Receptor-mediated Regulation of the Nonselective Cation Channels
TRPC4 and TRPC5*

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Mammalian transient receptor potential channels (TRPCs) form a family of Ca2+-permeable cation channels currently consisting of seven members, TRPC1–TRPC7. These channels have been proposed to be molecular correlates for capacitative Ca2+ entry channels. There are only a few studies on the regulation and properties of the subfamily consisting of TRPC4 and TRPC5, and there are contradictory reports concerning the possible role of intracellular Ca2+ store depletion in channel activation. We therefore investigated the regulatory and biophysical properties of murine TRPC4 and TRPC5 (mTRPC4/5) heterologously expressed in human embryonic kidney cells. Activation of Gq/11-coupled receptors or receptor tyrosine kinases induced Mn2+ entry in fura-2-loaded mTRPC4/5-expressing cells. Accordingly, in whole-cell recordings, stimulation of Gq/11-coupled receptors evoked large, nonselective cation currents, an effect mimicked by infusion of guanosine 5′-O-(thio)triphosphate (GTPγS). However, depletion of intracellular Ca2+ stores failed to activate mTRPC4/5. In inside-out patches, single channels with conductances of *42 and 66 picosiemens at ~60 mV for mTRPC4 and mTRPC5, respectively, were stimulated by GTPγS in a membrane-confined manner. Thus, mTRPC4 and mTRPC5 form nonselective cation channels that integrate signaling pathways from G-protein-coupled receptors and receptor tyrosine kinases independently of store depletion. Furthermore, the biophysical properties of mTRPC4/5 are inconsistent with those of 1Ca2+, the most extensively characterized store-operated current.

Mammalian homologues of the Drosophila cation channel TRP form a novel gene family within the superfamily of cation channels with six transmembrane segments (1). A first hint as to how mammalian TRPCs could be regulated came from the Drosophila visual signaling cascade. In Drosophila rhabdomeres, illumination is followed by G-protein-mediated activation of phospholipase C (PLC) and results in the stimulation of a light-induced current that is generated by TRP and TRPL (TRP-like) cation channels (2–4). The light-induced current may integrate several functions, including (i) depolarization of the membrane and generation of a receptor potential, (ii) augmentation of Ca2+-dependent signaling processes, and (iii) replenishment of internal calcium stores. Thus, mammalian homologues may serve similar functions.

In mammalian cells, activation of PLCs by extracellular signaling molecules leads to the production of inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol and couples to intracellular signaling cascades by increasing the cytosolic Ca2+ concentration (Ca2+). These changes in Ca2+, result from InsP3-mediated Ca2+ release from intracellular stores and/or Ca2+ entry from the extracellular space. Ca2+ entry may be regulated by different mechanisms. In many cell types, depletion of intracellular Ca2+ stores following Ca2+ release or inhibition of Ca2+ uptake leads to Ca2+ entry from the extracellular space, a process often called capacitative calcium entry that is mediated by store-operated channels (5, 6). The best characterized current through store-operated channels is the calcium release-activated Ca2+ current (I(CRAC)), but other, less Ca2+-selective channels have also been described (7). Other Ca2+ entry mechanisms downstream of receptor activation are mediated by second messengers, but independently of store depletion.

There is good agreement that TRPCs are activated downstream of G-protein-coupled receptors, which induce PLC-mediated phosphoinositide breakdown. However, the downstream signaling pathways that finally activate TRPCs remain highly controversial. For nearly all of the functionally expressed TRPCs, there is at least one report proposing a store-operated mechanism of activation (1, 8–13). On the other hand, there is growing evidence for the involvement of store-independent pathways in the regulation of TRPC3 (14–17), TRPC5 (18), TRPC6 (17, 19), and TRPC7 (20). For TRPL, TRPC3, and TRPC6, direct activators have been identified that stimulate the channels in a membrane-confined manner. Polysaturated fatty acids were shown to gate Drosophila TRPL (21) and diacylglycerols to activate TRP3, TRPC6, and TRPC7 (20).

Phylogenetic analysis of TRPCs revealed two subfamilies, namely the TRPC3/6/7 subfamily and the TRPC4/5 subfamily. From the close structural relationship of TRPC4 and TRPC5, similar regulatory and biophysical properties might be expected. Indeed, both bovine TRPC4 and rabbit TRPC5 have been reported to form store-operated, relatively Ca2+-selective channels with an inwardly rectifying current-voltage relation (9, 10). Despite relatively large whole-cell currents in cells

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expressing TRPC4, single-channel openings could not be resolved in cell-attached patches, suggesting that the single-channel conductance might be low (9). Similarly, rat TRPC4 has been reported to mediate store-operated Ca\(^{2+}\) entry when expressed in Xenopus oocytes (22). In contrast, however, mouse TRPC5 has been convincingly shown to be activated following receptor stimulation, but not by store depletion (18). Thus, the available data on TRPC4/5 cannot be integrated into a consistent model of signaling pathways leading to activation of these channels.

To study the activation mechanism and biophysical properties of TRPC4 and TRPC5, we cloned murine TRPC4 and TRPC5 (mTRPC4/5) and functionally expressed them in human embryonic kidney (HEK) cells. We provide evidence that mTRPC4 and mTRPC5 are nonselective cation channels regulated independently of the filling state of intracellular Ca\(^{2+}\) stores. Signaling pathways of G-protein-coupled receptors and receptor tyrosine kinases converge to stimulate mTRPC4/5, presumably downstream of phospholipases C. The regulatory and biophysical properties indicate that mTRPC4 and mTRPC5 form a novel subfamily of receptor-stimulated, nonselective cation channels predominantly expressed in neuronal tissues.

