Characterization and mapping of the 12 kDa FK506-binding protein (FKBP12)-binding site on different isoforms of the ryanodine receptor and of the inositol 1,4,5-trisphosphate receptor

Geert BULTYNCK^{*1}, Patrick DE SMET^{*}, Daniela ROSSI[†], Geert CALLEWAERT^{*}, Ludwig MISSIAEN^{*}, Vincenzo SORRENTINO[‡], Humbert DE SMEDT^{*} and Jan B. PARYS^{*}

^{*}Laboratorium voor Fysiologie, K.U.Leuven Campus Gasthuisberg O/N, Herestraat 49, B-3000 Leuven, Belgium, [†]Section of Molecular Medicine, Department of Neuroscience, University of Siena, I-53100 Siena, Italy, and [‡]DIBIT, San Raffaele Scientific Institute, I-20132 Milan, Italy

We investigated the interaction of the 12 kDa FK506-binding protein (FKBP12) with two ryanodine-receptor isoforms (RyR1 and RyR3) and with two *myo*-inositol 1,4,5-trisphosphate (IP₃) receptor isoforms (IP₃R1 and IP₃R3). Using glutathione S-transferase (GST)-FKBP12 affinity chromatography, we could efficiently extract RyR1 (42 ± 7% of the solubilized RyR1) from terminal cisternae of skeletal muscle as well as RyR3 (32 ± 4% of the solubilized RyR3) from RyR3-overexpressing HEK-293 cells. These interactions were completely abolished by FK506 (20 μM) but were largely unaffected by RyR-channel modulators. In contrast, neither IP₃R1 nor IP₃R3 from various sources, including rabbit cerebellum, A7r5 smooth-muscle cells and IP₃R-overexpressing Sf9 insect cells from *Spodoptera frugiperda*, were retained on the GST-FKBP12 matrix. Moreover, immunoprecipitation experiments indicated a high-affinity interaction of FKBP12 with RyR1 but not with IP₃R1. In order to determine the FKBP12-binding site, we fragmented both RyR1 and IP₃R1

by limited proteolysis. We obtained a 45 kDa fragment of RyR1 that bound to the GST-FKBP12 matrix, indicating that it retained all requirements for FKBP12 binding. This fragment was identified by its interaction with antibody m34C and must therefore contain its epitope (amino acids 2756–2803). However, no fragment of IP₃R1 was retained on the column. These molecular data are in agreement with the lack of correlation between FKBP12 and IP₃R1 expression in various cell types. The observation that FKBP12 did not affect IP₃-induced Ca²⁺ release but reduced caffeine-induced Ca²⁺ release also indicated that mature IP₃R1 and IP₃R3, in contrast to RyR1 and RyR3, did not display a specific, high-affinity interaction with FKBP12.

Key words: immunophilin, intracellular Ca²⁺-release channel, *myo*-inositol 1,4,5-trisphosphate receptor, protein–protein interaction.

INTRODUCTION

Ryanodine receptors (RyRs) and *myo*-inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃Rs) are large tetrameric intracellular Ca²⁺-release channels, both represented by three different isoforms (reviewed in [1]). The type-1 RyR (RyR1) is highly expressed in the terminal cisternae (TC) of striated muscle cells, whereas the type-3 isoform (RyR3) is expressed in many tissues at very low levels [2,3]. IP₃Rs are expressed ubiquitously and most cell types contain two or more isoforms [4–7].

Accessory proteins, such as the 12 kDa FK506-binding protein (FKBP12), are important regulators of the intracellular Ca²⁺-release channels [8–11]. FKBP12 is associated with the RyR1 of TC of skeletal-muscle cells [12,13] and also interacts with the RyR3 of diaphragm [14], whereas the related protein FKBP12.6 interacts with the cardiac RyR2 [15]. The functional effects of FKBP12 on RyR1 have been well investigated: FKBP12 stabilized the RyR1 channel, resulting in full-conductance openings [16], and promoted coupled gating of RyR1 clusters [17].

More recently, FKBP12 was proposed to interact with the cerebellar IP₃R1, thereby modulating IP₃-induced Ca²⁺ release [18] and targeting calcineurin to the IP₃R1 [19]. The putative FKBP12-binding site on IP₃R1 was determined by the yeast two-

hybrid technique. The interaction site corresponded to a leucylprolyl residue in the regulatory domain of the receptor (amino acids 1400–1401) [20]. This site is relatively well conserved among the different RyR and IP₃R isoforms. Recently, the interaction of FKBP12.6 with the homologous site on RyR2 was also demonstrated with the yeast and two-hybrid technique [21].

Until now, however, the localization of the FKBP12-binding site on either the RyR or the IP₃R has not been determined at the level of the mature protein in a direct way. Moreover, although the functional effects of FKBP12 on RyR1 are well described, its interaction with IP₃R1 is controversial. Several functional studies failed to support a physiological regulation of IP₃-induced Ca²⁺ release by FKBP12 [22–24].

We therefore investigated the interaction of FKBP12 with different types of intracellular Ca²⁺-release channels at the protein level and we characterized the localization and properties of the FKBP12-binding site. Our results indicate that both RyR1 and RyR3 efficiently bound FKBP12 and that the FKBP12-binding site on RyR1 is localized at or near the amino acids homologous to the FKBP12.6-binding site detected in RyR2 by the yeast two-hybrid technique [21]. However, neither IP₃R1 nor IP₃R3 did interact with FKBP12. Furthermore, the fragment of IP₃R1 containing the previously proposed FKBP12-binding site [20]

Abbreviations used: DTT, dithiothreitol; FKBP12, 12 kDa FK506-binding protein; GST, glutathione S-transferase; IP₃, *myo*-inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; IP₃R-FKBS, FKBP12-binding site of IP₃R1; RyR, ryanodine receptor; RyR-FKBS, FKBP12-binding site of RyR1; TC, terminal cisternae.

¹ To whom correspondence should be addressed (e-mail geert.bultynck@med.kuleuven.ac.be).

did not interact with FKBP12. Taken together, these results suggest that the FKBP12-binding site on the IP₃R is not functional in the mature protein or may only be accessible under specific conditions.

EXPERIMENTAL

Cell culture

SH-SY5Y human neuroblastoma cells, A7r5 rat aorta smooth-muscle cells, HEK-293 cells, RBL-2H3 rat mucosal mast cells, 16HBE14o – human bronchial epithelial cells and Sf9 insect cells from *Spodoptera frugiperda* were cultured as described previously [23,25–27]. C2C12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1% non-essential amino acids, 2 mM L-glutamine, 85 i.u./ml penicillin, 85 µg/ml streptomycin, 10% (v/v) foetal calf serum and 0.5% (v/v) chicken embryo extract. Differentiation was induced by reducing the foetal calf serum concentration to 0.5% (v/v). The expression of IP₃R1 and IP₃R3 in Sf9 insect cells [26,28] and of RyR3 in HEK-293 cells [27] was performed as described previously.

