Evaluation of GSK2487213A on $[^3\text{H}]$Ryanodine Binding to RyR1.

Final Report

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Summary

The effects of GSK2487213A (213A) on RyR1 function was assessed via skeletal muscle heavy sarcoplasmic reticulum (HSR) [3H]ryanodine binding. 213A did not affect HSR ryanodine binding when Ca\(^{2+}\) was the sole RyR1 regulator. Stripping HSR of FKBP12.0, via treatment with FK506, increased Ca\(^{2+}\)-activated ryanodine binding. Exogenous FKBP12.0 alone or in combination with 213A and or reducing agents failed to reverse the effects prior exposure to FK506. Including FK506 in the HSR [3H]ryanodine binding buffer dose-dependently increased ryanodine binding. 213A did not alter the FK506 dose response. Treating HSR with the nitric oxide donor, NOR-3, did not substantially reduce the FKBP12.0 content of the HSR. Prior exposure to NOR-3 enhanced Ca\(^{2+}\) stimulated HSR ryanodine binding. 213A reversed the NOR-3 activation of RyR1. These results suggest that 213A reverses nitrosylation-induced activation of RyR1 and the inhibition does not occur via stabilizing the interaction between RyR1 and FKBP12.0.

Introduction

Ryanodine receptors (RyR) are endo/sarcoplamic reticulum (SR) resident Ca\(^{2+}\) selective channels that form the efflux pathway for the release of Ca\(^{2+}\) from the SR to initiate muscle contraction. Isoform 1 (RyR1) is the predominate form in skeletal muscle, RyR2 is the predominate form in the heart while RyR3 has a widespread distribution. The channels are regulated by numerous endogenous effectors including ions primarily Ca\(^{2+}\) and Mg\(^{2+}\), metabolites such as the adenine nucleotides and accessory proteins including calmodulin and the FK506-binding proteins (FKBP). The sensitivity of the channel to these regulators can be modified by post-translational modifications such as phosphorylation and oxidation/reduction.

FKBPs are a family of cis-trans prolyl isomerases. RyR2 binds the 12.6 kD FKBP isoform (FKBP12.6; aka FKBP1B and calstabin 2) while RyR1 binds FKBP12.6 and the 12 kD isoform (FKBP12.0; aka FKBP1A and calstabin 1) with similar affinities [15]. FKBPs are receptors for the immunosuppressant drugs FK506 and rapamycin. Incubation of RyRs with either drug can remove FKBP from the channels. The functional effects of FKBP12.0 association with RyR1 have been suggested to include coordinating gating between RyR1 channel monomers to stabilize the full conductance state [1], coordinating coupled gating between neighboring channels [12] and coupling RyR1 with the transverse-tubule voltage sensor [10].

The loss of FKBP12.0/12.6 from RyR1/2 has been implicated in a number of pathologies including skeletal [16] and cardiac dysfunction [13] in heart failure, catecholaminergic polymorphic tachycardia.
seizures [11], muscular dystrophy [5] and skeletal muscle fatigue [6]. Chronic administration of S107, a drug designed to stabilize RyR/FKBP interaction has been reported it improve skeletal muscle function in models of skeletal muscle fatigue [6] and muscular dystrophy [5]. RyR1 channels from drug treated individuals exhibited reduced S-nitrosylation and increased FKBP12.0 binding compared to channels from non-drug treated individuals. Functionally, S107 treatment reduced the fatigue-induced increase in RyR1 channel activity. Although these studies show that chronic administration S107 mitigated some of the biochemical channel modifications and may have modified RyR1 function, the target of the drug remains to be determined. Thus, the goals of this project were to determine whether GSK2487213A, referred to as 213A for the remainder of this report, has direct affect on RyR1 function and whether it increases RyR1 affinity for FKBP12.0.

Methods

Materials. Pigs were obtained from Clemson University or Valley Brook Farms (Madison, GA). [3H]Ryanodine was purchased from PerkinElmer Life Sciences. Unlabeled ryanodine and (±)-(E)-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexene amide (NOR-3) were obtained from Calbiochem. Tacrolimus (FK506) was purchased from Sigma-Aldrich. FKBP12.0 was purchased from Sigma-Aldrich or provided by Glaxo-Smith Kline (GSK).

