Redox Sensitivity of the Ryanodine Receptor Interaction with FK506-binding Protein*

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The ryanodine receptor (RyR) calcium release channel functions as a redox sensor that is sensitive to channel modulators. The FK506-binding protein (FKBP) is an important regulator of channel activity, and disruption of the RyR2-FKBP12.6 association has been implicated in cardiac disease. In the present study, we investigated whether the RyR-FKBP association is redox-regulated. Using co-immunoprecipitation assays of solubilized native RyR2 from cardiac muscle sarcoplasmic reticulum (SR) with recombinant [35S]FKBP12.6, we found that the sulfydryl-oxidizing agents, H2O2 and diamide, resulted in diminished RyR2-FKBP12.6 binding. Co-sedimentation experiments of cardiac SR vesicles with [35S]FKBP12.6 also demonstrated that oxidizing reagents decreased FKBP binding. Matching results were obtained with skeletal muscle SR. Notably, H2O2 and diamide differentially affected the RyR2-FKBP12.6 interaction, decreasing binding to ~75 and ~50% of control, respectively. In addition, the effect of H2O2 was negligible when the channel was in its closed state or when applied after FKBP binding had occurred, whereas diamide was always effective. A cysteine-null mutant FKBP12.6 retained redox-sensitive interaction with RyR2, suggesting that the effect of the redox reagents is exclusively via sites on the ryanodine receptor. K201 (or JTV519), a drug that has been proposed to prevent FKBP12.6 dissociation from the RyR2 channel complex, did not restore normal FKBP binding under oxidizing conditions. Our results indicate that the redox state of the RyR is intimately connected with FKBP binding affinity.

Ryanodine receptors (RyRs)2 are tetrameric intracellular Ca2+ channels that mediate the release of Ca2+ from the sarcoplasmic reticulum in muscle and nonmuscle cells (1). Three genes coding for mammalian RyRs have been identified: RyR1 in skeletal muscle, RyR2 in heart and brain, and RyR3 in a number of tissues. The deduced primary structure of all RyRs suggests a hydrophobic C terminus forming the channel pore, with the remaining ~80% being cytoplasmic. RyR channel activity is regulated by Ca2+, Mg2+, ATP, phosphorylation and redox status, and a number of accessory proteins.

The immunophilin, FK506-binding protein (FKBP), a receptor protein for the immunosuppressants, FK506 and rapamycin, is an essential component of the RyR-Ca2+ release channel complex in both skeletal and cardiac muscle (2, 3). RyR1 binds to both FKBP12 and FKBP12.6 with similar affinities (4), whereas RyR2 associates specifically with the FKBP12.6 isoform (3, 5). The stoichiometry of the association is four molecules of FKBP per RyR tetrameric channel (i.e. one FKBP molecule for each RyR protomer) (3, 6). Mapping studies have failed to identify a unique FKBP-binding site, with evidence presented for three distinct RyR regions (N-terminal, central, and C-terminal domains) (7–12), suggesting that FKBP interaction may be stabilized by multiple physical contacts and/or may be conformation-sensitive. The functional effects of FKBP association with RyR have been suggested to include stabilization of the full conductance state (7, 13, 14), channel closure (5, 6, 15), and coupled gating between neighboring channels (16, 17). These effects are highlighted in abnormal or disease states, where defective regulation of the RyR-FKBP association has been implicated in cardiomyopathy (18), cardiac hypertrophy (19), heart failure (7, 20), and exercise-induced sudden cardiac death (21).

The RyR functions as a redox sensor that is sensitive to channel modulators (22), and channel activity has been correlated with the number of free sulfydryl groups (23). In general, oxidizing reagents activate, whereas reducing reagents inhibit, channel activity (24, 25). Pharmacological sulfydryl-reactive reagents, including thimerosal, dithiopyridines, N-ethylmaleimide, and diamide, have been shown to activate skeletal and cardiac muscle RyRs (26–28). This effect was reversed by reducing reagents, such as dithiothreitol (DTT), and reduced glutathione. Glutathione, which constitutes the major redox buffer system in eukaryotic cells, inhibits the channel in its reduced form (GSH), whereas its oxidized form (GSSG) is stimulatory (29). Reactive oxygen species, such as hydrogen peroxide (H2O2) and superoxide anion radical, activate the channel (30–33). A sarcoplasmic reticulum (SR)-associated NAD(P)H oxidase coupled to superoxide anion radical production has been shown to modulate RyR-mediated Ca2+ release in skeletal and cardiac muscle (33–36). Oxidation-induced RyR activation is due to increased sensitivity of the channel to Ca2+ activation as well as a decrease in Mg2+ inhibition (24, 25).

