Immunophilin FK506 binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux

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ABSTRACT The immunophilin FK506 binding protein 12 (FKBP12) is associated with and modulates the ryanodine receptor calcium release channel of skeletal muscle. Ryanodine receptor has amino acid homology and functional similarity with another intracellular Ca^{2+} release channel, the inositol 1,4,5-trisphosphate receptor (IP_3R). In the present study we show that highly purified preparations of IP_3R contain FKBP12. The complex of these two proteins is disrupted by the immunosuppressants FK506 and rapamycin, both of which are known to bind FKBP12 with high affinity. Disrupting the IP_3R –FKBP12 interaction increases Ca^{2+} flux through IP_3R , an effect that is reversed by added FKBP12. FKBP12 appears to be physiologically linked to IP_3R , regulating its Ca^{2+} conductance.

The immunophilins are a family of proteins that mediate the actions of immunosuppressant drugs such as cyclosporin A (CsA), FK506, and rapamycin. CsA is a cyclic undecapeptide of fungal origin that binds to members of the cyclophilin class of immunophilins (1). FK506 and rapamycin, two structurally related macrolide antibiotics, bind FK506 binding protein (FKBP) immunophilins. Numerous members of the immunophilin family have been identified, but only a few, especially cyclophilin A and B and FKBP12, appear responsible for immunosuppressant actions (2). Though the cyclophilins and FKBP display no amino acid sequence similarity, they both possess prolyl isomerase (rotamase) activity, associated with protein folding, which is inhibited by their respective immunosuppressant ligands. The immunosuppressant actions of these drugs are not derived from inhibition of immunophilin rotamase activity. Instead, the complex of CsA–cyclophilin or FK506–FKBP12 binds to the protein phosphatase calcineurin to inhibit its activity, thereby augmenting phosphorylated calcineurin substrates such as NFAT, a transcription factor that regulates interleukin 2 (IL-2) gene transcription (3–5). The immunosuppressant actions of rapamycin differ from those of CsA and FK506, influencing the actions of IL-2 rather than its synthesis (6). While rapamycin binds with high affinity to FKBP12 (and inhibits its rotamase activity), the rapamycin–FKBP complex does not interact with calcineurin, but with a recently identified target protein designated RAFT (rapamycin and FKBP12 target) or FRAP (FKBP–rapamycin-associated protein) (7, 8).

Recently Fleischer and associates (9) demonstrated that FKBP12 is physiologically associated with the ryanodine receptor (RyR) Ca^{2+} release channel of skeletal muscle. The RyR is a large, tetrameric calcium channel that mediates Ca^{2+} -induced Ca^{2+} release in skeletal muscle, cardiac muscle, and brain as well as other tissues (10). Whereas FK506 stimulates the binding of FKBP12 to calcineurin, FK506 causes a dissociation of FKBP12 and RyR (11). When FKBP12 is

“stripped” from RyR the Ca^{2+} channel becomes “leaky” such that net Ca^{2+} accumulation into RyR -gated stores is diminished and releasing agents such as caffeine provoke Ca^{2+} conductance at lower concentrations. FKBP12 stabilizes the full conductance states of RyR , making the channel harder to open but more stable once it is open, whereas in the absence of FKBP12, RyR displays multiple subconductance states (12).

Intracellular Ca^{2+} release is predominantly mediated by RyR and by inositol 1,4,5-trisphosphate (IP_3) acting through the IP_3 receptor (IP_3R) calcium release channel (13). IP_3R and RyR display extensive amino acid sequence homology and functional similarities (14, 15). We now demonstrate a close, physiologic association of FKBP12 with IP_3R . FKBP12 appears to stabilize the IP_3R Ca^{2+} channel.

MATERIALS AND METHODS

Materials. [^{32}P]ATP and $^{45}\text{Ca}^{2+}$ were purchased from Dupont/NEN. [^{32}P]FKBP12 was prepared as described (7). Antiserum to FKBP12 was a gift of K. Leach (Upjohn). FK506 was provided by S. Danishevsky and rapamycin was the gift of S. Sehgal at Wyeth–Ayerst. Bio-Gel P-30 gel resin was purchased from Bio-Rad. Human recombinant FKBP12 as well as all other reagents, unless otherwise specified, were purchased from Sigma.

Gel Filtration of IP_3R . IP_3R was purified as described (16) and concentrated in Amicon Centriprep-100 concentrators. Concentrated IP_3R was then run on an FPLC Superose 6 gel filtration column in a buffer of 250 mM NaCl/1% CHAPS/50 mM Tris, pH 7.4, at a flow rate of 0.2 ml/min and 1-ml fractions were collected. Samples were then loaded for SDS/PAGE and visualized by Coomassie stain or Western analysis (Fig. 1).

