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Analysis of Type 1 Ryanodine Receptor-12 kDa FK506-Binding Protein Interaction

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Although dissociation of the 12 kDa FK506 binding protein (FKBP12)-type 1 ryanodine receptor (RyR1) complex by macrolide immunosuppressants is well documented, effects of many solutes and drugs have not been quantitated. In the current study, the influence of these on binding between solubilised RyR1 and an FKBP12-glutathione-*S*-transferase fusion protein was analysed using a novel assay. Association between these two proteins is stable, and is not greatly altered by changes in temperature, pH, cations, and endogenous solutes over physiological ranges. Ascomycin, an FK506 analogue, was identified for the first time as a drug which can disrupt the FKBP12–RyR1 complex. © 2001 Academic Press

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The opening of channels which release Ca^{2+} from intracellular stores, namely the ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$), is modulated by a plethora of accessory proteins (1). FKBPs (FK506-binding proteins) are peptidylpropyl-*cis-trans* isomerases which act as binding sites for the immunosuppressants macrocyclic lactones FK506 and rapamycin (2). FKBP12 is a ubiquitous, predominantly cytosolic ~12 kDa isoform reported to bind to both RyRs and $InsP_3Rs$, altering their channel activity. Such association was initially postulated from bio-informatic analyses (3) and subsequently verified biochemically, with four molecules of FKBP12 binding each tetrameric type 1 RyR (RyR1) complex (4). Complex formation is independent of isomerase activity, since enzyme-deficient mutant FKBP12 proteins can both bind to and regulate the function of the RyR1 (5, 27). A candidate FKBP12-binding site in the $InsP_3R1$, identified by yeast two-hybrid analysis, is a leucyl-propyl dipeptide motif highly conserved amongst Ca^{2+} release channels (6). Controversially, recent biochemical evidence indicates that both RyR1 and RyR3 isoforms bind to FKBP12 with high affinity, whereas $InsP_3R1$ and $InsP_3R3$ subtypes do not (7).

Interaction between FKBP12 and Ca^{2+} release channels can be disrupted by treatment with the microbial macrolides FK506 or rapamycin, thereby permitting examination of the role of the isomerase in regulation these ion channel complexes. Removal of FKBP12 from RyRs results in enhanced sensitivity to channel agonists (4, 8, 9). At a single-channel level, RyR1 channels stripped of the isomerase (10), or expressed heterologously in insect cells which lack FKBP12 (8), display subconducting states of prolonged open duration. Readdition of FKBP12 to these systems restores channel gating to predominantly fully open and fully closed states. It has been suggested that FKBP12 anchors calcineurin, a Ca^{2+}-dependent protein phosphatase, to cerebellar $InsP_3Rs$ and RyRs (11). FKBP12 might also facilitate intercomplex coupling of Ca^{2+} release channels (12), or inward rectification in the RyRs (13). The RyR2 isoform interacts specifically with a distinct isomerase, called FKBP12.6 (14), which might also anchor calcineurin to this complex (15).

In the current study, we have developed a novel assay to quantify the interaction between RyR1 and an FKBP12-glutathione-*S*-transferase (FKBP12GST) fusion protein immobilised on glutathione agarose beads. We have employed this technique to analyse the influence of both endogenous and pharmacological species on binding of these two proteins.

MATERIALS AND METHODS

Materials. FK506 was kindly donated by Fujisawa (GmbH, Germany). Rapamycin, ascomycin, and ivermectin were from Calbiochem. Glutathione sepharose 4B beads and pGEX-2TK expression vector were from Unitech (Pharmacia, Ireland). Polychlorinated biphenyls (PCBs) were purchased from Metlab Supplies (UK) and

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Molecular Probes SYPRO Red stain was from Cambridge Biosciences (UK). Chaps (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), pregnanolone (5β-pregnan-3α-ol-20-one), protease inhibitors, horse-radish peroxidase antibody conjugates, palmitoyl coenzyme A, cyclic ADP ribose, cycloheximide, S100, and calmodulin were obtained from Sigma (Ireland).