Redox modulation of RyR channel activity may involve altered protein-protein interactions. Indeed, reactive sulfydryl groups mediate both intra- and intersubunit cross-links in

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2 The abbreviations used are: RyR, ryanodine receptor; DTT, dithiothreitol; FKBP, FK506-binding protein; IP, immunoprecipitation; SR, sarcoplasmic reticulum; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid.
RyR1 (27, 37), formation of high molecular weight complexes with triadin (38, 39), and calmodulin binding (32, 40–45). In this study, we present evidence that the critical interaction of the native skeletal and cardiac muscle RyR with FKBP12/12.6 is redox-sensitive.

**EXPERIMENTAL PROCEDURES**

**Materials**—Radioactive sulfur was obtained from Amersham Biosciences in the form of Pro-Mix containing ~70% L-[35S]methionine and 30% L-[35S]cysteine. Electrophoresis reagents were from Bio-Rad. CHAPS and rapamycin were obtained from Calbiochem, whereas all other reagents were from Sigma. Redox reagents were prepared at 100 mM stock solutions (in 20 mM Tris, pH 7.4), aliquoted, and stored frozen at −80 °C. H2O2 was always added fresh from 30% aqueous solution (stored at 4 °C). K201 (46), obtained from Aetas Pharma Co. Ltd. (Japan), was prepared at 1 mM stock solution (10% Me2SO in 10 mM Na2-Pipes, pH 7.4), aliquoted, and kept frozen at −20 °C.

**Plasmid Construction**—Preparation of plasmids expressing human FKBP12 and FKBP12.6, has been described previously (11). A double cysteine FKBP12.6 mutant (C23S/C77I) was generated using the QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. First, the C77I mutation was introduced using complementary oligonucleotide primers (forward, AGAGGGCGAAGCTGACAG; reverse, CTGTGTAGTGCACCACAG; and forward, CCAAGAAGGGCCAAACGTTGTGCACTACACAG; reverse, CTGTGTAGTGCACCACAG). Single and double mutant constructs were verified by direct DNA sequencing (BigDye; PerkinElmer Life Sciences).

**Cell-free Protein Expression**—In vitro cell-free protein expression was carried out using the TNT T7 quick coupled transcription and translation system (Promega). Reactions were carried out in 10-μl volumes by adding the TNT mix with 1 μg of plasmid DNA together with 1 μl (0.53 MBq or 14 μCi) of [35S]methionine (Pro-Mix; Amersham Biosciences). Reaction samples were incubated in a 30 °C water bath for 90 min and terminated by placing on ice. Hemoglobin was removed from the TNT reactions using one bed volume of Ni2⁺-nitriloacetic acid resin (Qiagen).

**Co-immunoprecipitation Assays**—Cardiac heavy SR vesicles (1 mg), prepared as described previously (12), were solubilized in 200 μl of IP buffer (20 mM Tris, 150 mM NaCl, 0.4% CHAPS, pH 7.4, and Complete protease inhibitors (Roche Applied Science)) by overnight incubation at 4 °C with continuous mixing. The insoluble material was pelleted at 20,000 g for 10 min at 4 °C, and the supernatant was withdrawn and treated with an appropriate redox reagent (2 mM DTT, 1 mM H2O2, 200 μM diamide) for 30 min at room temperature. In vitro synthesized, hemoglobin-free, radiolabeled FKBP was added and incubated for 6 h at 4 °C with continuous mixing. SR vesicles were recovered at 20,000 × g for 10 min at 4 °C, washed once, resuspended in SDS-PAGE loading buffer, heated at 85 °C for 5 min, and analyzed by SDS-PAGE and autoradiography.

