ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 7 & 8, 2005

Forum Original Research Communication

Effects of *S*-Glutathionylation and *S*-Nitrosylation on Calmodulin Binding to Triads and FKBP12 Binding to Type 1 Calcium Release Channels

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ABSTRACT

This study shows that the combination of glutathione (GSH) plus hydrogen peroxide (H_2O_2) promotes the *S*-glutathionylation of ryanodine receptor type 1 (RyR1) Ca^{2+} release channels, and confirms their joint *S*-glutathionylation and *S*-nitrosylation by *S*-nitrosoglutathione (GSNO). In addition, we show that ^{35}S-labeled 12-kDa FK506-binding protein ([^{35}S]FKBP12) bound with a K_d of 13.1 n*M* to RyR1 present in triads or heavy sarcoplasmic reticulum vesicles; RyR1 *S*-nitrosylation by NOR-3 or GSNO, but not *S*-glutathionylation, specifically increased by four- to fivefold this K_d value. RyR1 redox modifications also increased the K_d of [^{35}S]calmodulin binding to triads without affecting B_{max}. RyR1 *S*-glutathionylation (induced by GSH plus H_2O_2) or RyR1 *S*-nitrosylation (produced by NOR-3) increased by approximately six- or twofold, respectively, the K_d of apocalmodulin (apoCaM) or Ca^{2+}-calmodulin (CaCaM) binding to triads. Likewise, the combined *S*-glutathionylation and *S*-nitrosylation of RyR1 induced by GSNO increased by fourfold the K_d of CaCaM binding to triads and abolished apoCaM binding. As both FKBP12 and CaCaM inhibit RyR1, decreased FKBP12 binding to RyR1 and/or decreased CaCaM binding to either RyR1 or dihydropyridine receptor in triad preparations may cause the reported enhanced activation of Ca^{2+}-induced Ca^{2+} release kinetics mediated by *S*-glutathionylation/*S*-nitrosylation. We discuss possible consequences of these redox modifications on RyR1-mediated Ca^{2+} release in physiological or pathological conditions. *Antioxid. Redox Signal.* 7, 870–881.

INTRODUCTION

Ca^{2+}-RELEASE/RYANODINE RECEPTOR CHANNELS (RyR channels) are massive integral membrane proteins (~2.3 MDa) present in endo/sarcoplasmic reticulum membranes as homotetramers (9). Ca^{2+} release through these channels has a central role in Ca^{2+} signaling. Through Ca^{2+}-induced Ca^{2+} release (CICR), RyR channels can amplify Ca^{2+} entry signals and can also efficiently propagate Ca^{2+} signals to other cell locations or organelles, such as mitochondria or nuclei (35). These properties place RyR channels at the center of several important processes elicited by cellular Ca^{2+} signals. Some of them occur in all cells, such as apoptosis and necrosis, whereas others are cell-specific and include events as diverse as contraction, secretion, fertilization, and neuronal plasticity. Consequently, RyR channel function is extensively regulated by small ions and molecules (*e.g.,* Ca^{2+}, Mg^{2+}, or adenine nucleotides) and protein–protein interactions (*e.g.,* calmodulin) (10, 25, 31). Additionally, RyR channels are also regulated by phosphorylation/dephosphorylation events (28) and by redox modifications, as detailed below.

Mammalian RyR1 channels are likely to act as cellular redox sensors. They contain a few cysteine residues that react with pharmacological or endogenous redox-active molecules at physiological pH (6, 8, 32, 40, 46, 47). Moreover, RyR1 redox modifications have a profound effect on RyR1-mediated Ca^{2+} release and single-channel activity (14, 17, 27). Additionally, the RyR1 protein is susceptible to *S*-nitrosylation

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and *S*-glutathionylation (1, 8), joining thus the increasing number of proteins susceptible to these posttranslational modifications (12, 29). In the absence of Mg^{2+}, *S*-nitrosylation of the channel enhances its activation by Ca^{2+}, whereas channel *S*-glutathionylation decreases selectively its sensitivity to Mg^{2+} inhibition (1).

In mammalian skeletal muscle, RyR1 channels interact with several different accessory proteins, which contribute to the tight regulation of channel activity and hence modulate Ca^{2+} release. These proteins include the skeletal L-type Ca^{2+}channel [dihydropyridine receptor (DHPR)], which acts as voltage sensor during excitation–contraction coupling in skeletal muscle, triadin, calmodulin, FKBP12 (a 12-kDa FK506-binding protein), and calsequestrin (9, 25).

Oxidative-induced cross-linking of the RyR1 protein interferes with calmodulin binding, whereas *S*-alkylation prevents triadin binding to isolated sarcoplasmic reticulum vesicles (22, 48). In addition, incubation of RyR1 with either nitric oxide ($NO^{\bullet}$) or the $NO^{\bullet}$ donors NOC-12 and GSNO modulate RyR1 by different mechanisms; both $NO^{\bullet}$ and NOC-12 induce *S*-nitrosylation of Cys3635 (41, 42). In contrast, *S*-nitrosoglutathione (GSNO), a different $NO^{\bullet}$ donor that modifies a few highly reactive RyR1 cysteine residues other than Cys3635, has been proposed to activate RyR1 in a calmodulin-independent fashion (42). These results suggest that different *S*-nitrosylating agents activate RyR1 channels by reacting with different hyperreactive cysteine residues in the primary sequence.

Calmodulin in its apocalmodulin (apoCaM) form (not bound to Ca^{2+}) enhances RyR1 channel activity, whereas when present as Ca^{2+}-calmodulin (CaCaM) it behaves as a channel inhibitor (36, 37, 44). Both forms of calmodulin also bind to skeletal DHPR (39). Thus, by binding to both DHPR and RyR1, calmodulin may coordinate the RyR1/DHPR interactions that determine skeletal type excitation–contraction coupling (39).

In addition to calmodulin, FKBP12 is another RyR1 regulatory protein that may specifically coordinate RyR1 channel gating (11). Removal of this protein with rapamycin or FK506 leads to RyR1 activation and promotes the emergence of subconductance states in single RyR1 channels incorporated in lipid bilayers (20, 24). In addition, tissue-specific FKBP12 knockout mice exhibit altered orthograde and retrograde signaling between DHPR and RyR1, leading to altered L-type currents (43). These results suggest that FKBP12, together with calmodulin, is involved in skeletal muscle excitation–contraction coupling.

In the present work, we show that the RyR1 protein in native triads undergoes endogenous *S*-glutathionylation in addition to its reported endogenous *S*-nitrosylation (8). Incubation of triads with GSNO induced additional RyR1 *S*-glutathionylation and *S*-nitrosylation over the endogenous levels, whereas incubation with NOR-3 increased selectively RyR1 *S*-nitrosylation. We also show that RyR1 *S*-nitrosylation, but not *S*-glutathionylation, decreased the affinity of RyR1 for FKBP12, whereas both modifications decreased the affinity of RyR1 and/or DHPR for calmodulin. We propose that the differential effects of *S*-nitrosylation versus *S*-glutathionylation on FKBP12 and calmodulin binding to RyR1 reflect the differential reactivity of cysteine residues other than Cys3635 toward these modifications. Physiological and pathological consequences are discussed, including the possibility that modification of RyR1 by both *S*-nitrosylation and *S*-glutathionylation may promote diminished interaction between RyR1 and DHPR channels, leading to the loss of regulation in orthograde and/or retrograde signaling in skeletal muscle excitation–contraction coupling, characteristic features of malignant hyperthermia or central core disease (26).