Isolation of heavy sarcoplasmic reticulum vesicles. Skeletal muscle heavy SR vesicles (HSR) were prepared from porcine longissimus dorsi muscle [14]. Muscle (25g/100 mL) was homogenized in a Waring Blender (75% of full speed 2X30 sec with 30 sec rest) in ice-cold 0.1 M NaCl, 5 mM Tris maleate buffer, pH 6.8, and centrifuged for 30 min at 2,600g and 4°C. The supernatant was filtered through four layers of cheesecloth and centrifuged for 30 min at 15,000g and 4°C. Pelleted membranes were extracted in 1.1 M KCl, 5 mM Tris, pH 6.8, on ice for 60 min., centrifuged at 130,000g for 45 min at 4°C, and then resuspended in 0.3 M sucrose, 0.4 M KCl, 5 mM Tris, pH 6.8, buffer. SR was then centrifuged through a discontinuous sucrose gradient (22, 36, and 45% sucrose) at 130,000g for 16 hours at 4°C, and the heavy SR fraction was collected from the 36 and 45% sucrose interface. HSR vesicles were resuspended in a minimal volume (1-2 ml) of 0.3 M sucrose, 0.1 M KCl, 5 mM Tris, pH 6.8, frozen in liquid nitrogen, and stored at −80°C until use. All buffers contained a protease inhibitor mixture (1.0 µg/ml aprotinin, 1.0 µg/ml leupeptin, 1.0 µg/ml pepstatin, 0.2 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride).

Treatment of HSR with FK506. FK506 was dissolved at 10 mM in 100% ethanol and stored at -20°C until use. The protocol for removal of FKBP12 from HSR was based on work previously described by
Ahern et al [1]. HSR (2 mg/ml) was incubated for 15 minutes at 37°C in a buffer composed of 20 mM imidazole pH 7.4, 300 mM sucrose, the protease inhibitor mixture described in the previous section with 10 µM FK506 (FK506 treated) or 0.1% ethanol (control treated). SR was collected by centrifugation for 15 minutes at 4°C at 109,000 g (40,000 rpm) in a Beckman 70.1Ti. The pellet was resuspended in 300 mM sucrose buffer without FK506 and recentrifuged at 109,000 g. The washed pellet was resuspended in a minimal volume of 300 mM sucrose buffer without FK506, frozen in liquid N₂, and stored at -80°C until use.

Treatment of HSR with NOR-3. NOR-3 was dissolved at 45 mM in 100% dimethyl sulfoxide (DMSO) and stored at -20°C until use. Heavy SR (1 mg/ml) was incubated for 30 minutes at 25°C in buffer consisting of 150 mM KCl, 20 MOPS pH 7.0, 1 mM sodium EGTA, 0.93 mM calcium chloride (10 µM ionized Ca²⁺) with 0.2 mM NOR-3 (NOR-3 treated) or 0.44% DMSO (control treated). SR was collected by centrifugation for 15 minutes at 4°C at 109,000 g (40,000 rpm) in a Beckman 70.1Ti. The pellet was resuspended in 6 ml of sucrose buffer composed of 300 mM sucrose, 20 mM PIPES pH 7.0, the protease inhibitor mixture and centrifuged as again at 109,000 g. The pellet was resuspended in a minimal volume of 300 mM sucrose buffer, frozen in liquid N₂ and stored at -80°C until use.

GSK2487213A handling and storage. 213A was stored protected from the light in a desiccator at room temperature (21-23°C). Working solutions were made from an aqueous 10 mM stock solution. The stock solution was stored frozen at -20°C for no more than three days and thawed only once.

