Activation of TRPM7 Channels by Phospholipase C-coupled Receptor Agonists

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TRPM7 is a ubiquitously expressed nonspecific cation channel that has been implicated in cellular Mg2+ homeostasis. We have recently shown that moderate overexpression of TRPM7 in neuroblastoma N1E-115 cells elevates cytosolic Ca2+ and enhances cell-matrix adhesion. Furthermore, activation of TRPM7 by phospholipase C (PLC)-coupled receptor agonists caused a further increase in intracellular Ca2+ levels and augmented cell adhesion and spreading in a Ca2+-dependent manner (1). Regulation of the TRPM7 channel is not well understood, although it has been reported that PIP2 hydrolysis closes the channel. Here we have examined the regulation of TRPM7 by PLC-coupled receptor agonists such as bradykinin, lysophosphatidic acid, and thrombin. Using FRET assays for second messengers, we have shown that the TRPM7-dependent Ca2+ increase closely correlates with activation of PLC. Under non-invasive “perforated patch clamp” conditions, we have found similar activation of TRPM7 by PLC-coupled receptor agonists. Although we could confirm that, under whole-cell conditions, the TRPM7 currents were significantly inhibited following PLC activation, this PLC-dependent inhibition was only observed when [Mg2+]i was reduced below physiological levels. Thus, under physiological ionic conditions, TRPM7 currents were activated rather than inhibited by PLC-activating receptor agonists.

TRPM7 is a ubiquitously expressed nonspecific cation channel that, intriguingly, contains a C-terminal serine-threonine kinase domain. It belongs to the transient receptor potential (TRP)1 subfamily of TRP channels that transduce sensory signals (2). TRPM7 functioning appears essential for life in that both knock-out and overexpression of the channels cause growth arrest, loss of cell adhesion, and rapid cell death (3–5). We recently discovered that low overexpression of TRPM7 induces cell spreading and adhesion and that its activation by PIP2-hydrolyzing receptor agonists leads to the formation of adhesion complexes in a kinase-dependent manner (1).

Currents carried by TRPM7 channels exogenously expressed in mammalian cells have been analyzed by several groups. In physiological solutions, the channel conducts mainly Ca2+ and Mg2+ (6), but in the absence of these divalent cations, K+ and Na+ (3, 7) permeate efficiently. A characteristic feature is the inhibition of TRPM7 currents by physiological (1–2 mM) intracellular Mg2+ levels; in whole-cell patch clamp experiments, large outwardly rectifying TRPM7 currents (3, 7, 8) are evoked by perfusion with Mg2+-free pipette solutions. Furthermore, MgATP and MgGTP also inhibit the channels (3, 9), although some controversy was raised on this issue (10). TRPM7 currents have been termed MagNuM (for Mg2+-nucleotide-regulated metal ion (3)) or MIC (for Mg2+-inhibited cation (10)) currents. These terms will here be used interchangeably to reflect whole-cell currents evoked by internal Mg2+ depletion. MIC/MagNuM currents revert at about 0 mV and lack voltage- and time-dependent activation (3, 7). Inward currents are predominantly carried by divalent cations, whereas outward currents consist mainly of monovalent cations (at low [Mg2+]i). Outward rectification is most likely due to divalent permeation block of inward currents at negative potentials (3), because perfusion with divalent-free extracellular solutions augments inward currents and linearizes the I/V relationship. The activation of TRPM7 by internal perfusion with Mg2+-free solutions does not reflect the release of the permeation block, but the precise mechanism has not yet been solved (10). Several reports document that the set point for [Mg2+]i sensitivity is governed by the kinase domain; however, TRPM7 channels lacking the kinase domain can still be activated by internal Mg2+ depletion (4, 11, 12). Thus, the interactions of TRPM7 with Mg2+ are complex; Mg2+ is conducted through the channel pore, causes voltage-dependent permeation block, and influences gating at the cytosolic surface.

The exact mechanisms by which receptor agonists regulate TRPM7 are less well characterized, and the published data are, at least partly, conflicting. An initially claimed indispensable role for the kinase domain (7) was challenged in subsequent studies (4, 11–13). We have recently shown (1) that the TRPM7 α-kinase specifically phosphorylates the heavy chain of myosin-II, thereby strongly influencing cell adhesion. Importantly,
association with and subsequent phosphorylation of myosin-II depend on prior activation of the channel by phospholipase C (PLC)-coupled receptors, and influx of extracellular Ca^{2+} constitutes an essential step in this process (1). In accordance with this, TRPM7 binds directly to several PLC isoforms, including PLCγ and PLCβ (14). The stimulatory effect of PLC on TRPM7 observed in intact cells by biochemical, cell biological, and live cell-imaging studies contrasts with a report that, in human embryonic kidney 293 (HEK293) cells, whole-cell TRPM7 currents are inhibited by PIP_{2} hydrolysis (14). PIP_{2}-dependent gating also occurs in other TRP family members, including TRPV1 (15), TRPM4 (16, 17), TRPM5 (18), and TRPM8 (19, 20). Finally, Takezawa and colleagues (13) recently reported that, in HEK293 cells (expressing only endogenous muscarinic receptors), carbachol attenuated TRPM7 currents via the G_{α}-cAMP signaling pathway, whereas PLC activation was not involved.

