Regulation of *Drosophila* TRPL channels by Immunophilin FKBP59

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Running Title: Immunophilin regulates TRPL channels
SUMMARY

TRP and TRPL are Ca\(^{2+}\)-permeable cation channels found in *Drosophila* photoreceptor cells associated with large multimeric signaling complexes held together by the scaffolding protein, INAD. To identify novel proteins involved in channel regulation, *Drosophila* INAD was used as bait in a yeast two-hybrid screen of a *Drosophila* head cDNA library. Sequence analysis of one identified clone showed it to be identical to the *Drosophila* homolog of human FK506-binding protein, FKBP52 (previously known as FKBP59). To determine the function of dFKBP59, TRPL channels and dFKBP59 were co-expressed in Sf9 cells. Expression of dFKBP59 produced an inhibition of Ca\(^{2+}\) influx via TRPL in fura-2 assays. Likewise, purified recombinant dFKBP59 produced a graded inhibition of TRPL single channel activity in excised inside-out patches when added to the cytoplasmic membrane surface. Immunoprecipitations from Sf9 cell lysates using recombinant tagged-dFKBP59 and TRPL, showed that these proteins directly interact with each other, and with INAD. Addition of FK506 prior to immunoprecipitation resulted in a temperature-dependent dissociation of dFKBP59 and TRPL. Immunoprecipitations from *Drosophila* S2 cells and from fly head lysates demonstrated that dFKBP59, but not dFKBP12, interacts with TRPL *in vivo*. Likewise, INAD immunoprecipitates with dFKBP59 from S2 cell and head lysates. Immunocytochemical evaluation of thin sections of fly heads reveal specific FKBP immunoreactivity associated with the eye. Site-directed mutagenensis showed that mutations of P\(^{702}\)Q or P\(^{709}\)Q in the highly conserved TRPL sequence 701LPPPFNVP709, eliminated interaction of the TRPL with dFKBP59. These results provide strong support for the hypothesis that immunophilin dFKBP59 is part of the TRPL-INAD signaling complex and plays an important role in modulation of channel activity via interaction with conserved leucyl-prolyl dipeptides located near the cytoplasmic mouth of the channel.
INTRODUCTION

In *Drosophila* eye, photostimulation of rhodopsin leads to activation of the Ca\(^{2+}\)-permeable *transient receptor potential* (TRP\(^1\)) and *trp*-like (TRPL) channels (1-3). This response, which depends on phospholipase C, is extremely rapid and results in depolarization of the photoreceptor cell followed by Ca\(^{2+}\)-mediated feedback regulation of the visual signaling cascade. The biochemical or biophysical link between stimulation of PLC and channel activation remains unknown. Originally it was proposed that TRP and TRPL were store-operated channels (SOC)(2) i.e., channels activated by depletion of Ca\(^{2+}\) from inositol-1,4,5-trisphosphate(Ins(1,4,5)P\(_3\))-sensitive internal Ca\(^{2+}\) stores. More recent studies suggest that hydrolysis of phosphatidylinositol-4,5-bisphosphate and the concomitant generation of diacylglycerol, play a direct role in channel activation (4,5). Heterologous expression of TRPL gives rise to Ca\(^{2+}\)-permeable, non-selective cation channels that are unaffected by depletion of internal stores, but are activated following stimulation of membrane receptors linked to phosphoinositide turnover (6-11). In contrast, recombinant TRP is more selective for Ca\(^{2+}\) and is activated by depletion of internal stores by thapsigargin (9,12). These studies strongly support the hypothesis that TRP and TRPL proteins form the essential subunit structure of the channels responsible for light-induced conductance change in *Drosophila* photoreceptor cells.

Studies in photoreceptor cells have shown that TRP channels are held in a signaling complex (i.e., a signalplex) by a scaffolding protein called INAD (13-16). INAD contains five tandem PDZ domains which are thought to act as protein binding modules mediating the clustering of membrane and membrane-associated proteins. INAD has been shown to interact through the PDZ domains with itself and with a number of proteins involved in the
phototransduction cascade including PLC, PKC, rhodopsin, TRP, and TRPL, thus potentially creating clusters of signaling complexes (17). Studies have also shown that calmodulin (CaM) associates with the signalplex possibly by direct binding to INAD and/or TRP and TRPL. In *Drosophila* this signalplex appears to be an essential structural feature necessary for a normal photoresponse. When the TRP-INAD interaction is disrupted by a point mutation in the third PDZ domain of INAD, TRP is no longer spatially restricted to its normal subcellular compartment, i.e., the rhabdomere (14), and stimulation by light reveals a defect in deactivation of the light-induced current (13). Furthermore, genetic disruption of TRP-INAD interaction results in retinal degeneration (14). In *trp* mutant flies, INAD is also mislocalized within the photoreceptor cell suggesting that TRP-INAD interaction is necessary for mutual localization (18,19). Interestingly, approximately 25% of the INAD seen in the rhabdomere of wild-type flies, remains associated with the rhabdomere in *trp* mutants. This residual INAD may be necessary for light-activation of TRPL channels observed in *trp* mutant photoreceptor cells (19), presumably through INAD-mediated association of TRPL with the other members of the signalplex.

The biochemical similarity between *Drosophila* phototransduction and receptor-mediated Ca\(^{2+}\) signaling in mammalian cells led to the cloning of mammalian TRP genes. To date, seven primary mammalian TRP homologues have been identified (TRPC1-C7) which appear to be activated by receptor-dependent mechanisms at least when heterologously expressed (20-26). A mammalian gene bearing some homology to *Drosophila* INAD has also been identified and cloned (27), but there is no data to suggest that it interacts with mammalian TRP proteins. However, murine TRP4 and TRP5 bind to the first PDZ domain of the Na\(^+\)-H\(^+\) exchanger.
regulatory factor (NHERF) (28). Additionally, it has also been shown that PLCβ1 and PLCβ2 bind to the same PDZ domain indicating that NHERF may be capable of bringing together signaling molecules. Although the functional implications of NHERF interaction with either PLC or TRP channel proteins remain unknown, it seems reasonable to speculate that PDZ-containing proteins will form the scaffolding necessary for signalplex formation and localization in mammalian cells expressing the TRP channels. In this regard, the TRP channel signalplex may be localized to caveolar structures present in the plasmalemma of some mammalian cell types (29). However, the number or identity of accessory proteins associated with the signalplex in either mammalian or Drosophila cells remains unknown.