HSR [³H]ryanodine binding. [³H]Ryanodine selectively binds to the open RyR and therefore provides a useful indicator of channel activity [17]. HSR vesicles (0.2 mg/ml) were incubated at 37°C for 90 min in medium containing 150 mM KCl, 10 mM PIPES, pH 7.0, 100 nM [³H]ryanodine, and a Ca-EGTA buffer set to give the desired free Ca²⁺ concentration [8]. Estimates of maximal [³H]ryanodine binding capacity of each SR vesicle preparation were determined in medium that in addition contained 500 mM KCl, 5 mM ATP, and 100 µM Ca²⁺. Nonspecific binding was measured in the presence of 20 µM nonradioactive ryanodine and 0.01 µM Ca²⁺. [³H]Ryanodine binding is expressed as a percent of maximal [³H]ryanodine binding. Removal of FKBP12.0 from RyR1 appears to increase the rate of [³H]ryanodine binding, thus to increase the probability of detecting effects of FKBP12.0 on RyR1, the rate of SR vesicle [³H]ryanodine binding was slowed by incubation at room temperature (21-23°C) for 16 hrs. Binding media for the room temperature assays contained 150 mM KCl, 20 mM PIPES pH 7.0, 1.0 µg/ml aprotinin, 0.1 µg/ml leupeptin, 0.1 mg/ml bovine serum albumin and 20 nM [³H]ryanodine. In some experiments, described in the results section, SR was incubated with the reducing agent dithiothreitol (DTT) prior to addition to the assay buffer. Further, in some of these
experiments reduced glutathione (GSH) was included in the binding buffer. Estimates of maximal 
$[^3]H$ryanodine binding capacity of each SR vesicle preparation were determined in medium that in 
addition contained 500 mm KCl, 5 mM ATP, and 100 μM Ca$^{2+}$. Nonspecific binding was measured in 
the presence of 20 μM nonradioactive ryanodine, 10 mM MgCl$_2$ and 0.01 μM Ca$^{2+}$. SR vesicles were 
then collected by filtration on Whatman GF/B filters and washed with 8 ml ice-cold 100 mM KCl. After 
6 hrs incubation in scintillation fluid, radioactivity retained by the filters was determined by liquid 
scintillation counting using a Beckman LS-5000 scintillation counter.

**Electrophoresis and Western Blotting.** Forty micrograms of SR protein was denatured for 10 min at 
95° C in reducing sample buffer (12.5% v/v glycerol, 5% w/v SDS, 0.25% w/v bromophenol blue, 
2.5% (v/v) β-mercaptoethanol, 0.25 M Tris-HCl, pH 6.8) and separated by electrophoresis on a 5-20% 
sodium dodecyl sulfate polyacrylamined gel at 175 V using the BioRad Mini-Protean II system. 
Proteins were either visualized by staining with GelCode Blue (ThermoScientific, Rockford, IL) a 
coomassie based gel stain or transferred to polyvinylidene fluoride membranes using a buffer 
consisting of 192 mM glycine, 20% methanol and 25 mM Tris pH 8.3 at 30 V with a 0.1 A current limit 
for 18 hrs at 4° C. Voltage was then increased to 200 V for 1 hr with a current limit of 0.5 A. After 
transfer, membranes were cut at approximately the 75kD molecular weight marker. The membrane 
containing proteins >75kD was probed for RyR1. The membrane section containing proteins <75 kD 
was probed for FKBP12.0. Membrane were blocked for 60 min in phosphate-buffered saline, 0.1% 
Tween-20 (PBS-T) plus 5% bovine serum albumin (BSA) and washed 3X15 min in PBS-T. 
Membranes were probed overnight at 4° C with either mouse monoclonal anti-RyR antibody (clone 
34C, Iowa Developmental Studies Hybridoma Bank) at a dilution of 1:10,000 in PBS-T and 5% BSA 
or an affinity purified goat anti-FKBP12.0/12.6 antibody (R & D Systems AF4174) diluted 1:1,000 in 
PBS-T containing 5% BSA, and 0.5 M NaCl. The secondary antibodies is these experiments were a 
horseradish peroxidase conjugated goat anti-mouse IgG secondary antibody (12-249, Millipore) used 
at a 1:100,000 dilution for the RyR and a donkey anti-goat IgG (Santa Cruz sc-2020) also used at 
1:100,000. Antigen-antibody interactions were visualized using enchanced chemiluminence (GE 
Healthcare) and Kodak Biomax film.