Construction of glutathione S-transferase (GST) fusion proteins, encoding FKBP12 or the FKBP12-binding sites of IP₃R1 (GST-IP₃R-FKBS) and RyR1 (GST-RyR-FKBS)

cDNA encoding human FKBP12 was amplified using PCR with 5'-GCCGGATCCATGGGAGTGCAGGTGGAAACC-3' as the forward primer, with a 5'-flanking *EcoRI* restriction site (underlined), and 5'-CCAAGAATTCTCATTCCAGTTTTAG-AAGCTCC-3' as the reverse primer, with a 5'-flanking *BamHI* restriction site (underlined). PCR products were digested with *BamHI* and *EcoRI*. These fragments were purified by PAGE and ligated into the *BamHI*–*EcoRI* sites of pGEX-2T.

Amplification of the cDNA encoding the proposed FKBP12-binding site in the mouse IP₃R1 [20] (IP₃R-FKBS, amino acids 1349–1461) and the analogous domain in the rabbit RyR1 (RyR-FKBS, amino acids 2406–2520) was performed by PCR, using the full-length mouse IP₃R1 cDNA and the full-length rabbit RyR1 cDNA as templates.

Oligodeoxynucleotides were synthesized with a 5'-flanking *BamHI* restriction site (underlined below) for the forward primers and a 5'-flanking *EcoRI* restriction site (underlined below) for the reverse primers. For the construction of GST-IP₃R-FKBS, we used 5'-CAATGGATCCGCCTCTTCCAGACTCTGATCC-3' as a forward primer and 5'-TGTTGAATTCTCAG-GCCCTGCAGATCTCCACG-3' as a reverse primer. For the construction of GST-RyR-FKBS, we used 5'-GGCGCGGATCC TTTGGGAGGAGCC-3' as a forward primer and 5'-TCCA-GGAATTCTCAGCAGGAAGTCCTGG-3' as a reverse primer. PCR products were digested with *BamHI* and *EcoRI*. These fragments were purified by PAGE and ligated into *BamHI*–*EcoRI* restriction sites of pGEX-6p2. All plasmids were transformed into *Escherichia coli* DH5α for cloning and sequencing, followed by transformation into *E. coli* BL21 (DE3) expression host. The nucleotide sequences were confirmed by sequencing with the Automated Laser Fluorescent[®] sequencing system (Amersham Pharmacia Biotech, Uppsala, Sweden).

Purification of GST fusion proteins

Bacterial cultures containing plasmids encoding parental GST or GST fusion proteins were grown until the D_{600} reached a value between 0.6 and 1. Synthesis of the fusion proteins was induced with 1 mM isopropyl β-D-thiogalactoside and the cells were cultured for another 4 h at 28 °C. Cells were harvested and

resuspended in PBS supplemented with 77 nM aprotinin, 0.83 mM benzamidin, 1.1 µM leupeptin, 0.7 µM pepstatin A, 0.23 mM PMSF and 1% Triton X-100. After sonication at 20 kHz (nine times for 10 s using a probe sonicator; MSE, Crawley, Surrey, U.K.) and centrifugation, the soluble proteins were bound to glutathione–Sephacrose 4B. After washing with PBS, supplemented with benzamidin, PMSF, 2-mercaptoethanol and 0.1% (w/v) azide, the matrix-bound GST and GST-FKBP12 were stored at 4 °C. GST-IP₃R-FKBS and GST-RyR-FKBS were present in the insoluble pellet. These fusion proteins were solubilized with 1% (w/v) sarcosyl [29]. The sarcosyl-solubilized proteins were bound on glutathione–Sephacrose 4B. The GST fusion proteins were eluted with 10 mM reduced glutathione, dialysed in PBS with benzamidin, PMSF and 2-mercaptoethanol and stored at –80 °C.

SDS/PAGE and Western-blot analysis

FKBP12 and GST fusion proteins were analysed by SDS/PAGE (14 and 10% gels, respectively; 26 mA/gel, constant current), followed by semi-dry electrophoretic transfer to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, U.S.A.). Transfer was performed for 2 h in transfer buffer (25 mM Tris/192 mM glycine) supplemented with 0.0375% SDS and 20% methanol (0.8 mA/cm², constant current). Full-length IP₃Rs and RyRs were analysed by SDS/PAGE (3–12% gels; 50 mA/gel, constant current) followed by wet electrophoretic transfer performed for 16 h in transfer buffer (0.6 mA/cm², constant current). After blocking the membranes with PBS containing 1% (v/v) Tween-20 and 5% (w/v) non-fat dry milk powder, the membranes were subsequently incubated with primary antibody (Table 1) and alkaline phosphatase-conjugated secondary antibody. The immunoreactive bands were visualized after conversion of the Vistra[®] ECF substrate (Amersham Pharmacia Biotech) and analysed as described previously [30]. Values are given as means ± S.D. and statistical comparisons were made using the Student's *t* test. Commercial antibodies were anti-FKBP12 (Affinity BioReagents, Golden, CO, U.S.A.), m34C (Affinity BioReagents), XA7 (Upstate Biotechnology, Lake Placid, NY, U.S.A.), anti-IP₃R3 (Transduction Laboratories, Lexington, KY, U.S.A.) and anti-GST (Calbiochem, Darmstadt, Germany).

Microsomal preparations

TC were isolated as described previously [31], except for the addition of protease inhibitors (0.83 mM benzamidin/0.23 mM PMSF/2 µg/ml leupeptin) to the homogenization medium. Microsomes of rabbit cerebellum [32], Sf9 cells [26,28] and RyR3-transfected HEK-293 cells [27] were prepared as described.

GST-FKBP12 affinity chromatography

The GST-FKBP12 affinity-chromatography assay was adapted from a previously described procedure [33]. Microsomal pellets (2 mg of protein) were solubilized in 500 µl of solubilization buffer, i.e. 20 mM Tris/HCl, pH 7.4/1 M NaCl/2 mM dithiothreitol (DTT)/2% (w/v) CHAPS/1% (w/v) L-α-phosphatidylcholine in the presence of all the above-mentioned protease inhibitors. Solubilization (10 min, 4 °C) was arrested by centrifugation at 125000 *g*. The solubilized fraction was diluted 5-fold with 20 mM Tris/HCl, pH 7.4/2 mM DTT and incubated (40 min, 37 °C) under continuous agitation with GST-FKBP12, immobilized on glutathione–Sephacrose 4B. The beads were washed four times with PBS supplemented with 0.5% (w/v) CHAPS/2 mM DTT; in order to remove the actomyosin, NaCl

Table 1 Overview of the primary antibodies used in this study

The epitopes of the antibodies are indicated where available. Abbreviations used: pAb, polyclonal antibody; mAb, monoclonal antibody; TM, transmembrane domain. The indicated amino acids (aa) of IP₃R1 refer to the SI(+)/SI(-) splice variant from mouse cerebellum [41].

Name	Antigenic protein	Epitope	Dilution for Western blotting	Type	Reference
Anti-FKBP12	FKBP12	aa 1–13	1/1000	Rabbit pAb	—
m34C	RyR1	aa 2756–2803	1/3000–1/8000	Mouse mAb	[42,43]
XA7	RyR1	—	1/750–1/8000	Mouse mAb	[44]
Anti-RyR3	RyR3	TM4–TM5	1/3000	Rabbit pAb	[45]
Rbt03	IP ₃ R1	aa 2735–2749	1/8000	Rabbit pAb	[26,32]
Anti-loop17a-2	IP ₃ R1	aa 2463–2528	1/5000	Rabbit pAb	—
10A6	IP ₃ R1	aa 943–1237	1/750	Rat mAb	[46]
Anti-IP ₃ R3	IP ₃ R3	aa 22–230	1/1500	Mouse mAb	[7]
Anti-GST	Recombinant GST	—	1/1000	Rabbit pAb	—

(0.5 M) was added in the first two wash steps in the case of solubilized TC vesicles. Control conditions made use of either immobilized GST or of the addition of 20 μ M FK506 to immobilized GST-FKBP12. Protein complexes were eluted by the addition of Laemmli buffer.