Immunosuppressant Treatment of IP_3R . FPLC fractions containing IP_3R were pooled, further concentrated, and treated for 1 hr at room temperature with an ethanol control vehicle, 10 μM FK506, 10 μM CsA, or 10 μM rapamycin. All drugs were dissolved in ethanol to 0.1% final ethanol concentration. Following treatment, samples were individually loaded onto an IP_3 -agarose affinity column, washed, and eluted with inositol hexakisphosphate as described (17) to remove the FK506–FKBP12 complex, giving rise to “stripped” or “unstripped” IP_3R .

[^{32}P]FKBP12 Binding to IP_3R . Stripped or unstripped IP_3R (0.2 mg/ml, 0.8 μM) was incubated with 5 pM [^{32}P]FKBP12 (50,000 cpm) with or without FK506 (10 μM) or unlabeled human recombinant FKBP12 (1 μM) in a buffer of 50 mM NaCl/25 mM Tris, pH 7.4/2 mg of bovine serum albumin (BSA) per ml (Fig. 2). Reaction mixtures typically were 150 μl total volume incubated for 2 hr at 4°C. After incubation, 145 μl of reaction volume was passed over a Bio-Rad P-30 sizing

column and washed with 0.8 ml of buffer without BSA. IP₃R was recovered in this fraction (as determined by [³H]IP₃ binding, data not shown) whereas free FKBP12 was recovered in a later fraction (data not shown).

Effect of FKBP12 on Ca²⁺ Flux. Cerebellar microsomes were prepared from rat brain as described (18). One milliliter of microsome preparation (1 mg of protein per ml) was treated with FK506 or ethanol as above. Microsomes were then diluted with 10 ml of microsome buffer (20 mM Hepes, adjusted to pH 7.35 with KOH/0.25 M sucrose), and repelleted by centrifugation for 1 hr at 100,000 × *g* (Beckman TI-60 rotor). The supernatant was removed and microsome pellets were resuspended in 1 ml of microsome buffer. Samples were treated with 100 nM FKBP12 or vehicle for 2 hr at 4°C (see Fig. 4). Loading rate over 3 hr and response to IP₃ were monitored (see Figs. 4 and 5).

RESULTS

Association of FKBP12 and IP₃R. We purified IP₃R from rat cerebellum utilizing a heparin column followed by a concanavalin A column. Purified IP₃R was loaded onto an FPLC Superose 6 column, which separates proteins according to their molecular mass (Fig. 1). [³H]IP₃ binding is confined to FPLC fractions 9–12, which display protein bands of 260 kDa, reflecting the molecular mass of IP₃R. In Coomassie blue-stained fractions we do not detect a protein at the molecular mass of FKBP12. However, silver staining reveals a protein at about 14 kDa in fractions 9–12 (data not shown). Western blot analysis with an antibody selective for FKBP12 reveals an intense band in fraction 10 with lighter bands in fractions 9, 11, and 12 (Fig. 1). When pure FKBP12 is fractionated on the FPLC column, it elutes at fractions 19–21 (data not shown). Thus, FKBP12, with a molecular mass that is only 1% that of

FIG. 1. Electrophoresis on 12% SDS/PAGE of FPLC fractionation of IP₃R. The upper portion demonstrates visualization of IP₃R by Coomassie brilliant blue stain. MWM, molecular weight markers (from bottom to top): lysozyme (14.3 kDa), β -lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), BSA (68 kDa), phosphorylase B (97.4 kDa), and myosin (H chain) (200 kDa). [³H]IP₃ binding was routinely seen only in fractions 9–12 as was the band at 260 kDa. These fractions represent the elution point of proteins with a molecular mass around 1000 kDa, consistent with the IP₃R tetramer. The box insert in the lower portion demonstrates an alignment of identical SDS/PAGE of identical FPLC fractions subjected to Western analysis with an antibody specific for FKBP12 at a dilution of 1:2000. An intense band was seen in lane 10 with lighter bands in lanes 9, 11, and 12. When purified human recombinant FKBP12 was fractionated on the FPLC Superose 6 column, the protein as well as [³H]FK506 binding activity appeared in fractions 19–21 (data not shown).

IP₃R tetramer, is tightly associated with IP₃R, being retained with it following a series of fractionations.

To ensure that the immunoreactive FKBP12 is authentic FKBP12 protein, we pooled and concentrated fractions 9–12, conducted SDS/PAGE, and transferred to poly(vinylidene difluoride) paper. We then cut out and obtained sequence for the immunoreactive FKBP12 band. One major peptide is detected that yields N terminus sequence as follows (yields, in picomoles, are within parentheses, single-letter amino acid code is used, x refers to residues not positively identified): G(8.85), V(5.18), Q(4.86), V(4.08), E(3.68), T(3.64), I(2.40), S(1.60), P(3.83), G(4.27), D(2.87), G(3.96), R(3.50), x, F(1.16), x, x, x. ... Comparison with the amino acid sequence predicted from FKBP12 cloned from rat brain in our own laboratory (7) indicates a perfect match.

