ASSOCIATION OF IMMUNOPHILINS WITH MAMMALIAN TRPC CHANNELS

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Running Title: TRPC Channels bind FKBP
SUMMARY

*Drosophila* photoreceptor channels TRP and TRPL, are held in a large signalplex by the scaffolding protein, INAD. Immunophilin FKBP59, another member of the signalplex, binds to both INAD and TRPL. Mutation P^{702}Q or P^{709}Q in the highly conserved TRPL sequence LPPPFNVL^{709}, eliminates TRPL interaction with FKBP59. The first LP dipeptide in this region is conserved in mammalian TRPC channel proteins. However, the second LP is changed to IP in TRPC1, -C4, and -C5, and VP in TRPC3, -C6, and -C7. The purpose of the present study was to determine if mammalian FKBP12 or FKBP52 interact with TRPC channel proteins. Using TRPC-specific antibodies, immunoprecipitations from Sf9 cells individually co-expressing each of the TRPC proteins along with the immunophilins showed that TRPC3, -C6, and -C7 interact with FKBP12, whereas TRPC1, -C4, and -C5 interact with FKBP52. The binding of FKBP12 and FKBP52 was specific and could be displaced by the immunosuppressant drug FK506, at a concentration of 0.5 and 10 µM, respectively. To evaluate TRPC-immunophilin interactions *in vivo*, immunoprecipitations were performed using membrane lysates of rat cerebral cortex. FKBP12 co-immunoprecipitated with TRPC3, -C6, and -C7 from rat brain, whereas FKBP52 was found associated with TRPC1, -C4, and -C5. The association of immunophilins with the TRPC channels in rat brain lysates could be displaced by FK506. Receptor-mediated activation of TRPC6, stably expressed in HEK cells, was significantly inhibited by FK506 which also disrupted interaction between TRPC6 and the endogenous immunophilin found in HEK cells. P-to-Q mutations in the first LP dipeptide in the putative FKBP binding domain eliminated FKBP12 and FKBP52 interaction with TRPC3 and -C6, and TRPC1 and -C4, respectively. However, mutual swap of VP and IP in TRPC3 and TRPC5 did
not alter the association or the selectivity of the channels for their respective immunophilin binding partner. These results suggest that immunophilins are TRPC channel accessory proteins that play an important role in the mechanism of channel activation following receptor stimulation.
INTRODUCTION

The transient receptor potential channels, TRP and TRPL, are important molecular components of the phototransduction cascade in *Drosophila* (1-3). These channels are held in a large macromolecular ‘signalplex’ within the microvilli of the rhabdomere via interaction with the scaffolding protein, INAD (4-6). INAD contains 5 tandem PDZ domains that bind various players in the phototransduction cascade (e.g., PLC, PKC, calmodulin, rhodopsin), bringing them into close proximity with the channels, thus facilitating rapid signal response. Previous studies using INAD as bait in a yeast two-hybrid screen of a *Drosophila* head cDNA library, identified the immunophilin FKBP59 as an INAD binding partner (7). (FKBPs were named for their ability to bind the immunosuppressant drug, FK506.) Additional experiments revealed that FKBP59 not only interacts with INAD, but also binds tightly to TRPL and alters channel function when heterologously expressed in insect Sf9 cells. Immunoprecipitation experiments showed that TRPL associates with FKBP59, but not FKBP12, both *in vitro* and *in vivo* in the fly eye. These results led to the conclusion that FKBP59 is part of the *Drosophila* TRPL channel signalplex (7).

The first examples of immunophilin-channel interactions were found in skeletal and cardiac muscle (for review (8)). FKBP12 and FKBP12.6 tightly associate with the ryanodine-sensitive Ca$^{2+}$ release channels of the sarcoplasmic reticulum. In the absence of FKBP12, the ryanodine receptor channels exhibit fluctuations between four subconductance states, but in the presence of FKBP12, transitions to the full conductance level are highly favored (9). Additionally, FKBP12 appears to facilitate interaction between neighboring channels, so-called
‘coupled-gating’, thus providing a potential mechanism of signal propagation and/or amplification (10). Although the observation remains controversial (11-14), FKBP12 has been reported to interact with the inositol-1,4,5-trisphosphate receptor (IP₃R) in non-excitable cells and to regulate Ca²⁺ release from the endoplasmic reticulum (15,16). The mammalian homolog of FKBP59 is currently designated FKBP52 (8). FKBP52 is part of the steroid receptor-heat shock protein complex (17), but the actual function of this immunophilin in most cells remains largely unknown.

Immunophilins are peptidyl-prolyl cis-trans isomerases that recognize specific XP dipeptides in their target proteins (8). FKBP12 binds to a leucyl-prolyl (LP) dipeptide in the IP₃R (16) or to a valyl-prolyl (VP) dipeptide in the skeletal muscle ryanodine receptor, RYR1 (18). FKBP12.6 binds to a isoleucyl-prolyl (IP) dipeptide in the cardiac muscle ryanodine receptor, RYR2 (18). FK506 disrupts the interaction between FKBPs and target proteins by mimicking the XP-binding motif (19). In this regard, FK506 displaces FKBP59 from TRPL channels, and mutations P⁷⁰²Q or P⁷⁰⁹Q in the highly conserved TRPL sequence ⁷⁰¹LPPPFNVL⁷⁰⁹ eliminate TRPL interaction with FKBP59 (7). The corresponding sequence in Drosophila TRP is ⁶⁹⁴IPPPFNLCP⁷⁰². In contrast to TRPL, preliminary studies have shown that Drosophila TRP does not interact with FKBP59, but rather selectively binds the smaller immunophilin, FKBP12 (Goel and Schilling, unpublished observations). These results suggest that changing LP to either IP or CP alters immunophilin selectivity for the Drosophila channels in a fashion similar to that seen for the interaction of FKBP12 and FKBP12.6 with RYR1 and RYR2, respectively (18). However, a COOH-terminal truncation of TRPL that retains the putative immunophilin binding domain also results in loss of FKBP59 interaction (7). Thus,
additional regions of the COOH-terminus may play a role in defining channel-immunophilin interaction.

