The human TRPC6 channel was expressed in human embryonic kidney (HEK) cells, and activity was monitored using the giga-seal technique. Whole cell membrane currents with distinctive inward and outward rectification were activated by carbachol (CCh) in TRPC6-expressing cells, but not in lacz-transfected controls. The effect of CCh was steeply dose-dependent with a \( K_d \) of \(-10 \mu M \) and a Hill coefficient of \( 3-4 \). A steep concentration-response relationship was also observed when TRPC6 activity was measured using a fluorescence-based imaging plate reader (FLIPR) assay for membrane depolarization. Ionomycin, thapsigargin, and dialysis of the cell with inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) via the patch pipette had no effect on TRPC6 currents, but exogenous application of 1-oleoyl acetyl-sn-glycerol (OAG, 30–300 \( \mu M \)) produced a slow increase in channel activity. The PKC activator, phorbol 12-myristate 13-acetate (PMA, 0.5 \( \mu M \)) had no significant acute effect on TRPC6, or on the subsequent response to OAG. In contrast, the response to CCh was blocked >90% by PMA pretreatment. To further explore the role of DAG in receptor stimulation, TRPC6 currents were monitored following the sequential addition of CCh and OAG. Surprisingly, concentrations of CCh that produced little or no response in the absence of OAG, produced increases in TRPC6 currents in the presence of OAG that were larger than the sum of either agent alone. Likewise, the response to OAG was superadditive following prior stimulation of the cells with near threshold concentrations of CCh. Overall, these results suggest that generation of DAG alone may not fully account for activation of TRPC6, and that other receptor-mediated events act synergistically with DAG to stimulate channel activity. This synergy may explain, at least in part, the steep dose-response relationship observed for CCh-induced TRPC6 currents expressed in HEK cells.

The \( Ca^{2+} \) signaling cascade is initiated in a variety of excitable and non-excitable cells by the activation of phospholipase C (PLC) via agonist binding to G-protein-coupled membrane receptors (GPCR) (1, 2). PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI_2) leading to the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3). Ins(1,4,5)P_3 binds to its receptor on the endoplasmic reticulum causing a rapid release of stored \( Ca^{2+} \) and a transient increase in cytosolic free \( Ca^{2+} \) concentration ([\( Ca^{2+} \)]'). Through an unknown mechanism, the depletion of internal \( Ca^{2+} \) stores activates so-called store-operated channels (SOCs) present in the surface membrane. \( Ca^{2+} \) influx via SOCs causes a sustained increase in [\( Ca^{2+} \)]'. The rise in [\( Ca^{2+} \)]', combined with the production of DAG causes the activation and translocation of protein kinase C (PKC) to the plasma membrane where it presumably phosphorylates specific effector proteins and enzymes. When agonist dissociates from the GPCR, or following receptor desensitization, [\( Ca^{2+} \)] is returned to basal levels by the action of sarcoplasmic/endoplasmic reticulum \( Ca^{2+}\)-ATPase (SERCA) pumps which reload internal stores with \( Ca^{2+} \), and by the action of plasma membrane \( Ca^{2+}\)-ATPase (PMCA) pumps, which extrude \( Ca^{2+} \) from the cell, maintaining steady-state \( Ca^{2+} \) levels.

The mammalian homologues of the Drosophila transient receptor potential (TRP) channel have emerged as primary candidates for the ion channels responsible for \( Ca^{2+} \) influx following GPCR stimulation (for review see Refs. 3 and 4). In particular, the members of the TRPC subfamily have been shown in heterologous expression systems, and in some native settings to function as receptor-activated and/or store-operated channels. There are seven members of the TRPC subfamily designated TRPC1-TRPC7 (5). Although the exact composition of native TRPC channels and the rules governing subunit assembly remain largely unknown, various tissues and cell types express message for multiple TRPC channel proteins (6, 7), and there is growing evidence for heteromultimeric channel assembly (8–13). Recent studies in vitro (8) and in vivo (9) suggest that TRPC1, TRPC4, and TRPC5 associate, and that TRPC3, TRPC6, and TRPC7 associate, but that cross-association between these two groups does not occur. Likewise the mechanism of activation following receptor stimulation may differ for these two subgroups. In particular, the activity of heterologously expressed TRPC3, TRPC6, and TRPC7 is increased by exogenous application of OAG (14–18), whereas TRPC1, TRPC4, and TRPC5 are unaffected (14). This result suggests that generation of DAG may be the mechanism by which this subfamily of TRPC channels is activated following GPCR stimulation. In support of this hypothesis, the activity of TRPC3, TRPC6, and TRPC7 appears to be increased by inhibiting DAG-lipase with RHC80267 (14, 16, 19, 20), a maneuver that is
expected to increase DAG. Whether or not DAG is the physiological activator of this subfamily of TRPC channels or simply a modulator of channel activity remains an open question.