FK506 dissociates FKBP12 from RyR (11). We treated purified IP₃R with FK506, CsA, or rapamycin, repurified IP₃R over an IP₃-agarose affinity column to remove any drug-immunophilin complex, conducted SDS/PAGE analysis, and stained the gels by silver stain (Fig. 2). Untreated preparations of IP₃R possess a silver-stained band around 14 kDa, which reflects the mobility of FKBP12 in SDS/PAGE (electrophoretic properties of FKBP12 discussed in ref. 19). In samples treated with FK506 or rapamycin, the 14-kDa band is absent, while it is retained in samples treated with CsA. These results are consistent with the fact that FK506 and rapamycin bind to FKBP12 while CsA does not.

FIG. 2. Silver stain of IP₃R preparations treated with the immunosuppressants FK506, CsA, or rapamycin (Rapa) during further purification. Molecular weight markers (MWM) are the same as listed in Fig. 1; Untreated, ethanol control vehicle added (final concentration of ethanol, 0.1%); +FK506, 10 μ M FK506 added; +CsA, 10 μ M CsA added; +Rapa, 10 μ M rapamycin added. All drugs were dissolved in ethanol and final ethanol concentrations were 0.1% for all samples. Samples were analyzed by SDS/PAGE alongside 2 μ g of human recombinant FKBP12 (RecFKBP12). The bands visualized at 260 kDa correspond to the IP₃R; those at 100 kDa most likely correspond to an often observed breakdown product of the IP₃R. In the lanes treated with nothing or CsA, a protein with a molecular mass of around 14 kDa is observed, as indicated by the arrow. This band is not seen in samples treated with FK506 or rapamycin.

To directly evaluate interactions of FKBP12 with IP₃R we utilized [³²P]FKBP12 (Fig. 3). [³²P]FKBP12 binding to IP₃R is increased almost 5-fold when IP₃R is stripped of endogenous FKBP12 by treatment with FK506. Restoration of FKBP12 to the stripped IP₃R reduces binding to the levels of unstripped IP₃R. Similarly, FK506 inhibits binding of [³²P]FKBP12 to the stripped IP₃R. The substantially greater binding of stripped IP₃R establishes that binding of [³²P]FKBP12 to IP₃R is saturable. This conclusion is reinforced by the ability of unlabeled FKBP12 to compete with labeled FKBP12 for binding. The inhibition of binding by FK506 is consistent with the ability of FK506 to dissociate FKBP12 from IP₃R.

Influences of FKBP12 on Calcium Dynamics of IP₃R. To monitor the influence of FKBP12 on IP₃R flux of Ca²⁺, we utilized microsomal fractions of rat cerebellum enriched in endoplasmic reticulum (ER) and loaded with ⁴⁵Ca²⁺ (Fig. 4) as described (18, 20). Treatment of microsomes with FK506 reduces accumulation of ⁴⁵Ca²⁺ into IP₃-sensitive stores by about 50%. Adding FKBP12 to preparations treated with FK506 restores Ca²⁺ accumulation to control levels. We monitored the potency of IP₃ in stimulating Ca²⁺ flux in microsomes (Fig. 5). Treatment of preparations with FK506 shifts the concentration-response curve to the left rendering IP₃ about 10 times more potent in stimulating Ca²⁺ flux.

These findings resemble results with RyR (11) and indicate that stripping FKBP12 from IP₃R with FK506 renders the Ca²⁺ channel leaky. The reduced accumulation of Ca²⁺ following FK506 treatment may indicate that Ca²⁺ accumulated by the ER Ca²⁺ pump leaks out through the IP₃R calcium release channel. A similar IP₃R leakiness due to stripping of FKBP12 may account for the greater potency of IP₃ in releasing Ca²⁺ in FK506-treated microsomes.

FIG. 3. Specific and saturable binding of FKBP12 to IP₃R. Untreated or FK506-treated samples from Fig. 2 were designated as unstripped IP₃R or stripped IP₃R, respectively. [³²P]FKBP12 was prepared (20). Reaction mixtures contained stripped or unstripped IP₃R (0.2 mg/ml) with [³²P]FKBP12 (50,000 cpm, ~5 pM) with or without FK506 (10 μM) or unlabeled human recombinant FKBP12 (1 μM). [³²P]FKBP12 binding to purified IP₃R was assessed as described in the text. [³²P]FKBP12 binding to stripped IP₃R is significantly greater than to unstripped IP₃R ($P < 0.001$). The experiment was repeated four times with essentially the same result.

