Activation of M1 Muscarinic Acetylcholine Receptors Stimulates the Formation of a Multiprotein Complex Centered on TRPC6 Channels*

Ju Young Kim and David Saffen‡

From the Graduate Program in Molecular, Cellular, and Developmental Biology and the Departments of Pharmacology and Psychiatry, College of Medicine and Public Health, the Ohio State University Columbus, Ohio 43210

In this study we showed that stimulation of M1 muscarinic acetylcholine receptors (mACHRs) activates endogenous transient receptor potential-canonical, subtype 6 (TRPC6), channels in neuronal PC12D cells. Activation of TRPC6 channels is correlated with the formation of a multiprotein complex containing M1 mACHRs, TRPC6 channels, and protein kinase C (PKC). Formation of the M1 mACHR-TRPC6-PKC complex is transient, with highest levels reached ∼2 min after stimulation of M1 mACHRs. PKC in the complex phosphorylates TRPC6 on a conserved serine residue in the carboxy-terminal domain (Ser768 in the TRPC6A isoform and Ser714 in the TRPC6B isoform). The immunophilin FKBP12, the phosphatase calcineurin, and Ca2+-binding protein calmodulin are also recruited to the M1 mACHR-TRPC6-PKC complex following activation of M1 mACHRs and remain stably associated with the TRPC6 channels after M1 mACHRs and PKC have disassociated. Binding of FKBP12, calcineurin, and calmodulin to TRPC6 channels is blocked by the following: 1) inhibition of PKC; 2) mutation of the PKC phosphorylation site (Ser768 and Ser714 in the complexes); or 3) pretreatment with FK506 or rapamycin, immunosuppressants that directly bind FKBP12. Inhibition of FKBP12 binding blocks the dephosphorylation of TRPC6 channels and the disassociation of M1 mACHRs, without affecting disassociation of PKC. The calcineurin inhibitor cyclosporin A also blocks the dephosphorylation of TRPC6 and prevents the disassociation of M1 mACHRs. Together, these results show that activated TRPC6 channels form the center of a dynamic multiprotein complex that includes PKC and calcineurin, which respectively phosphorylate and dephosphorylate the channels. Phosphorylation of the TRPC6 channels by PKC is required for the binding of FKBP12, which in turn is required for the binding of calcineurin and calmodulin. Subsequent dephosphorylation of the channels by calcineurin is required for the disassociation of M1 mACHRs.

Stimulation of M1 mACHRs activates phospholipase C-β (PLCβ), resulting in increased production of (DAG) and inositol 1,4,5-trisphosphate (IP3) (1). DAG activates protein kinase C (PKC) (2), and IP3 binds to the IP3 receptor (IP3R) channels in the endoplasmic reticulum (ER), causing the channels to open and release stored Ca2+ (1–5). Depletion of these stores activates Ca2+ channels in the plasma membrane, increasing Ca2+ levels in the cytoplasm and facilitating the reuptake of Ca2+ into the ER (6, 7). Ca2+ channels that open in response to the depletion of ER Ca2+ stores are often referred to as store-operated channels (SOCs) (8). In addition to SOCs, there are Ca2+-permeable channels that are activated by Gq-coupled receptors independently of the depletion of ER Ca2+ stores. There is currently wide interest in identifying the molecular components of both types of channels and determining the mechanisms by which they are activated and inhibited.

During the last 10 years, cDNAs encoding seven mammalian homologs of Drosophila transient receptor potential and transient receptor potential-like channels have been isolated by molecular cloning (9, 10). These channels, designated TRPC (transient receptor potential-canonical), subtypes 1–7 (11), are currently the subject of intense investigation for their potential roles as mammalian SOCs and Ca2+ store-independent channels (12). Based upon similarities in amino acid sequences, the mammalian TRPCs can be classified into the following four groups: TRPC1, TRPC2, TRPC4/5, and TRPC3/6/7 (13). The TRPC1 subtype is widely expressed in tissues throughout the body, including heart and brain (14). It has been proposed to contribute to store-operated Ca2+ influx (15, 16), possibly functioning as heterotetrameric channels containing TRPC4 and/or TRPC5 subunits in some tissues (14, 17, 18). TRPC2 channels are not expressed in humans but have been shown to be expressed in the vomeronasal organ of rodents, where they function in sex pheromone signaling (19–21), and in the sperm of mice, where they function in fertilization (22). TRPC4 and -C5 channels are expressed in brain neurons and have variably been described as SOCs or Ca2+ store-independent channels (23). The TRPC3, -C6, and -C7 isoforms form homo- or heterotetrameric (24) channels with each other but not with other TRPC subtypes (18, 25). TRPC3/6/7 channels are activated by Gq protein-coupled receptors independently of Ca2+ stores, possibly by increases in intramembrane DAG (24, 26, 27).

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‡ To whom correspondence should be addressed: Dept. of Pharmacology, College of Medicine and Public Health, 5072C Graves Hall, 333 West 10th Ave., Columbus, OH 43210. Tel.: 614-688-4573; Fax: 614-292-7232; E-mail: saffen.1@osu.edu.

The abbreviations used are: mACHR, muscarinic acetylcholine receptor; PLCβ, phospholipase C-β; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; IP3R, IP3 receptor; ER, endoplasmic reticulum; SOC, store-operated channel; PMA, phorbol 12-myristate 13-acetate; GF109203X, 3-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)-maleimide; FKBP12, FK506 binding protein-12 kDa; PKC, protein kinase C; DMEM, Dulbecco’s modified Eagle’s medium; siRNA, small interfering RNA; RT, reverse transcriptase; EGFP, enhanced green fluorescent protein; Fura-2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2’-amino-5’-methoxyphenoxy)-ethane-N,N,N’,N’-tetraacetic acid.
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This activation does not depend upon PKC, and activation of PKC with phorbol esters inhibits the channels (28, 29). PC12D cells (30) are a rapidly differentiating subline of rat pheochromocytoma-derived PC12 (31). We chose PC12D cells for our studies because they express the M1 subtype of mACHRs, which robustly activate PLCβ and Ca2⁺ influx following exposure to the mACHR agonist carbachol (32). We showed previously that Ca2⁺ influx and PKC activation are the major upstream intracellular events required for the activation of the immediate-early genes zif268 and c-fos in these cells (33). These observations led us to investigate in more detail how M1 mACHRs regulate the influx of extracellular Ca2⁺.