Given the discrepancies between the documented whole-cell patch clamp results and our cell biological observations, we re-examined how PLC-activating receptor agonists affect TRPM7 currents using non-invasive techniques in cells that moderately overexpress TRPM7. To this end, we combined fluorescence resonance energy transfer (FRET) assays for second messengers with perforated patch experiments and Ca^{2+} fluorometry to show that opening of TRPM7 channels closely correlates with PLC activation but not with cAMP/cGMP signaling. We also showed that, in perforated patches, TRPM7 currents are evoked by treatment of intact cells with a membrane-permeable Mg^{2+} chelator (EDTA-AM) and that these currents are inhibited rather than augmented by PLC-activating receptor agonists. We conclude that PLC-coupled agonists activate rather than inhibit TRPM7 in intact cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Amphotericin B, MgATP, bradykinin, lysophosphatidic acid, spermine, La(NO_{3})_{2}, Gd_{2}(CO_{3})_{3}, sodium nitroprusside, prostaglandin E1, and niflumic acid were from Sigma. Oregon Green 488 BAPTA-1 AM, Fura Red AM, Indo-1 AM, o-nitrophenyl-EGTA AM, pluronic F127, EDTA-AM, and BAPTA-AM were from Molecular Probes. Ionomycin, 2-aminooxydiphosphoryl-borate (2-APB), SKF 96365, isobutylmethylxanthine, forskolin, and thapsigargin were from Calbiochem-Novabiochem. Dulbecco’s modified Eagle’s medium, fetal calf serum, penicillin, streptomycin, and neomycin were from Invitrogen, and FuGENE 6 was from Roche Diagnostics.

** Constructs**—TRPM7 constructs were as described previously (1). The PIP_{2} FRET sensor (eCFP-Phospholipase D1 and eYFP-Phospholipase D1) was previously generated in our laboratory (21). Sensors for cAMP and cGMP were kind gifts from Dr. M. Zaccaro (University of Padova, Padova, Italy) and Dr. W. Dostmann (University of Vermont, Burlington, VT), respectively, and Yellow Cameleon 2.1 was a gift from Dr. R. Tsien (University of California-San Diego, La Jolla, CA).

**Immunostaining and Kinase Reaction**—A TRPM7 antibody was raised to amino acids 1748–1862 in rabbits as detailed in Ref. 1. Preimmune serum was used as a control in all experiments. For immunolabeling, cells were fixed with 4% paraformaldehyde in phosphate buffer, permeabilized with 0.1% Triton X-100, and incubated with rabbit anti-TRPM7 sera (1:200) followed by horseradish peroxidase-conjugated anti-rabbit IgG (1:1000). Amplification was by tyramide-conjugated fluorescein isothiocyanate (PerkinElmer Life Sciences). For the in vitro kinase assay, TRPM7 was precipitated from lysed cells with anti-TRPM7 antibodies and assayed for kinase activity as published recently (1).

**Cell Culture, Fluorimetric Experiments**—Culture of mouse N1E-115 and Phoenix packaging cells was as described previously (1). Ratiometric and pseudoratiometric Ca^{2+} recordings on cells on glass coverslips were carried out essentially as published in Refs. 21–23 in HEPES-buffered saline, pH 7.2, at 37 °C. All traces were calibrated with ionomycin and BAPTA as published previously (23). Dynamic FRET essays were carried out as described previously (21, 24). Excitation was at 425 nm using an ND3 filter, and cyan and yellow fluorescent protein emissions were collected simultaneously at 470 ± 20 and 530 ± 25 nm, respectively. Data were acquired at 4 Hz, and FRET was expressed as the ratio of cyan to yellow fluorescent protein signals. This ratio was set to 1.0 at the onset of the experiments, and changes are expressed as the percent of deviation from this initial value.

**Patch Clamp Experiments**—Electrophysiological recordings were collected using the HEKA EPC9 system. Current recordings were digitized at 100 kHz (ramp and block pulse protocols) or 10 Hz (steady-state whole-cell currents). Borosilicate glass pipettes were fire-polished to 2–4 MΩ. After establishment of the G_{Ω} seal, the patched membrane was ruptured by gentle suction to obtain whole-cell configuration, or amphotericin B (240 μg/ml) was used to obtain the perforated patch configuration with the typical access resistance of 3–10 MΩ.

Solutions were (in mM) 120 whole-cell pipette potassium-glutamate, 30 KCl, 1 MgCl_{2}, 0.2 CaCl_{2}, 1 EGTA, 10 HEPES, pH 7.2, and 1 MgATP; 140 external solution NaCl, 5 KCl, 0–1 MgCl_{2}, 0–10 CaCl_{2}, 10 HEPES, and 10 glucose adjusted to pH 7.3 with NaOH; for perforated patch recordings, the pipette solution was complemented with 240 μg/ml amphotericin B, and MgATP was omitted.

**RESULTS**

**TRPM7 Expression Raises Basal and Agonist-induced Cyto
colic Ca^{2+} Levels**—Consistent with previous observations (3), we found that transient overexpression of TRPM7 channels was lethal to N1E-115 cells and several other cell lines tested within a few days (data not shown). Furthermore, at early time points after transfection, much of the TRPM7 protein was found localized to biosynthetic compartments. To circumvent these problems, we expressed TRPM7 in N1E-115 cells at low levels by retroviral transduction (1). These cells, termed N1E-115/TRPM7 cells, were viable, divided normally, and could be routinely kept in culture for several months (1). Using a TRPM7-specific antibody (1), the expression level in N1E-115/TRPM7 cells was found to be 2–3 times higher than that in the parental cells (Fig. 1a). Under these conditions, the TRPM7 mainly localized to the plasma membrane, as shown in Fig. 1b. Moreover, depletion of intracellular Mg^{2+} in whole-cell patch clamp recordings showed that N1E-115/TRPM7 has ~2.5 times higher current density of MIC/MagNuM currents than wild type (wt) cells (supplemental Fig. S1).
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![Image](https://i.imgur.com/3WQ8z.png)

