

## Classes of Thiols That Influence the Activity of the Skeletal Muscle Calcium Release Channel\*

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The skeletal muscle  $\text{Ca}^{2+}$  release channel/ryanodine receptor (RyR1) is a prototypic redox-responsive ion channel. Nearly half of the 101 cysteines per RyR1 subunit are kept in a reduced (free thiol) state under conditions comparable with resting muscle. Here we assessed the effects of physiological determinants of cellular redox state (oxygen tension, reduced (GSH) or oxidized (GSSG) glutathione, and  $\text{NO}/\text{O}_2^-$  (released by 3-morpholinopyridone) on RyR1 redox state and activity. Oxidation of ~10 RyR1 thiols (from ~48 to ~38 thiols/RyR1 subunit) had little effect on channel activity. Channel activity increased reversibly as the number of thiols was further reduced to ~23/subunit, whereas more extensive oxidation (to ~13 thiols/subunit) inactivated the channel irreversibly. Neither *S*-nitrosylation nor tyrosine nitration contributed to these effects. The results identify at least three functional classes of RyR1 thiols and suggest that 1) the channel may be protected from oxidation by a large reservoir of functionally inert thiols, 2) the channel may be designed to respond to moderate oxidative stress by a change in activation set-point, and 3) the channel is susceptible to oxidative injury under more extensive conditions.

$\text{Ca}^{2+}$  release channels/ryanodine receptors (RyRs)<sup>1</sup> are the largest known ion channels, consisting of four ~565-kDa RyR subunits and four associated 12-kDa FK506-binding protein subunits (1, 2). Following an action potential, the cardiac and skeletal muscle RyR isoforms release  $\text{Ca}^{2+}$  from an intracellular  $\text{Ca}^{2+}$  storing membrane compartment, the sarcoplasmic reticulum (SR), in a process known as excitation-contraction coupling. Numerous endogenous effectors are known to regulate RyR function and therefore muscle contractility. These effectors range from ions ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) to other small molecules (adenine nucleotides) to polypeptides such as calmodulin (1, 2). Recent work has also established RyRs as prototypic

redox-sensitive ion channels. The skeletal muscle isoform of RyR (RyR1) contains a large number of free thiols: as many as 50 out of a total of 101 cysteine residues/subunit (100 cysteines/RyR1 subunit (3) and 1 cysteine/FK506-binding protein subunit (4)) (5). RyR1 channel activity is dramatically altered by redox modifications of critical thiols (oxidation, *S*-nitrosylation, or alkylation) (5–14). Conversely, RyR1 has thiols whose redox potential is dependent on effectors that regulate RyR1 activity such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (15). In a physiological context, nitric oxide (NO) and reactive oxygen species are produced in contracting muscle and have been shown to modulate *in vitro* RyR redox state and channel activity (5, 12, 16–21). Remarkably, RyR1 redox state and function are dependent on  $\text{O}_2$  tension (5). Altering  $\text{O}_2$  tension alone dynamically reduced/oxidized as many as 6–8 thiols/RyR1 subunit and thereby modified channel responsiveness to physiological concentrations of NO (5).

In this study, we varied reducing and oxidizing conditions to explore in detail the effect of RyR1 redox state on channel function. At one end of the redox spectrum, RyR1 was maintained in a highly reduced state by GSH at low  $p\text{O}_2$  (~10 mm Hg). At the other end of the spectrum, we tested the effects of the strongly oxidizing conditions produced by high concentrations of  $\text{NO}/\text{O}_2^-$  (released by 3-morpholinopyridone SIN-1). Our studies demonstrate that 1) RyR1 thiols can be grouped into at least three distinct functional classes, 2) RyR1 channel function peaks in moderately oxidizing conditions (~23 free thiols/RyR1 subunit), 3) channel closure is favored by strongly reducing ( $\geq 38$  free thiols/RyR1 subunit) or oxidizing ( $\leq 15$  free thiols/RyR1 subunit) conditions, and 4) NO and  $\text{O}_2^-$ , which are produced *in vivo* (17), have concentration-dependent effects on channel function that are mediated by thiol oxidation and may have both physiological and pathological relevance.

### EXPERIMENTAL PROCEDURES

**Materials**—[<sup>3</sup>H]ryanodine was obtained from PerkinElmer Life Sciences; unlabeled ryanodine and monobromobimane (mBB) from Calbiochem; SIN-1 from Molecular Probes, Inc. (Eugene, OR); and CHAPS, leupeptin, and Pefabloc (a protease inhibitor) from Roche Molecular Biochemicals. All other chemicals were of analytical grade.

**Preparation of SR Vesicles**—“Heavy” rabbit skeletal muscle SR membrane fractions enriched in [<sup>3</sup>H]ryanodine binding, and  $\text{Ca}^{2+}$  release channel activities were prepared in the presence of protease inhibitors (100 nM aprotinin, 1  $\mu\text{M}$  leupeptin, 1  $\mu\text{M}$  pepstatin, 1 mM benzamide, 0.2 mM phenylmethylsulfonyl fluoride) as described (22).

**Quantification of RyR1 Thiol and *S*-Nitrosothiols Contents**—The number of free thiols in RyR1 was determined as described previously (5). Briefly, skeletal SR vesicles, treated with or without SIN-1 in the absence or presence of GSH or GSSG at  $p\text{O}_2$  of ~10 or 150 mm Hg, were centrifuged (100,000  $\times g$ ) at 4 °C for 1 h. The pellets were washed and resuspended and then probed with an excess (500  $\mu\text{M}$ ) of the lipophilic, thiol-specific agent mBB for 1 h in the dark at 24 °C. Following mBB treatment, SR vesicles were solubilized with 1.5% CHAPS, and the bimane-labeled RyR1 was isolated by sucrose density gradient centrifugation.

