S-Glutathionylation Decreases Mg$^{2+}$ Inhibition and S-Nitrosylation Enhances Ca$^{2+}$ Activation of RyR1 Channels*

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We have analyzed the effects of the endogenous redox-active agents S-nitrosoglutathione and glutathione disulfide, and the NO donor NOR-3, on calcium release kinetics mediated by ryanodine receptor channels. Incubation of triad-enriched sarcoplasmic reticulum vesicles isolated from mammalian skeletal muscle with these three agents elicits different responses. Glutathione disulfide significantly reduces the inhibitory effect of Mg$^{2+}$ without altering Ca$^{2+}$ activation of release kinetics, whereas NOR-3 enhances Ca$^{2+}$ activation of release kinetics without altering Mg$^{2+}$ inhibition. Incubation with S-nitrosoglutathione produces both effects; it significantly enhances Ca$^{2+}$ activation of release kinetics and diminishes the inhibitory effect of Mg$^{2+}$ on this process. Triad incubation with $^{[35]}$S-nitrosoglutathione at pCa 5 promoted $^{35}$S incorporation into 2.5 cysteine residues per channel monomer; this incorporation decreased significantly at pCa 9. These findings indicate that S-nitrosoglutathione supports S-glutathionylation as well as the reported S-nitrosylation of ryanodine receptor channels (Sun, J., Xu, L., Eu, J. P., Stamler, J. S., and Meissner, G. (2003) J. Biol. Chem. 278, 8184–8189).

The combined results suggest that S-glutathionylation of specific cysteine residues can modulate channel inhibition by Mg$^{2+}$, whereas S-nitrosylation of different cysteines can modulate the activation of the channel by Ca$^{2+}$. Possible physiological and pathological implications of the activation of skeletal Ca$^{2+}$ release channels by endogenous redox species are discussed.

Ca$^{2+}$-induced Ca$^{2+}$ release (CICR)$^3$ mediated by ryanodine receptors/Ca$^{2+}$ release channels (RyR channels) has a central role in very dissimilar processes. Among other processes, CICR mediates muscle contraction, neuronal plasticity, and secretion (1–4). Not surprisingly, these Ca$^{2+}$ release channels are extensively regulated by a variety of endogenous ions and molecules, as well as through interactions with other proteins (3, 5–8).

During the last 15 years, increasing evidence has accumulated supporting redox modulation of RyR channels (for reviews, see Refs. 9–11). Each of the four homologous 565-kDa protein subunits of the skeletal muscle type 1 ryanodine receptor channel (RyR1 channel) contains 100 cysteine residues (12). In the native channel, ~50 of these residues appear to be in the reduced state, and, of these, ~10–12 are highly susceptible to oxidation/modification by exogenous sulfhydryl (SH) reagents (13). Sulfhydryl modification enhances Ca$^{2+}$ release from skeletal SR vesicles (14–20) and activates RyR1 channels incorporated in planar lipid bilayers (18, 21–26). Sulfhydryl modification also increases $^3$Hryanodine binding to skeletal SR membranes (18, 21, 24, 27). Highly reactive SH residues of the RyR1 channel protein participate in interactions between homotetrameric channel subunits (24), participate in the formation of high molecular weight complexes with triadin (29, 30), and modulate calmodulin binding to the channel (31, 32).

Endogenous redox active molecules, such as O$_2$ and superoxide anion (33, 34), H$_2$O$_2$ (21, 35), and glutathione disulfide (GSSG) (13, 36–38), enhance RyR channel activity. Likewise, changes in the glutathione/glutathione disulfide ratio (GSH/GSSG) (26, 39, 40) or incubation with NO or NO donors also affect RyR1 channel activity (13, 33, 41, 42). In particular, S-nitrosoglutathione (GSNO) and NO donors modify RyR1 channel activity through S-nitrosylation of a few critical SH residues (13, 42). However, incubation of skeletal SR vesicles with 0.2–1.0 mM GSNO removes more free SH residues than those modified by S-nitrosylation (42). These findings indicate that GSNO induces additional SH modifications, which may include S-glutathionylation because in other systems GSNO acts both as S-nitrosylating and S-glutathionylating agent (43, 44). S-Glutathionylation, through the formation of a mixed disulfide between a protein SH residue and glutathione (45), and S-nitrosylation appear to be reversible post-translational protein modifications, which may modulate intracellular signaling pathways by targeting critical molecules (46). In fact, the activities of several signaling molecules including PP2A (47), Ras (48), and NFkB (49) are modified as a consequence of S-glutathionylation. This reaction is also markedly activated in response to oxidative stress, as shown by redox proteome analysis (50, 51).

