Glutathione Modulates Ryanodine Receptor from Skeletal Muscle Sarcoplasmic Reticulum

EVIDENCE FOR REDOX REGULATION OF THE Ca\textsuperscript{2+} RELEASE MECHANISM\textsuperscript{*}

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In this report, we demonstrate the ability of the cellular thiol glutathione to modulate the ryanodine receptor from skeletal muscle sarcoplasmic reticulum. Reduced glutathione (GSH) inhibited Ca\textsuperscript{2+}-stimulated \textsuperscript{[3H]}ryanodine binding to the sarcoplasmic reticulum and inhibited the single-channel gating activity of the reconstituted Ca\textsuperscript{2+} release channel. The effects of GSH on both the \textsuperscript{[3H]}ryanodine binding and single-channel measurements were dose-dependent, exhibiting an IC\textsubscript{50} of ~2.4 mM in binding experiments. Scatchard analysis demonstrated that GSH decreased the binding affinity of ryanodine for its receptor (increased K\textsubscript{D}) and lowered the maximal binding occupancy (B\textsubscript{max}). In addition, GSH did not modify the Ca\textsuperscript{2+} dependence of \textsuperscript{[3H]}ryanodine binding. In single-channel experiments, GSH (5–10 mM), added to the cis side of the bilayer lipid membrane, lowered the open probability (P\textsubscript{o}) of a Ca\textsuperscript{2+} (50 \mu M)-stimulated Ca\textsuperscript{2+} channel without modifying the single-channel conductance. Subsequent perfusion of the cis chamber with an identical buffer, containing 50 \mu M Ca\textsuperscript{2+} without GSH, re-established Ca\textsuperscript{2+}-stimulated channel gating. GSH did not inhibit channel activity when added to the trans side of the bilayer lipid membrane. Similar to GSH, the thiol-reducing agents dithiothreitol and \beta-mercaptoethanol also inhibited high affinity \textsuperscript{[3H]}ryanodine binding to sarcoplasmic reticulum membranes. In contrast to GSH, glutathione disulfide (GSSG) was a potent stimulator of high affinity \textsuperscript{[3H]}ryanodine binding and it also stimulated the activity of the reconstituted single Ca\textsuperscript{2+} release channel. These results provide direct evidence that glutathione interacts with reactive thiols associated with the Ca\textsuperscript{2+} release channel/ryanodine receptor complex, which are located on the cytoplasmic face of the SR, and support previous observations (Liu, G., Abramson, J. J., Zable, A. C., and Pessah, I. N. (1994) Mol. Pharmacol. 45, 189–200) that reactive thiols may be involved in the gating of the Ca\textsuperscript{2+} release channel.

In muscle cells, cytosolic Ca\textsuperscript{2+} levels are regulated by the intramuscular organelle, the sarcoplasmic reticulum (SR)\textsuperscript{1} (1–3). Following the arrival of an action potential at the surface membrane and subsequent depolarization of the transverse tubule, the SR releases its luminal store of Ca\textsuperscript{2+} through the Ca\textsuperscript{2+} release channel (CRC)/ryanodine receptor (RyR), thus triggering the contraction process. The interaction between the action potential at the transverse tubule and the release of Ca\textsuperscript{2+} from the SR has been termed excitation-contraction coupling (ECC). In skeletal muscle, the molecular mechanism underlying ECC has remained unclear. Following excitation, resting Ca\textsuperscript{2+} levels are reestablished through the active transport of the Ca\textsuperscript{2+} back into the lumen of the SR by the Ca\textsuperscript{2+},Mg\textsuperscript{2+}-ATPase.

A number of thiol reagents act as potent stimulators of the SR Ca\textsuperscript{2+} release channel. These compounds include heavy metals (4, 5), Cu\textsuperscript{2+}/cysteine (6), reactive disulfides (7), phthalocyanine dyes (8), anthraquinones (9), porphyrins (10), thimerosal (11), and the reactive oxygen species, H\textsubscript{2}O\textsubscript{2} (12, 13). Recently Lui \textit{et al.} (14, 15) have described the presence of a discrete class of highly reactive thiols associated with the SR CRCs and other junctionally related proteins, which were labeled by the fluorogenic coumaryl maleimide, CPM. During activation of Ca\textsuperscript{2+} release by a large class of non-thiol channel stimulators, a high molecular weight complex was formed. The addition of channel inhibitors resulted in the reduction of the disulfides formed, the dissociation of key SR proteins and the exposure of hyperreactive thiols (14, 15). Based on the results derived from fluorescence assays, ion flux measurements, single-channel experiments, and SDS-gel electrophoresis, the authors concluded that thiol oxidation-reduction chemistry plays a critical role in the channel gating of the SR CRC-RyR complex.

Endogenous and exogenous redox agents have been observed to have profound effects on a wide range of ion channel systems. In addition to the SR Ca\textsuperscript{2+} release channel, ion channels as varied as excitatory amino acid receptors, inositol 1,4,5-triphosphate (IP\textsubscript{3})\textsubscript{b}-gated Ca\textsuperscript{2+} release channels, and even certain K\textsuperscript{+} channels have been demonstrated to be modulated by redox agents. For example, the N-methyl-D-aspartate-sensitive excitatory amino acid receptor has been shown to be modulated by both thiol oxidants and reductants (16).