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<sup>1</sup> The abbreviations used are: RyR, ryanodine receptor; RyR1, skeletal muscle isoform of RyR; SR, sarcoplasmic reticulum; mBB, monobromobimane; NO, nitric oxide;  $\text{O}_2^-$ , superoxide anion; SIN-1, 3-morpholinopyridone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

ugation. The fluorescence intensity of bimane (*i.e.* the thiol content) in the sucrose gradient fraction most enriched in RyR1 (>95% purity) was determined and normalized for protein concentration as described (5). The S-nitrosothiol content of RyR1 was determined by isolating the receptor without prior mBB treatment and using a photolysis/chemiluminescence method (5, 12).

**[<sup>3</sup>H]Ryanodine Binding**—Unless otherwise indicated, skeletal SR vesicles, treated with various concentrations of SIN-1 in the absence or presence of GSH or GSSG at  $pO_2$  of ~10 or 150 mm Hg, were incubated with 5 nM [<sup>3</sup>H]ryanodine at 24 °C in media containing 0.125 M KCl, 20 mM imidazole, pH 7.0, 0.3 mM Pefabloc, 30 μM leupeptin, and the indicated concentrations of free Ca<sup>2+</sup>. Nonspecific binding was determined using a 1000-fold excess of unlabeled ryanodine. After 5 h, aliquots of the samples were diluted with 20 volumes of ice-cold water and placed on Whatman GF/B filters soaked with 2% (w/w) polyethyleneimine. Filters were washed with three 5-ml volumes of ice-cold buffer, and the radioactivity remaining on the filters was determined by liquid scintillation counting to obtain bound [<sup>3</sup>H]ryanodine.

**Single Channel Recordings**—Single channel measurements were performed by fusing skeletal SR vesicles with Mueller-Rudin type bilayers containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio 5:3:2 (25 mg of total phospholipid/ml *n*-decane) (5). The side of the bilayer to which the SR vesicles were added was defined as the *cis* (cytoplasmic) side. The *trans* (SR luminal) side of the bilayer was defined as ground. Single channels were recorded in a symmetric CsCH<sub>3</sub>SO<sub>3</sub> buffer solution (0.25 M CsCH<sub>3</sub>SO<sub>3</sub>, 10 mM Cs-HEPES, pH 7.3) containing the additions indicated (see Fig. 4). Measurement of the sensitivity of the channels to cytosolic Ca<sup>2+</sup> indicated that in a majority of recordings (>98%) the cytosolic side of the RyRs faced the *cis* side, and the luminal side faced the *trans* side of the bilayer. Electrical signals were filtered at 2 kHz, digitized at 10 kHz, and analyzed. Data acquisition and analysis were performed with a commercially available software package (pClamp 6.0.4; Axon Instruments, Burlingame, CA) with an IBM-compatible Pentium II computer and 12-bit A/D to D/A converter (Digidata 1200, Axon Instruments).

**Immunoblotting**—RyR1 and nitrotyrosine contents of SR vesicles were determined by immunoblot analysis. Samples were solubilized in nonreducing sample buffer, containing 62.5 mM Tris-HCl, pH 6.8, 20% glycerol (w/v), 1.2% SDS, and 0.05% bromophenol blue, and loaded onto 3–15% SDS-PAGE gradient gels. After electrophoresis, the proteins were transferred overnight to a nitrocellulose membrane (Schleicher and Schuell). The transferred membrane was blocked in Tris-buffered saline buffer, containing 0.05% Tween 20, 20 mM Tris-HCl, pH 7.4, 250 mM NaCl, and 5% nonfat milk, with agitation for 2 h at room temperature. RyR1 and nitrotyrosine proteins were identified using D110 anti-RyR1 monoclonal antibody (23) and anti-nitrotyrosine polyclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY), respectively. Secondary goat anti-mouse and anti-rabbit IgG-horseradish peroxidase-linked antibodies were used at 1:2000 dilution. Color development was accomplished using the DAB substrate kit (Roche Molecular Biochemicals).

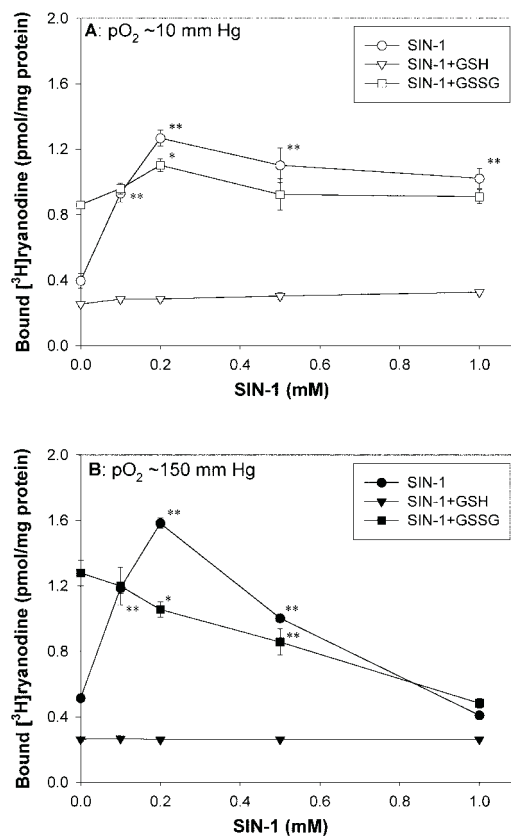
**Other Biochemical Assays**—Free Ca<sup>2+</sup> concentrations were obtained by including in the solutions the appropriate amounts of Ca<sup>2+</sup> and EGTA as determined using the stability constants and computer program published by Shoenmakers *et al.* (24). Free Ca<sup>2+</sup> concentrations >1 μM were verified with the use of a Ca<sup>2+</sup>-selective electrode (World Precision Instruments, Inc.). The protein concentrations were determined by the Amido Black method (25).