Preparation of skeletal muscle microsomes, FKBP12-glutathione-S-transferase (GST) and GST-only fusion proteins. Crude microsomal membranes were prepared from New Zealand white rabbit back-muscle as described previously (16). A cDNA encoding human FKBP12 was cloned and ligated into the pGEX2TK expression vector (Pharmacia), to create "FKBP12-pGEX2TK," as described previously by another laboratory (7). FKBP12-pGEX2TK and pGEX2TK plasmids were used to transfect Promega XL1-Blue competent cells according to the manufacturer's protocol. FKBP12GST and GST-only fusion proteins were isolated from bacterial cultures as described (7), except that these proteins were stored on glutathione sepharose 4B beads in FKBP12-wash buffer (FKBP-wash buffer: 300 mM sucrose, 170 mM NaCl, 0.1%$_{(w/v)}$ Chaps, 2 mM dithiothreitol, 20 mM Tris–HCl pH 7.4, and protease inhibitors: 2 mM iodoacetamide, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 0.5 mM PMSF) containing 0.05%$_{(w/v)}$ sodium azide. GST and FKBP12GST proteins were stable for at least 3 weeks at 4°C when stored in this manner.

Preparation of FKBP12GST–RyR1 complexes. Dissociation of RyR1 from the FKBP12GST fusion protein was analysed using an affinity purification procedure, modified from previously described protocols (7, 17). RyR1 was solubilised from skeletal muscle microsomes (2 mg protein/ml) by incubation in 8000 μl of 2%$_{(w/v)}$ Chaps, 500 mM NaCl, 2 mM dithiothreitol, 20 mM Tris–HCl pH 7.4, with protease inhibitors. After shaking (~100 rpm) at room temperature for 1 h, insoluble material was cleared by centrifugation at 113,000g_{max} for 60 min at 4°C (32,000 rpm, Beckman type 42.1 rotor). Solubilised proteins were diluted to 50 ml with FKBP-wash buffer, then incubated at 4°C for 14–16 h with 1000 μl of FKBP12GST or GST-only beads. Beads were pelleted by centrifugation at 200g_{max} for 2 min, then resuspended in 12 ml of FKWB. The resuspended beads were divided into 1 ml (2 μg of fusion protein) aliquots in 1.5 ml Eppendorf tubes immediately prior to assay of RyR-FKBP12GST dissociation.

Analysis of FKBP12GST–RyR1 interaction. Typically, aliquots (1 ml) of RyR-FKBP12GST beads in FKBP-wash buffer were incubated with test agents for 2 h at 37°C with shaking (~100 rpm), covered in aluminium foil in order to prevent exposure to light. Beads were subsequently pelleted at 11,600g_{max} for 20 s (12,000 rpm, Eppendorf microfuge), the supernatant removed by aspiration, then the beads were resuspended in 1 ml of FKWB. Centrifugation and resuspension were repeated three more times, then 40 μl of 1× SDS–PAGE loading buffer (16) was added to each tube. For analysis of the effect of Ca^{2+} or Mg^{2+} on RyR-FKBP12GST interaction, 100 μM EGTA was added as a buffer and free cation concentrations were estimated using the CABAD program (18). For pH titrations, 20 mM Tris–HCl pH 7.4 in FKBP wash buffer was replaced by the following buffer systems: pH 4.5, pH 5.0, 50 mM sodium acetate-acetic acid; pH 5.5, pH 6.0, pH 6.5, 50 mM MES-HCl; pH 7.0, pH 7.5, pH 8.0, 50 mM Tris–HCl; pH 8.5, pH 9.0, pH 9.5, pH 10.0, pH 10.5, 50 mM glycine-NaOH. In order to examine the effects of NaCl on RyR-FKBP12GST dissociation, appropriate concentrations of this salt were added to FKWB prepared without NaCl. The influence of temperature on this interaction was analysed by incubating reactions for 2 h on an MJ Research PTC-200 Peltier Thermal Cycler, using a gradient across the heating block. Reactions were placed on ice immediately after this incubation period prior to washing. All experiments were repeated at least three times in duplicate, with vehicle control reactions included in each where appropriate.