**Experimental Procedures**

**Molecular Biology—**TRPC4 and TRPC5 were isolated from mouse brain total RNA by specifically primed reverse transcription (SuperScript II, Life Technologies, Inc.) and polymerase chain reaction (Expand-HF, Roche Molecular Biochemicals, Mannheim, Germany). The primers for polymerase chain reaction were 5′-AGATGGCTGTCCTTTATCAGCA (mTRPC4 sense), 5′-CAACGGTAGAAGCAAGGACAG (mTRPC4 reverse), 5′-AACCTGGCCAGCTTACTAAAG (mTRPC5 sense), and 5′-GATACCGGGGATGGATTAG (mTRPC5 reverse). The amplified cDNAs were cloned in a eukaryotic expression vector (PCMV5, Clontech, Palo Alto, CA). Several clones containing the open reading frames of mTRPC4 or mTRPC5 were functionally expressed by intranuclear microinjection in CHO-K1 cells. Positive clones were selected for their ability to enhance Mn\(^{2+}\) entry in fura-2-loaded cells when stimulated with 100 μM ATP. By sequencing the positive clones, mTRPC5-4 was confirmed to be the wild-type mTRPC5. Two different sets of primers for mTRPC4 and for some exons of mTRPC5 were used (NdeI and XhoI cutting site by polymerase chain reaction-based primer), allowing the sequence determination. Sequencing was done on an ABI Prism 377 DNA sequencer.

**Electrophysiology**—HEK 293 cells (American Type Culture Collection, Manassas VA) were maintained according to the supplier’s recommendations. Cells were transiently transfected with a transfection reagent (Fugene 6, Roche Molecular Biochemicals) and subcultured on glass coverslips. The cDNA (4 μg) of the respective channel or the expression vector without insert was supplemented with 40–120 ng of pEGFP-C1 (CLONTECH, Palo Alto, CA). For experiments on excised patches, cells were seeded on poly-l-lysine-coated coverslips. The experiments were performed on HEK cells 2–3 days after transfection.

**Fluorescence Imaging—**Transfected HEK cells were loaded for 30 min with fura-2/AM (2–4 μM; Molecular Probes, Inc., Eugene, OR) in HEPES-buffered saline (pH 7.4) containing 135 mM NaCl, 6 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 5.5 mM glucose, 10 mM HEPES, and 0.2% (w/v) bovine serum albumin. Coverslips were mounted in a monochromator-equipped (PolyChrome II, TILL-Photonics, Martinsried, Germany) inverted microscope (Axiovert 100, Carl Zeiss, Göttingen, Germany). Fluorescence was recorded with a 12-bit CCD camera (IMAQ, TILL-Photonics). The fluorescence of fura-2 and eGFP was excited at 340, 358, 380, and 480 nm and filtered through a 516-nm long-pass filter. Fura-2 was not excited at 480 nm. However, eGFP contributed to fluorescence excited at 340–380 nm. By transfecting low amounts of reporter cDNA, eGFP-derived fluorescence was minimized. An additional control experiment was performed: the fluorescence of eGFP relative to the intensity at the 480-nm excitation wavelength was 9.3% at 380 nm, 4.5% at 358 nm, and 1.4% at 340 nm. After subtraction of background signals, eGFP-derived fluorescence at 340–380 nm was estimated from the fluorescence intensities at 480 nm and subtracted from the respective data sets. R\(_{\text{max}}\), R\(_{\text{min}}\), and F\(_{\text{max}}\) and F\(_{\text{min}}\) were determined by equilibrating fura-2-loaded HEK cells in HEPES-buffered saline containing 10 μM ionomycin and either 10 mM Ca\(^{2+}\) or 10 mM EGTA. Intracellular Ca\(^{2+}\) concentrations were calculated as described (23). GFP fusion proteins were visualized with an LSM510 confocal laser scanning microscope (Carl Zeiss) and a Plan-Apochromat 63×1.4 objective. eGFP was excited with the 488-nm line of an argon laser. Emitted light was filtered through a 505-nm long-pass filter, and pinhole held optical sections were set to 1 μm.

**Statistical Methods—**In each imaging experiment, data of 30–80 individual cells were sampled. After averaging the data of each single experiment, the means of five to eight independent experiments were calculated and expressed as mean ± S.E. The kinetics of fura-2 quench by Mn\(^{2+}\) was quantified after averaging the changes in F\(_{\text{380}\text{max}}\) over 5-s intervals and expressed as percentages over time. Mean basal and stimulated [Ca\(^{2+}\)]\(_{\text{I}}\), as well as quench of fura-2 fluorescence were compared by Student’s t test of unpaired data. Significance was accepted at p < 0.05. Mn\(^{2+}\) quench experiments in which thapsigargin was applied, the intra-assay S.D. is indicated by vertical lines on the mean values of F\(_{\text{380}\text{max}}\) and [Ca\(^{2+}\)]\(_{\text{I}}\).