FIGURE 1. Characterization of N1E-115/TRPM7 cells. a, retroviral transduction causes moderate overexpression of the TRPM7 channel kinase as judged from kinase autophosphorylation of immunoprecipitated protein. b, confocal image of N1E-115/TRPM7 cell showing localization of the protein to the plasma membrane. TRPM7 was detected using anti-TRPM7 antibodies followed by tyramide signal amplification. Scale bar, 10 μm. c, effects of retroviral TRPM7 expression on basal (B) and agonist-induced peak (P) and sustained (S, taken 2 min after the addition of BK) calcium levels. [Ca^{2+}]i concentrations were calibrated with ionomycin and BAPTA, and data of hundreds of cells were averaged. All of data are mean ± S.E. **, p < 0.01; ***, p < 0.001. d, ratiometric single-cell Ca^{2+} traces showing the effect of the addition of bradykinin (1 μM) in parental (left panel) and TRPM7-transduced cells (right panel). Note the characteristic abrupt end of the sustained phase after ~3–5 min. Shown are representative traces from hundreds of experiments.

Ratiometric analysis (see “Experimental Procedures”) showed that intracellular Ca^{2+} was elevated significantly in N1E-115/TRPM7 cells (108.9 ± 6.0 nM, n = 50) as compared with parental cells (85.3 ± 2.5 nM, n = 50; p < 0.05) (Fig. 1c). Thus, TRPM7 channel expression contributes to basal Ca^{2+} homeostasis, in agreement with reports that the channels are partly preactivated (1, 4, 11, 25, 26).

Because cell biological and biochemical data (1) indicate that TRPM7 channels are activated by bradykinin (BK), we monitored the effect of the addition of BK on TRPM7-mediated Ca^{2+} influx by comparing N1E-115/TRPM7 to parental cells. In parental cells, 1 μM BK induced a cytosolic Ca^{2+} increase (765.9 ± 9.7 nM, n = 80) that peaked within seconds and subsequently returned to values close to resting levels (92.9 ± 2.9 nM) after 60 s (Fig. 1d, left panel). This corresponds to a mean increase of 7.6 nM from basal levels, which was not statistically significant. In contrast, in N1E-115/TRPM7 cells, the BK-induced transient Ca^{2+} increase (peak 843.3 ± 12.7 nm) was followed by a prominent sustained Ca^{2+} elevation (141.0 ± 7.0 nM, n = 120) that lasted for several minutes before returning to resting levels rather abruptly (Fig. 1d, right panel; quantification in Fig. 1c). This corresponds to a mean increase in sustained Ca^{2+} levels of 32.1 nM (p < 0.001, paired t test) in accordance with the higher TRPM7 expression levels in these cells. Thus, in N1E-115/TRPM7 cells, the addition of BK elicits a sustained phase in the Ca^{2+} response, in good agreement with our cell biological data (1). In summary, the observed increase in Ca^{2+} influx is mediated by TRPM7 channels and is not due to the retroviral transduction procedure (supplemental Fig. S2).

TRPM7 Activation Is Downstream of PLC in Intact N1E-115 Cells—Endogenous B2 bradykinin receptors in N1E-115 cells signal predominantly via the Gαq-linked PLC pathway (27), but some reports suggest that, depending on the cell type, the B2 receptors may occasionally either inhibit (28) or stimulate (29) the production of cAMP. We set out to identify the signaling events responsible for BK-induced activation of TRPM7 in N1E-115/TRPM7 cells by correlating Ca^{2+} fluorometry with the activation of intracellular signaling pathways, as detected by various FRET assays.

BK caused rapid breakdown of a significant fraction (60–80%) of the plasma membrane PIP_{2} pool, as detected by a FRET assay (21) that reports membrane PIP_{2} content (Fig. 2a, upper panel, first trace). The Gα_{q}/PLC-coupled receptor agonist lysophosphatidic acid and thrombin receptor-activating peptide also activate PLC, although to a lesser extent (~20–30% of BK values; n > 200) (Fig. 2a, upper panel, second and third trace (21)). Similar to BK, the initial Ca^{2+} peak induced by these agonists was followed by sustained Ca^{2+} influx in N1E-115/TRPM7 cells (Fig. 2a, lower panel). In contrast, sustained Ca^{2+} influx was not seen when cells were stimulated with agonists of receptors that do not couple to PLC, such as prostaglandin E1 (Fig. 2a, 4th trace, and data not shown). Thus, TRPM7 activation correlates well with PLC activation/Ca^{2+} signaling.

To address the possible involvement of cAMP in the BK-induced opening of TRPM7, we monitored cAMP levels in intact N1E-115/TRPM7 cells using a genetically encoded cAMP sensor (30, 31). The addition of BK to N1E-115/TRPM7 cells had no effect on cAMP levels (n = 8), whereas forskolin (25 μM) readily raised cAMP levels (Fig. 2b, left panel). Furthermore, pretreatment of cells with pertussis toxin to specifically inhibit G_{i} and thereby block receptor-induced decreases in cAMP levels did not affect the BK-induced Ca^{2+} influx (data not shown). Therefore, in N1E-115/TRPM7 cells, changes in cAMP levels do not mediate the BK-induced opening of TRPM7. In addition, neither prostaglandin E1, which activates G_{i} to cause a rapid and sustained increase in [cAMP], (Fig. 2b, middle panel), nor sphingosine-1-phosphate (SIP, data not shown), which couples to G_{i} and G_{13}, but not to PLC in N1E-115 cells (32), had any effect on Ca^{2+} levels. We conclude that there is no evidence for a role of cAMP in BK-mediated Ca^{2+} influx in N1E-115/TRPM7 cells.

