Disparities in the association of the ryanodine receptor and the FK506-binding proteins in mammalian heart

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Accepted 17 November 2011
Journal of Cell Science 125, 1759–1769
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doi: 10.1242/jcs.098012

Summary

The FK506-binding proteins (FKBP12 and FKBP12.6; also known as FKBP1A and FKBP1B, respectively) are accessory subunits of the ryanodine receptor (RyR) Ca^{2+} release channel. Aberrant RyR2–FKBP12.6 interactions have been proposed to be the underlying cause of channel dysfunction in acquired and inherited cardiac disease. However, the stoichiometry of the RyR2 association with FKBP12 or FKBP12.6 in mammalian heart is currently unknown. Here, we describe detailed quantitative analysis of cardiac stoichiometry between RyR2 and FKBP12 or FKBP12.6 using immunoblotting and [^{3}H]ryanodine-binding assays, revealing striking disparities between four mammalian species. In mouse and pig heart, RyR2 is found complexed with both FKBP12 and FKBP12.6, although the former is the most abundant isoform. In rat heart, RyR2 is predominantly associated with FKBP12.6, whereas in rabbit it is associated with FKBP12 only. Co-immunoprecipitation experiments demonstrate RyR2-specific interaction with both FKBP isoforms in native cardiac tissue. Assuming four FKBP-binding sites per RyR2 tetramer, only a small proportion of available sites are occupied by endogenous FKBP12.6. FKBP interactions with RyR2 are very strong and resistant to drug (FK506, rapamycin and cyclic ADPribose) and redox (H_2O_2 and diamide) treatment. By contrast, the RyR1–FKBP12 association in skeletal muscle is readily disrupted under oxidative conditions. This is the first study to directly assess association of endogenous FKBP12 and FKBP12.6 with RyR2 in native cardiac tissue. Our results challenge the widespread perception that RyR2 associates exclusively with FKBP12.6 to near saturation, with important implications for the role of the FK506-binding proteins in RyR2 pathophysiology and cardiac disease.

Key words: Ryanodine receptor, FK506-binding protein, Cardiac muscle, Heart failure, Arrhythmias

Introduction

Ryanodine receptors (RyRs) are tetrameric intracellular Ca^{2+} channels that mediate the sarcoplasmic reticulum (SR) Ca^{2+} release required for muscle contraction (reviewed by Zissimopoulos and Lai, 2007). The immunophilins, FK506-binding proteins (FKBP12 and FKBP12.6; also known as FKBP1A and FKBP1B, respectively), receptor proteins for the immunosuppressants FK506 and rapamycin, are essential components of the RyR complex in skeletal and cardiac muscle (Jayaraman et al., 1992; Timerman et al., 1996). FKBP12 and FKBP12.6 consist of 108 amino acids (including a cleaved N-terminal methionine) and share ~85% similarity. Their X-ray structures are nearly identical and unaltered in their drug complexes (Deivanayagam et al., 2000; Michnick et al., 1991; Van Duyne et al., 1991). Northern blot analysis has revealed transcripts for both isoforms in all mammalian tissues, with significantly higher FKBP12 levels (Lam et al., 1995).

Skeletal muscle RyR1 binds both FKBP12 and FKBP12.6 with similar affinities (Qi et al., 1998; Timerman et al., 1996); however, native RyR1 is isolated as a complex with the more abundant FKBP12. RyR1–FKBP12 association in skeletal muscle is common to all five vertebrate classes, i.e. mammals (rabbit), birds (chicken), reptiles (turtle), fish (salmon and rainbow trout) and amphibians (frog). The binding stoichiometry involves four FKBP12 or FKBP12.6 units per RyR1 tetramer, i.e. one FKBP–RyR1 subunit. The cardiac RyR2 associates preferentially with FKBP12.6, also with a 1:4 stoichiometry (Jeyakumar et al., 2001; Timerman et al., 1996).

FKBP12.6 is considered to inhibit RyR2 activity, mediating channel closure. These FKBP12.6 effects have been investigated in heterologous systems expressing recombinant RyR2 channels, where FKBP12.6 coexpression suppressed spontaneous or agonist-induced Ca^{2+} release (George et al., 2003; Goonasekera et al., 2005). FKBP12.6 deficiency in cardiomyocytes (upon FK506 or rapamycin treatment, or gene-targeted deletion) results in increased intracellular Ca^{2+} transients and contractions, and also increased Ca^{2+} spark frequency, amplitude and duration (McCall et al., 1996; Xiao et al., 1997; Xin et al., 2002). FKBP12.6 overexpression in transgenic mice results in reduced Ca^{2+} spark frequency, enhanced contractility and cardiac output (Gellen et al., 2008; Huang et al., 2006). Adenovirus-mediated FKBP12.6 overexpression in isolated cardiomyocytes results in increased electrically evoked Ca^{2+} transients and SR Ca^{2+} content, but decreased Ca^{2+} spark frequency, amplitude and duration (Gomez et al., 2004; Loughrey et al., 2004; Prestle et al., 2001). Although the increased Ca^{2+} transients upon FKBP12.6 overexpression seem to contradict the results for FK506 and rapamycin treatment (where increased Ca^{2+} transients also observed), the former were due to an increased SR Ca^{2+} content, most probably resulting from suppression of the spontaneous RyR2 activity. In contrast to intact cell systems, studies of isolated RyR2 (or SR) have produced conflicting

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results. Single-channel recordings of FKBP12.6-deficient RyR2 channels (upon FK506 or rapamycin treatment, or gene-targeted deletion) have indicated sub-conductance behaviour and increased channel activity (Kaftan et al., 1996; Wehrens et al., 2003; Xiao et al., 1997), consistent with the effects observed in cell systems. However, other studies using similar experimental approaches reported no effects upon FKBP12.6 removal or addition (Barg et al., 1997; Timerman et al., 1996; Xiao et al., 2007).

Regulation of RyR2 by FKBP12 is unclear. FKBP12-deficient mice have severe cardiac defects and die in utero or shortly after birth, whereas RyR2 channels isolated from these mice display sub-conductance behaviour (Shou et al., 1998). Adenovirus-mediated FKBP12 overexpression in isolated cardiomyocytes increases SR Ca^{2+} content and decreases Ca^{2+} spark frequency (Seidler et al., 2007). However, FKBP12 fails to modulate recombinant RyR2 in heterologous expression systems (George et al., 2003; Goonasekera et al., 2005) or in permeabilised cardiomyocytes (Guo et al., 2010).

At present, our understanding of RyR2 regulation by FK506-binding proteins is hindered by the absence of information on the levels of endogenous FKBP12.6 and/or FKBP12 associated with RyR2 in the heart. Here, we characterise the interaction between RyR2 and FKBP12 or FKBP12.6 in mouse, pig, rabbit and rat heart. Using FKBP12- and FKBP12.6-specific antibodies and recombinant FKBP12 and FKBP12.6 protein standards, we assess the relative amounts of both isoforms present in cardiac SR by western blotting. In combination with [^{3}H]ryanodine binding assays, we determine the RyR2:FKBP12 and RyR2:FKBP12.6 molar ratio and observe significant differences between species. Surprisingly, we find high levels of FKBP12 especially in mouse heart, while the FKBP12.6 association is sub-stoichiometric.

Results

Assessment of FKBP antibodies

Ab^{ABR} was raised against the human FKBP12 N-terminus (residues 1–13), whereas Ab^{126} was raised against recombinant human FKBP12.6. Ab^{ABR} detects recombinant human and mouse FKBP12 and FKBP12.6, expressed in HEK293 cells, equally well (supplementary material Fig. S1A), whereas Ab^{126} detects FKBP12.6 (both human and mouse) with minimal FKBP12 cross-reactivity (supplementary material Fig. S1B). The specificity and potency of both antibodies was calibrated using recombinant, purified human FKBP12 and FKBP12.6. As shown in supplementary material Fig. S1C, the detection sensitivity of Ab^{ABR} is very similar for both FKBP12 and FKBP12.6 indicating that this antibody is equipotent towards the two FKBP isoforms. However, Ab^{126} preferentially selects FKBP12.6, but can also detect FKBP12 with a >20-fold lower affinity (supplementary material Fig. S1D). Ab^{126} displays much higher immunoreactivity than Ab^{ABR} towards FKBP12.6.

Both FKBP12 and FKBP12.6 are associated with RyR2 in native cardiac tissue

Native FKBP12 and FKBP12.6 in cardiac SR from mouse, pig, rabbit and rat heart, analysed using Ab^{ABR} revealed very low levels of FKBP12 in pig and rabbit, and no clearly detectable signal in rat (Fig. 1A, lanes 1–3). Surprisingly, Ab^{ABR} detected very high levels of FKBP12 in mouse cardiac SR (Fig. 1A, lane 4). The mouse protein detected by Ab^{ABR} is consistent with FKBP12 because its electrophoretic mobility is identical to FKBP12 from rabbit skeletal muscle (lane 7) and recombinant mouse FKBP12 (lane 5), and lower than recombinant mouse FKBP12.6 (lane 6). Importantly, Ab^{ABR} failed to detect endogenous FKBP12.6 in cardiac SR from any species, suggesting that it has much lower expression relative to FKBP12. The specificity of Ab^{ABR} is demonstrated in supplementary material Fig. S2A, where FKBP12 immunoreactivity was subjected to competition analysis with purified recombinant GST–FKBP12. At 100 nM GST–FKBP12 effectively abolished the signal from most samples with low FKBP12 expression (pig, rabbit and rat cardiac SR, HEK293 cell homogenate) but did not fully compete out the immunoreactivity for the high FKBP12 levels in rabbit skeletal muscle SR and, to a lesser extent, mouse cardiac SR.