Our preliminary studies revealed that stimulation of M1 mACHRs activates Ca2⁺ influx via both Ca2⁺ store-dependent and -independent pathways.2,3 The Ca2⁺ store-dependent pathway is the major Ca2⁺ influx pathway activated by carbachol and is also activated by thapsigargin, which empties intracellular Ca2⁺ stores by inhibiting the sarcoplasmic/endoplasmic reticulum calcium ATPase pump. This influx pathway is mediated by as-yet-unidentified SOCs that are (i) impermeable to Mn2⁺, (ii) impermeable to Ba2⁺, (iii) not inhibited by PKC, (iv) activated by the DAG analog 1-oleoyl-2-acetyl-sn-glycerol, (v) active for at least 30 min in the continuous presence of carbachol, and (vi) slow to inactivate following inhibition of M1 mACHR with atropine. The SOCs become inactive as the ER Ca2⁺ stores refill. By contrast, the Ca2⁺-store-independent pathway depends upon channels that are (i) impermeable to Mn2⁺, (ii) permeable to Ba2⁺, (iii) potently inhibited by PKC, (iv) activated by 1-oleoyl-2-acetyl-sn-glycerol, (v) active for only 2–3 min in the continuous presence of carbachol, and (vi) rapidly inactivated following inhibition of M1 mACHR with atropine. The Ca2⁺ store-independent channels, but not the SOCs, are blocked in PC12D cells expressing TRPC6 antisense RNA or a TRPC6 amino-terminal peptide, both shown previously (33) to inhibit TRPC6 channels exogenously expressed in COS-7 cells.

The goal of the experiments described in this paper was to elucidate molecular mechanisms by which M1 mACHRs regulate endogenous TRPC6 channels in PC12D cells. These studies show that activation of M1 mACHRs induces the transient formation of a multiprotein complex containing M1 mACHRs, TRPC6, and PKC. The immunophilin FKBP12, the phosphatase calcineurin, and the Ca2⁺-binding protein calmodulin are also recruited into this complex but maintain an association with TRPC6 after M1 mACHRs and PKC dissociate. TRPC6 channels in the complex are sequentially phosphorylated by PKC and dephosphorylated by calcineurin during the first 5 min following activation of M1 mACHRs. Phosphorylation of the channels by PKC is required for the recruitment of FKBP12, calcineurin, and calmodulin to the complex. The recruitment of activated calcineurin is required for channel dephosphorylation and the disassociation of M1 mACHRs. These novel findings provide a new framework for understanding the molecular mechanisms by which TRPC6 channels are regulated.

EXPERIMENTAL PROCEDURES

Materials—Carbamylcholine (carbachol), phorbol 12-myristate 13-acetate (PMA), GF19920X (3-[1-{3-(dimethylamino)propyl}-3-indolyl]-3-indolyl-maleimide), and atropine were purchased from Calbiochem. FK506, cyclosporin A, and rapamycin were obtained from LC Laboratories (Woburn, MA). Rabbit anti-TRPC6 antibodies raised against a 12-amino acid peptide (KQHKKGFPQDEAEKQ) at the carboxyl terminus of rat TRPC6 were purified by affinity chromatography as described previously (33). Antibodies specific for conventional PKC-α, mACHR, and calcineurin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Ser PKC substrate antibodies (specific for phosphoserine residues within proteins containing a hydrophobic amino acid at position +1 and arginine/lysine at positions −2 and/or +2) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-FKBP12 antibodies were from Pharmingen, and anti-calmodulin antibodies were from Upstate Biotechnology (Charlottesville, VA).

Cell Culture—PC12D cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 5% fetal bovine serum, 0.16% glucose, bicarbonate, 50 μg/ml streptomycin, 10 units/ml penicillin, and 45 μg/ml streptomycin at 37 ºC under 5% CO₂ as described previously (32).

Measurement of Ba²⁺ Influx— Fifty to 75% confluent PC12D cells were loaded with Fura-2 AM (Calbiochem; 2 μM in Krebs-Ringer/HEPES (KRH) buffer: 6 mM HEPES-NaOH, 125 mM NaCl, 5 mM KCl, 1.2 mM KHPO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, 2 mM glucose (pH 7.4)) for 90 min at room temperature in the dark. Cells in individual wells were gently washed four times with nominally Ca²⁺-free KRH buffer and immediately used for Ba²⁺ influx measurements. Changes in intracellular fluorescence were measured in individual cells or small groups of cells using a calcium imaging system comprising a Leica DMIRB inverted microscope equipped with a Lambda G-4 xenon light source (175 watts) and Lambda 12-2 filter wheel models (Novo, CA) and Orca-ER CCD camera (Hamamatsu Photonics). Hardware and date collection were performed using OPENLAB 3 imaging software (Improvement, Lexington, MA) running on a Macintosh G4 computer. Fura-2-loaded cells were stimulated with 340 nm light at 0.5-s intervals, and fluorescence was recorded at 510 nm. Data processing and graphing were carried out using Microsoft Excel.

Preparation of siRNAs and Transfection of PC12D Cells—Total cellular RNA from PC12D cells or rat brain was isolated using Trizol reagent (Invitrogen). For each siRNA preparation, 1 μg of purified RNA was treated with DNA-Free (Ambion, Austin, TX) to remove genomic DNA prior to use as a template for cDNA synthesis using the reagents in the SuperScript One-step PCR kit (Invitrogen), according to the directions of the manufacturers. RT and PCR amplifications were carried out in the same tubes using a Bio-Rad Thermocycler. PCR primers were designed to amplify segments of the TRPC2 and TRPC6 cDNAs that lack sequence homology to each other and to rat TRPC1, -3, -4, -5, and -7 cDNAs. A T7 promoter sequence (underlined) 5'-TAATACGACTCACTATAGGGTCTTGTTTCGACTCTAAAGA-3' (C2-C forward primer 2) and primer 1); 5'-ACTCACTATAGGGTCTTGTTTCGACTCTAAAGA-3' (C6-C reverse primer 2). Primers designed to amplify cDNA segments encoding amino-terminal and carboxyl-terminal regions of TRPC6 were as follows: 5'-TAATACGACTCACTATAGGGTTAAAGATGATGCTGTA-3' (C6-N forward primer 1); 5'-TAATACGACTCACTATAGGGTTAAAGATGATGCTGTA-3' (C6-N reverse primer 1); 5'-TAATACGACTCACTATAGGGTTAAAGATGATGCTGTA-3' (C6-C forward primer 2); and 5'-TAATACGACTCACTATAGGGTTAAAGATGATGCTGTA-3' (C6-C reverse primer 2). Reaction mixes contained the following: 1 μl of RT/Platinum Taq Mix, and nuclease-free water. siRNAs were generated from the double-stranded source (175 watts) and Lambda 12-2 filter wheel models (Novo, CA) and Orca-ER CCD camera (Hamamatsu Photonics). Hardware and data collection were performed using OPENLAB 3 imaging software (Improvement, Lexington, MA) running on a Macintosh G4 computer. Fura-2-loaded cells were stimulated with 340 nm light at 0.5-s intervals, and fluorescence was recorded at 510 nm. Data processing and graphing were carried out using Microsoft Excel.

