Signaling Mechanism for Receptor-activated Canonical Transient Receptor Potential 3 (TRPC3) Channels*

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Canonical transient receptor potential 3 (TRPC3) is a receptor-activated, calcium permeant, non-selective cation channel. TRPC3 has been shown to interact physically with the N-terminal domain of the inositol 1,4,5-trisphosphate receptor, consistent with a “conformational coupling” mechanism for its activation. Here we show that low concentrations of agonists that fail to produce levels of inositol 1,4,5-trisphosphate sufficient to induce Ca\(^{2+}\) release from intracellular stores substantially activate TRPC3. By several experimental approaches, we demonstrate that neither inositol 1,4,5-trisphosphate nor G proteins are required for TRPC3 activation. However, diacylglycerols were sufficient to activate TRPC3 in a protein kinase C-independent manner. Surface receptor agonists and exogenously applied diacylglycerols were not additive in activating TRPC3. In addition, inhibition of metabolism of diacylglycerol slowed the reversal of receptor-dependent TRPC3 activation. We conclude that receptor-mediated activation of phospholipase C in intact cells activates TRPC3 via diacylglycerol production, independently of G proteins, protein kinase C, or inositol 1,4,5-trisphosphate.

In non-excitable cells, depletion of internal Ca\(^{2+}\) stores activates store-operated channels (SOCs)\(^*\) mediating calcium entry across the plasma membrane, a process known as capacitative calcium entry (CCE) (1–3). Despite considerable attention, the molecular identity of SOCs and their gating mechanism(s) remain unknown. Two major hypotheses for the mechanism of activation of CCE have been proposed. The first involves the release of a soluble factor from the endoplasmic reticulum (4, 5). The second, the “conformational coupling” model, proposes a direct interaction of inositol 1,4,5-trisphosphate (IP\(_3\)) receptors in the endoplasmic reticulum with SOCs in the plasma membrane (2, 6). The latter hypothesis has gained widespread support in the last few years.

TRPC3 is a member of the canonical transient receptor potential (TRPC) family of Ca\(^{2+}\)-permeant, non-selective cation channels (7) whose members have been hypothesized to form, or contribute to the formation of the elusive SOC channel (7–9). TRPC3 and its close structural homologs, TRPC6 and TRPC7, in many expression systems, including HEK293 cells, behave as receptor-operated cation channels (10–12) that can be activated by exogenous application of diacylglycerols (DAG) independently of store-depletion (13, 14). However, Kiselyov et al. (15, 16) reported that TRPC3 channels in excised patches from TRPC3-expressing HEK293 cells could be stimulated with IP\(_3\) and IP\(_3\) receptors. These authors suggested that TRPC3 activation involves interaction with IP\(_3\) receptors in their ligand-bound state, consistent with the conformational coupling hypothesis. Ma et al. (17) also proposed a requirement of IP\(_3\) receptors for native SOC and TRPC3 channel activation based on experiments with the membrane-permeant IP\(_3\) receptor antagonist, 2-aminoethoxydiphenyl borane (2-APB). Finally, Boulay et al. (18) provided biochemical evidence for TRPC3 interaction with IP\(_3\) receptors by utilizing a glutathione S-transferase pull-down strategy to map the interacting domains on these two proteins.

Thus, conflicting results from different laboratories have generated a controversy as to the physiological mode of activation of TRPC3 channels. It is possible that different backgrounds of expression or different expression levels may produce different behaviors of TRPC3 (for example, see Refs. 19 and 20). In addition, studies examining single channel behavior in excised patches may suffer from loss of cellular components essential for cellular signaling. Therefore, in the current study, we have re-examined the role of IP\(_3\) in regulation of TRPC3 channels, by utilizing whole cell patch clamp and single cell microfluorimetry. We have also utilized two HEK293 cell lines stably expressing TRPC3, one of which has been used in previous studies by other groups to provide evidence for a role of IP\(_3\) and the IP\(_3\) receptor. Our findings indicate that receptor activation of TRPC3 in intact HEK293 cells does not require activation of heterotrimeric G-proteins or the formation of IP\(_3\). Rather, it appears that, in intact HEK293 cells, receptor activation of TRPC3 is mediated by DAG in a protein kinase C (PKC)-independent manner.