We have previously reported that thimerosal enhances single RyR channel activity both by increasing channel activation by micromolar [Ca$^{2+}$] and decreasing inhibition by 0.5 mM [Ca$^{2+}$] (23). Thimerosal, in a concentration- and time-dependent manner, also enhances CICR rates from SR vesicles from mammalian skeletal muscle and decreases or abolishes the
inhibition of CICR by 1 mM [Mg^{2+}] (20). Thimerosal, however, is an exogenous organomercurial compound that modifies SH residues most likely via S-alkylation (52). If endogenous GSNO had the potential to produce similar modifications of RyR channel activity as thimerosal, a significant enhancement of the CICR process would be expected in response to GSNO-induced endogenous redox modification of the channel protein. Therefore, we assessed the ability of GSNO to modify Ca^{2+} release kinetics and determined whether, in addition to its reported S-nitrosylating activity (42), GSNO also induced S-glutathionylation of RyR channels. We found that GSNO produced similar activation of CICR as thimerosal, and promoted Ca^{2+} release and S-glutathionylation of the RyR channel protein. In addition, we tested the effects on CICR of GSSG and of the NO donor NOR-3, as pure S-glutathionylation or S-nitrosylating agents, respectively. The present results suggest that S-glutathionylation modifies specific cysteine residues and, in doing so, either directly or indirectly modulates channel inhibition by Mg^{2+} whereas S-nitrosylation modifies other cysteines, thereby altering activation by Ca^{2+}. Possible physiological and pathological implications of these findings are discussed.

EXPERIMENTAL PROCEDURES

Materials—All reagents used were of analytical grade. Protease inhibitors (leupeptin, pepstatin A, benzamidine, and phenylmethylsulfonyl fluoride) and bovine serum albumin were obtained from Sigma. Calcium Green-2 and Calcium Green-5N were obtained from Molecular Probes, Inc. (Eugene, OR). The redox reagents GSH, GSSG, DTT, and NOR-3 were purchased from Calbiochem (La Jolla, CA). When stored, fresh GSH solutions were bubbled with N₂ and frozen at –20 °C. To avoid oxidation of GSH, aliquots were thawed only once and the unused portion was discarded. [35S]GSH and [3H]Ryanodine were purchased to avoid oxidation of GSH, aliquots were thawed only once and the unused portion was discarded. [35S]GSH and [3H]Ryanodine were purchased from PerkinElmer Life Sciences. Primary antibodies against RyR1 (MA3-925) or triadin (MA3-931) and the secondary antibody (SA1-100) were purchased from Affinity BioReagents, Inc. (Golden, CO).

Membrane Preparations—Triad-enriched SR vesicles (from non on triads) were isolated from rabbit fast skeletal muscle in the presence of 10 mM glucose and bovine serum albumin as standard (53). To avoid spontaneous oxidation, all membrane fractions were rapidly frozen and kept under liquid N₂. Experiments were performed within 24–48 h of membrane isolation to minimize possible oxidation during storage. Before use, the functionality of each preparation was verified. Native vesicles (0.2 mg/ml) that, following addition of 5 mM Mg-ATP, took longer than 2 min to 25 °C to actively decrease extravesicular free [Ca^{2+}] from 25 μM to <0.2 μM (measured with Calcium Green-2) were discarded. It is possible that, because of the steps taken to avoid oxidation, release rate constants reported in this work for native triads were somewhat lower than previously reported values (20). Protein concentration was determined according to Hartree (54) using bovine serum albumin as standard.

[3H]Ryanodine Binding—[3H]Ryanodine binding to triads was determined essentially as described (55). The composition of the solution was (in mM): 500 KCl, 0.5 adenosine 5'-[β,γ-imino]triphosphate, 20 MOPS-Tris, pH 7.2, pCa 5. Total binding was routinely measured in the presence of 10 nM [3H]Ryanodine and nonspecific binding in the additional presence of 10 μM ryanodine. The isolated triad preparations displayed, on average, Bₘ₉ values of ryanodine binding of 18 pmol/mg of protein.

Synthesis of GSNO—The synthesis of GSNO was performed as described (56). Briefly, equimolar quantities of GSH and NaNO₂ were incubated in 0.75 mM HCl at room temperature. After 5 min of incubation, the solution was neutralized by addition of solid Trizma (Tris base). Concentrations of GSNO were determined from its absorbance, using an extinction coefficient of 767 x 10⁻³ cm⁻¹ at 334 nm (44). Routinely, yields of GSNO were in the 90–95% range. Synthesis of [35S]GSNO was performed as above, using [35S]GSH at a specific activity of 600 mCi/ mmol, after extraction with ethyl acetate of the DTT present in this reagent.