SDS–PAGE, immunoblotting, and protein quantitation. Samples were resolved on 7.5% (w/v) SDS–PAGE minigels (Atto Corp.) with 3% (w/v) stacking gels. Myofibrillar proteins were employed as high molecular weight standards, as described by Murray and Ohlendieck (19). Immunoblotting was performed as described previously (16). Protein concentrations were estimated by the method of Bradford, calibrated using bovine serum albumin as a standard. Protein bands on SDS–PAGE gels were detected by SYPRO-Red staining (Molecular Probes) and laser scanning fluorimetry using a Molecular Dynamics Storm 860 device. Fluorescent intensities of protein bands were quantified using ImageQuant version 1.2 software for MacIntosh computers (Molecular Dynamics). Individual protein bands were isolated using the "spotfinder" application within this program, by increasing the minimum threshold values for data collection within selected regions of gels.

Data analysis. Skeletal muscle RyR ("% RyR remaining") associated with FKBP12GST beads was normalised to a maximal, untreated control value using the following equation:

$$\%\ \text{RyR remaining} = \frac{(\text{band density of RyR/band density of FKBP12GST}) \times 100\%}{(\text{control RyR band density/control FKBP12GST band density})}.$$

This normalisation eliminates variation in results due to loading errors, differences in RyR content of distinct microsomal preparations, differences in SYPRO-Red staining and potential effects of various agents on FKBP12GST-glutathione bead interaction. Dose-response relationships were fitted using GraphPad Prism software, according to the Hill equation, normalised to 100% RyR bound:

$$\%\ \text{RyR remaining} = 100\ (1 - (1/(1 + EC_{50}/[\log X])^{H}))$$

Where [log X] is the logarithmic molarity of test substance; EC_{50} the concentration of test substance necessary to elicit half-maximal effect; and ^{H} the Hill coefficient of the dose-response relationship.

RESULTS AND DISCUSSION

Although association between FKBP12 and the RyR1 has been recognised for almost a decade, there has been limited characterisation of exogenous and endogenous factors which can regulate this interaction. In the current investigation, a simple, direct assay was developed to quantitate the binding of these two proteins. An FKBP12GST fusion protein immobilised on glutathione agarose beads was employed to investigate factors influencing binding between FKBP12 and RyR1 solubilised from rabbit skeletal muscle. In order to determine if this binding was specific and saturable, increasing quantities of solubilised muscle protein were added to a fixed amount (2 μg) of the fusion protein. Detectable quantities of a high apparent molecular weight protein (~500 kDa) could be affinity purified from 100 μg of solubilised microsomal protein (Fig. 1A). Fusion protein beads were saturated with this ~500 kDa polypeptide by addition of 2000 μg of solubilised material. The affinity purified protein was identified as the RyR1 by immunoblot of a parallel gel (Fig. 1B) using the isoform-specific rabbit antiserum pAb 2141 (20). No detectable RyR1 was precipitated using GST-agarose beads alone (Figs. 1A and