MATERIALS AND METHODS

Reagents—Thapsigargin, methacholine, 1-oleoyl-2-acetyl-sn-glycerol (OAG), IP\(_3\), inositol 2,4,5-trisphosphate (2,4,5(\(\text{IP}_{3}\))), phorbol 12-myristoyl 13-acetate (PMA), and DAG kinase inhibitor II, R59022, were all purchased from Calbiochem. The 1-(\(\alpha\)-glycerophosphoryl)-\(\alpha\)-myo-inositol 4,5-bisphosphate (GPIP\(_2\)) was purchased from Roche Molecular Biochemicals, low molecular weight heparin and atropine from Sigma, the lipase inhibitor RHC-80267 from Alexis Biochemicals, and epidermal growth factor; BAPTA, 1,2-bis(\(\alpha\)-aminophenoxy)ethane-\(N,N,N,N\) tetraacetic acid; [\(\text{Ca}^{2+}\)], intracellular [\(\text{Ca}^{2+}\)].

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nal growth factor (EGF) from Upstate Biotechnology Inc.

Cell Culture and Transfection—HEK293 cells were obtained from ATCC and were transfected, using Superfect reagent (Qiagen), with pcDNA3 vector containing the green fluorescent protein (GFP) coding sequence added in-frame to the C terminus of human TRPC3 (11). Cells stably expressing TRPC3-GFP fusion protein (HEK-TRPC3) were selected first by antibiotic resistance and second by GFP fluorescence by flow cytometry. Cells were maintained in culture as described previously (21). An HEK293 cell line expressing human TRPC3 with the hemagglutinin epitope (HA) fused to its C terminus (HEK-T3, clone 9) were generated and grown as previously described (10).

Measurement of Intracellular Calcium Concentration—Coverslips with attached cells were mounted in a Teflon chamber and incubated at 37 °C and 5% CO2 in culture media (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum) containing 1 μM Fura-2 AM (Molecular Probes). Cells were then washed and bathed in Hepes-buffered saline solution (HBSS; in mM, 140 NaCl, 4.7 KCl, 10 CsCl2, 2 CaCl2, 1.13 MgCl2, 10 glucose, and 10 Hepes, pH 7.4) for at least 15 min before Ca2+ measurements were made.

In some experiments, reagents were introduced into the cell through patch pipettes (2–5 MΩ, Corning glass, 7052). The cells for these experiments had membrane capacitances of 10–25 pF and were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging Inc., Cincinnati, OH). Fura-2 fluorescence at an emission wavelength of 510 nm was induced by exciting the cells with a 510 nm wavelength light from a Deltascan D101 (Photon Technology International, Princeton, NJ) light source equipped with a 400 nm excitation filter (Nikon) and a 510 nm emission filter (Nikon). A constant holding potential of −65 mV was applied to the cells throughout the experiment. All experiments were conducted at room temperature. The data are expressed as the ratio of Fura-2 fluorescence because of excitation at 340 nm to that because of excitation at 380 nm.

For Ca2+ measurements after application of external stimuli only (i.e. not involving patch clamp), fluorescence images of the cells were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging Inc., Cincinnati, OH). The cells were excited alternately by 340 and 380 nm wavelengths from a Deltascan D101 (Photon Technology International, Princeton, NJ) light source equipped with a 400 nm excitation filter (Nikon) and a 510 nm emission filter (Nikon). A constant holding potential of −60 mV was applied to the cells throughout the experiment. All experiments were performed at room temperature.

Electrophysiology—For whole cell TRPC3 current measurements, coverslips of TRPC3-expressing HEK293 cells were prepared and mounted in a perfusion chamber. Electrophysiological measurements were made at room temperature 24–72 h after cells have been seeded on coverslips. The cells studied had membrane capacitances of 10–25 pF. Patch pipettes (Corning glass, 7052) with a resistance of 2–5 MΩ were generated using an automatic micropipette puller (model P-97, Sutter Instruments, Novato, CA). An agar bridge served as the electrical connection between the bath and the signal ground. Whole cell currents were elicited by voltage stimuli lasting 250 ms, delivered every 2 or 10 s, with voltage ramps from −120 to +90 mV. Data were sampled at 2 kHz, filtered at 1 kHz, recorded with an Axopatch 200B amplifier (Axon Instruments), and analyzed using pCLAMP 8.0 software (Axon Instruments). The internal pipette solution (P2) was buffered to 125 mM free Ca2+ and contained (in mM): 140 CsCl2, 10 BAPTA (cesium salt), 5 CaCl2, 2 MgATP, 0.1 GTP (sodium salt), 10 Hepes, pH 7.2, with Tris base. The bath solution was (in mM): 140 NaCl, 4.7 KCl, 10 MgCl2, 10 CaCl2, 10 glucose, and 10 Hepes, pH 7.4, with NaOH. The osmolality of these solutions was adjusted to 290–310 milliosmoles with bath solution. Ca2+ channel function was assayed by perfusion and the time required for a complete solution change was around 2 s. All voltages were corrected for liquid junction potential.