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Plasmid Construction—Rat TRPC6b cDNA lacking a stop codon was obtained by PCR amplification from pEFP-BOS-TRPC6b (33) using the
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forward primer 5'-ATAGACCGCGGGTATGAAAGC-3' and reverse primer 5'-TCTGCGGTTCTTCTGTTGTC-3'. The PCR product was subcloned into pCRII-TOPO vector (Invitrogen). The TRPC6B insert was excised by EcoR I digestion and cloned in EcoRI-digested pCMV-Tag5A (Stratagene), which encodes a "Myc" tag downstream from the EcoRI restriction site. Digestion with HindIII was used to screen for correct insertions of the insert, yielding the pCMV-TRPC6B-myc. The codon for Ser714 was converted to GCT (encoding alanine) using the "QuickChange" site-directed mutagenesis kit (Stratagene) with the forward primer 5'-CCCTTTACATGTGGCCAGTCCCAAAGTGCCGCTTTATCT-3' and reverse primer 5'-GAGGACACGGTGGATAGGAAAG-3'. Briefly, 50 ng of pCMV-TRPC6B-myc was used as template for PCR amplification with 125 ng (each) forward and reverse primers and 2.5 units of Phu Turbo DNA polymerase. The PCR conditions were as follows: 1x (95 °C for 30 s) and 16x (95 °C for 30 s, 55 °C for 1 min, and 68 °C for 8 min). The reaction mix was cooled to 22 °C for 1 min and treated with 10 units of DpnI at 37 °C for 1 h. One μl of DpnI-treated DNA was introduced into transformation-competent DH5-α cells (Invitrogen) and kanamycin-resistant colonies isolated by cultivating on agar plates containing 50 μg/ml kanamycin. Plasmids recovered from the bacteria were screened for the presence of the mutation by DNA sequencing. DNA sequencing of the entire TRPC6B-myc coding segment of pEF-CMV-TRPC6B-SH3 inserted plasmid confirmed that the sequence was identical to the wild-type TRPC6B isoform, except for the Ser714 codon (AGT, which was changed to a codon for alanine (GCT). Transfection of PC12D Cells—siRNAs were transfected into PC12D cells using Oligofectamine Transfection Reagent (Invitrogen), as recommended by the manufacturer. Briefly, cells were seeded to ~50% confluency in Cellstar 6-well tissue culture plates (Greiner Bio-One, Inc., Longwood, FL) were transfected with 0.5 μg of siRNA, 50 ng of pEGFP (enhanced green fluorescent protein expression vector, pEGFP-N2; Clontech). Two or 3 days following transfections, the cells were loaded with Fura-2 as described above. Ba2+ influx measurements were carried out on EGFP-expressing cells identified visually using a fluorescein fluorescence filter cube. Coimmunoprecipitations—Approximately 90–95% confluent cultures of undifferentiated PC12D cells were used for all of the experiments. Cells were transfected with inhibitors or vehicle at 37 °C for 10 h (GF109203X) or 30 min (FK506, cyclosporin A, and rapamycin) prior to stimulation with water or carbachol at room temperature for the indicated times. Cells were then rinsed once with cold phosphate-buffered saline and lysed by addition of 500 μl of lysis buffer (150 mM NaCl, 1.0% Nonidet P-40 (nonoxynolphenoxyethoxylate), 0.5% deoxycholate, 0.1% SDS, 0.1% TriCl (pH 5.0), 2 μM leupeptin, 1 μM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin). The lysed cells were collected in 1.5 ml microcentrifuge tubes and sonicated using a probe sonicator to the manufacturer's instructions. Visualized by enhanced chemiluminescence (ECL kit, Pierce) according to the manufacturer's instructions. RESULTS Activation of M1 mAChRs Activates Endogenous TRPC6 Channels in PC12D Cells—Fig. 1 shows that addition of carbachol to Fura-2-loaded PC12D cells in nominally Ca2+-free KRH buffer causes a transient increase in intracellular fluorescence, correlating with the release of Ca2+ from the ER and its rapid expulsion from the cells. Subsequent addition of 500 μM BaCl2 to the medium results in an increase in Fura-2 fluorescence due to the influx of Ba2+. Ba2+ is not a substrate for plasma membrane Ca2+ pumps and is therefore not expelled from the cells (34, 35). Thus, Ba2+ dependent increases in Fura-2 fluorescence reflect Ba2+ influx and accumulation only and not the balance of influx and efflux (as is the case for Ca2+). The release of Ca2+ from internal stores and Ba2+ influx are both mediated by M1 mAChRs, because both are blocked in cells pretreated with atropine. Based upon reverse transcriptase (RT)-PCR and Western blot analyses, we previously determined that PC12D cells express four TRPC channel subtypes: TRPC1, TRPC3, TRPC6, and TRPC7. The TRPC6 channel expressed in PC12D cells is the TRPC6B isoform, which lacks 54 amino acid residues present in the amino terminus of the TRPC6A isoform (33). Carbachol-stimulated Ba2+ influx in PC12D cells is blocked by exogenous expression of anti-TRPC6 mRNA or the amino-terminal domain of TRPC6B, which may act in a dominant-negative manner to block TRPC6 channel assembly (36, 37). Fig. 2A shows that carbachol-stimulated Ba2+ influx, but not release Ca2+ from ER stores, is blocked in PC12D cells transfected with siRNA specific for rat TRPC6 mRNA. By contrast, transfection of PC12D cells with anti-TRPC2 siRNA does not block carbachol-stimulated Ba2+ influx. Fig. 2B shows that PC12D cells transfected with anti-TRPC6 siRNA are selectively depleted in TRPC6 protein, with no decrease in levels of TRPC5 or TRPC7 protein. By contrast, TRPC6 protein is not depleted in cells transfected with anti-TRPC2 siRNA. Activation of M1 mAChRs Stimulates the Formation of a Complex between the Receptors and TRPC6—To investigate molecular mechanisms underlying the regulation of TRPC6 channels, we explored the possibility that activation of M1 mAChRs induces the formation of a complex that includes the receptors and channels. For this purpose, we examined whether exposure to carbachol allows the M1 mAChRs and TRPC6 to coimmunoprecipitate. Fig. 3A (top set) shows that stimulation of M1 mAChRs with carbachol for 2 min causes TRPC6 channels to coimmunoprecipitate with M1 mAChRs, and that this coimmunoprecipitation is blocked by pretreatment of the cells with atropine. Fig. 3A (bottom set) shows, conversely, that exposure to carbachol causes the M1 mAChRs to coimmunoprecipitate with TRPC6 channels. Again, coimmunoprecipitation is blocked by...
PKC Is Recruited into the M1 mAChR-TRPC6 Complex—DAG released following activation of M1 mAChRs would be expected to activate PKC. Because DAG is presumably generated in the proximity of M1 mAChRs, we investigated whether PKC is also recruited into the M1 mAChR-TRPC6 complex. Fig. 4A shows that PKC commnunoprecipitates with TRPC6 channels (top set) and M1 mAChRs (bottom set) following exposure to carbachol for 2 min. Fig. 4B shows that atropine blocks the formation of the M1 mAChR-PKC complex and causes the previously formed complex to disassociate. Identical results were obtained when anti-TRPC6 or anti-PKC antibodies was used for the initial immunoprecipitation and commnunoprecipitating proteins examined by Western blotting.4