**Analysis.** The Ca$^{2+}$ dependence of ryanodine binding was fit with Equation 1, which assumes a high 
affinity Ca$^{2+}$ binding site, which when bound will activate the RyR and a lower affinity Ca$^{2+}$ binding site 
which when bound will inhibit channel opening.
Equation 1:

\[ B = B_0 \left( \frac{[Ca^{2+}]}{([Ca^{2+]}+EC_{50})(1-\frac{[Ca^{2+]} }{([Ca^{2+]}+IC_{50})}+y_0.)} \right) \]

where \( B \) is the \([^3]H\)ryanodine bound, \( B_0 \) is the maximal \( Ca^{2+} \) stimulated \([^3]H\)ryanodine binding, \( EC_{50} \) and \( IC_{50} \) are the half-activating and half-inhibiting \( Ca^{2+} \) concentrations, respectively, and \( y_0 \) is the baseline \([^3]H\)ryanodine binding. The FK506 concentration dependence of HSR vesicle \([^3]H\)ryanodine binding were fit with a four-parameter Hill equation. Comparisons between two groups were made using Student t-test. Multiple comparisons were by one-way analysis of variance with a Holm-Sidak post-hoc test. Statistical analysis was performed using SigmaStat 3.0 (Systat Software, Point Richmond, CA). Data are reported as the mean±SEM. The level of significance was \( p<0.05 \).

**Results**

Initial experiments were designed to determine whether 213A affected RyR1 activity with \( Ca^{2+} \) as the sole regulator. The 213A concentration dependence of skeletal muscle HSR vesicle \([^3]H\)ryanodine binding were performed under our standard conditions (37° C for 90 minutes) in media containing either 1 or 30 \( \mu \)M free \( Ca^{2+} \).

Figure 1 shows that increasing buffer \( Ca^{2+} \) increased SR vesicle ryanodine binding, however, 213A, up to 100 \( \mu \)M, did not significantly alter binding. A control experiment shown in 1B demonstrates the well-characterized caffeine-induced increase in channel activation and the ruthenium red inhibition of the channel.

The effects of 213A on RyR1 regulation were further investigated by examining the \( Ca^{2+} \)-dependence of HSR \([^3]H\)ryanodine binding. Including 1 \( \mu \)M 213A in the binding media causes a small but non-significant reduction in the maximal \( Ca^{2+} \)-activated ryanodine binding (Figure 2, Table 1).
Further, addition of 213A to the assay had no effect on the Ca^{2+} concentration required to half-activate (EC_{50}) or half-inhibit (IC_{50}) the channel. In contrast, 5 mM caffeine increased while 100 µM ruthenium red reduced SR vesicle ryanodine binding (Figure 2, inset). Together these experiments indicate 213A has no significant effects on Ca^{2+} regulation of RyR1.

Next, the effects of removing FKBP12.0 from RyR1 on channel regulation were examined. As is clear from the representative Western blot shown in Figure 3, treating HSR with 10 µM FK-506 for 15 minutes at 37°C following by washing and centrifugation removed FKBP12.0 from the treated SR. SR treated in an identical manner without FK506 retained FKBP12.0.

To determine the functional effects of FKBP12.0 removal on RyR1 function, the Ca^{2+}-dependence of ryanodine binding by control and FK506 treated SR vesicles was determined. Initial studies, performed at 37°C for 90 minutes, failed to demonstrate an effect of FKBP12.0 removal on the Ca^{2+}-dependence of ryanodine binding (Figure 4). Slowing the rate of ryanodine binding by lowering the incubation temperature to room temperature (21-23°C; for 16 hrs) revealed a significant increase in the maximal Ca^{2+}-stimulated HSR ryanodine binding (Figure 5, Table 2) with no change in the Ca^{2+} EC_{50} or IC_{50}.

Activation of RyR1 by removal of endogenous FKBP12.0 from RyR1 should be reversible by the addition of exogenous FKBP12.0. Thus, addition of exogenous FKBP12.0 to the binding media should reduce ryanodine binding by FK506-treated HSR. Further, if 213A increases RyR1 affinity for FKBP12.0, this inhibition should occur at lower FKBP12.0 concentrations. Thus, experiments were undertaken to examine the effects 213A on the FKBP12.0 concentration dependence of FK506-treated HSR vesicle ryanodine binding.