There are seven mammalian TRP orthologs designated TRPC1-TRPC7 (see review (20)). Based on sequence homology, the TRPC family can be divided into two major subgroups consisting of TRPC1, -C4, and -C5, and TRPC3, -C6, and -C7. (TRPC2 is a pseudo gene in humans (21)). Various tissues and cell types express message for multiple TRPC channels (22) and there is growing evidence for heteromultimeric channel assembly. Recent in vitro (23,24) and in vivo (24) studies suggest that TRPC1, -C4, and -C5 associate and that TRPC3, -C6, and -C7 associate, but that cross association between the two major subgroups does not occur. The putative FKBP binding domain found in Drosophila TRPL is likewise found in all of the mammalian TRPCs proteins. The first LP dipeptide in this region is conserved in TRPC1, -C3, -C4, -C5, -C6, and -C7. However, the second LP is changed to IP in TRPC1, -C4, and -C5, and to VP in TRPC3, -C6, and -C7. Thus, the purpose of the present study was to determine if the mammalian immunophilins, FKBP12 or FKBP52, interact with TRPC channel proteins and to determine if the IP and VP dipeptides define immunophilin specificity. To accomplish these goals, FKBP12 and FKBP52 were cloned from human embryonic kidney (HEK293) cells and individually co-expressed along with each of the TRPC channel proteins in Sf9 insect cells using recombinant baculoviruses. Reciprocal immunoprecipitations from both Sf9 cells and from rat brain lysates showed that FKBP12 interacts with TRPC3, -C6, and -C7, whereas FKBP52 is found associated with TRPC1, -C4, and -C5. The immunophilins could be displaced from the TRPC channel proteins by FK506 in a concentration-dependent fashion. Furthermore, FK506 inhibited receptor-mediated activation of TRPC6 channels stably expressed in HEK cells. P-to-
Q mutations in the first LP dipeptide within the putative immunophilin binding domain eliminated channel-immunophilin interaction. However, mutual swap of IP and VP did not alter immunophilin binding or specificity. These results suggest that immunophilins FKBP12 and FKBP52 bind specifically and selectively to TRPC channel proteins via interaction with a conserved LP dipeptide located near the cytoplasmic mouth of the channel, but that other regions of the channel proteins are important determinants of immunophilin selectivity.
MATERIALS AND METHODS

Antibodies. The generation and characterization of rabbit polyclonal antibodies specific for each TRPC channel protein has been previously described (24). Anti-GST antibody was purchased from Uptstate Biotechnology (Lake Placid, NY), mouse anti-FKBP12 monoclonal antibody was from PharMingen/Transduction Laboratories (San Diego, CA), mouse anti-FKBP52 monoclonal antibody was from Stressgen (Victoria, British Columbia), and the pan-FKBP antibody was from Affinity Bioreagents (Golden, CO).

Cell Culture. Spodoptera frugiperda (Sf9) cells were obtained from ATCC and cultured as previously described (25) using Grace's Insect Medium supplemented with 2% lactalbumin hydrolysate, 2% yeastolate solution, 2mM L-glutamine, 10% heat-inactivated fetal bovine serum, and 1% penicillin-streptomycin-neomycin solution (Gibco). HEK 293 cells were grown at 37°C in monolayer culture in a humidified air atmosphere with 5% CO₂ using Minimal Essential Medium supplemented with L-glutamine, 10% heat-inactivated fetal bovine serum, and 1% penicillin-streptomycin-neomycin solution.

Cloning of immunophilins FKBP12 and FKBP52. Human FKBP12 and FKBP52 cDNAs were cloned from HEK293 cells using Advantage one step RT-PCR Kit (Clontech) according to the manufacturer’s instructions. Primers used for amplification of FKBP52 were: forward, ATG ACA GCC GAG GAG ATG A and reverse, CTA TGC TTC TGT TGT CTC CAC CT. Primers for amplification of FKBP12 were: forward, GATT CATGGAGTGCAGGTG and reverse,
GCGGCCGCTCACTCTAAGTTGAGC. FKBP52 and FKBP12 were sequenced and subcloned in pAcGHLT or pVL1393 baculovirus vectors using standard techniques.

**TRPC channel mutations.** Mutagenesis was performed using the Quickchange II XL kit (Stratagene) according to the manufacturer’s instructions. Mutagenic primer pairs, 39 nucleotides in length, were designed to span the desired codon and were synthesized by Invitrogen Life Technologies. TRPC3 and TRPC5 cDNAs were mutated while cloned in pCDNA 3, TRPC6 in pCDNA 3.1, TRPC7 in pCI-neo. Mutant cDNAs were subsequently transferred into baculovirus transfer vector, pVL1393. TRPC1 and TRPC4 were mutated while cloned in pVL1393, and were used for baculovirus production without further subcloning. All mutations were confirmed by sequence analysis.

