FKBP12 associates tightly with the skeletal muscle type 1 ryanodine receptor, but not with other intracellular calcium release channels

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Abstract This study compared the relative levels of ryanodine receptor (RyR) isoforms, inositol 1,4,5-trisphosphate receptor (IP_3R) isoforms, and calcineurin, plus their association with FKBP12 in brain, skeletal and cardiac tissue. FKBP12 demonstrated a very tight, high affinity association with skeletal muscle microsomes, which was displaced by FK506. In contrast, FKBP12 was not tightly associated with brain or cardiac microsomes and did not require FK506 for removal from these organelles. Furthermore, of the proteins solubilised from skeletal muscle, cardiac muscle and brain microsomes, only skeletal muscle RyR1 bound to an FKBP12–glutathione-*S*-transferase fusion protein, in a high affinity FK506 displaceable manner. These results suggest that RyR1 has distinctive FKBP12 binding properties when compared to RyR2, RyR3, all IP_3R isoforms and calcineurin.

Key words: FKBP12; Ryanodine receptor; Inositol 1,4,5-trisphosphate receptor; Calcineurin; Intracellular Ca^{2+} release channel; FK506; Skeletal muscle; Brain; Cardiac muscle

1. Introduction

The 12-kDa FK506 binding protein (FKBP12), a *cis–trans* prolyl isomerase, was identified originally as the cytoplasmic receptor for the immunosuppressant drugs FK506 and rapamycin [1,2]. This ubiquitously expressed protein was discovered subsequently to be a crucial regulator of Ca^{2+} release from both ryanodine receptors (RyRs) and more recently inositol 1,4,5-trisphosphate receptors (IP_3Rs) [3,4]. These receptors are large tetrameric structures, both represented by three isoforms [4] and FKBP12 or its close homologue FKBP12.6 are often described as integral subcomponents of these Ca^{2+} release channels. This description arises mainly from studies showing that FKBP12 binds very tightly to RyR1 from skeletal muscle, with sufficient affinity to co-purify with the receptor [5,6]. This tight association can be disrupted by treatment with high concentrations of FK506 and rapamycin [7]. The FKBP12–RyR1 interaction is required for normal and coupled gating [8,9] of RyR1 channels, where FKBP12 removal from RyR1 de-stabilises the channel, resulting in long-lasting sub-conductance states [8]. Cardiac RyR2 can also be found in association with FKBP12 [10] and is regulated by this immunophilin [11]. However, RyR2 co-purifies with the FKBP12 homologue FKBP12.6 [12,13], which has been demonstrated to bind tightly to RyR2 in canine sarcoplasmic reticulum (SR) and to regulate Ca^{2+} release from cardiac RyR2 in some [14,15] but not all [13,16] studies. The final RyR isoform, RyR3, interacts with FKBP12 [17,18], although FKBP12 has not been shown to co-purify or modulate Ca^{2+} release from RyR3 [17].

Subsequent to the studies showing that FKBP12 was an integral subcomponent of skeletal muscle RyR1, it was revealed that cerebellar IP_3R (predominantly the type 1 isoform) was also modulated by FKBP12 in a similar fashion to RyR1, with IP_3R1 and FKBP12 co-purifying through several chromatographic steps including size-exclusion [19]. Further studies showed that the phosphatase calcineurin bound tightly to the IP_3R complex in an FK506/rapamycin displaceable fashion, suggesting that FKBP12 anchored calcineurin to IP_3R1 [20]. However, other functional studies have failed to demonstrate a physiological regulation of IP_3R-induced Ca^{2+} release by FKBP12 [21]. Furthermore, very recent work has demonstrated that mature IP_3R1 does not display a specific, high affinity interaction with FKBP12 [18].

It is thus far from clear whether the tight association of FKBP12 with intracellular Ca^{2+} release channels is a global phenomenon found outside that demonstrated in skeletal and cardiac muscle between RyR1–FKBP12 and RyR2–FKBP12.6, respectively. Furthermore, with the exception of cerebellar IP_3R, nothing is known concerning the interaction or association of FKBP12 with intracellular Ca^{2+} release channels in the central nervous system. This is important as the brain has a very rich expression of RyR and IP_3R isoforms [22–25], and contains exceptionally high levels of FKBPs [26]. It is also unknown how widespread the described association between FKBP12 and IP_3R isoforms, as well as FKBP12 and calcineurin is in different tissue types, or other brain regions besides the cerebellum. Finally, previous studies have for the most part been performed on solubilised or purified preparations, or in systems overexpressing intracellular Ca^{2+} release channels. It has thus been difficult to gain an understanding of the real dynamics of FKBP12–membrane

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Abbreviations: CHAPS, 3-((cholamidopropyl)dimethylammonio)-1-propane-sulfonate; FKBP12, FK506 binding protein, 12 kDa; FKBP12.6, FK506 binding protein, 12.6 kDa; FKWB, FKBP wash buffer; GST, glutathione-*S*-transferase; IP_3R, inositol 1,4,5-trisphosphate receptor; PMSF, phenylmethyl-sulfonylfluoride; RyR, ryanodine receptor; SR, sarcoplasmic reticulum

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and FKBP12–Ca^{2+} release channel association in native tissue preparations.