Ca^{2+} Release Kinetics—Ca^{2+} release kinetics was measured in a SX.18MV fluorescence stopped flow spectrometer from Applied Photophysics Ltd. (Leatherhead, United Kingdom) as described (20, 57). Vesicles were actively loaded with Ca^{2+} before eliciting Ca^{2+} release.

RyR Activation by S-Glutathionylation

For this purpose triads (1 mg/ml) were incubated for 20 min at 25 °C in a solution containing (in mM): 0.05 CaCl₂, 100 KCl, 5 ATP, 5 MgCl₂, 10 phosphocreatine plus 15 units/ml creatine kinase, 20 imidazole-MOPS, pH 7.2. Calcium release was initiated by mixing 1 volume of Ca^{2+}-loaded triads with 10 volumes of releasing solution. Releasing solutions were designed to produce after mixing pCa 5 (unless indicated otherwise), 0.6–1.5 mM free [ATP], and variable free [Mg^{2+}]. The free [Ca^{2+}] (the lowest value attainable after active loading) to 900 μM. The increase in extravesicular [Ca^{2+}] was determined by measuring the fluorescence of the Ca^{2+} indicator Calcium Green-5N (20). The free [Ca^{2+}], free [Mg^{2+}] and free ATP concentration of releasing solutions were calculated with the WinMaxC program using the constants provided in the file cmc1002e.ccm (www.stanford.edu/~epaton/winmaxc2.html).

S-glutathionylation of the CICR process would be expected in response to GSNO-induced endogenous redox modification of the channel protein. Therefore, we assessed the ability of GSNO to modify Ca^{2+} release kinetics and determined whether, in addition to its reported S-nitrosylating activity (42), GSNO also induced S-glutathionylation of RyR channels. We found that GSNO produced similar activation of CICR as thimerosal, and promoted Ca^{2+} release and S-glutathionylation of the RyR channel protein. In addition, we tested the effects on CICR of GSSG and of the NO donor NOR-3, as pure S-glutathionylation or S-nitrosylating agents, respectively. The present results suggest that S-glutathionylation modifies specific cysteine residues and, in doing so, either directly or indirectly modulates channel inhibition by Mg^{2+} whereas S-nitrosylation modifies other cysteines, thereby altering activation by Ca^{2+}. Possible physiological and pathological implications of these findings are discussed.
Molecular Imager FX system (Bio-Rad). Screens were scanned, and the images were quantified using the Quantity One software (Bio-Rad).

**RESULTS**

**Fast Release Kinetic Measurements**—The time course of CICR, measured in the presence of 1.5 mM free ATP, from native and SH-modified triads is illustrated in Fig. 1. In native triads Ca\(^{2+}\) release followed a single exponential time course with an average rate constant (k) value of 11.7 s\(^{-1}\) at <25 μM free [Mg\(^{2+}\)] (Table I). The experimental record obtained in one preparation that displayed a k value of 11.4 s\(^{-1}\) is illustrated in Fig. 1, panel A. In agreement with previous results (20), increasing free [Mg\(^{2+}\)] to 400 μM produced a strong inhibition of CICR (Fig. 1, panel B), decreasing k in this case to 1.6 s\(^{-1}\), with an average value of 1.4 s\(^{-1}\) (Table I). At free [Mg\(^{2+}\)] < 25 μM, the same triad preparation incubated with 1 mM GSSG exhibited a k value similar to that for controls, 12 s\(^{-1}\) (Fig. 1, panel C); the average k value was 12.1 s\(^{-1}\) (Table I). However, in 400 μM free [Mg\(^{2+}\)], GSSG-treated vesicles had a 3-fold higher k value than control, 4.6 s\(^{-1}\) (Fig. 1, panel D), with an average value of 5.0 s\(^{-1}\) (Table I). At <25 μM free [Mg\(^{2+}\)], triads incubated with 100 μM GSNO displayed a higher k value than control vesicles, 17.2 s\(^{-1}\) (Fig. 1, panel E), with an average value of 15.8 s\(^{-1}\) (Table I). In 400 μM free [Mg\(^{2+}\)], these same triads also displayed higher k values than controls, 8.2 s\(^{-1}\) (Fig. 1, panel F), with an average k value of 8.9 s\(^{-1}\) (Table I). Incubation with the NO donor NOR-3 produced at <25 μM free [Mg\(^{2+}\)] a k value of 21 s\(^{-1}\) (Fig. 1, panel G), with an average value of 25.2 s\(^{-1}\), which is 2-fold higher than control (Table I). However, when release was measured in 400 μM free [Mg\(^{2+}\)], triads incubated with NOR-3 displayed a low k value of 1.9 s\(^{-1}\) (Fig. 1, panel H), with an average value of 2.3 s\(^{-1}\) (Table I). These results indicate that, in the incubation conditions used in this work, GSNO and NOR-3, but not GSSG, stimulated CICR when measured at <25 μM free [Mg\(^{2+}\)]. These results suggest that GSNO and NOR-3 promoted through S-nitrosylation an increase in the Ca\(^{2+}\) sensitivity of CICR. In addition, GSSG and GSNO, but not NOR-3, relieved the strong inhibitory effect on k exerted by Mg\(^{2+}\). To test the hypothesis that S-nitrosylation increases the