In the present study, we employed the yeast two-hybrid system to identify other proteins involved in regulation of TRP channels. Using INAD as bait to screen a Drosophila head cDNA library, the Drosophila immunophilin dFKBP59 was identified. To elucidate the possible role of dFKBP59 in channel regulation, TRPL and dFKBP59 were co-expressed in Sf9 insect cells using recombinant baculovirus, and Ca²⁺ influx via TRPL was measured using the fura-2 fluorescence assay. The effect of purified recombinant dFKBP59 on single TRPL channel activity was monitored in excised inside-out patches from TRPL-expressing Sf9 cells. Co-immunoprecipitation experiments were employed to demonstrate physical interaction between recombinant TRPL, INAD, and dFKBP59. Finally, to test for interactions in vivo, reciprocal co-immunoprecipitations of endogenous proteins were done from lysates of Drosophila S2 cells and fly heads. The results suggest that Drosophila immunophilin dFKBP59 is part of the TRPL-INAD signalplex and may be an important modulator of channel activity.
MATERIALS AND METHODS

Physiological solutions and reagents. MES-buffered saline (MBS) contained the following: 10 mM NaCl, 60 mM KCl, 17 mM MgCl₂, 10 mM CaCl₂, 4 mM D-glucose, 110 mM sucrose, 0.1% bovine serum albumin, and 10 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH adjusted to 6.2 at room temperature with Trizma-base. The total osmolarity of MBS was ~340 mosM. Thapsigargin and bradykinin (BK) were obtained from Calbiochem. Anti-GST antibody was obtained from Santa Cruz Biotechnology, anti-FLAG from Sigma, and anti-VR1 from Chemicon. Anti-FKBP59 and anti-FKBP12 were from Affinity BioReagents and Santa Cruz Biotechnology, respectively. Note that although the anti-FKBP antibodies are designated as specific, we have found that both commercially available antibody preparations recognize both FKBP12 and FKBP59. Anti-TRPL and anti-INAD antibodies were generous gifts from Dr. Craig Montell (Johns Hopkins University) and Dr. Bih-Hwa Shieh (Vanderbilt University), respectively.

Cell Culture: *Spodoptera frugiperda* (Sf9) cells were obtained from ATCC and cultured as previously described (30,31) 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 solution (Gibco).

Yeast Two Hybrid Screen. The LexA-interaction trap system was employed as previously described (32). Briefly, InaD was cloned as a LexA fusion into pEG202 and introduced into yeast strain EGY48 harboring the pSH18-34 lacZ reporter plasmid. InaD did not activate transcription of the LEU2 and lacZ reporters by itself and was also shown to enter the nucleus in
a repression assay. To screen for potential interactors, an activation-tagged *Drosophila* head cDNA library in pJG4-5 vector (a generous gift from Dr. Michael Rosbash, Brandeis University) was transformed *en masse* into the EGY48 selection strain and plated onto –ura/-his/-trp drop-out media to select for all three plasmids. Approximately $10^6$ c.f.u. were plated onto each 100 mm galactose/raffinose –ura/-his/-trp/-leu plate to induce expression of activation-tagged proteins and to select for Leu+ potential interactors. After shutting off expression on glucose master plates, individual colonies were replica patched onto selection media. Library transformants showing galactose-dependent Leu+ and lacZ phenotypes were picked for further characterization. Rescued library plasmids were re-introduced into the original selection strain and into strains harboring irrelevant baits to verify specificity of interaction. All plasmids and yeast strains were obtained from Dr. Roger Brent (Harvard University).

**Generation of full-length dFKBP59 cDNA by PCR.** A forward primer was designed to incorporate the nine amino acids missing from the amino terminus of dFKBP59 partial cDNA clone. The sequence of the primer pairs used were: Forward: 5'-CA CCA TGG ATG CCG GAA GGG AAT AAA ATC GAC TTG TCC GGG GAC GGT GGC GTC CTA AAG G-3', Reverse: 5'-GAG CGG CCG CTT AGA TCA TGA TTA TAT TGT CGC GCT CCA GCG TCA ATT CTG CTT CG-3'. Plasmids containing the partial cDNA of dFKBP59 were purified and used as a template for the PCR. The amplified product was subcloned into pGEMT easy (Promega) and sequenced to verify structure and to confirm that no errors were introduced during amplification.
Bacterial expression and purification of recombinant dFKBP59. Full-length dFKBP59 cDNA was subcloned in-frame with the GST start codon of the bacterial expression vector pET42a (Novagen) between the BamH1 and Not1 restriction sites and transformed into the protease-deficient BL21 E. coli strain. Protein expression was induced by adding IPTG to a final concentration of 1mM and incubating for 1.5 hrs at 37°C. Cells were lysed by freeze-thawing followed by centrifugation at 12,000 rpm for 15 min. Both soluble and particulate fractions were assessed for presence of fusion proteins by SDS-PAGE. The fusion protein, which was found mainly in the soluble fraction, was affinity purified using the B-PER 6xHis fusion protein purification kit (Pierce) following the manufacturer's recommendations. The GST-dFKBP59 fusion protein was further purified using the B-PER GST fusion purification kit (Pierce). The dFKBP59 was cleaved from the fusion protein by Factor Xa digestion. The cleaved dFKBP59 was concentrated and dialysed against a solution containing 10 mM Tris-HCl, pH 8.0 using a Ultra-free BioMax spin 30K filter. The final protein concentration was determined using Bradford reagent and protein purity was determined by SDS-PAGE followed by silver staining.

Generation of GST-TRPL COOH-terminal mutants. Mutants were generated by two-step PCR method as previously described (33). The sequence of the primers used for mutagenesis were as follows:

P702Q:

5'-GACAGTGCCACCCTGCAACACCCTTTCATGCTCTGTCCTGCCCTCGTGCAAGTGGGTC-3'

P709Q:

5'-GACAGTGCCACCCTGCGCCACCCTTTCATGCTCTGCAATCCGTCAAGTGGGTC-3'
The corresponding PCR products were digested with restriction enzymes Afl11 and Dra111 and subcloned into pVL1393 containing full length TRPL cDNA cut with the same enzymes. Mutations were verified by sequence analysis. For bacterial expression, the COOH-terminal domains of TRPL, TRPL:(P°702Q) and TRPL:(P°709Q) were PCR amplified using primer pairs: forward: 5'-CTCGTACAGCGTCATTAACGTGATTG-3', reverse: 5'-GCGGCCGCTTAGTTTCGATGCTTTGGCCGCTGGGGAC-3'. The primers amplified a region encompassing nucleotides 1951 to 3375 bp which encode amino acids 651 to 1124 of TRPL. The PCR fragments were purified and cloned in-frame with the GST start codon of the bacterial expression vector pET42a between EcoR1 and Not1 restriction sites. Clones in the correct orientations were transformed into BL21, induced with IPTG, and isolated using a glutathione affinity column.

**Generation of recombinant baculovirus.** The cDNAs encoding *Drosophila* TRPL, human B2 bradykinin receptor, dFKBP59, INAD, and the rat vanilloid receptor, VR1 were individually subcloned into baculovirus transfer vector, PVL1393 using standard techniques. The cDNA for INAD and VR1 were generous gifts of Drs. Bih-Hwa Shieh (Vanderbilt University) and David Julius (University of California, San Francisco), respectively. Recombinant baculoviruses were produced using 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 and used for infection of Sf9 cells as described previously (30,34).
Infection of Sf9 insect cells with recombinant baculovirus. Sf9 cells in Grace's medium were plated into 100 mm plastic tissue culture dishes or onto glass coverslips (~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 24-32 h post-infection.