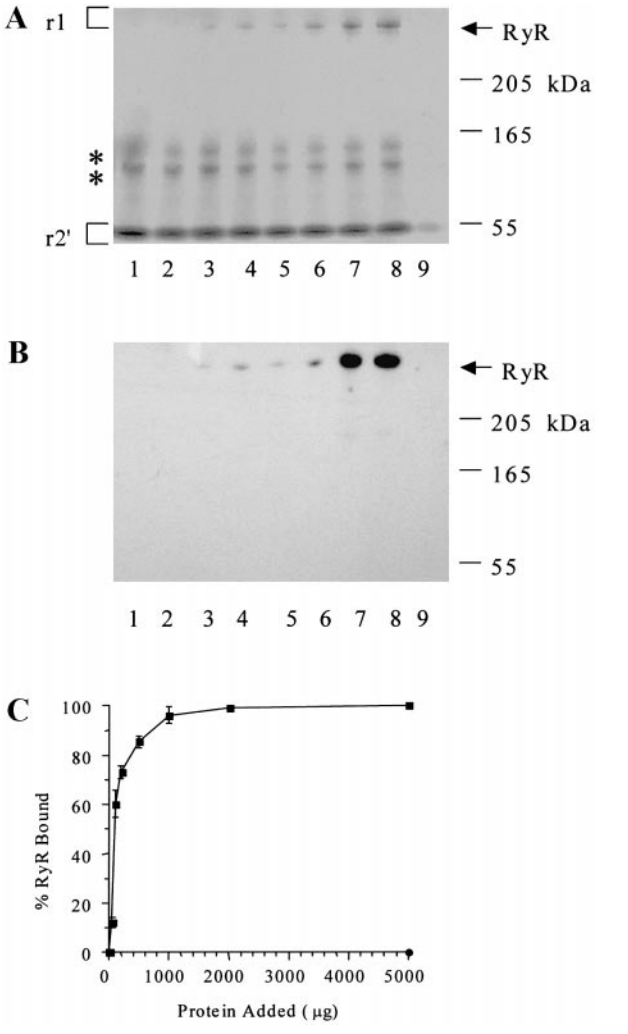

FIG. 1. FKBP12GST specifically interacts with RyR1 from skeletal muscle. Increasing amounts of protein solubilised from rabbit skeletal muscle microsomes were incubated with 2 μg of FKBP12GST fusion protein (lanes 1 to 8), or GST alone, immobilised on agarose beads. Following washing, proteins precipitated using this technique were resolved on SDS–PAGE gels, then stained with SYPRO-Red (A) or transferred onto nitrocellulose and detected with a RyR1-specific antiserum (B). The polypeptides marked by an asterisk (A) represent aggregates of the FKBP12GST fusion protein. Fluorescent staining intensities of proteins in regions "r1" and "r2′" were determined in order to estimate the relative amount of RyR precipitated from these solubilised microsomes. These data were normalised to the maximum amount of RyR isolated and plotted against the amount of solubilised protein added (C, mean ± SEM, $n = 4$). Amounts of solubilised protein added were: lane 1, 0 μg; lane 2, 50 μg; lane 3, 100 μg; lane 4, 200 μg; lane 5, 500 μg; lane 6, 1000 μg; lane 7, 2000 μg; lanes 8 and 9, 5000 μg.

1B). RyR protein bands within region "r1" of the SYPRO-Red stained gel, were digitally isolated, their densities measured and then divided by the densities of corresponding FKBP12GST bands in region "r2′". These ratios were normalised to the maximal value, then were plotted as a graph (Fig. 1C). In addition to a major polypeptide migrating close to the dye-front of these gels, the fusion protein alone contained two additional protein bands of ~72 kDa and ~110 kDa (Fig. 1A, asterisk). These probably represent dimers and trimers of the ~36 kDa fusion protein, since they are recognised on immunoblot by antibodies against GST, as well as by those recognising FKBP12 (data not shown). In agreement with this, formation of high molecular weight aggregates in SDS–PAGE gels by GST fusion proteins has been reported previously (21). Since RyR1 can be detected when affinity precipitated from microgram quantities of solubilised skeletal muscle microsomal protein, this technique might be employed to isolate these channel proteins from limited amounts of biological material, such as cultured cells, or tissue biopsies.