Ca<sup>2+</sup>-ATPase activity in SR vesicles was assayed by malachite green ATPase method (26) in the presence of a Ca<sup>2+</sup> ionophore (1 μM ionomycin) and the absence or presence of SIN-1. Mg<sup>2+</sup>-ATPase remaining in the SR preparations was subtracted from the total ATPase by adding 1 mM EGTA to assay media.

**Data Analysis**—Results are given as means ± S.D. with the number of experiments in parentheses. 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

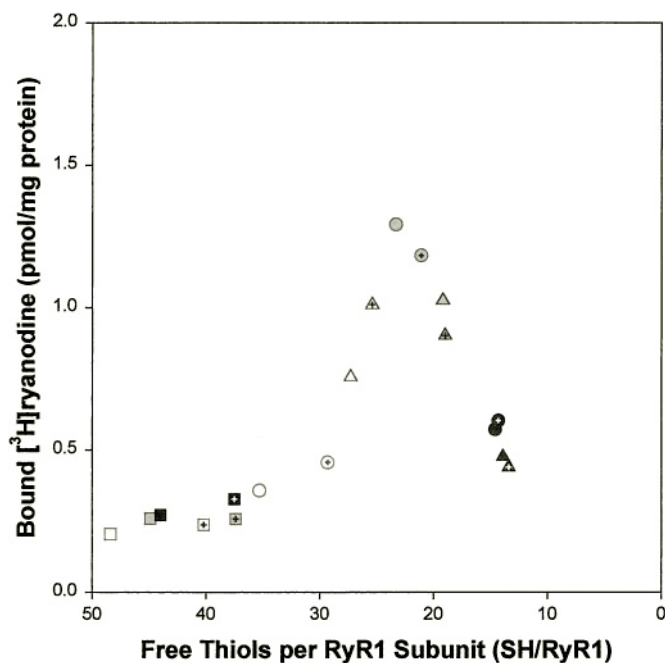
**Correlation of RyR1 Redox State and Activity**—The highly specific plant alkaloid ryanodine is widely used as a probe of RyR channel activity because of its preferential binding to open channel states (1, 2). In Fig. 1, [<sup>3</sup>H]ryanodine binding to rabbit skeletal muscle SR vesicles, and therefore RyR1 channel activity, was determined as a function of three different redox modifiers: glutathione (5 mM GSH *versus* 5 mM GSSG), SIN-1



**FIG. 1. Dose-dependent effects of SIN-1 on RyR1 channel activity in low and high O<sub>2</sub> tension.** Effects of SIN-1 on specific [<sup>3</sup>H]ryanodine binding to skeletal SR vesicles were determined either in the absence (circles) or presence of 5 mM GSH (triangles) or GSSG (squares) as described under "Experimental Procedures." A,  $pO_2$  ~10 mm Hg; B,  $pO_2$  ~150 mm Hg. Compared with each control, asterisks represent  $p < 0.05$ , and double asterisks represent  $p < 0.01$ .

(which releases NO/O<sub>2</sub><sup>-</sup> (27, 28) (0–1.0 mM)), and O<sub>2</sub> tension ( $pO_2$  ~10 *versus* ~150 mm Hg). Comparison of the *upper* and *lower* panels in Fig. 1 indicates that, in the absence of SIN-1 and glutathione, skeletal SR [<sup>3</sup>H]ryanodine binding is significantly higher ( $p < 0.05$ ) when measured in ambient  $pO_2$  (~150 mm Hg) than in muscle  $pO_2$  (10 mm Hg; Refs. 29 and 30). In both conditions, GSH (triangles) reduced [<sup>3</sup>H]ryanodine binding as compared with the controls without glutathione (circles), whereas GSSG (squares) increased [<sup>3</sup>H]ryanodine with the highest level observed in ambient air. These results confirm previous studies showing that reducing conditions (low O<sub>2</sub> tension and GSH) promote RyR1 channel closure, and oxidizing conditions (high oxygen tension and GSSG) favor channel opening (5, 9, 15).

The effects of SIN-1 on RyR1 activity were dependent on oxygen tension and the presence of GSH or GSSG. In ambient O<sub>2</sub> tension and the absence of glutathione, SIN-1 activated RyR1 channel activity maximally at 0.2 mM. Higher concentrations of SIN-1 reduced [<sup>3</sup>H]ryanodine binding ultimately back to base line (Fig. 1B). The biphasic concentration dependence indicates that moderate amounts of NO/O<sub>2</sub><sup>-</sup> activate RyR1, whereas excess amounts are probably damaging. Although 0.2 mM SIN-1 also maximally activated the channel at a  $pO_2$  of 10 mm Hg, simulating physiological conditions *in vivo* (29, 30), channel activity did not return to base line at 1.0 mM SIN-1 (Fig. 1A). The less pronounced effects of SIN-1 in muscle O<sub>2</sub> tension can be explained by the fact that the rate of release of NO and O<sub>2</sub><sup>-</sup> from SIN-1 is dependent on molecular oxygen concentration (27, 28). In the added presence of 5 mM GSSG, SIN-1 dose-dependently inactivated the channel in ambient