TRPC6 Channels Are Phosphorylated by PKC—Analysis of the rat TRPC6A and -B amino acid sequences using Prosite (us.expasy.org/tools/scanprosite) (38) revealed six potential PKC phosphorylation sites (that are conserved in human TRPC6A) within the predicted cytoplasmic domains of the

Fig. 2. M1 mAChR-mediated stimulation of Ba2+ influx requires the expression of TRPC6 channels. A, Ca2+- and Ba2+-dependent changes in intracellular fluorescence were measured in Fura-2-loaded PC12D cells in nominally Ca2+-free KRH buffer. Cells were transfected with pEGFP or pEGFP plus the indicated siRNAs 2 days prior to exposure to 500 μm carbachol and 500 μm BaCl2, at the times indicated by the arrows. Top pair of traces, PC12D cells were transfected with pEGFP alone (−siRNA-TRPC6) or pEGFP plus siRNA specific for rat TRPC6. Bottom pair of traces, PC12D cells were transfected with pEGFP alone (−siRNA-TRPC2) or pEGFP plus siRNA specific for rat TRPC2. Maximal Ba2+-dependent fluorescence corresponds to ~60% of the maximum fluorescence signal obtained by exposing the cells to 1 μM ionomycin (not shown). The results shown are representative of two independent experiments. B, EGFP-expressing cells that had been transfected with siRNA specific for TRPC6 (left) or TRPC2 (right) were isolated by fluorescence-activated cell sorting and TRPC6, TRPC3, TRPC7, and actin protein levels determined by Western blotting. Bands obtained from a comparable number of unsorted PC12D cells are shown in the lanes on the far right. siRNA-TRPC2 served as a control for nonspecific effects of siRNAs. PC12D cells do not express TRPC2 protein.5 GFP+ cells were isolated 2 days after transfection of siRNAs. The results shown (A and B) are representative of two independent experiments.

FIG. 3. Activation of M1 mAChRs stimulates the formation of a complex containing the receptors and TRPC6 channels; atropine causes the complex to disassociate. A, PC12D cells in nominally Ca2+-free KRH were pretreated with vehicle (−) or 10 μM atropine for 5 min prior to stimulation for 2 min with vehicle (−) or 500 μM carbachol. Immunoprecipitation (IP) with anti-TRPC6 antibodies (top set) or anti-M1 mAChR antibodies (bottom set) and Western blot analysis of the immunoprecipitates were performed as described under “Experimental Procedures.” In the top set, M1 mAChRs that coinmunoprecipitated with TRPC6 channels were detected by probing the transfer membranes with anti-M1 mAChR antibodies (top row). Immunoprecipitation of TRPC6 channels was confirmed by stripping the membranes and reprobing them with anti-TRPC6 antibodies (bottom row). In the bottom set, TRPC6 channels that coinmunoprecipitated with M1 mAChRs were detected by probing the membranes with anti-TRPC6 antibodies (top row). Membranes were then stripped and re-probed with anti-M1 mAChR antibodies (bottom row). The results shown are representative of two independent experiments. Identical results were obtained when PC12D cells in KRH buffer containing 2 mM CaCl2 or in DMEM were exposed to carbachol plus/minus atropine.4 B, PC12D cells in DMEM were treated with 500 μM carbachol for the indicated times. At 40 s the cells were exposed to vehicle (upper set) or 10 μM atropine (bottom set). Cell extracts were prepared in lysis buffer and subjected to immunoprecipitation using anti-M1 mAChR antibodies prior to Western analysis using anti-TRPC6 antibodies (top row in each set). The membranes were then stripped and reprobed with anti-M1 mAChR antibodies (bottom row in each set). The last band in each row is the indicated protein detected in PC12D cell extracts without immunoprecipitation. The results shown (A and B) are representative of two independent experiments.
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PKC Phosphorylates a Conserved Serine Residue Located Near the TRPC6 Channel Carboxyl Terminus—A recent study by Trebak et al. (29) showed that TRPC3 channels are phosphorylated by PKC on Ser712 in the carboxyl-terminal domain. TRPC6A and TRPC6B channels each contain a homologous PKC consensus phosphorylation site, Ser765 in TRPC6A and Ser714 in TRPC6B.

To determine whether the carbachol-stimulated phosphorylation of TRPC6 channels in PC12D cells takes place on these
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Following exposure of TRPC6-myc and TRPC6B-S714A-myc transfected cells to carbachol. Analysis of TRPC6A-S768A-myc yielded similar results.\(^4\) Nearly identical results were also obtained with TRPC6A and -B channels containing glycine in place of Ser\(^{768/714}\).\(^4\) Taken together, these data show that PKC phosphorylates TRPC6 channels on Ser\(^{768/714}\). Because the anti-phospho-Ser PKC substrate antibodies do not recognize phosphothreonine residues, we could not determine whether PKC phosphorylates Thr\(^{628/677}\). Our results show, however, that Ser\(^{768/714}\) is the major site for serine phosphorylation by PKC in the TRPC6A and -B isoforms.

Activation of M1 mAChRs Recruits Immunophilin FKBP12, Calcineurin, and Calmodulin to the M1 mAChR-TRPC6-PKC Complex—A recent study by Sinkins et al. (40) showed that TRPC6 exogenously expressed in Sf9 insect cells or endogenously expressed in rat brain immunoprecipitates with FKBP12 (FK506-binding protein, 12 kDa), an “immunophilin” that is best known as the molecular target of the immunosuppressants FK506 and rapamycin (41).

The cellular functions of FKBP12 are poorly understood, but several studies provide evidence for a role in regulating Ca\(^{2+}\) release from the sarcoplasmic reticulum and endoplasmic reticulum. FKBP12 binds to type 1 ryanodine receptor (RyR1) calcium channels in sarcoplasmic reticulum of striated muscle (42–44) and to the IP\(_R\) Ca\(^{2+}\) channels in the endoplasmic reticulum (45–47), and has been proposed to regulate Ca\(^{2+}\) release through those channels (42–47). FKBP12, an isoform of FKBP12, binds to the type 2 ryanodine receptor (RyR2) of cardiac smooth muscle and regulates its channel properties (48). FKBP12-binding sites in these three channels have been identified, and each contains a conserved leucyl-prolyl (LP) or vanyl-prolyl (VP) dipeptide.

Mutagenesis experiments by Sinkins et al. (40) showed that FKBP12 also binds to the carboxyl-terminal domain of TRPC6A at the amino acid sequence 759LPVPFLNPV767. The LP dipeptide and the Pro at position +9 in this sequence are conserved in TRPC1–C7 and in the TRPC6B isoform. The VP dipeptide is conserved in TRPC3, -C6, and -C7, which binds FKBP12, whereas a isoleucyl-propyl (IP) dipeptide is found at the homologous positions in TRPC1, -C4, and -C5, which bind to a distinct immunophilin, FKBP52 (40). Binding between TRPC6 and FKBP12 is disrupted by low concentrations of FK506 (0.5-3 μM) (40).