<FIGURE 1>

**Effect of redox agents on calcium release kinetics**. Triads (1 mg/ml) actively loaded with calcium were mixed (1:10) in a stopped flow fluorescence spectrometer with solutions that contained after mixing 0.6–1.5 mM free [ATP], pCa 5, and different free [Mg\(^{2+}\)]. Kinetic measurements are shown for free [Mg\(^{2+}\)] < 25 μM (left panels) and free [Mg\(^{2+}\)] = 400 μM (right panels). Panels A and B show the time course of calcium release from native triads. Panels C and D illustrate release kinetics from triads incubated with 100 μM GSSG, and panels G and H, from triads incubated with 50 μM NOR-3. Changes in fluorescence of Calcium Green-5N with time were adjusted to single exponential functions, characterized by the release rate constant k. Records represent the average of 6–8 determinations.

<FIGURE 2>

**Effect of redox agents on calcium release rate constants**

Release was measured in the presence of 1.5 mM free ATP, pCa 5, and the indicated free [Mg\(^{2+}\)]. Data are given as mean ± S.D. The number of determinations is in parentheses. For other experimental details, see “Experimental Procedures.”

| Condition | Release rate constant |
|-----------|-----------------------|
| 0.4 mM [Mg\(^{2+}\)] | 11.7 ± 0.8 (4) | 1.4 ± 0.2 (4) |
| 1 mM GSSG | 12.1 ± 0.8 (3) | 5.0 ± 0.6 (3) |
| 100 μM GSNO | 15.8 ± 3.8 (5) | 8.9 ± 3.0 (5) |
| 500 μM GSNO | 26.9 ± 7.3 (2)* | 26.9 ± 7.3 (2)* |
| 50 μM NOR-3 | 25.2 ± 5.0 (3) | 2.3 ± 0.6 (3) |

*The error range of only two determinations is given.
activation of CICR by Ca\(^{2+}\)), we measured CICR kinetics at pCa 6, 5.6, and 5 in triads incubated with NOR-3. A comparison of the \(k\) values obtained in control and NOR-3 incubated triads is given in Fig. 2. Triads incubated with NOR-3 displayed higher \(k\) values than controls at pCa 6, 5.6, and 5, with the highest stimulation at pCa 6 (Fig. 2, hatched columns) indicating that NOR-3 increased significantly the affinity of the release channels for Ca\(^{2+}\).

The inhibitory effects of free [Mg\(^{2+}\)] on release rate constants for native and redox modified triads are compared in Fig. 3. In agreement with previous results (20), increasing free [Mg\(^{2+}\)] inhibited calcium release rate constants in native triads with \(K_{0.5} = 67 \pm 15 \mu M\) (Fig. 3A). Incubation with either GSSG (Fig. 3B) or GSNO (Fig. 3C) resulted in an important reduction of the inhibitory effect of Mg\(^{2+}\). Triads incubated with 1 mM GSSG had a \(K_{0.5}\) value for Mg\(^{2+}\) inhibition of 275 ± 52 \mu M, which increased to >2 \mu M for triads incubated with 5 mM GSSG (Fig. 3B). The corresponding \(K_{0.5}\) value for triads incubated with 100 \mu M GSNO was 400 ± 96 \mu M (Fig. 3C). In contrast, release rate constants in triads incubated with NOR-3 were strongly inhibited by increasing free [Mg\(^{2+}\)], with a \(K_{0.5} = 60 \pm 15 \mu M\) (Fig. 3D). This value is comparable to the \(K_{0.5}\) value of 67 ± 15 \mu M determined in native vesicles. Incubation of triads with GSH did not modify calcium release rate constants or \(K_{0.5}\) for Mg\(^{2+}\) inhibition when compared with control triads (data not shown).