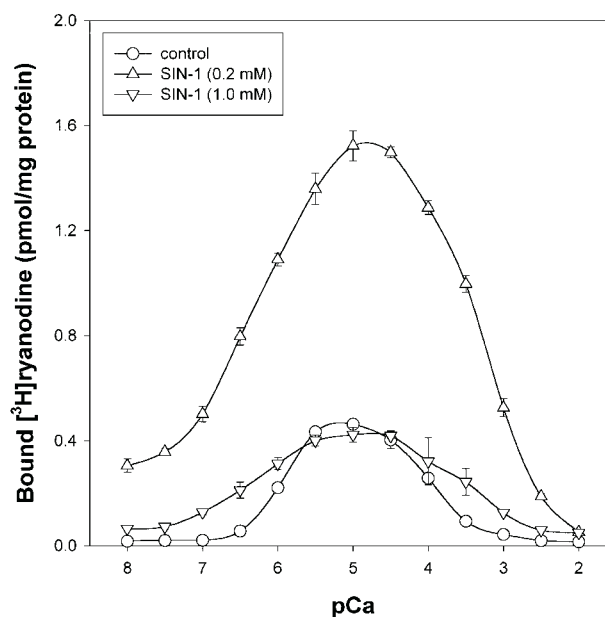


**FIG. 2. RyR1 channel activity is controlled by redox state.**  $[^3\text{H}]$ Ryanodine binding and free thiol content were determined as described under "Experimental Procedures" either in the absence (circles) or presence of 5 mM GSH (squares) or GSSG (triangles) in either  $p\text{O}_2 \sim 10$  mm Hg (symbols without cross) or  $\sim 150$  mm Hg (symbols with cross) and in the absence (open symbols) or presence of 0.2 mM (gray symbols) or 1 mM (dark symbols) SIN-1.  $[^3\text{H}]$ Ryanodine binding was determined by incubating SR vesicles for 1 h at 24 °C with 25 nM  $[^3\text{H}]$ ryanodine in medium containing 0.125 M KCl, 20 mM imidazole, pH 7.0, 0.3 mM Pefabloc, 30  $\mu\text{M}$  leupeptin, and 10  $\mu\text{M}$  free  $\text{Ca}^{2+}$ . The free thiol content of RyR1 was determined by the mBB fluorescence method in the same conditions. Data are the averages of at least three experiments done in duplicate with the S.D. being less than 20% for the  $[^3\text{H}]$ ryanodine binding and less than 15% for the free thiol content determinations.

oxygen tension (Fig. 1B) but had only moderate effects at  $p\text{O}_2 \sim 10$  mm Hg (Fig. 1A). Finally, in the presence of 5 mM GSH, SIN-1 effects on  $[^3\text{H}]$ ryanodine binding were almost entirely suppressed. This latter observation is consistent with GSH being a potent scavenger for reactive nitrogen and oxygen species (31).

Recent studies have indicated that oxidation and *S*-nitrosylation affect channel activity by altering RyR1's interaction with calmodulin (5, 13). SR vesicles contain 0.10–0.15 calmodulin/RyR1 subunit (5). To eliminate the effects of endogenous calmodulin, SR vesicles were pretreated with 2  $\mu\text{M}$  myosin light chain kinase-derived calmodulin binding peptide, followed by centrifugation through a layer of 0.3 M sucrose to remove complexed calmodulin and calmodulin binding peptide. After centrifugation, the endogenous SR-associated calmodulin content was reduced to  $\sim 5\%$  of the control value, as determined by a phosphodiesterase activation assay (5). The effects of SIN-1 on RyR1 activity, as measured by  $[^3\text{H}]$ ryanodine binding under the conditions in Fig. 1, were then studied by exposing control and pretreated vesicles to 0, 0.2, and 1.0 mM SIN-1 at  $p\text{O}_2 \sim 150$  mm Hg. An essentially identical activation by 0.2 mM SIN-1 and inactivation by 1.0 mM SIN-1 for vesicles pretreated and not pretreated with the calmodulin binding peptide (data not shown) indicated that SIN-1 did not transduce its effects in Fig. 1 via the small amounts of calmodulin associated with the SR vesicles.

The oxidation or reduction of a large number of thiols is the principle mechanism by which  $\text{O}_2$  tension and reducing agents such as glutathione modulate RyR1 channel activity in SR vesicles. A specific free thiol-labeling agent, mBB, was used to

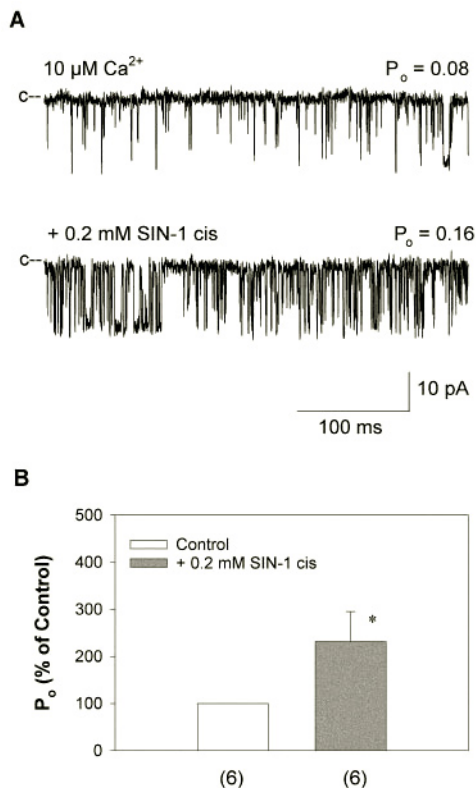


**FIG. 3. Effects of SIN-1 on  $\text{Ca}^{2+}$  dependence of  $[^3\text{H}]$ ryanodine binding to SR vesicles.** Vesicles were incubated for 30 min at 24 °C with either 0, 0.2, or 1.0 mM SIN-1 and then incubated for 5 h with 5 nM  $[^3\text{H}]$ ryanodine at the indicated  $\text{Ca}^{2+}$  concentrations. Data are the means  $\pm$  S.D. of three experiments.