**Generation of recombinant baculovirus for expression of mammalian TRPC channel proteins.** As previously described (24), the cDNAs for the TRPC channel proteins were individually subcloned into baculovirus transfer vector, pVL1393 using standard techniques. Recombinant baculoviruses were produced using the BaculoGold™ Transfection Kit (Pharmingen, San Diego, CA, USA) as described in the instructions provided by the manufacturer. Recombinant viruses were plaque purified and amplified to obtain a high titer viral stock solution. The virus was stored at 4°C under sterile conditions.

**Infection of Sf9 insect cells with recombinant baculovirus.** Sf9 cells in Grace's medium were plated onto 100 mm plastic tissue culture dishes at a density of ~10^5 cell/cm^2. Following incubation for 30 min, an aliquot of viral stock was added (multiplicity of infection was ~10) and
the cells were maintained at 27°C in a humidified air atmosphere. Unless otherwise indicated, cells were used at 28 hr post-infection.

**Isolation of rat brain lysates.** Rat cerebral cortex was isolated, minced, and suspended in homogenization buffer containing 150mM NaCl, 10mM Tris-Cl (pH 7.5) and 1% triton X100. The brain suspension was homogenized using a Brinkman PT10/35 Polytron fitted with a 10 mm generator (3 x 10 sec pulses at a power setting of 5). Homogenates/lysates were centrifuged at 6000 x g at 4°C for 10 min to remove the tissue debris. The resulting supernatant was incubated at 4°C for 30 min and subsequently subjected to centrifugation at 50,000 x g for 60 min at 4°C to remove unsolubilized membranes. The resulting brain lysates were used immediately for immunoprecipitation experiments.

**Expression of TRPC6 in mammalian cells.** The generation and characterization of HEK 293 cells stably-expressing human TRPC6 has been described (26). All experiments reported in this manuscript were performed using TRPC6-clone 14 cells which were serially cultured under continuous selection pressure using G418.

**Immunoprecipitations and immunoblots.** Sf9 cells infected with baculovirus or TRPC6 clone 14 cells were incubated in 1ml of lysis buffer containing 150mM NaCl, 10mM Tris-Cl (pH 7.5) and 1% triton X100 at 4°C for 30 min. The protein extracts were centrifuged at 50,000 x g for 60 min at 4°C to remove cellular debris and partially solubilized membrane fragments. Cell or brain lysates (~500 µg of protein) were precleared by adding control IgG together with protein A/G agarose beads for 1 hr at 4°C. Pre-cleared lysates were incubated with the indicated antibodies
Immunocomplexes were captured by incubating with protein A/G agarose beads at 4°C for 12 hrs. Beads were pelleted, washed four times with lysis buffer, resuspended in 2x SDS sample buffer (100 µl), and boiled for 3 min. Cell lysates and immunoprecipitated proteins were fractionated by SDS-PAGE (5 µg of lysate protein or 10 µl of immunoprecipitate per lane) and electrotransferred to PVDF membrane (100V for 1hr) in Tris-glycine buffer. Blots were probed with the indicated primary antibody and detected, following incubation with HRP-conjugated anti-rabbit IgG, by SuperSignal® West Pico chemiluminescent substrate (Pierce). The figures show representative results from at least 3 independent experiments. For the co-expression experiments in Sf9 cells, this represents at least three independent infections. Evaluation of FKBP52 by Western blot analysis was complicated by the fact that FKBP52 migrates to the same position on the gel as the heavy chain of IgG used for immunoprecipitation. For this reason, a GST-FKBP52 fusion protein (predicted molecular weight of 89 kDa) was employed for in vitro co-immunoprecipitation assays. Control experiments at the outset of this project showed that the commercially available anti-FKBP12 antibody recognized FKBP12 protein in Western blot analysis, but was unable to immunoprecipitate FKBP12 from cell lysates. Therefore, a GST-FKBP12 fusion protein was used in our initial experiments. However, over the course of this study, a mouse monoclonal antibody directed against FKBP12 became available and was subsequently used for direct immunoprecipitation experiments.

**Electrophysiological techniques.** The giga-seal technique for current recording was utilized in the whole-cell mode. All experiments were performed on HEK TRPC6-clone14 cells attached to circular glass coverslips which were transferred to a perfusible recording chamber on the stage of a Nikon inverted microscope immediately before use. The extracellular solutions contained...
(in mM) 160 NaCl, 4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, and 5 HEPES (pH 7.4). The pipette solution contained (in mM) 145 cesium aspartate, 2 MgCl$_2$, 0.3 CaCl$_2$, 10 EGTA, and 10 HEPES (pH 7.2 and pCa 8). Cesium was used in the pipette solution to block K$^+$ channels. Data were obtained using an Axopatch 200A amplifier (Pacer Scientific, Los Angeles, CA) and sampled on-line using pCLAMP 8.0 software. The ground electrode was an Ag-AgCl wire connected to the bath via an agar bridge containing 150 mM NaCl. All recordings were made at room temperature (~22°C). To generate current-voltage ($I$-$V$) relations, voltage ramps from -120 to +120 mV over 200 milliseconds were repetitively applied at 15 second intervals. Use of different ramp rate or voltage-step protocols did not alter the TRPC6 $I$-$V$ relationship. Unless otherwise indicated, the holding potential between ramps was -50 mV. All figures show representative traces corrected for liquid junction potential. Channel activation rates were determined from the normalized initial slope of current change with time immediately following addition of agonist to the bath solution and are shown as pA pF$^{-1}$ s$^{-1}$. Comparisons between groups were performed using a Students $t$-test with $p < 0.05$ considered significant. Where indicated, “n” equals the number of individual cells tested under each condition.
RESULTS