MATERIALS AND METHODS

Materials

All reagents used were of analytical grade. Bovine serum albumin (BSA), *N*-ethylmaleimide (NEM), 2,4-dithiothreitol (DTT), 3-(*N*-morpholino)propanesulfonic acid (MOPS), hydrogen peroxide (H_2O_2), and protease inhibitors (leupeptin, pepstatin A, aprotinin, aminobenzamidine, and phenylmethylsulfonyl fluoride) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acrylamide/*bis*-acrylamide solution (30%/2.6% cross-linker), ammonium persulfate, Coomassie Brilliant Blue R-250, sodium dodecyl sulfate (SDS), bromophenol blue, Tween-20, and polyvinylidene difluoride membranes were obtained from Bio-Rad (Hercules, CA, U.S.A.). Glutathione (GSH), and (±)-(*E*)-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR-3) were from EMD Biosciences (Darmstadt, Germany). GSH stock solutions were bubbled with N_2 and stored in aliquots at −80°C to prevent spontaneous oxidation; once thawed, aliquots were discarded. SuperBlock® Blocking Buffer in phosphate-buffered saline (PBS) was from Pierce (Rockford, IL, U.S.A.). Cold calmodulin and [^{35}S]-calmodulin were obtained as described (36). Cold and ^{35}S-radiolabeled FKBP12 were obtained according to a protocol to be described elsewhere (Tang *et al.,* manuscript in preparation).

Antibodies

Rabbit polyclonal anti-*S*-nitrosocysteine (anti-CysSNO) antibody was obtained from Calbiochem (La Jolla, CA, U.S.A.). Mouse monoclonal anti-glutathione (anti-GSH) antibody was from Virogen (Watertown, MA, U.S.A.), and mouse monoclonal anti-FKBP12 was from Affinity Bioreagents (Golden, CO, U.S.A.). Goat polyclonal anti-mouse IgG conjugated with Alexa-Fluor 680 was from Molecular Probes (Eugene, OR, U.S.A.). Goat polyclonal anti-rabbit IgG conjugated with IR800Dye was from Rockland Immunochemicals (Gilbertsville, PA, U.S.A.). The use of anti-CysSNO and anti-GSH antibodies to detect cysteine *S*-nitrosylation or *S*-glutathionylation, respectively, has been described in the literature (for examples, see 18 and 30).

Membrane isolation

Sarcoplasmic reticulum preparations enriched in triads (from now on, triads) were isolated from rabbit fast skeletal muscle as described previously (16). Isolated triads contain on average 10% attached transverse tubule membranes, as determined by their [^{3}H]ouabain binding density (3, 5). Terminal-cisternae sarcoplasmic reticulum vesicle prepara-

tions (from now on, HSR vesicles), which in contrast to triads do not contain attached transverse tubule membranes, were isolated from rabbit fast skeletal muscle as described (15). Both triads and HSR vesicles displayed high [^{3}H]ryanodine-binding densities, in the range of 30 pmol/mg of protein. Membrane fractions were stored in liquid N_2 for up to 30 days. Protein concentration was determined (23) using BSA as standard.

GSNO synthesis

Synthesis of GSNO was performed according to Rossi *et al.* (38); in brief, equimolar quantities of GSH and $NaNO_2$ were mixed in 0.75 *M* HCl at room temperature. After 5 min, the mixture was neutralized with solid Tris-base. The resulting GSNO concentration was determined in a spectrophotometer at 334 nm, using the molar extinction coefficient of 767 M^{-1} cm^{-1} (19); routine yields were 90–95%.

Incubation of vesicles with redox modifying agents

Triads or HSR vesicles (1 mg/ml) were incubated for 15 min at 25°C with one of the following reagents: 4 m*M* GSH plus 100 μ*M* H_2O_2, 125 μ*M* NOR-3, or 250 μ*M* GSNO. Unless otherwise indicated, redox modification was stopped by centrifugation at 100,000 *g* for 30 min at 4°C, followed by resuspension of HSR vesicles to 10 mg/ml either in nonreducing SDS–polyacrylamide gel electrophoresis (PAGE) solution or in FKBP12 or calmodulin binding buffer (see below for compositions).

Detection of S*-nitrosylated or* S*-glutathionylated protein bands by western blotting*

Samples, previously denatured at 60°C for 30 min in nonreducing SDS-PAGE solution [1% (wt/vol) SDS, 5 m*M* NEM, 0.005% (wt/vol) bromophenol blue, 50 m*M* NaH_2PO_4, 170 m*M* Na_2HPO_4], were separated in 4% stacking/7.5% resolving Laemmli gels (21). Proteins were transferred to polyvinylidene difluoride membranes at 22 V for 16 h at 4°C. Membranes were blocked for 1 h at 25°C under constant rocking with 2% (wt/vol) BSA in PBS (120 m*M* NaCl, 2.7 m*M* KCl in 10 m*M* phosphate buffer, pH 7.2) or Super Block Blocking Buffer in PBS, for the anti-CysSNO or anti-GSH antibodies, respectively, and probed with these antibodies (1:1,000 dilution in blocking buffer) for 1 h at 25°C under constant rocking. After three washes of 5 min each with PBS solution supplemented with 0.1% Tween-20 (PBS-T), membranes were incubated with the respective secondary antibodies. Following three washes of 5 min each with PBS-T and an additional wash of 5 min with PBS, fluorescence at 700 or 800 nm of membranes was monitored in a near-infrared Odyssey® scanner (Li-Cor Biosciences, Lincoln, NE, U.S.A.). Membranes were stained subsequently with Coomassie Blue and analyzed for optical density; the RyR1 subunit band was identified by parallel western blot assays (shown in Fig. 1). Densitometric analysis of band fluorescence or Coomassie Blue staining was carried out with the Quantity One® software (Bio-Rad). *S*-Nitrosylation, detected with anti-CysSNO, produced a fluorescent signal measurable at 800 nm. *S*-Glutathionylation, detected with anti-GSH, yielded a fluorescence signal at 700 nm. The resulting fluorescence detected on the RyR1 subunit band was normalized to its respective Coomassie Blue optical density. To estimate the degree of stimulation by redox agents, the ratio between the normalized fluorescence obtained from redox-treated and untreated RyR1 (displaying only endogenous redox modification) was calculated.

Equilibrium [^{35}S]FKBP12 binding assay

Native or redox-treated triads (10 μg per assay) were incubated with different concentrations of [^{35}S]FKBP12 (2.5–100 n*M*) for 2 h at 25°C in FKBP12 binding buffer [0.3 *M* NaCl, 1.2 m*M* $CaCl_2$, 1 m*M* EGTA, 0.1 mg/ml BSA, and 0.1% (wt/vol) CHAPS in 50 m*M* MOPS-NaOH, pH 7.4]. Nonspecific binding was determined in the presence of 10 μ*M* cold FKBP12. Bound FKBP12 was separated from the free ligand by vacuum filtration using Whatman GF/F fiber glass filters. After five washes with 3 ml of ice-cold washing buffer [0.3 *M* NaCl, 1.2 m*M* $CaCl_2$, 1 m*M* EGTA, 0.1% (wt/vol) CHAPS in 50 m*M* MOPS-NaOH, pH 7.4], the radioactivity associated to the filters was quantified by liquid scintillation counting.