correlate RyR1 free thiol content and activities in the presence of redox active molecules. As reported previously (5), RyR1 in SR vesicles exposed to 5 mM GSH had  $\sim 48$  and  $\sim 40$  free thiols/subunit at  $p\text{O}_2 \sim 10$  or  $\sim 150$  mm Hg, respectively. Exposure of the vesicles to variable glutathione,  $\text{O}_2$ , and/or  $\text{NO}/\text{O}_2^-$  concentrations resulted in the oxidation of up to 35 thiols/RyR1 subunit. A plot of  $[^3\text{H}]$ ryanodine binding against the number of free thiols per subunit revealed three principle redox states of the channel (Fig. 2): state 1 involving oxidation of  $\sim 10$  thiols ( $\sim 48$  to  $\sim 38$  thiols/RyR1 subunit), which had little effect on channel activity; state 2 corresponding to the total loss of  $\sim 25$  thiols (38–23 thiols/RyR1 subunit), which maximally activated the channel; and state 3 corresponding to the oxidation of an additional  $\sim 10$  thiols (23–13 thiols/RyR1 subunit), which decreased  $[^3\text{H}]$ ryanodine binding to levels observed under strongly reducing conditions. Thus, RyR1 channel activity can be variably affected by the oxidation of an enormous number of thiols,  $\sim 35$ /subunit or 140/RyR1 tetramer.

**$\text{Ca}^{2+}$  Dependence of Redox Modulation of RyR1 by SIN-1/(NO/O<sub>2</sub><sup>-</sup>)**—The regulation of the RyR1 by  $\text{Ca}^{2+}$ , as measured by  $[^3\text{H}]$ ryanodine binding, was determined at the three function-linked redox states of the channel corresponding to  $\sim 30$  (control), 23 (0.2 mM SIN-1), and 13 (1.0 mM SIN-1) residual thiols/RyR1 subunit (studies done in ambient  $\text{O}_2$  tension, Fig. 2). Moderate oxidation by 0.2 mM SIN-1 activated the RyR1 across the whole range of  $\text{Ca}^{2+}$  concentrations (from  $p\text{Ca}$  8 to  $p\text{Ca}$  2; Fig. 3) with a greater degree of activation observed at low  $\text{Ca}^{2+}$  concentrations ( $p\text{Ca} \leq 7$ ). Extensive oxidation by 1.0 mM SIN-1 resulted in reduced  $[^3\text{H}]$ ryanodine binding and a broadened  $\text{Ca}^{2+}$  activation/inactivation profile. These results suggest that SIN-1 activates and inactivates the RyR1, altering the  $\text{Ca}^{2+}$  dependence of channel activity.

The effects of SIN-1 on RyR1 activity were also explored in single channel measurements using maximally activating concentrations of  $\text{Ca}^{2+}$  (10  $\mu\text{M}$ ; Fig. 3) and SIN-1 (0.2 mM; Fig. 1). Skeletal SR vesicles were incorporated into planar lipid bilayers, and RyR1 single channel activity was recorded at ambient oxygen tension with  $\text{Cs}^+$  as the current carrier to eliminate other ion currents also present in SR vesicles (32). In the

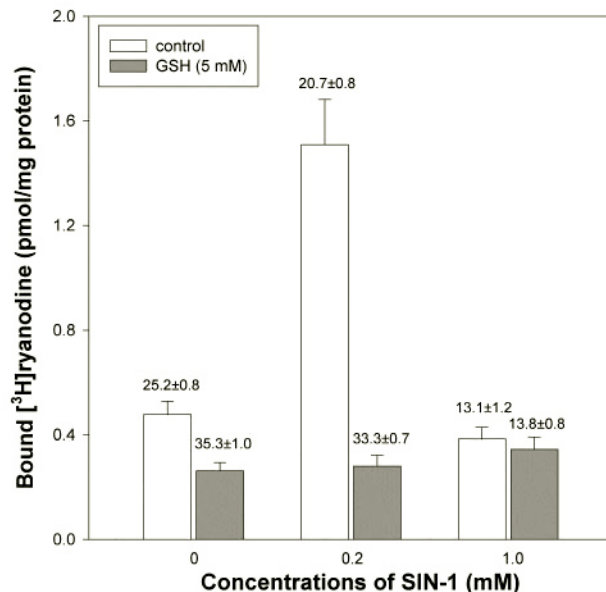


**FIG. 4. Effect of SIN-1 on RyR1 single channel activities.** Skeletal SR vesicles were fused with lipid bilayers at  $pO_2 \sim 150$  mm Hg. *A*, single RyR1 channel currents, shown as downward deflections from closed (*c*) levels, were recorded in symmetrical 0.25 M  $CsCH_3SO_3$ , pH 7.3 buffer at a holding potential of  $-35$  mV. *Top trace*, control with  $10 \mu M$  free  $Ca^{2+}$ ,  $P_o = 0.08$ ; *second trace*, immediately after the addition of  $0.2$  mM SIN-1 to the cytosolic side of the bilayer,  $P_o = 0.16$ . *B*, quantitative presentation of changes in  $P_o$ . Values of controls were normalized as 100%, and the changes were expressed as percentage of the controls. Normalized  $P_o$  before (*open columns*) and after the addition (*filled columns*) of  $0.2$  mM SIN-1 ( $n = 6$ ) is shown. Data are the means  $\pm$  S.D. of six experiments. Compared with control, *asterisks* represent  $p < 0.05$ .

presence of an optimally activating  $Ca^{2+}$  concentration of  $10 \mu M$ , channel open probability ( $P_o$ ) increased 2–3-fold ( $n = 6$ ) after the addition of  $0.2$  mM SIN-1 to the cytosolic (*cis*) chamber of the bilayer apparatus (Fig. 4). Thus, in agreement with the [ $^3H$ ]ryanodine binding measurements, single channel recordings show that a moderately oxidizing concentration of SIN-1 activates the RyR1.