The amount of RyR1 associated with FKBP12GST beads was maximal at 4°C and decreased to 18.1 (± 3.7, $n = 3$)% (mean ± standard error in mean, n = number of experiments) of this value at 65°C (Fig. 2A). The effects of higher temperatures could not be investigated, since the interaction between FKBP12GST and glutathione beads became unstable. Temperature does not have a major effect over a pathophysiological range, with 70.9 (± 6.7, $n = 3$)% of the RyR remaining associated at 35.4°C compared with 45.0 (± 3.7)% at 41.7°C. Binding of the RyR1 to FKBP12GST displays a biphasic dependence of NaCl concentration (Fig. 2B). No association between these two proteins was observed in FKBP-wash buffer without added NaCl. Binding is optimal between 100 and 200 mM of NaCl, decreasing at greater concentrations of this salt. These results suggest that the RyR1–FKBP12 interaction depends on both hydrophobic and electrostatic bonding. The RyR1–FKBP12GST complex is not very sensitive to buffer conditions between pH 4.5 and pH 9.0, with marked dissociation only occurring under more basic conditions (Fig. 2C).

Numerous solutes and myoplasmic proteins which regulate the activity of the RyR1 channel (1) were tested for their ability to dissociate FKBP12. Ca^{2+}, which exerts a biphasic effect on the gating of these Ca^{2+} release channels, had no significant influence over the range tested (0.1 nM to 100 μM). Likewise, Mg^{2+} inhibits RyRs at low millimolar levels, but did not disrupt the FKBP12–Ca^{2+} release-channel complex at concentrations of between 100 nM and 5 mM. These results indicate that FKBP12 can interact with the RyR1 in both its open and closed channel conformations (7). Cyclic ADP ribose is a candidate second messenger which can activate ryanodine-sensitive Ca^{2+} release channels, as well as dissociating FKBP12.6 from RyR2 in pancreatic islet cells (22). This signalling molecule had no effect on RyR1–FKBP12GST binding over a 1 nM to 100 μM range. Palmitoyl coenzyme A is a major lipid metabolism intermediate, reported to cause