<table>
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<tr>
<th>Extent of Activation (B0) (%)</th>
<th>Control Treated</th>
<th>FK506 Treated</th>
<th>p</th>
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<tr>
<td>EC_{50} (µM)</td>
<td>1.32±0.32</td>
<td>1.10±0.23</td>
<td>0.59</td>
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<tr>
<td>IC_{50} (µM)</td>
<td>83±20</td>
<td>92±19</td>
<td>0.76</td>
</tr>
<tr>
<td>y_0 (%Bmax)</td>
<td>0.04±0.5</td>
<td>0.5±0.6</td>
<td>0.58</td>
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binding. Surprisingly, FK506-treated HSR ryanodine binding was not reduced by the addition of exogenous FKBP12.0, either in the absence or in the presence of 1 µM 213A.

Both RyR1 and FKBP12.0 contain oxidizable cysteine residues, 100 in RyR1 and 1 in FKBP12.0. RyR1 FKBP12.0 binding is reduced under oxidizing conditions. In addition, binding of FKBP12.0 to RyR1 appears to protect a sensitive RyR1 cysteine residue from oxidative modification [19]. Prior to FK506 treatment, RyR1 is bound with FKBP12.0 and is unlikely to be oxidized at residues critical for the RyR1-FKBP12.0 interaction. However, the channel may be modified during the FK506 treatment or alternatively the exogenous FKBP12.0 may be modified. In an attempt to reverse any oxidative channel modification that may have occurred during the FK506 treatment, SR was incubated in 10 mM DTT for 30 minutes at room temperature prior to use. After addition to the binding buffer, HSR solution makes up 10% of the assay solution volume, thus the concentration of DTT in the assay buffer is 1 mM. Further, 1 mM reduced glutathione (GSH) was included in the binding media. As shown in Figure 7, treatment with DTT and GSH reduced HSR vesicle ryanodine binding in the absence and presence of FKBP12.0. However, FKBP12.0, up to 10 µM did not alter SR vesicle ryanodine binding, either in the presence or absence of DTT and GSH.

A similar experiment was performed with the addition of 1 or 2 mM GSH to the binding buffer and either incubating HSR with or without DTT (Figure 8). In this experiment, there was dose-dependent inhibition of ryanodine binding by the reducing compounds but no effect of FKBP12.0.
The FKBP12.0 used in the previous experiments was obtained from two different sources, Sigma-Aldrich and GSK. The Sigma-Aldrich FKBP12.0 was shipped in a solution containing 0.5 mM DTT and thus is unlikely to have been significantly oxidized. To determine whether reduction of GSK FKBP12.0 altered its ability to inhibit FK506-treated SR, FKBP12.0 was incubated in 2 mM DTT for 30 minutes prior to use in a binding assay. Reduced GSK FKBP12.0 did not inhibit FK506-treated SR vesicle ryanodine binding (Figure 9). The results presented in figures 6-9 suggest that an easily reversible oxidative modification of either RyR1 or FKBP12.0 does not underlie the lack of FKBP12.0 inhibition of the channel in these preparations.

As an alternative approach to determine whether 213A stabilizes FKBP12.0 binding to RyR1, the FK506 concentration dependences of SR vesicle ryanodine binding in media containing either no 213A or 1 µM 213A were compared (Figure 10). Including 213A in the binding media did not alter either the FK506 EC$_{50}$ (No 213A: 0.13±0.05 µM; 213A: 0.13±0.02 µM) or the Hill coefficient (No 213A: 1.19±0.47 213A: 1.48±0.34).

RyR1 nitrosylation has been reported to decrease the affinity of the channel for FKBP12.0 [1]. Therefore, we examined the effects NOR-3 on the HSR FKBP12.0 content and the Ca$^{2+}$-dependence of HSR ryanodine binding (Figure 11, Table 3). NOR-3 treatment did not substantially reduce the FKBP12 content of our HSR preparations, it did however, enhance Ca$^{2+}$-activated HSR ryanodine binding. Although the Ca$^{2+}$-dependence of control treated HSR was not affected by including 1 µM 213A in the binding buffer, 213A reduced Ca$^{2+}$-activation of NOR-3 treated RyR1 such that there was no difference between the Ca$^{2+}$-dependence of ryanodine binding by NOR-3 treated HSR+123A and control treated HSR.