Direct in Vitro Interaction of Immunophilins FKBP12 and FKBP52 with TRPC Channel Proteins. To determine if the TRPC channel proteins physically associate with FKBP52, the TRPC proteins were individually co-expressed with a GST-FKBP52 fusion protein in Sf9 insect cells using recombinant baculovirus. The association was first examined by subjecting total cell lysates to immunoprecipitation with antibodies specific for each TRPC channel protein followed by separation of precipitated proteins by SDS-PAGE and immunoblotting using an anti-GST antibody. When proteins were immunoprecipitated with anti-TRPC1, -C4, or -C5, the GST-FKBP52 polypeptide (predicted molecular weight 89 kDa) was detected (Fig 1), but when proteins were immunoprecipitated with anti-TRPC3, -C6, or -C7, GST-FKBP52 was not observed (data not shown). Association was also examined by immunoprecipitating with anti-GST antibody followed by immunoblotting with the specific anti-TRPC antibodies. TRPC1, -C4, and -C5 were found in the immunocomplexes precipitated with anti-GST (Fig 1), whereas TRPC3, -C6, and -C7 were not (data not shown). To determine if FKBP12 physically associates with TRPC channel proteins, identical experiments were performed on Sf9 cell lysates co-expressing each of the TRPC proteins with GST-FKBP12. When proteins were immunoprecipitated with anti-TRPC3, -C6, or -C7, the GST-FKBP12 polypeptide (predicted molecular weight 40 kDa) was detected (Fig 2), but when proteins were immunoprecipitated with anti-TRPC1, -C4, or -C5, GST-FKBP12 was not observed (data not shown). Likewise, TRPC3, -C6, and -C7 were found in the immunocomplexes precipitated with anti-GST (Fig 2), whereas TRPC1, -C4, and -C5 were not (data not shown). These results demonstrate that the
interaction of immunophilins is specific and selective; FBPB52 interacts with TRPC1, -C4, and -C5, whereas FKBP12 interacts with TRPC3, -C6, and -C7.

Effect of FK506 on the Association of FKBP52 and FKBP12 with the TRPC Channel Proteins. The immunophilins FKBP12 and FKBP52 are peptidyl-prolyl cis-trans isomerases that recognize and bind to specific XP dipeptides in target proteins (for review see (19)). FK506 acts as a prolyl-peptidomimetic and disrupts the interaction of FKBPs with their target proteins by mimicking the XP binding motif. If the interaction of FKBPs with the TRPC channels involves an XP binding motif, FK506 should attenuate the interaction. To determine the effect of FK506, total cell lysates, prepared from Sf9 cells individually co-expressing TRPC1, -C4, or -C5 along with GST-FKB52, were subjected to immunoprecipitation using anti-TRPC antibodies. The immunocomplexes were incubated with increasing concentrations of FK506, and both the beads and supernatants were analyzed for the presence of GST-FKB52. Following immunoprecipitation with anti-TRPC1, -C4, or -C5, all of the FKBP52 is found associated with the agarose beads in the absence or in the presence of 0.5 or 1 µM FK506 (Fig 3A). However, FKBP52 was displaced from the beads and appeared in the supernatant when lysates were incubated with 10 and 20 µM FK506. Similar experiments were performed to determine the effect of FK506 on FKBP12 interaction with TRPC3, -C6, and -C7. As seen in Fig 3B, 0.5 µM FK506 was sufficient to completely displace FKBP12 from each of the channel proteins. In parallel experiments, we also found that preincubation of Sf9 cell lysates with FK506 (30 µM) prior to immunoprecipitation with anti-TRPC antibodies, prevented the co-immunoprecipitation of FKBP12 or FKBP52 with their respective TRPC binding partners (data not shown).
Direct in Vivo Interaction of FKBP12 and FKBP52 with TRPC Channel Proteins. Direct immunoprecipitation experiments from rat brain could not be performed for FKBP52 because its presence would be masked by IgG heavy-chain which has a similar molecular mass. Therefore, to demonstrate the association of FKBP52 with native TRPC channels, rat brain lysates were first immunoprecipitated with the individual TRPC antibodies. The immunocomplexes attached to the beads were then incubated with 30 µM FK506 to displace FKBPs. FKBP52 was easily detected in the supernatant from TRPC1,-C4, or -C5 immunoprecipitates, but not from TRPC3, -C6, or -C7 (Fig 4A). Control experiments showed that FK506 did not displace the immunoprecipitating IgG from the beads (data not shown). Similar experiments were performed to determine if FKBP12 can be displaced from native TRPC channel proteins by FK506. Rat brain lysates were first immunoprecipitated with the individual TRPC antibodies. The immunocomplexes attached to the beads were then incubated with 30 µM FK506 to displace FKBPs. FKBP12 was detected in the supernatant from TRPC3,-C6, or -C7 immunoprecipitates, but not from TRPC1, -C4, or -C5 (Fig 4B). Direct immunoprecipitation experiments from rat brain lysates revealed that FKBP12 co-immunoprecipitated with TRPC3, -C6 and -C7, but not with TRPC1, -C4, or –C5 (data not shown). Overall, these results demonstrate that 1) FKBP52 specifically and selectively associates with TRPC1, -C4, and -C5, whereas FKBP12 associates with TRPC3, -C6, and -C7 in rat brain, and 2) the association between FKBP52 and FKBP12 with their respective TRPC binding partners can be disrupted by FK506, suggesting that interaction occurs via a XP dipeptide motif.