Mapping of the FKBP12-binding site on RyR1 and IP₃R1

TC vesicles (1 mg of protein) or microsomes of IP₃R1-over-expressing Sf9 cells (2 mg of protein) were partially trypsinized as described previously [34,35]. In short, microsomal fractions were washed twice with 20 mM Tris/HCl, pH 7.4/150 mM NaCl. TC vesicles were treated with trypsin (1:12, w/w) for 6 min and Sf9 microsomes with trypsin (1:1000, w/w) for 10 min, both at room temperature. Trypsinization was stopped by the addition of a 10-fold excess of soya bean trypsin inhibitor and 0.23 mM PMSF. Trypsinized membranes were solubilized in the above-described solubilization buffer. The solubilized fraction was incubated for 2 h (4 °C) with GST-FKBP12, immobilized on glutathione-Sepharose 4B. During this incubation, 0.2% ethanol (vehicle) or 20 μ M FK506 was added. The beads were subsequently washed four times with PBS containing 0.5% (w/v) CHAPS, 2 mM DTT and protease inhibitors. Protein complexes were eluted with Laemmli buffer.

Immunoprecipitation

The microsomal pellets (0.8 and 2 mg of protein for TC and rabbit cerebellar microsomes, respectively) were solubilized for 4 h at 4 °C in 20 mM Tris/HCl, pH 7.4/150 mM NaCl/0.25% (v/v) Triton X-100 and all the above-mentioned protease inhibitors. The solubilization was arrested by centrifugation at 125000 g. The supernatants were incubated subsequently for 2 h at 4 °C with anti-RyR1 (m34C, 1/20) or anti-IP₃R1 (Rbt03, 1/40) and allowed to react with Protein G-Sepharose or Protein A-Sepharose, respectively (2 h, 4 °C). After four washes with PBS containing 0.25% (v/v) Triton X-100, the immune complexes were dissociated with Laemmli buffer.

Unidirectional ⁴⁵Ca²⁺ fluxes on saponin-permeabilized cells

Unidirectional ⁴⁵Ca²⁺-efflux measurements on permeabilized A7r5 cells were performed exactly as described previously [36]. For C2C12 cells, the efflux medium contained in addition 5 mM MgCl₂/5 mM ATP/150 nM free Ca²⁺ in order to activate RyRs.

RESULTS

Expression of intracellular Ca²⁺-release channels and FKBP12

The protein levels of IP₃R1, RyR1 and FKBP12 were quantified in microsomal fractions from different cell types by Western-blot analysis with isoform-specific antibodies. The relative expression levels for IP₃R1 and FKBP12 are shown in Figure 1. IP₃R1-

Figure 1 Expression of IP₃R1 and FKBP12 in different cell types

The microsomal fraction of rabbit cerebellum (RCM), of SH-SY5Y, A7r5, RBL-2H3 and 16HBE14o— cells or of TC from skeletal muscle (TC) were subjected to SDS/PAGE and immunoblotted with specific antibodies (A) against IP₃R1 (Rbt03) or (B) against FKBP12 (anti-FKBP12). The results were normalized to the amount of protein loaded on the gel and calculated relative to the signal obtained in RCM. Each result is the mean $\pm$ S.D. from at least three independent experiments. Note the logarithmic scale.

Figure 2 Affinity purification of RyR1 using GST-FKBP12 immobilized on glutathione-Sepharose 4B

TC vesicles were solubilized and incubated with GST-FKBP12 or GST immobilized on glutathione-Sepharose 4B. The retained proteins were eluted with Laemmli buffer and subjected to SDS/PAGE. (A) Protein staining with Sypro Orange[®]; (B) immunoblotting with anti-RyR1 (XA7). Lane P, molecular-mass markers; lanes 1, TC vesicles (5 μ g); lanes 2, solubilized TC vesicles; lanes 3, fraction bound to immobilized GST; lanes 4, fraction bound to immobilized GST-FKBP12; lanes 5, fraction bound to immobilized GST-FKBP12 in the presence of 20 μ M FK506. The arrows on the left indicate the molecular masses of the protein standards. The arrows on the right indicate the RyR1, GST-FKBP12 and GST. The identities of the GST fusion proteins were confirmed by an anti-GST antibody. Equivalent amounts of samples were used in lanes 1 and 2 and a 4-fold higher amount in lanes 3–5.

specific bands were detected in the microsomal fraction of rabbit cerebellum, SH-SY5Y, A7r5, RBL-2H3 and 16HBE14o– cells, but not in TC vesicles of skeletal-muscle cells (Figure 1A), which are highly enriched in RyR1 (results not shown). FKBP12 was present in the microsomes of rabbit cerebellum, SH-SY5Y and 16HBE14o– cells and in TC vesicles (Figure 1B). Since TC vesicles express 8-fold higher levels of FKBP12 than rabbit cerebellar microsomes, and since no FKBP12 was detected in, for example, A7r5 cells expressing relatively high levels of IP₃R1, one can conclude that FKBP12 expression was not correlated with IP₃R1 expression.

The relation between expression of FKBP12 and of RyR and IP₃R isoforms was therefore investigated further during differentiation of C2C12 muscle cells (results not shown). Undifferentiated C2C12 cells did not express RyRs. After 7 days of differentiation, C2C12 cells expressed high levels of both RyR1 and RyR3. Expression of IP₃R1, as well as that of IP₃R2 and IP₃R3, decreased significantly during differentiation ($P < 0.05$). In contrast, FKBP12 expression was up-regulated similarly to that of RyR1 and RyR3 ($P < 0.05$).

Table 2 Purification efficiency of the different receptors with GST-FKBP12 affinity chromatography

The binding procedure is described in the Experimental section. The values indicate the amount of bound receptor relative to the amount of solubilized receptor, set at 100%. Binding to GST was used as a negative control. The difference between the amounts bound to GST-FKBP12 and to GST is considered to indicate specific binding. Each result is the mean $\pm$ S.D. from at least three to six independent experiments.

Preparation	Receptor	GST (%)	GST-FKBP12 (%)
TC	RyR1	0	42 $\pm$ 7
Rabbit cerebellum	IP ₃ R1	$\approx$ 2	$\approx$ 2
IP ₃ R1-overexpressing Sf9 cells	IP ₃ R1	$\approx$ 2	$\approx$ 2
IP ₃ R3-overexpressing Sf9 cells	IP ₃ R3	$\approx$ 1	$\approx$ 1
RyR3-overexpressing HEK-293 cells	RyR3	0	32 $\pm$ 4

Table 3 Influence of different modulators of RyR1 and RyR3 on FKBP12 binding

The values were normalized to the amount of RyR1 or RyR3 bound under control conditions, set at 100%. Each result is the mean $\pm$ S.D. of at least three to five independent experiments. * $P < 0.05$, † $P < 0.001$ compared with the respective control. n.d., not determined.