The effects of increasing [GSNO] and [GSSG] on the rate constant values \(k\) of CICR, measured at low or at high free [Mg\(^{2+}\)], are compared in Fig. 4. Near-maximal release rate constant stimulation was obtained at 100 \mu M GSNO in release solution containing <25 \mu M free [Mg\(^{2+}\)] (Fig. 4A, open squares). In contrast, when measured in ~15 \mu M free [Mg\(^{2+}\)], increasing [GSSG] up to 5 mM did not affect significantly release rate constants (Fig. 4B, open circles). In the presence of 0.3 \mu M free [Mg\(^{2+}\)], near-maximal stimulation was obtained at 500 \mu M GSNO (Fig. 4A, solid squares) or 5 mM GSSG (Fig. 4B, solid circles).

Glutathionylation ([\(^{35}\)S Labeling) of RyR Channels Incubated with [\(^{35}\)S]GSNO—We used two independent approaches to determine the incorporation of \(^{35}\)S from [\(^{35}\)S]GSNO into the RyR1 channel protein. First, RyR1 channels were solubilized with CHAPS from triads previously incubated with 4 mM [\(^{35}\)S]GSNO. First, RyR1 channels were solubilized with CHAPS from triads previously incubated with 4 mM [\(^{35}\)S]GSNO. Second, CHAPS-containing sucrose density gradients (see “Experimental Procedures”). As illustrated in Fig. 5, a minor protein peak containing significant \(^{35}\)S radioactivity migrated to the same position as the peak labeled with \(^{35}\)H]-ryanodine. This result suggests that incubation with GSNO produced RyR1 channel glutathionylation, i.e., covalent attachment of the [\(^{35}\)S]GS moiety of GSNO to channel protein SH residues.
To ascertain specific glutathionylation of RyR channels and to quantify this incorporation, it is essential to rule out the presence of other proteins that may contaminate RyR1-enriched gradient fractions. Accordingly, we carried out an additional set of experiments to detect $^{35}$S incorporation into RyR1 channels separated by gel electrophoresis under non-reducing conditions. A Coomassie Blue-stained non-reducing gel of triads incubated with varying concentrations of $[^{35}$S]GSNO, from 12.5 µM to 500 µM, is shown in Fig. 6A. The corresponding phosphorimage is shown in Fig. 6B. Incubation of triads with 12.5–500 µM $[^{35}$S]GSNO at pCa 5 produced increasing $^{35}$S incorporation into RyR1 channels and other triadic proteins (Fig. 6B). Some radioactive material found at the top of the gel stained faintly with Coomassie Blue and may represent a minor component of aggregated proteins. The second band of radioactivity corresponded to the RyR1 monomer. Lower molecular weight bands had apparent molecular weights consistent with triadin and triadin oligomers. These assignments of band identities were confirmed with Western blots using specific antibodies, as illustrated in Fig. 7. Whereas the RyR1-containing band did not shift under non-reducing conditions, the single triadin-containing band observed in reducing gels appeared as multiple higher molecular weight bands in non-reducing gels. However, the RyR1-containing protein band did not contain associated triadin after separation in non-reducing gels (Fig. 7). This migration pattern was not affected by incubation of triads with up to 1 mM GSNO (data not shown). The incorporation of radioactivity from $[^{35}$S]GSNO into RyR1 increased with increasing concentrations of $[^{35}$S]GSNO; half-maximal incorporation was obtained at 157 ± 29 µM (Fig. 6C). The half-time for radioactive labeling of RyR1 channels was 8 min (data not shown). In the following experiments, we incubated triads with GSNO for 20 min as done in kinetic experiments.

To quantify the number of S-glutathionylated SH residues per channel, RyR1-containing bands were sliced from the gels and their $^{35}$S radioactivity was measured in a liquid scintillation counter. Incubation of triads with 500 µM $[^{35}$S]GSNO during 20 min resulted in 180 ± 26 pmol of $[^{35}$S]GS incorporated/mg of total triad protein. We investigated next the effect of changing free $[\text{Ca}^{2+}]$ and of adding Mg$^{2+}$ on the incorporation of $^{35}$S into RyR1 channels. Results are summarized in Table II. We found no differences in the radioactivity incorporated into RyR1 channels at pCa 5 or at pCa 7, either in the absence or in the presence of 0.8 mM free $[\text{Mg}^{2+}]$. Further decreasing free $[\text{Ca}^{2+}]$ to pCa 9 reduced to 67% the incorporation of $^{35}$S. The NO scavenger PTIO reduced the incorporation of $^{35}$S into RyR channels; 50 or 25% of the incorporation observed at pCa 5 was attained in the presence of 100 or 300 µM PTIO, respectively (Table II). In the time frame of our experiments (20 min), 100 or 300 µM PTIO did not produce a significant decrease of the concentration of $[^{35}$S]GSNO. Thus, the observed decrease in $^{35}$S incorporation into RyR1 channels was most likely the result of the NO scavenging action of PTIO.