Immunoprecipitations and immunoblots. Frozen *Drosophila* heads were homogenized in 0.5 ml of buffer A (30 mM NaCl, 1 mM EDTA, 20 mM HEPES pH 7.5, plus protease inhibitors), using a glass-Teflon homogenizer. The homogenate was subjected to centrifugation at 4000 x g for 1 min. A microsomal fraction was isolated from the resultant supernatant by centrifugation at 50,000 x g for 60 min at 4°C. The membrane pellet was resuspended in 0.1 ml of buffer A containing 1% Triton X-100 (fly head lysate). Sf9 cells infected with baculovirus or S2 cells were lysed in 1ml of buffer containing 0.2% n-dodecyl-β-maltoside, 0.2 mM sodium vanadate, 50 mM NaF, 2.7 mM KCl, 10mM Tris-Cl (pH 7.5), 150mM NaCl, 1 mM EDTA, pH 8.0 and incubated at 4°C for 30 min. The protein extracts from Sf9 and S2 cells and fly head lysates were centrifuged at 50,000 x g for 60 min at 4°C to remove the cell debris and partially solubilized membrane fragments. Lysates (~400 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 for 3 hrs. Immunocomplexes were captured by incubating with protein A/G agarose beads at 4°C for 12 hrs. Beads were pelleted, washed four times with buffer A, resuspended in 2x SDS sample buffer (100 µl), and boiled for 3 min. Cell lysates and immunocomplexes were fractionated by SDS-PAGE (5 µg of lysate protein or 10 µl of immunoprecipitate per lane) and electrotransfered to PVDF membrane (100V for 1hr) in Tris-
glycine buffer. Blots were probed with the indicated primary antibody at 1:2000 to 1:7000 dilution and detected using ECL-Plus (Amersham Life Sciences).

**Immunofluorescence in fly head sections.** Heads from wild type *Drosophila* were fixed in Bouin’s fixative (Electron Microscopy Sciences, PA) for 3 hr at room temperature. After dehydration by consecutive incubation in 50%, 70% and 90% acetone, the heads were embedded in paraffin. Cross-sections of 10 µm thicknesses were cut and placed on microscope slides. The slides were subsequently treated with xylene to dissolve the paraffin. Following the rehydration by consecutive incubations in 90%, 70% and 50% ethanol the sections were blocked in PBS containing 1% BSA, 1% goat serum for 30 min at room temperature and incubated with anti-FKBP antibodies (1:300) overnight at 4°C. For negative controls, the sections were incubated with FITC-conjugated goat anti-rabbit IgG (1:300). The sections were washed in PBS, incubated 1 h with FITC-conjugated goat anti-rabbit IgG (1:500) at room temperature and washed with PBS. Fluorescence was visualized using a FITC-HYQ filter cube (excitation and emission wavelength 480/505 nm) on a Nikon Eclipse E600 microscope. The images were obtained using Spot RT camera and analyzed using spot advanced software (Spot Diagnostic Instruments, Inc.).

**Measurement of free cytosolic Ca\(^{2+}\) concentration.** \([\text{Ca}^{2+}]_i\) was measured in Sf9 cells using the fluorescent indicator, fura-2, as previously described (30,34). Briefly, cells were dispersed, washed and resuspended at a concentration of 1.5-2 x 10\(^6\) cell/ml in MBS containing 2 µM fura-2/AM. Following 30 min incubation at room temperature (22°C), the cell suspension was subjected to centrifugation, resuspended in an equal volume of MBS and incubated for an additional 30 min. The cells were twice washed and fluorescence was measured using an SLM...
8100 spectrophotofluorimeter. Excitation wavelength alternated between 340 and 380 nm and fluorescence intensity was monitored at an emission wavelength of 510 nm. All measurements on Sf9 cells were performed at 22°C. Calibration of the fura-2 associated with the cells was accomplished using triton lysis in the presence of a saturating concentration of Ca\(^{2+}\) followed by addition of EGTA (pH 8.5). [Ca\(^{2+}\)]\(_i\) was calculated by the equation of Grynkiewicz et al. (35) using a \(K_d\) value for Ca\(^{2+}\) binding to fura-2 of 278 nM for 22°C (36). Unless otherwise indicated, the results shown are representative of at least three independent experiments.

**Electrophysiological techniques.** The patch clamp technique was utilized in cell-attached and excised inside-out recording modes (9,37). All experiments were performed on single Sf9 cells at room temperature (~22°C). Unless otherwise indicated, the bath and pipette solutions contained 100 mM Na-gluconate, and 10 mM MES, pH 6.5. The osmolarity was adjusted to 340 mosM with mannitol. Free Ca\(^{2+}\) concentration in this solution, determined using fura-2 fluorescence, was 1.6 \(\mu\)M. Data was obtained using an Axopatch 1-D amplifier (Pacer Scientific, Los Angeles, CA, USA) using pClamp 8.0 software, and recorded on VCR tape via a VR-10B Digital Data Recorder interface (Instrutech Corp., Great Neck, NY, USA) for subsequent computer analysis. Single channel records were filtered at 2 kHz, digitized and analyzed using pClamp8 and EDA (Event Dynamic Analysis utility, (38)). Open probability of multi-channel patches are calculated from the idealized events binned at one second intervals. Where indicated, \(n\) equals the number of cells examined under each condition. Statistical differences were determined by \(t\)-test with Bonferroni’s correction for multiple comparisons where appropriate (39); \(p<0.05\) was considered significant.
RESULTS

Identification of *Drosophila* immunophilin as an INAD-binding protein. To identify novel proteins involved in regulation of TRP channels, INAD was used as bait in a yeast two-hybrid screen. Yeast harboring LexA-INAD fusion and LacZ reporter plasmids were transformed with an activation-tagged *Drosophila* head cDNA library. Screening of $10^5$ individual clones for galactose-dependent leucine phototrophy and β-galactosidase activity resulted in more than 40 positive clones. Library plasmids were rescued from positive clones and individually retransformed into the original yeast selection strain or into strains harboring irrelevant baits to confirm specificity of interaction with INAD. This secondary screening step identified 10 positive clones. The 10 clones were partially sequenced and compared to known proteins in the database. Sequence analysis revealed that one of the identified INAD-binding clones was a novel *Drosophila* protein homologous to mammalian immunophilin-p59. Immunophilin-p59 belongs to a large family of proteins that bind the immunosuppressant drug, FK506 and has been designated as FK506-binding protein-59, or FKBP59 (the mammalian protein is currently designated FKBP52 (40)). An additional BLAST search showed that the clone identified in the yeast screen was localized on *Drosophila* chromosome 2L, but was not full-length, lacking nine amino acids at the NH$_2$-terminus. The full-length clone of *Drosophila* immunophilin was obtained by PCR as described in *Materials and Methods*. The full-length sequence included an open reading frame of 1317 nucleotides encoding a protein of 439 amino acids with a predicted molecular weight of 48,877 kDa. **Fig. 1** shows the amino acid comparison of *Drosophila* immunophilin, designated dFKBP59 with human, mouse, and rat FKBP52. dFKBP59 shows 46, 42 and 43% identity and 68, 60, and 66% similarity to human, mouse, and rat FKBP52,
respectively. During the course of this study, the cloning of an identical *Drosophila* immunophilin was reported by another investigator (Accession No. AF163664) (41). In accordance with this previous report, we will use the designation dFKBP59 when referring to the *Drosophila* protein and FKBP52 when referring to the mammalian isoform.

Two other members of the immunophilin family, FKBP12 and FKBP12.6, are found tightly associated with the ryanodine-sensitive Ca$^{2+}$ release channels of skeletal (RYR1) and cardiac (RYR2) muscle, respectively (40). Single channel measurements revealed that subconductance states of ryanodine receptor channels are observed more frequently in the absence of FKBP12, whereas transitions to the full conductance level are favored by tight association of FKBP12 with the channel (42). FKBP12 also associates with, and alters Ca$^{2+}$ release via the Ins(1,4,5)P$_3$ receptor found in non-excitable cells (43). FKBP52 appears to be part of the steroid receptor/heat-shock protein complex (44), but the actual function of FKBP52 in most cells remains unclear. Based on binding of dFKBP59 to INAD, we reasoned that dFKBP59 may affect the function of *Drosophila* photoreceptor channels. To test this hypothesis, the effect of dFKBP59 on TRPL channel activity was determined in Sf9 cells co-expressing both TRPL and dFKBP59. We first created recombinant baculovirus containing the dFKBP59 cDNA under control of the polyhedrin promoter and evaluated protein expression following infection of Sf9 insect cells. We next examined the effect of dFKBP59 on TRPL channel activity in Sf9 cells using the fura-2 fluorescence assay.