Conditions	RyR1 (%)	RyR3 (%)
Control	100	100
20 μ M FK506	0 $\pm$ 0†	0 $\pm$ 0†
50 μ M Ca ²⁺	91 $\pm$ 13	88 $\pm$ 9
50 μ M Ca ²⁺ + 10 μ M calmodulin	96 $\pm$ 10	n.d.
1 mM EGTA	101 $\pm$ 9	64 $\pm$ 11*
25 μ M Ruthenium Red	99 $\pm$ 10	n.d.
1 mM ATP	92 $\pm$ 14	n.d.
2 μ M Ryanodine	98 $\pm$ 7	n.d.
50 μ M Ryanodine	95 $\pm$ 6	n.d.
7.5 mM Caffeine	91 $\pm$ 12	n.d.
10 μ M cADP ribose	105 $\pm$ 16	n.d.
10 mM Mg ²⁺	126 $\pm$ 10*	n.d.

Interaction of GST-FKBP12 with RyR1

In order to investigate the interaction between FKBP12 and the different intracellular Ca²⁺-release channels, we implemented an assay based on the high-affinity interaction of RyR1 with FKBP12 [33]. The intracellular Ca²⁺-release channels were gently solubilized and any endogenously bound FKBP12 was allowed to exchange with GST-FKBP12 immobilized on glutathione-Sepharose 4B. Figure 2 shows the extraction of RyR1 on a GST-FKBP12 matrix. In lane 4 of Figure 2(A), the purified RyR1 is shown after separation on SDS/PAGE and staining with Sypro Orange[®]. The retained RyR1 was specifically bound to GST-FKBP12, since parental GST (Figure 2A, lane 3) did not bind any RyR1. Furthermore, the interaction of RyR1 with GST-FKBP12 was completely disrupted by the addition of FK506 (20 μ M) during the exchange reaction (Figure 2A, lane 5). We identified the RyR1 by immunoblotting with a RyR1-specific antibody (XA7; Figure 2B). About 42 $\pm$ 7% of the solubilized RyR1 was retained by this GST-FKBP12 affinity assay (Table 2), a yield identical to that obtained by others [33]. These data support the earlier observations that the RyR1 and FKBP12 are tightly associated with each other.

Influence of different modulators of the RyR on the interaction of GST-FKBP12 with RyR1

We have subsequently investigated whether activators or inhibitors of the RyR can modulate the interaction of FKBP12 with

Figure 3 GST-FKBP12 affinity chromatography applied for IP₃R1, IP₃R3, RyR1 and RyR3 from different sources

Microsomes were solubilized and incubated with GST-FKBP12 or GST immobilized on glutathione-Sepharose 4B. The retained proteins were eluted with Laemmli buffer. After SDS/PAGE, the proteins were analysed by immunoblotting. (A) IP₃R1 from rabbit cerebellar microsomes (10 μg , Rbt03, 1/8000); (B) RyR1 from rabbit cerebellar microsomes (500 μg , XA7, 1/750); (C) IP₃R1 from microsomes of IP₃R1-overexpressing Sf9 insect cells (10 μg , Rbt03, 1/8000); (D) IP₃R3 from microsomes of IP₃R3-overexpressing Sf9 insect cells (10 μg , anti-IP₃R3, 1/1500); (E) IP₃R1 from A7r5 microsomes (20 μg , Rbt03, 1/8000); (F) IP₃R3 from A7r5 microsomes (20 μg , Rbt03, 1/1500); (G) RyR3 from microsomes from RyR3-overexpressing HEK-293 cells (10 μg , anti-RyR3, 1/2000). Lanes 1, microsomal fraction; lanes 2, solubilized microsomal fraction; lanes 3, fraction bound to immobilized GST; lanes 4, fraction bound to immobilized GST-FKBP12. Equivalent amounts of samples were used in lanes 1 and 2. In lanes 3 and 4, 4-fold (A–D), 10-fold (E, F) or 20-fold (G) higher amounts were loaded.

RyR1. Table 3 indicates that the interaction was dependent on neither Ca^{2+} nor any other activating or inhibiting ligands. Only, the addition of 10 mM Mg^{2+} induced a small but significant increase in the amount of RyR1 that was retained. These results therefore indicate that FKBP12 interacted with both the open and closed state of the RyR1, suggesting that FKBP12 remained associated with RyR1 during Ca^{2+} release.

Analysis of a possible interaction between GST-FKBP12 and the different intracellular Ca^{2+} -release channels

The same method as described above was used to study the interaction with other intracellular Ca^{2+} -release channels, i.e. IP₃R1, IP₃R3 and RyR3 (Figure 3). As is apparent from Figures 3(A), 3(C) and 3(D), no specific interaction of either IP₃R1 solubilized from rabbit cerebellum or of IP₃R1 or IP₃R3

Figure 4 Co-immunoprecipitation experiments of RyR1 and IP₃R1 with FKBP12

(A) RyR1 from TC vesicles and (B) IP₃R1 from rabbit cerebellar microsomes were solubilized and immunoprecipitated with m34C and Rbt03, respectively. Immunoprecipitates were separated on SDS/PAGE and immunoblotted with XA7 (A, upper panel) or anti-loop17a-2 (B, upper panel) and anti-FKBP12 (A and B, lower panels).

solubilized from heterologously overexpressing Sf9 cells could be detected under conditions that allowed the specific binding and purification of RyR1. When the gel was 20-fold overloaded, some non-specific binding of the IP₃R to the matrix was observed (results not shown), which however never exceeded 2% of the solubilized amount (Table 2). The addition of ligands of IP₃R, such as IP₃ or adenophostin A (10 μM), or of Ca^{2+} (100 μM), Ca^{2+} -calmodulin (10 μM) or EGTA (1 mM) could also not induce binding (results not shown). Moreover, the GST-FKBP12 affinity assay allowed the specific extraction of the RyR1 from cerebellum, although its expression level in this tissue was much lower than that of the IP₃R1 (Figure 3B).

A similar experiment was performed on A7r5 cells, which express both IP₃R1 and IP₃R3 but no immunodetectable FKBP12. Neither IP₃R1 nor IP₃R3 was retained on the GST-FKBP12 affinity matrix (Figures 3E and 3F). This ascertained that the lack of specific interaction of GST-FKBP12 with IP₃Rs in different samples was not due to a slow exchange of the endogenously bound FKBP12 for immobilized GST-FKBP12. Finally, in differentiated C2C12 cells expressing both RyRs and IP₃Rs, no IP₃Rs could be retained on the GST-FKBP12 matrix, although RyR1 was retained (results not shown). Taken together, these results imply that the full-size