**RESULTS**

**A. Effect of incubation with varying [GSNO] and [GSSG] on the rate constants of calcium release.** A, CICR from vesicles incubated with varying concentrations of GSNO was determined in releasing solutions containing <25 µM free $[\text{Mg}^{2+}]$ (open squares) or 300 µM free $[\text{Mg}^{2+}]$ (solid squares); B, CICR from vesicles incubated with varying concentrations of GSSG was determined in releasing solutions containing <25 µM free $[\text{Mg}^{2+}]$ (open circles) or 300 µM free $[\text{Mg}^{2+}]$ (solid circles). Rate constants of $\text{Ca}^{2+}$ release were calculated from fluorescence records such as those shown in Fig. 1. Data represent mean ± S.E. from independent determinations in two to six different preparations.

**B. Separation of CHAPS-solubilized RyR1 channels incubated with $[^{35}$S]GSNO on sucrose density gradients.** Triads (1 mg/ml) were incubated with 4 mM $[^{35}$S]GSNO for 10 min. In parallel, an equivalent triad fraction was incubated with non-radioactive GSNO plus 2 µM $[^{3}$H]ryanodine. Both samples were solubilized with CHAPS and further fractionated in separate sucrose density gradients as detailed under “Experimental Procedures.” Twenty-five 1-ml fractions were collected from the gradients, and aliquots of each fraction were assayed for $^{35}$S (closed circles) or $[^{3}$H (open squares) radioactivity and for protein concentration (open circles). Each data point corresponds to the mean ± S.E. of three independent experiments.
Control experiments showed that incubation of triads with 500 μM [35S]GSNO in the presence of 5 mM DTT reduced 35S incorporation into RyR channels by 92% relative to the controls. Likewise, incubation of triads with 500 μM [35S]GSH produced negligible incorporation of 35S into RyR1 channels (4%).

**DISCUSSION**

Recent reports reveal RyR channels as highly sensitive redox-sensing proteins (33, 39, 40, 60). We have shown previously that thimerosal stimulates RyR1 single-channel activity and modifies the pattern of RyR1 activation by Ca^{2+}, shifting to the left the Ca^{2+} activation curve of channel open probability (23) even in purified RyR channels (61). We have also reported that incubation of triad vesicles from skeletal muscle with 250 μM thimerosal enhances CICR kinetics when measured at low free [Mg^{2+}] (25 μM) and decreases the inhibition of CICR exerted by 1 mM free [Mg^{2+}] (20). Furthermore, although incubation with 500 μM thimerosal does not enhance CICR at low free [Mg^{2+}], it completely abolishes CICR inhibition by 1 mM free [Mg^{2+}] (20). These combined results suggest that thimerosal reacts with different SH residues of the channel protein to regulate either channel activation by Ca^{2+} or inhibition by Mg^{2+}.

Several endogenous redox molecules, including H_2O_2, GSH, GSSG, GSNO, and CysNO, modify RyR channel activity in vitro (21, 36, 37, 39, 41, 42). Consequently, we determined whether the intracellular redox agents GSNO and GSSG stimulate CICR kinetics mediated by skeletal RyR1 channels. We also assessed the effects of NOR-3, an NO donor that, in analogy with other NO donors (38), presumably promotes S-nitrosylation of RyR1 channels. In addition, we determined whether RyR1 channels undergo S-glutathionylation following incubation with [35S]GSNO. Results obtained in kinetic experiments will be discussed first.

**Differential Effects of GSNO, GSSG, and NOR-3 on CICR Kinetics**—Our studies show that GSSG, an S-glutathionylating agent, and NOR-3, which S-nitrosylates protein SH residues, have different effects on CICR kinetics. We also found that GSNO produced effects similar to the combined effects of NOR-3 and GSSG, and modified CICR kinetics in a similar fashion as thimerosal (20), suggesting that GSNO and thimerosal may modify the same free SH residues. Both NOR-3 and GSNO, but not GSSG, stimulated CICR kinetics when measured in <25 μM free [Mg^{2+}], as reflected in a 2-fold increase of Ca^{2+} release rate constants k (see Table I). This stimulation