**Electrophysiological Techniques—**For whole-cell experiments, the standard extracellular solution contained 140 mM NaCl, 5 mM CaCl\(_2\), 2 mM MgCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). For Na-′-free solutions, Na\(^{+}\) was replaced by N-methyl-D-glucamine (NMDG\(^{+}\)). For Ca\(^{2+}\)-free solutions, Ca\(^{2+}\) was replaced by 1 mM MgCl\(_2\). The standard intracellular solution contained 110 mM cesium methanesulfonate, 25 mM CsCl, 2 mM MgCl\(_2\), 0.362 mM CaCl\(_2\), 1 mM EGTA, and 30 mM HEPES (pH 7.2 with CsOH) with a calculated [Ca\(^{2+}\)]\(_{\text{I}}\) of 0.1 μM. Some experiments were performed in 10 mM Ca\(^{2+}\) solutions with a higher Ca\(^{2+}\) buffer capacity (3.62 mM CaCl\(_2\) and 10 mM EGTA) were used. For experiments with 1 and 10 mM Ca\(^{2+}\), the solutions contained 0.851 mM CaCl\(_2\) and 1 mM EGTA, and 0.998 mM CaCl\(_2\) and 1 mM HEDTA, respectively. Whole-cell recordings were made with an EPC-7 amplifier using Pulse software (HEKA, Lambrecht, Germany).

**Supplementary Materials—**The patch pipettes, made of borosilicate glass, had resistances of 3–5 MΩ when filled with a standard intracellular solution. Cells were held at a potential of ~60 mV, and current-voltage (I–V) relations were obtained from voltage ramps from −100 to +100 mV with a duration of 400 ms applied at a frequency of 0.2 Hz. Ramp data were acquired at a frequency of 4 kHz after filtering at 1 kHz. The holding current was acquired at 30 Hz. Series resistance compensation of ≥50% was used in most experiments. Fluuctuation analysis was done as described (24). Briefly, the single-channel current (i) and total number of channels in the patch (N) were estimated by fitting the equation \( i \sim \frac{1}{N} \sqrt{V} \) to plots of current variance (\( \sigma^2 \)) against mean current amplitude (\( \langle I \rangle \)). Single-channel experiments were performed as described previously (17). Bath solutions (pH 7.4) contained 120 mM sodium isethionate, 2 mM magnesium gluconate, 0.575 mM calcium gluconate, 1 mM EGTA, 10 mM glucose, and 10 mM HEPES (solution B); 120 mM CsCl, 0.62 mM calcium gluconate, 2 mM HEDTA, and 20 mM HEPES (solution B2); and 120 mM CsCl, 2 mM magnesium gluconate, 1.87 mM calcium gluconate, 2 mM EGTA, and 20 mM HEPES (solution B3). The pipette solution (pH 7.4) contained 120 mM CsCl, 1 mM magnesium gluconate, 1.8 mM calcium gluconate, and 10 mM HEPES (solution P1). All solutions were adjusted to 290–310 mosmol/liter with mannitol. In experiments with 10 mM ruthenium red in the pipette solution, the ground electrode was connected to the bath through an agar bridge. Unless otherwise indicated, single-channel data were filtered at 2.5 kHz and analyzed with pCLAMP7 software (Axon Instruments, Inc., Foster City, CA). Channel activity is expressed as NP (the product of the number (N) of channels in the patch and the open probability (P\(_{\text{open}}\)) calculated for consecutive 5-s periods. For analysis of mean open time,
currents were filtered at 5 kHz and sampled at 50 kHz. Openings with durations shorter than 0.1 ms were excluded from the analysis.

Chemicals—Carbachol, thapsigargin, ionomycin, GTP$\gamma$S, heparin, arachidonic acid, linoleic acid, linolenic acid, DL-threo-dihydrosphingosine, Triton X-100-reduced form, indomethacin, and wortmannin were from Sigma (Deisenhofen, Germany). Diacylglycerols, monoacylglycerols, inositol phosphates, phorbol esters, U73122, U73343, RHC-80267, staurosporine, genistein, and bisindolylmaleimide I were from Calbiochem (Bad Soden, Germany).

RESULTS

Subcellular Localization of mTRPC4 and mTRPC5 Fused to Green Fluorescent Protein—To visualize the subcellular localization of the presumptive cation channels in living HEK cells, cDNA constructs encoding mTRPC4, mTRPC5, and hTRPC6 C-terminally fused to eGFP were generated. All fusion proteins were functional channels with regulatory and biophysical properties indistinguishable from the respective channels without eGFP (data not shown). The subcellular distribution of mTRPC4-eGFP and mTRPC5-eGFP was followed by confocal fluorescence microscopy and compared with that of hTRPC6-eGFP. The most prominent mTRPC4-eGFP and hTRPC6-eGFP signals were found in the plasma membrane, whereas a considerable amount of mTRPC5-eGFP was retained in a perinuclear compartment, probably the Golgi apparatus (Fig. 1E). In the plasma membrane, mTRPC4-eGFP and mTRPC5-eGFP showed a clustered appearance, thus contrasting sharply with the homogeneous membrane expression pattern of hTRPC6-eGFP (Fig. 1C, F, and I). In HEK cells, a similar punctate distribution pattern has been described for correctly targeted L-type calcium channels (25).