To determine whether FKBP12 also associates with TRPC6 in PC12D cells, we performed reciprocal immunoprecipitation assays using antibodies specific for both proteins. The Western blots depicted in Fig. 7, A and B (1st rows), show that immunoprecipitation of FKBP12 with TRPC6 is not observed in unstimulated cells or cells pretreated with atropine but is robustly detected 2 and 10 min following activation of M1 mAChRs with carbachol. Fig. 7, C and D (1st rows), shows that FKBP12 coimmunoprecipitates with M1 mAChRs at 2 min, but not at 10 min, after stimulation of mAChRs with carbachol.

The molecular structure of part of FK506 closely resembles the LP dipeptide. Based upon this resemblance and the fact that the FKBP12-FK506 complex binds calcineurin, Cameron et al. (47) proposed that the FKBP12-LP complex in the IP\(_R\) functions as a binding site for calcineurin. Unlike the FKBP12-FK506 complex, which potently inhibits calcineurin, FKBP12 bound to an LP-containing segment of the IP\(_R\) was proposed to serve as an anchor for calcineurin without inhibiting its phosphatase activity. In fact, calcineurin bound to the FKBP12-IP\(_R\) complex was shown to dephosphorylate PKA-phosphorylated MAP-2 and PKC-phosphorylated IP\(_R\) (46).

Our findings that PKC phosphorylates TRPC6 following M1 mAChR activation combined with the discovery by Sinkins et al. (40) showed that FKBP12 also binds to the carboxyl-terminal domain of TRPC6A at the amino acid sequence 759LPVPFLNPV767. The LP dipeptide and the Pro at position +9 in this sequence are conserved in TRPC1–C7 and in the TRPC6B isoform. The VP dipeptide is conserved in TRPC3, -C6, and -C7, which binds FKBP12, whereas a isoleucyl-propyl (IP) dipeptide is found at the homologous positions in TRPC1, -C4, and -C5, which bind to a distinct immunophilin, FKBP52 (40). Binding between TRPC6 and FKBP12 is disrupted by low concentrations of FK506 (0.5-3 μM) (40).
al. (40) that TRPC6 binds FKBP12 suggested that calcineurin might also be targeted to and dephosphorylate TRPC6 channels. To test this idea, we investigated whether calcineurin is recruited into the M1 mAChr-TRPC6-PKC complex following exposure of the cells to carbachol. Because calcineurin is activated by the binding of the Ca$^{2+}$-bound form of calmodulin, we also investigated whether calmodulin is recruited to the complex. As shown in Fig. 7, A and B (2nd and 3rd rows), no coimmunoprecipitation of calcineurin or calmodulin with TRPC6 is observed in unstimulated cells or cells pretreated with atropine, but robust coimmunoprecipitation is observed 2 and 10 min after activation of M1 mAChRs with carbachol in the absence of atropine. Fig. 7, C and D (2nd and 3rd rows), shows that calcineurin and calmodulin coimmunoprecipitate with M1 mAChRs at 2 min, but not 10 min, after exposure to carbachol.

To determine whether the association of FKBP12, calcineurin, and calmodulin with M1 mAChRs depends upon TRPC6 channels, we examined the coimmunoprecipitation of these proteins with M1 mAChRs in cells transfected with anti-TRPC6 siRNA. As shown in Fig. 8, A and B (1st to 4th rows), depletion of TRPC6 channels prevents carbachol-stimulated association of FKBP12, calcineurin, and calmodulin with M1 mAChRs. Surprisingly, association between PKC and M1 mAChRs was also blocked in anti-TRPC6 siRNA-treated cells (Fig. 8, A and B, 5th row). Fig. 8C shows that TRPC6 protein is not detected in cells pretreated with anti-TRPC6 siRNA. Taken together, these data suggest that TRPC6 channels form the center of the carbachol-stimulated protein complex.

The differences in associations of TRPC6 channels and M1 mAChRs with FKBP12, calcineurin, and calmodulin at 2 and 10 min after exposure to carbachol (Figs. 7 and 8) led us to examine the time course of these associations in more detail. Fig. 9A (top set of blots) shows the time course of association of FKBP12, calcineurin, calmodulin, M1 mAChRs, and PKC with TRPC6 channels during the first 10 min after activation of M1 mAChRs. Although it is not possible to directly compare levels of each protein in the complex (because of likely differences in the affinity between the antibodies used in the Western blot analysis), it is possible to compare times where maximal binding of each protein occurs. Thus, maximal association of M1 mAChRs and PKC with TRPC6 channels occurs ~2 min after the addition of carbachol, a time when levels of coimmunoprecipitating FKBP12, calcineurin, and calmodulin are still increasing. M1 mAChR and PKC levels are significantly reduced or absent at 5 min and undetectable at 10 min, at which time the binding of FKBP12, calmodulin, and calcineurin is still robust. These results suggest that M1 mAChRs and PKC associate with TRPC6 channels at the same time or slightly earlier than FKBP12, calcineurin, and calmodulin and that the later proteins remain in the TRPC6 complex after M1 mAChRs and PKC have disappeared.

As predicted from the results of Sinkins et al. (40), pretreatment of PC12D cells with FK506 blocks the association of FKBP12 with TRPC6 (Fig. 9A, bottom set of blots). FK506 also blocks the association of calcineurin and calmodulin with TRPC6, consistent with the proposed role for the FKBP12-TRPC6 complex as an anchor for calcineurin/calmodulin. By contrast, FK506 does not disrupt the interaction between TRPC6 and PKC. Most significantly, pretreatment with FK506 allows the coimmunoprecipitation of M1 mAChRs and TRPC6 at late (5 and 10 min) time points, suggesting that the binding...
FKBP12 and/or calcineurin is required for the release of M1 mAChRs from the complex. Nearly identical results were obtained in experiments examining the coimmunoprecipitation of the FKBP12, calcineurin, and calmodulin with TRPC6 is stable for at least 10 min after stimulation of M1 mAChRs; FK506 blocks the association of FKBP12, calcineurin, and calmodulin with TRPC6. PC12D cells in DMEM culture medium were pretreated with 0.1% Me2SO (top sets) or 1 µM FK506 (bottom sets) for 30 min and stimulated with water (−) or 500 µM carbachol for the indicated times prior to preparation of cell extracts and immunoprecipitation (IP) with anti-TRPC6 (A), M1 mAChRs (B), PKC (C), or calcineurin (D) antibodies. Coimmunoprecipitating proteins were analyzed by Western blotting. The last band in each row is the indicated protein detected in PC12D cell extracts, without immunoprecipitation. The results shown in A–D are representative of two independent experiments.