Effect of immunophilins on TRPC channel function. To begin to evaluate the effect of immunophilins on TRPC channel activity, we took advantage of a recently characterized HEK
cell line stably expressing human TRPC6 (26). As previously reported, stimulation of endogenous muscarinic receptors by carbachol (100 µM) in this cell line, produces a dramatic increase in both inward and outward currents via TRPC6 channels (Fig 5). Currents peak within 30 sec and subsequently decline with time to a sustained, but elevated level in the continuous presence of receptor agonist. The current-voltage relationship (Fig 5, inset, upper panel) shows the distinctive inward and outward rectification properties indicative of TRPC6 whole-cell currents. Incubation of the cells with 10 or 30 µM FK506 had no effect on basal TRPC6 channel activity, but significantly decreased the rate of channel activation by carbachol. In control fura-2 experiments, FK506 had no effect on carbachol-induced release of Ca$^{2+}$ from internal stores (data not shown) demonstrating that this drug does not interfere with agonist binding to receptor, activation of PLC, or the ability of Ins(1,4,5)P$_3$ to release Ca$^{2+}$. Interestingly, the current density 3 mins after carbachol addition was not significantly different in the presence or absence of FK506 (Fig 5), suggesting that immunophilins may primarily modulate the kinetics of channel activation following receptor stimulation.

Although the message for FKBP12 and FKBP52 is present in the TRPC6-expressing HEK cell line, preliminary immunoprecipitation experiments showed that neither are detectable at the protein level. However, following immunoprecipitation of TRPC6, an immunoreactive band at a molecular mass of approximately 13 kDa was observed by Western blot analysis using a pan-FKBP antibody. This protein was displaced from TRPC6 by incubation of the immunoprecipitated proteins with FK506 at a concentration of 10 to 50 µM (Fig 5, inset, lower panel) confirming that the immunoreactivity reflects the endogenous FK506-binding protein expressed in these cells. Importantly, the concentration of FK506 that inhibits channel activity is
similar to the concentration needed to displace the FKBP from the TRPC6 channel protein. These results suggest that endogenous immunophilins interact with TRPC channels and play an important role in receptor-mediated channel activation.

Identification of the putative FKBP binding domain on TRPC channel proteins. Previous studies on Drosophila TRPL showed that mutations in a unique, highly-conserved, proline-rich sequence of amino acids, COOH-terminal to S6, the last transmembrane segment, can disrupt immunophilin interaction with the channel (7). As seen in Table 1, the first LP dipeptide in this region is conserved in each of the TRPC channel proteins, whereas the second LP dipeptide is VP in TRPC1, -C4, and -C5, and IP in TRPC3, -C6, and -C7. For Drosophila TRPL, P-to-Q mutations at either the first or second LP eliminated FKBP59 interaction with the TRPL channel protein. To determine if this region represents the FKBP binding domain for the mammalian TRPC homologs, the first LP in TRPC1, -C3, -C4, and -C6 was mutated to LQ. Wild-type and mutant TRPC1 and -C4 were individually co-expressed in Sf9 cells along with FKBP52, and TRPC3 and -C6 were co-expressed with FKBP12. Reciprocal immunoprecipitations showed that P-to-Q mutants of TRPC1 (Fig 6A) and TRPC4 (not shown) did not interact with FKBP52. Likewise, P-to-Q mutants of TRPC3 (Fig 6B) and TRPC6 (not shown) did not interact with FKBP12.

As mentioned above, FKBP12 interacts with VP in RYR1, whereas FKBP12.6 interacts with IP in RYR2. Interestingly, changing VP to IP in RYR1 eliminates binding of FKBP12, but allows binding of FKBP12.6 (18). Thus, the amino acid preceding the proline residue appears to play a role in defining immunophilin specificity. To determine if the second IP and VP in the
proline-rich domain of the TRPC proteins plays an important role in determining specificity for FKBP12 and FKBP52, the VP in TRPC5 was mutated to IP, and the IP in TRPC3 was changed to VP. The mutated channels were individually co-expressed with FKBP12 or FKBP52. Mutual swap of VP and IP had no effect on the ability of TRPC5 and TRPC3 to interact with FKBP52 or FKBP12, respectively (Fig 7). Furthermore, swapping VP and IP did not confer binding of FKBP52 and FKBP12 on TRPC3 and TRPC5, respectively (Fig 7).
DISCUSSION

The results of the present study show that the immunophilins FKBP12 and FKBP52 interact with mammalian TRPC channel proteins in a specific and selective fashion. FKBP12 interacts with TRPC3, -C6, and -C7, whereas FKBP52 interacts with TRPC1, -C4, and -C5. The interaction was observed in vitro when the immunophilins and channel proteins were co-expressed in Sf9 insect cells and in vivo in rat brain lysates. The immunophilin-channel interaction could be disrupted by the immunosuppressant drug FK506 which, in functional assays, attenuated the ability of receptor stimulation to activate TRPC6 stably expressed in HEK cells. Mutations of a highly conserved proline residue found in the COOH-terminal domain, a region predicted to be cytoplasmic, eliminated immunophilin-channel interaction. Taken together, these results provide strong support for the hypothesis that immunophilins are TRPC channel accessory proteins.