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**Fig. 6.** 35S labeling of triad proteins incubated with [35S]GSNO. A, non-reducing SDS containing polyacrylamide gradient gel (3.5–8%) stained with Coomassie Blue. Lanes 1–6 contained 30 μg of triad protein incubated with varying concentrations of [35S]GSNO as follows: lane 1, 12.5 μM; lane 2, 25 μM; lane 3, 50 μM; lane 4, 100 μM; lane 5, 250 μM; lane 6, 500 μM. See "Experimental Procedures" for further details. B, phosphorimaged scan of the same gel shown in A, C, left axis, the radioactivity associated to RyR1 bands was determined from densitometric scans such as those illustrated in B. Right axis, the number of SH residues per RyR1 monomer labeled with 35S was calculated as detailed in the text (see "Discussion").
suggestions that NOR-3 and GSNO S-nitrosylate SH residues that either directly or indirectly participate in RyR1 channel activation by Ca\(^{2+}\). The marked stimulation of release rate constants observed at pCa 6 in triads incubated with NOR-3 support this proposal. Additionally, both GSSG and GSNO, but not NOR-3, reduced the strong inhibitory effect of [Mg\(^{2+}\)] on CICR, increasing significantly the free [Mg\(^{2+}\)] needed to inhibit \(k\) values by half. These findings suggest that, under our experimental conditions, GSSG and GSNO both S-glutathionylate SH residues and alter the low affinity Mg\(^{2+}\) inhibitory site. Whereas, in addition to its S-glutathionylating properties, GSSG produces changes in redox status through changes in the GSSG/GSH ratio (40, 62), our results clearly show that GSNO induces cysteine S-glutathionylation. Previous reports indicate that both skeletal and cardiac RyR channels are endogenously S-nitrosylated (33, 63). Skeletal RyR1 channels can also be exogenously S-nitrosylated with GSNO, which enhances \([^{[3]}\text{H}]\text{ryanodine binding in a dose-dependent manner both at atmospheric and at reduced O}_2\) concentration (42). GSNO decreases RyR1 free sulphydryl content, partly through S-nitrosylation of cysteines other than Cys-3635 and partly through other oxidation reactions (42).

S-Glutathionylation of Skeletal RyR Channels—Several recent reports have described covalent modification of protein SH residues by GSSG or GSNO through the formation of S-glutathionyl derivatives. The growing list of these proteins include glyceraldehyde-3-phosphate dehydrogenase, e-Jun, SERCA1, creatine kinase, H-Ras, protein phosphatase 2A, NFXB, thioredoxin, tyrosine hydroxylase, mitochondrial complex I, and actin (47–49, 64–72). Cells generate both GSSG and GSNO from GSH, which is present in m\(\text{M}\) concentrations in the cytoplasm (62). Oxidation of GSH generates GSSG, whereas GSNO is formed by reaction of GSH with NO (73, 74). It has been proposed that GSNO, which has a longer half-life in cells than NO (75), serves as a storage molecule of likely physiological relevance because it can either act as an S-nitrosylating or an S-glutathionylating agent (46, 76). However, except from our previous short report (37), S-glutathionylation of RyR1 channels has not been reported.

The present results, which show that incubation of skeletal triads with [\(^{[3]}\text{S}\)]GSNO resulted in \(^{35}\text{S}\) incorporation into RyR1 channels, constitute a direct demonstration of S-glutathionylation of RyR1 channel SH residues. To our knowledge, this is the first report to describe S-glutathionylation of an ion channel.

Incubation with 500 \(\mu\text{M}\) GSNO resulted in near-maximal S-glutathionylation, with values of 180 pmol/mg of triad protein (see “Results”). To convert this number into incorporation per RyR1 channel monomer, we considered the average maximal density of \([^{[3]}\text{H}]\text{ryanodine binding sites of our triad preparations, 18 pmol/mg of protein. On the assumption that each RyR homotetramer binds only one molecule of \([^{[3]}\text{H}]\text{ryanodine (77, 78), this B}_\text{max}^\ast\) value yields 18 pmol of RyR1 homotetramer/mg of protein. According to the 180 pmol of SH residues S-glutathionylated/mg of protein obtained following incubation with 500 \(\mu\text{M}\) GSNO would correspond to 10 SH residues S-glutathionylated per tetramer, or 2.5 residues/monomer.