Glutathione is one of the most abundant low molecular weight peptides in eukaryotic cells and the most prevalent intracellular thiol. Depending on the cell type, glutathione levels have been estimated to range from 1 to 10 mM (17, 18). In the cell, glutathione acts as both a reducing agent and an antioxidant. Among its many physiological roles, glutathione

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\textsuperscript{1} The abbreviations used are: SR, sarcoplasmic reticulum; CRC, Ca\textsuperscript{2+} release channels; RyR, ryanodine receptor; RyR\textsubscript{1}, ryanodine receptor (skeletal isoform); ECC, excitation-contraction coupling; CPM, \textsuperscript{7-dithioflumin-3-(4'-maleimidylphenyl)-4-methylcoumarin; DT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); BME, \beta-mercaptoethanol; CsMS, cesium methanesulfonate or CsCH\textsubscript{3}SO\textsubscript{3}; RyR\textsubscript{2}, ryanodine receptor (cardiac isoform); NMDA, N-methyl-D-aspartate; IP\textsubscript{3}, inositol 1,4,5-triphosphate.
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plays an important role in the protein folding process and serves to protect intracellular constituents from oxidation by scavenging reactive oxygen species produced during normal cell metabolism (19). In the cell, glutathione is present in both the reduced (GSH) and oxidized (GSSG or glutathione disulfide) form.

Glutathione disulfide is capable of undergoing thiol-disulfide exchange with reactive protein thiol residues to form a mixed disulfide complex, or it can completely oxidize endogenous sulfhydryls to form disulfides (20). In this capacity, GSSG has been demonstrated to modulate a number of ion channel systems. Gilbert et al. (21) observed that GSSG inhibited NMDA and glycine evoked [Ca\(^{2+}\)]\textsubscript{i}, increases in embryonic rat neurons. This demonstrated that GSSG interacted with the NMDA receptor-associated redox site. Furthermore, Rendard-Rockey et al. (22) has shown that IP\(_3\) receptors are sensitized to IP\(_3\), in the presence of GSSG. These reports, along with others, demonstrate the ability of GSSG alone to regulate the function of redox-sensitive protein complexes.

The present investigation describes the interaction between glutathione, both the reduced and oxidized forms, as well as other thiol-reducing agents, and key reactive thiol groups associated with the SR Ca\(^{2+}\) release mechanism. It is demonstrated that the thiol-reducing agents dithiobrityl (DTT), GSH, and \(\beta\)-mercaptoethanol (BME) inhibited, whereas GSSG stimulated SR CRC activity. These results were observed in \(^{3}H\)ryanodine binding assays as well as in single-channel experiments. The evidence in this report demonstrates that ligand-gated channel activity associated with the RyR complex in skeletal muscle is subject to both inhibition via thiol reduction and activation by thiol oxidation. Furthermore, at physiologically relevant levels, GSH and GSSG modulate SR Ca\(^{2+}\) release channel and \(^{3}H\)ryanodine binding activity.

**EXPERIMENTAL PROCEDURES**

**Preparation of SR Vesicles**—For all studies, crude sarcoplasmic reticulum vesicles were prepared from rabbit hind leg and back white skeletal muscle according to the method of MacLennan (23). The protein concentration was determined by absorption spectroscopy (24). Prior to use, all SR preparations were suspended at 15–25 mg/ml in decane, were formed across a 150–µm hole drilled in a polystyrene cup separating two chambers of 0.7 ml each. The cis chamber contained 500 mM CsCl (or CsMS), 100 mM HEPES, pH 7.0, while the trans side contained 100 mM CsCl (or CsMS), 20 mM HEPES pH 7.0. SR vesicles, suspended in 0.3 mM sucrose, were added to the cis chamber at a final concentration of 5–20 µg/ml. Following the fusion of a single vesicle, 150 µM EGTA, pH 7.0, was added to the cis chamber to stop further fusions. The cis chamber was then perfused with an identical buffer containing no added Ca\(^{2+}\) or EGTA. Channel activity was then measured at a holding potential of +25 mV with respect to the trans (ground) side. A Warner Instruments Bilayer Clamp Amplifier (model BC-525A) was used to amplify picomperme currents. The data were processed with an Instratech Digital Data Recorder (model VR-10), stored unfiltered on a VCR tape and subsequently analyzed for channel activity. For analysis, the data were passed through a Krohn-Hite low pass filter (model 3202) at 1.5 kHz, digitized with a Scientific Solutions analog to digital converter and analyzed using the pCLAMP software package (version 5.5, Axon Instruments, Burlington, CA). See figure captions for specific experimental conditions.

**Materials**—All reagents were analytical grade. HEPES was obtained from Research Organics (Cincinnati, OH). \(^{3}H\)Ryanodine was purchased from DuPont NEN, and ryanodine-dehydroxyanodine was purchased from Agrisystems Int. (Windy Gap, PA). All other chemicals were obtained from Sigma.