We also investigated the effects of the nitric oxide donor nitroprusside, because nitric oxide was reported to activate TRPM7-mediated Ca^{2+} influx in cultured cortical neurons (33). In intact parental and N1E-115/TRPM7 cells, nitroprusside triggered the production of cGMP (Fig. 2c, left panel (24)) without affecting [Ca^{2+}]i, (Fig. 2c, right panel). In conclusion, TRPM7 opening closely correlates with PLC activation but not with other G protein-linked signals.

Activation of PLC Inhibits Whole-cell TRPM7 Currents in N1E-115/TRPM7 Cells—In whole-cell patch clamp experiments using HEK-293 cells overexpressing M1 muscarinic
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FIGURE 2. Activation of TRPM7 by GPCRs: involvement of the Gq/PLC signaling pathway. a, representative FRET traces showing PIP2 hydrolysis (upper panel) induced by BK, lysophosphatidic acid, and thrombin receptor-activating peptide (TRP) and the ensuing sustained Ca2+ elevations in N1E-115/TRPM7 cells (lower panel). Prostaglandin E1 (PGE1), which potently raises [cAMP], does not activate PLC and fails to evoke sustained Ca2+ entry (right-most traces). b, BK receptor activation does not alter cAMP levels in N1E-115/TRPM7 cells as detected by a protein kinase A-based FRET probe (left panel). The addition of the adenylyl cyclase activator forskolin together with the phosphodiesterase inhibitor isobutylmethylxanthine serves as a positive control. Conversely, prostaglandin E1 strongly activates the Gq/CAMP pathway through its cognate G protein-coupled receptor (middle panel) but fails to elicit the sustained Ca2+ elevation (right panel) that is typically seen after Gq/PLC activation. c, in N1E-115/TRPM7 cells, BK fails to alter cGMP levels, whereas the nitric oxide donor nitroprusside readily activates this pathway, as assessed using Cygnet, a FRET sensor for cGMP (41). The addition of nitroprusside had no effect on [Ca2+]i in these cells (right panel).

FIGURE 3. MIC/MagNuM currents in N1E-115/TRPM7 cells are inhibited by BK. a, TRPM7 currents, evoked with Mg2+-free pipette solution in a N1E-115/TRPM7 cell, are strongly inhibited after activation of PLC. Currents evoked by ±100-mV ramps (b) from a holding potential of 0 mV are quantified at −80 mV (circles) and +80 mV (squares). Traces depicted in b represent currents before (1) and during (2) TRPM7 activation and after (3) BK stimulation. Note that the small inward currents are due to divalent block in Mg2+- and Ca2+-containing extracellular medium. Shown are representative data from 10 individual experiments.

Pressing by PLC activation in our cells. Indeed, BK rapidly suppressed outward-rectifying whole-cell TRPM7 currents both in native N1E-115 cells (data not shown) and N1E-115/TRPM7 cells (Fig. 3a). Although inward currents were completely abolished (99.7 ± 1.0% of control values, n = 9), small outward currents were still observed at high depolarizing potentials (Fig. 3b, trace 3 and see Fig. 6b). Other PLC-coupled agonists, including TRP and lysophosphatidic acid, also inhibited the currents, although to a lesser extent. Therefore, it appears that the original observations on PIP2 dependence of whole-cell TRPM7 currents hold true for N1E-115/TRPM7 cells.

PLC-coupled Receptor Agonists Activate TRPM7 Currents in Intact Cells—The above observations leave us with a paradox: PLC activation inhibits TRPM7 currents when using whole-cell electrophysiology, whereas it opens the channels when assayed by Ca2+ fluorometry. What causes this difference? In whole-cell patch clamp experiments, diffusible signaling molecules will be rapidly diluted, which could influence channel gating properties. We therefore analyzed the electrophysiological effects of PLC activation using the perforated patch configuration (34, 35), which provides electrical access to the cell without disturbing the cytosolic composition. Voltage-clamped perforated patches were held at −70 mV, and membrane conductance was monitored from currents evoked by a biphasic block pulse protocol (+10 and −10 mV from resting potential (Fig. 4). In unstimulated cells, currents were small (0.46 ± 0.16 pA/pF, n = 10), corresponding to a membrane conductance of 23 pS/pF. Note that because Mg2+ levels remain intact in this configuration, the magnitude of the currents should match those of whole-cell experiments when Mg2+ is included in the pipette. Indeed, as shown in supplemental Fig. S1a, lower panel, the currents induced by 120-mV voltage steps in unstimulated whole cells measured 3.1 ± 0.4 pA/pF (n = 5), which corresponds to ∼26 pS/pF (in close agreement with the perforated patch data). As expected, spontaneously developing currents were never observed in perforated patches (n = 12) (see Fig. 5a).