DISCUSSION

The primary goal of this project was to determine whether 213A affects the functional interaction between FKBP12.0 and RyR1. RyR1 channel activity was assessed via HSR vesicle [3H]ryanodine binding. Ryanodine binding to open RyR channels occurs with high affinity and selectivity and is
therefore a sensitive indicator of the number of open channels. Although changes in ryanodine binding are the results of regulatory ligand interaction with the channel, binding of the putative regulator to the RyR is not measured.

Initial experiments indicate that 213A, up to 100 μM did not affect RyR1 channel activation at low (3 μM) to moderate (30 μM) Ca²⁺. Further, 1 μM 213A did not significantly alter ryanodine binding at free Ca²⁺ between 0.01 and 10,000 μM. This is in contrast with the recently published effects of the related benzothiazepine K201 (JTV519). Blayney et al [7] reported that at 3 μM Ca²⁺, 0.1 μM K201 significantly reduced FKBP12-stripped RyR1 [³H]ryanodine binding. Binding assays were performed under similar conditions as used in experiments shown figures 1 and 2 (37° C for 90 minutes). While dephosphorylation or PKA phosphorylation of the channel did not alter the Ca²⁺ dependence of ryanodine binding, the authors report that K201 reduced Ca²⁺ activation of the channels regardless of the phosphorylation status. However, the effect on the phosphorylated channels was quite small and of questionable significance. Further, the use of paired t-tests on ryanodine binding data is unusual. Not only does the K201 data differ from the current 213A results, it appears to be inconsistent with itself. Figure 5A from that paper shows inhibition of ryanodine binding by 1 μM K201 but figure 5D shows no effect of 1 μM K201.

Almassy et al [2] examined the effects of K201 on RyR1 channels incorporated into planar lipid bilayers. In this work, channels were more fully inhibited by K201; 10 μM K201 reduced RyR1 open probability approximately 10% and channels were nearly closed by 100 μM K201. K201 also had the
interesting effect of inducing a channel sub-conductance state at all drug concentration examined. A characteristic of FKBP12.0 stripped RyR1 channels is the appearance of multiple sub-conductance states [9]. The lack of channel sub-conductance states in the control recordings strongly suggests that channels were bound with FKBP12.0. Thus, K201 had direct effects on RyR1 and the inhibition was not due to stabilizing the FKBP12.0- RyR1 interaction.

Removal of FKBP12.0 from our porcine HSR preparations significantly increased ryanodine binding at optimal Ca\(^{2+}\) concentrations when binding was performed at room temperature but not when binding was performed at 37\(^\circ\) C. However, the addition of exogenous FKBP12.0 did not reduce ryanodine binding. This is surprising given that recombinant human FKBP12.0 has previously been shown to inhibit ryanodine binding by FK506-treated porcine SR under virtually identical conditions (150 mM KCl, 20 mM PIPES pH 7.0, BSA and protease inhibitors, 20 nM \[^{3}\text{H}\]ryanodine, 16 hr room temperature incubation; Guo et al, 2010 Supplemental Data). The amino acid sequence of the human and rabbit FKBP12.0s used are identical and differ only slightly from the pig FKBP12.0 (see sequence comparison below). The amino acid sequence of the human and rabbit FKBP12.0s used are identical and differ only slightly from the pig FKBP12.0 (see sequence comparison below). The one difference major difference in the assay conditions was the use of 5 mM GSH in the Guo et al study and only 1-2 mM GSH in the current work.