**RESULTS**

The measurement of high affinity ryanodine binding to sarcoplasmic reticulum vesicles has been demonstrated to be an effective probe of Ca\(^{2+}\) release channel activity (25). High affinity ryanodine binding to SR membranes increases under conditions in which Ca\(^{2+}\) release channels are activated, whereas channel inhibitors diminish binding. With few exceptions, Ca\(^{2+}\) release channel agonists stimulate ryanodine binding to RyRs, while compounds that inhibit channel activity decrease binding. A major exception is the effect of Ag\(^{+}\), which acts as a potent SR Ca\(^{2+}\)-releasing agent at micromolar concentrations (4, 5), to increase ryanodine binding by rapidly displacing bound ryanodine from its receptor (25).

The effects of thiol-reducing agents on high affinity ryanodine binding to SR membranes were examined in Fig. 1. In this figure, Ca\(^{2+}\) (50 µM)-stimulated ryanodine binding was inhibited by increasing concentrations of reducing agent (DTT, GSH, or BME). For all three reducing agents, binding was decreased...
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High affinity ryanodine binding experiments were performed as described in Fig. 1. Results are the mean ± standard deviation of at least three experiments performed in duplicate on separate days. Hill analyses were applied as described under “Experimental Procedures.”

| Reducing agent | IC$_{50}$ (mM) | Apparent Hill coefficient (n) |
|----------------|----------------|-------------------------------|
| DTT           | 0.1 ± 0.1      | 1.7 ± 0.3                    |
| BME           | 0.4 ± 0.1      | 0.9 ± 0.2                    |
| GSH           | 2.4 ± 0.4      | 2.1 ± 0.7                    |

| Oxidizing agent | EC$_{50}$ (mM) | Apparent Hill coefficient (n) |
|-----------------|----------------|-------------------------------|
| GSSG            | 0.09 ± 0.05    | 0.8 ± 0.1                    |

The effects of channel inhibitors and activators on GSSG-stimulated ryanodine binding are examined in Fig. 3. In Fig. 3A, ryanodine binding was measured with and without GSSG (2 mM) in the presence of the known channel inhibitors ruthenium red and Mg$^{2+}$. Ruthenium red (20 μM) completely inhibited ryanodine binding in both the presence and absence of GSSG, whereas 1 mM Mg$^{2+}$ decreased binding for control and GSSG treated vesicles 75% and 60%, respectively. The observation that oxidation stimulated channel activity is inhibited by known channel antagonists has been similarly observed in previous reports (6–11).

Ryanodine binding was measured in the presence of known channel activators, with or without GSSG, in Fig. 3B. In the absence of GSSG, both caffeine (5 mM) and cAMP (1 mM) stimulated binding significantly, when compared to control conditions. Caffeine stimulated ryanodine binding by 37%, while the presence of cAMP increased binding by 95%. GSSG was shown to increase ryanodine binding under all three activating conditions. However, the percent increase of binding due to the agonists was attenuated in the presence of GSSG. Furthermore, comparison of experiments carried out in the absence and presence of GSSG, under activating conditions, demonstrates that GSSG increased binding is diminished as the level of control binding increased.

Lineweaver-Burk analysis was performed to determine whether or not GSSG stimulation and GSH inhibition of ryanodine binding are examined in Fig. 3. The effects of known channel inhibitors are compared at various GSH concentrations. The data were fitted to a modified Hill equation, as described under “Experimental Procedures.” The results of the Hill analyses are summarized in Table I. As shown in this table, the inhibition of alkaloid binding due to either DTT or GSH exhibited positive cooperativity ($n > 1$). The logit slope for BME inhibition of ryanodine binding was near unity. Furthermore, as observed in Fig. 2, both in the presence of inhibitors (Fig. 2A) or channel activators (Fig. 2B), reduced glutathione inhibited high affinity [H]ryanodine binding. Similar results were obtained with DTT or BME (data not shown).

GSH levels as a function of time were measured using absorbance spectroscopy to verify that significant oxidation of GSH was not occurring during the course of the ryanodine binding assays. In these measurements, GSH was suspended at 0.1, 1.0, 5.0, and 10.0 mM in standard binding buffer and incubated at 34°C in either the absence or presence of SR (0.5 mg/ml). At each time point, the GSH was diluted to 50 μM, based on the original concentration, into a similar buffer containing 1 mM DTNB, pH 7.1. The absorbance increase due to the interaction between DTNB and free thiol groups on GSH or thiols associated with the SR was monitored at 412 nm (data not shown). To calibrate the DTNB-SH response, measurement of absorbance as a function of either freshly prepared GSH or BME were performed, in the concentration range between 10 and 80 μM, where the response was linear. From these dose-response (calibration) curves, the extinction coefficient was determined to be in the range of 13,200–13,800 M$^{-1}$ cm$^{-1}$. In the absence of SR vesicles, following a 4.5-h incubation at 34°C, the [GSH] dropped significantly from an original concentration of 100 μM to 35 μM, a decrease of 65%. At 100 μM, two-thirds of the original GSH was oxidized to GSSG during the incubation period. At GSH levels of 1.0, 5.0, and 10.0 mM, the percentage of GSH oxidized after incubation at 34°C for 4.5 h was approximately 20%, 13%, and 9%, respectively. However, in the presence of SR, an insignificant decrease in GSH levels was observed at all concentrations measured. Following a 4.5-h incubation at 34°C, GSH levels were never less than 95% of the original starting concentration of GSH (0.1, 1.0, 5.0, and 10.0 mM). No significant decrease in GSH levels was observed during the time course of the ryanodine binding assays presented in this report.