Submicromolar concentrations of FK506 were sufficient to displace FKBP12 from TRPC3, -C6, and -C7 channels at room temperature, whereas 10 µM FK506 was needed to displace FKBP52 from TRPC1, -C4, and -C5. This result suggests that the affinity of FKBP52 for TRPC1, -C4, and -C5 is higher than that of FKBP12 for TRPC3, -C6, and -C7. In this regard, FKBP52 is composed of three tandem FKBP-like domains (17,27). Each of the domains may interact with specific XP dipeptides in the TRPC channels and thus strengthen the interaction between FKBP52 and the TRPCs. Alternatively, it has been reported that the second FKBP-like domain may contain a nucleotide binding sequence and the COOH-terminal segment following the third FKBP-like domain contains a consensus calmodulin binding domain (17).
Preliminary studies have shown that FKBP52 binds calmodulin in a Ca^{2+}-independent fashion (Goel and Schilling, unpublished observations). Thus, additional modifiers may also influence the affinity of FKBP52 for the channel proteins.

Previous studies showed that P-to-Q mutations in the LP dipeptides in the putative FKBP binding domain on *Drosophila* TRPL channels eliminated channel-immunophilin interaction (7). Likewise, mutation of the first conserved LP in each of the TRPC channels disrupted binding. This proline-rich region is conserved in all of the TRPC and TRPM channel proteins, but is not present in the TRPVs. Thus, FKBP12 and/or FKBP52 may also interact with the TRPM subfamily. The proline-rich region has recently been implicated in binding of the adapter protein, Homer, to TRPC1 channels (28). Homer is thought to link TRPC1 channels in the plasmalemma to Ins(1,4,5)P_3 receptors present in the endoplasmic reticulum. Interestingly, it was found that P-to-L mutations in the first LP-dipeptide greatly enhanced binding of Homer to TRPC1 in GST-pull-down experiments, and deletion mutants lacking the proline-rich region retained Homer binding, leading to identification of a second Homer binding site in the NH_{2}-terminal region of TRPC1. Although the presence of FKBP5s in these experiments is unknown, it seems possible that binding of FKBP52 at the proline-rich region in the COOH-terminus of TRPC1 may attenuate Homer interaction with the NH_{2}-terminal site of TRPC1 and that binding of Homer is enhanced in the P-to-L mutant following displacement of FKBP52. Likewise, the possibility that Homer binds directly to FKBP52 cannot be eliminated at this time.
Reciprocal immunoprecipitation studies both in vitro and in vivo showed that TRPC1, -C4, and -C5 associate, and the TRPC3, -C6, and -C7 associate, but no cross association is observed between the two major subgroups of TRPC channel proteins (23,24). Furthermore, P-to-Q mutation at the second LP dipeptide in Drosophila TRPL channels eliminates binding of FKBP59. Thus, the observation that the second LP is changed to IP in TRPC1, -C4, and -C5, and VP in TRPC3, -C6, and -C7 was intriguing. However, mutual swap of VP and IP in TRPC3 and TRPC5 did not eliminate or alter the selectivity of the immunophilins for their respective binding partner. Thus, it seems likely that other regions of the TRPC channel proteins play an important role in defining this interaction. In this regard, target proteins may interact with immunophilins at binding domains not structurally associated with the active site. It is well established that upon binding of FK506 to the active site of FKBP12, the drug-immunophilin complex forms a composite surface that binds with high affinity to calcineurin, inhibiting phosphatase activity (8,19). Thus, when FKBP12 or FKBP52 are attached to the TRPC channel proteins via the LP dipeptide, the immunophilin may participate in additional protein-protein interactions with other regions of the TRPC channel protein itself or perhaps with calcineurin. It has been reported that FKBP12 anchors calcineurin to both the Ins(1,4,5)P3 receptor and the ryanodine receptor altering receptor phosphorylation status and regulating Ca2+ flux (16,29,30). The possible interaction of calcineurin with TRPC channels awaits further investigation.

The effect of FKBPs on native TRPC channel function remains unknown, however it is well established that FKBP12/12.6 stoichiometrically bind to the RYR in both skeletal and cardiac muscle and alter channel gating (8-10,18,27). The function of FKBP52 is less clear. FKBP52 is part of the steroid receptor-heat shock protein complex and as such may have a
chaperone function. Recent studies on *Drosophila* TRPL have shown that addition of recombinant FKBP59 inhibited TRPL single channel activity in excised inside-out patches (7). Thus, FKBP52 may also have direct effects on channel gating and play a fundamental role in signal transduction. However, the effect of FKBP52 on TRPC channels is expected to be rather complex given that FKBP52 may bind ATP, GTP, and/or calmodulin (17). As mentioned above, the presence or absence of FKBP12 or FKBP52 could also influence the binding of other proteins such as Homer. To begin to evaluate the effect of immunophilins on mammalian TRPC channel function, we utilized an HEK cell line stably expressing TRPC6. This cell line exhibits robust expression of TRPC6 channel protein that can be activated by stimulation of endogenous muscarinic receptors (26). FK506 produced an inhibition of receptor-induced activation of TRPC6. Furthermore, FK506 disrupted immunophilin-channel interaction in immunoprecipitation experiments from HEK cells. Although the endogenous immunophilin in HEK cells is probably FKBP13, these results suggest that immunophilins may be required for activation of TRPC6 by receptor stimulation at least when heterologously expressed.