Ca$^{2+}$ Signaling and Mn$^{2+}$ Entry in HEK Cells—The effects of mTRPC4/5 expression on [Ca$^{2+}$], were studied by fluorescence imaging of fura-2-loaded cells transiently transfected with cDNAs encoding mTRPC4 and mTRPC5 or, as a control, with the empty expression vector (pcDNA3). In the presence of extracellular Ca$^{2+}$ (1 mM), the mean basal [Ca$^{2+}$] was higher in mTRPC4-expressing (82 ± 40 nM) and mTRPC5-expressing (124 ± 30 nM) cells than in pcDNA3-transfected control cells (70 ± 4 nM). The difference between control and channel-expressing cells was largest and statistically significant for mTRPC5-expressing cells (Fig. 2, A-C). The mean basal [Ca$^{2+}$] in eGFP-negative cells was 60–95 nM in all experiments. The elevated basal [Ca$^{2+}$] in mTRPC4/5-expressing cells rapidly decreased to below 60 nM when external Ca$^{2+}$ was replaced by 1 mM EGTA. Re-addition of external Ca$^{2+}$ was followed by a minor rise in [Ca$^{2+}$] in untransfected or pcDNA3-transfected cells, but resulted in a larger increase in mTRPC4-expressing (Fig. 3) and mTRPC5-expressing (data not shown) cells. The effect of receptor activation on mTRPC4/5 was investigated in HEK cells by stimulation of the endogenous muscarinic acetylcholine receptor with carbachol (100 μM). In pcDNA3-transfected control cells, [Ca$^{2+}$] transients reached peak values of 242 ± 20 nM (315 cells in $n = 6$ independent experiments). In mTRPC4- and mTRPC5-transfected cells, peak values of [Ca$^{2+}$] after carbachol treatment were 733 ± 121 nM (357 cells, $n = 7$) and 607 ± 40 nM (315 cells, $n = 5$), respectively.

To discriminate between Ca$^{2+}$ release from intracellular stores and Ca$^{2+}$ entry from the external medium, the quench of fura-2 fluorescence by extracellularly applied Mn$^{2+}$ was monitored at the isosbestic wavelength. Following the addition of
Mn$^{2+}$ to the bath, a significant acceleration of the basal decrease in fura-2 fluorescence was observed only in mTRPC5-expressing cells ($-0.1 \pm 0.03\%/s$, $n = 6$ independent experiments), but not in pcDNA3-transfected ($-0.02 \pm 0.02\%/s$, $n = 5$) or mTRPC4-transfected ($-0.04 \pm 0.01\%/s$, $n = 8$) cells. The addition of carbachol resulted in an immediate decrease in fura-2 fluorescence in mTRPC4-expressing ($-4.6 \pm 0.3\%/s$, 308 cells, $n = 8$) and mTRPC5-expressing ($-2.5 \pm 0.6\%/s$, 278 cells, $n = 6$) cells, but not in control cells ($-0.16 \pm 0.08\%/s$, 334 cells, $n = 5$) (Fig. 2, D-F). At a higher temporal resolution, a significant stimulation of Mn$^{2+}$ influx in mTRPC4-expressing cells was detectable within 50–200 ms after the initial increase in [Ca$^{2+}$], (data not shown). The kinetics of Mn$^{2+}$ influx indicated a transient increase in membrane permeability. Increases in [Ca$^{2+}$], and Mn$^{2+}$ influx like those obtained in HEK cells were also observed in CHO-K1 cells expressing mTRPC4 and mTRPC5 following activation of a coexpressed histamine H$_1$ receptor or an endogenous P2Y purinoreceptor.

Because some TRPCs have been reported to mediate capacitative Ca$^{2+}$ entry, we tested whether mTRPC4 and mTRPC5 are regulated by the filling state of intracellular Ca$^{2+}$ stores. Passive depletion of internal Ca$^{2+}$ stores by thapsigargin (2.5 $\mu$M) strongly reduced or abolished carbachol-induced [Ca$^{2+}$]$_{i}$ transients in control cells in the presence (Figs. 3 and 4A) and absence (data not shown) of extracellular Ca$^{2+}$. Re-addition of Ca$^{2+}$ after store depletion resulted in larger increases in [Ca$^{2+}$]$_{i}$ in mTRPC4-expressing cells than in pcDNA3-transfected control cells (Fig. 3). However, the same cells already displayed higher recalcification signals prior to thapsigargin...
EGF or carbachol. Other blockers that did not affect the stimulation of mTRPC4/5 by carbachol include the sphingosine kinase inhibitor dihydrospingosine (30 \(\mu M\)), the serine/threonine kinase inhibitors staurosporine (10 \(\mu M\)) and bisindolylmaleimide I (1 \(\mu M\)), the tyrosine kinase inhibitor genistein (10 \(\mu M\)), the phosphatase inhibitor sodium orthovanadate (1 \(mM\)), the phosphatidylinositol 3-kinase inhibitor wortmannin (10 \(\mu M\)), the cyclooxygenase and phospholipase A2 inhibitor indomethacin (up to 300 \(\mu M\)), and the diacylglycerol kinase inhibitor RHC-80267 (50 \(\mu M\)). The membrane-permeant protein kinase C activators phorbol 12-myristate 13-acetate (1–10 \(\mu M\)), phorbol didecanoate (1–10 \(\mu M\)), oleoylactetyl-sn-glycerol (up to 300 \(\mu M\)), dioctanoylglycerol (up to 100 \(\mu M\)), and 1-monooenoyl-rac-glycerol (300 \(\mu M\)) did not activate mTRPC4/5. Because polyunsaturated fatty acids activate Drosophila TRPL (20), we tested the effects of arachidonic, linoleic, linolenic, and oleic acids on mTRPC4/5. At concentrations up to 300 \(\mu M\), none of these compounds stimulated \(\text{Mn}^{2+}\) entry through mTRPC4/5 or prevented the channels from being activated by carbachol. A minor (<2-fold) and delayed acceleration of \(\text{Mn}^{2+}\) entry into pcDNA3-transfected or mTRPC4/5-expressing cells was promoted by arachidonic acid and may reflect another non-capacitative \(\text{Ca}^{2+}\) entry mechanism recently described for HEK cells (26).