Calcineurin Dephosphorylates TRPC6 Channels—As mentioned above, calcineurin that is presumably anchored to FKBP12 on the IP3R was found to dephosphorylate IP3Rs previously phosphorylated by PKC (46, 47). If calcineurin recruited to the M1 mAChR-TRPC6-PKC complex plays a role in the dephosphorylation of TRPC6 by PKC, pretreatment with calcineurin inhibitors would be expected to prolong TRPC6 phosphorylation. The experiments depicted in Fig. 10 show that this is the case. Pretreatment of PC12D cells with FK506, which binds FKBP12 to form a potent calcineurin inhibitor, prevents the dephosphorylation of TRPC6 channels, which usually occurs 3–10 min after activation of M1 mAChR with carbachol (Fig. 10, A and B). Similarly, pretreatment with cyclosporin A, which binds ubiquitously expressed cyclophilin to form a potent calcineurin inhibitor (49, 50), also blocks the...
dephosphorylation of PKC-phosphorylated TRPC6 (Fig. 10C). By contrast to FK506, the immunosuppressant rapamycin binds to FKBP12, but the resulting complex does not inhibit calcineurin (51, 52). Pretreatment of the cells with rapamycin, however, is also effective in preventing the dephosphorylation of PKC-phosphorylated TRPC6 (Fig. 10D). This result suggests that efficient dephosphorylation of the TRPC6 channels requires not only the activation of calcineurin but also the targeting of activated calcineurin to FKBP12 in the M1 mAChR-TRPC6-PKC complex.

To obtain additional insights into the actions of cyclosporin A and rapamycin, we examined the effects of these inhibitors on the formation of the multiprotein complexes. The results depicted in Fig. 11A show that pretreatment of the cells with cyclosporin A prevents the recruitment of calcineurin and calmodulin to the M1 mAChR-TRPC6-PKC complex without interfering with the recruitment of FKBP12 (Fig. 11A, 1st to 3rd rows). As observed for FK506, cyclosporin A also blocks the dissociation of M1 mAChRs and TRPC6 (Fig. 11A, 4th row), without changing the transient association between PKC and TRPC6 (Fig. 11A, 5th row).

Rapamycin is similar to FK506 in that it blocks the recruitment of FKBP12, calcineurin, and calmodulin (Fig. 10B, 1st to 3rd rows). It also blocks the dissociation of M1 mAChR and TRPC6 without affecting the transient association of PKC with TRPC6 (Fig. 11B, 1st to 5th rows). Taken together, these results suggest that recruitment of activated calcineurin to the M1 mAChR-PKC complex is required for the dephosphorylation of PKC-phosphorylated TRPC6 and release of M1 mAChRs from the complex.

**FIG. 10.** Dephosphorylation of TRPC6 channels is blocked by inhibitors of calcineurin or FKBP12 binding. PC12D cells in DMEM culture medium were pretreated with 0.1% Me2SO (A), 1 μM cyclosporin A (C), or 1 μM rapamycin (D) for 30 min and stimulated with water (−) or 500 μM carbachol for the indicated times prior to preparation of cell extracts and immunoprecipitation (IP) with antibodies specific for TRPC6. Western blots were probed with anti-phospho-Ser PKC substrate antibodies (top rows), after which the membranes were stripped and reprobed with anti-TRPC6 antibodies (bottom rows). The last band in each row is the indicated protein detected in extracts of carbachol-treated PC12D cells. The results shown in A–D are representative of two independent experiments.

**FIG. 11.** Cyclosporin A blocks the binding of calcineurin and calmodulin to the TRPC6 complex but not the binding of FKBP12; rapamycin blocks the binding of FKBP12, calcineurin, and calmodulin to the complex; both cyclosporin A and rapamycin prevent the dissociation of M1 mAChRs from TRPC6 channels. PC12D cells in DMEM culture medium were pretreated with 0.1% Me2SO (−), 1 μM cyclosporin A (A) or 1 μM rapamycin (B) for 30 min and stimulated with water (−) or carbachol for the indicated times. TRPC6 was immunoprecipitated (IP) from cell extracts, and coimmunoprecipitating proteins were detected by Western blotting. The last band in each row is the indicated protein detected in PC12D cell extracts. The results shown in A and B are representative of two independent experiments.

**PKC Phosphorylation of TRPC6 Channels Is Required for Binding of FKBP12, Calcineurin, and Calmodulin—** Fig. 12A (1st to 3rd rows) shows that inhibiting PKC with GF109203X blocks the coimmunoprecipitation of FKBP12, calcineurin, and calmodulin with the TRPC6 channels. Similar to FK506, GF109203X has no effect on coimmunoprecipitation of M1 mAChRs and TRPC6 channels at 2 min but inhibits the dissociation of the M1 mAChRs and the channels normally seen at 10 min (Fig. 12A, 4th row). GF109203X has no effect on the association of TRPC6 channels and PKC (Fig. 12A, 5th row). Fig. 12, B and C, shows that coimmunoprecipitation of FKBP12, calcineurin, and calmodulin with TRPC6B channels is blocked when Ser714 is replaced with alanine. TRPC6B-S714A-myc channels also maintain an association with M1 mAChRs at 10 min after addition of carbachol, even in the absence of pretreatment with GF109203X (compare 4th rows in Fig. 12, B and C). Nearly identical results to those shown in Fig. 12C were obtained with TRPC6A and -B channels containing glycine in place of Ser714.4 These observations, together with the results described above, imply that dissociation of M1 mAChRs from TRPC6 channels requires three sequential events as follows: 1) phosphorylation of TRPC6 channels on Ser708/714 by PKC, 2) binding of FKBP12 to a site immediately adjacent to Ser708/714, and 3) dephosphorylation of Ser708/714 by calcineurin targeted to the TRPC6/FKBP12 anchor.

**DISCUSSION**

In this paper we describe a multiprotein complex containing M1 mAChRs, TRPC6 channels, PKC, immunophilin FKBP12, calcineurin, and calmodulin that forms following the activation of M1 mAChRs in PC12D cells. To our knowledge, this is the
first study to show that TRPC6 channels physically associate with M1 mAChRs and the first to show that the channels are phosphorylated by PKC and dephosphorylated by calcineurin. Our working model for the regulation of TRPC6 channels is depicted in Fig. 13.