The present study therefore initially investigated the relative distribution of RyR and IP_3R isoforms with FKBP12 and calcineurin, in crude microsomes and native membranes prepared from rabbit skeletal muscle, cardiac muscle and brain. We then determined the nature of the association between FKBP12 and microsomes prepared from these tissues. Finally, we investigated the FKBP12 binding ability of protein solubilised from brain, cardiac and skeletal muscle microsomes, using FKBP12–glutathione-*S*-transferase (GST) fusion affinity precipitation. Results found differing tissue distributions of the key Ca^{2+} release control proteins in these tissue types, with only skeletal muscle preparations and skeletal muscle RyR1 found to be tightly associated with FKBP12, in an FK506 displaceable manner. These results indicate that RyR1 has distinctive FKBP12 binding properties when compared to RyR2, RyR3, calcineurin and all IP_3R isoforms.

2. Materials and methods

2.1. Preparation of crude microsomal and membrane fractions

Crude microsomal fractions from various adult rabbit tissues were prepared by differential centrifugation using a slight modification of a method described previously [27]. Fresh skeletal muscle, cardiac muscle and brain were dissected free of excess fat and blood vessels and were then cut into approximately 1 cm^3 pieces. Tissues were homogenised with three 30 s bursts in an Ultra-Turrax homogeniser (T-25 probe, maximum speed) in four volumes (per wet weight) of ice cold homogenisation buffer consisting of 0.3 M sucrose, 20 mM imidazole–HCl, pH 7.4, 0.8 mM benzamidine, 1 mM iodoacetamide, 1 μg/ml of aprotinin, pepstatin A and leupeptin, and 0.5 mM phenylmethyl-sulfonylfluoride (PMSF). Homogenates were centrifuged at $5000\times g_{max}$ for 10 min at 4°C in a Beckman Avanti J-25 (JLA-16.250 rotor). Particulate fractions were resuspended in four volumes of homogenisation buffer and further centrifuged at $5000\times g_{max}$ for 10 min at 4°C. The two resulting supernatants were combined and centrifuged in a Beckman Ultracentrifuge at $100\,000\times g_{max}$ for 60 min at 4°C (Beckman Type 42.1 rotor), to obtain the crude microsomal membranes. Membranes were resuspended in two volumes of homogenisation buffer by gentle homogenisation using a loose fitting glass-Teflon homogeniser. Both the crude microsomal membranes and $100\,000\times g_{max}$ derived supernatant fractions were aliquoted and stored at −70°C. Total membrane and cytosolic fraction were also prepared from the original homogenates by centrifugation at $100\,000\times g_{max}$ (Beckman Type 42.1 rotor). The derived membrane and supernatant fractions were aliquoted and stored at −70°C. The protein concentration of the membrane and microsomal fractions was determined using a modified version of the Lowry procedure with bovine serum albumin as standard [28].