RESULTS

Low Concentrations of Gαi-linked Receptor Agonists Activate TRPC3—TRPC3 channels expressed in HEK293 cells have been shown in a number of laboratories (10, 12) including our own (11, 21) to behave as a receptor or second messenger operated cation channel, which is not activated by store-depletion (but see Ref. 15). However, the exact mechanism through which agonists activate TRPC3 remains controversial. To gain further insights into the mechanism of TRPC3 activation, we explored the concentration dependence of TRPC3 activation by the muscarinic receptor agonist methacholine, which activates PLC-β through a heterotrimeric G-protein-dependent pathway. HEK293 cells express muscarinic receptors, and respond to muscarinic agonists with a robust IP3-mediated release of Ca2+, and subsequent store-operated Ca2+ entry (23). In these
Fig. 2. IP$_3$ does not activate TRPC3. 

(a) TRPC3-GFP-expressing HEK293 cells (HEK-TRPC3) were incubated in the absence of added Ca$^{2+}$. 5 μM Gd$^{3+}$ was present throughout the experiment to inhibit endogenous CCE. 400 μM IP$_3$ in solution P1 (see “Materials and Methods”) was included in the patch pipette and the whole cell mode was established. After the Ca$^{2+}$ release phase, 2 mM external Ca$^{2+}$ and 100 μM methacholine (MeCh) were sequentially added as indicated. 

(b) Similar experiment to (a), except that external application of methacholine was performed before Ca$^{2+}$ addition. 

(c) Similar to (a), except that 1 mM (2,4,5)IP$_3$ was included in the patch pipette instead of 400 μM IP$_3$. 

(d) Similar to (a), except that external application of methacholine was performed before Ca$^{2+}$ addition. 

(e) HEK-T3-9 cells instead of HEK-TRPC3 cells. 

(f) Similar to (e), except that external application of methacholine was performed before Ca$^{2+}$ addition. 

experiments, an automated real-time fluorescence plate reader system was utilized (see “Materials and Methods”), and background controls (i.e. Ca$^{2+}$ additions in the absence of agonist) were carried out in parallel and subtracted from the conditions with agonist. Gadolinium (Gd$^{3+}$, 5 μM) was included in the media in some wells to inhibit endogenous agonist-induced Ca$^{2+}$ entry, as described previously (21). As shown previously (21), TRPC3 expressing cells showed a larger influx of Ca$^{2+}$ than did wild-type cells in response to muscarinic receptor activation. This TRPC3-dependent influx was unaffected by Gd$^{3+}$, whereas it was completely blocked in the wild-type cells. The concentration-effect relationships for release of Ca$^{2+}$ in both cell types, as well as for the entry of Ca$^{2+}$ in the wild-type cells, were very similar. However, interestingly, the relationship for TRPC3-dependent Ca$^{2+}$ entry was left-shifted, such that concentrations of methacholine that produced little or no Ca$^{2+}$ store release were able to substantially activate TRPC3-dependent Ca$^{2+}$ entry (Fig. 1). This is not likely because of a temporal delay in accumulation of IP$_3$, because this should have resulted in a leftward shift of the wild-type cells as well. This result means either that, unlike SOCs, TRPC3 is sensitive to very low concentrations of IP$_3$, or that IP$_3$ is not involved in TRPC3 activation. To address this issue, we next decided to directly introduce IP$_3$ into cells and determine its effect on TRPC3-mediated Ca$^{2+}$ entry. 