The relative abundance of FKBP12 in mouse heart SR was an unexpected finding that was consistently observed in two different mouse strains, C57BL6/J and BALB/c (not shown). Possibly, FKBP12 is expressed at much higher levels specifically in mouse heart. However, we found a lower abundance of FKBP12 in cardiac homogenates from mouse compared with that in pig and similar levels to that in rabbit (supplementary material Fig. S3). Notably, FKBP12 levels in rat cardiac homogenate were substantially lower than those in other species, consistent with the negligible amount found in rat cardiac SR. We should emphasise that with the exception of the mouse, where FKBP12 is concentrated on the SR, most of this protein is not SR-bound in pig, rabbit and rat heart.

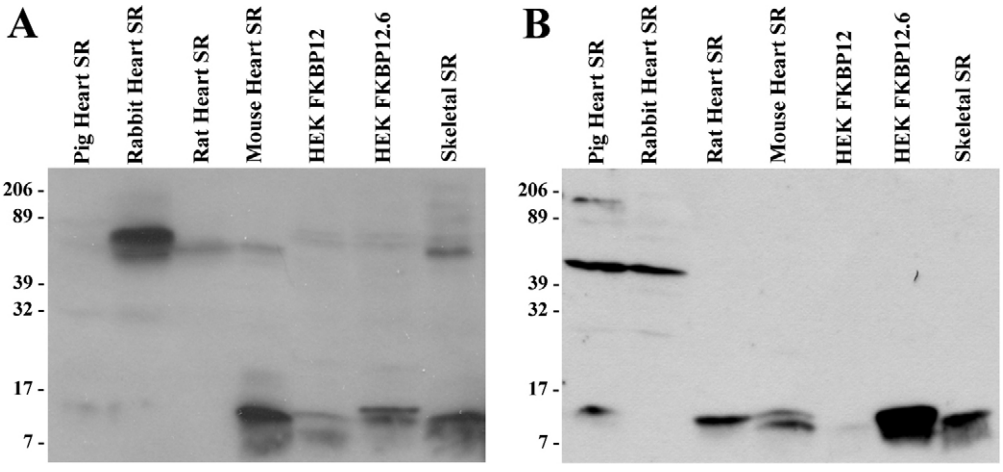

Fig. 1. Both FKBP12 and FKBP12.6 are present in mammalian heart SR. Western blot analysis of subcellular fractions using Ab^{ABR} (**A**) or Ab^{126} (**B**). The following amounts were loaded onto 15% SDS-PAGE gels: cardiac SR, pig (100 µg), rabbit (100 µg), rat (100 µg), mouse (100 µg), rabbit skeletal muscle SR [50 µg (A) or 100 µg (B)], HEK293 cell homogenate expressing mouse FKBP12 [100 µg (A) or 10 µg (B)] and HEK293 cell homogenate expressing mouse FKBP12.6 [100 µg (A) or 10 µg (B)].

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The presence of SR-associated FKBP12.6 was monitored using Ab^{126}. Fig. 1B shows low levels of FKBP12.6 in pig, rat and mouse (lanes 1, 3, 4), and it was undetectable in rabbit cardiac SR (lane 2). The protein recognised by Ab^{126} is consistent with FKBP12.6 because it has identical mobility to recombinant mouse FKBP12.6 (lane 6). Note that Ab^{126} detects a doublet in mouse heart SR (lane 4). The lower band has identical mobility to FKBP12 from rabbit skeletal muscle (lane 7) and recombinant mouse FKBP12 (lane 5), and therefore is attributable to the FKBP12 isoform. It is unlikely to be a FKBP12.6 degradation product because the whole procedure of SR preparation was carried out at 4℃ in the presence of protease inhibitors, whereas the doublet was consistently observed in different mouse cardiac SR preparations but never in pig, rabbit or rat preparations. Given that Ab^{126} has a low affinity for FKBP12 (supplementary material Fig. S1), the relative abundance of SR-associated FKBP12 in mouse heart might be much higher than that of FKBP12.6, as also indicated by Ab^{ABR} (Fig. 1A). Supplementary material Fig. S2B demonstrates Ab^{126} specificity, where FKBP immunoreactivity is competed out by 100 nM purified recombinant GST–FKBP12.6. We could not detect FKBP12.6 in cardiac whole-cell homogenates from all four species (not shown) suggesting that the very low levels of FKBP12.6 being expressed are concentrated on the SR.

The immunoblot analysis demonstrates expression of both FKBP isoforms in mammalian cardiac SR but not any direct RyR2 interaction. Association of RyR2 with FKBP12 and FKBP12.6 was monitored by co-immunoprecipitation experiments from CHAPS-solubilised cardiac SR. RyR2 was immunoprecipitated with Ab^{1093} and the presence of co-precipitated FKBP12 and FKBP12.6 was analysed by western blotting using Ab^{ABR} and Ab^{126}. Negative controls included addition of 20 μM rapamycin. Fig. 2A,B shows that endogenous FKBP12 (Ab^{ABR}-immunoreactive) was recovered in immunoprecipitated RyR2 samples from mouse and pig cardiac SR, but was nearly absent from negative controls (rapamycin-treated samples). We could not detect FKBP12 co-immunoprecipitated

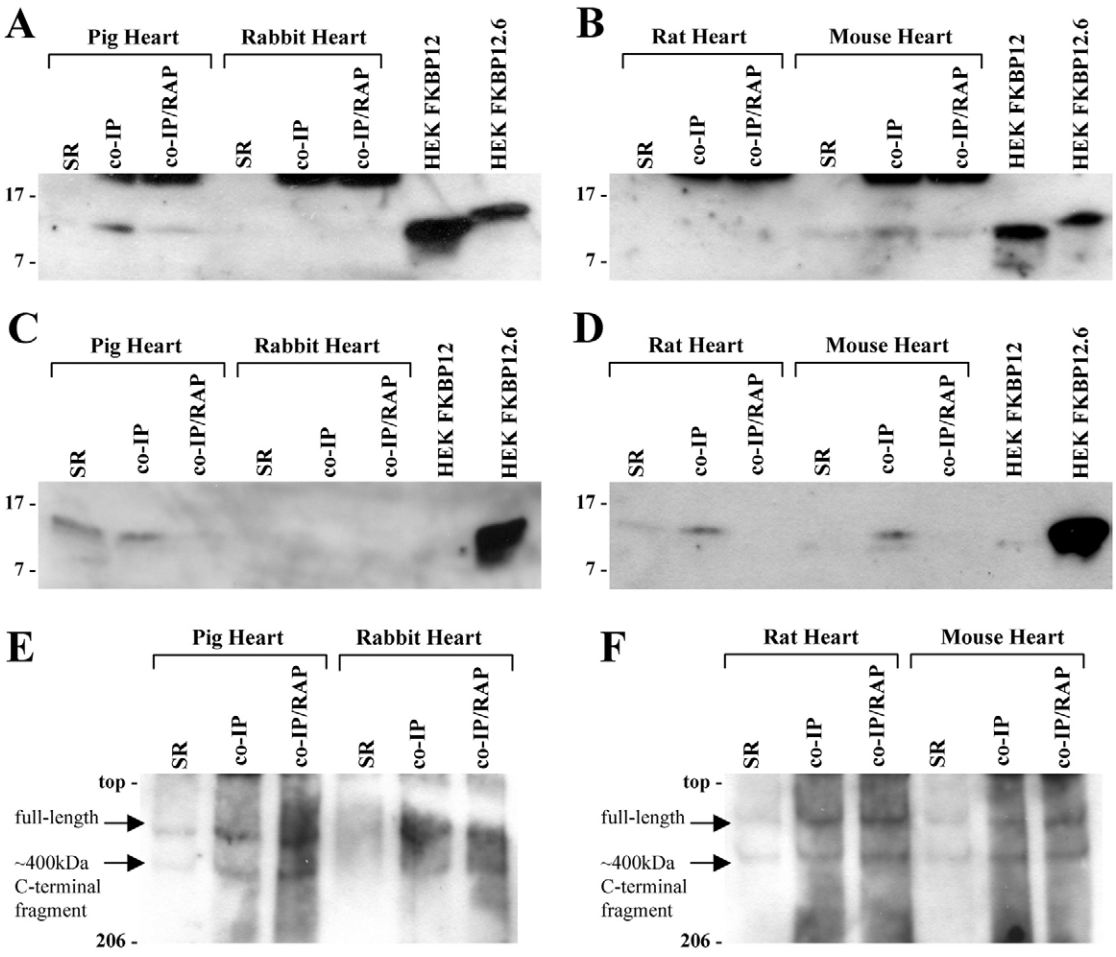

Fig. 2. Both FKBP12 and FKBP12.6 are associated with RyR2 in mammalian heart. Co-immunoprecipitation assays from pig and rabbit (**A**,**C**), or rat and mouse cardiac SR (**B**,**D**) and subsequent western blot analysis using Ab^{ABR} (A,B) or Ab^{126} (C,D). RyR2 was immunoprecipitated with Ab^{1093} from CHAPS-solubilised cardiac SR in the presence or absence of 20 μM rapamycin (RAP) and the presence of associated FKBP12 and FKBP12.6 were analysed by SDS-PAGE (15% gels) and western blotting. Cardiac SR, pig [25 μg (A) or 50 μg (C)], rabbit (50 μg), rat [50 μg (B) or 25 μg (D)], mouse [10 μg (B) or 25 μg (D)], and HEK293 cell homogenate expressing mouse FKBP12 [100 μg (A,B) or 10 μg (C,D)] or mouse FKBP12.6 [100 μg (A,B) or 10 μg (C,D)], were also included to serve as molecular mass standards. The intense dark bands at the top edge of the blots in panels A and B are the IgG light chain of the immunoprecipitating Ab^{1093}. The presence of precipitated RyR2 (a tenth of the immunoprecipitation sample) was analysed by SDS-PAGE (4% gels) and western blotting using RyR2 Ab^{1093} (**E**,**F**). Cardiac SR (10 μg) was also included to serve as a molecular mass standard. RyR2 is partially cleaved by the endogenous calpain protease to generate a ~400 kDa C-terminal fragment as indicated.