2.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

Proteins (20–30 μg of protein depending on antisera used) from membranes, crude microsomes (and when appropriate their respective cytosolic fractions), and FKBP12–GST affinity precipitations were resolved on 15% SDS–PAGE for FKBP12 and calcineurin detection, 8% SDS–PAGE for IP_3R detection and 6 or 5% SDS–PAGE for RyR detection. Proteins were transferred electrophoretically to nitrocellulose membranes (Schleicher and Schuell, 0.45 μM) using a semi-dry blotting apparatus (32 V, 1 h) for calcineurin and FKBP12 immunoblotting, and a wet transfer blotting apparatus (80 V, 1 h for IP_3R detection, and 72 V, 2 h for RyR detection). The transfer buffer for FKBP12, calcineurin and IP_3R immunoblots consisted of 48 mM Tris, 39 mM glycine and 20% ethanol whereas for RyR immunoblots the transfer buffer was supplemented with 0.137% SDS and ethanol was omitted. Nitrocellulose membranes were blocked for 1 h in 150 mM NaCl, 10 mM Tris–HCl, pH 8.0, 0.1% Tween 20 (TBS-T) and 5% non-fat milk. Immunoblots were incubated overnight at 4°C with the various primary antibody solutions diluted in TBS-T with 5% non-fat milk. Antisera dilutions and sources were as follows: FKBP12 (1:600 dilution, Santa Cruz); calcineurin Aα (1:850 dilution, Transduction Laboratories); IP_3R type 3 (1:1000, Transduction Laboratories); IP_3R types 1 and 2, CT-1 and CT-2 (1:100) were raised against the C-terminus of each subtype as described and characterised previously [17]; RyR types 1–3 (1:3000) raised against the region of low homology situated between the predicted transmembrane domains 4 and 5 for RyR1 and RyR2 and against the D1 region of RyR3, as characterised and described previously [23,29]. Nitrocellulose membranes were washed three times in TBS-T for 30 min, incubated with a 1:1000 dilution of horseradish peroxidase-linked anti-rabbit, anti-mouse or anti-goat IgG (Dako) in TBS-T for 1 h. Blots were washed as described above, then immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Pharmacia Biotech) and quantified by densitometry (GeneGenius Gel Documentation and Analysis System/Syngene), with results obtained analysed as absolute values (total integrated volume).

2.3. Treatment of crude microsomes and membranes with FK506

Crude microsomes and total membrane fractions were treated with FK506 using a slight modification of the method described by Timerman et al. [7], to dissociate FKBP from terminal cisternae vesicles. Membranes (3.5 mg protein/ml) and microsomes (3.5 mg protein/ml) from the three tissue types were incubated in a final volume of 1 ml of 10 mM Tris-maleate pH 7.0, 0.1 mM NaCl, 0.3 M sucrose, 10 μg/ml aprotinin, leupeptin and pepstatin, containing 10 μM FK506 (diluted in 0.1% methanol) at 22°C or 37°C, for 30 min. The samples were then centrifuged at $100\,000\times g_{max}$ for 30 min at 4°C in a Beckman Ultracentrifuge (Beckman Type 65 rotor) to yield a pellet and supernatant (SN-1) fraction. This supernatant should contain the soluble FKBP–FK506 complex. To ensure complete removal of FKBP12 the pellet was washed by resuspension in 1 ml of the above buffer, resedimented at $100\,000\times g_{max}$ for 30 min at 4°C (Sorval TY-65 rotor) and the resulting pellet (P) and supernatant (SN-2) collected. This pellet was washed superficially and resuspended in 1 ml of the above buffer. All tissue preparations were also subjected to the above procedure with vehicle (0.1% methanol). Fractions were analysed by SDS–PAGE and Western immunoblot analysis where equal volumes of pellet (P) and supernatant (SN-1; SN-2) fractions were solubilised in sample buffer before resolution on SDS–PAGE (FK506 was a generous gift from Fujisawa GmbH, Munich, Germany).

2.4. Preparation of FKBP12–GST and GST fusion proteins

An FKBP12–GST fusion protein was prepared from a pGEX2TK plasmid containing human FKBP12 cDNA, as described previously [22]. In brief, 1 l XL-1 blue *Escherichia coli* transfected with this FKBP12-pGEX2TK construct, or with pGEX2TK alone, was grown at 37°C in LB broth to an optical density at 600 nm of ~0.5, then was induced by addition of 1 mM isopropyl β-D-thiogalactoside, followed by a further 2 h incubation at 25°C. Bacteria were pelleted at $5000\times g_{max}$ for 10 min at 4°C (5400 rpm, Sorval GS3 rotor), then were resuspended in 25 ml of 0.1% Triton X-100, 100 μg/ml lysozyme, phosphate-buffered saline (PBS). Suspensions were sonicated, centrifuged at $20\,000\times g_{max}$ for 1 h at 4°C (12 500 rpm, Sorval SS34 rotor), and resultant supernatants were mixed with 1000 μl (unpacked volume) of glutathione Sepharose 4B beads in PBS for 12–16 h at 4°C. Beads were pelleted at $200\times g_{max}$ for 2 min, were resuspended in 2 ml of FKBP wash buffer (FKWB: 300 mM sucrose, 170 mM NaCl, 0.1% CHAPS (3-((cholamidopropyl)dimethylammonio)-1-propane-sulfonate), 2 mM dithiothreitol, 20 mM Tris–HCl pH 7.4 and protease inhibitors) containing 0.05% sodium azide, then were stored for up to 3 weeks at 4°C.