Equilibrium [^{35}S]calmodulin binding assay

Native or redox-treated triads (10 μg per assay) were incubated with different concentrations of [^{35}S]calmodulin (2.5–100 n*M*) for 2 h at 25°C in apoCaM binding buffer [0.3 *M* NaCl, 1 m*M* EGTA, 0.1 mg/ml BSA, and 0.1% (wt/vol) CHAPS in 50 m*M* MOPS-NaOH, pH 7.4] or in CaCaM binding buffer [0.3 *M* NaCl, 1.2 m*M* $CaCl_2$, 1 m*M* EGTA, 0.1 mg/ml BSA, and 0.1% (wt/vol) CHAPS in 50 m*M* MOPS-NaOH, pH 7.4]. Nonspecific binding was determined in both cases in the presence of 10 μ*M* unlabeled calmodulin. Bound calmodulin was separated from the free ligand by vacuum filtration using Whatman GF/F fiber glass filters. After five washes with 3 ml of ice-cold washing buffer for apoCaM [0.3 *M* NaCl, 1 m*M* EGTA, 0.1% (wt/vol) CHAPS in 50 m*M* MOPS-NaOH, pH 7.4] or for CaCaM [0.3 *M* NaCl, 1.2 m*M* $CaCl_2$, 1 m*M* EGTA, 0.1% (wt/vol) CHAPS in 50 m*M* MOPS-NaOH, pH 7.4], the radioactivity associated to the filters was quantified by liquid scintillation counting.

Statistical analysis

Data are presented as means ± SD of at least three independent determinations. For statistical comparisons, the ANOVA test was performed with Bonferroni post-analysis using the GraphPad® Prism software (version 3.0).

RESULTS

Endogenous and exogenously promoted RyR1 S*-glutathionylation and* S*-nitrosylation*

We developed a western blot assay using anti-GSH and anti-CysSNO antibodies to investigate RyR1 *S*-glutathionylation or *S*-nitrosylation, respectively. We utilized GSH + H_2O_2 to induce RyR1 *S*-glutathionylation and NOR-3 to promote *S*-nitrosylation; we also used GSNO, which has the potential to induce both redox modifications (1, 42). As shown in Fig. 1A and B, incubation with these agents modified only a few of the many protein bands present in triad vesicles; prominent

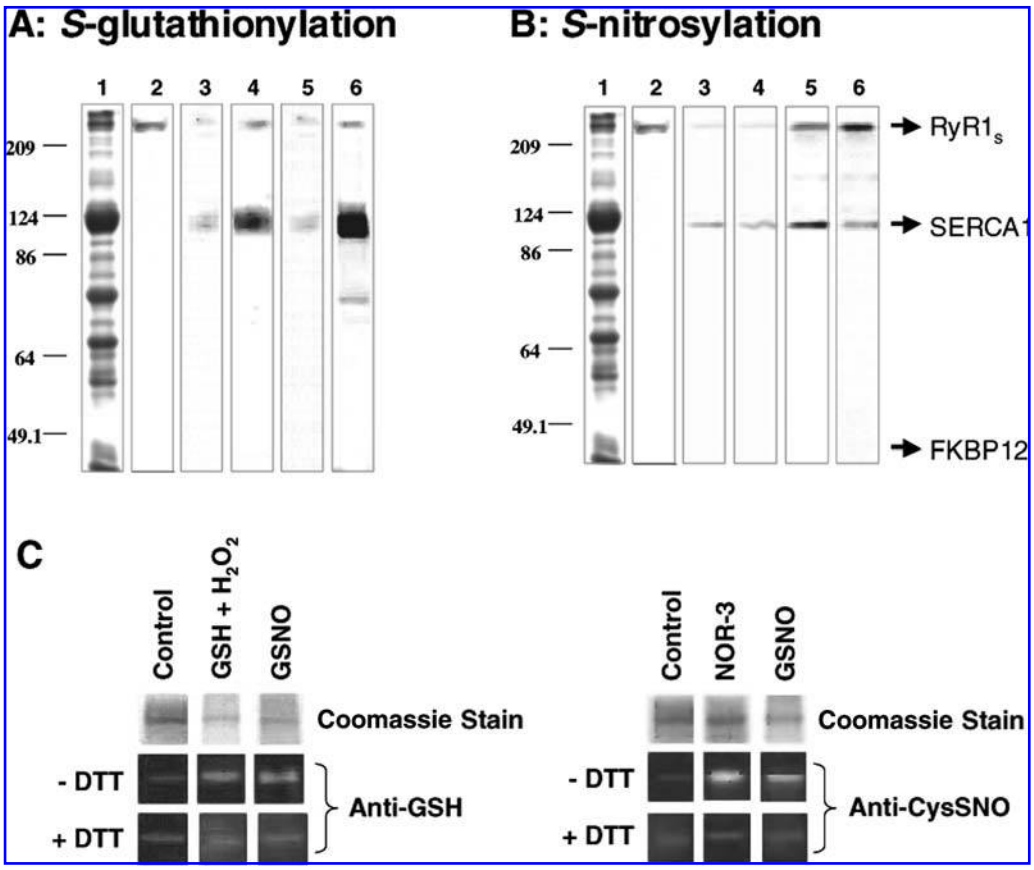

FIG. 1. Endogenous and exogenously induced *S*-glutathionylation and *S*-nitrosylation of triad proteins. Triads (1 mg/ml) were incubated for 15 min at 25°C with one of the following: 4 m*M* GSH plus 100 μ*M* H_2O_2, 125 μ*M* NOR-3, or 250 μ*M* GSNO. Reactions were stopped by addition of nonreducing SDS-PAGE denaturing solution, supplemented with 5 m*M* NEM. (**A** and **B**) Parallel western blots (see Materials and Methods) were probed with anti-RyR1 (lane 2, A and B), with anti-GSH (lanes 3–6, A) or anti-*s*-CysSNO (lanes 3–6, B), followed by fluorescent secondary antibodies. Lanes 1 (in both A and B): triads stained with Coomassie Blue; lanes 2: triads probed with anti-RyR1; lanes 3: control triads; lanes 4: triads incubated with GSH + H_2O_2; lanes 5: triads incubated with NOR-3; lanes 6: triads incubated with GSNO. Images correspond to representative western blots, run in parallel, of at least three independent experiments. Numbers on the left indicate relative migration of molecular weight standards. (**C**) After incubation with the redox agents, samples were further incubated with or without 25 m*M* DTT for 30 min at 25°C. Samples were subjected to western blot as above. Only the RyR1 bands are shown.

among them was the SERCA1 and the RyR1 subunit, which due to its very high molecular weight (570 kDa) migrates as a single band close to the top of nonreducing gels in a region devoid of other triadic proteins (see also 1). Redox modifications of SERCA1 have been previously reported (45) and they are outside the scope of the present study. All three redox reagents (GSH + H_2O_2, NOR-3, or GSNO) did not induce detectable redox modification of FKBP12 (Fig. 1A and B). Subsequent incubation of redox-modified triads with 2 m*M* DTT completely reversed both *S*-glutathionylation and *S*-nitrosylation (Fig. 1C).

As depicted in Fig. 2A and B, incubation of triads or HSR vesicles with GSH + H_2O_2 increased significantly RyR1 *S*-glutathionylation over the endogenous level, but did not promote RyR1 *S*-nitrosylation. In contrast, NOR-3 produced a significant increase of RyR1 *S*-nitrosylation over the endogenous level, but did not promote its *S*-glutathionylation. Scan analysis of the RyR1 band by densitometry indicated that incubation of triads or HSR vesicles with GSH + H_2O_2 or NOR-3 enhanced RyR1 *S*-glutathionylation (2.5-fold) or *S*-nitrosylation (3.5-fold), respectively, over the endogenous levels (Fig. 2C and D). Likewise, incubation with GSNO enhanced simultaneously RyR1 *S*-glutathionylation (1.9-fold) and *S*-nitrosylation (2.8-fold) over the endogenous levels (Fig. 2C and 2D).