In the present study we examine the regulation of heterologously expressed human TRPC6 channels by receptor stimulation. The TRPC6 currents recorded in HEK 293 cells were similar to those reported by other groups, but the dose-response relationship for activation by receptor stimulation was very steep, exhibiting a Hill coefficient of 3–4. The response to exogenous application of OAG was slow and relatively modest when compared with receptor stimulation. Interestingly, the response to OAG was unaffected by prior activation of PKC by phorbol ester, whereas the response of TRPC6 to receptor stimulation was blocked. Lastly, concentrations of agonist that produce little or no effect in the absence of OAG produced substantial stimulation of TRPC6 in the presence of this DAG analog. Together these results suggest that although DAG may modulate TRPC6 channel activity via a PKC-independent mechanism, some other GPCR-mediated event acts in a synergistic fashion to activate this channel. This synergy may explain, at least in part, the steep dose-response relationship observed.

MATERIALS AND METHODS

Cell Culture—HEK 293 cells were grown at 37 °C in monolayer culture in a humidified atmosphere with 5% CO₂ using Minimal Essential Medium supplemented with 10% heat-inactivated fetal bovine serum, and 1% penicillin-streptomycin solution.

Expression of TRPC6 in Mammalian Cells—To generate mammalian cell lines stably expressing human TRPC6, the TRPC6 cDNA was subcloned into the bicistronic vector, pIRE2-EGFP. This vector directs the production of a single mRNA that produces both the EGFP and TRPC6 protein, allowing for the direct identification of transfected cells by fluorescence. The pIRE2-TRPC6 plasmid was introduced into HEK 293 cells with the aid of the cationic lipid, FuGENE 6. After transfection, 400 μg/ml G418 was added to the growth media to select stable transfectants. Twenty-four single clones, selected for expansion based on green fluorescence, were serially cultured under continuous selection pressure. Control HEK cells were transfected with the gene for β-galactosidase, and stable clones were similarly selected. Membrane preparations were isolated from each HEK clone and subjected to Western blot analysis for the evaluation of TRPC6 protein expression. 16 of 24 clonal cell lines were TRPC6-positive, and 1 clone in particular (i.e. clone 14) showed extensive and uniform GFP fluorescence and substantial expression of TRPC6 protein. All experiments reported in this manuscript were performed using TRPC6 clone 14, which were serially cultured under continuous selection pressure by G418.

Membrane Preparation and Immunoblotting—HEK membranes were collected from confluent cultures. Briefly, cells were harvested, resuspended in ice-cold lysis buffer (20 mM Tris, 5 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.5), and sonicated for 30 s on ice. Cell homogenates were centrifuged at 60 000 × g to remove nuclei, and a crude membrane fraction was isolated from the resultant supernatant by centrifugation at 40 000 × g. The membrane pellet was resuspended in lysis buffer at a concentration of 5–10 mg/ml. Following electrophoresis (7.5% polyacrylamide), proteins were transferred to polyvinylidene difluoride membranes. The membranes were probed with an affinity-purified rabbit TRPC6-specific polyclonal antibody (a) and detected, following incubation with horseradish peroxidase-conjugated anti-rabbit IgG, by SuperSignal® West Pico chemiluminescent substrate (Pierce).