In contrast to the reducing agents, the oxidized form of glutathione was a potent stimulator of ryanodine binding. As shown in Fig. 1, glutathione disulfide increased binding from 2.1 to 3.3 pmol/mg, roughly 60%. Hill analysis yielded an EC$_{50}$ of −90 μM and an apparent Hill coefficient (n) of −0.8 (see Table I).

The effects of channel inhibitors and activators on GSSG-stimulated ryanodine binding are examined in Fig. 3. In Fig. 3A, ryanodine binding was measured with and without GSSG (2 mM) in the presence of the known channel inhibitors ruthenium red and Mg$^{2+}$. Ruthenium red (20 μM) completely inhibited ryanodine binding in both the presence and absence of GSSG, whereas 1 mM Mg$^{2+}$ decreased binding for control and GSSG treated vesicles 75% and 60%, respectively. The observation that oxidation stimulated channel activity is inhibited by known channel antagonists has been similarly observed in previous reports (6–11).

Ryanodine binding was measured in the presence of known channel activators, with or without GSSG, in Fig. 3B. In the absence of GSSG, both caffeine (5 mM) and cAMP (1 mM) stimulated binding significantly, when compared to control conditions. Caffeine stimulated ryanodine binding by 37%, while the presence of cAMP increased binding by 95%. GSSG was shown to increase ryanodine binding under all three activating conditions. However, the percent increase of binding due to the agonists was attenuated in the presence of GSSG. Furthermore, comparison of experiments carried out in the absence and presence of GSSG, under activating conditions, demonstrates that GSSG increased binding is diminished as the level of control binding increased.

Lineweaver-Burk analysis was performed to determine whether or not GSSG stimulation and GSH inhibition of ryanodine binding were due to an interaction with the same critical thiols/redox sites associated with the RyR (Fig. 4). GSSG dose-response curves were generated in the presence of various amounts of GSH. The resultant data were plotted as 1/ryanodine bound) as a function of 1/[GSSG]. The linear regression of the resulting Lineweaver-Burk plot, for the three concentrations of GSH, intersect at a y intercept of 0.26 ± 0.04 mg/pmol.
This suggests that the mechanisms of GSSG oxidation and GSH reduction interact via a competitive interaction. It is likely that GSH and GSSG compete for the same reactive thiol sites, reducing endogenous disulfides or oxidizing free -SH groups, depending on the thiol status of the channel. From the y intercept, the theoretical \( B_{\text{max}} \) for oxidation-stimulated binding was determined to be 3.8 ± 0.6 pmol/mg.

To better describe their effects on the RyR1, Scatchard analyses were performed in the presence of the reducing agents (GSH, BME, and DTT), in Fig. 5 (A and B), and oxidized glutathione (GSSG), in Fig. 6 (A and B). In Fig. 5A, ryanodine saturation binding experiments were performed in the absence or presence of reducing agents. The raw data were plotted as bound/free versus bound ryanodine (Fig. 5B). Linear regression of the resultant Scatchard plots, in Fig. 5B, show that the reducing agents decreased the binding affinity of the alkaloid for its receptor, increasing the \( K_d \) by 2–5-fold. DTT (2 mM), demonstrated the most potent effect on the binding affinity, followed by BME, then GSH. In addition to decreasing the binding affinity, the maximum number of binding sites (or \( B_{\text{max}} \)) decreased slightly for each of the reducing agents, from 8.7 to 8.0 (for GSH), 7.7 (for BME), or 7.2 (for DTT) pmol/mg. The decrease in high affinity ryanodine binding induced by the addition of reducing agents (Fig. 1) is caused by an increase in the \( K_d \) and a slight decrease in the \( B_{\text{max}} \) for ryanodine binding.

