12 (0.1 and 1.0 mM) produced virtually the same signal as NO (6) but in ambient pO_2, thus increasing the level of *S*-nitrosylation of native and WT RyR1s but not of C3535A RyR1. Specificity of S-nitrosylation was proven by showing that prior treatment with $HgCl_2$ nearly eliminated the signal (not shown). In contrast, 0.2 and 1.0 mM GSNO did not noticeably increase the low levels of endogenous immunoreactivity. Taken together, the data of Figs. 4–6 suggest that NOC-12 and GSNO affect the RyR1 by two different mechanisms, *i.e.* NOC-12 by

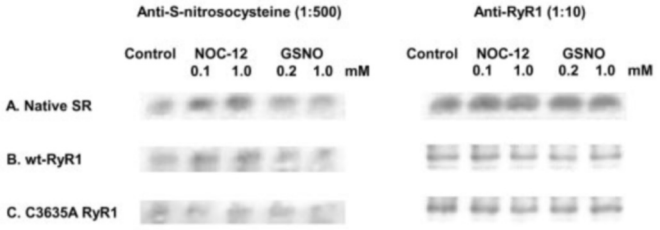


FIG. 6. **Immunoblots for nitrosocysteine and RyR1.** Skeletal SR vesicles (*A*, 10 μg protein/lane) and cell membrane fractions (*B* and *C*, 20 μg protein per lane) containing WT RyR1 (*B*) or C3635A RyR1 (*C*) were incubated for 1 h at 24 °C in pO_2 ~150 mm Hg with 8 μM free Ca^{2+} in the absence or presence of indicated concentrations of NOC-12 or GSNO. The proteins were separated by 3–12% gradient SDS-PAGE and transferred to polyvinylidene difluoride membranes overnight at 4 °C. A polyclonal anti-*S*-nitrosocysteine antibody (*left panel*, 1:500 dilution) was used to detect an *S*-nitrosylation signal in the protein band region of RyR1 probed with D110 monoclonal anti-RyR1 (*right panel*, 1:10 dilution). The data are representative of three experiments.

S-nitrosylation of Cys-3635 and GSNO by *S*-nitrosylation and/or oxidation of an additional/alternative class of RyR1 thiols.

DISCUSSION

The massive (~2,200 kDa) ryanodine receptors contain numerous allosteric sites subserving multiple levels of control (25). It has been firmly established that all three mammalian ryanodine receptor isoforms are redox sensitive, *i.e.* the channels contain regulatory thiols whose oxidation or covalent modification alters their activities (1, 2, 29–32). These thiols (~50/subunit) have been grouped according to their differential reactivities toward NO, O_2, and glutathione, which in turn may be linked to binding of allosteric effectors (1, 2). We have recently shown that NO, at low pO_2, selectively modifies Cys-3635 (6). PO_2 is dynamically linked to the redox state of a class of 6–8 thiols. However, the identities of these regulatory thiols and the mechanistic basis of the pO_2 regulation of NO binding (homotropic *versus* heterotropic) remain to be determined. Here we have probed this question by taking advantage of the different reactivities and properties of alternative classes of NO donors.