2.5. FKBP12–GST affinity precipitations

Proteins that interact with FKBP12 were isolated from solubilised microsomal membranes by a modification of a previously described procedure [30]. Protein (16 mg) from skeletal muscle, cardiac muscle or brain crude microsomes was solubilised in 8 ml of 2% CHAPS, 500 mM NaCl, 2 mM dithiothreitol, 0.5 mM PMSF, 200 mM Tris–HCl pH 7.4 for 1 h at 22°C with shaking. Reactions were centrifuged at $100\,000\times g_{max}$ for 1 h (Beckman Type 42.1 rotor, 32 000 rpm, 4°C), then the solubilised material incubated with 40 μg of FKBP12–GST or GST protein, immobilised on agarose beads, overnight at 4°C. Beads were then pelleted, resuspended in 10 ml of FKWB, and divided into 10×1 ml aliquots. Complexes were incubated in the presence of 10 μM FK506, or vehicle (0.1% methanol) for 2 h at 37°C, then

were washed six times with FKWB by repeated centrifugation and resuspension of the beads (12 000 rpm for 10 s, Eppendorf microfuge). Beads were finally resuspended in 20 μl of 1×SDS–PAGE loading buffer and were stored at −20°C prior to resolution of 10 μl of each sample by SDS–PAGE.

3. Results

The distribution and relative levels of FKBP12, calcineurin Aα, RyR isoforms and IP_3R isoforms were analysed in native membranes and crude microsomes prepared from skeletal muscle, heart and brain by Western immunoblot using protein- and isoform-specific antisera. FKBP12 was expressed ubiquitously in these tissues, co-migrating with pure recombinant FKBP12 at 14 kDa (Fig. 1A). Densitometric analyses of the combined immunoreactivity from total membrane and cytosolic fractions revealed highest FKBP12 expression in brain, which was approximately 3.0- and 2.5-fold higher than that observed in skeletal and cardiac muscle, respectively (Fig. 1A). FKBP12 was detected predominantly in skeletal and cardiac muscle cytosol with minimal amounts evident in total membrane fractions, in contrast, proportionally higher levels of FKBP12 were observed in brain membranes (Fig. 1A). Approximately equal levels of FKBP12 were present in skeletal muscle and brain microsomes, with much lower expression observed in cardiac microsomes (Fig. 1A). A higher mobility FKBP12 immunoreactive band was detected in brain microsomes and derived cytosolic fractions (Fig. 1A, lanes 4 and 7). This band, which was also apparent in cardiac microsomes when higher protein concentrations were analysed, has been determined by other work using this antiserum (raised against a C-terminal region of FKBP12, shared by FKBP12.6) to be FKBP12.6 [10]. Calcineurin Aα was detected in all tissues (Fig. 1B), brain fractions had the richest expression levels of this phosphatase, followed by skeletal muscle with lowest expression observed in cardiac muscle preparations.

Studies examining the distribution of the different RyR and IP_3R isoforms among the rabbit tissues revealed the greatest RyR1 levels in skeletal muscle membranes and microsomes, with approximately 20-fold lower expression levels observed in cardiac membranes and microsomes, and no expression evident in brain (Fig. 1C). On the other hand, the greatest