Figure 5 Localization of the putative FKBP12-binding site on RyR1 and IP₃R1

After partial fragmentation of RyR1 (**A**) and IP₃R1 (**C**) by limited trypsin digestion, the TC vesicles (**A**) and Sf9 microsomes (**C**) were solubilized and incubated with GST-FKBP12 immobilized on glutathione–Sephacrose 4B. Affinity-purified fragments were separated on SDS/PAGE and analysed by immunoblotting with site-specific anti-RyR1 (**A**, m34C, 1/2500) and anti-IP₃R1 (**C**, 10A6, 1/750). Lanes 1, TC vesicles (**A**, 5 µg) or Sf9 microsomes (**C**, 10 µg); lanes 2, trypsinized microsomes; lanes 3, supernatant after centrifugation of trypsinized microsomes; lanes 4, CHAPS-solubilized fraction of the trypsinized microsomes; lanes 5, fraction bound to immobilized GST-FKBP12; lanes 6, fraction bound to immobilized GST-FKBP12 in the presence of 20 µM FK506. Equivalent amounts of samples were used in lanes 1–4, whereas 4-fold (**A**) and 20-fold (**C**) higher amounts were used in lanes 5 and 6. The arrows on the left indicate the molecular masses of the protein standards. The upper arrows on the right indicate the full-size RyR1 (**A**) and IP₃R1 (**C**). The other arrows on the right indicate proteolytic fragments of RyR1 (**A**) or the 76-kDa proteolytic fragment of IP₃R1 (**C**). The arrow labelled FKBP12-BD indicates the 45 kDa fragment, which is the smallest proteolytic fragment that still bound FKBP12. (**B**) Schematic presentation of rabbit RyR1 (5037 amino acids) with indications of the Ca²⁺-binding sites, the calmodulin-binding sites (CaM), the ATP-binding sites, the phosphorylation site Ser-2843 (P), the FKBP12-binding site (FKBP12) and the m34C epitope. FKBP12-BD indicates the localization of the identified 45 kDa proteolytic fragment.

IP₃R1 and IP₃R3 did not interact with FKBP12, although, using the same protocol, RyR1 did (Figure 2).

RyR3, solubilized from RyR3-overexpressing HEK-293 cells, was also specifically retained by GST-FKBP12 (Figure 3G). The efficiency of purification of RyR3 was 32 ± 4% (Table 2). The binding of RyR3 to GST-FKBP12 was partially dependent on Ca²⁺, as 1 mM EGTA reduced the purification efficiency to about ≈ 64% compared with control conditions (Table 3).

Immunoprecipitation of RyR1 and IP₃R1

It is conceivable that, at least in cerebellar microsomes, the endogenous FKBP12 is tightly bound to IP₃R1 and cannot readily be exchanged for exogenously added GST-FKBP12. We therefore analysed whether FKBP12 could co-immunoprecipitate with IP₃R1. RyR1 and IP₃R1 were immunoprecipitated from the Triton X-100-solubilized fraction of TC and rabbit cerebellar microsomes with m34C and Rbt03, respectively (Figure 4). FKBP12 co-immunoprecipitated with RyR1 but not with IP₃R1,

indicating that FKBP12 is associated as an endogenous protein with RyR1 but not with IP₃R1.

Identification of the FKBP12-binding site on RyR1

GST-RyR-FKBS and GST-IP₃R-FKBS, expressing the proposed FKBP12-binding sites of RyR1 (amino acids 2406–2520) and IP₃R1 (amino acids 1349–1461) [20], were constructed. However, these proteins were expressed in the inclusion bodies of *E. coli* and were therefore solubilized in 1% (w/v) sarcosyl before binding to glutathione–Sephacrose 4B. The immobilized proteins were subsequently incubated with exogenous FKBP12, but neither of the GST fusion proteins interacted (results not shown). This indicates that either the sequence or the conformation of these recombinant proteins is insufficient for stable interaction with FKBP12.

To identify the domain that is necessary and sufficient for FKBP12 binding, we performed GST-FKBP12 affinity chromatography on partially trypsinized RyR1 and IP₃R1. TC vesicles were trypsinized (Figure 5A, lane 2) and solubilized (Figure 5A,

Figure 6 Functional effects of FKBP12 on Ca^{2+} release

(A) Non-mitochondrial Ca^{2+} stores of permeabilized A7r5 cells were loaded first to steady state with $^{45}\text{Ca}^{2+}$ for 45 min and subsequently incubated in efflux medium in the presence of 2.5 μM FKBP12 (■), 2.5 μM GST-FKBP12 (▲) or 1% (v/v) PBS as control (□). After 10 min of efflux, 1 μM IP_3 was added for 2 min. (B and C) Non-mitochondrial Ca^{2+} stores of permeabilized C2C12 cells, differentiated for 7 days, were loaded first to steady state with $^{45}\text{Ca}^{2+}$ for 45 min. During the $^{45}\text{Ca}^{2+}$ -loading phase, 0.5 μM FK506 was added in order to dissociate endogenous FKBP12. After an additional wash step, the monolayers were incubated in efflux medium in the presence of 2.5 μM FKBP12 (■) or 1% (v/v) PBS as control (□). After 4 min of efflux, 1 μM IP_3 (B) or 10 mM caffeine (C) was added for 2 min. The efflux in control conditions (○) was determined in the absence of the activators. Each data point is the mean $\pm$ S.D. of three measurements. The results shown are typical of three independent experiments.

lane 4). The proteolytic pattern obtained was very similar to that described previously [34]. After GST-FKBP12 affinity chromatography, 75 and 45 kDa polypeptides, both recognized by the m34C antibody, were retained on the column (Figure 5A, lane 5). Moreover, the interaction of these fragments with GST-FKBP12 was FK506-sensitive (Figure 5A, lane 6). The epitope of m34C (amino acids 2756–2803) is localized in the proximity of amino acids 2461–2462, which is the putative FKBP12-binding site based on the analogy with the FKBP12-binding site proposed for $\text{IP}_3\text{R1}$ [20] (Figure 5B). The existence of this 45 kDa proteolytic fragment of RyR1, containing all determinants for FKBP12 binding, therefore allows in a direct way the identification of the FKBP12-binding pocket on the native protein (Figure 5B).

Whereas the full-length RyR1 as well as the RyR1 domain, containing the FKBP12-binding site, could interact with FKBP12, the full-length $\text{IP}_3\text{R1}$ could not. Since the FKBP12-binding site is conserved in $\text{IP}_3\text{R1}$, it remained to be elucidated whether a fragment of the $\text{IP}_3\text{R1}$ containing the putative FKBP12-binding site (amino acids 1400–1401) could interact

with FKBP12. For this, a similar procedure to that for the RyR1 was used (Figure 5C). The proteolytic fragments were identified by the site-specific antibody 10A6, from which the epitope is localized in the proximity of the putative FKBP12-binding site. Limited trypsinization of the $\text{IP}_3\text{R1}$ led to a limited number of fragments, including a 76 kDa fragment [35] containing the proposed FKBP12-binding site [20]. This domain was however not retained by GST-FKBP12 affinity chromatography (Figure 5C, lane 5). Taken together, these results indicate that neither the full-size $\text{IP}_3\text{R1}$ nor the 76 kDa fragment of $\text{IP}_3\text{R1}$ containing the potential FKBP12-binding site interacted with FKBP12.