**Expression of dFKBP59 in Sf9 insect cells.** dFKBP59 protein expression was determined by Western blot analysis on cell lysates obtained from control Sf9 cells and from cells expressing
dFKBP59. Using a pan-FKBP polyclonal antibody, uninfected Sf9 cells, and cells infected with either TRPL-baculovirus (TRPL-cells) or with the human B₂ bradykinin receptor baculovirus (BK-cells) express a small, but detectable amount of endogenous immunoreactivity at a molecular mass of approximately 48 kDa, presumably reflecting endogenous FKBP associated with Sf9 cells (Fig. 2A). Sf9 cells co-infected with TRPL and dFKBP59 virus (TRPL:dFKBP59-cells), or co-infected with BK and dFKBP59 virus (BK:dFKBP59-cells) greatly overexpress dFKBP59 at 28 hrs post-infection time compared to levels of endogenous immunophilin. Importantly, the results show that 1) baculovirus proteins are not recognized by FKBP antibodies, 2) FKBP antibodies recognize the recombinant Drosophila protein on Western blot, and 3) dFKBP59 is expressed to similar levels in both TRPL:dFKBP59- and BK:dFKBP59-cells.

Effect of dFKBP59 expression on TRPL channel activity. Fura-2-loaded Sf9 cells were suspended in MES-buffered saline (MBS), placed in a cuvette at 22°C, and the fluorescence ratio was recorded as a function of time. Addition of bradykinin to the cuvette produced an immediate increase in [Ca²⁺]ᵢ from a mean ± SE basal value of 171 ± 29 nM to a peak level of 970 ± 64 nM (n=3; Fig 3A). [Ca²⁺]ᵢ subsequently returned slowly towards basal resting levels. As in mammalian cells, the response of Sf9 cells to bradykinin reflects an initial Ca²⁺ release from internal stores via Ins(1,4,5)P₃ receptors and Ca²⁺ entry from the extracellular space via endogenous SOCs (30). Neither the basal [Ca²⁺]ᵢ nor the response to bradykinin was significantly affected by co-expression of dFKBP59 along with the bradykinin receptor (Fig 3A). Addition of thapsigargin to control BK-cells produced an initial increase in [Ca²⁺]ᵢ that reflects Ca²⁺ release from stores subsequent to pump inhibition, and a more slowly rising phase that
reflects Ca\(^{2+}\) entry via SOCs. The [Ca\(^{2+}\)]\(_i\) increase in response to thapsigargin was slightly reduced in cells co-expressing dFKBP59 (643 ± 75 versus 777 ± 83 nM at 300 sec after thapsigargin; Fig. 3B), but the difference was not significant. Additionally, no significant difference in either the time course or magnitude of the thapsigargin response was detected between cells infected with recombinant dFKBP59 baculovirus alone and BK-cells (n=3; data not shown). These results suggest that overexpression of dFKBP59 has little or no effect on endogenous Ca\(^{2+}\) signaling in Sf9 insect cells.

Our previous studies have shown that TRPL channels are constitutively active when expressed in Sf9 cells (7,34). The constitutive activity gives rise to an elevated basal [Ca\(^{2+}\)]\(_i\). We have also shown that TRPL channels are not activated by depletion of internal Ca\(^{2+}\) stores by thapsigargin per se, but are stimulated by a rise in Ca\(^{2+}\) that occurs via endogenous SOCs (45). As expected, expression of TRPL in Sf9 cells resulted in an elevated basal [Ca\(^{2+}\)]\(_i\) (Fig 3C) compared to control BK-cells (Fig. 3A and 3B). Addition of thapsigargin to TRPL cells produced a rapid increase in [Ca\(^{2+}\)]\(_i\), from a resting level of 521 ± 2 nM to a peak value of 1.8 ± 0.07 µM 3-5 min after thapsigargin addition (Fig.3C). In the cells co-expressing both dFKBP59 and TRPL, the basal [Ca\(^{2+}\)]\(_i\) was not significantly different from that observed for TRPL-cells, but addition of thapsigargin produced a biphasic response that was similar in time course and magnitude to the response produced in control BK-cells (compare Fig. 3B with 3C). These results suggest that expression of dFKBP59 has no effect on constitutive TRPL channel activity, but either inhibits or prevents Ca\(^{2+}\)-induced stimulation of TRPL.
**Purification of recombinant dFKBP59.** In order to determine if dFKBP59 has a direct membrane delimited effect on TRPL single channels, we purified recombinant dFKBP59 protein following bacterial expression. The dFKBP59 cDNA was subcloned in-frame into a bacterial expression vector containing the coding sequence for glutathione-S-transferase (GST), followed by poly-histidine and a Factor Xa cleavage site. The resulting fusion protein was recovered from bacterial cultures and purified using Ni²⁺ and glutathione affinity columns as described in *Material and Methods*. Coomassie staining following SDS-PAGE revealed that the GST-dFKBP59 fusion protein (~75 kDa) was the predominant protein present following the initial purification steps, but that several minor protein bands were also observed in this preparation (Fig. 2B). This fraction therefore, could not be used for patch clamp experiments. However, only two proteins were observed following purification on the Ni²⁺ column; GST-dFKBP59 at ~75 kDa and a lower molecular weight protein at ~55 kDa. Cleavage of the fusion protein with Factor Xa and purification using a 30K spin filter resulted in a single band on silver staining which corresponds to the full-length dFKBP59 as indicated by Western blot analysis using a pan-FKBP antibody (Fig 2). This final preparation was employed for subsequent patch clamp studies.

**Effect of recombinant dFKBP59 on single TRPL channel activity.** TRPL channel activity was monitored electrophysiologically in excised inside-out patches from TRPL-expressing Sf9 cells. As seen in Fig. 4, TRPL channel activity was low in cell-attached mode and remained relatively low following excision of an inside-out patch into nominally Ca²⁺-free bath solution ([Ca²⁺]_free in nominally Ca²⁺-free bath solution was estimated by fura-2 to be 1-2 μM). Addition of buffer containing 1mM EGTA ([Ca²⁺]_free in EGTA buffer was ~10 nM) during this period led
to an increase in TRPL channel activity. This effect of EGTA on TRPL activity was irreversible, as channel activity remained high even after extensive washing by bath exchange with nominally Ca\(^{2+}\)-free buffer (i.e., without EGTA). This result suggests that activation of TRPL by chelation of Ca\(^{2+}\) probably reflects displacement of a protein or factor necessary for maintaining the TRPL channel in a low activity state. This factor does not appear to be Ca\(^{2+}\) or CaM, since addition of 1\(\mu\)M CaM to the bath solution during re-addition of Ca\(^{2+}\) did not reduce TRPL channel activity (n=2, data not shown). However, following activation of TRPL by EGTA, addition of purified, recombinant dFKBP59 (0.3\(\mu\)g/ml) to the bath solution resulted in a rapid inhibition of channel activity as indicated by a reduction in \(nP_o\) (Fig. 5). This response was dose-dependent as an increase in concentration of purified dFKBP59 from 0.3 to 3\(\mu\)g/ml produced a further inhibition of TRPL channel activity (Fig. 5). Subsequent washing of excised inside-out patches with buffer containing EGTA again resulted in an increase in TRPL channel currents and re-addition of dFKBP59 in the presence of nominally Ca\(^{2+}\)-free solution again resulted in inhibition. Application of comparable amounts of protein from other non-dFKBP59 wash fractions from the affinity columns (see Material and Methods) had no effect on TRPL channel activity (data not shown). Thus, the effect of recombinant dFKBP59 on TRPL is specific, graded, and reversible. These results suggest that dFKBP59 inhibits TRPL in a membrane-delimited fashion, possibly by direct interaction with the channel protein. The apparent \(K_{0.5}\) for dFKBP59 inhibition of TRPL was ~60 nM (Fig. 5, inset).