Cysteine 3635 is part of a predicted hydrophobic motif for *S*-nitrosylation (33) located within the RyR1 CaM binding domain (7, 8); NO regulation of RyR1 activity is thus CaM dependent (1, 6). We posited that the inability of NO to *S*-nitrosylate Cys-3635 at ambient O_2 tension is either due to Cys-3635 being oxidized (*i.e.* Cys-3635 is one of the 6–8 thiols) or to a change in channel conformation brought about by the oxidative posttranslational modification. As a first step to address this question, we determined the dependence of NOC-12 and GSNO on Cys-3635 and pO_2. NOC-12 and GSNO had very similar effects on RyR1 channel activity (at concentrations matched for NO release), and neither compound showed O_2 dependence (pO_2 ~10 mm Hg *versus* pO_2 ~150 mm Hg). However, the underlying mechanism of activation was quite different in each case. GSNO activated RyR1 via poly-*S*-nitrosylation and/or oxidation of RyR1 thiols. Cys-3635 and CaM were not essential for activation. These data are highly reminiscent of the effects of GSNO on cardiac muscle isoform of RyR (RyR2), except that O_2 and CaM dependence were not explored at that time (20). NO and NOC-12 have little effects on RyR2.[2] In stark contrast, NOC-12 activates RyR1 via *S*-nitrosylation of Cys-3635, and the increases in activity ([^{3}H]ryanodine binding in intact SR) is CaM dependent, as seen with NO. Specifically, the only modification of RyR1 by NOC-12 was nitrosylation of a single thiol,

[2] J. Sun and G. Meissner, unpublished studies.

and full-length, heterogeneously expressed RyR1 with a C3635A mutation was not activated. Unlike NO, however, S-nitrosylation by NOC-12 is seen at high pO_2. Thus we conclude that Cys-3635 of RyR1 is not oxidized at ambient O_2 tension.

Why NO and NOC-12 mediated *S*-nitrosylation differ in their pO_2-dependence remains unclear. NOC-12 evidently depends on released NO, because its RyR1-activating effect was inhibited by C-PTIO (a NO scavenger) and was not reproduced by the spent compound. It is unlikely that differences in half-life of NOC-12 *versus* NO (>6 h *versus* 10 min, Table I) provide an explanation, because the effect of NOC-12 in single channel recordings was seen within 1 min. Instead, we favor the idea that access of NO to the cysteine thiol is responsible for the differences. Hydrophobic domains that concentrate nitrosylating equivalents and the quaternary structure of the target site are both important determinants for *S*-nitrosylation (33, 34). NOC-12 may interact with the RyR1 (ionic interactions of these compounds are seen with other proteins),[3] perhaps in a way that is conducive to nitrosylation irrespective of RyR conformation. An interaction with the protein would also have the effect of increasing the effective molarity of the NONOate, thereby potentiating nitrosylation chemistry involving O_2. In contrast, access of solution NO (*i.e.* through the protein) to Cys-3635 might be available in the low pO_2 conformation but blocked at high pO_2. The hydrophobic pocket where Cys-3635 resides may even serve to concentrate NO/O_2 to produce nitrosylating equivalents. In this scenario, the allosteric function subserved by low pO_2 is 2-fold: 1) to produce a nitrosylation-responsive conformation of the RyR1; and 2) to catalyze nitrosylation chemistry (micellar catalysis).

An intriguing finding was that NOC-12 and GSNO operate by different mechanisms. NO release from GSNO is evidently necessary for activation of the RyR1, because the NO scavenger C-PTIO blocked the effects of GSNO on the RyR1. Nonetheless, it is premature to exclude transnitrosylation reactions of GSNO, acting alone or in concert with released NO, in the activating mechanism. Other explanations for GSNO effects include the possibility that GSNO-mediated oxidation favors the *S*-nitrosylation of a specific class of thiols (or *vice versa*). It has recently been shown that proteins may have specific binding sites for GSNO, which would direct the chemistry to thiols in its vicinity (35).

In summary, NO, NOC-12 and GSNO all activate RyR1 to comparable degrees in [^{3}H]ryanodine binding and single channel measurements. However, only the effect of NO is pO_2-dependent. Activation by both NO and NOC-12 involves the CaM-dependent *S*-nitrosylation of Cys-3635, whereas GSNO mediated activation (involving *S*-nitrosylation/oxidation of up to seven RyR1 thiols) can occur independent of Cys-3635 and CaM. Thus O_2, NO, and GSNO react with different classes of thiols, and the role of pO_2 in RyR1 *S*-nitrosylation is likely mediated through allostery.

[3] J. S. Stamler, unpublished observations.

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