Measurement of Membrane Potential by Fluorescence—The functional expression of TRPC6 was determined using a fluorescence imaging platform (FLIPR) and a proprietary expression of a voltage-sensitive dye (Molecular Devices Corp., Sunnyvale, CA, product number R-8034) as previously described (21–23). HEK 293 transfectants (TRPC6 clone 14 or lacZ control) were plated at 6 × 10⁶ per well in black 96-well clear bottom poly-d-lysine-coated plates (Biocoat, Becton Dickinson) and incubated overnight at 37 °C, 5% CO₂ to achieve 80–100% confluence. The cell culture medium was then removed, and cells of each well were washed once with 100 μl of assay buffer. NaCl assay buffer contained (in mM) 140 NaCl, 0.15 CaCl₂, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 10.0 n-glucose, 20.0 HEPES (pH 7.4). Na⁺−free N-methyl-d-glucamine (NMDG)-Cl assay buffer contained (in mM) 140 NMDG-Cl, 0.15 CaCl₂, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 10.0 n-glucose, 20.0 HEPES (pH 7.4). 100 μl of the membrane potential dye supplemented with 30 μl 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (tetraacetylmethylester) was added to each well. The plates were incubated at 37 °C, 5% CO₂ for 20 min before each experiment to allow the membrane potential dye to equilibrate across the cell membranes and to allow for cleavage of BAPTA-AM by cellular esterases. This Ca²⁺ chelator was included to minimize attenuation of TRPC6 channel activity by receptor-mediated increases in [Ca²⁺] (see “Results”). Fluorescence was recorded using the FLIPR instrument at an excitation wavelength of 488 nm and an emission wavelength of 540–590 nm by a bandpass filter obtained from Molecular Devices (FLIPR Filter Kit, catalog 0310-4077). After baseline readouts for ~10 s, 50 μl of a 3X solution of carbachol in either NaCl or NMDG-Cl assay buffer was added, and changes in fluorescence were recorded for 300 s. In those experiments where pharmacological agents were tested, the cells were preincubated for 10 min with each compound prior to stimulation with carbachol. All assays were carried out at room temperature.

Electrophysiological Techniques—The giga-seal technique for current recordings was utilized in the whole cell mode. All experiments were performed on HEK 293 cells attached to circular glass coverslips, which were transferred to a perfusable recording chamber on the stage of a Nikon inverted microscope immediately before use. The extracellular solutions contained (in mM) 160 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, and 5 HEPES (pH 7.4). The normal pipette solution contained (in mM) 145 cesium aspartate, 2 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, i.e. HEPES (pH 7.2 and pCa 8). The high EGTA pipette solution contained (in mM) 145 cesium aspartate, 2 MgCl₂, 0.3 CaCl₂, 10 EGTA, and 10 HEPES (pH 7.2 and pCa 8). In some experiments, the Na⁺ in the bath solution was iso-osmotically replaced with NMDG. Data were obtained using an Axopatch 200A amplifier (Pacer Scientific, Los Angeles, CA) and sampled on-line using pCLAMP 8.0 software. The ground electrode was an Ag-AgCl wire connected to the bath via an agar bridge containing 150 mM NaCl. All recordings were made at room temperature (~22 °C). To generate current-voltage (I-V) relations, voltage ramps from −120 to +120 mV over 200 ms were repetitively applied at 15-s intervals. Unless otherwise indicated, the holding potential between ramps was −95 mV. Representative traces of concentration-dependent response relationships were determined from the normalized initial slope of current change with time immediately following addition of agonist to the bath solution and are shown as pA pF² s⁻¹.

Curve Fitting and Statistics—The nonlinear curve-fitting module in ORIGIN graphics software was used to fit a logistic equation to the concentration-response data sets of the electrophysiology studies, whereas Graphpad Prism was the graphics software used for the membrane potential data. Comparisons between groups were performed using a Student’s t test, and p < 0.05 was considered significant. Where indicated, n equals the number of individual cells tested under each condition. Slight variations in channel expression levels, densities, were noted over the course of this study. For this reason, control responses were interspersed within each data set. Where comparisons are made for each condition, the data sets were obtained on the same batch of cells and within the same time frame.

RESULTS

Activation of Human TRPC6 by Receptor Stimulation—Exposure of HEK cells stably expressing human TRPC6 (TRPC6 clone 14 cells) to carbachol (CCh) resulted in a time-dependent activation of whole cell membrane current with distinctive inward and outward rectification properties (Fig. 1). Current increased to a peak value within ~1 min and subsequently declined slowly with time. The time course of current decay after the peak was variable from cell to cell. With Na⁺ as the major cation in the extracellular solution and Cs⁺ in the pipette, the current reversed at about −10 mV. Iso-osmotic replacement of Na⁺ in the extracellular solution with the large impermeable cation, NMDG⁺ resulted in a shift of the reversal potential to −50mV and a substantial reduction in inward current (Fig. 1A, inset), consistent with activation of a non-selective cation channel. Perfusion of the cell with a divalent-free extracellular solution after stimulation by CCh produced an immediate increase in both inward and outward current, but had no effect on the rectification properties (data not shown). Similar currents were activated in >95% of the TRPC6.
clone 14 cells tested, but were never observed in lacZ-transfected controls. TRPC6 currents were also activated by extracellular ATP (100 µM) and blocked by La³⁺ (IC₅₀ = 13 µM) and by 50 µM SK&F 96365 (data not shown). These results demonstrate that the currents recorded in TRPC6 clone 14 cells are similar to TRPC6 currents reported by other investigators (14, 16, 17).