We previously showed that Jurkat T lymphocytes predominantly express TRPC3 and TRPC6, and rat basophilic leukemia cells predominantly express TRPC1 and TRPC5 (22). In a recent study, knockout of TRPC1 in DT40 B lymphocytes decreased B-cell receptor-mediated changes in \([\text{Ca}^{2+}]\), leading to reduced NF-AT activity (31). Thus, the immunosuppressant effect of FK506 may, at least in part, reflect an acute change in the activity of TRPC channel proteins in cells responsible for the immune response. With regard to immunophilin actions in the brain, it is clear that TRPC channels are highly, but differentially expressed in both central and peripheral nervous systems (22,32,33). Furthermore, immunophilins are highly expressed in
nervous tissue (34). Studies have also shown that immunophilin ligands including FK506 exhibit potent neuroprotective and neuroregenerative activities (35-37). The neurotrophic action of FK506 appears to be mediated by FKBP52 (38). Thus, it is tempting to speculate that the effect of immunophilin ligands on neuronal survival and growth may involve TRPC channel function.
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FOOTNOTES

Abbreviations Used:

FKBP, FK506 binding protein
GST, glutathione-S-transferase
HEK, human embryonic kidney
HEPES, 4,(2-hydroxyethyl)-1-piperazinethanesulfonic acid
INAD, protein responsible for the inactivation-no-after-potential Drosophila mutant D
Ins(1,4,5)P$_3$, inositol-1,4,5-trisphosphate
IP$_3$R, inositol-1,4,5-trisphosphate receptor
PLC, phospholipase C
PKC, protein kinase C
RYR, ryanodine-sensitive Ca$^{2+}$ release channel
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sf, Spodoptera frugiperda
TRP, protein responsible for the transient receptor potential Drosophila mutant
TRPC, mammalian ortholog of Drosophila TRP
TRPL, Drosophila TRP-like protein
Table 1. **Putative FKBP binding domain of Drosophila TRPL and mammalian TRPC channels.**

|       | Drosophila TRPL   | LPPFNLPSVK |
|-------|-------------------|-------------|
| dTRPL |                   | LPPFNLPSVK  |
| dTRP  |                   | LPPFNLCPNMK |
| TRPC1 |                   | LPPFNIIPSPK |
| TRPC3 |                   | LPPFSLVPSPK |
| TRPC4 |                   | LPPFNLVPSK  |
| TRPC5 |                   | LPPFNIIPSPK |
| TRPC6 |                   | LPPFNLVPSK  |
| TRPC7 |                   | LPPFNLVPSK  |
Fig. 1. **TRPC1, TRPC4, and TRPC5 co-immunoprecipitate from Sf9 cell lysates with FKBP52.** Immunoprecipitations, from total lysates prepared from Sf9 cells individually co-expressing TRPC1, TRPC4, or TRPC5 with GST-FKBP52, were performed as described in Materials and Methods. Protein aliquots (indicated above each lane as Lysate or IP) were subjected to SDS-PAGE and Western blotting. The antibodies used for immunoprecipitation and immunoblotting are indicated below each lane.

Fig. 2. **TRPC3, TRPC6, and TRPC7 co-immunoprecipitate from Sf9 cell lysates with FKBP12.** Immunoprecipitations, from total lysates prepared from Sf9 cells individually co-expressing TRPC3, TRPC6, or TRPC7 with GST-FKBP12, were performed as described in Materials and Methods. Protein aliquots (indicated above each lane as Lysate or IP) were subjected to SDS-PAGE and Western blotting. The antibodies used for immunoprecipitation and immunoblotting are indicated below each lane.

Fig. 3. **FK506 disrupts the interaction of FKBP52 and FKBP12 with the TRPC channel proteins.** Panel A. Total lysates, prepared from Sf9 cells individually co-expressing TRPC1, TRPC4, or TRPC5 along with GST-FKBP52, were subjected to immunoprecipitation for 12 hr with the anti-TRPC antibody indicated to the right of each blot. The beads with attached immunocomplexes were washed and resuspended in lysate buffer containing FK506 at the concentration shown below each lane and incubated for 30 min at 20°C. The beads with immunocomplexes were subjected to centrifugation and aliquots of beads and supernatants were
probed using anti-GST antibody. *Panel B.* Protocol was the same as in panel A with total lysates prepared from Sf9 cells individually co-expressing TRPC3, TRPC6, or TRPC7 along with FKBP12. The blots were probed using anti-FKBP12 antibody.

**Fig. 4. FKBP52 and FKBP12 co-immunoprecipitate with TRPC channel proteins from rat brain lysates.** Total lysates prepared from rat cerebral cortex were subjected to immunoprecipitation using the indicated anti-TRPC antibody. The beads with immunocomplex attached were incubated with 30 µM FK506 at 20°C for 30 min to displace bound FKBP. Beads were separated by centrifugation and aliquots of the supernatant were subjected to Western blot analysis using anti-FKBP52 antibody (*Panel A*) or anti-FKBP12 (*Panel B*).