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with RyR2 from rat heart SR whereas in rabbit, a rapamycin-sensitive interaction was occasionally seen. This is not due to inefficient RyR2 immunoprecipitation; indeed, Ab^{1093} successfully immunoprecipitated RyR2 from all four species (Fig. 2E,F).

In parallel co-immunoprecipitation experiments examining RyR2–FKBP12.6 association, Ab^{126} detected endogenous FKBP12.6 in RyR2 immunoprecipitated samples from mouse, pig and rat cardiac SR in a rapamycin-sensitive manner (Fig. 2C,D). FKBP12.6 was not observed in RyR2 immunoprecipitated samples from rabbit heart SR, whereas in mouse, co-immunoprecipitated FKBP12 was also detected. However, native rabbit RyR2 is able to interact with exogenous [^{35}S]FKBP12.6, as shown by co-immunoprecipitation assays (supplementary material Fig. S4).

Assessment of RyR2:FKBP12 and RyR2:FKBP12.6 stoichiometry

We determined the molar ratio of FKBP12 and FKBP12.6 to RyR2 in heart SR by quantitative measurements on three different SR preparations for each species. RyR2 concentration was determined by [^{3}H]ryanodine-binding assays in the presence of 100 μM $CaCl_2$ and 10 mM caffeine to ensure maximal activation and therefore maximal ryanodine binding (Table 1).

To measure cardiac SR FKBP12 and FKBP12.6 levels, we performed extensive immunoblot and densitometry analysis using recombinant purified FKBP12 and FKBP12.6 as protein concentration standards. Representative blots are shown in Fig. 3 and cumulative data in Table 1. FKBP12 expression in rat cardiac SR and FKBP12.6 in rabbit was below the sensitivity of our detection system. Table 1 presents the derived RyR2:FKBP12.6 and RyR2:FKBP12 stoichiometry, which indicates there is a disparity in the RyR2 association with FKBP12.6 and/or FKBP12 that is species dependent. To validate our analysis we performed similar experiments with rabbit skeletal muscle SR and observed a RyR1:FKBP12 stoichiometry of 1:5.2 (Fig. 3; Table 1), near the expected 1:4 molar ratio.

Native RyR2–FKBP12 and –FKBP12.6 association is resistant to drug and redox treatment

To gain insights into the RyR2–FKBP12 and –FKBP12.6 association, we investigated the effects of drug treatment. Cardiac SR, incubated with an appropriate reagent [20 μM FK506, 20 μM rapamycin, 1 μM purified GST–FKBP12.6 or 100 μM cyclic ADP-ribose (cADPR)], was precipitated and the presence of associated FKBP12 and FKBP12.6 was analysed by western blotting using Ab^{ABR} and Ab^{126}. In initial experiments, no substantial FKBP12 or FKBP12.6 dissociation was apparent upon 30 minutes drug treatment (not shown). Surprisingly, even after 6 hours of incubation at room temperature with FK506 and rapamycin, there was minimal FKBP12 or FKBP12.6 dissociation from cardiac SR irrespective of species (supplementary material Fig. S5). Incubation with GST–FKBP12.6 or cADPR was also largely not disruptive. Fig. 4 presents cumulative densitometry analysis of three separate experiments for each species. Although cADPR resulted in ~20% FKBP removal in some species, the effect did not reach statistical significance ($P>0.05$). In contrast to cardiac SR, FKBP12 was readily removed from skeletal muscle SR by FK506, rapamycin and GST–FKBP12.6 but not by cADPR (supplementary material Fig. S5; Fig. 4).

The redox sensitivity of the RyR1 or RyR2 association with FKBP12 and FKBP12.6 was also investigated. Skeletal or cardiac SR was incubated for 30 minutes or 6 hours with the appropriate redox reagent (2 mM DTT, 1 mM H_2O_2 or 200 μM diamide). SR vesicles were precipitated and the presence of associated FKBP12 and FKBP12.6 was analysed by western blotting using Ab^{ABR} and Ab^{126}. Representative blots are shown in supplementary material Fig. S6, and Fig. 5 provides cumulative densitometry analysis of three independent experiments for each species. The oxidising reagent diamide resulted in rapid (by 30 minutes) FKBP12 dissociation from skeletal muscle SR, whereas H_2O_2 was less effective, requiring a longer treatment (6 hours). In cardiac SR however, FKBP12 and FKBP12.6 removal was minimal even after prolonged oxidation, irrespective of species.

RyR2 interaction with exogenous FKBP12 is negligible

The mouse FKBP12 sequence is different in three residues from human (or rabbit) with a unique Pro79Ser substitution (supplementary material Fig. S7). This potentially enables mouse FKBP12 to have higher RyR2-binding affinity, thus explaining the enhanced FKBP12 levels observed in mouse heart SR.

We examined the interaction of recombinant human FKBP12 and FKBP12.6 with native RyR2 from pig cardiac SR using co-immunoprecipitation, co-sedimentation and 'pull-down' assays. As a negative control we included rapamycin, which forms a complex with exogenous FKBP when incubated simultaneously, thereby preventing interaction with RyR2. [^{35}S]FKBP12 binding was undetectable in co-immunoprecipitation assays and extremely weak binding was observed in co-sedimentation assays, whereas [^{35}S]FKBP12.6 binding to cardiac SR was robust (Fig. 6A,B). By contrast, pull-down assays using GST–FKBP12 or –FKBP12.6

Table 1. RyR:FKBP12 and RyR:FKBP12.6 stoichiometry

SR source	FKBP12 (pmol per mg of SR)	FKBP12.6 (pmol per mg of SR)	RyR (pmol per mg of SR)	FKBP12:RyR	FKBP12.6:RyR
Rabbit skeletal muscle	37.7±2.7	–	7.2±0.4	5.2	–
Mouse heart	48.6±3.6	0.48±0.05	0.35±0.07	138.9	1.4
Pig heart	1.74±0.24	0.30±0.01	0.16±0.01	10.8	1.8
Rabbit heart	0.58±0.11	–	0.20±0.03	2.9	–
Rat heart	–	0.63±0.08	0.35±0.10	–	1.8

FKBP12 and FKBP12.6 levels were determined by western blotting as described in the legend to Fig. 3 followed by densitometry analysis and production of a standard curve using the purified FKBP12 and FKBP12.6 concentration standards. Three different SR preparations from each species were assayed in triplicate. The amounts of FKBP12.6 from rabbit heart SR and of FKBP12 from rat heart SR were beyond the sensitivity of our detection system. RyR levels were determined by [^{3}H]ryanodine binding in the presence of 100 μM $CaCl_2$ and 10 mM caffeine as described in Materials and Methods. Three different SR preparations from each species were assayed five times. Data are given as means±s.e.m.