**IP$_3$ Does Not Activate TRPC3**—TRPC3-mediated Ca$^{2+}$ entry in response to introduction of IP$_3$ into stable TRPC3-GFP-expressing HEK293 cells (HEK-TRPC3) was assessed by Fura-2 imaging of single cells as indicated under “Materials and Methods.” Five μM Gd$^{3+}$ was used throughout these experiments to fully inhibit the endogenous CCE pathway in response to store depletion induced by intracellular introduction of IP$_3$ or external application of methacholine. Surprisingly, IP$_3$ did not activate TRPC3-mediated Ca$^{2+}$ entry over a wide range of concentrations (2–400 μM). Even at very high concentrations (400 μM; Fig. 2a), IP$_3$ failed to activate TRPC3 while subsequent addition of methacholine (100 μM) consistently activated TRPC3 in the same cells (n = 58). The Ca$^{2+}$ rise seen after methacholine addition resulted entirely from Ca$^{2+}$ entry rather than release from internal stores, because when we added methacholine after IP$_3$ introduction into the cell in the absence of external calcium, we observed no additional Ca$^{2+}$ release (Fig. 2b). Thus, these concentrations of IP$_3$ were sufficient to deplete intracellular Ca$^{2+}$ stores completely (n = 10; Fig. 2b). Similar concentrations of IP$_3$ in the absence of external Gd$^{3+}$ maximally activated CCE in wild-type cells, as methacholine did not induce a further increase of Ca$^{2+}$ entry, and also induced a complete depletion of internal Ca$^{2+}$ stores in wild-type HEK293 cells (data not shown).

To rule out possible complications because of metabolism of IP$_3$, we also used high concentrations of (2,4,5)IP$_3$ and GPIP$_2$, two non-metabolized analogues of IP$_3$ (22, 24). When 1 mM
Fig. 3. TRPC3 conductance is not increased by IP3. a, mean whole cell inward currents measured at −60 mV in wild-type cells, TRPC3-expressing cells, and TRPC3-expressing cells with 400 μM IP3, in the patch pipette. b, representative inward current (at −60 mV) from HEK293-Wt and HEK-TRPC3-GFP cells, before and after application of 100 μM methacholine (MeCh), are plotted as a function of time. Only the TRPC3-expressing cell shows inward current developing after MeCh addition. Basal currents (as summarized in a) have been subtracted. c, representative currents elicited by 250-ms voltage ramps every 2 s ranging from −120 to +80 mV from wild-type cells, and from HEK-TRPC3 cells before and after addition of 100 μM MeCh. d, inward current (at −60 mV) from a TRPC3-expressing HEK293 cell after internal application of 400 μM IP3 and subsequent external addition of 100 μM MeCh, is plotted as a function of time. e, representative currents elicited by 250-ms voltage ramps ranging from −120 to +80 mV, after introduction of IP3, and after external application of MeCh. The data were derived from a total of 9 (wild-type), 25 (TRPC3), and 17 (TRPC3 + IP3) experiments. The experiments in b–e are representative of at least 8 separate experiments.

(2,4,5)IP3 (Fig. 2c) or 1 mM GPIP2 (Fig. 2d) were introduced into HEK-TRPC3 cells, no TRPC3-mediated Ca2+ entry was seen when Ca2+ was restored to the external medium, while again methacholine (100 μM) activated TRPC3 in the same cells.

These findings are clearly inconsistent with the previously published findings of Kiselyov et al. (15, 16). These authors observed activation of TRPC3 single channel currents in the cell-attached configuration after stimulation with carbachol. These TRPC3 currents declined when the patch was excised, and were restored upon addition of IP3 and the IP3 receptor to the patch (but not in the IP3 receptor alone), leading to the conclusion that IP3 was the mediator of TRPC3 activation. These authors used a clonal HEK293 cell line stably expressing TRPC3 (T3-9) (10). To ensure that our apparently discrepant findings could not be attributed to the use of a different cell line, we examined the activation of TRPC3 in response to introduction of IP3 into T3-9 cells using the same protocol in Fig. 2a. As shown in Fig. 2e, 400 μM IP3 did not induce any TRPC3-mediated Ca2+ entry in T3-9 cells, whereas methacholine substantially activated TRPC3 in the same cells (n = 9). This concentration of IP3 was sufficient to induce complete store depletion in T3-9 cells, showing that the Ca2+ signal in response to methacholine was because of entry rather than Ca2+ release from internal stores (n = 4, Fig. 2f).