No activation of TRPC6 was seen in response to ionomycin or thapsigargin (data not shown), suggesting that the TRPC6 channels were not activated by depletion of the internal Ca²⁺ stores. However, the subsequent response of ionomycin-treated cells to CCh was significantly reduced compared with nontreated cells suggesting that a rise [Ca²⁺], may cause feedback inhibition. Inactivation by a rise in [Ca²⁺], has been reported for mouse TRPC6 channels expressed in HEK cells (16). To test this hypothesis more directly, EGTA in the pipette solution was increased from 1.1 to 10 mM with a corresponding change in the added Ca²⁺ such that the initial calculated free Ca²⁺ level

**Fig. 1. Effect of receptor stimulation on membrane current in TRPC6 clone 14 cells.***

*A*, whole cell membrane currents were recorded in TRPC6 clone 14 cells as described under “Material and Methods.” Voltage ramps were applied every 15 s, and the outward current at +80 mV and inward current at −80 mV during each ramp is plotted as a function of time after rupture of the patch for whole cell recording. At the time indicated by the lower horizontal bar, the bath solution was changed to one containing CCh (100 µM). At the time indicated by the upper horizontal bar, the bath solution was changed to one in which the Na⁺ was iso-osmotically replaced with NMDG⁺. Inset, I-V obtained during voltage ramps at the times indicated in A (a–d). *B*, whole cell currents were recorded in TRPC6 clone 14 (circles) or in lacZ-transfected controls (triangles) with either 1.1 (solid circles) or 10 mM EGTA (open circles, triangles) in the pipette solution with pCa held constant at 8. C, mean ± S.E. (bars) and individual experimental (cell) values for the peak inward current at −80 mV following CCh addition are shown for the same conditions as in *B*. Numbers in parentheses indicate the number of individual cells tested.
remained constant (pCa = 8; Fig. 1B). A 5-fold increase in CCh-induced TRPC6 currents was observed with high EGTA in the pipette solution, i.e. with a greater Ca\textsuperscript{2+} buffer capacity (Fig. 1C).

The activation of TRPC6 by CCh was dose-dependent (Fig. 2). The major effect of increasing agonist concentration was a change in the rate of current activation. A plot of current activation rate as a function of CCh concentration yielded an apparent K\_0.5 of 8.9 and 8.8 \mu M and a Hill coefficient of 3.2 and 4.3 for outward and inward TRPC6 currents, respectively. To more fully explore the dose-response relationship, a high throughput fluorescence-based assay was developed for the measurement of membrane potential using a FLIPR. As noted above, a rise in [Ca\textsuperscript{2+}], appears to attenuate receptor-mediated activation of TRPC6. Therefore, these experiments were performed in extracellular buffer containing low Ca\textsuperscript{2+}, and the cells were preloaded with the Ca\textsuperscript{2+} chelator, BAPTA. As seen in Fig. 3A, addition of KCl under these conditions to the extracellular solution of cells equilibrated with the potential sensitive dye, caused a time- and concentration-dependent increase in fluorescence in TRPC6 clone 14. The normalized change in fluorescence was a linear function of the log[K\textsuperscript{+}]\textsubscript{o} consistent with membrane depolarization (Fig. 3B). Addition of CCh caused a similar time- and concentration-dependent increase in fluorescence in TRPC6 clone 14 cells, but not in lacZ-transfected controls (Fig. 3, C and D). The CCh response was blocked by iso-osmotic replacement of the Na\textsuperscript{+} in the extracellular buffer with NMDG\textsuperscript{+} (Fig. 3D) and by SK&F 96365 (IC\textsubscript{50} = 5 \mu M), but not by nifedipine (data not shown). A plot of the normalized peak dye response as a function of CCh concentration yielded an apparent K\_0.5 of −1 \mu M and a Hill coefficient of 1.9 (Fig. 3D). These results are consistent with an agonist-
induced, TRPC6-dependent membrane depolarization, and provide additional evidence for the steep dose-response relationship observed in the whole cell current recordings.