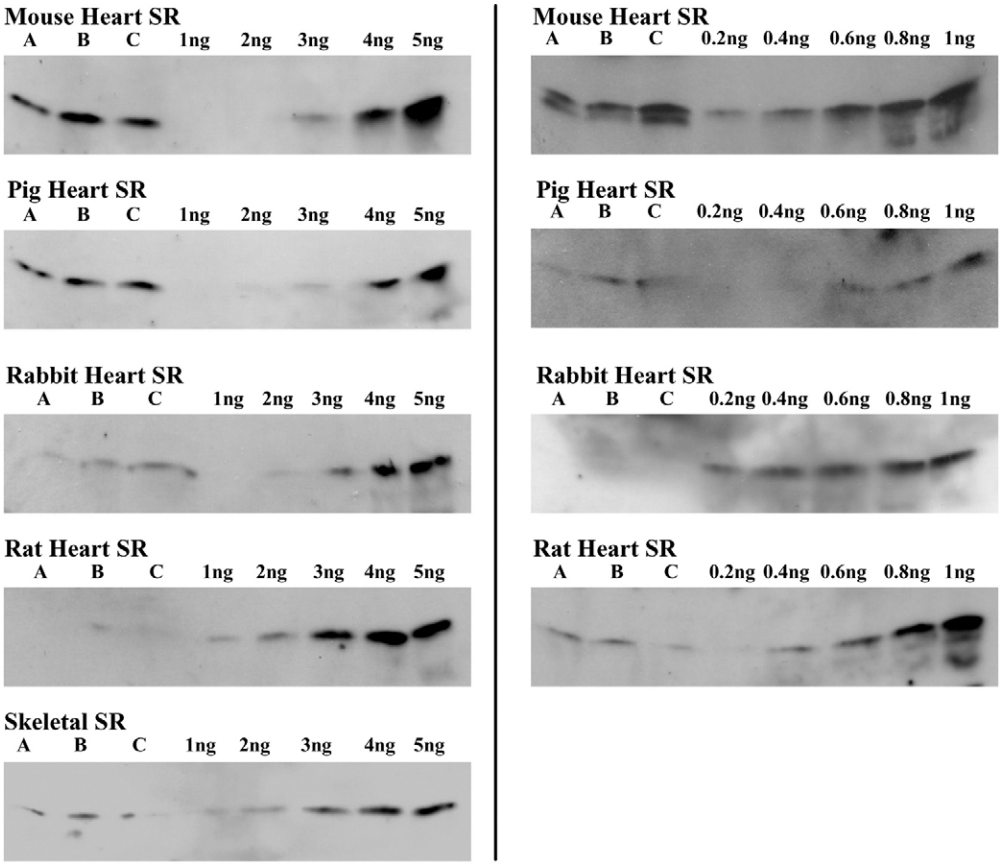

Fig. 3. Assessment of cardiac SR FKBP12 and FKBP12 levels. Western blot analysis using Ab^{ABR} (left panel) or Ab^{126} (right panel) of three different cardiac SR preparations (lanes A, B, C) for each species. The following amounts were loaded onto 15% SDS-PAGE gels: cardiac SR, pig (150 μg), rabbit (200 μg), rat [200 μg (left) or 50 μg (right)], mouse [7 μg (left) or 100 μg (right)], and rabbit skeletal muscle SR (5 μg). The indicated amounts of recombinant purified human FKBP12 and FKBP12.6 were also included to serve as concentration standards.

revealed that RyR2 interaction with GST–FKBP12 was comparable to that with GST–FKBP12.6 (Fig. 6C). Similar results were obtained with FKBP12 and FKBP12.6 fusions with a biotin-acceptor domain (BAD) (Fig. 6D). The protein fusion partner (GST or BAD) could be causing conformational changes that enable FKBP12 to structurally resemble FKBP12.6, thus endowing GST– and BAD–FKBP12 with a high-affinity RyR2 interaction.

Relative RyR2-binding affinities of exogenous human and mouse FKBP12 were assessed in mouse, pig, rabbit and rat cardiac SR using co-sedimentation assays. Cardiac SR was incubated with in-vitro-synthesised [^{35}S]FKBP12 for prolonged times (20 hours at room temperature) to enable exchange with endogenous FKBP12 or FKBP12.6. SR vesicles were precipitated and the bound [^{35}S]FKBP12 was analysed by SDS-PAGE followed by autoradiography (supplementary material Fig. S8), revealing very low RyR2 binding for both human and mouse [^{35}S]FKBP12, irrespective of the RyR2 species. Table 2 presents the cumulative data from densitometry analysis of four separate experiments. FKBP12 interaction is recorded as rapamycin-sensitive binding; FKBP12 bound in the presence of rapamycin (supplementary material Fig. S8, lanes 4, 8) was subtracted from total (supplementary material Fig. S8, lanes 3, 7), yielding a specific binding value. Specific FKBP12 binding is normalised against input [in-vitro-expressed protein (TnT 1/100th, supplementary material Fig. S8, lanes 2, 6)] to account for variable expression and/or [^{35}S]-labelling of the human or mouse FKBP12. This analysis indicates that mouse FKBP12 has a somewhat higher RyR2 affinity than human FKBP12. Notably, a very low RyR2 binding for both mammalian FKBP12 isoforms compared with that of FKBP12.6 was observed, irrespective of RyR2 origin (mouse, pig, rabbit or rat heart SR). These results suggest that the relatively high FKBP12 levels in mouse heart SR are not due to an enhanced RyR2 affinity for mouse FKBP12. Interestingly, rat cardiac SR, which has a miniscule amount of endogenous FKBP12 (Fig. 1A, Fig. 3), displayed the lowest binding of exogenous [^{35}S]FKBP12, indicating that rat RyR2 has a very weak affinity for this FKBP isoform. This suggests that very low binding of exogenous FKBP12 to heart SR from any species is not due to saturation by, or slow exchange with, the endogenous protein but an intrinsically low affinity for RyR2.

We further investigated whether exogenous FKBP12 binding occurs at the FKBP12.6 sites by performing co-sedimentation experiments using pig cardiac SR as above, but in the presence of purified GST–FKBP12.6 (supplementary material Fig. S9). GST–FKBP12.6 dose-dependently inhibited RyR2 binding of both human and mouse [^{35}S]FKBP12 (supplementary material Table S1), suggesting that exogenous FKBP12 and FKBP12.6 share a common or overlapping binding site(s).

Discussion

In this study, we report on the RyR2 association with FK506-binding proteins in native cardiac tissue. Our principal findings include: (1) RyR2–FKBP12.6 association is sub-stoichiometric, (2) FKBP12 is the predominant isoform associated with cardiac SR in most mammals, (3) RyR2–FKBP12 and –FKBP12.6 association is resistant to FK506, rapamycin and redox treatment, and (4) in skeletal muscle, the RyR1–FKBP12 association is easily disrupted under oxidative conditions.

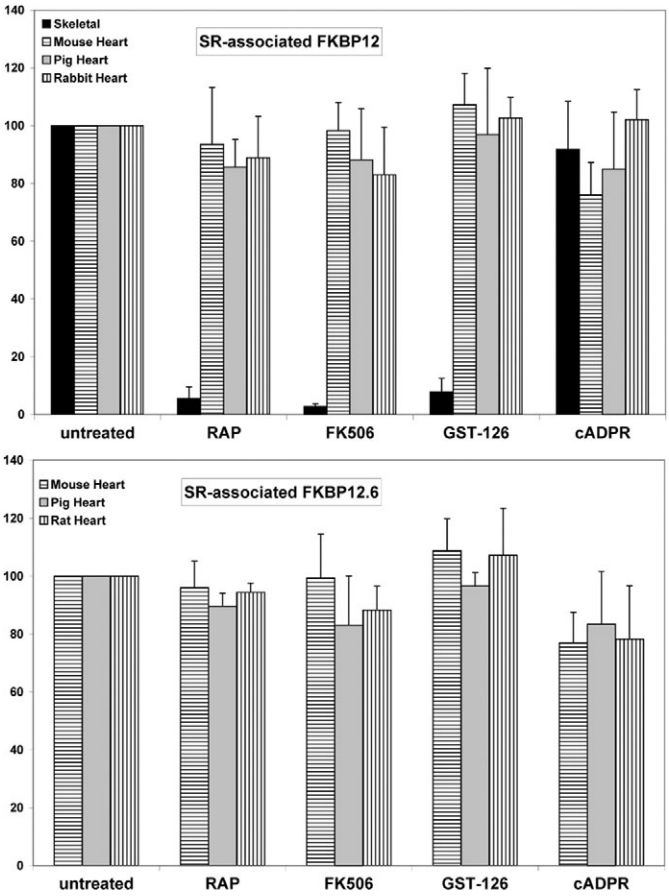

Fig. 4. Effects of drug treatment on RyR–FKBP12 and –FKBP12.6 association. SR vesicles, incubated with 20 μM rapamycin (RAP), 20 μM FK506, 1 μM purified GST–FKBP12.6 (GST-126) or 100 μM cADPR for 6 hours at room temperature were precipitated by centrifugation and the presence of associated FKBP12 and FKBP12.6 was analysed by SDS-PAGE and western blotting using Ab^{ABR} and Ab^{126}, respectively. Densitometry analysis ($n=3$) was carried out and SR-associated FKBP was normalised against control samples (untreated). The changes seen in rabbit skeletal muscle SR treated with rapamycin, FK506 and GST–FKBP12.6 are statistically significant ($P<0.01$). Data are given as means+s.e.m.