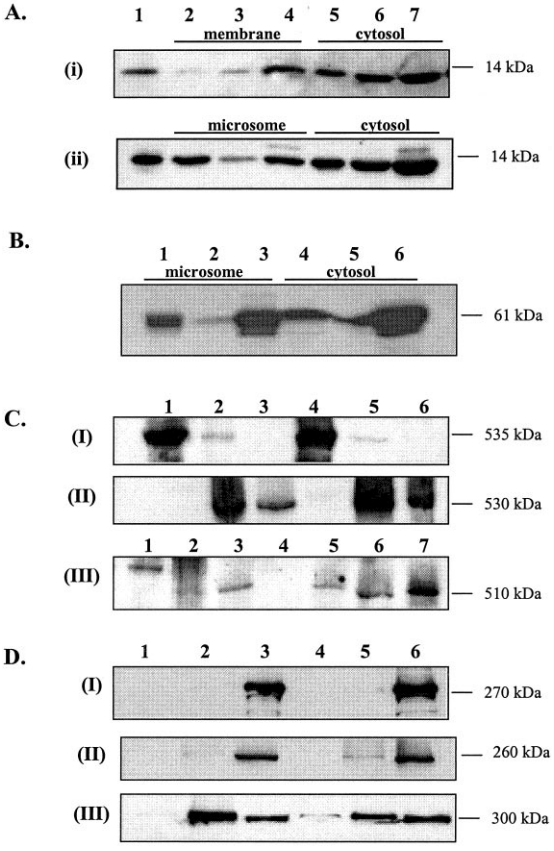

Fig. 1. Representative immunoblots showing the relative expression levels of (A) FKBP12, (B) calcineurin Aα, (C) RyR isoforms and (D) IP_3R isoforms in various fractions prepared from skeletal muscle, cardiac muscle and brain. A: Lane 1 contains pure human recombinant FKBP12, lanes 2–7 contain fractions indicated, prepared from skeletal muscle (lanes 2, 5), cardiac muscle (lanes 3, 6) and brain (lanes 4, 7). B: Lanes 1–6 contain fractions indicated, derived from skeletal muscle (lanes 1, 4) cardiac muscle (lanes 2, 5) and brain (lanes 3, 6), respectively. C: (I) RyR1, (II) RyR2 and (III) RyR3 immunoblots of skeletal muscle, cardiac muscle, brain membranes (lanes 1–3) and derived microsomes (lanes 4–6), respectively. For RyR3 bovine diaphragm was loaded (lane 7) as an internal control. D: (I) IP_3R1, (II) IP_3R2 and (III) IP_3R3 immunoblots of skeletal muscle, cardiac muscle, brain membranes (lanes 1–3) and derived microsomes (lanes 4–6), respectively. Representative data from three separate determinations are shown.

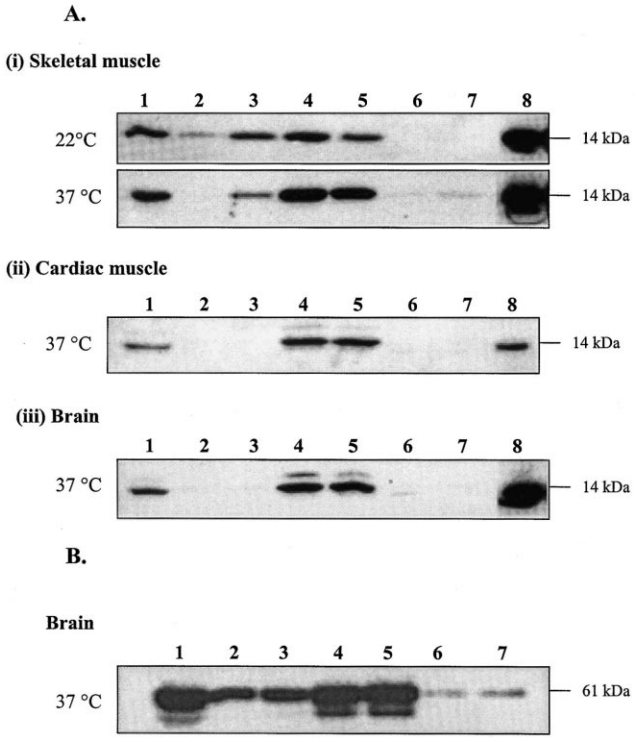

Fig. 2. Representative immunoblots showing the effect of 10 μM FK506 treatment (plus FK506) or vehicle alone (minus FK506) on the distribution of (A) FKBP12 in skeletal muscle, cardiac muscle and brain fractions and (B) calcineurin Aα in brain fractions. Microsomes were treated with FK506 (10 μM) for 1 h at 22°C or 37°C as indicated, and derived final pellet (P) and initial (SN-1) and final supernatant (SN-2) analysed for FKBP12 or calcineurin immunoreactivity. A,B: Lane 1, original microsomes; lane 2, P plus FK506; lane 3, P minus FK506; lane 4, SN-1 plus FK506; lane 5, SN-1 minus FK506; lane 6, SN-2 plus FK506; lane 7, SN-2 minus FK506 and lane 8, pure human recombinant FKBP12. Data shown are representative of two identical experiments.