**Western Blot Analysis**—Protein samples were resuspended in SDS-PAGE loading buffer (60 mM Tris, 2% SDS, 10% glycerol, 5 mM EDTA, 2% β-mercaptoethanol, 0.01% bromophenyl blue, pH 6.8) and heated at 85 °C for 5 min, and proteins were separated through a 4% SDS-polyacrylamide gel, strengthened with 0.5% agarose (47). Proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) using a semidry transfer system (Trans-Blot SD; Bio-Rad) in buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS) at 22 V for 4 h. The membrane was blocked with 5% nonfat milk protein in TBS-T buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.4). Primary antibodies were applied overnight at 4 °C: RyR1-specific antibody at 1:1000 dilution (Ab2142, rabbit polyclonal antibodies raised to unique RyR1 residues 4454–4474), RyR2-specific antibody at 1:1000 dilution (Ab1099, rabbit polyclonal antibodies raised to unique RyR2 residues 4454–4474). Immuno-reactive protein bands were visualized by ECL (Amersham Biosciences).

**RESULTS**

Glutathione-induced Aggregation of RyR and FKBP—Initially, we tested the effect of redox reagents (i.e. 2 mM DTT, 5 mM GSH, 2 mM GSSG, 1 mM H2O2, 200 μM diamide) on the solubilization of the RyR. Heavy SR vesicles were treated with...
redox reagents for 1 h at room temperature, pelleted to remove reagents, and then incubated overnight in IP buffer followed by a centrifugation step to remove insoluble material. We estimate that under our conditions, ~50% of the RyR is solubilized in control or DTT-treated samples. As shown in Fig. 1A, the sulfydryl-oxidizing reagents H₂O₂ and diamide reduces RyR solubilization from both skeletal and cardiac SR. This could be due to increased protein aggregation, since such oxidants have been shown to induce intra- and intermolecular disulfide bonds within RyR (37, 48) and with the SR integral protein triadin (38, 39). Surprisingly, RyR could not be solubilized from SR samples that had been treated with glutathione. No soluble RyR1 was detected from skeletal muscle SR treated with either reduced or oxidized glutathione or soluble RyR2 treated with GSH, whereas there was modest solubilization of RyR2 from cardiac SR treated with GSSG (Fig. 1A). We subsequently examined the effect of redox reagents on RyR, postsolubilization. Solubilized RyR remained soluble following treatment with DTT, H₂O₂, or diamide but became insoluble with GSH or GSSG (Fig. 1B). Some soluble RyR2 remained after treatment with GSSG.

We also tested for any glutathione effects on FKBP12/12.6. In vitro synthesized [³⁵S]FKBP12.6 was incubated with 5 mM GSH or 2 mM GSSG, in the presence or absence of 10 μM rapamycin, followed by centrifugation at 14,000 × g for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in SDS-PAGE loading buffer and analyzed by SDS-PAGE (15% gel) and autoradiography. An aliquot of the TNT reaction, equivalent to the volume processed as described above, was included in the first lane of the autoradiogram.

FIGURE 1. Glutathione induces RyR and FKBP aggregation. A, skeletal (200 μg) or cardiac heavy SR (1 mg) was treated with redox reagents (2 mM DTT, 5 mM GSH, 2 mM GSSG, 1 mM H₂O₂, 200 μM diamide) for 1 h at room temperature and recovered by centrifugation at 20,000 × g for 10 min at 4 °C. SR vesicles were incubated overnight in 200 μl of IP buffer (20 mM Tris, 150 mM NaCl, 0.4% CHAPS, pH 7.4) followed by centrifugation at 20,000 × g for 10 min at 4 °C to remove insoluble material. The soluble supernatant was withdrawn, the pellet was resuspended in 200 μl of IP buffer, and 40 μl of each was loaded on 4% SDS-polyacrylamide gels for Western blot analysis. B, skeletal (200 μg) or cardiac heavy SR (1 mg) was solubilized in the absence of any redox reagent as described above, and the insoluble material was removed. Solubilized SR was treated with redox reagents (2 mM DTT, 5 mM GSH, 2 mM GSSG, 1 mM H₂O₂, 200 μM diamide) for 6 h at 4 °C followed by centrifugation at 20,000 × g for 10 min at 4 °C. The soluble supernatant was withdrawn, the pellet was resuspended in 200 μl of IP buffer, and 40 μl of each was loaded on 4% SDS-polyacrylamide gels for Western blot analysis. C, in vitro synthesized, hemoglobin-free [³⁵S]FKBP12.6 in IP buffer was incubated for 6 h at 4 °C with 5 mM GSH or 2 mM GSSG, in the presence or absence of 10 μM rapamycin, followed by centrifugation at 14,000 × g for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in SDS-PAGE loading buffer and analyzed by SDS-PAGE (15% gel) and autoradiography. An aliquot of the TNT reaction, equivalent to the volume processed as described above, was included in the first lane of the autoradiogram.
and therefore the effect of the latter on RyR-FKBP association cannot be investigated. Also, in subsequent IP experiments, DTT, H$_2$O$_2$, and diamide were added once SR vesicles had been solubilized, in order to ensure that identical amounts of soluble RyR are processed in all IP samples examined.