Activation of M1 mAChRs in PC12D cells stimulates a rapid and robust influx of extracellular Ba\(^{2+}\) (Fig. 1), which we have shown here (Fig. 2) and elsewhere\(^3\) to be mediated by TRPC6 channels. Stimulation of M1 mAChRs form a complex with TRPC6 channels and PKC. The formation of this complex correlates with the onset of carbachol-stimulated Ca\(^{2+}\) influx (Ba\(^{2+}\) was used as a surrogate for Ca\(^{2+}\) in our experiments). Both carbachol-stimulated complex formation and Ba\(^{2+}\) influx are blocked by the mAChR antagonist atropine. 3) PKC phosphorylates TRPC6A/B channels on serine (768/714); phosphorylation is blocked by the PKC inhibitor GF109203X. 4) Immunophilin FKBP12 binds to the TRPC6A/B channels at a site immediately adjacent to the phosphoserine (768/714), creating a site for the binding of calcineurin/calmodulin. 5) Calcineurin/ calmodulin dephosphorylates phosphoserine (768/714) and binds to the FKBP12-TRPC6 complex. (It is unclear whether dephosphorylation of TRPC6A/B takes place before or after the binding of calcineurin/calmodulin to FKBP12/TRPC6.) Dephosphorylation of TRPC6A/B correlates with the dissociation of M1 mAChRs from TRPC6 channels. Dissociation of PKC from TRPC6 channels is not dependent upon the dephosphorylation of the TRPC6 channels. FK506 blocks the binding of FKBP12 to the TRPC6 channels and inhibits calcineurin. Rapamycin blocks the binding of FKBP12 to TRPC6 channels, without inhibiting calcineurin. Cyclosporin A inhibits calcineurin, without blocking the binding of FKBP12 to TRPC6 channels.

Addition of carbachol to the cells stimulates the formation of a complex between M1 mAChRs and TRPC6 channels with a time course that parallels the activation of Ba\(^{2+}\) influx (Fig. 3). The formation of this complex depends upon the continuous stimulation of M1 mAChRs and is reversible, because addition of atropine rapidly blocks the formation of new complexes and disrupts previously formed complexes (Fig. 3). Atropine also rapidly blocks carbachol-stimulated Ba\(^{2+}\) influx.

Activation of M1 mAChRs recruits PKC to the M1 mAChR-TRPC6 complex with a time course identical to that for M1 mAChRs and TRPC6 channels (Fig. 4). This was somewhat surprising because activation of PKC is correlated with inactivation of TRPC6 channels (28, 29, 33). To reconcile these two observations, we hypothesize that DAG produced by stimulation of M1 mAChRs activates TRPC6 channels more rapidly than DAG-activated PKC can inhibit the channels. A similar bimodal regulation by DAG has been proposed recently to explain the transient activation of exogenously expressed TRPC3...
channels following stimulation of mACHRs in HEK293 cells (28).

By using antibodies that specifically recognize phosphoserine residues in PKC substrates, we demonstrated that TRPC6 channels are phosphorylated following exposure to phorbol ester or carbachol (Fig. 5). Phosphorylation of TRPC6 channels is detected as early as 20 s after activation of M1 mACHRs, peaks at about 2 min, and then declines to undetectable levels by 5 min. These observations are consistent with a recent report showing that PKC phosphorylates TRPC3 channels on a conserved serine residue, Ser714, in the carboxyl-terminal domain (29). Substitution of alanine for Ser714 in the terminal domain (29). Substitution of alanine for Ser714 in the terminal domain (29). Substitution of alanine for Ser714 in the terminal domain (29).

Phosphorylation of TRPC6 channels by PKC is correlated with channel inhibition. Evidence for this includes the following observations: 1) TRPC6 channels are inhibited in PC12D cells exposed to 100 nM PMA; and 2) this inhibition is blocked by pretreating the cells with 2 μM GF109203X. Also, Myc-tagged TRPC6B-S714A channels exogenously expressed in COS-7 cells are activated normally following stimulation of M1 mACHRs but are not inhibited in cells pretreated with 100 nM PMA. By contrast, Myc-tagged TRPC6B wild-type channels are completely inhibited by pretreatment with PMA. In addition to inhibition of the channels by PKC, there must also be PKC-independent mechanisms by which TRPC6 channels are inactivated. Evidence for this includes the observation that pretreating the cells with GF109302X does not increase the rate or extent of carbachol-stimulated Ba2+ influx. Instead, similar or slightly reduced rates of Ba2+ influx are observed in GF109203X-pretreated cells. As discussed below, a possible PKC-independent mechanism for the inactivation of TRPC6 channels is Ca2+-dependent binding of calmodulin to the carboxyl terminus, as proposed by Zhu and co-workers (54, 55).

The recent report by Sinkins et al. (40) showing that TRPC6 channels bind FKBP12 led us to investigate whether this immunophilin is a component of the M1 mAChr-TRPC6-PKC complex. Figs. 7–9 show that FKBP12 does not associate with TRPC6 channels prior to activation of M1 mAChRs but rapidly binds to the M1 mAChr-TRPC6-PKC complex following exposure to carbachol. FKBP12 enters the complex at the same time or slightly later than M1 mAChRs and PKC and remains after M1 mAChRs and PKC have disassociated. In addition to FKBP12, calcineurin and calmodulin are also recruited into the M1 mAChr-TRPC6-PKC complex with a similar time course (Figs. 7–9). Formation of a complex between these proteins and M1 mAChRs is dependent upon TRPC6, because no association is observed in TRPC6-depleted cells (Fig. 8). As predicted by the model of Cameron and co-workers (47), the association of calcineurin and calmodulin with TRPC6 channels is dependent upon the binding of FKBP12, because their binding is blocked by FK506 (Fig. 9).

Our findings differ from those of Sinkins et al. (40) in that we observe coimmunoprecipitation of FKBP12 and TRPC6 channels only after activation of M1 mAChRs, whereas they found FKBP12 and the channels to be constitutively associated. This difference may reflect differences in our experimental systems. Whereas we studied endogenous TRPC6 and FKBP12 in PC12D cells, Sinkins et al. (40) examined exogenously expressed TRPC6 channels and FKBP12 in Sf9 cells. It is possible that high levels of exogenously expressed proteins favor association in the absence of TRPC6 channel activation. Sinkins et al. (40) also showed that FKBP12 and TRPC6 channels coimmunoprecipitate from rat brain lysates. It is possible that the immunoprecipitated FKBP12 from brain was bound to a subset of activated TRPC6 channels. A third possibility is that factors controlling the binding of FKBP12 to TRPC6 channels are different in different types of cells.

As mentioned above, the observation that FK506 blocks the binding of FKBP12 to the TRPC6 channels is reminiscent of the FK506-sensitive binding of FKBP12 to IP3Rs (45–47). Based upon the resemblance between FKBP12/FK506 and FKBP12 bound to a conserved LP dipeptide in the IP3Rs, Cameron et al. (47) proposed that the binding of FKBP12 creates a docking site for calcineurin on the IP3Rs and that calcineurin anchored at this site dephosphorylates IP3Rs that had been phosphorylated by PKC. This model is controversial, however, and alternative schemes have been proposed (56). Because our data seemed to fit the model of Cameron et al. (47), we decided to investigate whether calcineurin targeted to TRPC6/FKBP12 can dephosphorylate TRPC6 channels that had been phosphorylated by PKC.