Whereas the reducing agents increased the \( K_d \) and decreased the \( B_{\text{max}} \), GSSG had an opposite effect. In Fig. 6A, ryanodine saturation binding curves were generated in the absence or presence of 2 mM GSSG. Standard assay conditions were the same as described in Fig. 1, with 15 nM \[^{3}H\]ryanodine and 50 \( \mu \)M Ca\(^{2+}\) at pH 7.1. In A, binding was increased from 3.0 (untreated) to 5.0 pmol/mg when incubated with 2 mM GSSG. Channel inhibitors, 20 \( \mu \)M ruthenium red or 1 mM Mg\(^{2+}\), decreased binding in the presence (to <0.1 or 2.0 pmol/mg, respectively) or absence (to <0.1 or 0.8 pmol/mg, respectively) of 2 mM GSSG. In B, 1 mM cAMP or 5 mM caffeine increased binding, compared to control values, in the presence (5.5 or 6.6 pmol/mg, respectively) and absence (4.1 or 5.9 pmol/mg, respectively) of GSSG. Data are the averages of experiments performed in duplicate. The fractional error for each measurement was less than 5%.
perform in duplicate. Yielding similar results. The data are the averages of experiments performed three times using two separate SR preparations. Alteration of the apparent sensitivity of receptor to Ca$^{2+}$ inhibition and GSSG stimulation of ryanodine binding does not diminish by GSH, the receptor sensitivity to Ca$^{2+}$ was retained. Hill analysis of the data in Fig. 7 yielded apparent Hill coefficients ($n$) of 1.3 ± 0.3 (control), 1.5 ± 0.3 (+2 mM GSSG), and 1.8 ± 0.2 (+5 mM GSH). The EC$_{50}$ values for Ca$^{2+}$ activation were calculated to be: 1.54 ± 0.43 μM (control), 1.65 ± 0.31 μM (+2 mM GSSG), and 1.96 ± 0.24 μM (+5 mM GSH). Similar results were observed in experiments performed in the presence of 1 mM Mg$^{2+}$ (free). The observation that GSH inhibition and GSSG stimulation of ryanodine binding does not alter the apparent sensitivity of the receptor to Ca$^{2+}$ activation suggests that neither the reduced or oxidized form of glutathione bind Ca$^{2+}$. Direct measurements of free Ca$^{2+}$ levels, upon addition of GSH or GSSG, using a Ca$^{2+}$-selective electrode (World Precision Instruments, Inc.) verified that at concentrations as high as 20 mM glutathione only a negligible amount of Ca$^{2+}$ was bound (less than 2%; data not shown). These measurements were performed in standard binding buffer in the absence of SR vesicles.

To better characterize the interaction between GSH, GSSG, and the SR, single-channel experiments were performed on SR Ca$^{2+}$-release channels reconstituted into an artificial lipid membrane. This experimental procedure allows the visualization of channel current fluctuations across the SR membrane and facilitates the direct observation of modifications to channel open probability ($P_o$) and conductance. Fig. 8 illustrates two Ca$^{2+}$ channels reconstituted into a planar artificial membrane. Using Cs$^+$ as the carrier current in a 5 to 1 cis-trans gradient, channel gating was recorded as a function of time at a holding potential of +25 mV with respect to the trans side of the channel. In trace i, channel open probability was very low following channel fusion ($P_o = 0.05$ for $n = 2$ channels). In trace ii, 50 μM Ca$^{2+}$ was added to the cis chamber and channel gating was stimulated, $P_o = 0.75$. Addition of 2 mM GSH to the cis chamber lowered the channel $P_o$ to 0.15, as shown in trace iii. Subsequent additions of 4 mM GSH aliquots to the cis chamber further reduced the channel open probabilities to 0.10 and <0.05, respectively. This experiment verifies the effects of GSH observed in Fig. 1. Furthermore, GSH inhibits Ca$^{2+}$-release channel activity by decreasing channel open probability without affecting unitary channel conductance (data not shown).

Additional single-channel experiments were performed to determine the sidedness of GSH inhibition, as shown in Fig. 9. In the first trace of this figure (i), channel gating was recorded in 5:1 cesium methanesulfonate gradient. The single-channel open probability was initially very low, $P_o = 0.01$. Addition of 50 μM cis Ca$^{2+}$ increased channel gating, $P_o = 0.45$, in trace ii. As shown in trace iii, addition of 5 mM GSH to the trans side was observed to have no effect on channel open probability. However, when 5 mM GSH was added to the cis side (trace iv), channel gating activity was lowered ($P_o = 0.30$). Raising the cis GSH concentration to 10 mM, in trace v, decreased the $P_o$ to

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**FIG. 4. GSSG and GSH interact competitively with the same thiol redox site.** Lineweaver-Burk analysis was performed on data obtained from GSSG dose-response curves for equilibrium [$^3$H]ryanodine binding at various concentrations of GSH. The standard assay conditions were the same as in Fig. 1, in the presence of 50 μM Ca$^{2+}$, 7.5 nM [$^3$H]ryanodine and various GSH concentrations ([GSH] = 3 mM (circles), 5 mM (squares), or 10 mM (triangles)) at pH 7.1. The experiment was performed three times using two separate SR preparations yielding similar results. The data are the averages of experiments performed in duplicate.

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**FIG. 5. Scatchard analysis of [$^3$H]ryanodine binding in the presence of thiol-reducing agents.** Equilibrium binding experiments were carried out in the presence of 0.5 nM [$^3$H]ryanodine and various concentrations of unlabeled ryanodine (0.5–32 nM) at 50 μM Ca$^{2+}$ (pH 7.1). The experiments were performed under control conditions (circles) or with the addition of 5 mM GSH (squares), 5 mM BME (triangles), or 2 mM DTT (inverted triangles). The binding data are shown in A, and the corresponding Scatchard plots are shown in B. In B, the data were fit to a one-site model using linear regression. The results are summarized in Table II. Data are the average of representative experiments performed in duplicate. The experiments were repeated three times with similar results.
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FIG. 6. Scatchard analysis of GSSG-stimulated [3H]ryanodine binding. Equilibrium binding experiments were carried out as described in Fig. 5 under control conditions (circles) or in the presence of 0.5 mM Ca\(^{2+}\) (squares) or 2 mM GSSG (triangles). The binding data are shown in A, and the corresponding Scatchard plots are shown in B. In B, the data were analyzed as described in Fig. 5 and the results are presented in Table II. Data are the average of representative experiments performed in duplicate. Experiments were repeated three times with similar results.