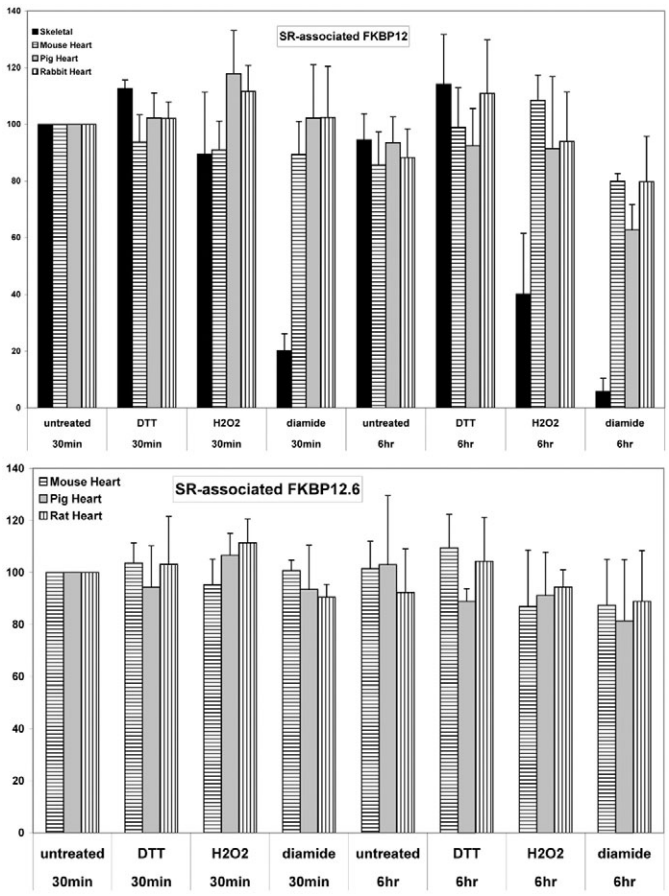

Fig. 5. Effects of redox treatment on RyR–FKBP12 and –FKBP12.6 association. SR vesicles, incubated with 2 mM DTT, 1 mM H_2O_2, or 200 μM diamide for 30 minutes or 6 hours at room temperature were precipitated by centrifugation and the presence of associated FKBP12 and FKBP12.6 was analysed by SDS-PAGE and western blotting using Ab^{ABR} and Ab^{126}, respectively. Densitometry analysis ($n=3$) was carried out and SR-associated FKBP was normalised against control samples (untreated, 30 minutes). The changes seen in rabbit skeletal muscle SR treated with diamide (30 minutes and 6 hours) and H_2O_2 (6 hours) are statistically significant ($P<0.01$ and $P<0.05$ respectively). Data are given as means+s.e.m.

Variable RyR2:FKBP12 and RyR2:FKBP12.6 stoichiometry in mammalian heart

Previous work suggested that RyR2 from dog heart associates exclusively with FKBP12.6 (Timerman et al., 1996), although in subsequent studies the presence of both FKBP12 and FKBP12.6 isoforms in the heart of other species was identified (Jeyakumar et al., 2001). In vitro binding assays with exogenous [^{35}S]FKBP12 and FKBP12.6 has suggested that there are four FKBP sites (FKBP12 or FKBP12.6) per RyR2 tetrameric channel (Jeyakumar et al., 2001; Timerman et al., 1996). However, the molar ratio of endogenous FKBP12 and FKBP12.6 to RyR2 in native cardiac tissue had not previously been directly assessed.

Using [^{3}H]ryanodine-binding and immunoblot analysis, we now provide quantitative measurements of RyR2 concentration and of both FKBP isoforms in heart SR from four mammalian species. This analysis reveals significant differences in the FKBP complement of cardiac RyR2 from different mammals (Figs 1, 3; Table 1). In mouse and pig heart, RyR2 forms complexes with both FKBP12 and FKBP12.6, but the former is the predominant isoform. In rabbit and rat heart, RyR2 associates with a single but different FKBP isoform, FKBP12 for rabbit and FKBP12.6 for rat.

Assuming four FKBP-binding sites per RyR2 tetramer, the amount of endogenous FKBP12.6 bound to RyR2 in mouse, pig and rat heart is sub-stoichiometric (a FKBP12.6:RyR2 molar ratio of 1.4, 1.8 and 1.8, respectively; Table 1). This means that only ~35% of available sites are occupied in mouse RyR2, ~45% in pig and rat RyR2, and virtually zero in rabbit. This finding might explain the beneficial effects on SR Ca^{2+} handling upon FKBP12.6 overexpression in isolated cardiomyocytes (Gomez et al., 2004; Loughrey et al., 2004; Prestle et al., 2001) or in transgenic animals (Gellen et al., 2008; Huang et al., 2006; Seidler et al., 2011). If RyR2 channels were already saturated by FKBP12.6, then overexpression of this protein should be ineffective in improving SR Ca^{2+} handling, as previously suggested (Gellen et al., 2008). Our results also concur with recent findings by Bers and colleagues, who indirectly estimated

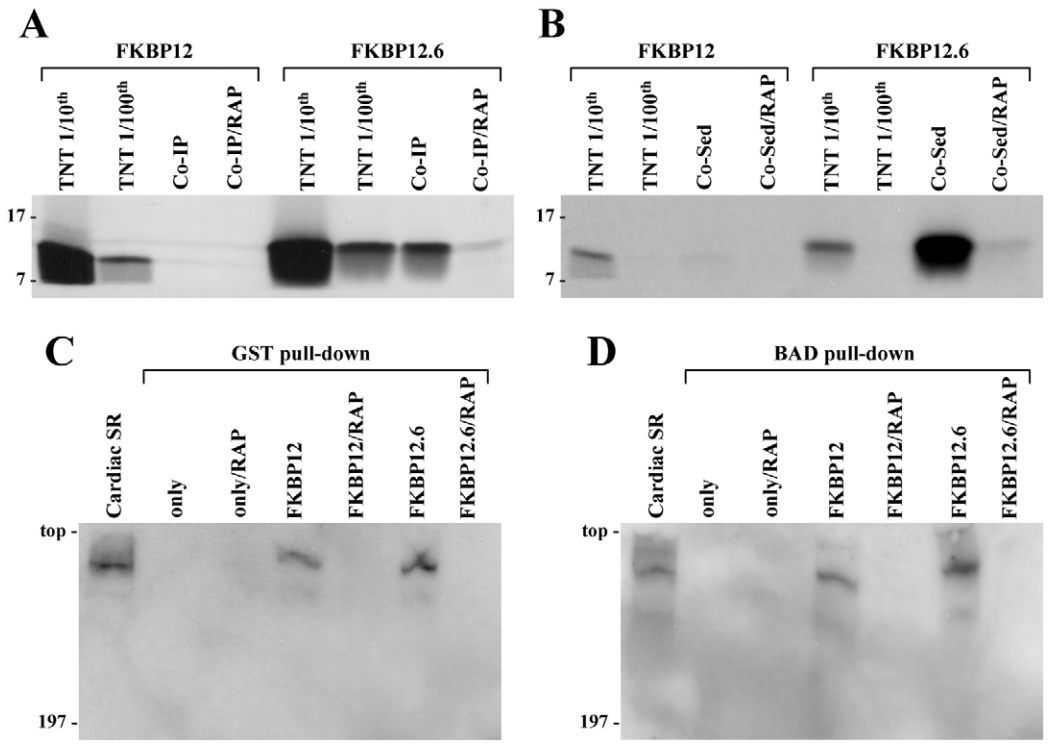

Fig. 6. Assessment of exogenous FKBP12 and FKBP12.6 interaction with RyR2. (**A**) Co-immunoprecipitation assays of RyR2 with exogenous human [^{35}S]FKBP12 and [^{35}S]FKBP12.6. Solubilised pig cardiac SR vesicles were incubated with in-vitro-synthesised [^{35}S]FKBP12 and [^{35}S]FKBP12.6 in the presence or absence of 20 μM rapamycin (RAP). RyR2 was immunoprecipitated with Ab^{1093} and the presence of co-precipitated FKBP12 and FKBP12.6 was analysed by SDS-PAGE (15% gel) and autoradiography. Aliquots of the TnT reaction, 10% and 1% of the volume processed in co-immunoprecipitation assays, were included in the autoradiogram. (**B**) Co-sedimentation assays of RyR2 with exogenous human [^{35}S]FKBP12 and [^{35}S]FKBP12.6. Pig cardiac SR vesicles were incubated with in-vitro-synthesised [^{35}S]FKBP12 and [^{35}S]FKBP12.6 in the presence or absence of 20 μM rapamycin (RAP). Vesicles were precipitated by centrifugation and the presence of co-precipitated FKBP12 and FKBP12.6 was analysed by SDS-PAGE (15% gel) and autoradiography. Aliquots of the TnT reaction, 10% (1/10th) and 1% (1/100th) of the volume processed in co-sedimentation assays, were included in the autoradiogram. (**C**,**D**) Pull-down assays of RyR2 with recombinant purified GST–FKBP12 and –FKBP12.6 (C) or BAD–FKBP12 and –FKBP12.6 (D). Solubilised pig cardiac SR vesicles were incubated with GST–FKBP12 and –FKBP12.6 (C), captured on glutathione beads, or BAD–FKBP12 and –FKBP12.6 (D), captured on NeutrAvidin beads, in the presence or absence of 20 μM rapamycin (RAP). Negative controls were also carried out with GST or BAD alone. Beads were precipitated by centrifugation and the presence of co-precipitated RyR2 was analysed by SDS-PAGE (4% gels) and western blotting using Ab^{1093}. Pig cardiac SR (50 μg) was also loaded on the gels.

the proportion of FKBP12.6 saturated sites at 10–20% by measuring the binding of exogenous fluorescently labelled protein (Guo et al., 2010). Given the high affinity of the RyR2–FKBP12.6 interaction (Guo et al., 2010; Jeyakumar et al., 2001; Timerman et al., 1996), such low FKBP12.6 occupancy probably results from low FKBP12.6 expression. In addition, some of the available FKBP-binding sites might be occupied by FKBP12, at least in mouse and pig heart. Interestingly, FKBP12.6 is completely absent from rabbit heart SR (Fig. 1B, Fig. 3), although rabbit RyR2 binds strongly to exogenous FKBP12.6 (supplementary material Fig. S4). It is implausible that the antibody does not detect rabbit FKBP12.6, as the rabbit and human sequences are nearly identical (supplementary material Fig. S7). Alternatively, rabbit heart FKBP12.6 expression could be extremely low; however, we were unable to test this because FKBP12.6 was undetectable in cardiac homogenates from all four species (not shown).