**Fig 5. FK506 attenuates receptor-mediated activation of TRPC6 channel activity.** Whole-cell membrane currents were recorded in HEK cells stably expressing TRPC6 as described in Material and Methods. Voltage ramps were applied every 15 sec from a holding potential of –50 mV. Outward currents at +80 mV (*upper left panel*) and inward currents at –80 mV (*lower left panel*) are plotted for representative cells as a function of time after rupture of the patch for whole-cell recording. At the time indicated by the horizontal bar, the bath solution was changed to one containing vehicle (ethanol, ●), 10 µM FK506 (V) or 30 µM FK506 (■). Carbachol (CCh; 100 µM) was added to the bath solution at 4 min. *Inset, upper left panel.* Current-voltage relationships (I-V) obtained during voltage ramps at the times indicated in the main panel (a, b, c). *Inset, lower left panel.* Total lysates, prepared from TRPC6-expressing HEK cells were subjected to immunoprecipitation for 12 hr with the anti-TRPC6 antibody. The beads with attached immunocomplexes were washed and resuspended in lysate buffer containing FK506 at
the concentration shown below each lane and incubated for 3 hrs at 20°C. The beads with immunocomplexes were isolated by centrifugation and aliquots from beads and supernatants were subjected to Western blot analysis using pan-FKBP antibody. Note that as the concentration of FK506 increases, FKBP immunoreactivity is displaced from the beads and appears in the supernatant. **Right panels.** Mean ± se outward (Upper panel) and inward (Lower panel) values for current activation rates recorded in the presence of CCh alone, or CCh with the concentration of FK506 indicated below each bar. The values in parentheses indicates the number of individual cells tested under each condition.

**Fig 6.** **P-to-Q mutations in the first LP dipeptide of the putative FKBP binding domain on TRPC3 and TRPC5 eliminates FKBP-channel interaction.** **Panel A.** Immunoprecipitations were performed from total lysates prepared from Sf9 cells individually co-expressing GST-FKBP52 along with either wild-type TRPC1 or with mutant TRPC1 in which the first LP dipeptide (see Table 1) was changed to LQ. **Panel B.** Same as in panel A with total lysates prepared from Sf9 cells co-expressing FKBP12 along with either wild-type TRPC3 or mutant TRPC3 in which the first LP dipeptide (see Table 1) was changed to LQ. The antibodies used for immunoprecipitation and immunoblotting are indicated below each lane.

**Fig 7.** **Swap of IP and VP in TRPC5 and TRPC3 does not eliminate or alter immunophilin-channel interaction.** **Panel A.** Immunoprecipitations were performed from total lysates prepared from Sf9 cells co-expressing mutant TRPC5 in which the IP dipeptide was changed to VP (see Table 1) along with either GST-FKBP52 (left panel) or FKBP12 (right panel). **Panel B.** Immunoprecipitations were performed from total lysates prepared from Sf9 cells individually co-
expressing mutant TRPC3 in which the VP dipeptide was changed to IP along with either FKBP12 (left panel) or GST-FKBP52 (right panel). The antibodies used for immunoprecipitation and immunoblotting are indicated below each lane.
Fig. 1

IP: anti-TRPC1 -- +
Blot: anti-GST + +

IP: anti-TRPC1 -- -- +
Blot: anti-TRPC1 + + +

IP: anti-TRPC4 -- +
Blot: anti-GST + +

IP: anti-TRPC4 -- -- +
Blot: anti-TRPC4 + + +

IP: anti-TRPC5 -- +
Blot: anti-GST + +

IP: anti-TRPC5 -- -- +
Blot: anti-TRPC5 + + +
Fig. 2
Fig. 3
Fig. 4
Fig. 6
Fig. 7
Association of immunophilins with mammalian TRPC channels
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Pil1p and Lsp1p negatively regulate the 3-phosphoinositide-dependent protein kinase-like kinase Pkh1p and downstream signaling pathways Pkc1p and Ypk1p.

Xiping Zhang, Robert L. Lester, and Robert C. Dickson

In our study, the genotype of strain DL523 should be MATa ura3Δ0 trp1Δ his4Δ can1R pck1::LEU2 YCp50 [pck1ts], not MATa ura3Δ0 trp1Δ his4Δ can1R pck1::LEU2. Thus, strains cited in the paper including DL523, RCD504, RCD505, and RCD506 carry a chromosomal pck1::LEU2 allele and a temperature-sensitive PKC1 allele [pck1ts] on plasmid YCp50. This correction does not alter the data or conclusions about the function of Pil1p and Lsp1p.

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The functional interaction of the hepatitis C virus helicase molecules is responsible for unwinding processivity.

Mikhail K. Levin, Yuh-Hwa Wang, and Smita S. Patel

We use the incomplete gamma function (Equation 4) to model the multistep kinetics of helicase DNA unwinding. We wish to make a clarification that this method was developed independently in our laboratory but was first described in Lucius et al. (Lucius, A. L., Vindigni, A., Gregorian, R., Ali, J. A., Taylor, A. F., Smith, G. R., and Lohman, T. M. (2002) J. Mol. Biol. 324, 409–428 and Lucius, A. L., Maluf, N. K., Fischer, C. J., and Lohman, T. M. (2003) Biophys. J. 85, 2224–2239). These papers were referenced as (37) and (35), respectively, in conjunction with the modeling of the helicase kinetics.

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Association of immunophilins with mammalian TRPC channels.

William G. Sinkins, Monu Goel, Mark Estacion, and William P. Schilling

Page 34525, right-hand column, sentence beginning 19 lines from the bottom: “IP” and “VP” were transposed. The correct sentence should read: “As seen in Table I, the first LP dipeptide in this region is conserved in each of the TRPC channel proteins, whereas the second LP dipeptide is IP in TRPC1, -C4, and -C5 and VP in TRPC3, -C6, and -C7.”

Page 34526, left-hand column, sentence beginning 3 lines from the top: TRPC3 and TRPC5 were transposed. The correct sentence should read: “To determine if the second IP and VP in the proline-rich domain of the TRPC proteins plays an important role in determining specificity for FKBP12 and FKBP52, the VP in TRPC3 was mutated to IP, and the IP in TRPC5 was changed to VP.”