These combined results show that the RyR1 protein has cysteine residues that readily react with either *S*-glutathionylating or *S*-nitrosylating reagents (see Discussion). These results also show that the presence of transverse tubule membranes in triads, with the consequent association between RyR1 and DHPR, does not affect RyR1 redox modifications because when present in triads or HSR vesicles RyR1 exhibited similar levels of *S*-glutathionylation and *S*-nitrosylation (Fig. 2).

Effects of S*-glutathionylation or* S*-nitrosylation on FKBP12 binding to triads*

We have previously shown that *S*-glutathionylation of RyR1 decreases Mg^{2+} inhibition of CICR, whereas *S*-nitrosylation enhances its activation by Ca^{2+} (1). To identify other possible consequences of these redox modifications on RyR1

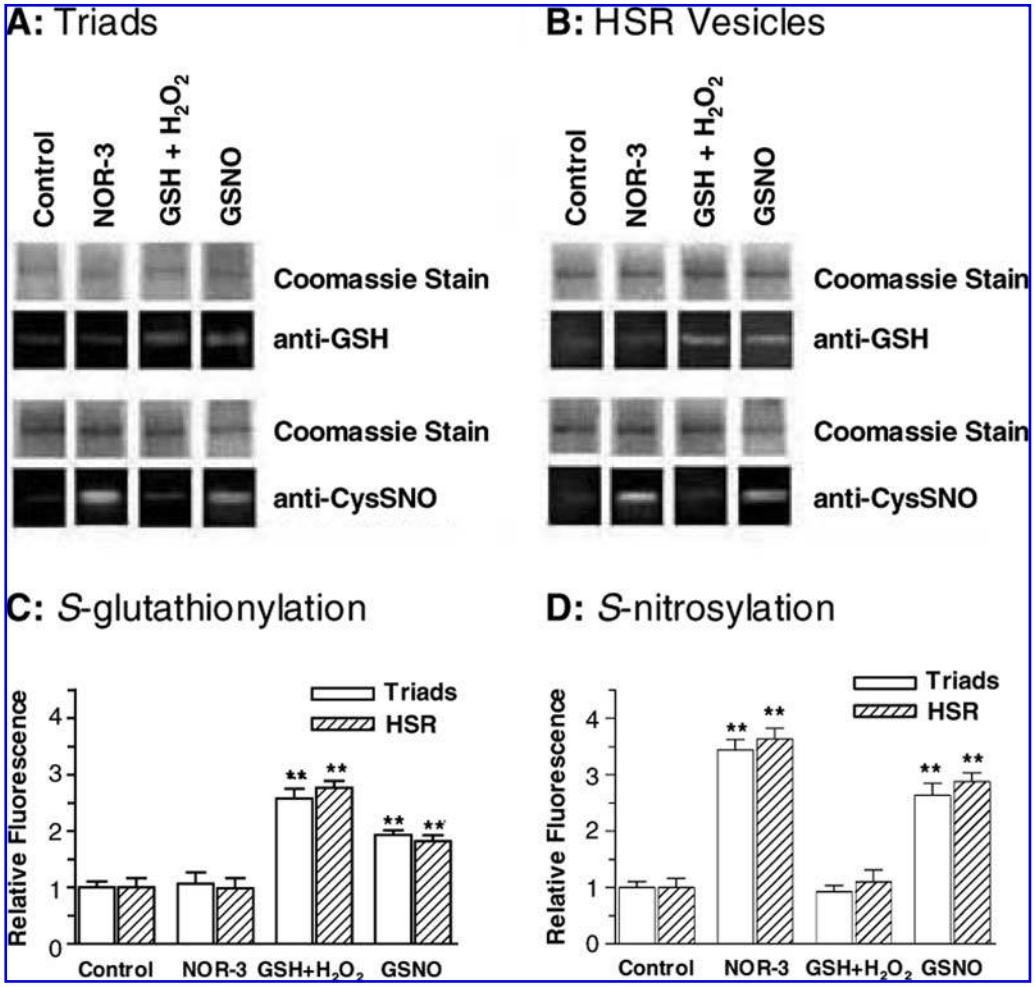

FIG. 2. Endogenous and exogenously induced *S*-glutathionylation and *S*-nitrosylation of RyR1 channels present in triads or HSR vesicles. Triads or HSR vesicles (1 mg/ml) were incubated for 15 min at 25°C with one of the following: 4 m*M* GSH plus 100 μ*M* H_2O_2, 125 μ*M* NOR-3, or 250 μ*M* GSNO. Reactions were stopped by addition of nonreducing SDS-PAGE denaturing solution, supplemented with 5 m*M* NEM. The corresponding western blots (see Materials and Methods) were probed with anti-GSH or anti-CysSNO antibodies, followed by fluorescent secondary antibodies. (**A** and **B**) Representative images of the Coomassie Blue and fluorescence staining of the RyR1 channel protein band from triads or HSR vesicles, respectively (only RyR1 bands are shown). (**C** and **D**) Densitometric scan analysis of the above signals obtained in triads (open bars) or HSR vesicles (hatched bars). Data are given as means ± SD ($n = 3$) of the corrected relative fluorescence intensity (redox-modified over controls; see Materials and Methods). $^{**}p < 0.01$ compared with the control.

function, we examined the effects of GSH + H_2O_2, NOR-3, or GSNO on [^{35}S]FKBP12 binding to RyR1 in triads. For this purpose, triads were incubated with these reagents, and after excess reagent was washed away, equilibrium [^{35}S]FKBP12 binding was assayed as described in Materials and Methods. FKBP12 was not *S*-nitrosylated or *S*-glutathionylated by these agents either when forming part of triads (Fig. 1) or when incubated with these agents after its purification (Fig. 3). As shown in Fig. 4, [^{35}S]FKBP12 binding to native triads increased hyperbolically, with a maximal binding value of 118.6 pmol/mg of protein and K_d of 13.1 n*M* (Table 1). Incubation of triads with GSH + H_2O_2 did not alter significantly these binding parameters (Table 1), suggesting that *S*-glutathionylation does not modify the interaction of FKBP12 with RyR1. In contrast, incubation of triads with NOR-3 or GSNO significantly increased the K_d of [^{35}S]FKBP12 binding without affecting maximal binding (Table 1, Fig. 4). These findings suggest that RyR1 *S*-nitrosylation alters selectively its interactions with FKBP12.