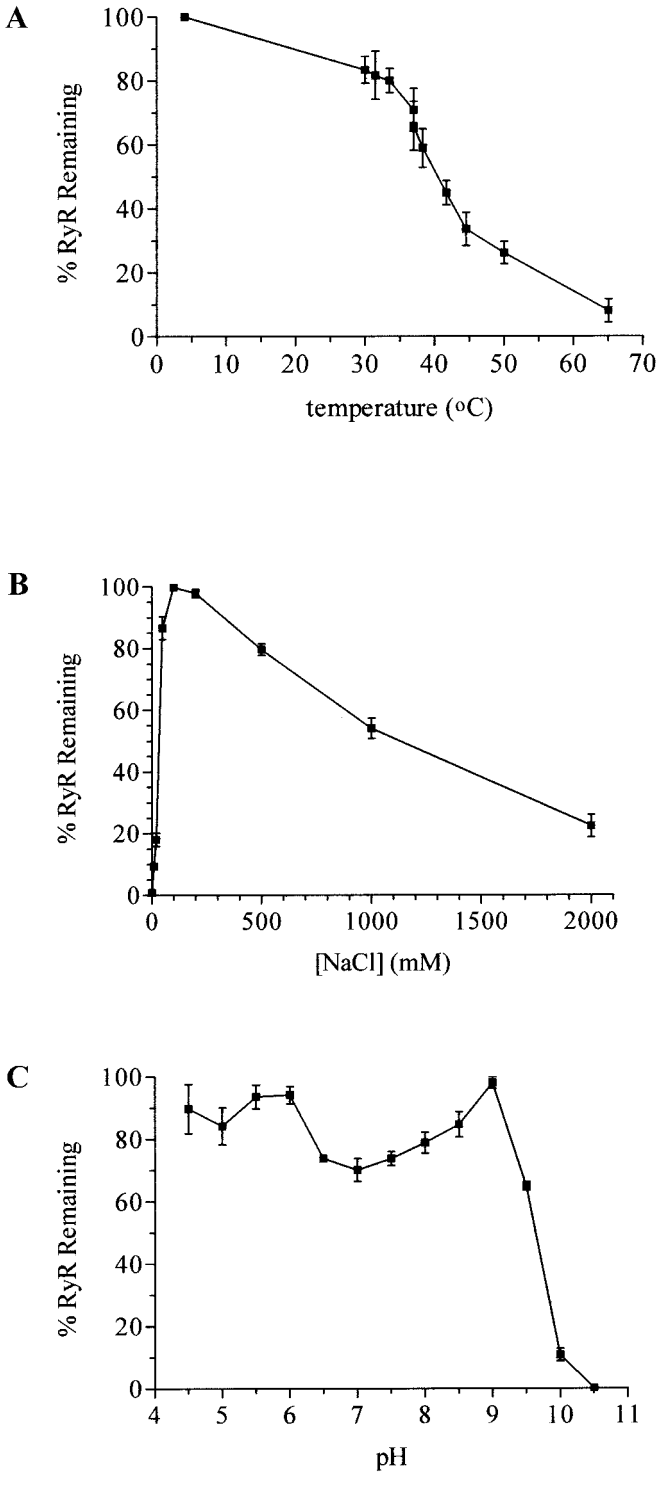

FIG. 2. Effects of temperature, sodium chloride, and pH on the RyR1–FKBP12GST complex. The influence of temperature (A), NaCl (B), and pH (C) on the interaction between FKBP12GST fusion protein and RyR1 solubilised from rabbit skeletal muscle microsomes was analysed. Data are normalised to maximum values and represent the mean of three separate experiments performed in duplicate. Error bars depict the standard errors in these mean values.

dissociation of FKBP12 from the RyR1 in rabbit skeletal muscle (23). However, this metabolite (0.5–200 μM) did not modify the binding of these proteins in the assay employed in the current study. Calmodulin and S100 are myoplasmic Ca^{2+} binding proteins which regulate the channel activity of Ca^{2+} release channels (1). Neither of these regulatory proteins (2–2000 nM) disrupted the RyR–FKBP12GST complex, with or without the addition of 10 μM free Ca^{2+}. It is possible that these candidate signalling species exert their effects via accessory proteins, absent in the assay system presented here. Indeed, efficacy of acyl CoA in stimulating the opening of the RyR1 channel is greatly enhanced by the presence of an acyl CoA-binding protein (23). Immunoblot investigations indicate that at least one regulatory protein, calcineurin, is absent from RyR1 complex isolated by Chaps-solubilisation and FKBP12GST affinity precipitation (manuscript in preparation).

The macrolide immunosuppressant FK506 causes an apparent increase in the dissociation of RyR1 from FKBP12GST. The apparent dissociation constant (k) was increased from 0.0029 ± 0.0012 min^{-1} (n = 3) in vehicle-treated controls, to 0.0317 ± 0.0034 min^{-1} (n = 3) in complexes treated with 10 μM FK506, corresponding to half-times of 324.0 ± 97.8 min and 25.4 ± 3.2 min, respectively. In steady-state assays, FK506 dose-dependently dissociated the RyR1–FKBP12GST complex, with an apparent half-maximal effective concentration (EC_{50}) of 18.7 ± 0.8 nM and a Hill slope (n^H) of −0.906 ± 0.027 (Fig. 3, n = 3). This EC_{50} value is considerably lower than previously reported (5). Rapamycin, another immunosuppressant macrolide, dissociates these proteins with an EC_{50} of 82.1 ± 8.7 nM, again a lower value than previously reported (10), with a Hill slope of −1.111 (± 0.142) (Fig. 3, n = 3). The reason for these discrepancies in EC_{50} values might be a consequence of comparing data obtained from complex, membrane based-models to the current isolated protein system. FK506 and rapamycin are known to interact with several components of the sarcoplasmic reticulum, including the sarcoplasmic/endoplasmic Ca^{2+}-ATPase and lipid constituents of the membrane (24). This binding could lead to an increase in the apparent half-maximal effective concentration for the action macrolide lactones on the FKBP12–RyR1 complex.