Strikingly, stimulation with bradykinin significantly increased the membrane conductance (250 ± 53 pS, n = 5).
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![Image](https://example.com/fig4.png)

**Figure 4.** TRPM7 currents detected using perforated patch clamping. Mean membrane conductance changes (black line; shaded area depicts S.E.) in N1E-115 wt (a) and N1E-115/TRPM7 cells (b) deduced from responses to ±10-mV block pulses from a holding potential of −70 mV. BK (1 μM) was added at t = 0; note the sustained activation of an additional conductance. To verify that the small BK-evoked current would suffice to increase \([Ca^{2+}]_i\), significantly, we point out that an influx of just 10 pA of current carried by \([Ca^{2+}]_i\) equals \(10 \times 10^{-12} F / F = 5 \times 10^{-3} \text{ mol} / \text{F} \times \text{valence of ion \(z\)} \times \text{volume \(V\)}\) in 1 s, if no buffer systems are in place. Right panels, weakly rectifying currents induced by BK in wt and N1E-115/TRPM7 (black, closed squares) cells obtained by subtracting unstimulated (gray, open squares) from stimulated (gray, open circles) I/V plots.

In all cases, currents were transient, reverting to baseline with somewhat variable kinetics (range, 2–8 min). Similarly, the addition of BK slightly but significantly augmented the endogenous TRPM7 current in parental cells (Fig. 4a). Perforated patch I/V plots of BK-induced currents in wt and N1E-115/TRPM7 cells revealed almost linear currents (Fig. 4, a and b, right panels), probably because of the divalent permeation block of outward currents. The magnitude of these currents correlates with the TRPM7 expression levels, and they are inhibited by the same panel of inhibitors (see Table S1). We found that the distinction between PLC-induced inhibition (in whole cells) versus stimulation (in perforated patches) holds true for TRPM7-transfected HEK-293 cells as well (see supplemental Fig. S3). We conclude that a prototypic PLC-activating receptor agonist (bradykinin) activates TRPM7 channels under perforated patch clamp conditions.

**Chelation of Intracellular Mg^{2+} Mimics Whole-cell TRPM7 Currents in Perforated Patches**—To further investigate the differences in TRPM7 current behavior under different recording conditions, we sought to lower \([Mg^{2+}]_i\) in intact cells. Cells were pretreated with EDTA-AM, a membrane-permeable precursor of the \(Mg^{2+}/Ca^{2+}\) chelator EDTA. Upon removal of its lipophilic AM moiety by cytosolic esterases, the compound becomes trapped in the cytosol and attains its chelating properties. Although in the perforated patch no spontaneous run-up of TRPM7 currents was observed (Fig. 5a), exposure of N1E-115/TRPM7 cells to 10 μM EDTA-AM consistently evoked large currents with outward-rectifying I/V relationships, identical to those obtained in whole cells (Fig. 5b). The slow onset of this current likely reflects the slow development of Mg^{2+} buffering during buildup of the intracellular EDTA concentration. As a control, treatment with BAPTA-AM, a highly \(Ca^{2+}\)-selective membrane-permeable chelator, had no effect (Fig. 5d). Physiological and pharmacological properties of EDTA-AM-evoked perforated patch currents were identical to whole-cell TRPM7 currents (see supplemental Fig. S4 and Table S1).

To date, most studies on TRPM7 have been performed on cells transiently overexpressing the channels, commonly HEK-293 or Chinese hamster ovary cells. We therefore tested whether EDTA-AM also activates TRPM7 channels in perforated patch experiments on HEK-293 cells. Indeed, heterologously expressed as well as endogenous TRPM7 channels were strongly activated by EDTA-AM treatment (Fig. 5c; for statistics, see the legend).

**Effects of Agonist-induced PIP2 Breakdown on EDTA-AM-induced and Whole-cell MIC/MagNuM Currents**—How does PLC activation affect EDTA-AM-induced TRPM7 currents in perforated patches? Strikingly, under these conditions, stimulation with BK caused a large fraction of TRPM7 channels to close rapidly (Fig. 6, a and b). This effect was rapid and transient rather than sustained in all cells tested. Rapid inhibition was also observed in parental N1E-115 cells, and it was not specific for BK, in that lysophosphatidic acid and thrombin receptor-activating peptide had the same effect (data not shown).

In contrast, in the whole-cell experiments of Runnels et al. (14), PIP2 hydrolysis caused irreversible closure of whole-cell TRPM7 currents. We therefore compared the effects of the addition of G protein-coupled receptor agonists on PIP2 levels in whole-cell and perforated patch experiments in N1E-115/TRPM7 cells. Automated analysis of confocal time lapse images (21) show that the PIP2 indicator GFP-PH(PLCδ1) was retained at the plasma membrane during intracellular accumulation of EDTA-AM (data not shown). Subsequent stimulation with BK resulted in fast translocation of GFP-PH to the cytosol (Fig. 6c, right panel) followed by its relocation to the plasma membrane.
within a few minutes. The quantification in Fig. 6d shows that 5 min after BK stimulation recovery is almost complete. In contrast, in whole-cell experiments with N1E-115/TRPM7 cells, the BK-induced GFP-PH translocation was sustained (Fig. 6c, left panel), in good agreement with published data for HEK-293 cells (14).