<table>
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<tr>
<th>Human – Pig FKBP12.0 Sequence Comparison</th>
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<tr>
<td>Human/Rabbit  MGVQETISPJDGRTPKRGQTCVHYTGMLGEDKKFDSSRDNKPFKFMLGKQEVIRGW 60</td>
</tr>
<tr>
<td>Pig          MGVQETISPJDGRTPKRGQTCVHYTGMLGEDKKFDSSRDNKPFKFVLGKQEVIRGW 60</td>
</tr>
<tr>
<td>Human/Rabbit  EEGVAQMSVQRKLETISPDYAYGATGHPGIIIPPHATLVFDVELLKLE 108</td>
</tr>
<tr>
<td>Pig          EEGVAQMSVQRKLETISPDYAYGATGHPGIIIPPATLVFDVELLKLE 108</td>
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RyR1 contains 100 cysteine residues and FKBP12.0 contains a single cysteine, thus oxidation of either protein may have prevented channel inhibition by exogenous FKBP12.0. In general, oxidation activates RyR1 while reduction inhibits the channel. Binding of FKBP12.0 by RyR1 is sensitive to redox state and the interaction between FKBP12.0 and RyR1 appears to protect an oxidizable cysteine residue that is critical for FKBP12.0 binding [19]. It is possible that this critical cysteine residue is protected in our HSR preparations while endogenous FKBP12.0 is bound and is oxidized during the FK506 treatment. This would explain the channel activation by FK506 and the inability of exogenous FKBP12.0 to inhibit the channel. Attempts were made to reverse cysteine oxidation by incubating SR with DTT prior to use and by including GSH in the binding assay. The decrease in SR ryanodine binding under these conditions indicates these treatments reduced a number of cysteine residues. In spite of reduced conditions of the SR and assay buffer, the channel remained insensitive inhibition by exogenous FKBP12.0. The FKBP12.0 purchased from Sigma-Aldrich contains DTT and is unlikely to be oxidized. GSK FKBP12.0 pretreated with DTT and added to binding buffer containing GSH also failed to inhibit FK506-treated HSR. Channel phosphorylation has been suggested to
reduce FKBP12.0 binding to RyR1, however, this possibility is rejected as ATP is not present at any point in the experiment. A trivial explanation is the incomplete removal of FK506 from the HSR prior to use in the ryanodine binding assay. This is unlikely. Considering the small volume of HSR treated (~0.1 ml), the volume of washes (6 ml) and the greater than 10X dilution of the HSR into the ryanodine binding buffer the concentration of FK506 in the assay buffer would less than 0.1 nM. Thus, the more likely cause of the lack of exogenous FKBP12.0 inhibition is an irreversible modification of either the channel or the FKBP.

As an alternative to the FKBP12.0 add-back experiments, the effects of 213A on the FK506 concentration dependence of channel activation was investigated. The addition of FK506 to the assay buffer increased HSR ryanodine binding in a concentration-dependent manner. The activator occurred over approximately a 10-fold range of FK506 concentration. The dependence of ryanodine binding on FK506 concentration was not altered by the inclusion of 213A in the binding media suggesting 213A does not stabilize the binding of FKBP12.0 to RyR1.

Aracena et al [3,4] reported that incubation of SR with NOR-3 results in RyR1 S-nitrosylation, enhanced Ca$^{2+}$ activation of the channel and reduced affinity of the channel for FKBP12.0. Thus, we investigated the effects of 213A on NOR-3 treated SR ryanodine binding. Although we did not quantify RyR1 S-nitrosylation, our NOR-treatment protocol was based on that of Aracena et al [4] and likely resulted in channel nitrosylation. While we did not observe a substantial loss of FKBP12.0 from our NOR-3 treated SR preparations, we did observe the expected increase in Ca$^{2+}$-activated ryanodine binding. Therefore, the NOR-3 induced channel activation was independent of FKBP12.0 loss. Interestingly, 213A did not reduce ryanodine binding when RyR1 was activated by Ca$^{2+}$ alone but the drug did reverse NOR-3 activation of the channel. This preliminary finding should be confirmed by 213A ryanodine binding dose-response curves and RyR1 S-nitrosylation should be verified. Binding results should be replicated using a second methodology such as single channel recording. However, these experiments are beyond the scope of the current project. If these results are verified the findings would indicate that 213A may not affect RyR1 under basal conditions, but may blunt the effects of specific channel activators. This characteristic of 213A would be beneficial as it may target pathological rather than physiological activation of RyR1.
REFERENCES