**Mechanism of Redox Modification of RyR1 by SIN-1/(NO/O $_2^-$ )**—In the presence of molecular oxygen, SIN-1 generates one molecule each of NO and O $_2^-$ , which probably combine rapidly (rate constant of  $3.7 \times 10^7 M^{-1} s^{-1}$ ) to form peroxynitrite (27, 28). Therefore, peroxynitrite is probably a dominant oxidative species in experiments involving SIN-1. Rapid formation of peroxynitrite was supported by the finding that a NO electrode with a high sensitivity (5) failed to detect any NO release from SIN-1 in our assay conditions at either oxygen tension (data not shown).

Peroxyntirite oxidizes thiols reversibly to disulfide bonds or sulfenic (SOH) acids or irreversibly to sulfinic (SO $_2$ H) or sulfonic acids (SO $_3$ H) (33–35). The reversibility of RyR1 oxidation was determined at ambient oxygen tension from the thiol content of SR vesicles that were first treated with 0, 0.2, or 1.0 mM SIN-1 at  $24^\circ C$  for 5 h and then exposed to 5 mM GSH (experimental group) or no reducing equivalent (control) for another 5 h. [ $^3H$ ]Ryranodine binding and free RyR1 thiol content were determined after the final 5 h of incubation. The results are summarized in Fig. 5, with the number of free thiols per RyR1



**FIG. 5. Reversibility of SIN-1 induced changes in RyR1 channel activity and free thiol content.** SR vesicles were treated for 5 h at  $24^\circ C$  at  $pO_2 \sim 150$  mm Hg with 0, 0.2, or 1.0 mM SIN-1 and then incubated for another 5 h in the absence (*open columns*) or presence of 5 mM GSH (*filled columns*) either in the absence (free thiol quantification) or presence of 5 nM [ $^3H$ ]ryanodine ([ $^3H$ ]ryanodine binding). The free thiol content (thiols per RyR1 subunit) is given *above* the *columns*. Data are the means  $\pm$  S.D. of at least three experiments.

subunit (mean  $\pm$  S.D.,  $n \geq 3$ ) labeled at the *top* of each *column*. Channel activation and thiol oxidation by  $0.2$  mM SIN-1 were nearly completely reversed by 5 mM GSH. In contrast, 5 mM GSH could not reverse the effects of  $1.0$  mM SIN-1 on RyR1 channel activity or redox state (Fig. 5, *third pair of columns*). These results suggest, but do not prove, that at high concentrations of peroxynitrite, numerous RyR1 thiols ( $\sim 10$ /RyR1 subunit) proceeded to high degrees of oxidation.

Peroxyntirite may also *S*-nitrosylate free thiols in proteins (33, 34). We have shown previously that *S*-nitrosylation of a single thiol per RyR1 can activate the channel (5). To see if RyR1 is *S*-nitrosylated by SIN-1, control and SIN-1 ( $0.2$  mM)-treated samples were assayed for *S*-nitrosothiol content using a photolysis/chemiluminescence method (5, 12). In the control group, there were  $\sim 0.4$  *S*-nitrosothiol/RyR1 subunits, a level comparable with the endogenous amount of *S*-nitrosylation found in our previous study (5). SIN-1/peroxyntirite did not *S*-nitrosylate any additional RyR1 thiols (Table I). Therefore, unlike NO (5), SIN-1/peroxyntirite did not activate RyR1 by *S*-nitrosylation.

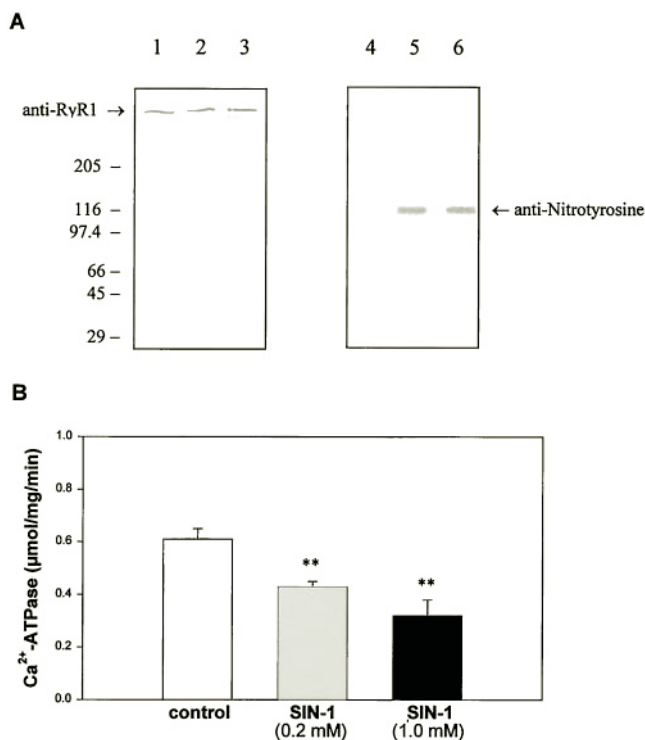
Peroxyntirite can also modify proteins by the addition of a nitro group to the ortho position of tyrosine to form nitrotyrosine (33, 34). In the case of the SR  $Ca^{2+}$ -ATPase, *in vitro* exposure of skeletal SR vesicles to peroxynitrite resulted in both *S*-nitrosylation (36) and nitrotyrosine formation (37). Both modifications contributed to inhibition of SR  $Ca^{2+}$ -ATPase activity. Nitrotyrosine formation could therefore represent an additional mechanism by which SIN-1 modulates RyR1, and this possibility was examined in immunoblots. A polyclonal antibody recognizing nitrotyrosine (Fig. 6A, *lanes 4–6*) did not detect any nitrotyrosine formation in the region of the blots corresponding to RyR1 (Fig. 6A, *lanes 1–3*) in controls and SR vesicles exposed to 0.2 or 1.0 mM SIN-1. In contrast, the anti-nitrotyrosine antibody revealed a weak band in the control sample (*lane 4*) and two stronger bands in the samples treated with  $0.2$  mM SIN-1 (*lane 5*) and  $1.0$  mM SIN-1 (*lane 6*), corresponding to a protein with an apparent molecular mass of  $\sim 100$