Currents Activated by Agonists of \(G_{q/11}\)-coupled Receptors—In whole-cell recordings, the holding current at −60 mV immediately following patch rupture in HEK cells expressing mTRPC4 (−1.4 ± 0.3 pA/pF, \(n = 13\); \(C_m = 13.8 ± 0.4 \text{ pF}\)) was similar to that in control cells (−0.8 ± 0.2 pA/pF, \(n = 7\); \(C_m = 10.8 ± 0.6 \text{ pF}\)). In contrast, many cells expressing mTRPC5 exhibited larger (−4.9 ± 0.7 pA/pF, \(n = 10\); \(C_m = 13.7 ± 0.9 \text{ pF}\)), noisy, NMDG−-sensitive currents on break-in with an I−V relation of a form characteristic for activated mTRPC5 (see below). These data, together with the increased basal [\(\text{Ca}^{2+}\)]\(_i\) and \(\text{Mn}^{2+}\) influx in TRPC5-expressing cells, suggest that mTRPC5 displays more basal activity than mTRPC4.

The activation of either endogenous muscarinic receptors or coexpressed histamine \(H_1\) receptors by the respective addition of carbachol or histamine (100 \(\mu M\)) to the extracellular solution resulted in the rapid, transient activation of an inward current at a holding potential of −60 mV in mTRPC4- or mTRPC5-expressing cells (Fig. 6, A and B). In some pcDNA3-transfected control cells, a small inward current (−3.8 ± 1.5 pA/pF, \(n = 7\); \(C_m = 10.8 ± 0.6 \text{ pF}\)) was activated by carbachol. However, this did not resemble the currents of mTRPC4 and mTRPC5. In cells expressing mTRPC4 or mTRPC5, the current amplitudes were highly variable, but reached peak values of several nanoamperes in many experiments (Fig. 6E). At −60 mV, the mean peak inward current densities in response to carbachol (100 \(\mu M\)) were −232.0 ± 75.3 pA/pF (\(n = 13\); \(C_m = 13.8 ± 0.4 \text{ pF}\)) and −228.1 ± 57.1 pA/pF (\(n = 10\); \(C_m = 13.7 ± 0.9 \text{ pF}\)) for mTRPC4- and mTRPC5-expressing cells, respectively. Exchange of the extracellular solution for a Na+−free (NMDG+−) or Na+−Ca2+-free solution (NMDG+ and EGTA) almost completely abolished the inward current. The I−V relations of the agonist-induced currents had a characteristic doubly rectifying form and displayed reversal potentials close to 0 mV (Fig. 6, C and D). Currents with identical properties were measured in CHO-K1 cells expressing mTRPC4/5 after activation of coexpressed histamine receptors.

Effects of Store Depletion Protocols and Inositol Phosphates—To test for a role of intracellular store depletion in the activation of mTRPC4/5, protocols were used that have previously been shown to activate store-operated channels (27). Infusion of InsP_3 (10−100 \(\mu M\)) in a Ca2+-free 10 mM EGTA pipette solution resulted in no increase in holding current in cells.
expressing mTRPC4 (n = 11) and a slight, slow increase in current at −60 mV (approximately a doubling over 5 min) in most mTRPC5-transfected cells showing spontaneous activity (n = 7). Similar results were obtained with InsP3 in solutions containing 100 nM free Ca2+ buffered with 1 (n = 3 and 7 for mTRPC4 and mTRPC5, respectively) or 10 (n = 7 for TRPC4/5) mM EGTA (Fig. 7A). In Jurkat T lymphocytes, infusion of the latter solution fully activated ICrAC within 30–60 s after patch rupture (data not shown). Slow increases in holding current in mTRPC5-expressing cells showing spontaneous activity were also observed using the same pipette solutions without InsP3. After 5 min of InsP3 infusion, the effect of agonist application on whole-cell currents was tested. No response to carbachol or histamine was observed in six cells expressing mTRPC4 using a Ca2+-free intracellular solution with 10 mM EGTA. With the same solution in mTRPC5-expressing cells, three out of seven cells responded to agonist. In a solution with 10 mM EGTA and 100 nM free Ca2+, three out of seven cells expressing mTRPC4 and four out of seven cells expressing mTRPC5 responded. With 1 mM EGTA and 100 nM free Ca2+, three out of three cells expressing mTRPC4 and six out of seven cells expressing mTRPC5 responded to agonist application. The addition of thapsigargin (1 μM) to the extracellular solution during whole-cell recording did not result in an activation of mTRPC4 (n = 4) or mTRPC5 (n = 5) with an intracellular solution containing 10 mM EGTA and no Ca2+ (Fig. 7B). In cells showing spontaneous

**Fig. 6.** Carbachol induces transient, NMDG+-sensitive currents in mTRPC4- and mTRPC5-expressing cells. A, shown are whole-cell currents of mTRPC4-transfected HEK cells recorded at a holding potential of −60 mV. The addition of carbachol (CCh; 100 μM) to the extracellular solution resulted in the transient activation of large inward currents that were completely abolished in Na+/Ca2+-free (NMDG+) extracellular solution. B, the same experiment as in A was performed on mTRPC5-expressing cells. C and D, shown are the responses to voltage ramps from −100 to 100 mV applied at the start of whole-cell recordings and close to the maximum inward current in A and B. E, shown are the whole-cell current densities at −60 mV following break-in and at the peak of carbachol-stimulated currents for all control (pcDNA3-transfected) cells and mTRPC4- and mTRPC5-expressing cells.