Effect of FKBP12 or GST-FKBP12 on IP_3 - and caffeine-induced Ca^{2+} release

Finally, we wanted to investigate the potential effects of FKBP12 on intracellular Ca^{2+} release. We therefore measured the effect of exogenously added FKBP12 or GST-FKBP12 on IP_3 -induced Ca^{2+} release in A7r5 cells, which contain no endogenous FKBP12

(Figure 6A). We previously demonstrated that FK506 did not influence the IP_3 -induced Ca^{2+} release in these cells [23]. Permeabilized A7r5 monolayers loaded to steady state with $^{45}\text{Ca}^{2+}$ slowly lost their accumulated $^{45}\text{Ca}^{2+}$ during efflux. A 2 min exposure to $1\ \mu\text{M}$ IP_3 , which is the EC_{50} for IP_3 -induced Ca^{2+} release under these conditions, strongly accelerated the rate of Ca^{2+} release. FKBP12 or GST-FKBP12 ($2.5\ \mu\text{M}$) were added during loading of the Ca^{2+} stores with $^{45}\text{Ca}^{2+}$ (45 min, $25\ ^\circ\text{C}$) and continued during the efflux period to allow efficient binding of the proteins to the IP_3 Rs in the permeabilized A7r5 monolayers. Despite these precautions, no increase or decrease in the efficiency of IP_3 for inducing Ca^{2+} release was observed (Figure 6A). A similar experiment was performed in differentiated C2C12 cells, which express both RyRs and IP_3 Rs. Since these cells contained endogenous FKBP12, we first depleted the permeabilized C2C12 cells of FKBP12 by the addition of $0.5\ \mu\text{M}$ FK506 during the $^{45}\text{Ca}^{2+}$ -loading phase. The FKBP12-stripped cells were subsequently challenged with agonist ($1\ \mu\text{M}$ IP_3 or $10\ \text{mM}$ caffeine). Addition of FKBP12 ($2.5\ \mu\text{M}$) did not significantly affect the IP_3 -induced Ca^{2+} release (Figure 6B). However, FKBP12 reduced the caffeine-induced Ca^{2+} release through the RyR to $61 \pm 6\%$ ($P < 0.05$; Figure 6C). These data are in complete agreement with the molecular observations that FKBP12 is a regulator of the RyR isoforms but not of the IP_3 R isoforms.

DISCUSSION

Many regulatory proteins are proposed to interact with intracellular Ca^{2+} -release channels, including the immunophilin FKBP12 [8]. Although the effect of FKBP12 on RyR1 function is very well documented [12,16,17,21,23], its interaction site has not yet been directly determined and localized. Moreover, data concerning the association of the IP_3 R1 with FKBP12 are still controversial, since reports concerning the functional regulation of the IP_3 R by FKBP12 [18,19] or the molecular interaction of FKBP12 with IP_3 R1 [20] were not confirmed in other studies. In particular, there is much less evidence for a functional role of FKBP12 in the regulation of IP_3 -induced Ca^{2+} release. First, we previously demonstrated that FK506 had no functional effect on IP_3 -induced Ca^{2+} release in different cell types, including SH-SY5Y cells and A7r5 cells [23]. Moreover, in the present study, we demonstrated that also the addition of FKBP12 did not influence IP_3 -induced Ca^{2+} release in permeabilized A7r5 cells. Second, effects of FK506 on IP_3 -induced Ca^{2+} release in intact cells are controversial. It was suggested that the dissociation of FKBP12 of the IP_3 R1 by FK506 might potentiate Ca^{2+} flux through this channel [18]. However, FK506 strongly suppressed ATP-induced Ca^{2+} oscillations in tracheal epithelium cells [22], which is probably due to an inhibition of the SERCA (sarcoplasmic/endoplasmic reticulum $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase) pumps by FK506 [23]. Third, IP_3 -induced Ca^{2+} release in IP_3 R1-overexpressing COS-7 cells was not affected by co-transfection of FKBP12 [24]. Fourth, transgenic mice lacking FKBP12 had severe dilated cardiomyopathy and had altered RyR function. However, no IP_3 R-related effects were described in these mice [37].

The analysis of the expression patterns already demonstrated that FKBP12 expression was related to the expression of RyR isoforms but not to that of IP_3 Rs, suggesting that FKBP12 is not necessary for the function of the IP_3 R. An affinity assay was used to investigate further the interaction of FKBP12 with the intracellular Ca^{2+} -release channels. A direct interaction with RyR1 and RyR3, in the open as well as in the closed state, was demonstrated. Moreover, we localized the FKBP12-binding site

on RyR1 using limited proteolysis. FKBP12 was shown to interact with a proteolytic domain of $\approx 45\ \text{kDa}$ (amino acids 2401–2840 [38]) recognized by antibody m34C. The $45\ \text{kDa}$ fragment contained the conserved region, which was identified as the binding site for FKBP12 on IP_3 R1 and for FKBP12.6 on RyR2 by the yeast two-hybrid assay [20,21]. Although this conserved FKBP12-binding site is present in both IP_3 R1 and IP_3 R3, no specific interaction with FKBP12 was observed. The exchange reaction is very well characterized for the RyR1 and is complete within 40 min at $37\ ^\circ\text{C}$ [33]. It is unlikely that the lack of interaction with the IP_3 R is due to a tight association and too slow an exchange reaction, as no interaction was observed using preparations of cell types that did not contain detectable levels of endogenous FKBP12. Moreover, FKBP12 could be co-immunoprecipitated with RyR1 but not with IP_3 R1. This indicates that FKBP12 is endogenously associated with RyR1 and that it can be exchanged readily for GST-FKBP12, but that no such interaction occurs between FKBP12 and IP_3 R1.

The proteolytic fragment of IP_3 R1 containing the proposed FKBP12-binding site was not retained by GST-FKBP12 chromatography. This indicates that in contrast to the observation in the RyR1, this FKBP12-binding site in IP_3 R1 is not readily accessible. This result does not necessarily contradict the yeast two-hybrid study [20], since the proposed binding domain is very hydrophobic and poorly soluble [35]. The yeast two-hybrid technique, being an extremely sensitive assay, may be activated by a very small amount of soluble protein. Moreover, the solubility of the domain may be higher in a yeast nucleus. However, the lack of an interaction with either the full-length IP_3 R or a proteolytic fragment raises the question of the significance of the FKBP12-binding site on the IP_3 R.

One possibility is that the FKBP12-binding site is functionally active during the synthesis of the IP_3 R proteins. It is known that all immunophilins have *cis-trans* peptidylprolyl isomerase activity [39]. This isomerase activity is important for protein folding, since it is thought that FKBP12 stabilizes the high-energy, twisted-amide transition state of peptidylprolyl bonds during *cis-trans* isomerization [39]. FKBP12 will bind with high affinity to this transition state, catalyse the isomerization and eventually be released [40]. It is conceivable that steric hindrance constrains the valylprolyl residue (amino acids 2461–2462), which is the probable FKBP12-binding site on RyR1, in a high-energy, unstable transition state, preventing completion of the isomerization reaction [11]. Binding of FKBP12 to this site will then lower the energy of this intermediate and stabilize the structure of the receptor [11]. In the case of the IP_3 R isoforms, FKBP12 may bind to the leucylprolyl residue at amino acids 1400–1401 and catalyse the *cis-trans* isomerization of the peptide bonds (e.g. during protein synthesis). As a consequence, FKBP12 will be released from this low-energy, stabilized state of the substrate. The steric hindrance, which prevents the peptidylprolyl bond achieving the *cis* or *trans* conformations in the RyR isoforms, may be absent or insufficient in the IP_3 R isoforms.