We found barely detectable amounts of FKBP12 in rat heart SR (Fig. 1A, Fig. 3). Rat RyR2 also displayed the lowest binding for exogenous FKBP12 compared with that of other RyR2 mammalian isoforms (supplementary material Fig. S8; Table 2), suggesting that it has the lowest affinity for FKBP12. With the exception of rat, FKBP12 was present in pig, rabbit and especially abundant in mouse heart SR (Fig. 1A, Fig. 3; Table 1). Quantitative analysis indicated that FKBP12 levels exceeded the available RyR2 sites in mouse and pig (Table 1), assuming one site per RyR2 subunit. This raises the possibility of additional binding sites per subunit. Previous studies have presented evidence for three distinct RyR regions (N-terminal, central and C-terminal domains) capable of supporting FKBP interaction (Bultynck et al., 2001; Gaburjakova et al., 2001; Marx et al., 2000; Masumiya et al., 2003; Zissimopoulos and Lai, 2005a; Zissimopoulos and Lai, 2005b). Alternatively, additional non-RyR, FKBP12-binding targets might exist in cardiac SR, although no such protein(s) has been identified. We also found that exogenous FKBP12 displays very low binding to cardiac SR (Fig. 6A,B), arguing against the presence of additional high-affinity FKBP12-interacting protein(s). Nonetheless, we note that endogenous FKBP12 indeed associates with native RyR2, as shown by co-immunoprecipitation assays (Fig. 2A,B). The validity of our assays was apparent because applying our quantitative [^{3}H]ryanodine binding and immunoblot analysis to skeletal muscle SR yielded a 1:5 molar ratio for RyR1:FKBP12, close to the expected 1:4 stoichiometry (Table 1). Thus, it

Table 2. RyR2 binding of exogenous FKBP12

	Human [^{35}S]FKBP12		Mouse [^{35}S]FKBP12	
SR source	TnT (1%)	Bound	TnT (1%)	Bound
Mouse heart	100	36.3±8.9	100	60.3±9.2
Pig heart	100	49.1±7.1	100	76.8±11.1
Rabbit heart	100	52.6±7.0	100	70.9±11.8
Rat heart	100	14.1±6.9	100	27.5±7.9

Cardiac SR vesicles, incubated with in vitro synthesised (TnT) human or mouse [^{35}S]FKBP12 in the presence or absence of 20 μM rapamycin, were precipitated by centrifugation and the presence of associated [^{35}S]FKBP12 was analysed by SDS-PAGE and autoradiography. Densitometry analysis (n=4) was carried out, and SR-associated FKBP12 was normalised against the [^{35}S]FKBP12 input processed in co-sedimentation assays (TnT, 1%). 'Bound' refers to the amount of [^{35}S]FKBP12 specifically bound, corrected for rapamycin-resistant binding. Data are given as means ± s.e.m.

appears that FKBP12 is the predominant isoform in mouse, pig and rabbit heart SR. Importantly, excess cytosolic FKBP12 concentration alone cannot account for our findings. Recently, the endogenous cellular FKBP12 and FKBP12.6 concentrations were estimated at 1 μM and ≤100 nM, respectively, whereas the exogenous affinity of FKBP12 for RyR2 was ~150-fold lower than that for FKBP12.6 (Guo et al., 2010). This suggests that FKBP12 levels associated with cardiac SR would maximally be equal to that of FKBP12.6. This is clearly not occurring because Ab^{ABR}, which detects both isoforms equally well, fails to detect FKBP12.6 in cardiac SR (Fig. 1A).

Our data for the RyR2 interaction with exogenous FKBP12 and FKBP12.6 suggest that it has a lower affinity for FKBP12 compared with that for FKBP12.6 (Fig. 6A,B), in agreement with previous findings (Guo et al., 2010; Timerman et al., 1996). Weak RyR2 interaction with exogenous FKBP12 appears incompatible with apparently reasonably high affinity for endogenous protein, implied by the high FKBP12 levels in cardiac SR. Endogenous protein might form oligomers that retain their RyR2 interaction and therefore increase the FKBP12:RyR2 molar ratio because FKBP12 can form dimers and trimers (Schories et al., 2007). Alternatively, post-translational modifications within cardiomyocytes might enhance the binding of endogenous FKBP12 to RyR2. FKBP12 post-translational modification might induce structural changes so that it resembles the FKBP12.6 isoform and confers high-affinity binding for RyR2, as we found for FKBP12 fusion proteins with glutathione-S-transferase or biotin-acceptor domain (Fig. 6C,D). Modified FKBP12 might also bind to RyR2 sites other than the FKBP12.6-binding site. Notably, in vitro FKBP12 phosphorylation by protein kinase C, as well as arginine methylation, has previously been reported (Chen et al., 2004; Cryan et al., 1991).

Little is known about FKBP12-mediated regulation of RyR2. FKBP12 failed to modulate recombinant RyR2 when co-expressed in CHO cells or skeletal myotubes (George et al., 2003; Goonasekera et al., 2005). Similarly, exogenous FKBP12 had no effects in permeabilised cardiomyocytes (Guo et al., 2010). However, isolated RyR2 channels from FKBP12-knockout mice display sub-conductance behaviour (Shou et al., 1998). Furthermore, adenovirus-mediated FKBP12 overexpression in isolated cardiomyocytes reduces excitation–contraction coupling gain and decreases Ca^{2+} spark frequency (Seidler et al., 2007). Assuming that a cardiac-specific FKBP12 post-translational modification is required for RyR2 interaction would explain the latter two studies.

FK506 and rapamycin do not disrupt the native RyR2–FKBP12 and –FKBP12.6 association

The immunosuppressants, FK506 and rapamycin disrupt RyR1–FKBP12 association in skeletal muscle (Ahern et al., 1997; Timerman et al., 1993). We also found FK506 and rapamycin very effective in removing endogenous FKBP12 from skeletal muscle SR (supplementary material Fig. S5; Fig. 4). The effectiveness of FK506 and rapamycin in cardiac tissue is assumed to be similar to that in skeletal muscle, because FK590 (an FK506 analogue) displaces exogenous [^{35}S]FKBP12.6 pre-bound to RyR2 (Timerman et al., 1996).

Surprisingly, we found that neither drug significantly disrupts the native RyR2–FKBP12 and –FKBP12.6 association, even after prolonged incubation (6 hours at room temperature) (supplementary material Fig. S5; Fig. 4). This was evident for both FKBP isoforms from all four mammalian species and suggests that there is a strong endogenous RyR2–FKBP12 and –FKBP12.6 interaction characterised by a very slow off-rate. FKBP has to spontaneously dissociate from RyR, then the FKBP–drug complex needs to form to prevent FKBP re-binding RyR. Our data are consistent with recent observations showing that rapamycin was unable to remove exogenous FKBP12.6 pre-bound to RyR2 (Guo et al., 2010). However, a previous report indicated that FK590 was effective in dissociating pre-bound, exogenous [^{35}S]FKBP12.6 from RyR2 (Timerman et al., 1996). A notable difference in the latter study is the experimental temperature of 37℃. Importantly, we identified a rapamycin-sensitive, endogenous FKBP12 and FKBP12.6 interaction with RyR2 in co-immunoprecipitation experiments (Fig. 2). Thus, certain conditions, such as higher (i.e. physiological) temperature or the presence of detergents (e.g. CHAPS), might alter the native RyR2–FKBP interaction and accelerate dissociation rates. Our data could explain previously reported discrepancies, where the FKBP12.6 functional effects in mammalian hearts were studied following application of FK506 (or rapamycin), assuming it would strip the FKBP12.6 from RyR2. It is imperative that such studies assess the degree of FKBP12 and FKBP12.6 dissociation achieved by drug treatment.

cADPR has been proposed to be a physiological ligand for FKBP12.6, dissociating it from RyR2 and resulting in Ca^{2+} leak (Noguchi et al., 1997; Zhang et al., 2009). However, other studies have been unable to reproduce these results (Copello et al., 2001; Thomas et al., 2001). Our cADPR data are consistent with the latter studies (supplementary material Fig. S5; Fig. 4). Although we found ~20% FKBP dissociation in some cases, the effect was not statistically significant and required a high, non-physiological cADPR concentration (100 μM). Moreover, cADPR did not affect the RyR2 interaction with exogenous [^{35}S]FKBP12.6 in co-immunoprecipitation or co-sedimentation assays (not shown).