Thus, the time course of the inhibition of Mg\(^{2+}\)/H\(_{11001}\) depletion-induced TRPM7 currents correlates well with that of the loss of PIP\(_2\) following receptor activation. The data also strongly suggest that lack of recovery of BK-induced TRPM7 currents in whole-cells is due to impaired PIP\(_2\) resynthesis in this configuration.

with EDTA-AM (10 \(\mu\)M) causes development of outward-rectifying MIC/MagNuM (trace 2) currents after an initial lag period (trace 1) that does not run down for over an hour. c, EDTA-AM evokes MIC/MagNuM currents in wild type HEK-293 cells (trace 1) and HEK-293 heterologously expressing TRPM7 channels (trace 2). EDTA-AM caused TRPM7 currents to increase from 57 \(\pm\) 16 to 173 \(\pm\) 65 pA, \(n = 4\), in wt HEK-293 cells and from 146 \(\pm\) 15 to 783 \(\pm\) 29 pA, \(n = 4\), in TRPM7-transfected HEK-293 cells, as detected at \(+80\) mV (\(p < 0.001\)). d, application of EDTA-AM (black trace) but not of BAPTA-AM (gray trace) increases [Ca\(^{2+}\)], as detected with Yellow Cameleon-CAAX, a plasma-membrane targeted FRET sensor for Ca\(^{2+}\). Shown are representative traces of experiments performed at least 5-fold.

FIGURE 6. Transient inactivation of TRPM7 channels by bradykinin in non-invasive patch clamping. a, upon activation of TRPM7 currents by EDTA-AM in perforated patches, BK causes transient inhibition. Shown is a typical example of 12 experiments. b, quantification (mean \(\pm\) S.E.) of BK-induced inhibition of TRPM7 currents at the indicated time points, detected at \(+80\) mV for whole cells (WC; circles, \(n = 20\)) and perforated patches (PP, squares, \(n = 12\)). TRPM7 currents before BK was added (at \(t = 0\)) were set to 100%. *, \(p < 0.05\) and **, \(p < 0.01\). c, kinetics of PIP\(_2\) responses to BK, monitored by GFP-PH(PLC\(_{1}\)) imaging, show sustained PIP\(_2\) breakdown in whole-cell experiments (left panel). In contrast, in EDTA-AM pretreated N1E-115/TRPM7 cells (right panel) responses are transient and very similar to those of untreated cells (compare Fig. 2a). Shown are typical examples of 5–8 experiments. PM/Cyt denotes the ratio of fluorescence intensities at the plasma membrane and cytosol, as determined using an automated image analysis routine (21). Note that the time span of the BK-mediated PIP\(_2\) drop correlates well with TRPM7 channel inhibition. d, quantification of GFP-PH(PLC\(_{1}\)) membrane localization, expressed as percentage of control, for whole-cells (circles, \(n = 5\)) and EDTA-AM pretreated perforated patches (squares, \(n = 8\)) at the indicated time points. At \(t = 0\), 1 \(\mu\)M BK was added. Data are mean \(\pm\) S.E.; *, \(p < 0.05\). All data are from N1E-115/TRPM7 cells.

FIGURE 5. The membrane-permeable Mg\(^{2+}\) chelator EDTA-AM activates TRPM7 in perforated patches. a, perforated patch recording does not evoke activation of spontaneous currents in N1E-115/TRPM7 cells. b, acute treatment...
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DISCUSSION

In this study, we have addressed the regulation of TRPM7 channels in intact cells. Transient overexpression of TRPM7 was avoided because this caused mislocalization of TRPM7 to endomembranes and cell death because of the constitutive activity of TRPM7. Furthermore, transient overexpression is also more likely to titrate out essential binding partners and thereby may influence signaling events. Indeed, Takezawa et al. (13) note that TRPM7 overexpression abolishes the activation of PLC by carbachol receptors. Recently, Kim et al. (36) have also noted that regulation of native TRPM7 channels by PLC differs from the results obtained with transfected HEK-293 cells (14). We therefore used perforated patch clamping and Ca<sup>2+</sup> imaging to avoid the disturbances in cell signaling that likely occur in the whole-cell configuration.

Physiological Properties—Our results reveal that retroviral transduction results in the appearance of a conductance with all the expected properties of TRPM7 (see supplemental “Discussion”). At first sight, it may seem disturbing that the observed perforated patch currents are relatively small and lack strong outward rectification, a feature that has been termed “TRPM7 signature.” However, small amplitude basal and evoked currents were to be expected, because TRPM7 was expressed at low levels, and the currents were recorded at non-depolarized voltages to allow comparison with the Ca<sup>2+</sup> data. Moreover, in HEK-293 overexpression studies, the TRPM7 inward currents (mainly Ca<sup>2+</sup> and Mg<sup>2+</sup>) were also minor (3, 7).

Strong outward rectification is seen under conditions of low [Mg<sup>2+</sup>], (whole cells; EDTA-AM-treated perforated patches). Interestingly, similar to the perforated patch currents, the whole-cell currents evoked by PLC activation in TRPM7-transfected HEK-293 cells with 1 mM Mg<sup>2+</sup> in the pipette showed only weak outward rectification (supplemental Fig. S3c). Most likely, this reflects an anomalous mole fraction effect, whereby intracellular Mg<sup>2+</sup> attenuates outward monovalent currents. However, separation of this effect from the inhibitory effect of intracellular Mg<sup>2+</sup> on channel gating would require systematic analysis (preferably in inside-out patches), which to our knowledge has not been performed thus far. In addition, intracellular Mg<sup>2+</sup> levels may act through the TRPM7 protein itself, in view of the reported function of the kinase domain in determining the set point for Mg<sup>2+</sup> inhibition (4, 9) as well as through additional mechanisms (37). The use of EDTA-AM to activate the channels in intact cells should present an important new experimental paradigm for studies in this direction.