**Direct in vitro interaction of dFKBP59 with TRPL and INAD.** To determine if dFKBP59 physically associates with TRPL *in vitro*, we co-expressed GST-dFKBP59 with FLAG-tagged TRPL in Sf9 cells. In immunoblotting, anti-GST antibodies recognized a ~75 kDa polypeptide
in total lysates prepared from infected Sf9 cells (Fig. 6A). Similarly, FLAG monoclonal antibody recognized a ~128 kDa polypeptide which is the predicted molecular weight of TRPL (Fig. 6A). The association of dFKBP59 with TRPL was first examined by immunoprecipitating the total extracts by anti-FLAG antibody followed by separation of precipitated proteins by SDS-PAGE and immunoblotting using anti-GST antibody; GST-dFKBP59 polypeptide (~75 kDa) was detected in the precipitated immunocomplex (Fig. 6A). In addition, association was also examined by immunoprecipitating the total extracts using anti-GST antibody and immunoblotting with anti-FLAG antibody. TRPL polypeptide at ~128 kDa was detected in the precipitates (Fig. 6A). These results demonstrate that dFKBP59 and TRPL interact in vitro.

In order to determine whether this interaction between dFKBP59 and TRPL was specific, we co-expressed the vanilloid receptor, VR1, along with GST-dFKBP59 in Sf9 cells. Recent studies have shown that Sf9 cells expressing the VR1 receptor exhibit large increases in [Ca\(^{2+}\)]\(_i\) following stimulation by capsaicin, consistent with functional expression of VR1 in the plasmalemma (46). Immunoblotting with anti-VR1 recognized a polypeptide with molecular mass of ~95 kDa which is the predicted molecular weight of VR1 protein (Fig 6B). Immunoprecipitation experiments using anti-VR1 antibody and immunoblotting with anti-VR1 antibody showed precipitation of VR1 protein from whole cell lysates. However, no dFKBP59 signal was detected in the immunocomplexes precipitated by anti-VR1 antibody (Fig. 6B). Similarly, no VR1 signal was found in blots of immunocomplexes precipitated from cell lysate using anti-GST antibody. These results demonstrate that VR1 and dFKBP59 do not interact and suggest that the interaction between dFKBP59 and TRPL is specific.
Since dFKBP59 interacts with INAD in the yeast two-hybrid assay, we sought to examine this association by immunoprecipitation. Sf9 cells were co-infected with recombinant baculoviruses expressing INAD and GST-dFKBP59. Immunoblotting with anti-INAD antibody recognized two polypeptides at ~77 and ~75 kDa (Fig. 7A). The predicted molecular weight of INAD protein is 74.3 kDa. Similarly, immunoblotting with anti-GST antibody recognized a single polypeptide at ~75 kDa. GST-dFKBP59 co-precipitated with INAD in immunocomplexes obtained using anti-INAD antibody (Fig. 7A), and INAD co-precipitated along with GST-dFKBP59 when anti-GST antibody was used for immunoprecipitation. Interestingly, both polypeptides recognized by anti-INAD antibodies were co-precipitated with dFKBP59. Thus, INAD and dFKBP59 appear to associate with each other, confirming the results obtained with yeast two-hybrid.

Previous studies have shown that INAD functions in Drosophila vision by directly binding to the light-sensitive ion channels, TRP and TRPL (13-16). Furthermore, it has been proposed that residual INAD observed in the rhabdomeres of trp mutant flies is important for light-induced activation of TRPL (19). However, there has been one report suggesting that the majority of TRPL found in the photoreceptor cell is not part of the INAD signalplex (47). Since dFKBP59 associates with both INAD and TRPL, we re-examined whether INAD also binds to TRPL. FLAG-tagged TRPL and INAD were co-expressed in baculovirus-Sf9 system. Immunoblotting with anti-INAD antibody recognized a polypeptide of ~75 kDa in immunoprecipitates obtained using anti-FLAG antibody (Fig. 7B). Likewise, TRPL co-precipitated with INAD in immunocomplexes obtained using anti-INAD antibody. These results indicate that INAD associates with TRPL. As additional controls, lysates from Sf9 cells
expressing either FLAG-TRPL, GST-dFKBP59, or INAD alone were subjected to immunoprecipitation by anti-FLAG, anti-GST, and anti-INAD antibodies (Fig. 7C). The corresponding proteins were immunoprecipitated only from the cell type expressing each protein confirming antibody specificity. Together these results suggest that dFKBP59 is part of the same complex that contains INAD and TRPL.

**Direct in vivo interaction of dFKBP59 with TRPL and INAD.** In order to determine whether TRPL and dFKBP59 proteins also co-assemble in cells that normally express them, we performed immunoprecipitations from *Drosophila* S2 cell and fly head lysates. Significant amounts of TRPL and dFKBP59 were detected in S2 cells by immunoblotting with anti-TRPL and anti-FKBP antibodies, respectively (Fig. 8). Immunoprecipitation experiments showed that anti-TRPL antibody precipitated both dFKBP59 and INAD from S2 cells. Likewise, reciprocal co-immunoprecipitation experiments showed that dFKBP59 immunoprecipitates with TRPL and INAD from *Drosophila* heads (Fig. 8). Lastly, specific FKBP immunofluorescence was detected in thin sections of fly heads with clear staining of the eye (Fig. 9). These results provide strong support for the hypothesis that TRPL and dFKBP59 interact *in vivo* and that dFKBP59 is part of the INAD signaling complex. To determine specificity of the TRPL-dFKBP59 interaction we examined the ability of anti-TRPL antibodies to immunoprecipitate dFKBP12. As seen in Fig. 8, both *Drosophila* S2 cell and fly head lysates have significant amounts of dFKBP12 that does not co-immunoprecipitate with TRPL. Thus, the interaction between TRPL and dFKBP59 *in vivo* is specific.
Effect of FK506 on the association of dFKBP59 with TRPL. The immunophilins FKBP12 and FKBP52 are peptidyl-prolyl cis-trans isomerases. FKBP12 appears to bind the Ins(1,4,5)P$_3$ receptor by interaction with a region of the channel protein that contains a leucyl-prolyl (LP) dipeptide (48). FK506 disrupts the interaction between FKBP and target proteins by mimicking the LP binding motif (49). To determine the effect of FK506 on the interaction of dFKBP59 with TRPL, total cell lysates prepared from Sf9 cells co-expressing FLAG-TRPL and GST-dFKBP59 were incubated with increasing amounts of FK506. The interactions were examined by immunoprecipitation with anti-GST antibody followed by detection with anti-FLAG antibody or by immunoprecipitation with anti-FLAG antibody followed by detection with anti-GST antibody (Fig. 10). It is important to note that immunoprecipitation reactions were performed for ~12 hr at 4°C and that FK506 was present for the entire time. The addition of FK506 to the cell lysates during immunoprecipitation reduced the association of dFKBP59 and TRPL in a concentration-dependent fashion (Fig. 10A and 9B). The threshold concentration for this effect was ~1 µM and substantial displacement was observed at 50 µM FK506. The apparent displacement of dFKBP59 by FK506 was not related to a decrease in the amount of precipitated immunocomplexes as the same amount of GST-dFKBP59 protein was detected in immunocomplexes precipitated with anti-GST in the absence or presence of FK506 (not shown). Likewise, equal amounts of TRPL were precipitated from lysates using the anti-FLAG antibody in the absence or presence of FK506 (not shown). These results suggest that dFKBP59 is an FK506-binding protein and that FK506 binding to dFKBP59 prevents interaction with TRPL.