Oxidative conditions disrupt the RyR–FKBP association in skeletal but not in cardiac muscle

We previously reported that the RyR–FKBP interaction is redox-sensitive, with oxidised RyR2 displaying reduced affinity for exogenous FKBP12.6 (Zissimopoulos et al., 2007). Here, we find

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that the interaction of endogenous FKBP12 and FKBP12.6 with RyR2 is not readily disrupted by oxidants, but requires harsh conditions (prolonged diamide exposure) (supplementary material Fig. S6; Fig. 5). These findings, similar to those above with FK506 and rapamycin treatment, suggest that the native RyR2–FKBP12 and –FKBP12.6 association is very robust. In addition, FKBP12- and FKBP12.6-bound RyR2 might protect the channel from oxidation. The two datasets for RyR2 binding to endogenous (present work) and exogenous FKBP12.6 (Zissimopoulos et al., 2007) support the conclusion that FKBP12.6 pre-bound to RyR2 does not readily dissociate under oxidative conditions; however, once removed its re-association with oxidised RyR2 is substantially decreased. In contrast to cardiac muscle, endogenous FKBP12 was easily removed from RyR1 in skeletal muscle SR upon treatment with H_2O_2 and diamide (supplementary material Fig. S6; Fig. 5). This finding might have important implications in skeletal muscle physiology, particularly in fatigue and ageing, conditions characterised by oxidative stress (Reid and Durham, 2002).

Implications for RyR2 pathophysiology

Our data indicate that both the specific FKBP isoform and mammalian species employed are important considerations when studying the RyR2–FKBP–Ca^{2+}-release-channel complex. Importantly, FKBP12 is the more abundant isoform in cardiac SR (except rat) (Fig. 1A). The rat would enable studies of RyR2 regulation by FKBP12.6, given that there are trivial amounts of FKBP12. Rabbits might be preferred for studies of FKBP12, owing to the virtual absence of FKBP12.6. Unfortunately, the precise FKBP12 and FKBP12.6 complement of RyR2 in human heart is unknown at present.

Use of the mouse to study RyR2 regulation by FKBP12 and FKBP12.6 might be problematic. Mouse heart SR displays very high levels of FKBP12, ~100-fold higher than those of FKBP12.6 (Table 1, Fig. 1B, Fig. 3). The mouse heart is distinctive among mammals because of its very high beat rate and the major contribution of SR-mediated Ca^{2+} release (~95%) in excitation–contraction coupling (Li et al., 1998; Yao et al., 1998). Whether high SR-associated FKBP12 levels are required for reliable heartbeat remains to be clarified. Notably, FKBP12-knockout mice have severe cardiac defects, and although this might be due to mechanisms other than Ca^{2+} signalling, their RyR2 channels display sub-conductance states and increased channel activity (Shou et al., 1998). The mouse is the animal of choice for genetic manipulation studies, including those of RyR2, FKBP12 and FKBP12.6 (reviewed by Kushnir et al., 2010), and has been used to confirm (or deny) the involvement of FKBP12.6 in RyR2 function. However, the presence of, and putative co-regulation by, the FKBP12 isoform requires further consideration.

The fact that FKBP12 is the predominant isoform in complex with RyR2, whereas FKBP12.6 occupancy is sub-stoichiometric, has important implications for RyR2 pathophysiology and cardiac disease. Numerous publications by Marks and co-workers have placed FKBP12.6 dissociation at the centre stage for explaining RyR2 channel dysfunction and associated SR Ca^{2+} leak in heart failure and catecholaminergic polymorphic ventricular tachycardia (Kushnir and Marks, 2010). However, even in normal heart, RyR2 is not saturated with FKBP12.6; only 35–45% of the available RyR2 sites are occupied in mouse, pig and rat heart, and virtually zero in rabbit (Table 1). Therefore, FKBP12.6 saturation is not crucial for normal RyR2 function. Whether or not FKBP12.6 levels are even lower in acquired or inherited cardiac disease might be unlikely to have a major impact in RyR2 function. The rabbit in particular, would be very vulnerable to cardiac arrhythmias even in the absence of other precipitating factors. Moreover, the presence of FKBP12 in cardiac SR at a much higher concentration than FKBP12.6, adds an extra layer of complexity. Hence, the precise role of FKBP12 in RyR2 regulation and pathophysiology remains to be elucidated.

What is the role of FKBP12.6 in the heart then? The finding that FKBP12.6 occupancy of RyR2 is low (or near absent in rabbit) suggests that this protein might not be vital for normal heart function. However, at full saturation FKBP12.6 has the potential to further stabilise RyR2 function and reduce diastolic SR Ca^{2+} leak, as indicated by overexpression studies in isolated cardiomyocytes (Gomez et al., 2004; Loughrey et al., 2004; Prestle et al., 2001) and transgenic animals (Gellen et al., 2008; Huang et al., 2006; Seidler et al., 2011). Recent work has demonstrated that FKBP12.6 levels are increased in the late preconditioned myocardium and that this protects against myocardial stunning (Lucats et al., 2007). Increased FKBP12.6 expression has also been reported in animal models of ischaemia–reperfusion (Astrom-Olsson et al., 2009) and spontaneous dilated cardiomyopathy (Oyama et al., 2009). Thus, it appears that the heart might employ FKBP12.6 upregulation as an adaptive response to ameliorate cardiac dysfunction.

Materials and Methods

Materials

Anti-FKBP12 antibody (PA1-026A, rabbit polyclonal; Ab^{ABR}) was from Affinity Bio-Reagents (Thermo Scientific) and anti-FKBP12.6 antibody (goat polyclonal; Ab^{126}) was from R&D Systems. [^{3}H]ryanodine and L-[^{35}S]methionine (EasyTag Expre^{35}S^{35}S) were obtained from Perkin-Elmer. Electrophoresis reagents were from Bio-Rad and protease inhibitor cocktail (Complete) from Roche. Factor Xa was obtained from Novagen (Merck), CHAPS and rapamycin from Calbiochem (Merck), DTSSP from Pierce (Thermo Scientific), and FK506, cADPR and all other reagents were from Sigma.

Plasmid construction

Preparation of plasmids for expression of human FKBP12 and FKBP12.6 in mammalian cell lines has been described previously (Zissimopoulos and Lai, 2005a). Mouse FKBP12 and FKBP12.6 cDNA were obtained from GeneService (Life-Sciences). The entire ORF was amplified by PCR and cloned into pCR3 (Invitrogen) for expression in mammalian cells. Plasmids for bacterial expression of human FKBP12 and FKBP12.6 fused with a biotin-acceptor domain were generated by sub-cloning into PinPointXa1 (Promega). Plasmids for bacterial expression of GST fusions of human FKBP12 and FKBP12.6 were generated by sub-cloning into a modified pGEX5X2 vector (GE Healthcare). An *Nru*I restriction site at the 3′ end of the Factor Xa cleavage site (identical to the PinPointXa1 sequence) was generated using the Quik-Change XL site-directed mutagenesis kit (Stratagene, Agilent Technologies) and complementary oligonucleotide primers (forward: 5′-ATC-TGATCGAAGGTCGCGAGATCCCCGGAATTCCC-3′, reverse: 5′-GGGAA-TTCCGGGGATCTCGCGACCTTCGATCAGAT-3′). Human FKBP12 and FKBP12.6 were sub-cloned into this modified vector at the *Nru*I site, which results in a fusion protein with the initiating FKBP methionine immediately following the Factor Xa cleavage site. All plasmids were verified by direct DNA sequencing (BigDye, Perkin-Elmer).

Preparation of cardiac heavy SR vesicles

Cardiac and (rabbit) skeletal muscle heavy SR vesicles were prepared as described previously (Meissner and Henderson, 1987) with the modifications of Sitsapesan and Williams (Sitsapesan and Williams, 1990). Typically, we processed ~200 g from one pig heart, ~2 g from 16 mouse adult hearts (C57BL6/J), ~2.5 g from three adult rat hearts (Wistar), ~4 g from one adult rabbit heart (New Zealand white). Ventricle muscle was homogenised in four volumes of homogenisation buffer (10 mM Na_2PIPES pH 7.4, 0.3 M sucrose, and protease inhibitors, 2 mM benzamidine, 0.2 mM AEBSF and 10 µM leupeptin) with the use of a blender (or hand-held blender Ultra-Turrax T25), and the homogenate was centrifuged at 8000 ***g*** for 10 minutes at 4℃. The pellet was re-homogenised in two volumes of homogenisation buffer, and the centrifugation step was repeated. The two supernatants were combined and centrifuged at 30,000 r.p.m. using a Beckman 45Ti or 50.2Ti rotor for 1 hour at 4℃. The pellet representing the crude cardiac microsomal fraction was resuspended in gradient buffer (10% sucrose, 10 mM Na_2PIPES pH 7.0, 0.4 M KCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM EGTA)

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and layered onto discontinuous sucrose gradients consisting of three layers of 20%, 30% and 40% sucrose (in the salt solution used for the gradient buffer). The gradients were centrifuged at 20,000 r.p.m. (Beckman SW28.1 rotor) for 14 hours at 4℃ and the 30%–40% interface containing the heavy SR vesicles was collected and diluted with an equal volume of 10 mM Na_2PIPES pH 7.4. Cardiac heavy SR vesicles were pelleted at 30,000 r.p.m. (Beckman 50.2Ti rotor) for 1 hour at 4℃, resuspended in homogenisation buffer and stored at −80℃ in small aliquots.