Table II
Thiol reducing agents and GSSG modulate ryanodine binding kinetics of the skeletal muscle SR ryanodine receptor

| Conditions | \(K_d\) | \(B_{\text{max}}\) |
|------------|--------|-----------------|
| Control (50 \(\mu\)M Ca\(^{2+}\)) | 11.5 ± 0.9 | 8.7 ± 0.7 |
| +5 mM GSH | 27.1 ± 4.2 | 8.0 ± 1.3 |
| +5 mM BME | 42.5 ± 8.1 | 7.8 ± 1.5 |
| +2 mM DTT | 54.9 ± 11.4 | 7.2 ± 1.5 |
| +0.5 mM GSSG | 9.7 ± 0.7 | 9.5 ± 0.7 |
| +2 mM GSSG | 6.2 ± 0.2 | 10.6 ± 0.4 |

Thus GSH appeared to inhibit channel gating from the cytoplasmic, but not the luminal side of channel. While the GSH inhibitory site(s) on the RyR reside(s) on the cytoplasmic face of the channel, perfusion of the cis chamber with identical buffer, minus GSH, fully restored channel gating (data not shown).

The interaction between GSSG and the reconstituted Ca\(^{2+}\) release channel was demonstrated in Fig. 10. In the presence of 10 \(\mu\)M Ca\(^{2+}\) (cis), addition of 0.5 mM GSSG to the cis chamber stimulated channel activity resulting in an increased open probability, in trace ii. In trace iii, a second addition of 0.5 mM GSSG further stimulated channel gating. In traces i–iii, the measured \(P_o\) values were 0.05 ± 0.02, 0.09 ± 0.02, and 0.15 ± 0.03, respectively. Subsequent additions of GSH (5 mM) to the cis chamber resulted in decreased channel open probability, as shown in traces iv and v. The presence of GSSG did not affect the unitary conductance of the reconstituted Ca\(^{2+}\) release channel and did not stimulate channel activity when added to trans chamber (data not shown). GSSG stimulation of the Ca\(^{2+}\) release channel resulted in a decreased mean closed time. The mean closed times in this experiment were 10.0 ms (no GSSG), 6.4 ms (+0.5 mM GSSG), and 4.9 ms (+1.0 mM GSSG) with no significant change in mean open time (0.7 ms in the absence or presence of either 0.5 or 1.0 mM GSSG). These findings suggest that treatment with GSSG may stimulate channel gating by increasing the on rate associated with channel activation, without affecting the off rate. Furthermore, the ability of GSSG to stimulate channel gating was observed to be highly Ca\(^{2+}\)-dependent. GSSG was unable to stimulate single-channel activity at [Ca\(^{2+}\)]\(_{\text{cis}}\) below 2 \(\mu\)M. Moreover, if GSSG was observed to stimulate channel activity, and the Ca\(^{2+}\) concentration was then decreased below 2 \(\mu\)M, all channel activity was completely inhibited (data not shown).

FIG. 7. [Ca\(^{2+}\)] dependence of ryanodine binding in the presence of GSH and GSSG. Equilibrium [3H]ryanodine binding experiments were carried out in standard binding buffer containing 7.5 nM [3H]ryanodine as described under "Experimental Procedures," in the absence (circles) or presence of 5 mM GSH (triangles) or 2 mM GSSG (squares). [Ca\(^{2+}\)] levels were maintained, ranging from 0.03 to 100 \(\mu\)M, by the addition of EGTA or Ca\(^{2+}\). Data were fit to Hill equation, as described under "Experimental Procedures." The results of the Hill analysis are presented in the text. Samples were incubated for 4.5 h at 34°C. Data are the average of experiments performed in duplicate.

DISCUSSION
In this report, the interactions between thiol-reducing agents, DTT, BME, the reduced and oxidized forms of glutathione (GSH and GSSG), and the SR Ca\(^{2+}\) release mechanism have been examined. It has been demonstrated that thiol reductants decrease ryanodine binding stimulated by Ca\(^{2+}\), caffeine, and the adenine nucleotide, cAMP. Ca\(^{2+}\)-stimulated binding was particularly susceptible to inhibition by thiol reductants. While the reducing agents inhibited ryanodine binding in experiments carried out at 50 \(\mu\)M Ca\(^{2+}\), GSH did not appear to affect the sensitivity of ryanodine binding to Ca\(^{2+}\) activation. The reducing agents both decreased the binding affinity of the SR membranes to the alkaloid and lowered the available number of receptor binding sites. Similar results were observed at the single-channel level, where GSH was shown to reversibly decrease channel open probability, without affecting unitary...
channel conductance. Furthermore, the apparent location of the GSH interaction site was determined to be on the cytosolic side of the membrane.