TABLE I

SNO/RyR1 and [<sup>3</sup>H]ryanodine binding with or without SIN-1

The amounts of *S*-nitrosothiols (SNO) of RyR1 were assayed by the photolysis/chemiluminescence method described under "Experimental Procedures." [<sup>3</sup>H]Ryanodine binding was determined under the same conditions (with 10 μM free Ca<sup>2+</sup>). Data are the means ± S.D. of the number of experiments given in parentheses.

| Preparations   | SNO/RyR1 subunit | Bound [ <sup>3</sup> H]ryanodine |
|----------------|------------------|----------------------------------|
|                |                  | <i>pmol / mg protein</i>         |
| Control        | 0.41 ± 0.02 (6)  | 0.49 ± 0.10 (7)                  |
| + 5 mM GSH     | 0.09 ± 0.05 (4)  | 0.25 ± 0.04 (4)                  |
| SIN-1 (0.2 mM) | 0.39 ± 0.10 (5)  | 1.41 ± 0.16 (7)                  |
| + 5 mM GSH     | 0.23 ± 0.07 (5)  | 0.35 ± 0.04 (3)                  |



**FIG. 6. Immunoblots and Ca<sup>2+</sup>-ATPase activity of SR vesicles treated with SIN-1.** *A*, SR vesicles were incubated for 1 h at 24 °C either in the absence (*lanes 1 and 4*) or presence of 0.2 mM (*lanes 2 and 5*) or 1.0 mM (*lanes 3 and 6*) SIN-1. Proteins separated by 3–15% gradient SDS-PAGE were transferred to nitrocellulose membranes and probed with an anti-RyR1 (*lanes 1–3*) or anti-nitrotyrosine (*lanes 4–6*) antibody. The anti-nitrotyrosine antibody did not detect nitrotyrosines in RyR1. However, a protein with a molecular mass of ~100 kDa was recognized by the antibody, and SIN-1 significantly increased the level of nitrotyrosines. *B*, SIN-1 (0.2 or 1.0 mM) significantly decreased SR Ca<sup>2+</sup>-ATPase activity. Data are the means ± S.D. of three experiments. Compared with control, *double asterisks* represent *p* < 0.01.

kDa. The results suggested that a 100-kDa protein such as the skeletal muscle SR Ca<sup>2+</sup>-ATPase has endogenous nitrotyrosine(s) and that its level is increased by exogenous SIN-1/peroxynitrite (37). In favor of this interpretation, SIN-1 (0.2 or 1.0 mM) inhibited SR Ca<sup>2+</sup>-ATPase activity by about ~30 and 50%, respectively (Fig. 6B).

Taken together, the results suggest that SIN-1 acts in our assay conditions as an oxidant rather than a NO donor. In the absence of glutathione, NO/O<sub>2</sub><sup>-</sup> reversibly activates RyR1 at low concentrations and irreversibly inactivates the channel at high concentrations. Furthermore, modulation of RyR1 by NO/O<sub>2</sub><sup>-</sup> appears to be due to oxidation of thiols, since neither *S*-nitrosylation nor tyrosine nitration of RyR1 was detected.

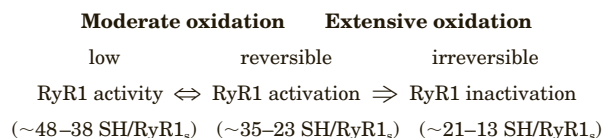
## DISCUSSION

Contracting muscle produces reactive oxygen and nitrogen species (16, 17, 38, 39). A functional role of these molecules is indicated by the finding that force development in muscle is affected using nitric-oxide synthase inhibitors and scavengers of superoxide. The data we have presented here imply that the effects of NO/O<sub>2</sub><sup>-</sup>-related species are mediated, at least in part, through the SR Ca<sup>2+</sup> release channel in skeletal muscle.

The RyR1 is exquisitely sensitive to redox modulation. Ample evidence indicates that RyRs are activated or inactivated or both by sulfhydryl-modifying molecules (5–15, 18–21, 40, 41). We previously uncovered a striking plasticity of redox state in RyR1 channel activity (5). RyR1 free thiol content and channel activity were dynamically controlled by GSH/GSSG, oxygen tension, and NO. Other recent studies have shown that RyR1 responds to changes in transmembrane glutathione redox potential (14) and contains thiols whose redox potential is dependent on ligands (Ca<sup>2+</sup>, Mg<sup>2+</sup>) that control RyR1 activity (15).