Alternatively, the FKBP12-binding site on the IP_3 R may have a function in the regulation of intracellular Ca^{2+} signalling, but the interaction with FKBP12 might not be constitutive, as in the case of the RyR1, but dependent on critical intracellular conditions and/or on the association of other regulatory proteins. In this respect, it is interesting to mention that FKBP12.6 binding to RyR2 is dependent on the correct phosphorylation state of the receptor [21].

In summary, we have localized the FKBP12-binding site on RyR1 using limited proteolysis and GST-FKBP12 affinity chromatography. Although the FKBP12-binding site is conserved in IP_3 R as well as in RyR isoforms, no evidence was found

for a direct protein–protein interaction between FKBP12 and IP₃R1 or IP₃R3. It is conceivable that the FKBP12-binding site is required for *cis*–*trans* isomerization during protein processing, but that it is not functional in the mature protein.

We thank L. Bauwens, M. Crabbé, A. Florizoone, L. Heremans, Y. Parijs, J. Renders, H. Van Weijenbergh and I. Willems for their skilful technical assistance. We acknowledge Dr A. Friedrich and Dr K. Murato (Fujisawa GmbH, Munich, Germany) for providing us with FK506, Dr K. Mikoshiba for providing us with the 10A6 antibody and the IP₃R1 cDNA, Dr D. H. MacLennan for providing us with the RyR1 cDNA and Dr I. Pecht and Dr C. Gruenert for the gift of the RBL-2H3 and 16HBE14o– cell lines, respectively. We thank Dr B. Ehrlich and Dr I. Bezprozvanny for interesting discussions. G. B. has a predoctoral fellowship of the Vlaams Instituut voor de Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie (I. W. T.). P. D. S. is Research Assistant of the Foundation for Scientific Research–Flanders (F.W.O.). This work was supported by grants 3.0207.99 of the F. W. O. (to H. D. S. and J. B. P.), by grant P4/23 of the Program on Interuniversity Poles of Attraction (to J. B. P., G. C., H. D. S. and L. M.), by grant 99/08 of the Concerted Actions of the K.U.Leuven (to L. M., H. D. S., G. C. and J. B. P.) and by grants from Telethon and Murst (to V. S.).

REFERENCES

- Berridge, M. J. (1993) Inositol trisphosphate and calcium signalling. *Nature* (London) **361**, 315–325.
- McPherson, P. S. and Campbell, K. P. (1993) The ryanodine receptor/Ca²⁺ release channel. *J. Biol. Chem.* **268**, 13765–13768.
- Sorrentino, V. and Reggiani, C. (1999) Expression of the ryanodine receptor type 3 in skeletal muscle. A new partner in excitation-contraction coupling? *Trends Cardiovasc. Med.* **9**, 54–61.
- De Smedt, H., Missiaen, L., Parys, J. B., Bootman, M. D., Mertens, L., Van Den Bosch, L. and Casteels, R. (1994) Determination of relative amounts of inositol trisphosphate receptor mRNA isoforms by ratio polymerase chain reaction. *J. Biol. Chem.* **269**, 21691–21698.
- Newton, C. L., Mignery, G. A. and Südhof, T. C. (1994) Co-expression in vertebrate tissues and cell lines of multiple inositol 1,4,5-trisphosphate (InsP₃) receptors with distinct affinities for InsP₃. *J. Biol. Chem.* **269**, 28613–28619.
- Wojcikiewicz, R. J. (1995) Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *J. Biol. Chem.* **270**, 11678–11683.
- De Smedt, H., Missiaen, L., Parys, J. B., Henning, R. H., Sienaeert, I., Vanlingen, S., Gijssens, A., Himpens, B. and Casteels, R. (1997) Isoform diversity of the inositol trisphosphate receptor in cell types of mouse origin. *Biochem. J.* **322**, 575–583.
- Mackrill, J. J. (1999) Protein–protein interactions in intracellular Ca²⁺-release channel function. *Biochem. J.* **337**, 345–361.
- Bultynck, G., Missiaen, L., Callewaert, G., Parys, J. B. and De Smedt, H. (1999) Do inositol 1,4,5-trisphosphate receptors function as multiprotein complexes? *Curr. Top. Biochem. Res.* **1**, 193–204.
- Patel, S., Joseph, S. K. and Thomas, A. P. (1999) Molecular properties of inositol 1,4,5-trisphosphate receptors. *Cell Calcium* **25**, 247–264.
- Marks, A. R. (2000) Cardiac intracellular calcium release channels: role in heart failure. *Circ. Res.* **87**, 8–11.
- Timerman, A. P., Ogunbumni, E., Freund, E., Wiederrecht, G., Marks, A. R. and Fleischer, S. (1993) The calcium release channel of sarcoplasmic reticulum is modulated by FK-506-binding protein. Dissociation and reconstitution of FKBP-12 to the calcium release channel of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **268**, 22992–22999.
- Timerman, A. P., Jayaraman, T., Wiederrecht, G., Onoue, H., Marks, A. R. and Fleischer, S. (1994) The ryanodine receptor from canine heart sarcoplasmic reticulum is associated with a novel FK-506 binding protein. *Biochem. Biophys. Res. Commun.* **198**, 701–706.
- Murayama, T., Oba, T., Katayama, E., Oyama, H., Oguchi, K., Kobayashi, M., Otsuka, K. and Ogawa, Y. (1999) Further characterization of the type 3 ryanodine receptor (RyR3) purified from rabbit diaphragm. *J. Biol. Chem.* **274**, 17297–17308.
- Timerman, A. P., Onoue, H., Xin, H. B., Barg, S., Copello, J., Wiederrecht, G. and Fleischer, S. (1996) Selective binding of FKBP12.6 by the cardiac ryanodine receptor. *J. Biol. Chem.* **271**, 20385–20391.
- Brillantes, A. B., Ondrias, K., Scott, A., Kobrin, S., Ondriasová, E., Moschella, M. C., Jayaraman, T., Landers, M., Ehrlich, B. E. and Marks, A. R. (1994) Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* **77**, 513–523.
- Marx, S. O., Ondrias, K. and Marks, A. R. (1998) Coupled gating between individual skeletal muscle Ca²⁺ release channels (ryanodine receptors). *Science* **281**, 818–821.
- Cameron, A. M., Steiner, J. P., Sabatini, D. M., Kaplin, A. I., Walensky, L. D. and Snyder, S. H. (1995) Immunophilin FK506 binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1784–1788.
- Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V. and Snyder, S. H. (1995) Calcineurin associated with the inositol 1,4,5-trisphosphate receptor-FKBP12 complex modulates Ca²⁺ flux. *Cell* **83**, 463–472.
- Cameron, A. M., Nucifora, F. C., Fung, E. T., Livingston, D. J., Aldape, R. A., Ross, C. A. and Snyder, S. H. (1997) FKBP12 binds the inositol 1,4,5-trisphosphate receptor at leucine-proline (1400–1401) and anchors calcineurin to this FK506-like domain. *J. Biol. Chem.* **272**, 27582–27588.
- Marx, S. O., Reiken, S., Hisamatsu, Y., Jayaraman, T., Burkhardt, D., Roseblat, N. and Marks, A. R. (2000) PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* **101**, 365–376.
- Kanoh, S., Kondo, M., Tamaoki, J., Shirakawa, H., Aoshima, K., Miyazaki, S., Kobayashi, H., Nagata, N. and Nagai, A. (1999) Effect of FK506 on ATP-induced intracellular calcium oscillations in cow tracheal epithelium. *Am. J. Physiol.* **276**, L891–L899.
- Bultynck, G., De Smet, P., Weidema, A. F., Ver Heyen, M., Maes, K., Callewaert, G., Missiaen, L., Parys, J. B. and De Smedt, H. (2000) Effects of the immunosuppressant FK506 on intracellular Ca²⁺ release and Ca²⁺ accumulation mechanisms. *J. Physiol. (London)* **525**, 681–693.
- Boehning, D. and Joseph, S. K. (2000) Functional properties of recombinant type I and type III inositol 1,4,5-trisphosphate receptor isoforms expressed in COS-7 cells. *J. Biol. Chem.* **275**, 21492–21499.
- Vanlingen, S., Sipma, H., Missiaen, L., De Smedt, H., De Smet, P., Casteels, R. and Parys, J. B. (1999) Modulation of type 1, 2 and 3 inositol 1,4,5-trisphosphate receptors by cyclic ADP-ribose and thimerosal. *Cell Calcium* **25**, 107–114.
- Sipma, H., De Smet, P., Sienaeert, I., Vanlingen, S., Missiaen, L., Parys, J. B. and De Smedt, H. (1999) Modulation of inositol 1,4,5-trisphosphate binding to the recombinant ligand-binding site of the type-1 inositol 1,4,5-trisphosphate receptor by Ca²⁺ and calmodulin. *J. Biol. Chem.* **274**, 12157–12162.
- Manunta, M., Rossi, D., Simeoni, I., Butelli, E., Romanin, C., Sorrentino, V. and Schindler, H. (2000) ATP-induced activation of expressed RyR3 at low free calcium. *FEBS Lett.* **471**, 256–260.
- Maes, K., Missiaen, L., De Smet, P., Vanlingen, S., Callewaert, G., Parys, J. B. and De Smedt, H. (2000) Differential modulation of inositol 1,4,5-trisphosphate receptor type 1 and type 3 by ATP. *Cell Calcium* **27**, 257–267.
- De Waard, M., Witcher, D. R., Pragnell, M., Liu, H. and Campbell, K. P. (1995) Properties of the alpha 1-beta anchoring site in voltage-dependent Ca²⁺ channels. *J. Biol. Chem.* **270**, 12056–12064.
- Vanlingen, S., Parys, J. B., Missiaen, L., De Smedt, H., Wuytack, F. and Casteels, R. (1997) Distribution of inositol 1,4,5-trisphosphate receptor isoforms, SERCA isoforms and Ca²⁺ binding proteins in RBL-2H3 rat basophilic leukemia cells. *Cell Calcium* **22**, 475–486.
- Saito, A., Seiler, S., Chu, A. and Fleischer, S. (1984) Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. *J. Cell Biol.* **99**, 875–885.
- Parys, J. B., De Smedt, H., Missiaen, L., Bootman, M. D., Sienaeert, I. and Casteels, R. (1995) Rat basophilic leukemia cells as model system for inositol 1,4,5-trisphosphate receptor IV, a receptor of the type II family: functional comparison and immunological detection. *Cell Calcium* **17**, 239–249.
- Xin, H. B., Timerman, A. P., Onoue, H., Wiederrecht, G. J. and Fleischer, S. (1995) Affinity purification of the ryanodine receptor/calcium release channel from fast twitch skeletal muscle based on its tight association with FKBP12. *Biochem. Biophys. Res. Commun.* **214**, 263–270.
- Chen, S. R., Airey, J. A. and MacLennan, D. H. (1993) Positioning of major tryptic fragments in the Ca²⁺ release channel (ryanodine receptor) resulting from partial digestion of rabbit skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **268**, 22642–22649.
- Yoshikawa, F., Iwasaki, H., Michikawa, T., Furuichi, T. and Mikoshiba, K. (1999) Trypsinized cerebellar inositol 1,4,5-trisphosphate receptor. Structural and functional coupling of cleaved ligand binding and channel domains. *J. Biol. Chem.* **274**, 316–327.
- Missiaen, L., De Smedt, H., Droogmans, G. and Casteels, R. (1992) Ca²⁺ release induced by inositol 1,4,5-trisphosphate is a steady-state phenomenon controlled by luminal Ca²⁺ in permeabilized cells. *Nature* (London) **357**, 599–602.
- Shou, W., Aghdasi, B., Armstrong, D. L., Guo, Q., Bao, S., Charrng, M. J., Mathews, L. M., Schneider, M. D., Hamilton, S. L. and Matzuk, M. M. (1998) Cardiac defects and altered ryanodine receptor function in mice lacking FKBP12. *Nature* (London) **391**, 489–492.