To ensure that the failure of IP3 to activate TRPC3 was not because of the loss of some necessary co-factor because of diffusion from the patch-clamped cells, we also microinjected (2,4,5)IP3 into TRPC3-GFP expressing HEK293 cells as described earlier (22). In TRPC3 cells, (2,4,5)IP3 activated Ca2+ entry in the absence of Gd3+, indicating that microinjection of this poorly metabolized inositol trisphosphate efficiently discharges stores and activates CCE, but fails to activate TRPC3 channels (data now shown).

We next investigated the regulation of TRPC3 expressed in HEK293 cells by examining ionic currents with the whole cell patch clamp technique. After establishing the whole cell configuration, cell membrane potential was clamped to a holding potential of +30 mV, and 250-ms voltage ramps from −120 to +80 mV were applied every 2 s. Following break-in, the whole cell currents measured at −60 mV were greater in TRPC3 expressing cells than in wild-type (Fig. 3b). The failure to detect agonist-activated currents in wild-type cells allows us to examine the activation of TRPC3 in the absence of the CCE blocker, Gd3+. A typical current-voltage relationship curve (I-V curve) before and after addition of methacholine to a HEK-TRPC3 cell is shown in Fig. 3c. We examined the ability of IP3 to induce TRPC3 current in HEK-TRPC3 cells by including 400 μM IP3 in the patch pipette. Consistent with the Fura-2 experiments in Fig. 2, no current developed after IP3 introduction into HEK-TRPC3 cells, and the steady-state current was not significantly different from TRPC3 cells without IP3 introduction. Again, 100 μM methacholine induced large inward currents in the IP3-treated cells (Fig. 3d). A lower, perhaps more physiological concentration of IP3 (20 μM), as used by Kiselyov et al. (16), was also inefficient in
activating TRPC3 currents (data not shown, and see Fig. 6c). Methacholine increased inward and outward whole cell currents as seen in the I-V curves (Fig. 3, c and e). These effects were highly reproducible; IP$_3$ introduction into HEK-TRPC3 cells never resulted in the development of current, whereas methacholine consistently yielded a substantial increase in TRPC3 current amplitude.

**IP$_3$ Receptor Activation Is Not Required for TRPC3 Activation**—One point of evidence supporting the requirement of IP$_3$ receptors in TRPC3 activation was the sensitivity of TRPC3 to the membrane permeant IP$_3$ receptor antagonist 2-APB (17). 2-APB was subsequently shown to block CCE in response to store depletion by ionomycin in the DT40 B-cell line, and this compound was equally effective on DT40 cells lacking the three isoforms of the IP$_3$ receptor (25). Furthermore, 2-APB was shown to be more efficient from the outside of the cell suggesting that it is likely inhibiting SOC channels through a direct action (26).

Heparin is a competitive IP$_3$ receptor antagonist that has been previously reported to inhibit Ca$^{2+}$ release and Ca$^{2+}$ entry in response to agonist or to direct introduction of IP$_3$ into the cell, but has no effect on CCE induced by passive depletion of Ca$^{2+}$ stores by thapsigargin or ionomycin (22, 25). Unlike 2-APB, heparin is membrane-impermeant and so we introduced it into the cell through the patch pipette. An effective concentration clearly entered the cell, because ~300 s after forming the whole cell mode, external application of methacholine failed to release stored Ca$^{2+}$ or activate CCE in wild-type HEK293 cells (Fig. 4a). In HEK-TRPC3 cells, Ca$^{2+}$ release because of methacholine was also blocked (Fig. 4, b and d), whereas thapsigargin-induced release was unaffected (Fig. 4d). The same protocol was used to examine the effect of heparin on TRPC3 activation in HEK-TRPC3 and T3-9 cells. In these experiments, 5 $\mu$m Gd$^{3+}$ was included in all solutions to inhibit endogenous CCE. 1 $\mu$m EGF was added followed by sequential addition of 2 mM external Ca$^{2+}$ and 100 $\mu$m methacholine (MeCh) when indicated. b, HEK-TRPC3 cells were treated in a similar way to the protocol in a, except that EGF was omitted.