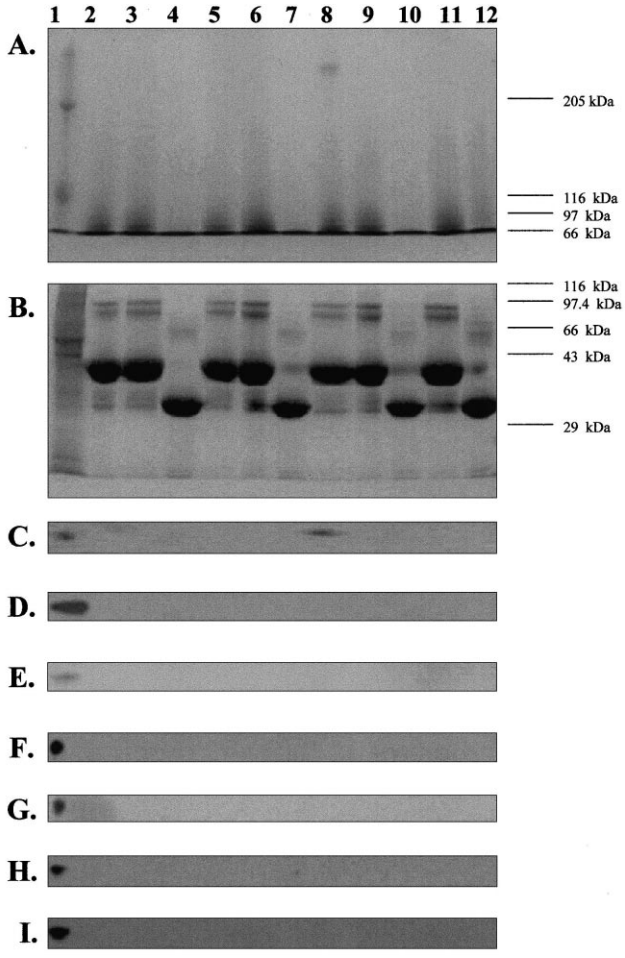

Fig. 3. CHAPS-solubilised membrane proteins from brain (lanes 2–4), cardiac (lanes 5–7) or skeletal muscle (lanes 8–10) microsomes were incubated with GST beads (lanes 4, 7, 10, 12) or with FKBP12–GST beads (all other lanes). Reactions were subsequently treated with 10 μM FK506 (lanes 3, 6, 9) or methanol vehicle (lanes 2, 5, 8). The resulting complexes were washed, resolved on 6% (A, C–H) or 15% (B and I) SDS–PAGE gels, then stained with Coomassie R250 (A and B), or blotted onto nitrocellulose for immunostaining with the following antibodies: C, anti-RyR1; D, anti-RyR2; E, anti-RyR3; F, anti-IP_3R1; G, anti-IP_3R2; H, anti-IP_3R3; and I, anti-calcineurin Aα. Lanes 11 and 12 are FKBP12–GST (lane 11) and GST (lane 12) incubated in the absence of solubilised microsomal proteins. Positive controls for immunoblots were 10 μg of solubilised microsomal protein from skeletal muscle (A and C) or brain (all other panels), loaded into lane 1 of these gels. Positions of molecular weight markers for SDS–PAGE are given on the right hand side of A and B. Representative data of three separate experiments are shown.

expression of RyR2 was apparent in cardiac membranes and microsomes, with substantial expression evident in brain (approximately 2.5-fold less) and no expression in skeletal muscle (Fig. 1C). Finally, RyR3 levels were present at maximum levels in brain microsomes with lower levels detected in cardiac microsomes, and no expression in skeletal muscle (Fig. 1C). A higher RyR3 immunoreactive band was detected in skeletal muscle membranes but not in derived microsomes. All IP_3R isoforms were expressed to relatively high levels in brain microsomes and membrane fractions (Fig. 1D). IP_3R2 was identified in cardiac fractions (Fig. 1D) and IP_3R3 was present in all tissues examined, with highest expression in cardiac and brain membranes and microsomes, and minimal expression observed in skeletal muscle microsomes (Fig. 1D).

It has been shown previously that high concentrations of FK506 (10 μM) need to be incubated with solubilised terminal cisternae prepared from skeletal muscle, to enable complete removal of FKBP12 from this SR fraction [7]. The dissociation of FKBP12 from microsomes prepared from the three tissue types was thus monitored, in the presence and absence of this previously described FK506 treatment procedure. Western blots with FKBP12 antisera revealed a different profile of FKBP12 removal when comparing skeletal muscle microsomes with brain or cardiac microsomes. Results in Fig. 2A show that FK506 treatment was required to totally remove FKBP12 from skeletal muscle microsomes where significant differences in FKBP12 removal from microsomes were observed in the absence and presence of FK506. FKBP12 removal from skeletal muscle microsomes was temperature dependent, with complete FKBP12 removal observed at 37°C in the presence of FK506, and partial removal observed at 22°C (Fig. 2A). In striking contrast to the situation observed in skeletal muscle, FKBP12 was removed completely and equally effectively, from both brain and cardiac microsomes, in the presence and absence of FK506. Similar results were observed when incubations were performed at 22°C, and also with membrane fractions (results not shown). In fact FKBP12 was found to dissociate from both brain and cardiac microsomes and membranes during simple high-speed centrifugation ($100\,000 \times g_{max}$) alone. Higher levels of FKBP12.6 immunoreactivity were apparent in the supernatants derived from FK506-treated brain and cardiac samples compared to vehicle-treated preparations. Results in Fig. 2B demonstrate that identical amounts of calcineurin Aα remain in the brain microsomal fractions and are removed from it in the presence and absence of FK506. Similar results were observed for skeletal muscle microsomes (results not shown). The relationship of calcineurin Aα with cardiac microsomes was more difficult to monitor, owing to its lower levels of expression in this tissue.