Effects of S*-glutathionylation or* S*-nitrosylation on calmodulin binding to triads*

We examined next the effects of GSH + H_2O_2, NOR-3, or GSNO on the binding of [^{35}S]calmodulin to triads. In order to assess apoCaM or CaCaM binding, assays were carried out after redox modifications of vesicles either in Ca^{2+}-free buffer (containing 1 m*M* EGTA) or in the presence of 0.2 m*M* Ca^{2+} (1.2 m*M* $CaCl_2$ plus 1 m*M* EGTA), respectively (see Materials and Methods). Mammalian skeletal muscle calmodulin does not contain cysteine residues in its primary sequence; thus, this protein cannot undergo *S*-nitrosylation or *S*-glutathionylation by residual contamination with redox reagents after incubation of vesicles. [^{35}S]ApoCaM binding to native

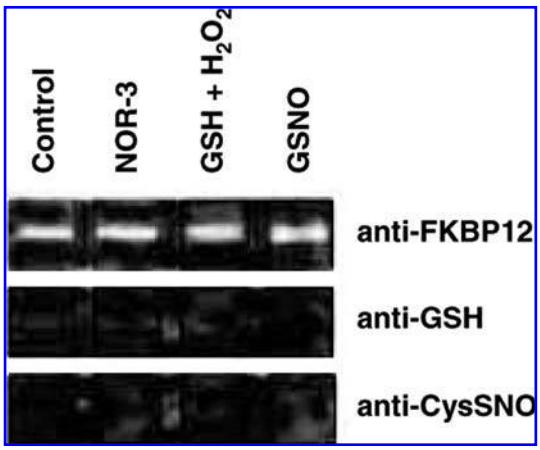

FIG. 3. Lack of redox modification of FKBP12. Purified FKBP12 (0.1 mg/ml) was incubated for 15 min at 25°C with either 125 μ*M* NOR-3, 4 m*M* GSH plus 100 μ*M* H_2O_2, or 250 μ*M* GSNO. The reaction was stopped by addition of nonreducing sample buffer, the protein sample was subjected to nonreducing electrophoresis (10 μg per lane), and parallel western blots were probed with anti-FKBP12, anti-GSH, or anti-CysSNO antibodies as described in Materials and Methods. A representative image of two independent experiments is illustrated in the figure.

TABLE 1. EFFECT OF GSH + H_2O_2, NOR-3, OR GSNO ON THE PARAMETERS OF FKBP12 BINDING TO TRIADS

	K_d (*n*M)	B_{max} (*pmol/mg*)
Control triads	13.1 ± 1.3	118.6 ± 2.7
Triads + 4 m*M* GSH + 100 μ*M* H_2O_2	14.1 ± 1.7	120.9 ± 3.2
Triads + 125 μ*M* NOR-3	52.4 ± 8.4*	112.9 ± 4.7
Triads + 250 μ*M* GSNO	67.7 ± 9.5*	133.3 ± 9.4

Triads (1 mg/ml) were incubated with the redox agents listed above for 15 min at 25°C; reactions were stopped by centrifugation and washing (see Materials and Methods for further details). The equilibrium FKBP12 binding parameters K_d and B_{max} were obtained from hyperbolic binding curves such as those illustrated in Figs. 3 and 4. Values are shown as means ± SD from at least three independent experiments.

*$p < 0.01$ compared with control (untreated) triads.

triads increased hyperbolically as a function of calmodulin concentration, with a maximal binding value of 66.2 pmol/mg of protein and K_d of 3.9 n*M* (Table 2, Fig. 5). Incubation with GSH + H_2O_2 significantly increased the K_d of [^{35}S]apoCaM binding without affecting maximal binding (Table 2, Fig. 5A), suggesting that *S*-glutathionylation decreases the affinity of interaction of apoCaM with triad pro-

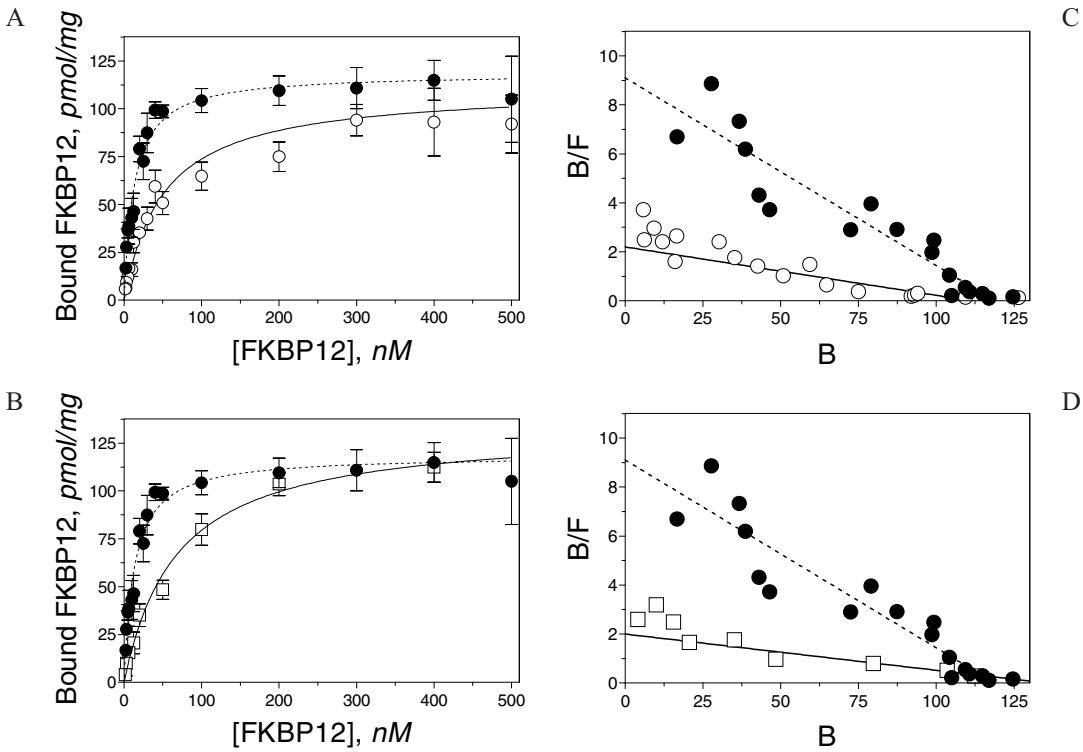

FIG. 4. Effect of NOR-3 or GSNO on FKBP12 binding to triads. Triads (1 mg/ml) were incubated for 15 min at 25°C with either 125 μ*M* NOR-3 or 250 μ*M* GSNO. After the reaction was stopped, samples were assayed for equilibrium FKBP12 binding, as detailed in Materials and Methods. (**A**) Values of specific FKBP12 binding to triads incubated with NOR-3 (open circles) or control triads (solid circles). (**B**) Scatchard analysis of experimental data plotted in A. (**C**) Values of specific FKBP12 binding to triads incubated with GSNO (open squares) or control triads (solid circles). (**D**) Scatchard analysis of experimental data plotted in C. Data are given as means ± SD of at least three independent experiments. Solid lines represent nonlinear data fit to a hyperbolic binding function with a single class of binding sites. Straight lines in Scatchard analyses were calculated from the nonlinear regression analysis of data illustrated in A and C.

TABLE 2. EFFECT OF GSH + H_2O_2, NOR-3, OR GSNO ON THE PARAMETERS OF CALMODULIN BINDING TO TRIADS

	K_d (*n*M)	B_{max} (*pmol/mg*)
Apocalmodulin		
Control triads	3.9 ± 0.7	66.2 ± 2.6
Triads + 4 m*M* GSH + 100 μ*M* H_2O_2	22.4 ± 4.0*	68.4 ± 4.6
Triads + 125 μ*M* NOR-3	25.8 ± 4.8*	73.0 ± 5.3
Triads + 250 μ*M* GSNO	ND	ND
Ca^{2+}-calmodulin		
Control triads	11.4 ± 1.5	69.1 ± 2.9
Triads + 4 m*M* GSH + 100 μ*M* H_2O_2	21.8 ± 3.1†	69.1 ± 3.6
Triads + 125 μ*M* NOR-3	25.0 ± 3.4†	74.1 ± 3.9
Triads + 250 μ*M* GSNO	43.7 ± 6.3*	72.3 ± 4.8

Triads (1 mg/ml) were incubated with the redox agents listed above for 15 min at 25°C; reactions were stopped by centrifugation and washing (see Materials and Methods for further details). The equilibrium calmodulin binding parameters K_d and B_{max} were obtained from hyperbolic binding curves such as those illustrated in Figs. 5 and 6. Values are shown as means ± SD from at least three independent experiments. ND, not detectable.