**Growth Factor Receptor Activates TRPC3**—To determine whether heterotrimeric G proteins play an obligatory role in TRPC3 activation, we tested whether TRPC3-mediated Ca$^{2+}$ entry can be activated through the tyrosine kinase-PLC-$\gamma$ pathway. We serum-starved HEK-TRPC3 cells to boost EGF receptor expression. Stimulation of the EGF receptor by EGF activates PLC-$\gamma$ through receptor tyrosine kinase (27). Application of Gd$^{3+}$ was involved in agonist-induced activation of TRPC3.
of EGF (50–200 ng/ml) to wild-type HEK293 cells induced neither Ca\(^{2+}\) release nor Ca\(^{2+}\) entry upon restoration of external Ca\(^{2+}\). When higher concentrations of EGF were applied (1 \(\mu\)g/ml), only 4 cells of 45 cells studied (−8%) showed Ca\(^{2+}\) release and subsequent Ca\(^{2+}\) entry (data not shown). Stimulation of HEK-TRPC3 cells with 1 \(\mu\)g/ml EGF also resulted in Ca\(^{2+}\) release in a small fraction of the cells studied (6%; \(n = 96\)). However, all cells showed substantial TRPC3-mediated Ca\(^{2+}\) entry when Ca\(^{2+}\) was restored to the external medium regardless of Ca\(^{2+}\) release (Fig. 5a). The extent of TRPC3-mediated Ca\(^{2+}\) entry was similar when lower concentrations of EGF (100 ng/ml) were used (data not shown). The control experiment performed in the absence of EGF is shown in Fig. 5b. These results demonstrate that TRPC3 can be activated in a G protein-independent manner. Fig. 5, a and b, shows the results of individual imaging experiments showing average data from 29 and 19 cells, respectively; S.E. values were very small, and not visible at this graphic resolution.

Methacholine and OAG Cause Non-additive Activation of TRPC3—Our results to this point would seem to essentially rule out IP\(_3\) as the signal for TRPC3 activation. Membrane-permeant analogues of DAG have been shown to stimulate TRPC3 and TRPC6 expressed in Chinese hamster ovary cells, providing a possible mechanism of activation of these channels.

**Fig. 6. Methacholine (MeCh) and OAG activate TRPC3 non-additively.** a, HEK-TRPC3 cells were incubated in the presence of 2 mM Ca\(^{2+}\). 5 \(\mu\)M Gd\(^{3+}\) was present throughout the experiment to inhibit endogenous CCE. 100 \(\mu\)M MeCh was added to the cells and when a sustained plateau of Ca\(^{2+}\) entry was reached, 300 \(\mu\)M OAG was added as indicated. b, HEK-TRPC3 cells were treated in a similar protocol to a, with OAG added first followed by MeCh as indicated. c, inward TRPC3 currents, measured every 10 s (at −60 mV) are plotted as a function of time. 20 \(\mu\)M IP\(_3\) was included in the patch pipette and 100 \(\mu\)M MeCh was added to the cells followed by 300 \(\mu\)M OAG (red trace) or inversely, OAG was added before MeCh (blue trace). A control trace (black) where only MeCh was added is also shown. d, representative currents elicited by 250-ms voltage ramps ranging from −120 to +80 mV, after the whole cell mode was established with IP\(_3\) in the pipette (black), after MeCh addition (red), and after OAG addition (blue).
by PLC-linked receptors (13). Thus, we tested whether receptor activation and OAG activation of TRPC3 occur through independent pathways. Stimulation of HEK-TRPC3 cells with methacholine in the presence of external Ca\(^{2+}\)/H11001 and 5\(\mu\)M Gd\(^{3+}\)/H11001 leads to an [Ca\(^{2+}\)]\(_i\) rise that reaches a stable plateau, corresponding to sustained Ca\(^{2+}\)/H11001 entry through TRPC3 channels. Addition of OAG to these methacholine-activated cells did not induce any further increase of Ca\(^{2+}\)/H11001 entry, rather, a slight but reproducible decrease was consistently observed after addition of OAG (\(n=274\); Fig. 6a). Application of methacholine to OAG-stimulated HEK-TRPC3 cells induced Ca\(^{2+}\)/H11001 release, but did not produce any further increased Ca\(^{2+}\)/H11001 entry (\(n=177\); Fig. 6b). Fig. 6, a and b, are individual imaging experiments showing average data and S.E. of 50 and 27 cells, respectively; S.E. values are smaller than the line thickness. Patch clamp experiments showed that whole cell currents elicited by methacholine were not further increased by OAG and vise versa (Fig. 6, c and d; in the experiment shown, 20\(\mu\)M IP\(_3\), which did not affect current, was present in the pipette; similar results were obtained in the absence of IP\(_3\), data not shown). The current-voltage relationship after establishing the whole cell configuration with 20\(\mu\)M IP\(_3\) in the patch pipette after subsequent addition of methacholine (100\(\mu\)M) and after further addition of OAG (300\(\mu\)M) to the same HEK-TRPC3 cell (Fig. 6d) shows that methacholine and OAG have no additive effect on TRPC3 current amplitude. These results suggest that the same TRPC3 channels are activated by OAG and through PLC-coupled receptors. The lack of additivity is also consistent with a mechanism whereby DAG, formed as a consequence of PLC activation, is the signal for TRPC3 activation.