 Effects of GTPγS Infusion on Whole-cell Currents—To determine whether activation of G-proteins can stimulate mTRPC4/5, GTPγS (500 μM) was added to the intracellular solution. With various delays following GTPγS infusion, a slow, transient stimulation of mTRPC4/5 immediately following break-in in many cells. In cells that later responded to carbachol, the responses to carbachol were 5–94-fold larger than those to Ca2+ (n = 7 for both channels). In two cells with 10 μM Ca2+, however, larger, rapid responses, like those to carbachol or GTPγS (see below), were observed. Similar results to those obtained with 1 or 10 μM Ca2+ alone were obtained after infusion of InsP3 in 10 μM Ca2+ (n = 5 for both channels).

**Fig. 7.** Depletion of intracellular Ca2+ stores fails to activate mTRPC4 and mTRPC5. A, infusion of InsP3 does not activate mTRPC4/5. Currents were recorded from mTRPC4- and mTRPC5-expressing cells during infusion of InsP3 (10 μM) through the patch pipette. The pipette solution had a calculated free Ca2+ concentration of 100 mM buffered with EGTA (10 mM). B, effects of thapsigargin (1 μM) on currents from mTRPC4- and mTRPC5-expressing cells. Currents were recorded at −60 mV using a pipette solution without InsP3. Arrows indicate the break-in to the whole-cell mode.
A and B, currents recorded at -60 mV during GTPγS stimulation (500 μM) in cells expressing mTRPC4 and mTRPC5, respectively. Exchange of the extracellular solution for a Na⁺/Ca²⁺-free solution (NMDG⁺) during the falling phase abolished the inward current. C and D, I-V relations obtained during voltage ramps from -100 to +100 mV following break-in and at the peak of GTPγS stimulation for the experiments in A and B. E and F, I-V relations during GTPγS stimulation recorded in a 140 mM Na⁺ and Ca²⁺-free solution (140 Na⁺) and a 10 mM Ca²⁺ and Na⁺-free solution (10 Ca⁺) for cells expressing mTRPC4 and mTRPC5, respectively.

![Graphs showing current-voltage (I-V) relations during GTPγS stimulation for mTRPC4 and mTRPC5.](image)

and mTRPC5 close to maximum GTPγS stimulation (Fig. 8, C and D) were indistinguishable from those obtained during receptor stimulation of the channel currents (Fig. 6, C and D). For mTRPC4, the current decayed rapidly and completely, whereas for mTRPC5, decay was incomplete, and a very low level of activity was maintained. Like receptor-activated currents, GTPγS-activated inward currents were abolished in Na⁺-free or Na⁺/Ca²⁺-free bathing solutions. Current activation by GTPγS was associated with a clear increase in current noise. Using fluctuation analysis, the estimated values of single-channel currents at -60 mV were -1.6 ± 0.1 pA for mTRPC4 and -1.8 ± 0.2 pA for mTRPC5 (n = 4 for both channels).

Because currents following GTPγS stimulation decayed less rapidly than agonist-induced currents, we determined the ion selectivity under these conditions. Reversal potentials were estimated from current ramps during the decaying phase of the current. The extracellular solution was first changed from the standard solution to a Na⁺-containing, Ca²⁺-free solution and then to a Ca²⁺-containing (10 mM) Na⁺-free (replaced by NMDG⁺) solution. The mean reversal potentials were -1.5 ± 1.5 mV in Na⁺ alone and -39.7 ± 3.3 mV in 10 Ca²⁺-containing, Na⁺-free solution (n = 4) for mTRPC4 and -3.3 ± 4.1 and -29.2 ± 5.3 mV (n = 5) for the respective solutions recorded for mTRPC5 (Fig. 8, E and F). From the shifts of the reversal potentials, the P_Ca/P_Na values calculated using the Goldman equation for divalent and monovalent cations (29) were 1.05 for mTRPC4 and 1.79 for mTRPC5.

The activation of mTRPC4/5 by GTPγS was dependent on extracellular Ca²⁺. No clear activation of mTRPC4 was observed during GTPγS infusion in a Ca²⁺-free extracellular medium. The addition of Ca²⁺ to the extracellular solution after 5 min of GTPγS infusion resulted in a small increase in current with an I-V shape typical for mTRPC4. In contrast, infusion of GTPγS into mTRPC5-expressing cells resulted in a clear, but small activation of current in the absence of extracellular Ca²⁺. Upon re-addition of Ca²⁺ after 5 min of GTPγS infusion, a large increase in current was observed. Interestingly, a similar result was reported for mTRPC5 following agonist activation (18). Raising the extracellular Ca²⁺ concentration from 2 to 10 mM (by replacing Na⁺) close to the peak of current activation also potentiated the current response in mTRPC4/5-expressing cells.

One characteristic biophysical feature of I_{CRAC} is a specific block by low micromolar concentrations of La³⁺. A surprising finding was that 100 μM La³⁺ did not inhibit, but, like 10 mM Ca²⁺, even potentiated GTPγS-induced currents through mTRPC4/5 without changing the reversal potential. The addition of La³⁺ to the extracellular solution after the peak of inward current led to large, rapid, and reversible current increases. Analysis of voltage ramps indicated that the current at both positive and negative membrane potentials was increased by La³⁺. Likewise, carbacol-induced Mn²⁺ entry through mTRPC4 and mTRPC5 in fura-2-loaded HEK cells was not impaired by La³⁺ at concentrations up to 300 μM.