Previous studies on FKBP12 association with RYR1 indicate that the immunophilin slowly dissociates from the receptor at room temperature (22°C), but remains tightly associated
when kept at 0-4°C (50). Since FK506 acts as a surrogate for the LP binding motif, FKBP12 must first dissociate from the channel in order for FK506 to gain access to the binding site. Thus, the effect of FK506 on dFKBP59 should be temperature sensitive. To test this hypothesis, total cell lysates prepared from Sf9 cells co-expressing FLAG-TRPL and GST-dFKBP59 were incubated with increasing concentrations of FK506 for 3 hr at 20°C prior to immunoprecipitation for ~12 hr at 4°C. As seen in Fig. 10C, much lower concentrations of FK506 prevent the interaction of dFKBP59 with TRPL when the drug is present at the higher temperature, presumably reflecting the faster dissociation of dFKBP59 from TRPL at 22°C. These results provide further support for the hypothesis that dFKBP59 binds to TRPL with characteristics previously described for FKBP-channel interactions.

**Identification of the putative dFKBP59 binding residues on TRPL.** TRPL contains a unique proline-rich region approximately 32 amino acids COOH-terminal to S6, the last transmembrane spanning segment. This region, \(^{702}\text{LPPPFNVL}^{709}\), is predicted to be cytoplasmic and would be positioned near the mouth of the channel pore. To determine if this region represents the dFKBP59 binding site, a GST-fusion protein construct, designated GST-TRPL\(_{C\text{-term}}\), was created with amino acids 652-1124 of TRPL fused in-frame with GST. GST-TRPL\(_{C\text{-term}}\) was bacterially expressed, purified, and employed for GST pull-down experiments on glutathione affinity columns. As seen in Fig. 11A (left panel), GST-TRPL\(_{C\text{-term}}\) binds to the glutathione column and can be eluted with free glutathione. When lysates from Sf9 cells expressing dFKBP59 were allowed to equilibrate with the GST-TRPL\(_{C\text{-term}}\) columns, dFKBP59 was also eluted by free glutathione (Fig. 11A, middle panel). However, incubation of dFKBP59 cell lysates with 1 µM FK506 prior to application to the GST-TRPL\(_{C\text{-term}}\) column, prevented dFKBP59 binding (Fig.
These results demonstrate that a binding site for dFKBP59 resides in the COOH-terminal region of TRPL and that FK506 prevents interaction, presumably by mimicking the LP binding motif.

To test the hypothesis that the proline-rich region is required for dFKBP59 binding to TRPL, two site-directed mutants were created, P\textsuperscript{702}Q and P\textsuperscript{709}Q. Both GST-TRPL\textsubscript{C-term} mutants bind to and can be eluted from the glutathione columns (Fig. 11B and 10C, left panels). However, dFKBP59 did not bind to either of the GST-TRPL\textsubscript{C-term} mutants (Fig. 11B and 10C, right panels). We also examined the potential interaction of the full-length FLAG-TRPL\textsubscript{C-term} mutants with GST-dFKBP59 in immunoprecipitation experiments following co-expression in Sf9 cells. Although both mutants were present in the total cell lysates, neither immunoprecipitated with anti-GST antibodies (Fig. 11D). Together these results suggest that both LP dipeptides in this proline-rich region are required for dFKBP59 interaction with TRPL.

We previously reported the functional expression of a TRPL construct in which 322 amino acids were deleted from the COOH-terminus (45). This construct, designated TRPL\textsubscript{trunc}, has an intact LP binding domain. To determine if other regions of the COOH-terminal domain of TRPL are important for interaction with dFKBP59, co-immunoprecipitations were performed on Sf9 cells co-expressing dFKBP59 and FLAG-tagged TRPL\textsubscript{trunc}. As seen in Fig. 11D, FLAG-TRPL\textsubscript{trunc} does not immunoprecipitate with anti-GST antibodies. Thus, in addition to the LP binding motif, other sites in the COOH-terminus of TRPL appear to be required for interaction with dFKBP59.
DISCUSSION

The signal transduction cascade in *Drosophila* photoreceptors, which is spatially restricted to the microvilli of the rhabdomere, is extremely rapid. The temporal and compartmental requirements of the photoresponse are maintained in part by the scaffolding protein, INAD which tethers TRP, TRPL, and presumably TRPγ, to the microvilli in close proximity to other players in the signaling pathway, thus forming a large multimeric signalplex. Included in the signalplex are proteins necessary for rapid channel activation (e.g. rhodopsin, Gqα, PLC), and for Ca^{2+}-dependent feedback regulation (e.g., PKC, CaM) of the photoresponse. Some of these proteins may be constitutively bound to INAD, whereas others may interact in a more dynamic fashion (17). The results of the present study identify dFKBP59 as a novel regulator of TRPL channel activity and a new member of the signalplex. dFKBP59 binds to both INAD and TRPL, both *in vitro* and *in vivo*. The interaction is specific, reversible, of high affinity, and attenuated by FK506 in a temperature-dependent manner. Furthermore, dFKBP59 inhibits TRPL-mediated Ca^{2+} influx in fura-2 assays, and produces direct membrane-delimited effects on TRPL single channel activity recorded in excised insideout patches. Together these results support the hypothesis that dFKBP59 is a physiological regulator of TRPL channels. Additionally, the results of mutagenesis studies suggest that the site of interaction of dFKBP59 on TRPL is a unique, highly conserved proline-rich region present in the COOH-terminal domain of TRPL. Thus, dFKBP59 appears to be located in close proximity to the mouth of the channel pore, a position that may facilitate regulation of channel gating.
Drosophila dFKBP59 is a member of the immunophilin family with high degree of similarity to mammalian FK506 binding protein, FKBP52. Although the actual function of FKBP52 in mammalian cells remains largely unknown, it is well established that FKBP12 binds to and regulates the activity of intracellular Ca\textsuperscript{2+} release channels. Specifically, FKBP12 binds to RYR1 and to the Ins(1,4,5)P\textsubscript{3} receptor, whereas FKBP12.6 binds to RYR2. RYR1 and RYR2 are important molecular components of the excitation-contraction coupling machinery in skeletal and cardiac muscle, respectively, although their mechanism of activation during muscle contractions differs. RYR1, present in the terminal cisternae of the sarcoplasmic reticulum (SR), physically interacts with the α-subunit of the voltage-operated Ca\textsuperscript{2+} channel (Ca\textsubscript{v}) present in the transverse-tubule of the plasma membrane. During depolarization, a conformational change in the Ca\textsubscript{v} is transmitted to RYR1, which in turn releases Ca\textsuperscript{2+} from the SR for delivery to the contractile elements. FKBP12 is tightly and stoichiometrically associated with the channel structure and biochemically co-purifies with the ryanodine receptor (51). The binding of FKBP12 to RYR1 has rather dramatic effects on the single channel activity. In the absence of FKBP12, the RYR1 opens more frequently to subconductance levels, whereas in the presence of FKBP12, probability of opening is reduced, but the channel opens to the full conductance state (42). This has led investigators to speculate that FKBP12 is needed for subunit-subunit interaction, allowing for a concerted contribution of the individual subunits of the tetrameric structure to channel conductance, presumably via allosteric mechanisms. Furthermore, recent studies suggest that FKBP12 may also serve to facilitate tetramer-tetramer interactions, thus providing a mechanism for signal propagation from RYR1 tethered to plasmalemmal Ca\textsubscript{v} to adjacent non-tethered ryanodine receptors (52). Recent patch clamp recordings obtained from isolated Drosophila rhabdomereal membrane fragments showed evidence of coordinated gating.
of TRP-dependent channels suggesting that groups of photoreceptor channels open and close in a concerted fashion (53). By analogy to the FKBP12-RYR interaction, dFKBP59 may facilitate communication between TRP and/or TRPL channels which could play a fundamental role in response amplification or propagation.