This laboratory has investigated the role of thiol oxidation in the skeletal muscle SR Ca\(^{2+}\) release mechanism. It has been previously demonstrated that thiol reactive compounds can initiate rapid Ca\(^{2+}\) release from SR vesicles via mercaptidation (4, 5), thiol-disulfide exchange (6, 7), and direct thiol oxidation (8–10, 12). In addition, the results of this report suggest that physiologically controlled levels of GSH, within the muscle fiber, may serve to down-regulate the gating state of the SR Ca\(^{2+}\) release channel, reconstituted from isolated SR vesicles (14). In this latter investigation, hyperreactive thiols were labeled with the fluorogenic maleimide, CPM, under conditions that promoted Ca\(^{2+}\) channel closure. Under stimulatory conditions these reactive thiols were unavailable for CPM labeling. These observations strongly suggested that during stimulation of Ca\(^{2+}\) release, highly reactive thiols were oxidized to disulfides while under inhibitory conditions these thiols existed in the reduced form.

It has previously been proposed that during normal Ca\(^{2+}\) channel activation, a high molecular weight disulfide-linked complex is formed between key SR proteins (15). If this hypothesis is correct, reduction of these disulfide linkages by treatment with reducing agents would be expected to inhibit Ca\(^{2+}\) channel activity. In Figs. 1 and 2, it was observed that thiol-reducing agents inhibited Ca\(^{2+}\), caffeine, and cAMP-stimulated ryanodine binding and single-channel activity (Figs. 8 and 9).

Furthermore, the relative order of potency (DTT, BME, and GSH, respectively) agrees with predictions based on redox potential and accessibility arguments. The reduction potential for DTT, \(V_{\text{redox}}\) has been measured to be \(-330 \text{ mV}\) (28), which is significantly lower than that of either BME or GSH (31). Furthermore, although the reduction potentials of BME and GSH are comparable, the smaller size and lipophilic nature of BME suggests that it would probably have better accessibility to redox sites than the larger more hydrophilic GSH (31).

On a more general level, the results of this report suggest that physiologically controlled levels of GSH, within the muscle fiber, may serve to down-regulate the gating state of the SR Ca\(^{2+}\) release channel. Based on the data presented, such a system would assist in keeping SR Ca\(^{2+}\) permeability low, particularly during the resting state of the cell. In addition, the effects of cellular GSH may act in unison with Mg\(^{2+}\) levels to counteract the stimulatory effects of in situ adenine nucleotide levels under resting conditions. It is interesting that estimated cytosolic levels of GSH within skeletal muscle are maintained at about the concentration of the IC\(_{50}\) for GSH inhibition of the RyR. Given this observation, it is likely that small perturbations of the cellular GSH level may have profound implications on RyR function. 

**FIG. 8.** GSH inhibits single-channel gating of the reconstituted SR Ca\(^{2+}\) release channel. The Ca\(^{2+}\) release channel, reconstituted into an artificial membrane, was treated with various concentrations of GSH added to the cis side of the membrane. Details of these experiments are described under “Experimental Procedures.” The cis chamber contained 500 mM CsCl, 10 mM HEPES, 100 \(\mu\)M CaCl\(_2\), at pH 7.1, while the trans chamber contained 100 mM CaCl\(_2\), 10 mM HEPES, at pH 7.1. In trace i, Ca\(^{2+}\) channel gating was recorded as a function of time, following vesicle fusion and perfusion with identical buffer, with no added SR or Ca\(^{2+}\) (\(P_o = 0.05, n = 2\) channels). Following addition of 50 \(\mu\)M Ca\(^{2+}\) (ii) (cis) channel gating was stimulated (\(P_o = 0.75\)). Subsequent additions of GSH, to final concentrations (cis) of 2 mM (iii), 6 mM (iv), and 10 mM (v), inhibited channel gating. In traces iii–iv, the channel \(P_o\) values were determined to be 0.15, 0.10, and <0.05, respectively. Data were analyzed using pCLAMP 5.0 and are representative of similar experiments, performed five times using three separate SR preparations. Each trace is 500 ms in duration, solid lines denote the closed state, and arrows represent open states of the channel(s). For each trace, the \(P_o\) was determined from at least 1 min of continuous recording.

**FIG. 9.** GSH inhibits reconstituted SR Ca\(^{2+}\) release channel from the cis but not the trans side of the channel complex. Single-channel experiments were carried out as described under “Experimental Procedures.” The cis chamber contained 250 mM CsMS, 10 mM HEPES, at pH 7.2, while the trans chamber contained 50 mM CsMS, 10 mM HEPES, at pH 7.2. In trace i, Ca\(^{2+}\) channel gating was recorded as a function of time, following vesicle fusion (\(P_o = 0.01\)), at +25 mV holding potential with respect to the trans side. Following addition of 50 \(\mu\)M Ca\(^{2+}\) (cis) channel gating was stimulated (ii) (\(P_o = 0.45\)). Addition of 5 mM GSH to the trans side had no effect on channel gating (iii) (\(P_o = 0.50\)). However, addition of 5 mM GSH to the cis chamber lowered channel open probability to 0.30 (iv). A second addition of 5 mM GSH (cis) further lowered channel gating (v) (\(P_o = 0.20\)). Experiment was performed three times, using two SR preparations, with similar results. The solid line represents the closed state, and the arrow denotes the open state of the channel. For each trace, \(P_o\) was determined from at least 1 min of continuous recording.
of GSSG on channel gating. In muscle fibers, while cytoplasmic levels of GSSG are kept at very low levels, in the range of 30–50 μM, GSSG is a by-product of GSH depletion during oxidative stress. In the presence of reactive oxygen species, decreased [GSH] accompanied by increased [GSSG] would be expected to increase the Ca\(^{2+}\) permeability of the SR. Higher resting Ca\(^{2+}\) concentrations could disrupt ECC signaling, distinct from the direct effects of the oxygen radicals on SR membrane proteins.