Signaling Pathways—Takezawa et al. (13) recently reported that activation of PLC through endogenous M1-muscarinic receptors had little effect on MIC/MagNuM currents in TRPM7-overexpressing HEK-293 cells. Perhaps the discrepancy between the Runnels et al. (14) and our study on the one hand and that of Takezawa et al. (13) on the other hand is a difference in potency of the PLC-activating receptors involved, as in their (and our) hands, endogenous M1 receptors cause only minor PIP<sub>2</sub> breakdown. Furthermore, unlike in our low level retroviral overexpression studies, in HEK-293 cells, TRPM7 overexpression inhibited carbachol-induced PLC signaling (13). Their study also relied on the use of the “PLC inhibitor” U-73122, a compound with many known side effects (38, 39). Rather, Takezawa et al. (13) suggest that the modulatory effects of carbachol are mediated by cAMP, as lowering [cAMP], attenuated whole-cell TRPM7 currents in HEK-293 cells. However, in N1E-115 cells, Bradykinin had no effect on cAMP levels, and conversely, potent cAMP-raising agonists did not trigger sustained Ca<sup>2+</sup> influx or TRPM7 currents detected in the perforated patch. Rather, our analysis indicates a key role for PLC-derived signals.

Dual Regulation by Phosphoinositide Signals—At odds with a previous report (14), we observed that stimulation of PLC-activating receptors caused TRPM7 channel opening rather than closure. This discrepancy is not cell type-dependent nor does it depend on TRPM7 expression levels. How can the paradoxical effects of PLC activation on TRPM7 currents in whole-cell and perforated patch experiments be reconciled? We speculate that fundamentally different modes of regulation mediate stimulation and inhibition.

On the one hand, in unperturbed cells, currents increase upon PLC activation, and therefore we propose that this must be the more physiological mode of action. It is reassuring that this concurs with our recent biochemical and cell biological data, which unequivocally demonstrate the activation of TRPM7 by PLC-activating agonists in intact cells (1), and with evidence from a very recent study by Kim et al. (36). However, the precise mechanism of this activation pathway remains elusive, although we can exclude the involvement of the PIP<sub>2</sub>-derived messengers diacylglycerol (because the addition of membrane-permeable analogues did not activate TRPM7, data not shown) and cytosolic Ca<sup>2+</sup> (supplemental Fig. S2a).

On the other hand, PIP<sub>2</sub> undoubtedly is an important cofactor for normal TRPM7 functioning (14, 37, 40). In analogy to other TRP family channels (17, 20), C-terminal stretches of positive amino acids may bind PIP<sub>2</sub> to impose a proper tertiary structure. In TRPM7, positive stretches are present in the TRP consensus domain and at amino acids 1147–1154 and 1196–1218 in the C terminus. Loss of PIP<sub>2</sub> could inhibit normal opening of TRPM7, but this would only be revealed following prior full activation of the channel by Mg<sup>2+</sup> depletion. In line with this notion, spontaneous rundown of MIC/MagNuM currents in whole-cell and inside-out experiments is accompanied by loss of PIP<sub>2</sub> (37, 40). Our data show that, in whole cells but not in perforated patches, PLC-activating agonists cause a sustained PIP<sub>2</sub> drop due to impaired resynthesis. Thus, the effects of PLC activation on whole-cell TRPM7 currents could be viewed as “accelerated rundown.” Alternatively, the opposing effect of PIP<sub>2</sub> depletion on PLC-mediated activation may reflect a subtle feedback mechanism whereby ongoing loss of PIP<sub>2</sub> counteracts or limits TRPM7 activation. A similar mechanism was proposed recently for TRPM8 (20). In strong support of this dual regulatory model, kinetic analysis of BK-mediated stimulatory and inhibitory effects shows that activation proceeds distinctly faster than inactivation.

In summary, our experiments reveal a second mode of activation for TRPM7; not only can the channel be activated by depletion of intracellular Mg<sup>2+</sup> and Mg<sup>2+</sup> nucleotides but also by stimulation of endogenous PLC-activating receptors.
PLC Activates TRPM7 Channels