**Inhibitors of DAG Metabolism Activate TRPC3 and Slow the Inactivation of Agonist-activated TRPC3**—To assess the role of endogenously generated DAG in activation of TRPC3, we treated TRPC3 expressing cells with a combination of two inhibitors of DAG metabolism: DAG kinase inhibitor II (28) and the DAG lipase inhibitor, RHC80267 (29, 30). As shown in Fig. 7, a, a combination of DAG kinase inhibitor II (30\(\mu\)M) and DAG lipase inhibitor (RHC80267, 75\(\mu\)M) was added to HEK-TRPC3 cells, followed by 100\(\mu\)M methacholine (MeCh), and 50\(\mu\)M atropine (Atro, a and b), or the medium was changed from one containing Ca\(^{2+}\)/H11001 to a Ca\(^{2+}\)/H11001-free medium (0-Ca\(^{2+}\), c and d) as indicated. The time course of the [Ca\(^{2+}\)]\(_i\) changes in the absence of the inhibitors is shown in b and d. 5\(\mu\)M Gd\(^{3+}\)/H11001 was present throughout. In e and f, the time course of the decline in [Ca\(^{2+}\)]\(_i\), after atropine addition (e) or Ca\(^{2+}\)/H11001 removal (f) has been scaled between 100% (immediately before atropine addition or Ca\(^{2+}\)/H11001 removal) and 0% (value at the end of the experiment). In a, also shown is the response to the inhibitors (or lack thereof) in wild-type cells (in the absence of Gd\(^{3+}\), red trace).
Fig. 7a, the combination of inhibitors caused a rise in [Ca$^{2+}$]. This increase was not seen in wild-type cells (Fig. 7a, red trace), and was not seen in the absence of extracellular Ca$^{2+}$ (data not shown). Interestingly, in wild-type cells, the inhibitors substantially inhibited both release and entry because of methacholine, likely because of PKC activation that commonly inhibits the PLC pathway (31, 32). However, addition of 100 µM methacholine to the inhibitor-treated TRPC3 cells resulted in an increase in [Ca$^{2+}$], beyond that seen with the inhibitors alone, consistent with the finding that only minimal PLC activation is required to stimulate TRPC3 (i.e., Fig. 1). Subsequent addition of 50 µM atropine to block the muscarinic receptor caused Ca$^{2+}$ to decline to a level similar to that seen with the inhibitors alone. The rate of decline was considerably slower than that for the [Ca$^{2+}$], signal in methacholine-stimulated cells that had not been treated with the inhibitors (Fig. 7b), as shown by the normalized data from the two experiments (Fig. 7c). This effect was not because of changes in Ca$^{2+}$ pumping across the plasma membrane, because the rate of decline following removal of extracellular Ca$^{2+}$ was not affected by the inhibitors (Fig. 7, d, and f).

OAG Activation of TRPC3 Is PKC-independent—We next tested whether the effect of OAG on TRPC3 activation is mediated through PKC. TRPC3 activation by OAG was completely blocked by the PKC activator, PMA (n = 311; Fig. 8a), suggesting rather that PKC negatively regulates TRPC3 channels. This result is consistent with data in Fig. 6a showing a decrease of methacholine-induced activation of TRPC3 after OAG addition; this decrease may be because of slight activation of PKC by OAG. OAG apparently does not maximally activate PKC, because it does not block Ca$^{2+}$ release due to muscarinic receptor activation (i.e., see Fig. 6b) while phorbol ester drugs do (data not shown). Down-regulation of PKC isoforms by treatment with 1.6 µM PMA for 20 h did not prevent OAG-induced activation of TRPC3 but did prevent blockade of the response by PMA (n = 244; Fig. 8b).