RYR1 and RYR2 are both activated by an increase in cytosolic Ca\(^{2+}\). Indeed, the primary mechanism of excitation-contraction coupling in cardiac muscle involves Ca\(_v\)-mediated Ca\(^{2+}\) entry and activation of RYR2 via a Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) mechanism. Interestingly, dissociation of FKBP12/12.6 from RYR1/2 increases the sensitivity of the channel to activation by Ca\(^{2+}\) (42,54). The effect of recombinant dFKBP59 on TRPL is similar to the regulation of RYR1/2 by FKBP12/12.6. Co-expression of dFKBP59 with TRPL greatly attenuates activation of TRPL by the rise in Ca\(^{2+}\) that occurs subsequent to thapsigargin treatment as measured in fura-2 assays. Likewise, addition of dFKBP59 to the cytoplasmic membrane surface during single channel recording in excised patches produced a graded reduction in the probability of opening. Thus, the functional effect of dFKBP59 interaction with TRPL has characteristics typical of FKBP-channel interactions.

Immunophilins, which are all peptidyl-prolyl \emph{cis-trans} isomerases, are targets of immunosuppressant drugs, FK506 and rapamycin. Although the isomerase activity apparently is not required for functional effects of FKBP12 on the Ca\(^{2+}\) release channels (50), the active site of FKBP12 interacts with a region of the Ins(1,4,5)P\(_3\) receptor that contains a leucyl-prolyl (LP) dipeptide (48). Recent studies have shown that FKBP12.6 interacts with a region of RYR2 that contains a isoleucyl-prolyl (IP) dipeptide, and FKBP12 interacts with a region of RYR1 that
contains a valyl-prolyl (VP) dipeptide (55). FK506 apparently acts as a prolyl-peptidomimetic and prevents interaction between FKBP12 and the intracellular release channels. (49). Previous studies on FKBP52 have shown that it is composed of 3 tandem FKBP-like domains (40). Likewise, dFKBP59 appears to have a similar structure (Fig 11A). Moreover, of the 12 amino acid residues involved in high-affinity interaction between FKBP12 and FK506 (56), all are conserved within the first and second FKBP-like domains of dFKBP59 (Fig 11A). The degree of conservation in the third FKBP-like domain is less than that seen in the first two domains. Thus, dFKBP59 has at least two highly conserved FK506 binding domains. One of the domains may be necessary for binding of dFKBP59 to TRPL, whereas the other may be involved in the interaction of dFKBP59 with INAD. The presence of multiple FK506 binding domains on dFKBP59 however, raises the intriguing possibility that FK506 may bind to dFKBP59 at low concentrations (i.e., <1 µM) and modulate channel function without displacing dFKBP59 from its binding site on the TRPL channels. Although the effect of FK506 on TRPL channel activity requires further investigation, this would represent a novel mechanism of action for the immunosuppressant drugs which could mediate acute effects of these agents on ion channel activity.

The LP dipeptides important for dFKBP59 binding to TRPL are located in a proline-rich region of the channel protein that is highly conserved in all of the primary TRP homologs (Fig. 12B). Thus, it seems likely that dFKBP59 will interact with Drosophila TRP, TRPL, and TRPγ. Likewise, mammalian FKBP12 or FKBP52 may interact with the primary mammalian TRP homologs (i.e., TRPC1-7). 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 (55). Thus, there appears to be specific structural features of the FKBPs that recognize the conserved change from IP to VP. In this regard, TRPL interacts specifically with dFKBP59, but not with dFKBP12. This result suggests that the individual *Drosophila* and mammalian TRP channels may be regulated by different members of the FKBP family. These predictions, derived from the results of the present study, await further investigation.
FOOTNOTES

Abbreviations Used:

BK-cells, cells heterologously expressing the human B<sub>2</sub> bradykinin receptor

[Ca<sup>2+</sup>]<sub>i</sub>, cytosolic free Ca<sup>2+</sup> concentration

dFKBP59, Drosophila immunophilin p48

EGTA, [ethylenebis(oxyethylenenitrile)]tetraacetic acid

FKBP, FK506 binding protein

GST, glutathione-S-transferase

HEPES, 4,(2-hydroxyethyl)-1-peperazineethanesulfonic acid

INAD, protein responsible for the inactivation-no-after-potential Drosophila mutant

Ins(1,4,5)P<sub>3</sub>, inositol-1,4,5-trisphosphate

MES, 2-[N-morpholino]ethanesulfonic acid

NHERF, Na<sup>+</sup>-H<sup>+</sup> exchange regulatory factor

nPo, number of channels x probability of opening

PLC, phospholipase C

PKC, protein kinase C

RYR, ryanodine-sensitive Ca<sup>2+</sup> release channel

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sf, Spodoptera frugiperda

SOC, store-operate channel

TRP, protein responsible for the transient receptor potential Drosophila mutant

TRPL, Drosophila TRP-like protein

TRPL-cells, cell heterologously expressing TRPL channels
ACKNOWLEDGEMENTS

This work was supported in part by grant GM52019 from General Medicine, National Institutes of Health (WPS), and by a postdoctoral fellowship award 20381B from the American Heart Association, Northeast Ohio Affiliate (MG). We thank Mr. Brian Wisnoskey for providing recombinant baculovirus containing the VR1 receptor, and Dr. William G. Sinkins for helpful discussions.
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*Science* **252**, 839-842
FIGURE LEGENDS

Fig. 1. Amino acid comparison of Drosophila immunophilin (dFKBP59) with rat (ra), mouse (mo) and human (hu) FKBP-52. Protein sequences of dFKBP59, human, mouse and rat FKBP-52 were assembled using Gene Doc program. Identical amino acids are shown in black and similar amino acids are shaded in grey.