**Sulfydryl-oxidizing Reagents Weaken the RyR-FKBP Interaction**—The redox sensitivity of the RyR2-FKB12.6 interaction was tested by co-immunoprecipitation assays. Solubilized cardiac heavy SR was treated with an appropriate redox reagent (2 mM DTT, 1 mM H$_2$O$_2$, 200 mM diamide) for 30 min at room temperature followed by incubation with exogenous $[^{35}S]$FKBP12.6. RyR2 was immunoprecipitated with the isof orm-specific Ab$[^{995}]$, and the presence of co-precipitated $[^{35}S]$FKBP12.6 was analyzed by SDS-PAGE (15% gels) and autoradiography. Aliquots of the TNT reactions, 10 and 1% of the volume processed in co-IP assays, were included in the first two lanes of the autoradiogram.

**TABLE 1**

| Control DTT | H$_2$O$_2$ | Diamide |
|-------------|------------|---------|
| Atmosphere (n = 5) | 100 | 105.9 ± 8.6 | 77.1 ± 10.5 | 54.1 ± 7.1 |
| 2mM EGTA (n = 3) | 100 | 106 ± 8.4 | 92.9 ± 9.5 | 54.9 ± 6.8 |
| 100 mM CaCl$_2$ (n = 4) | 100 | 102.5 ± 5.1 | 75.1 ± 7.8 | 49.4 ± 5.8 |
| 2mM MgCl$_2$ (n = 4) | 100 | 106.7 ± 6.3 | 92.2 ± 9.8 | 52.7 ± 4.1 |

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The effects of H$_2$O$_2$ and diamide on the binding interaction could be due to cysteine(s) modification of the RyR2 and/or FKB12.6. In order to test whether the two FKB12.6 cysteine residues are involved, we generated the double mutant protein, FKB12.6$^{C23S/C77I}$. Cys$^{77}$ was replaced by Ile, the corresponding residue present in FKB12, whereas Ser was substituted for Cys$^{23}$ to preserve the size and polarity of the side chain at that position. Both cysteines are located on the outer surface of the protein, away from the FK506-binding pocket, and they have not been previously implicated in the interaction with the RyR. Using co-IP assays, we found that the C23S/C77I double mutant FKB12.6 retains binding to the RyR2 and that binding is weakened by H$_2$O$_2$ and diamide (Fig. 2B). FKB12.6$^{C23S/C77I}$ binding to RyR2 was 102.2 ± 7.8% for DTT, 74.7 ± 6.3% for H$_2$O$_2$, and 47.5 ± 7.1% for diamide compared with control (n = 5). These data with FKB12.6$^{C23S/C77I}$ are identical to data obtained for the wild-type protein (Table 1), suggesting that FKB12.6 cysteine modification is not directly involved in the interaction with RyR.

The redox sensitivity of the RyR2 interaction with wild-type FKB12.6 was also tested by co-sedimentation assays. The centrifugation-based binding assay is conducted in a detergent-free environment with the RyR resident in the SR membrane. Thus, the effect of redox reagents on RyR2 in its native conformation can be studied, as opposed to with solubilized RyR2, where normally inaccessible cysteine residue(s) may be exposed to redox reagents. Intact cardiac heavy SR was treated with an appropriate redox reagent (2 mM DTT, 1 mM H$_2$O$_2$, 200 µM diamide) for 30 min at room temperature followed by incubation with exogenous $[^{35}S]$FKBP12.6. SR vesicles were pelleted, and the presence of co-sedimenting FKB12.6 was analyzed by SDS-PAGE and autoradiography. We observed less FKB12.6 co-sedimenting with cardiac SR treated with H$_2$O$_2$ and diamide compared with control or DTT-treated SR (Fig. 3A). Cumulative data demonstrate that H$_2$O$_2$ decreased FKB12.6 binding by ~10% and diamide by ~35% (Table 2). These values are lower than those obtained by co-IP assays, which could be due to increased accessibility of cysteine(s) or modification of additional residue(s) in detergent-solubilized RyR2.