FIG. 4. Effect of FKBP12 on loading of IP₃-sensitive stores. Cerebellar microsomes were treated with FK506 and/or FKBP12. Each preparation was then assessed for loading of ⁴⁵Ca²⁺ into their IP₃-sensitive stores. Values for samples treated with FK506 without added FKBP12 differ significantly from all other values ($P < 0.005$).

DISCUSSION

A major finding of this paper is the intimate association of FKBP12 with IP₃R. Evidence for a physiologic interaction includes the continued association of the two proteins through an extensive purification procedure. Additionally, radiolabeled FKBP12 binds saturably and specifically to IP₃R.

The dissociation of FKBP12 from IP₃R elicited by FK506 or rapamycin further reflects the high specificity of interaction between the two proteins. Both FK506 and rapamycin inhibit the prolyl isomerase activity of FKBP12, suggesting that IP₃R interacts with FKBP12 at the rotamase active site or an allosteric site that is conformationally altered by immunosuppressant binding.

The association of IP₃R and FKBP12 and its disruption by rotamase inhibitors imply that the isomerase activity of FKBP12 alters the conformation of IP₃R. This conforma-

FIG. 5. Effect of FK506 on IP₃R response to IP₃. Submaximal concentrations of IP₃ were added to stripped or unstripped microsomes and ⁴⁵Ca²⁺ loading of IP₃-sensitive stores was assessed. Untreated microsomes fluxed 50% of total Ca²⁺ at IP₃ concentrations of around 100 nM, with maximum flux at ~10 μM. FK506 increased the potency of IP₃ about 10-fold. This experiment was repeated four times with essentially the same results.

FIG. 6. The IP₃R is a target of the immunophilin FKBP12. The diagram illustrates either of the two intracellular calcium release channels (IP₃R or RyR) and their interaction with FKBP12. This interaction can be disrupted by the immunosuppressant FK506 (and rapamycin), resulting in suboptimal subunit cooperativity and leaky calcium channels.

tional change may mediate FKBP12's regulation of IP₃R Ca²⁺ conductance. Our finding that FK506 decreases Ca²⁺ accumulation into IP₃R-gated stores in microsomes and increases the potency of IP₃ in releasing Ca²⁺ is analogous to findings that FK506 stripping of FKBP12 decreases accumulation of Ca²⁺ by RyR and augments the potency of caffeine in releasing Ca²⁺ from RyR-gated stores (11). Biophysical studies indicate that FKBP12 stabilizes RyR in a full conductance state, eliminating the multiple subconductance states seen in RyR preparations lacking FKBP12 (12). This suggests that reports of subconductance states for RyR reflect RyR from which FKBP12 has been dissociated during tissue preparation. The multiple subconductance states observed in such preparations may arise from the failure of RyR's four subunits to interact with maximal cooperativity. Presumably with IP₃R, FKBP12 also stabilizes the Ca²⁺ channel, promoting optimal cooperativity between the channel's four subunits. It is likely that the prolyl isomerase activity of FKBP12 optimizes the conformation of IP₃R to maximize channel stability. Consistent with this notion is our demonstration that inhibitors of rotamase activity such as FK506 and rapamycin cause leakiness in the channel's gating properties.

The prolyl isomerase activity of cyclophilin and FKBP12 has been proposed to mediate correct folding of proteins as they emerge from ribosomes (21). Some experimental evidence indicates that this prolyl isomerase activity contributes to the physiologic conformation of collagen (22) and transferrin (23). The present findings suggest that a major role of the immunophilins is to stabilize the optimal physiological conformations of already synthesized membrane proteins. For IP₃R and RyR (12), this isomerase activity appears particularly critical for regulation of ion channel activity. Recently, Patrick and associates (24) observed influences of CsA upon the channel activity of nicotinic acetylcholine receptors and 5-hydroxytryptamine type 3 receptors. This suggests that regulating ion channels of membrane proteins is a major function of the immunophilins. Yeast two hybrid studies have revealed an association of FKBP12 with the receptor for transforming growth factor β (TGF- β) (25). How FKBP12 modulates the function of the TGF- β receptor is not clear.

Because of the extraordinary selectivity and high affinity of immunosuppressant drugs for immunophilins, investigators have speculated about the existence of endogenous ligands for the immunophilins. Endogenous ligands are normally conceptualized as small molecules, such as hormones or neurotransmitters, that bind to a large receptor, altering its conformation to induce changes in ion conductances or second messengers. Perhaps the immunophilins, relatively small proteins, are ligands for large membrane proteins such as IP₃R and RyR. In

this instance, binding of FKBP12 to IP₃R would elicit a conformational change through the prolyl isomerase activity of FKBP12 and would alter ion conductance of the target IP₃R (Fig. 6).

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