Western blot analysis

Protein samples were resuspended in SDS-PAGE loading buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5 mM EDTA, 2% β-mercaptoethanol, 0.01% Bromophenol Blue), heated at 85℃ for 5 minutes and centrifuged at 14,000 ***g*** for 3 minutes before loading on SDS-PAGE gels. 4% SDS-PAGE gels for RyR separation were strengthened with 0.5% agarose (Tatsumi and Hattori, 1995). Proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore) using a semi-dry transfer system (Trans-Blot SD, Bio-Rad) in buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS and 20% methanol) at 20 V for 1 hour (for FKBP) or at 24 V for 4 hours (for RyR, methanol omitted). The membrane was blocked with 5% non-fat milk protein in TBS-T buffer (20 mM Tris-HCl pH 7.4, 137 mM NaCl and 0.1% Tween-20). Primary antibodies were applied overnight at 4℃: anti-FKBP12 antibody at 4 μg/ml (Ab^{ABR}, Affinity Bio-Reagents), anti-FKBP12.6 antibody at 2 μg/ml (Ab^{126}, R&D Systems) and anti-RyR2 antibody at 1:1000 dilution (Ab^{1093}, rabbit polyclonal antibodies raised to RyR2 residues 4454–4474). Immunoreactive protein bands were visualised by enhanced chemiluminescence detection (Pierce ECL, Thermo Scientific).

Co-immunoprecipitation assays

Cardiac SR vesicles (400 μg) were solubilised in 200 μl of IP buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.4% CHAPS and protease inhibitors) by overnight incubation at 4℃ with continuous mixing. The insoluble material was pelleted at 20,000 ***g*** for 10 minutes at 4℃ and the supernatant was withdrawn. In the meantime, RyR2-specific Ab^{1093} (2 μl, 1:100 dilution) was captured on 20 μl nProtein-A–Sepharose beads (GE Healthcare) in 200 μl of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) for 2 hours at room temperature. Antibodies were crosslinked on protein A with addition of 2 mM DTSSP (Pierce) and incubation for 30 minutes at room temperature. Beads were recovered at 1500 ***g*** for 2 minutes at 4℃ and reaction was stopped with addition of IP buffer (which contains 20 mM Tris) for 1 hour at 4℃. Beads were washed twice more with IP buffer. Solubilised SR proteins were then transferred into tubes with crosslinked RyR2 antibody–protein-A beads, and incubated for 6 hours at 4℃ with mixing. Beads were recovered at 1500 ***g*** for 2 minutes at 4℃ and washed once with IP buffer for 10 minutes. Immunoprecipitated proteins were eluted with SDS-PAGE loading buffer (without reducing reagent), heated at 50℃ for 5 minutes, and analysed by SDS-PAGE and western blotting using an anti-FKBP antibody.

Co-immunoprecipitation experiments with in-vitro-synthesised [^{35}S]FKBP12 and [^{35}S]FKBP12.6 were carried out as described previously (Zissimopoulos et al., 2007).

Co-sedimentation assays

Rabbit skeletal muscle (20 μg) or mouse (20 μg or 100 μg for FKBP12 and FKBP12.6 detection, respectively), pig (100 μg), rabbit (100 μg) and rat (100 μg) cardiac SR vesicles were resuspended in 200 μl of buffer (10 mM Na_2PIPES pH 7.4, 120 mM KCl and protease inhibitors). SR vesicles were incubated with an appropriate reagent (20 μM FK506, 20 μM rapamycin, 1 μM purified GST–FKBP12.6 or 100 μM cADPR) for 6 hours at room temperature with continuous mixing. SR vesicles were recovered at 20,000 ***g*** for 10 minutes at 4℃, washed once, resuspended in SDS-PAGE loading buffer, heated at 85℃ for 5 minutes, and analysed by SDS-PAGE and western blotting using an anti-FKBP antibody.

For the redox treatment experiments, SR vesicles were treated with an appropriate redox reagent (2 mM DTT, 1 mM H_2O_2 or 200 μM diamide) for 30 minutes or for 6 hours at room temperature, and processed as above.

For the experiments with [^{35}S]FKBP12, cardiac SR vesicles (200 μg) were resuspended in 200 μl of buffer (10 mM Na_2PIPES, 120 mM KCl, pH 7.4, and protease inhibitors). In-vitro-synthesised radiolabelled FKBP12 was added and incubated for 20 hours at room temperature with continuous mixing. SR vesicles were recovered at 20,000 ***g*** for 10 minutes at 4℃, washed once, resuspended in SDS-PAGE loading buffer, heated at 85℃ for 5 minutes, and analysed by SDS-PAGE and autoradiography.

Bacterial expression, purification and cleavage of GST–FKBP12 and –FKBP12.6 fusion proteins

Bacteria (*E.coli* Rosetta, Novagen) carrying the appropriate expression plasmid were grown in 400 ml of LB medium at 30℃. Protein expression was induced at 25℃ with 0.1 mM isopropyl β-D-thiogalactoside when the optical density (at 600 nm) reached 0.5. Cells were harvested 3 hours later and the pellet was resuspended in 20 ml of PBS buffer supplemented with protease inhibitors. Cells were permeabilised by a combination of freeze–thaw cycles (in liquid nitrogen) and brief sonication bursts, and the insoluble material was removed by centrifugation at 10,000 ***g*** for 10 minutes at 4℃. GST fusion proteins were isolated by incubation of the bacterial supernatant with glutathione–Sepharose-4B beads (1 ml bead volume, GE Healthcare) for 2 hours at 4℃ with gentle mixing. Beads were washed twice with PBS containing 0.5% Triton X-100 and once with PBS. GST fusion proteins were eluted with 10 mM reduced glutathione (in 50 mM Tris-HCl pH 8) following incubation for 30 minutes at room temperature. Reduced glutathione was removed with the use of a Vivaspin 20 concentrator (10,000 kDa molecular mass cut-off, Sartorius Stedim Biotech) and the buffer was exchanged for PBS. Purified GST fusion proteins were aliquoted and stored at −80℃ until use. Bacterial expression and purification of biotin-acceptor domain (BAD) fusion proteins was carried out as above, using NeutrAvidin agarose beads (Pierce, Thermo Scientific).

Proteolytic cleavage of GST fusion proteins to liberate the FKBP12 and FKBP12.6 proteins (resulting in 'free' FKBP12 and FKBP12.6 with no additional amino acids at the N-terminus due to cloning) was performed with Factor Xa. Briefly, 100 μg of GST–FKBP12.6 was incubated with 10 units of Factor Xa in 500 μl of buffer (100 mM NaCl, 50 mM Tris-HCl pH 8 and 5 mM $CaCl_2$) for 20 hours at room temperature. Cleaved GST was removed with glutathione–Sepharose-4B beads, and purified 'free' FKBP12.6 was stored in small aliquots at −80℃. Cleavage of GST–FKBP12 was carried out similarly, using 100 μg of the protein together with 5 units of Factor Xa in 1 ml of buffer supplemented with 0.2% Triton X-100. Protein concentration was determined by densitometry analysis of Coomassie-stained gels and BSA protein concentration standards.

GST pull-down assays

Pig cardiac SR vesicles (500 μg) were solubilised in 200 μl of IP buffer by overnight incubation at 4℃ with continuous mixing, and the insoluble material was pelleted at 20,000 g for 10 minutes at 4℃. In the meantime, 0.2 nmol of GST fusion protein (5.2 μg of GST alone, 7.6 μg of the GST-tagged FKBP12 or FKBP12.6) was captured on 20 μl of glutathione–Sepharose-4B beads in 200 μl of IP buffer overnight at 4℃. Beads were recovered at 1500 ***g*** for 2 minutes at 4℃ and the supernatant removed. Solubilised SR proteins were then transferred into tubes with pre-captured GST fusion protein on glutathione beads, and incubated for 6 hours at 4℃ with mixing. Beads were recovered at 1500 ***g*** for 2 minutes at 4℃ and washed twice for 10 minutes with IP buffer. Precipitated proteins were eluted with SDS-PAGE loading buffer, heated at 85℃ for 5 minutes, and analysed by SDS-PAGE and western blotting using an anti-RyR2 antibody.

Pull-down assays using BAD–FKBP12 and –FKBP12.6 fusion proteins were carried out as above, using NeutrAvidin agarose beads.

[^{3}H]Ryanodine binding assays

[^{3}H]Ryanodine binding was performed using 10 nM [^{3}H]ryanodine (Perkin-Elmer) and 200 μg of cardiac SR or 20 μg of skeletal muscle SR. Incubation was for 2 hours at 37℃ in buffer (25 mM PIPES pH 7.4, 1 M KCl) supplemented with 100 μM $CaCl_2$ and 10 mM caffeine. Non-specific binding was measured in the presence of 10 μM unlabelled ryanodine. Bound [^{3}H]ryanodine was separated from unbound by vacuum filtration through glass filters (Whatman GF/F). Radioactivity was quantified by liquid scintillation counting.

Other methods

In vitro cell-free protein expression [carried out using the TnT T7 Quick coupled transcription and translation system (Promega)] and autoradiography were performed as described previously (Zissimopoulos et al., 2007). HEK293 cell culture and transfection were performed as described previously (Zissimopoulos and Lai, 2005b). Densitometry analysis was performed using a GS-700 scanner (Bio-Rad) and Quantity One software (Bio-Rad). Statistical analysis of densitometry data was performed using an unpaired Student's *t*-test.

Funding

This work was supported by a British Heart Foundation Fellowship [grant number FS/08/063 to S.Z.]; an EU FP6 STREP grant (Control of Intracellular Calcium and Arrhythmias project); and Wales Heart Research Institute Placement Scholarship to S.S.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.098012/-/DC1

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