Ascomycin is an analogue of FK506, with an ethyl rather than an allyl substitution at position C21. A novel finding presented here is that ascomycin also dissociates the FKBP12GST–RyR1 complex, albeit with a higher EC_{50} value of 4362 ± 89.5 nM than FK506, with an n^H of −0.799 ± 0.065 (Fig. 3, n = 3). This increased EC_{50} value indicates the importance of hydrophobic side-groups in mediating this effect. All macrolides which stripped RyR1 from the FKBP12GST fusion protein did so with a negative Hill slope. This

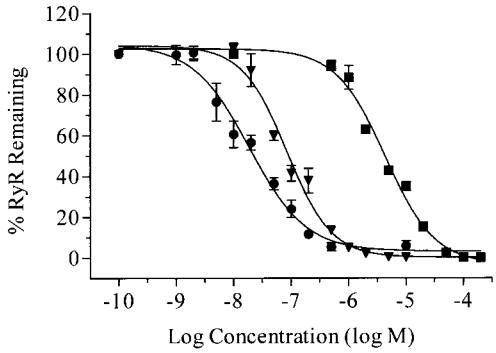

FIG. 3. FK506, rapamycin, and ascomycin cause dissociation of the RyR–FKBP12GST complex in a dose-dependent manner. RyR–FKBP12GST complexes were incubated for 2 h with different concentrations of FK506 (●), rapamycin (▼), or ascomycin (■). Relative amounts of RyR remaining associated were determined, normalised to the maximum value, then the mean values (± SEM, $n = 3$) were plotted against the logarithmic concentration of each macrolide.

indicates that the four FKBP12 binding sites (5) on this channel complex are allosterically coupled. The neutral plant alkaloid ryanodine also interacts with low affinity binding sites on RyR1 in a manner displaying negative cooperativity (25).

Ivermectin, a macrolide with antihelminthic properties, had no detectable effect on this complex at concentrations between 1 nM and 100 μM. Cycloheximide is reported to inhibit the isomerase activity of FKBP12 with micromolar efficacy (26). Data from another laboratory using FKBP12-mutants (27) indicated that isomerase activity was not necessary for the effects on RyR1 function. In agreement, cycloheximide (0.5–500 μM) did not promote FKBP12GST–RyR1 dissociation. Molecular modelling analysis has indicated that several steroid hormones could bind FKBP12 and were subsequently demonstrated to bind this isomerase with low micromolar affinity (28). This raises the possibility that steroid-like compounds could be endogenous modulators of the FKBP12–RyR1 complex. However, one of these compounds, pregnanolone (1 nM–100 μM), had no detectable influence on the binding between these proteins. Neither the RyR agonist caffeine (0.1–20 mM), nor its antagonist ruthenium red (0.5–200 μM), had detectable effects on the interaction between this channel and FKBP12, over pharmacological ranges used to modify the function of this Ca^{2+} release channel. Polychlorinated biphenyls (29) promote subconductance channel states in RyR1, which resemble Ca^{2+} release channels stripped of FKBP12 by treatment with macrolide immunosuppressants. However, in the study presented polychlorinated biphenyl congeners PCB4 (0.5–200 μM) and PCB95 (0.5–200 μM) did not cause isomerase-channel dissociation over concentration ranges known to exert such effects.

In summary, the assay described in this report has proved useful in characterising the interaction between FKBP12 and RyR1 in a quantitative manner. This technique will permit screening of pharmacological agents, signalling molecules and accessory proteins for their ability to influence binding between these two proteins, thereby modulating RyR1 channel activity. Such studies will aid the identification of structural features of exogenous reagents critical for causing FKBP12–RyR1 dissociation, as well as assisting in the discovery of endogenous which might regulate this interaction.

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