**DISCUSSION**

In the present study, we have shown that when expressed in HEK293 cells, TRPC3 activation through PLC-coupled receptors is not mediated by IP$_3$ intracellular application of IP$_3$ did not activate TRPC3, and maximally effective concentrations of the IP$_3$ receptor antagonist, heparin, had no effect on the activation of TRPC3 by methacholine. Ga$_s$-linked receptors or tyrosine kinase receptors, activating PLC-β or PLC-γ, respectively, both increase TRPC3 channel activity. External application of a DAG analogue (OAG) or an increase in cellular DAG by use of DAG metabolism inhibitors stimulates TRPC3 activity. TRPC3 channel activation through receptor stimulus cannot be further increased by external application of DAG analogues. Reciprocally, DAG-mediated activation of TRPC3 cannot be augmented by agonist stimulation. The turn-off of receptor-activated TRPC3 following application of a receptor antagonist is significantly slowed by inhibitors of DAG metabolism. We conclude that receptor-mediated activation of PLC activates TRPC3 through DAG production in a PKC- and G protein-independent fashion.

These results were unexpected given the substantial published evidence that IP$_3$ receptors liganded with IP$_3$ are required for TRPC3 activation (15–17). Kiselyov et al. (15–17) studied IP$_3$ effects on single channels in excised patches, from cells previously stimulated with carbachol, whereas our findings were obtained in intact cells stimulated with a wide range of intracellular IP$_3$ concentrations. Thus, it may be that TRPC3 channels can be activated by IP$_3$ receptors in some cells, under some conditions. However, we conclude that the physiological mechanism in HEK293 cells involves signaling through DAG.

The pharmacological evidence provided by Ma et al. (17) was based on experiments with the IP$_3$ receptor antagonist 2-APB shown, in subsequent studies to be a relatively nonspecific inhibitor of other ion channels (25, 26, 33–36). A report by Venkatachalam et al. (12) demonstrated that receptor activation of TRPC3 in DT40 B-lymphocytes was unimpaired in a line lacking IP$_3$ receptors, a result consistent with the findings in this study.

TRPC3 is structurally similar to TRPC7 and TRPC6 and the three constitute a subfamily of the TRPC family. All three members of this subfamily have been shown to be DAG-activated channels (13, 14, 37). Furthermore, TRPC6 could not be activated when IP$_3$ was included in the patch pipette (13, 37). The efficacy of DAG analogues and drugs that cause an increase in intracellular DAG in activating TRPC3-mediated Ca$^{2+}$ entry, the inability of DAG analogues to have an additive effect on receptor-activated TRPC3, and the ability of DAG metabolism inhibitors to slow the reversal of agonist-depend-
ent TRPC3 activation suggest that DAG generated by stimulation of PLC-coupled receptors is the activator of TRPC3. DAG activation of TRPC6 and TRPC7 was shown to be PKC-independent (13, 14). This is consistent with our results obtained with TRPC3. In light of these findings, it is very likely that members of the TRPC3/6/7 subfamily share the same mode of activation, through DAG production independently of IP3.

Agonist concentrations insufficient to produce IP3-induced Ca2+ release from stores induce significant TRPC3-mediated Ca2+ entry. Furthermore, TRPC3 could be activated through receptor tyrosine kinase activation of PLC-γ, even in the absence of detectable IP3-induced Ca2+ release from stores. It seems therefore that TRPC3 is activated by relatively small amounts of DAG generated from low PLC activity, probably in a membrane-delimited fashion, as previously proposed by Hofmann et al. (13).

In conclusion, receptor-activated TRPC3 channels are activated by DAG production independently of IP3 and G proteins. These data support a similar activation mechanism for the TRPC3/6/7 subfamily and call into question the previously proposed role of IP3 in activating TRPC3 channels, as well as the proposed conformational coupling mechanism for this ion channel. Accumulating experimental evidence suggests that members of the TRP family of ion channels are requisite components of native Ca2+-permeant channels activated through physiological stimuli. Clearly, further work is needed to understand how TRP channels are gated and regulated and how their activation participates in complex physiological functions.

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