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REFERENCES

1. Clark, K., Langeslag, M., van Leeuwen, B., Ran, L., Ryazanov, A. G., Figdor, C. G., Moolenaar, W. H., Jalink, K., and van Leeuwen, F. N. (2006) EMBO J. 25, 290–301
2. Clapham, D. E. (2003) Nature 426, 517–524
3. Nadler, M. J., Hermosura, M. C., Inabe, K., Perraud, A. L., Zhu, Q., Stokes, A. J., Kuroskii, T., Kinét, J. P., Penner, R., Scharenberg, A. M., and Fleig, A. (2001) Nature 411, 590–595
4. Schmitz, C., Perraud, A. L., Johnson, C. O., Inabe, K., Smith, M. K., Penner, R., Kuroski, T., Fleig, A., and Scharenberg, A. M. (2003) Cell 114, 191–200
5. Su, L. T., Agapito, M. A., Li, M., Simonson, W. T., Huttenlocher, A., Habas, R., Yue, L., and Runnels, L. W. (2006) J. Biol. Chem. 281, 11260–11270
6. Voets, T., Nilius, B., Hoefs, S., van der Kemp, A. W., Droogmans, G., Bindels, R. J., and Hoenderop, J. G. (2004) J. Biol. Chem. 279, 19–25
7. Runnels, L. W., Yue, L., and Clapham, D. E. (2001) Science 291, 1043–1047
8. Kerschbaum, H. H., Kozak, J. A., and Cahalan, M. D. (2003) Biophys. J. 84, 2293–2305
9. Demeuse, P., Penner, R., and Fleig, A. (2006) J. Gen. Physiol. 127, 421–434
10. Kozak, J. A., Kerschbaum, H. H., and Cahalan, M. D. (2002) J. Gen. Physiol. 120, 221–235
11. Matsushita, M., Kozak, J. A., Shimizu, Y., McLachlin, D. T., Yamaguchi, H., Wei, F. Y., Tomizawa, K., Matsuji, H., Chait, B. T., Cahalan, M. D., and Nairn, A. C. (2005) J. Biol. Chem. 280, 20793–20803
12. Schnitz, C., Dorovkov, M. V., Zhao, X., Davenport, B. J., Ryazanov, A. G., and Perraud, A. L. (2005) J. Biol. Chem. 280, 37763–37771
13. Takezawa, R., Schnitz, C., Demeuse, P., Scharenberg, A. M., Penner, R., and Fleig, A. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 6009–6014
14. Runnels, L. W., Yue, L., and Clapham, D. E. (2002) Nat. Cell Biol. 4, 329–336
15. Prescott, E. D., and Julius, D. (2003) Science 300, 1284–1288
16. Zhang, Z., Okawa, H., Wang, Y., and Liman, E. R. (2005) J. Biol. Chem. 280, 39185–39192
17. Nilius, B., Maihie, F., Prenen, J., Janssens, A., Owssianik, G., Vennekens, R., and Voets, T. (2006) EMBO J. 25, 467–478
18. Liu, D., and Liman, E. R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 15160–15165
19. Liu, B., and Qin, F. (2005) J. Neurosci. 25, 1674–1681
20. Rohacs, T., Lopes, C. M., Michalidis, I., and Logothetis, D. E. (2005) Nat. Neurosci. 8, 626–634
21. van der Wal, J., Habets, R., Varnai, P., Balla, T., and Jalink, K. (2001) J. Biol. Chem. 276, 15337–15344
22. Rasmussen, U., Brooogher, C. S., and Sandberg, F. (1978) Acta Pharm. Suec. 15, 133–140
23. Jalink, K., van Corven, E. J., and Moolenaar, W. H. (1990) J. Biol. Chem. 265, 12232–12239
24. Ponsioen, B., Zhao, J., Riedl, J., Zwartkruis, F., van der Kortg, G., Zaccolo, M., Moolenaar, W. H., Bos, J. L., and Jalink, K. (2004) EMBO Rep. 5, 1176–1180
25. Kozuk, J. A., and Cahalan, M. D. (2003) Biophys. J. 84, 922–927
26. Hanano, T., Hara, Y., Shi, J., Morita, H., Umebayashi, C., Mori, E., Sumimoto, H., Ito, Y., Mori, Y., and Inoue, R. (2004) J. Pharmacol. Sci.
27. Coggan, J. S., and Thompson, S. H. (1995) Am. J. Physiol. 269, C841–C848
28. Hanke, S., Nurnberg, B., Groll, D. H., and Liebmann, C. (2001) Mol. Cell. Biol. 21, 8452–8460
29. Albert, O., Ancellin, N., Preisser, L., Morel, A., and Corman, B. (1999) Life Sci. 64, 859–867
30. Zaccolo, M., De Giorgi, F., Cho, C. Y., Feng, L., Knapp, T., Negulescu, P. A., Taylor, S. S., Tsien, R. Y., and Pozzan, T. (2000) Nat. Cell Biol. 2, 25–29
31. Bacskai, B. J., Hochner, B., Mahaut-Smith, M., Adams, S. R., Kaang, B. K., Kandel, E. R., and Tsien, R. Y. (1993) Science 260, 222–226
32. Postma, F. R., Jalink, K., Hengeveld, T., Offermans, S., and Moolenaar, W. H. (2001) Curr. Biol. 11, 121–124
33. Aarts, M., Iihara, K., Wei, W. I., Xiong, Z. G., Arundine, M., Cervinski, W., MacDonald, J. F., and Tymianski, M. (2003) Cell 115, 863–877
34. Rae, J., Cooper, K., Gates, P., and Watsky, M. (1991) J. Neurosci. Methods 37, 15–26
35. Postma, F. R., Jalink, K., Hengeveld, T., Bot, A. G., Alblas, J., de Jonge, H. R., and Moolenaar, W. H. (1996) EMBO J. 15, 63–72
36. Kim, B. J., Lim, H. H., Yang, D. K., Jun, Y. J., Chang, I. Y., Park, C. S., So, I., Stanfield, P. R., and Kim, K. W. (2005) Gastroenterol. 129, 1504–1517
37. Kozuk, J. A., Matsushita, M., Nairn, A. C., and Cahalan, M. D. (2005) J. Gen. Physiol. 126, 499–514
38. Balla, T. (2001) Curr. Pharm. Des. 7, 475–507
39. Horowitz, L. F., Hirdes, W., Suh, B. C., Hilgemann, D. W., Mackie, K., and Hille, B. (2005) J. Gen. Physiol. 126, 243–262
40. Gwanyanya, A., Sidipo, K. R., Vereecke, J., and Mubagwa, K. (2006) Am. J. Physiol. 291, C627–C635
41. Honda, A., Adams, S. R., Sawyer, C. L., Lev-Ram, V., Tsien, R. Y., and Dostmann, W. R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2437–2442