We also tested the redox sensitivity of the RyR1 interaction with FKB12 and FKB12.6 using co-sedimentation assays and skeletal muscle heavy SR. Binding of both FKB12 and FKB12.6 to RyR1 was reduced when skeletal muscle SR was treated with H$_2$O$_2$ and diamide (Fig. 3, B and C). FKB12 binding to RyR1 was 108 ± 1.5% for DTT, 85.3 ± 5.6% for H$_2$O$_2$, and 39.5 ± 2.9% for diamide compared with control (n = 3), whereas FKB12.6 binding to RyR1 was 104.8 ± 5% for DTT, 91.9 ± 5.2% for H$_2$O$_2$, and 63.8 ± 1.5% for diamide compared with control (n = 3). Thus, the RyR1-FKB12 interaction is more sensitive to redox conditions than with FKB12.6, which is possibly related to the lower FKB12 binding affinity for RyR1 compared with FKB12.6 (11, 49).

**Effect of H$_2$O$_2$ Depends on Activation State of the Channel**—We next examined whether the open or closed state of the RyR2 channel affects the redox sensitivity of the interaction with FKB12.6. The RyR is known to undergo conformational changes in its transition from the open to the closed state (50, 51). It is plausible that such conformational changes may expose cysteine residue(s), thereby providing additional targets for sulphydryl-oxidizing agents. Alternatively, conformational...
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changes may bury cysteine(s), thereby blunting the effect of oxidants.

In order to distinguish between these two possibilities, we carried out co-IP and co-sedimentation assays under conditions that should promote a predominantly open (100 μM CaCl2) or closed RyR2 channel (2 mM EGTA or 2 mM MgCl2). These results, summarized in Tables 1 and 2, show that DTT treatment is largely without any effect, whereas diamide reduced FKBP12.6 binding to the same extent (by ~50 or ~35%, depending on the assay), irrespective of the RyR2 activa-

### Table 2

| Redox-sensitive co-sedimentation of RyR2 with FKBP12.6 |
|-----------------------------------------------|
| SR vesicle co-sedimentation experiments to determine the redox sensitivity of the RyR-FKBP interaction were carried out as described in the legend to Fig. 3, followed by quantitation of the [35S]FKBP12.6 band by densitometric analysis of autoradiograms and normalization against control. |
| Control | DTT | H2O2 | Diamide |
|---------|-----|------|---------|
| Ambient (n = 5) | 100 | 105.5 ± 4.1 | 91.1 ± 3.9 | 64.6 ± 6.4 |
| 2 mM EGTA (n = 3) | 100 | 105.8 ± 5.0 | 99.6 ± 8.5 | 68.9 ± 9.5 |
| 100 μM CaCl2 (n = 3) | 100 | 101.2 ± 2.6 | 91.7 ± 1.3 | 65.9 ± 6.4 |
| 2 mM MgCl2 (n = 3) | 100 | 107.6 ± 7.1 | 101.8 ± 9.2 | 68.7 ± 5.8 |

FIGURE 3. Redox-sensitive co-sedimentation of RyR1/2 with FKBP12/12.6. Intact heavy SR was treated with an appropriate redox reagent (2 mM DTT, 1 mM H2O2, 200 μM diamide) for 30 min at room temperature, followed by incubation with in vitro synthesized, hemoglobin-free [35S]FKBP. SR vesicles were pelleted, and the presence of co-sedimenting FKBP was analyzed by SDS-PAGE (15% gels) and autoradiography. A, cardiac SR with [35S]FKBP12/12.6; B, skeletal SR with [35S]FKBP12; C, skeletal SR with [35S]FKBP12.6. Aliquots of the TNT reactions, 10 and 1% of the volume processed in co-sedimentation assays, were included in the first two lanes of the autoradiogram.
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K201 Does Not Restore RyR2-FKBP12.6 Association under Oxidative Conditions—K201, also known as JTV519, is a drug that has recently been proposed to stabilize RyR2 function by promoting FKBP12.6 association (53, 54). We tested whether K201 can restore FKBP12.6 binding to diamide-oxidized RyR2 using co-IP assays. Solubilized cardiac heavy SR was treated with 2 mM DTT or 200 μM diamide for 30 min at room temperature in the presence or absence of 10 μM K201, followed by incubation with exogenous [35S]FKBP12.6. RyR2 was immunoprecipitated with Ab1093, and the presence of co-precipitated [35S]FKBP12.6 was analyzed by SDS-PAGE (15% gels) and autoradiography. Aliquots of the TNT reactions, 10 and 1% of the volume processed in co-IP assays, were included in the first two lanes of the autoradiogram.