*$p < 0.01$ and †$p < 0.05$, compared with control (untreated) triads.

teins. A similar effect was found after incubating triads with NOR-3 (Table 2, Fig. 5B), suggesting that *S*-nitrosylation also decreases the affinity of the interaction of apoCaM with triad proteins. It is noteworthy that incubation of triads with GSNO completely abolished [^{35}S]apoCaM binding (Fig. 5C). These findings suggest that combined *S*-glutathionylation and *S*-nitrosylation of triad proteins (most likely RyR1; see Discussion) produce additive effects on apoCaM binding.

Binding of [^{35}S]CaCaM also increased hyperbolically with calmodulin concentration, reaching a maximal binding value of 69.1 pmol/mg of protein and K_d of 11.4 n*M* (Table 2, Fig. 6). Incubation with GSH + H_2O_2 or NOR-3 induced a similar K_d increase of [^{35}S]CaCaM binding without affecting maximal binding density (Table 2, Fig. 6A and B). These results suggest that both RyR1 *S*-glutathionylation and *S*-nitrosylation decrease the interaction of CaCaM with triads (see Discussion). Although triad incubation with GSNO did not entirely abolish [^{35}S]CaCaM binding, its effect was more marked than that of GSH + H_2O_2 or NOR-3 (Table 2, Fig. 6C). These observations suggest that apoCaM–triad interaction is more sensitive to *S*-glutathionylation and *S*-nitrosylation than CaCaM–triad interaction.

DISCUSSION

Redox modifications of RyR1

The present results show that RyR1 displays endogenous *S*-glutathionylation and corroborate the *S*-glutathionylating properties of GSNO (1). This work shows, in addition, that incubation of triads or HSR vesicles with GSH + H_2O_2 promotes RyR1 *S*-glutathionylation. This is an important observation because H_2O_2 may be generated in skeletal muscle during activity (34), and in combination with cytoplasmic GSH it may enhance RyR1 *S*-glutathionylation. Furthermore, we have confirmed experimentally that NOR-3, as well as GSNO, *S*-nitrosylates RyR1 cysteine residues as proposed in our previous study (1). Based on the present findings, we propose that the combination of GSH + H_2O_2 produces solely RyR1 *S*-glutathionylation, that NOR-3 (a NO· donor) induces only *S*-nitrosylation, whereas GSNO promotes both RyR1 *S*-glutathionylation and *S*-nitrosylation, as reported previously (1, 42). Of the several protein bands present in triads and HSR vesicles, only RyR1 and SERCA1 revealed significant reaction with the above redox agents. SERCA1 redox modifications, which have been already described (45), are outside the scope of the present study. Additionally, this work shows that RyR1 present in triads (which presumably retain the RyR1–DHPR interactions) or in HSR vesicles exhibited similar levels of endogenous redox modifications and underwent similar additional redox modifications by *S*-glutathionylating or *S*-nitrosylating agents. Accordingly, we propose that the association of RyR1 with the transverse tubule DHPR voltage sensors does not affect their susceptibility to undergo modifications by the redox agents studied here.

We have previously shown that RyR1 *S*-glutathionylation and *S*-nitrosylation have different effects on the kinetics of CICR mediated by RyR1 channels (1). Most likely, these effects reflect redox modifications of cysteines located in different RyR1 regions that share the common property of reacting at physiological pH with the redox agents used in this work. Recently, seven hyperreactive cysteine residues on RyR1 (including Cys3635) were identified by mass spectrometry following incubation of sarcoplasmic reticulum vesicles with a sulfhydryl-directed coumarin derivative (46). The reactivity of these cysteines (excepting Cys3635) toward *S*-glutathionylating and/or *S*-nitrosylating agents remains to be investigated.

RyR1 S-nitrosylation decreases the apparent affinity of FKBP12 binding to the channels

FKBP12 is a protein tightly associated to the RyR1 that reportedly modulates channel gating (11). In this work, we report for the first time that FKBP12 binds to native triads with a K_d of 13.1 ± 1.3 n*M*. As FKBP12 binds only to RyR1 and not to other triadic or HSR proteins (4), this apparent high affinity is compatible with the reported tight FKBP12–RyR1 interaction (4). The apparent B_{max} of 118.6 pmol/mg of native triad protein suggests that four FKBP12 protein units bind to each RyR1 homotetramer, in agreement with previous reports (9). This calculation is based on our measurements of ~30 pmol/mg for the B_{max} of [^{3}H]ryanodine binding to triads.

Additionally, we found that *S*-glutathionylation and *S*-nitrosylation of RyR1 have distinct effects on FKBP12 binding. Incubation of triads or HSR vesicles with NOR-3 or GSNO, but not with GSH + H_2O_2, reduced to the same extent the apparent affinity of FKBP12 binding without significantly modifying B_{max}. We propose, accordingly, that *S*-nitrosylation of some RyR1 cysteines that presumably are not susceptible to *S*-glutathionylation interferes with FKBP12 binding. Thus, *S*-nitrosylation of RyR1 would selectively decrease FKBP12