An FKBP12–GST fusion protein, immobilised on agarose beads, was allowed to interact with protein solubilised from brain, cardiac and skeletal muscle microsomes. Resulting complexes were then probed with various antibodies in immunoblot assays (Fig. 3C–I) or stained with Coomassie R250 (Fig. 3A,B). Of all proteins tested, only a high apparent molecular weight protein (~500 kDa, Fig. 3A, lane 8) from skeletal muscle bound to these beads. This protein was immunoreactive with the anti-type 1 RyR antibody (Fig. 3C) and was specifically associated with FKBP12, since it did not bind to GST-only beads (Fig. 3A,C) and was displaced from the FKBP12–GST fusion protein by treatment with 10 μM FK506 (Fig. 3A,C). All other Ca^{2+} release channels were not detectable in FKBP12–GST affinity precipitations from tissues in which they are expressed (Fig. 3D–H). Similarly, calcineurin Aα was not detectable in such precipitates (Fig. 3I). A variety of conditions were used to determine whether differing buffer components (e.g. pH, NaCl, concentrations, Ca^{2+} and Mg^{2+}) could induce a high affinity association of the other Ca^{2+} release channels or calcineurin with FKBP12. However, no matter what conditions were employed only skeletal muscle RyR1 was found to bind tightly with FKBP12.

4. Discussion

In initial experiments we measured the complement of RyR isoforms, IP_3R isoforms, FKBP12 and calcineurin in native membranes and microsomes from brain, skeletal and cardiac tissue, thus determining the range of endogenous Ca^{2+} release proteins that could potentially form a tight association with FKBP12/12.6 in tissues which rely heavily on intracellular Ca^{2+} signalling. FKBP12 was detected predominantly in the cytosol of all tissues, as previously described [3], with greatest levels present in brain, in agreement with [^{3}H]FK506 binding studies of Steiner et al. [26]. High FKBP12 levels were also evident in skeletal muscle and brain microsomes, with lower expression apparent in cardiac microsomes. Much lower levels of FKBP12.6 were detected compared to FKBP12, which was identified predominantly in brain microsomes, with minimal expression in cardiac microsomes. Interestingly, FKBP12.6 expression has not been described previously in brain. The low levels of FKBP12.6 compared to FKBP12 in rabbit cardiac microsomes were at first sight surprising, considering that FKBP12.6 has been reported to be the major isoform in cardiac microsomal membranes from canine heart [12,13]. However, recent studies have shown FKBP12.6 to be undetectable in rabbit cardiomyocytes, with FKBP12 being the predominant isoform [31], and other work has shown that FKBP12 and FKBP12.6 are expressed in cardiac microsomes from diverse vertebrate species, the exception being canine microsomes, which only express FKBP12.6 [10].

The expression of Ca^{2+} release channel isoforms in the various tissue types agreed with the previous literature finding RyR1, RyR2 and RyR3 to be expressed predominantly in skeletal muscle, heart and brain, respectively, with high levels of RyR2 detected in brain [22,23,27], low levels of RyR1 and RyR3 detected in heart [32,33], and RyR3 protein detected in diaphragm [33]. Comparative levels of IP_3R protein isoforms have been analysed previously in rat tissue membranes, excluding skeletal muscle [25], but have for the most part been analysed at an mRNA level [24,34,35]. It has been inferred from mRNA estimations that IP_3R2 is present at high levels in skeletal muscle. However, this isoform was not detected in rabbit skeletal microsomes, which only expressed minimal levels of the IP_3R3 isoform. Brain and cardiac microsomes and membranes expressed all three IP_3R isoforms to differing degrees [25] with IP_3R1 expression found to very minimal levels in cardiac tissue. The calcineurin Aα expression profile agrees with previous studies, with lowest expression evident in cardiac tissue which predominantly expresses the calcineurin Aβ isoform [36].