**Activation and Properties of mTRPC4/5 in Membrane Patches**—Since fluctuation analysis suggested that unitary currents through mTRPC4/5 should be resolvable, we looked for their presence in membrane patches. In cell-attached patches from HEK cells cotransfected with the histamine H₁ receptor and mTRPC4, little channel activity was observed prior to agonist application, whereas some activity was observed in patches from mTRPC5-expressing cells (Fig. 9A). The addition of histamine (80 μM) to the extracellular solution resulted in transient 3–50-fold increases in channel activity in six out of eight patches from mTRPC4-expressing cells (Fig. 9A). For mTRPC5, seven out of nine patches responded to histamine with a 10–200-fold increase in activity (Fig. 9A). No currents were observed in control cells transfected with pcDNA3 and the histamine H₁ receptor.

The properties of the unitary events were studied in detail in inside-out membrane patches. Following patch excision from mTRPC4- and mTRPC5-expressing cells, single-channel events with a low frequency of opening were observed at a patch potential of -60 mV. The addition of GTPγS (10 μM) to the internal membrane surface resulted in large increases in N_p (Fig. 9B) in 23 out of 29 patches for mTRPC4 (5–300-fold) and in 28 out of 32 patches for mTRPC5 (10–1000-fold). The stimulatory effect of GTPγS, although strongest in the first minutes after application, was maintained for the length of the recording (Fig. 9B). In all 14 patches isolated from pcDNA3-transfected cells, GTPγS failed to activate single-channel events. Raising the [Ca²⁺] from 100 nM to 10 μM at the internal face of the membrane had a stimulatory effect in the absence of GTPγS (up to 50-fold; n = 5 for each channel). Under symmetrical buffer conditions (solution B3), the single-channel I-V relations for mTRPC4 and mTRPC5 closely resembled those for whole-cell currents, showing a doubly rectifying shape and a reversal potential close to 0 mV (Fig. 9C). The single-channel chord conductances at -60 mV were 41 ± 1 (n = 10) and 63 ± 1 (n = 7) picosiemens for mTRPC4 and mTRPC5, respectively. In an attempt to explain the doubly rectifying I-V relation, we tested the possibility that Mg²⁺ blocks the channel. In symmetrical Mg²⁺-free solutions (solution B2), inward and outward currents through mTRPC4 and mTRPC5 channels were increased, and the I-V relation displayed slight outward rectification (Fig. 9D). In the absence of Mg²⁺, the chord conductances at -60 mV were 55 ± 2 (n = 9) and 88 ± 2 (n =...
FIG. 9. Stimulation and characterization of unitary currents through mTRPC4/5. A, currents in cell-attached patches of mTRPC4- and mTRPC5-expressing cells cotransfected with the histamine H4 receptor. Histamine (80 µM) was applied to the bathing solution. Recording was done at a pipette potential of +80 mV, and channel activity is expressed as NPo, averaged over 5-s intervals. B, currents recorded in inside-out patches of mTRPC4- and mTRPC5-expressing cells (solutions P1 and B1). GTPγS (10 µM) was applied to the bath as indicated. The insets show current traces before (1) and after (2) the addition of GTPγS. C and D, I-V relations obtained by changing the patch potential from −90 to +90 mV in steps of 20 mV or by applying continuous voltage ramps from −90 to +90 mV to patches prestimulated with GTPγS (10 µM) in symmetrical solutions with 2 mM Mg2+ (solution B3; C) or stimulated with Ca2+ (1 µM) in the absence of Mg2+ (solution B2; D). For steady potentials, mean single-channel current amplitudes of mTRPC4 and mTRPC5 were calculated from the current amplitude histograms. Note the different scaling of the currents. Examples of current traces at patch potentials of −60 and +60 mV are given in the upper left insets. In the lower right insets, superimposed current traces (n = 4–8) during application of continuous voltage ramps from −90 to +90 mV are shown.

PLC activation is a necessary step. Carbachol-induced channel activation is prevented by the phospholipase C inhibitor U73122, and channels are activated following EGF receptor stimulation. The signaling pathways downstream of EGF receptor tyrosine kinase include phosphoinositide breakdown via PLCγ independent of heterotrimeric G-proteins. We conclude that mTRPC4 and mTRPC5 are dually activated via PLCβ or PLCγ. Since both signaling cascades converge at the level of phosphoinositide breakdown, it is likely that activation of mTRPC4/5 depends on this process. However, the steps leading to channel activation following PLC activation remain unclear. Consistent with a PLC-dependent (30, 31), but InsP3 receptor-independent (32) activation of light-induced current in the Drosohila rhabdomere, our results indicate that InsP3 is not involved in the pathways leading to activation of mTRPC4/5. None of the protocols used to deplete Ca2+ stores resulted in increased Mn2+ entry or activated cation currents. The sphingosine kinase inhibitor dl-threo-dihydrosphingosine was shown to block ICRAC in rat basophilic leukemia cells (33). Involvement of second messenger formation by sphingosine kinase was ruled out for mTRPC4/5 by the inability of dl-threo-dihydrosphingosine to prevent channel activation.