Fig. 2. Expression and purification of dFKBP59 using GST and His tags. Panel A. Aliquots of total cell lysates prepared from uninfected Sf9 cells (lane 1, 40µg), Sf9 cells expressing TRPL, (lane 2, 40µg), Sf9 cells expressing the bradykinin receptor (BK; lane 3, 40µg), or from Sf9 cells co-expressing TRPL and dFKBP59 (lane 4, 10µg) or BK and dFKBP59 (Lane 5, 10µg) were subjected to SDS-PAGE and immunoblotted with anti-FKBP antibodies. Panel B, Left. Coomassie stained gel showing the overexpression and purification of GST-dFKBP59 fusion protein using glutathione and Ni²⁺ affinity columns. The last lane shows by silver stain the purified dFKBP59 obtained after cleavage of the fusion protein with Factor Xa and dialysis using a 30K spin filter. Panel B, Right. Purified GST-dFKBP59 fusion protein and Factor Xa-digested protein were subjected to SDS-PAGE and immunoblotted with anti-FKBP antibodies.

Fig. 3. Effect of dFKBP59 on calcium influx via TRPL. Fura-2 loaded Sf9 cells were suspended in MBS. Two traces shown are superimposed in each panel. Panel A. Bradykinin (BK, 50nM) was added at the indicated time to cells expressing the bradykinin receptor (BK alone), or co-expressing BK and dFKBP59 (BK: dFKBP59). Panel B: At the indicated time, thapsigargin (TG, 200nM) was added to the cuvette. Panel C: TG (200nM) was added at the
indicated time to cells expressing TRPL (TRPL alone), or co-expressing TRPL and dFKBP59 (TRPL: dFKBP59).

Fig. 4. **Effect of EGTA on TRPL single channel activity.** *Upper panel.* Single channels were recorded in excised insideout patches from TRPL-cells. The normal bath and pipette solutions contained 100 mM sodium gluconate, 10mM MES (pH 6.5; \([\text{Ca}^{2+}]_{\text{free}} = 1.6 \text{ uM}\)). The average open probability (nPo) binned at 5s intervals is shown as a function of time after seal formation. At the indicated time, the bath solution was changed to one containing EGTA (\([\text{Ca}^{2+}]_{\text{free}} = 10 \text{ nM}\)). *Lower panel.* Histogram showing the mean ± SE nPo before, during, and after changing the bath solution to one containing EGTA (n=12). Individual experiments are shown between the bars.

Fig. 5. **Effect of dFKBP59 on TRPL single channel activity.** Single channels were recorded in excised inside out patches from TRPL-cells. The normal bath and pipette solutions contained 100 mM sodium gluconate, 10mM MES (pH 6.5; \([\text{Ca}^{2+}]_{\text{free}} = 1.6 \text{ uM}\)). The open probability (nPo) binned at 5s intervals is shown as a function of time after seal formation. At the indicated times, the bath solution was changed to one containing EGTA, or different amounts of purified dFKBP59 in normal bath solution. The inset shows mean ± SE nPo, normalized to the value before addition of recombinant dFKBP59, as a function of dFKBP59 concentration in the bath solution (n=3-8 for each).
Fig. 6. **Association of dFKBP59 with TRPL.** *Panel A.* Immunoprecipitations, from total lysates prepared from Sf9 cells co-expressing GST-dFKBP59 and FLAG-TRPL, 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. *Panel B.* Total lysates and immunoprecipitates were prepared from Sf9 cells co-expressing GST-dFKBP59 and VR1.

Fig. 7. **Association of INAD with dFKBP59 and TRPL.** *Panel A.* Immunoprecipitations, from total lysates prepared from Sf9 cells co-expressing GST-dFKBP59 and INAD, 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. *Panel B.* Total lysates and immunoprecipitates were prepared from Sf9 cells co-expressing FLAG-TRPL and INAD. *Panel C.* Total lysates and immunoprecipitates were prepared from cells individually expressing FLAG-TRPL, GST-dFKBP59, and INAD as indicated above each lane. The only protein bands observed are those shown; i.e., no bands at other molecular weights are seen.

Fig. 8. **Association of TRPL with dFKBP59 and InaD in Drosophila heads and S2 cells.** Immunoprecipitations, from total lysates prepared from *Drosophila* heads (*Panel A*) or *Drosophila* S2 cells (*Panel B*), 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. 9. **FKBP immunofluorescence in Drosophila eye.** Thin sections from adult *Drosophila* heads were fixed, labeled, and visualized as described in *Materials and Methods*. **Panel A.** Representative image of a tissue section labeled with anti-FKBP antibodies followed by FITC-conjugated secondary antibody. **Panel B.** Section labeled with secondary antibody only. Identical magnification and camera settings were employed for both images.

Fig. 10. **Effect of FK506 on the interaction between TRPL and dFKBP59.** Total lysates, prepared from Sf9 cells co-expressing FLAG-TRPL and GST-dFKBP59 fusion protein, were incubated at 4°C with FK506 at the concentration shown above each lane, and immunoprecipitated for 12 hr with anti-GST (**Panel A**) or anti-FLAG antibodies (**Panel B**). The precipitated proteins were probed with anti-FLAG or anti-GST antibodies as indicated. **Panel C.** The above lysates were incubated with increasing amounts of FK506 for 3 hr at 20°C followed by immunoprecipitation with anti-FLAG antibodies for 12 hr at 4°C. The precipitated proteins were probed with anti-GST antibodies.

Fig. 11. **dFKBP59 association with the COOH-terminal region of TRPL requires conserved proline residues.** **Panel A.** The COOH-terminal 474 amino acids of TRPL, overexpressed as a GST fusion protein (GST-TRPLC-term), was bound to a glutathione affinity column. Following wash of the column, the bound protein was eluted with buffer containing free glutathione, subjected to SDS-PAGE and immunoblotting with anti-GST antibodies (**left panel**). Total lysates, prepared from Sf9 cells overexpressing dFKBP59 were incubated with GST-TRPLC-term column (**middle panel**) or pretreated with 1 μM FK506 at 20°C before incubating with GST-TRPLC-term column (**right panel**). The retained proteins were eluted with
buffer containing free glutathione, subjected to SDS-PAGE and immunoblotting with anti-FKBP antibodies. Panel B and C. Single amino acid substitutions, P^{702}Q and P^{709}Q, were created in the GST-TRPL_{C-term}. The mutant fusion proteins were bound to a glutathione affinity column, eluted with buffer containing free glutathione, and detected with anti-GST antibodies (left panels). Lysates from Sf9 cells overexpressing dFKBP59 were incubated with GST-TRPL_{P702Q} and GST-TRPL_{P709Q} bound to affinity columns. The retained proteins were eluted with buffer containing free glutathione and analyzed by immunoblotting with anti-FKBP antibodies (right panels). Panel D. Cell lysates and immunoprecipitates were prepared from Sf9 cells co-expressing GST-dFKBP59 and full-length FLAG-TRPL_{P702Q}, FLAG-TRPL_{P709Q}, or FLAG-TRPL-trunc.

Fig. 12. Structural comparison of putative FKBP binding sites and FK506 binding domains. Panel A shows the amino acid comparison of the three FK506 binding domains of dFKBP59 with human FKBP12 and FKBP12.6. Amino acids 1-122 of dFKBP59 comprise the first domain (dFKBP59a), amino acids 123-239 comprise the second domain (dFKBP59b), and 240-439 comprise the third domain (dFKBP59c). Amino acids critical for FK506 binding are shown in black. Panel B shows the proline-rich region of Drosophila and mammalian TRP channels i.e., the putative FKBP binding domain.
Figure 1
Figure 2
Figure 3.
Figure 5
Figure 6
Figure 7
A. Drosophila heads

B. S2 cells

Figure 8
Figure 9

A

Anti-FKBP

B

Secondary antibody only
Figure 10
Figure 11
Figure 12