While GSH and GSSG are shown to be potent modulators of RyR function, these compounds did not affect the [Ca\(^{2+}\)] dependence of high affinity ryanodine binding (Fig. 7). In the presence of millimolar GSH, maximum binding levels as a function of [Ca\(^{2+}\)] are markedly decreased, while the EC\(_{50}\) for activation remained unchanged. Additionally, millimolar levels of GSSG increased peak binding levels with little effect on the [Ca\(^{2+}\)] EC\(_{50}\). This evidence suggests that glutathione may act as an amplifier for channel activity but does not serve as a primary trigger for Ca\(^{2+}\) release. However, the glutathione balance within the cytosol, may serve to modulate Ca\(^{2+}\)-stimulated channel activity following an EC coupling event.

The interaction between GSSG and RyR is not an overly surprising observation. In fact, a number of other groups have reported GSSG interaction with various membrane protein systems. For instance, Gilbert et al. reported that GSSG inhibited NMDA- and glycine-stimulated [Ca\(^{2+}\)]\(_{i}\) levels in neurons (21), by interacting with an NMDA receptor-associated redox site. In addition, the authors noted that GSSG inhibited [Ca\(^{2+}\)]\(_{i}\) changes produced by KCl depolarization, possibly through an interaction with N- and L-type Ca\(^{2+}\) channels. In another report, Renard-Rooney et al. (22) observed that GSSG, under certain conditions, stimulated IP\(_{3}\) binding to hepatic IP\(_{3}\) receptors by increasing the available number of binding sites (B\(_{max}\)), while not affecting the binding affinity. IP\(_{3}\)Rs share certain sequence homologies with RyRs, which include cysteine residues near the C terminus. It has been suggested that this common thiol-containing motif may play a common role in the redox regulation of the respective protein complexes (31). Furthermore, Park et al. (32) observed that K\(_{ca}\) channel activity, from either pulmonary or ear arterial smooth muscle cells, was increased by treatment with GSSG.

It has been reported previously that the cardiac RyR isoform (RyR\(_{2}\), like RyR\(_{1}\), is subject to regulation via thiol oxidation and reduction. Boraso et al. (33) reported that SR isolated from cardiac muscle was susceptible to modulation by the reactive oxygen species peroxide. The authors demonstrated that millimolar levels of peroxide were capable of stimulating cardiac CBC gating in single-channel experiments. In addition, it was observed that Ca\(^{2+}\)-stimulated RyR\(_{2}\) channel gating was inhibited by 10 mM DTT. This latter report demonstrated that RyR\(_{2}\) exhibits sensitivity to redox modulation similar to the skeletal isoform. As stated above, the skeletal RyR has also been shown to be sensitive to direct stimulation by peroxide (12). It is highly likely that both RyR\(_{1}\) and RyR\(_{2}\) are, to some degree, regulated by intracellular levels of GSH and GSSG. This evidence suggests that, in both cardiac and skeletal muscle, oxidative stress is induced by both a direct interaction between oxygen-derived radicals and key SR protein components, and by an alteration of normal myoplasmic GSH levels. Either of these mechanisms could lead to a disruption of normal cellular Ca\(^{2+}\) homeostasis.

GSH appears to be protected from autooxidation in the presence, but not in the absence of the SR. Micromolar concentrations of heavy metals (Cu\(^{2+}\) or Fe\(^{3+}\)) are known to catalyze the oxidation of GSH to GSSG. The SR may be preventing the oxidation of GSH by binding contaminating heavy metals, or the SR may contain an endogenous reductase that is capable of reducing GSSG back to GSH. This latter explanation is unlikely since no reduced substrate (i.e. NADH or NADPH) is
present in these assays. Irrespective of the explanation, in the presence of SR vesicles, GSH levels remain constant during the 4.5 h that the SR was incubated with [3H]ryanodine at 34 °C.

In summary, we have demonstrated that thiol-reducing agents inhibit both ryanodine binding and single-channel gating stimulated by known channel agonists. These compounds appear to inhibit ryanodine binding primarily by decreasing the binding affinity of ryanodine for its receptor. At the single-channel level, thiol-reducing agents were also shown to decrease the open channel probability ($P_o$). In addition, it was shown that GSSG stimulated ryanodine binding and single-channel gating by directly oxidizing thiols associated with the channel complex. The interaction between GSSG stimulation and GSH inhibition modulate ryanodine binding via a competitive mechanism. One explanation for this observation is that there exists a discrete set of reactive thiol groups that are subject to both GSSG oxidation and GSH reduction, depending on the activation state of the channel. Single-channel measurements indicate that the thiol groups associated with GSH inhibition of the channel are located on the cytoplasmic face of the SR membrane. These observations support previous work from this laboratory, suggesting that oxidation-reduction of protein thiols within the ryanodine receptor complex plays an important role in SR Ca$^{2+}$ release channel function.

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