In this study, we used SIN-1 to probe the effect of redox state on RyR1 activity and have done so under a spectrum of redox conditions that are encountered in muscle, by further varying GSH/GSSG and *p*O<sub>2</sub>. SIN-1 spontaneously generates NO and O<sub>2</sub><sup>-</sup> (27), both of which rapidly combine in a 1:1 stoichiometry to form the highly oxidative species peroxynitrite, although NO/O<sub>2</sub><sup>-</sup> chemistry may produce other species as well (28, 33). While NO and O<sub>2</sub><sup>-</sup> are modulators of muscle contractility (16, 17, 38–40), we consider it unlikely that NO reacted with the RyR1, since we were unable to detect it with a NO electrode. Furthermore, SIN-1 and free NO modulate the skeletal muscle Ca<sup>2+</sup> release channel by two very different redox-related mechanisms. NO activates the channel at *p*O<sub>2</sub> ~10 mm Hg by *S*-nitrosylation of a single cysteine per RyR1 subunit (5), whereas SIN-1 affected channel activity by the oxidation of a large number of thiols. NO/O<sub>2</sub><sup>-</sup> generation is likely to be encountered in normally functioning muscle and in pathological states. Indeed, immunoblotting with an anti-nitrotyrosine antibody revealed endogenous and SIN-1-mediated nitration in a protein that co-migrated with the SR Ca<sup>2+</sup>-ATPase, confirming a previous report (37). Notably, the anti-nitrotyrosine antibody failed to detect endogenous or SIN-1-mediated nitration of tyrosines in RyR1.

Exposure to the three redox variables O<sub>2</sub>, glutathione, and SIN-1 revealed three distinct redox states of RyR1. The free thiol content ranged from as low as 13 to as high as 48 thiols/RyR1 subunit (SH/RyR1<sub>s</sub>) (see scheme below).



SCHEME 1

RyR1 free thiol content was high and channel activity was low in the presence of 5 mM GSH at *p*O<sub>2</sub> ~10 mm Hg; *i.e.* there are ~48 free thiols/RyR1 subunit under conditions encountered in normally functioning skeletal muscle. Remarkably, oxidation of ~10 free thiols/RyR1 subunit (SIN-1 in the presence of 5 mM GSH) had virtually no effect on RyR1 activity, suggesting that RyR1 has a large buffer capacity against oxidants such as O<sub>2</sub><sup>-</sup> or peroxynitrite. In addition, these thiols probably subserve the O<sub>2</sub> sensor function that tunes the NO response in muscle (5). Moderate oxidative activation (5 mM GSSG, 0.2 mM SIN-1 in the absence of 5 mM GSH) was accounted for by the loss of ~15 extra thiols and was reversible. Thus, RyR1 can probably respond to moderate changes in cellular redox state with changes in basal setpoint (*i.e.* a change in activity is maintained across

the whole  $\text{Ca}^{2+}$  concentration range). Extensive oxidative inactivation (1.0 mM SIN-1 in the absence and presence of 5 mM GSSG) was attributed to the loss of up to 10 additional thiols (~13 free thiols/RyR1 subunit remain) and was irreversible, making it likely to be of pathological rather than physiological significance. Therefore, RyR1 redox state and function appear to be intimately linked.

This analysis of RyR1 is reminiscent of our previous studies of RyR2 (12). In the case of RyR1, however, the oxidation of up to 25 thiols was reversible (suggesting the formation of disulfides and/or sulfenic acids), whereas only 5–6 thiols could be oxidized in the cardiac channel before irreversible changes were encountered. Irreversible thiol oxidation (suggesting oxidation to sulfinic or sulfonic acids) was dependent on the concentration of the oxidant (1.0 mM SIN-1) and required the absence of GSH. Although the (patho)physiological correlative of thiol oxidation remains to be elucidated, the finding of endogenous nitration in the SR (Ref. 37 and this study) indicates that such oxidative modifications are likely (*i.e.* thiols are generally more reactive toward  $\text{NO}/\text{O}_2^-$  and peroxyxynitrite than tyrosines (42)).

Intracellular  $\text{Ca}^{2+}$  concentration, the main determinant of skeletal muscle contractile function, is controlled by the RyR1 and an ATP-driven  $\text{Ca}^{2+}$  pump, with the former releasing the stored  $\text{Ca}^{2+}$  from SR to initiate contraction and the later sequestering  $\text{Ca}^{2+}$  back in SR to initiate relaxation. We previously showed that  $\text{O}_2$  tension dynamically reduced/oxidized 6–8 thiols/RyR1 subunit. The alteration of channel redox state determined its responsiveness to *S*-nitrosylation by NO of one cysteine per RyR1 subunit, and the effect of *S*-nitrosylation on channel activity was transduced via calmodulin (5). We believe that such regulation may impact on excitation-contraction coupling. On the other hand, we now show that  $\text{NO}/\text{O}_2^-$  can transduce its effects on RyR1 independently of calmodulin, thus providing an additional means by which the redox state of the cell may influence RyR1 function. But these findings probably bear on situations characterized by oxidative stress, where we envision that the RyR1 would counter impairments of force production imposed by oxidizing conditions with oxidation-induced activation. Ultimately, however, irreversible oxidation of RyR1 thiols may contribute to dysfunction of muscle. The relevance of these findings to conditions such as in fatigue, spasm, and rhabdomyolysis remains to be shown.

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