Table 3

| Control | DTT | H2O2 | Diamide |
|---------|-----|------|---------|
| %       | %   | %    | %       |
| 30 min (n = 4) | 100 | 101.8 ± 6.5 | 93.6 ± 4.4 | 92.9 ± 1.6 |
| 6 h (n = 4)    | 100 | 79.3 ± 3.6  | 94.9 ± 2.9  | 65.5 ± 6.5  |

DISCUSSION

The RyR and its accessory protein FKBP12.6 have attracted much attention in recent years because of their involvement in the pathogenesis of heart failure. It has been proposed that heart failure is caused by a chronic hyperadrenergic state that results in “hyperphosphorylation” of RyR2 mediated by cAMP-dependent protein kinase at a unique site (Ser2809), which in turn promotes the dissociation of FKBP12.6 from the RyR2 channel complex (7). Although specific details of this mechanism remain highly controversial (55–59), the loss of the stabilizing FKBP12.6 subunit is expected to result in a constitutively increased Ca2+ leak from the SR, leading to decreased SR Ca2+ content and increased diastolic Ca2+ release. However, additional or alternative mechanisms may account for FKBP12.6 dissociation from the RyR2 Ca2+ release channel complex. In the present study, we demonstrate that sulfhydryl-oxidizing agents may provide a novel form of regulation due to their ability to disrupt the RyR-FKBP interaction.

Using co-immunoprecipitation assays with detergent-solubilized RyR, we found that FKBP12.6 binding was decreased by ~25 or ~50% when RyR2 was treated with H2O2 or diamide, respectively (Fig. 1, Table 1). Identical results were obtained with a cysteine-null mutant version of FKBP12.6 (Fig. 2), indicating that the effect of the oxidizing reagents is via modification of one or more RyR2 cysteine residue(s). SR vesicle co-sedimentation assays (Fig. 3) confirmed that H2O2 and diamide reduce FKBP12.6 binding, although to a lesser extent (by ~10 and ~35%, respectively) (Table 2). Nevertheless, the two sets of data are compatible with each other and demonstrate that oxidized RyR2 has reduced binding affinity for FKBP12.6.

Treatment of RyR2 with DTT did not affect FKBP12.6 binding compared with control, despite DTT reducing any sulfhydryls that could have been oxidized by air exposure. However, this is not surprising, since oxidation of up to ~10 thiols from the physiological redox state (muscle O2 tension 10 mm Hg and 5 mM GSH) was shown to have little effect on channel activity (23).

H2O2 and diamide target disparate sulfhydryl(s) and/or a distinct number of sulfhydryl(s), since they decreased FKBP12.6 binding to a different extent. This conclusion is supported by the observation that H2O2 was almost ineffective under conditions that result in a closed RyR2 channel, whereas diamide was equally effective under open or closed conditions (Fig. 4). Differences in the action of H2O2 and diamide should be attributable to differences in redox chemistry, redox potential, and/or hydrophobic nature. Indeed, it has been shown that both agents induce RyR activation mediated by formation of intersubunit disulfide bonds, which increases with the more potent oxidizing agents (e.g. diamide), and the latter notably also induces additional intrasubunit cross-linking (27, 37, 48). A recent report also suggested that the type of redox modification differentially affects FKBP12 binding to RyR1, since RyR1 S-nitrosylation reduced binding affinity, whereas S-glutathionylation (induced by a combination of H2O2 and GSH) was without any effect (45). In the present study, H2O2 and diamide are expected to induce disulfide bond formation within the RyR rather than mixed sulfydryls with glutathione, whereas the effect of NO donors was not investigated.

