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 Ca2+ release channel, the inositol 1,4,5-trisphosphate receptor (IP3R). In the present study we show that highly purified preparations of IP3R 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 IP3R-FKBP12 interaction increases Ca2+ flux through IP3R, an effect that is reversed by added FKBP12. FKBP12 appears to be physiologically linked to IP3R, regulating its Ca2+ 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's 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 RAF (rapamycin–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) Ca2+ release channel of skeletal muscle. The RyR is a large, tetrameric calcium channel that mediates Ca2+-induced Ca2+ 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 Ca2+ channel becomes “leaky” such that net Ca2+ accumulation into RyR-gated stores is diminished and releasing agents such as caffeine provoke Ca2+ 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 Ca2+ release is predominantly mediated by RyR and by inositol 1,4,5-trisphosphate (IP3) acting through the IP3 receptor (IP3R) calcium release channel (13). IP3 and RyR display extensive amino acid sequence homology and functional similarities (14, 15). We now demonstrate a close, physiologic association of FKBP12 with IP3R. FKBP12 appears to stabilize the IP3R Ca2+ channel.

MATERIALS AND METHODS

Materials. [32P]ATP and 86Ca2+ were purchased from Du Pont/NEN. [32P]FKBP12 was prepared as described (7). Antibodies to FKBP12 was a gift of K. Leach (Upjohn). FK506 was provided by S. Danishefsky and rapamycin was the gift of S. Seghal 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 IP3R. IP3R was purified as described (16) and concentrated in Amicon Centricon-100 concentrators. Concentrated IP3R 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 IP3R. FPLC fractions containing IP3R 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 IP3-agarose affinity column, washed, and eluted with inositol hexakisphosphate as described (17) to remove the FK506–FKBP12 complex, giving rise to “stripped” or “unstripped” IP3R.

[32P]FKBP12 Binding to IP3R. Stripped or unstripped IP3R (0.2 mg/ml, 0.8 μM) was incubated with 5 pM [32P]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 were typically 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.

Abbreviations: BSA, bovine serum albumin; FKBP, FK506 binding protein; IP3, inositol 1,4,5-trisphosphate; IP3R, inositol 1,4,5-trisphosphate receptor; CsA, cyclosporin A; RyR, ryanodine receptor. ‡To whom reprint requests should be addressed.
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 Ca\(^{2+}\) Flux. Cerbellar 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 pelleted by centrifugation for 1 hr at 100,000 \(\times\) 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\(^\circ\)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 IP3R tetramer, is tightly associated with IP3R, being retained with it following a series of fractionations.

To ensure that the immuno reactive 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 immuno reactive 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.
To directly evaluate interactions of FKBP12 with IP$_3$R we utilized [32P]FKBP12 (Fig. 3). [32P]FKBP12 binding to IP$_3$R is increased almost 5-fold when IP$_3$R is stripped of endogenous FKBP12 by treatment with FK506. Restoration of FKBP12 to the stripped IP$_3$R reduces binding to the levels of unstripped IP$_3$R. Similarly, FK506 inhibits binding of [32P]FKBP12 to the stripped IP$_3$R. The substantially greater binding of stripped IP$_3$R establishes that binding of [32P]FKBP12 to IP$_3$R 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 IP$_3$R.

**Influences of FKBP12 on Calcium Dynamics of IP$_3$R.** To monitor the influence of FKBP12 on IP$_3$R flux of Ca$^{2+}$, we utilized microsomal fractions of rat cerebellum enriched in endoplasmic reticulum (ER) and loaded with $^{45}$Ca$^{2+}$ (Fig. 4) as described (18, 20). Treatment of microsomes with FK506 reduces accumulation of $^{45}$Ca$^{2+}$ into IP$_3$-sensitive stores by about 50%. Adding FKBP12 to preparations treated with FK506 restores Ca$^{2+}$ accumulation to control levels. We monitored the potency of IP$_3$ in stimulating Ca$^{2+}$ flux in microsomes (Fig. 5). Treatment of preparations with FK506 shifts the concentration-response curve to the left rendering IP$_3$ about 10 times more potent in stimulating Ca$^{2+}$ flux.

These findings resemble results with RyR (11) and indicate that stripping FKBP12 from IP$_3$R with FK506 renders the Ca$^{2+}$ channel leaky. The reduced accumulation of Ca$^{2+}$ following FK506 treatment may indicate that Ca$^{2+}$ accumulated by the ER Ca$^{2+}$ pump leaks out through the IP$_3$R calcium release channel. A similar IP$_3$R leakiness due to stripping FKBP12 may account for the greater potency of IP$_3$ in releasing Ca$^{2+}$ in FK506-treated microsomes.

**DISCUSSION**

A major finding of this paper is the intimate association of FKBP12 with IP$_3$R. 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 IP$_3$R.

The dissociation of FKBP12 from IP$_3$R 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 IP$_3$R interacts with FKBP12 at the rotamase active site or an allosteric site that is conformationally altered by immunosuppressant binding.

The association of IP$_3$R and FKBP12 and its disruption by rotamase inhibitors imply that the isomerase activity of FKBP12 alters the conformation of IP$_3$R. This confor-
Fig. 6. The IP$_3$R is a target of the immunophilin FKBP12. The diagram illustrates either of the two intracellular calcium release channels (IP$_3$R 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 IP$_3$R Ca$^{2+}$ conductance. Our finding that FK506 decreases Ca$^{2+}$ accumulation into IP$_3$R-gated stores in microsomes and increases the potency of IP$_3$ in releasing Ca$^{2+}$ is analogous to findings that FK506 stripping of FKBP12 decreases accumulation of Ca$^{2+}$ by RyR and augments the potency of caffeine in releasing Ca$^{2+}$ from RyR-gated stores (11). Biochemical 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 had 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 IP$_3$R, FKBP12 also stabilizes the Ca$^{2+}$ channel, promoting optimal cooperativity between the channel's four subunits. It is likely that the prolyl isomerase activity of FKBP12 optimizes the conformation of IP$_3$R 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 IP$_3$R 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 IP$_3$R and RyR. In this instance, binding of FKBP12 to IP$_3$R would elicit a conformational change through the prolyl isomerase activity of FKBP12 and would alter ion conductance of the target IP$_3$R (Fig. 6).

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References