Near-maximal effects on release rate constants measured in 0.3 mM free [Mg\(^{2+}\)] were obtained with 500 \(\mu\text{M}\) GSNO (Fig. 4B). This finding suggests that S-glutathionylation of SH residues per RyR1 monomer (Fig. 6C) is enough to decrease Mg\(^{2+}\) inhibition of CICR. On the other hand, near-maximal effects on CICR kinetics measured in <25 \(\mu\text{M}\) free [Mg\(^{2+}\)] were already observed at 100 \(\mu\text{M}\) GSNO (Fig. 4A). Whereas we did not determine the number of SH residues S-nitrosylated by GSNO, we estimate from published data (42) that S-nitrosylation of no more than 2 SH residues/RyR1 monomer is responsible for the enhancement of channel activity by Ca\(^{2+}\). The present results, however, do not rule out the possibility that redox modifications of RyR1 channel associated proteins, such as triadin, may contribute to the effects of GSNO on CICR kinetics. Our previous findings (61) indicate that redox modification of purified RyR channels with thimerosal makes single channels more responsive to activation by micromolar [Ca\(^{2+}\)] and less susceptible to inhibition by 0.5 mM [Ca\(^{2+}\)], which at this high concentration occupies the Mg\(^{2+}\) inhibitory site. On this basis, we propose that the stimulation of CICR kinetics by GSNO reported in this work is caused by redox modifications of the RyR1 channel itself.

The same extent of S-glutathionylation was obtained when triads were incubated at pCa7 or at pCa5; addition of 0.8 mM free [Mg\(^{2+}\)] did not affect \(^{35}\text{S}\) labeling. However, S-glutathionylation decreased Mg\(^{2+}\) inhibition of CICR. These results suggest that Mg\(^{2+}\) binding to its low affinity inhibitory site does not interfere with the ability of GSNO to react with the SH residues involved in controlling the affinity of the site for Mg\(^{2+}\). In contrast, covalent attachment of the glutathionyl group to these SH residues would hinder the accessibility of Mg\(^{2+}\) to this site, either because these SH residues are directly present in the site or because, as a consequence of S-glutathionylation, they induce a channel conformational change that hinders Mg\(^{2+}\) access.
The above findings suggest that under resting conditions, i.e. pCa 7 plus 0.8 mM [Mg2+]2, skeletal muscle RyR1 channels can readily undergo S-glutathionylation incorporation. Only a substantial decrease in free [Ca2+]p to pCa 9 produced a significant decrease of label incorporation. Therefore, we propose that very low free [Ca2+]p hinders the accessibility of hyperreactive SH groups to GSNO presumably by inducing RyR1 channel conformational changes. Similar effects of low free [Ca2+]p have been reported for other RyR channel redox-active modulators (22, 40, 79).

Comparison between S-Glutathionylation and S-Nitrosylation of RyR Channels—Previous reports indicate that skeletal muscle RyR1 channels are activated by NO-induced S-nitrosylation of a single SH residue at reduced but not at atmospheric O2 concentrations (33). The S-nitrosylation reaction was specific for RyR1 channels among SR proteins, and its effects depended on the presence of calmodulin. Moreover, NO and NO-donors S-nitrosylate Cys3635 (38, 42) whereas GSNO-induced S-nitrosylation, is calmodulin-independent, takes place either at reduced or at atmospheric O2 concentrations and does not involve Cys3635 (42). Interestingly, incubation with GSNO removes more free SH residues from skeletal muscle RyR1 channels than the residues actually S-nitrosylated (42), indicating that GSNO induces other SH modifications. Here, we report as a novel finding S-glutathionylation by GSNO of RyR1 channel SH residues as well as the functional consequences of GSNO-induced redox modification. If GSNO also induced S-nitrosylation and S-glutathionylation of cardiac RyR channels, this double feature would explain why GSNO removes more free SH residues from purified cardiac RyR channels than those actually S-nitrosylated (63).

Physiological Implications—Redox modulation of cellular signal transduction processes has received increased attention during the last few years (46, 80–83). The intracellular redox state is mainly controlled by the GSH/GSSG ratio (62). Nevertheless, redox species, and hence cellular redox state, may vary in response to physiological stimuli. Currently, S-nitrosothiols are regarded as intracellular nitric oxide transporters/donors and S-nitrosylating/thionylating agents (82, 84). In particular, GSNO-donors are specific for RyR1 channels among SR proteins, and its effects concerning that GSNO induces other SH modifications. Here, we propose that very specific and low free SH residues from skeletal muscle RyR1 channel SH residues as well as the functional consequences of GSNO-induced redox modification. If GSNO also induced S-nitrosylation and S-glutathionylation of cardiac RyR channels, this double feature would explain why GSNO removes more free SH residues from purified cardiac RyR channels than those actually S-nitrosylated (63).

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