Interestingly, H2O2 could not induce FKBP12.6 dissociation when this protein was already bound to RyR2 (Table 3). Thus, FKBP12.6 binding to the RyR2 protects the cysteine residue(s) reacting with H2O2, either directly, if such residue(s) are within the primary FKBP-binding site, or indirectly, by stabilizing a RyR2 conformation that occludes such residue(s). A similar observation has been made for calmodulin, with the bound cal-

FIGURE 4. Diamide, but not H2O2, induces FKBP12.6 dissociation from RyR2. Solubilized cardiac heavy SR was incubated with in vitro synthesized, hemoglobin-free [35S]FKBP12.6 and the RyR2 protein complex was immunoprecipitated with Ab1093. A redox reagent (2 mM DTT, 1 mM H2O2, 200 μM diamide) was then applied for 30 min at room temperature (A), or for 6 h at 4°C (B). Protein immunocomplexes were isolated, and the presence of co-precipitated [35S]FKBP12.6 was analyzed by SDS-PAGE (15% gels) and autoradiography. Aliquots of the TNT reactions, 10 and 1% of the volume processed in co-IP assays, were included in the first two lanes of the autoradiogram.
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modulin protein protecting Cys3615, thereby preventing RyR1 intersubunit disulfide bond formation and oxidation-induced activation (40, 41). In the case of FKBP12.6, we can only speculate about the identity of the protected cysteine(s). Sulphur atoms in the cytoplasm have been excluded from mediating the action of H2O2, and intramembranous cysteines have been implicated as the targeted residues (60), in particular the two cysteines within transmembrane segment M6 (nomenclature of the 10TM model (61)). We have previously presented evidence that FKBP12.6 binds at the C terminus of the RyR2, and we suggested that a Val-Pro-Leu-Val motif (amino acids 4594–4597 for human RyR2) within transmembrane segment M6 (nomenclature of the 10TM model (61)). We have previously presented evidence that FKBP12.6 binds at the C terminus of the RyR2, and we suggested that a Val-Pro-Leu-Val motif (amino acids 4594–4597 for human RyR2) within transmembrane segment M6 could constitute part of the FKBP-binding core (12). Therefore, it is plausible that the two cysteines within M6, which are the only predicted intramembranous sulphurcontaining proteins in the interaction with FKBP12.6.

In contrast to H2O2, diamide did result in partial FKBP12.6 loss from RyR2 (Table 3). Oxidation-induced FKBP removal is consistent with oxidation-induced RyR activation, since interaction with this accessory protein promotes channel closure (5, 6, 15). This raises the possibility that FKBP dissociation from the RyR channel complex may be a causative mechanism in oxidative stress-related disease and also in heart failure. Oxidative stress, a condition where the production of reactive oxygen species overcomes the scavenging effects of the antioxidant defense system and the associated intracellular Ca2+ overload, has been implicated in the genesis of various cardiac diseases, including ischemia-reperfusion injury, diabetic and catecholamine- and doxorubicin-induced cardiomyopathies, and the transition from cardiac hypertrophy to heart failure (62–64). A role for FKBP12.6 has been proposed in hypoxia-induced, RyR2-mediated Ca2+ signaling in vascular smooth muscle, since FKBP12.6 deficiency (gene knock-out or FK506 exposure) enhanced hypoxia-induced Ca2+ release (65). Heart failure is a condition characterized by increased activity of the sympathetic nervous system and increased catecholamine levels. Elevated catecholamine levels produce reactive oxygen species due to autoxidation, leading to cardiomyopathy as well as age-related neurodegeneration (62, 64, 66). It is noteworthy that a recent study reported on reduced RyR2-FKBP12.6 association in an animal model of heart failure that was corrected with the administration of an antioxidant (67).

In these studies, we used redox reagent concentrations believed to mimic the oxidative conditions caused by localized, highly reactive oxygen free radicals produced in certain pathophysiological responses (68). The intracellular level of H2O2 has been estimated to reach 100 μM in pathological cases, whereas diamine is a nonphysiological redox reagent. Under oxidative stress conditions, H2O2 is not the primary reactive oxygen species produced, since the extremely reactive superoxide anion radical (’O2−) is the most prominent and more toxic (68). The ‘O2− is converted to H2O2 through the action of superoxide dismutase, and the H2O2 can in turn give rise to the highly reactive hydroxyl radical (‘OH) in the presence of heavy metal ions (e.g. Fe2+). In addition, an SR-associated NAD(P)H oxidase coupled to ‘O2− production has been shown to regulate RyR channel activity (34–36, 69). It will be of interest to assess the Ry-R-FKBP association following stimulation of the NAD(P)H oxidase; however, these experiments are beyond the scope of the present work.

In conclusion, we have presented direct evidence that the fundamental RyR-FKBP regulatory protein interaction is redox-sensitive. Our results suggest that oxidative conditions may result in FKBP12.6 dissociation from the RyR2 channel complex, leading to altered Ca2+ release, and this phenomenon may contribute to aberrant Ca2+ signaling-mediated cardiac disease.

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