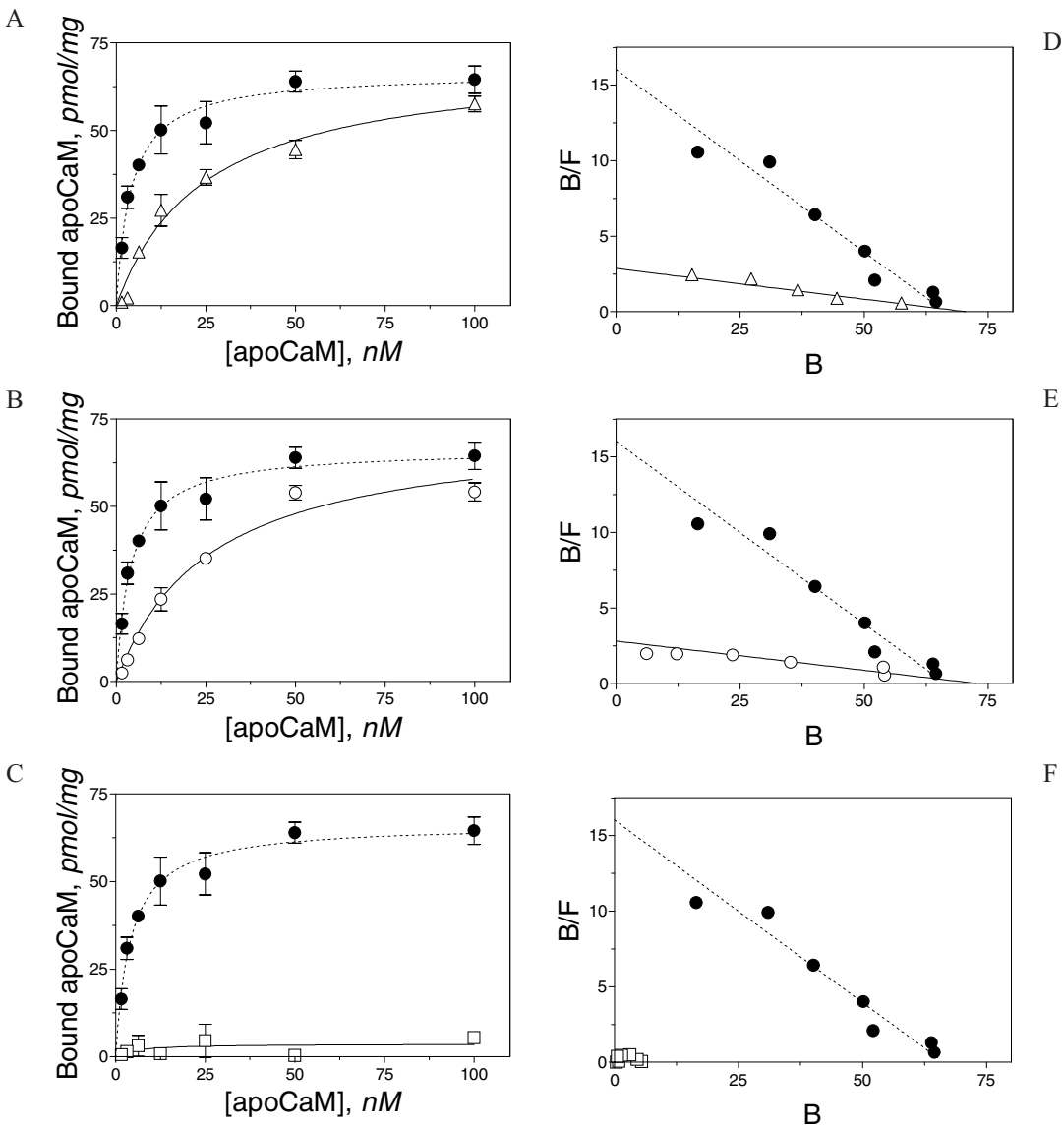

FIG. 5. Effect of GSH + H_2O_2, NOR-3, or GSNO on apoCaM binding to triads. Triads (1 mg/ml) were incubated for 15 min at 25°C with either 4 m*M* GSH plus 100 μ*M* H_2O_2, 125 μ*M* NOR-3, or 250 μ*M* GSNO. After the reaction was stopped, samples were assayed for equilibrium calmodulin binding in a low Ca^{2+} buffer (1 m*M* EGTA), as detailed in Materials and Methods. (**A**) Values of specific apoCaM binding to triads incubated with GSH + H_2O_2 (open triangles) or control triads (solid circles). (**B**) Scatchard analysis of experimental data plotted in A. (**C**) Values of specific apoCaM binding to triads incubated with NOR-3 (open circles) or control triads (solid circles). (**D**) Scatchard analysis of experimental data plotted in C. (**E**) Values of specific apoCaM binding to triads incubated with GSNO (open squares) or control triads (solid circles). (**F**) Scatchard analysis of experimental data plotted in E. Data are given as means ± SD of at least three independent experiments. Solid lines represent nonlinear data fit to a hyperbolic binding function with a single class of binding sites. Straight lines in Scatchard analyses were calculated from the nonlinear regression analysis of data illustrated in A, C, and E.

binding, presumably through an allosteric effect on the actual FKBP12 binding site.

The available information indicates that FKBP12 modulates RyR1 channel gating and excitation–contraction coupling (2, 11, 43). Removal of this protein from RyR1 incorporated in lipid bilayers, using rapamycin or FK506, increases channel activity and promotes subconductance states (20, 24), whereas skeletal muscle-specific FKBP12 deficiency alters excitation–contraction coupling (43). The present study shows, for the first time, that *S*-nitrosylation of RyR1 cysteines perturbs RyR1–FKBP12 interactions. It is likely that *S*-nitrosylation of Cys3635 is not involved in this effect because GSNO does not *S*-nitrosylate this cysteine (42), yet it decreased the affinity of FKBP12 binding to RyR1. As *S*-nitrosylation increases selectively RyR1 sensitivity to Ca^{2+} activation, the present results suggest that this activation may be due, at least partially, to *S*-nitrosylation-induced decrease in the interaction of the channel with FKBP12.