Although FKBP12 is predominantly cytosolic, it has been found to be very tightly associated with solubilised skeletal muscle terminal cisternae, but not heart SR [7,13]. This FKBP12 terminal cisternae association requires high concentrations of FK506 for disruption, and is assumed to occur through the well described integral association of RyR1 with FKBP12 [7–9]. Our results showed that this tight FKBP12–membrane association was also evident in unsolubilised native microsomes, where identical temperature dependent FK506 treatments as those described by Timerman et al. [7,13] were essential to enable total dissociation of FKBP12 from skeletal muscle but not cardiac microsomes. The nature of the association between FKBP12 and brain microsomes has been assumed to be similar to that displayed between FKBP12 and skeletal muscle, owing to studies showing that FKBP12 associates tightly with the cerebellar IP_3R1 complex, requiring FK506 for removal [19,20]. However, our work found that FKBP12 was very readily displaced from brain microsomes in an FK506 independent manner, suggesting that FKBP12 was not an integral subcomponent of the array of Ca^{2+} release channels expressed in brain. Much lower FKBP12.6 levels were detected in cardiac and brain fractions, and it appeared that FK506 was more effective in removing FKBP12.6 from both these fractions, however this was difficult to quantify as FKBP12.6 expression was extremely low compared to FKBP12. It has been suggested that association of FKBPs with intracellular Ca^{2+} release channels may favour the more abundant FKBP isoform present in the cytosol [37]. More work needs to be performed to investigate the relevance of the modulation of Ca^{2+} release channels in brain by endogenous FKBP12.6 in the presence of vastly higher levels of endogenous FKBP12, as observed in this study.

Yeast two-hybrid studies have found that FKBP12 binds to IP_3R1 at a leucyl-prolyl dipeptide motif, which anchors calcineurin to this hydrophobic IP_3R–FKBP12 complex [38]. Analogous FKBP12/12.6 dipeptide binding domains are conserved through all types of IP_3R and RyR isoforms. Quantification of the affinity of interaction between FKBP12 and all endogenously expressed RyR isoforms, IP_3R isoforms and calcineurin among excitable tissue types has not been analysed. This is important in order to understand the dynamics of FKBP12 control of intracellular Ca^{2+} signalling, and the constitutive subunit composition of RyR and IP_3R isoforms in these different tissue types. Results of the present study showed that skeletal muscle RyR1 bound tightly to FKBP12 affinity beads in an FK506 displaceable manner but that in sharp contrast, none of the other Ca^{2+} release channels or calcineurin Aα expressed in brain, heart or skeletal muscle bound specifically to FKBP12–GST under a variety of conditions. These data agree with our findings on the association of FKBP12 with the diverse native tissue types, and the many studies describing high affinity RyR1–FKBP12 association. However, once again they are in contrast to studies describing tight FKBP12 association with the IP_3R–calcineurin complex in brain. It is possible that these discrepancies result from species differences, different preparations or methodologies used. Although the tight binding of calcineurin and FKBP12 has not been broadly explored, some controversy does however exist with respect to the described integral association of FKBP12 with IP_3Rs. In this respect a very recent study detected no direct interaction between cerebellar IP_3R1 and FKBP12, and showed that the proteolytic fragment of IP_3R1 containing the proposed FKBP12 binding site is not retained by GST–FKBP12, in contrast to the situation with RyR1 [18]. The work described in the present study further extends this strongly suggesting that none of the endogenous IP_3R isoforms expressed in brain, heart, or skeletal muscle is tightly associated with FKBP12. This also agrees with studies that have been unable to demonstrate a functional effect of FK506 and FKBP12 on IP_3-induced Ca^{2+} release in different cell types [21]. We proposes that a constitutive tight binding association between IP_3R isoforms and FKBP12 may be tenuous at best. We furthermore suggest a lack of integral association between FKBP12 and RyR3 in brain and heart, which agrees with recent work hypothesising that comparative differ-

ences in RyR1 and RyR3 gating behaviour may be due to the lower affinity of RyR3 for FKBP12 [39].

The results of this study do not imply that FKBP12 does not functionally associate or modulate intracellular Ca^{2+} release channels besides RyR1. Differential structural constraints of these receptors compared to RyR1 may impede constitutive high affinity FKBP12 association with these Ca^{2+} release channels. Modulation of receptor conformation induced by cellular events such as protein folding, phosphorylation, or the association of other accessory proteins may induce both low and high affinity FKBP12 receptor association causing altered channel function. In this respect it is interesting to note that FKBP12.6 has been found to dissociate from cardiac RyR2 in response to RyR2 phosphorylation [15]. In conclusion, the results of the present study suggest that the properties of skeletal muscle RyR1 are unique among intracellular Ca^{2+} release channel isoforms with respect to their tight constitutive association with FKBP12. This integral association may underlie exclusive features of RyR1-induced skeletal muscle excitation–contraction coupling. The findings of this work are important in gaining an understanding of the differential mechanisms by which the FKBP12 family interacts with and regulates endogenous intracellular Ca^{2+} channels in different tissue types.

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