The lack of involvement of store depletion in the activation of TRPC4/5 is in conflict with a number of reports. Studies on
bovine and rat TRPC4 and rabbit TRPC5 describe an activation by depletion of intracellular Ca\(^{2+}\) stores with thapsigargin or InsP\(_3\) infusion (9, 10, 22, 34). However, receptor stimulation was not tested in these studies. In contrast, our data on the regulatory properties of both mTRPC4 and mTRPC5 showed activation following stimulation of G-protein-coupled receptors, but not after store depletion. In addition, InsP\(_3\) infusion was without effect in cells expressing mTRPC4 or mTRPC5. Thus, channel gating by conformational coupling to InsP\(_3\) receptors as suggested for TRPC3 (11) is unlikely for mTRPC4/5. Okada et al. (18) demonstrated that, after passively depleting the internal Ca\(^{2+}\) stores with thapsigargin, recalcification did not increase Ca\(^{2+}\) influx in mTRPC5-expressing cells compared with untransfected control cells. We confirmed the store-independent activation of mTRPC5 with the exception that mTRPC5-overexpressing cells displayed a higher basal Ca\(^{2+}\) influx than control cells. A potentiation of [Ca\(^{2+}\)]\(_i\) transients after re-addition of external Ca\(^{2+}\) to thapsigargin-treated cells does not necessarily support a store-dependent mechanism of activation. It may also reflect basal activity of a Ca\(^{2+}\)-permeable cation channel.

The properties of bovine TRPC4 have been reported to resemble those of store-operated channels like I\(_{\text{CRAC}}\) (9, 35). These include regulation by store depletion, a high selectivity for Ca\(^{2+}\), and an inwardly rectifying I-V relation. The inability to resolve single-channel events, despite relatively large whole-cell currents, was taken as an indication that the single-channel conductance might be low (9), like that of I\(_{\text{CRAC}}\) (35). In contrast, the present study shows that mTRPC4 and mTRPC5 form cation channels with similar permeabilities for Na\(^{+}\), Ca\(^{2+}\), and Ca\(^{2+}\) and respective single-channel conductances of 41 and 63 picoamperes in the presence of Mg\(^{2+}\). The I-V relations for both whole-cell and single-channel currents have a characteristic doubly rectifying shape and reversal potentials close to 0 mV. The conductance and the I-V relation shape of single-channel currents depended on the extra- and intracellular Mg\(^{2+}\) concentrations. In the absence of Mg\(^{2+}\) on both sides of the membrane, double rectification was lost, and both channels showed outward rectification and higher chord conductances. Unitary currents through channels mediating I\(_{\text{CRAC}}\) were observed only in the absence of extracellular divalent cations (36), whereas mTRPC4 and mTRPC5 were highly conductive also in the presence of divalent cations. Moreover, I\(_{\text{CRAC}}\) is strongly inhibited by La\(^{3+}\) at low micromolar concentrations, whereas currents through mTRPC4/5 were potentiated by La\(^{3+}\) at concentrations up to 1 mM. Thus, the regulatory and biophysical hallmarks of mTRPC4/5 are clearly different from those of I\(_{\text{CRAC}}\).

The reasons for the marked differences in regulatory and biophysical properties between our data and those obtained with bovine TRPC4 (9) and rabbit TRPC5 (10) remain unclear. As clones were obtained from different species, there are variations in the primary sequences that might explain the obvious differences in functional properties. Most strikingly, sequence alignments of murine, bovine, and rat TRPC4 reveal a gap in the primary sequence of the rat orthologue (GenBank\textsuperscript{TM}/EBI Data Bank accession number AB008889) that comprises the entire predicted second transmembrane segment. It remains to be clarified whether the deletion of this segment affects correct folding and the functional integrity of the protein. In addition, multiple frameshift mutations in the submitted sequence of rat TRPC4 as compared with murine and bovine TRPC4 make the results of functional characterization highly questionable. Nevertheless, rat TRPC4 was functionally characterized as a store-operated channel by expression in Xenopus oocytes (22).

The TRPC3/6/7 subfamily of the TRPCs has been reported to be activated subsequent to PLC stimulation, either as a result of InsP\(_3\)-mediated signaling cascades (11, 12) or by the lipid second messenger diacylglycerol (17, 20). In contrast to TRPC3 and TRPC6, no stimulation by diacylglycerols was evident for mTRPC4/5 (17), suggesting a different regulatory mechanism. Besides the differential regulation by diacylglycerols, TRPC3/6 and TRPC4/5 may be discriminated by biophysical differences. Openings of hTRPC3/6 were short, making open levels difficult to resolve. In contrast, longer open events were evident with mTRPC4/5. Furthermore, mTRPC4 and mTRPC5 were less selective for Ca\(^{2+}\) over Na\(^{+}\) than hTRPC3/6.

Both mTRPC4 and mTRPC5 are expressed in distinct areas of the brain (10, 18, 37). Although nonselective cation channels have been described in neurons of the regions where mTRPC4 and mTRPC5 are localized, their biophysical characterization is insufficient to allow a direct comparison with mTRPC4/5. Surprisingly, a current in native tissues with the clearest similarities, both in its regulation and functional properties, is one present in ileal smooth muscle (38). This channel is activated by acetylcholine through a G-protein and is also activated by GTPyS infusion. It is nonselective, dependent on Ca\(^{2+}\) for its activation, and potentiated by La\(^{3+}\) (39). It remains to be seen whether this or other currents that have been described in native tissues are formed by TRPC4 or TRPC5. Considering that the TRPC4/5 and TRPC3/6/7 subfamilies are regulated independently of the filling state of intracellular Ca\(^{2+}\) stores, the molecular correlates of capacitative calcium entry and I\(_{\text{CRAC}}\) are still obscure. Thus, the conclusion that any of the mammalian TRPCs are primarily regulated by store depletion has to be critically revised.

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