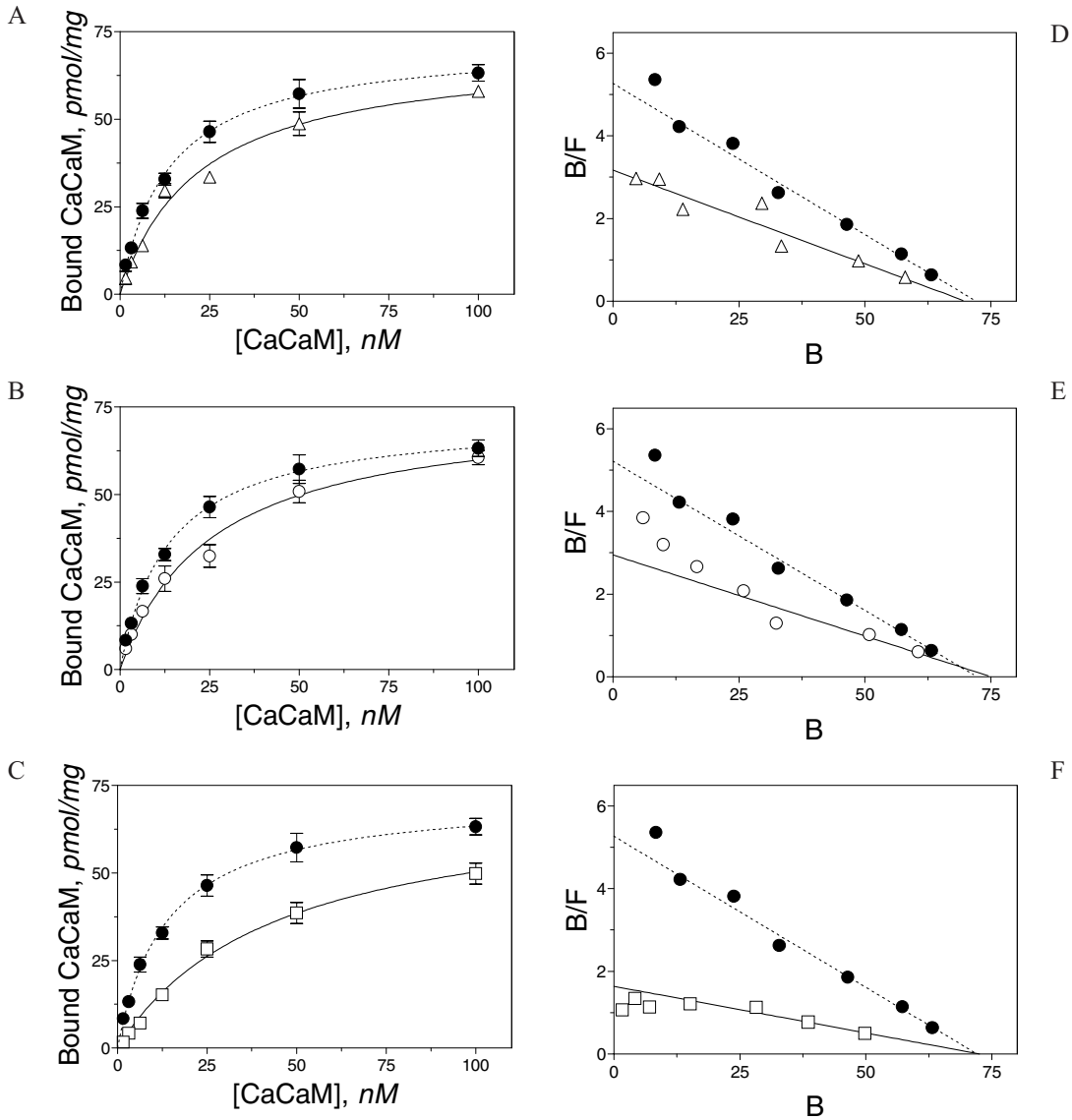

FIG. 6. Effect of GSH + H_2O_2, NOR-3, or GSNO on CaCaM binding to triads. Triads (1 mg/ml) were incubated for 15 min at 25°C with either 4 m*M* GSH plus 100 μ*M* H_2O_2, 125 μ*M* NOR-3, or 250 μ*M* GSNO. After the reaction was stopped, samples were assayed for equilibrium calmodulin binding in a high Ca^{2+} buffer (1 m*M* EGTA and 1.2 m*M* $CaCl_2$), as detailed in Materials and Methods. (**A**) Values of specific CaCaM binding to triads incubated with GSH + H_2O_2 (open triangles) or control triads (solid circles). (**B**) Scatchard analysis of experimental data plotted in A. (**C**) Values of specific CaCaM binding to triads incubated with NOR-3 (open circles) or control triads (solid circles). (**D**) Scatchard analysis of experimental data plotted in C. (**E**) Values of specific CaCaM binding to triads incubated with GSNO (open squares) or control triads (solid circles). (**F**) Scatchard analysis of experimental data plotted in E. Data are given as means ± SD of at least three independent experiments. Solid lines represent nonlinear data fit to a hyperbolic binding function with a single class of binding sites. Straight lines in Scatchard analyses were calculated from the nonlinear regression analysis of data illustrated in A, C, and E.

RyR1 S-*glutathionylation and/or* S-*nitrosylation decrease the apparent affinity of calmodulin binding to triads*

Calmodulin binds only to two skeletal triadic proteins, DHPR and RyR1 (39). Thus, we propose that RyR1 *S*-glutathionylation or *S*-nitrosylation increased the K_d of calmodulin binding to triads because the DHPR did not exhibit significant redox modifications. The SERCA1, the only other triadic protein significantly redox-labeled in this work, does not bind calmodulin (7, 13) and is not located at the site of DHPR/RyR1 interaction where calmodulin binds. These features make unlikely a participation of the redox-modified SERCA1 on redox modifications of calmodulin binding to triads. Both forms of calmodulin (apoCaM and CaCaM) bind to RyR1 and DHPR in mammalian skeletal muscle triads; remarkably, apoCaM enhances whereas CaCaM inhibits RyR1 (36, 37, 44). Accordingly, we studied both apoCaM and

CaCaM binding to redox-modified triads. We found that either *S*-glutathionylation or *S*-nitrosylation (promoted by GSH + H_2O_2 or NOR-3, respectively) decreased to the same extent the apparent affinity of apoCaM binding. After treatment with GSNO, however, triads displayed no detectable apoCaM binding, suggesting that joint *S*-glutathionylation and *S*-nitrosylation of different RyR1 cysteine residues abolished apoCaM binding. Comparable results were obtained when we analyzed CaCaM binding to triads; in this case, however, treatment of triads with the different redox agents decreased the binding of CaCaM to a lesser extent than the binding of apoCaM. The finding that GSNO treatment of triads did not abolish CaCaM binding, as it did with apoCaM binding, supports this proposal.

Taken together, these data suggest that both *S*-glutathionylation and *S*-nitrosylation of RyR1 hinder calmodulin binding to triads; they also suggest that these modifications do so in an independent fashion, because their effects appear to be additive. As proposed for FKBP12, these modifications may decrease calmodulin binding through an allosteric effect on the actual calmodulin binding site. The identity of the RyR1 cysteines modified by GSNO, which hinder calmodulin binding, remains to be determined. Likewise, the identity of the cysteines modified by GSH + H_2O_2, the other physiologically relevant redox agent used in this work, has to be established. It has been reported that NO•-induced *S*-nitrosylation of Cys3635 mediates CaM-dependent modulation of RyR1 by NO• (41). In contrast, GSNO *S*-nitrosylates RyR1 cysteines other than Cys3635, albeit the effects of GSNO-induced modifications on calmodulin binding have not been assessed (42).

The present results suggest that the activation of CICR kinetics produced by joint RyR1 *S*-glutathionylation and *S*-nitrosylation reported earlier (1) may be due, at least partially, to decreased interactions of RyR1 with CaCaM. Physiological modifications of RyR1 by redox agents such as the ones used in this work are likely to decrease both apoCaM and CaCaM binding to the junctional RyR1–DHPR complex. Furthermore, by binding to both DHPR and RyR1, calmodulin may coordinate the RyR1/DHPR interactions that determine skeletal type excitation–contraction coupling (39). Thus, endogenous redox modifications that decrease the affinity of calmodulin binding to triads may affect the excitation–contraction process in skeletal muscle.

Perspectives

Physiological conditions that enhance RyR1 *S*-nitrosylation through the intracellular generation of NO• or GSNO may decrease FKBP12 binding to RyR1; similarly, conditions enhancing RyR1 *S*-nitrosylation or *S*-glutathionylation, or both, may decrease calmodulin binding to the RyR1–DHPR complex. These redox modifications could lead to significant activation of RyR1 channel-mediated Ca^{2+} release in skeletal muscle fibers. Noteworthy, a recent study has shown that NO• donors decrease voltage-induced Ca^{2+} release and produce significant increases in resting Ca^{2+} concentration in mouse skeletal muscle fibers in a use-dependent fashion; as the function of neither the SERCA1 nor the DHPR was altered (33), these effects are presumably caused by RyR1 redox modifications. Accordingly, conditions of combined nitrosative and oxidative stress may result in removal of FKBP12 from RyR1 and of calmodulin from the RyR1–DHPR complex, causing substantial Ca^{2+} release from intracellular stores that may eventually lead to skeletal muscle cell death.

ACKNOWLEDGMENTS

This study was supported by FONDAP CEMC, grant 15600001, and by NIH AR050503.

ABBREVIATIONS

anti-CysSNO, rabbit polyclonal anti-*S*-nitrosocysteine antibody; anti-GSH, mouse monoclonal anti-glutathione antibody; apoCaM, apocalmodulin; BSA, bovine serum albumin; CaCaM, Ca^{2+}-calmodulin; CICR, Ca^{2+}-induced Ca^{2+} release; DHPR, dihydropyridine receptor; DTT, 2,4-dithiothreitol; FKBP12, 12-kDa FK506-binding protein; GSH, glutathione; GSNO, *S*-nitrosoglutathione; H_2O_2, hydrogen peroxide; HSR, heavy sarcoplasmic reticulum; MOPS, 3-(*N*-morpholino) propanesulfonic acid; NEM, *N*-ethylmaleimide; NO•, nitric oxide; NOR-3, (±)-(*E*)-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RyR1, type-1 ryanodine receptor/Ca^{2+} release channel; SDS, sodium dodecyl sulfate.

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Received for publication October 25, 2004; accepted January 19, 2005.

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