As predicted by the model by Cameron et al., dephosphorylation of TRPC6 channels, which is usually observed 3–10 min after activation of M1 mAChRs, is blocked by the calcineurin inhibitors FK506 and cyclosporin A (Fig. 10). Interpretation of the effects of FK506 is complicated, however, by the fact that FK506 inhibits both the phosphatase activity of calcineurin and the binding of FKBP12 to TRPC6 channels (Fig. 9). By contrast, the calcineurin inhibitor cyclosporin A does not block the binding of FKBP12 to the channels (Fig. 11A). The fact that cyclosporin A blocks the dephosphorylation of PKC-phosphorylated TRPC6 channels therefore provides strong evidence for the involvement of calcineurin. Significantly, dephosphorylation of TRPC6 channels is also blocked by the rapamycin, which blocks the binding of FKBP12 to TRPC6 channels (Fig. 11B) but does not inhibit calcineurin (51, 52). The observation that rapamycin blocks the dephosphorylation of TRPC6 channels suggests that the dephosphorylation of TRPC6 channels requires not only calcineurin activation but also the targeting of the activated calcineurin to the channels.

Because calcineurin is activated by binding the Ca2+-bound form of calmodulin, we investigated whether calmodulin is recruited to the TRPC6-centered protein complex. As expected, calmodulin enters the complex with the same time course as calcineurin (Fig. 9, A–D). Binding of both proteins to TRPC6 channels is also blocked by FK506 (Fig. 9), rapamycin (Fig. 11B), GF109203X (Fig. 12A), and the S714A mutation (Fig. 12B and C), each of which blocks the binding of FKBP12. Calmodulin coimmunoprecipitates with FKBP12 and calcineurin in the presence of FK506, consistent with the binding of FKBP12/FK506 to the activated (i.e. calmodulin-bound) form of calcineurin (Fig. 9D). Taken together, these results show that calmodulin and calcineurin bind to TRPC6 in a correlated manner, and the association of both proteins with TRPC6 is dependent on the binding of FKBP12.

By using in vitro binding assays and patch clamp recordings, Zhu and co-workers (54, 55) showed that calmodulin can regulate TRPC3 and TRPC4 channel activity by directly binding to a specific site, the CIRB (calmodulin/IP3 receptor binding) domain, located in the carboxyl-terminal domain of each channel. The site of calmodulin binding in the CIRB domain overlaps with the binding site for IP3Rs, leading the authors to propose that calmodulin modulates binding of IP3Rs to TRPC channels. Specifically, Zhu and co-workers (54, 55) hypothesize that activation of TRPC channels involves the displacement of loosely bound (“tethered”) calmodulin by an amino-terminal segment of the IP3Rs. Influx of Ca2+ then allows calmodulin to bind tightly to the TRPC channels, preventing further activation by IP3Rs.
As described above, we did not observe the coimmunoprecipitation of calmodulin with TRPC6 prior to activation of M1 mACHRs, and binding of calmodulin after activation was dependent upon the presence of FKBP12 in the complex. Our immunoprecipitation data, however, are not necessarily in conflict with the model of Zhu and co-workers (54, 55). Weakly bound calmodulin may have been displaced from the TRPC6 under our immunoprecipitation conditions, and tightly bound calmodulin may be difficult to detect using our antibodies. Thus, although our results do not provide direct support for the direct binding of calmodulin to TRPC6, they also do not exclude this possibility. Because the FKBP12-binding site, YQKIMKRLIKRYVLQAQID872 (54), TRPC6 channels could accommodate the binding of calmodulin at both sites. As mentioned above, the Ca\(^{2+}\)-dependent binding of calmodulin to the carboxyl-terminal segment of TRPC6 is an attractive mechanism for PKC-independent inactivation of the channel.

The observation that inhibition of PKC or substitution of alanine for Ser\(^{714}\) in TRPC6B blocks the binding of FKBP12, calcineurin, and calmodulin to the channels (Fig. 12, A–C) suggests that phosphorylation of this conserved serine, which is located immediately adjacent to the FKBP12-binding site, is required for the binding of FKBP12. We are currently attempting to directly test the effects of PKC phosphorylation of Ser\(^{768/714}\) on FKBP12 binding to TRPC6 channels using purified proteins.

The observation that dissociation of M1 mACHRs from TRPC6 channels is inhibited by blocking PKC phosphorylation of the channels with GF109203X or by substitution of alanine for Ser\(^{768/714}\) (Fig. 12, A–C) is probably related to the requirement for phosphorylation of Ser\(^{768/714}\) for the binding of FKBP12, i.e. phosphorylation of the channels by PKC is required for the binding of FKBP12, and the binding of FKBP12 is required for targeting calcineurin to the complex. As described for calcineurin-mediated dephosphorylation of the TRPC6 channels, release of M1 mACHRs from the complex requires both the activation of calcineurin by calmodulin and the targeting of the activated calcineurin to the complex. The requirement for activated calcineurin is shown by the fact that the calcineurin inhibitor cyclosporin A blocks the dissociation of M1 mACHRs from TRPC6 channels without blocking the binding of FKBP12 (Fig. 11A). The requirement for targeting activated calcineurin to the complex is shown by the fact that rapamycin blocks the dissociation of M1 mACHRs (Fig. 11B). We are currently attempting to determine whether dephosphorylation of TRPC6 channels is required for the dissociation of M1 mACHRs.

Taken together, the results obtained in this study are consistent with the following sequence of events (Fig. 13). 1) Activation of M1 mACHRs stimulates the formation of a multiprotein complex containing the M1 mACHRs, TRPC6 channels, and PKC. 2) PKC phosphorylates the channels on a conserved serine located adjacent to the FKBP12-binding site. 3) FKBP12 binds to the phosphorylated channels. 4) Activated calcineurin/calmodulin binds to the FKBP12/TRPC6 anchor, either before or after calcineurin dephosphorylates the channels. 5) Dephosphorylation of the channels (or perhaps another protein in the complex) by calcineurin triggers the release of M1 mACHRs from the TRPC6 channels.

This study provides several new insights into the molecular rearrangements and covalent modifications undergone by TRPC6 channels following their activation. Additional studies will be required to determine whether the multiprotein complex detected by coimmunoprecipitation results from direct interactions between the various protein components or depends upon indirect interactions through an as-yet-unidentified molecular scaffold. The *Drosophila* transient receptor potential channels in the rhabdome of the photoreceptor cells of the eye are known to be linked to PLC and PKC via the molecular scaffold INAD (57–59). TRPC1 channels have recently been shown to function within a molecular complex containing PLC\(\beta\), Go\(^{q11}\), IP\(_R\), calmodulin, caveolin, and the molecular adapter homer (60–63). TRPC4 and TRPC5 channels have been shown to bind the molecular scaffold Na\(^{+}/H^+\) exchanger regulatory factor (53), which may be considered one of the functional equivalents of INAD in mammals. Na\(^{+}/H^+\) exchanger regulatory factor does not bind TRPC6 channels, however, and alternative candidate scaffolding proteins have not yet been described.

Many more experiments will be required to understand the molecular mechanisms by which TRPC6 channels are regulated and the physiological significance of this regulation. The present study shows, however, that a satisfactory explanation for channel activation and inhibition must take into account the roles of multiprotein complex formation and channel phosphorylation and dephosphorylation.

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