- 38 Callaway, C., Seryshev, A., Wang, J. P., Slavik, K. J., Needleman, D. H., Cantu, C., 3rd, Wu, Y., Jayaraman, T., Marks, A. R. and Hamilton, S. L. (1994) Localization of the high and low affinity [^3H]ryanodine binding sites on the skeletal muscle Ca^{2+} release channel. *J. Biol. Chem.* **269**, 15876–15884
- 39 Kay, J. E. (1996) Structure-function relationships in the FK506-binding protein (FKBP) family of peptidylprolyl cis-trans isomerases. *Biochem. J.* **314**, 361–385
- 40 Fischer, G., Tradler, T. and Zarnt, T. (1998) The mode of action of peptidyl prolyl cis/trans isomerases *in vivo*: binding vs. catalysis. *FEBS Lett.* **426**, 17–20
- 41 Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. (1989) Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P400. *Nature (London)* **342**, 32–38
- 42 Airey, J. A., Beck, C. F., Murakami, K., Tanksley, S. J., Deerinck, T. J., Ellisman, M. H. and Sutko, J. L. (1990) Identification and localization of two triad junctional foot protein isoforms in mature avian fast twitch skeletal muscle. *J. Biol. Chem.* **265**, 14187–14194
- 43 Tong, J., Oyamada, H., Demareux, N., Grinstein, S., McCarthy, T. V. and MacLennan, D. H. (1997) Caffeine and halothane sensitivity of intracellular Ca^{2+} release is altered by 15 calcium release channel (ryanodine receptor) mutations associated with malignant hyperthermia and/or central core disease. *J. Biol. Chem.* **272**, 26332–26339
- 44 Campbell, K. P., Knudson, C. M., Imagawa, T., Leung, A. T., Sutko, J. L., Kahl, S. D., Raab, C. R. and Madson, L. (1987) Identification and characterization of the high affinity [^3H]ryanodine receptor of the junctional sarcoplasmic reticulum Ca^{2+} release channel. *J. Biol. Chem.* **262**, 6460–6463
- 45 Giannini, G., Conti, A., Mammarella, S., Scrobogna, M. and Sorrentino, V. (1995) The ryanodine receptor/calcium channel genes are widely and differentially expressed in murine brain and peripheral tissues. *J. Cell Biol.* **128**, 893–904
- 46 Maeda, N., Niinobe, M., Nakahira, K. and Mikoshiba, K. (1988) Purification and characterization of P400 protein, a glycoprotein characteristic of Purkinje cell, from mouse cerebellum. *J. Neurochem.* **51**, 1724–1730

Received 29 August 2000/5 December 2000; accepted 3 January 2001