**Activation of TRPC6 by Diacylglycerol**—Muscarinic receptor stimulation by CCh activates PLC and produces two second messengers, DAG and Ins(1,4,5)P3. Previous investigators have shown that inclusion of Ins(1,4,5)P3 in the pipette solution has no effect on TRPC6 channel activity (14, 16, 24). Preliminary experiments confirmed this result in TRPC6 clone 14 cells (n/H11005) suggesting the Ins(1,4,5)P3 alone is not sufficient to activate TRPC6. Previous investigators have also reported that exogenous application of the membrane permeant DAG analogue, oleoyl acetyl glycerol (OAG), activates TRPC3, TRPC6, and TRPC7 channels (14, 15). As shown in Fig. 4, 100 μM OAG caused a small (compared with CCh), but statistically significant activation of TRPC6 current. The currents activated by OAG exhibited the characteristic current-voltage relationship indicative of TRPC6. Increasing OAG to 300 μM had no further effect on TRPC6 channel activity (Fig. 4), and OAG had no effect on membrane current in the absence of TRPC6 channel expression (not shown). We were surprised by the relatively poor activation of TRPC6 by OAG, as other groups had reported rather robust activation of TRPC3 and TRPC6 (14, 20, 25).

However, use of OAG from different vendors (Sigma and Calbiochem) gave the same activation profiles. Likewise, application of OAG to HEK cells transiently transfected with pIRES-TRPC6 yielded currents of low amplitude relative to CCh controls (n/H11005 6, data not shown). Thus, it appears that exogenous OAG is at best, a modest activator of TRPC6 under the conditions used here.

To determine whether the small effect of OAG on TRPC6 clone 14 was because of the activation of PKC, cells were pretreated with the PKC activator PMA. PMA had no acute effect on membrane current in TRPC6 cells or on the ability of OAG to activate TRPC6 channels (Fig. 5). In fact, the response to OAG was slightly but not significantly (p = 0.3) enhanced by PMA pretreatment. In striking contrast, the response to 100 μM CCh was reduced on average 93% by pretreatment with PMA. Thus, as previously reported (14, 16), it seems unlikely that PKC is involved in activation of TRPC6 by OAG. However, the differential effect of PMA on OAG- and CCh-induced TRPC6 currents suggests that the inhibitory action of PMA occurs either at a step proximal to production of DAG or that receptor stimulation and DAG activate TRPC6 via different mechanisms.

If receptor stimulation activates TRPC6 channels by the
generation of DAG, then the response to low concentrations of CCh and OAG should be additive. To test this hypothesis, TRPC6 clone 14 cells were challenged with CCh followed after 2 min by the addition of OAG. As seen in Fig. 6A, 0.5 M CCh produced only a small increase in outward and inward TRPC6 current. However, the subsequent addition of OAG (100 μM) produced a large increase in current that was greater than expected if the responses were simply additive. A similar result was observed when the order of addition was reversed, i.e. OAG added before CCh (Fig. 6B). The I-V relationships show that the currents activated are indeed characteristic of TRPC6 channels. The average responses in the presence of CCh alone, OAG alone, and in the simultaneous presence of both agonist are shown in Fig. 7, A and A’. In the presence of both agonists, inward TRPC6 currents were 4.9-fold greater than the sum of either agent alone. Likewise, outward currents were 2.6-fold greater in the simultaneous presence of both agents compared with the sum of either agonist alone. Thus, receptor stimulation and exogenous OAG act in a synergistic fashion to increase TRPC6 channel activity. The synergistic activation of TRPC6 was also observed in the presence of 100 μM OAG with concentrations of CCh as low as 100 nM (Fig. 7, B and B’) and 10 nM (not shown). Synergy was also observed between 30 μM OAG and 0.5 μM CCh (Fig. 7, C and C’). A study by Albert and Large (26) has shown that the activation rate and magnitude of TRPC6-like currents in rabbit portal vein myocytes stimulated by exogenous OAG are greatly enhanced by the presence of Ins(1,4,5)P3 in the pipette. The activation of outward currents (at +80 mV) in TRPC6 clone 14 by 100 μM OAG in the absence and presence of 10 μM Ins(1,4,5)P3 in the pipette was 0.18 ± 0.07 (n = 6) and 0.23 ± 0.06 (n = 15) pA pF−1 s−1, respectively; inward current activation rates (at −80 mV) were 0.050 ± 0.03 and 0.039 ± 0.01 pA pF−1 s−1, respectively. Thus, it seems unlikely that a receptor-induced increase in Ins(1,4,5)P3 alone can explain the synergistic effects with OAG, at least under the recording conditions used in the present study.

DISCUSSION

Although it has been known for some time that TRPC6 channels can be activated by exogenous application of DAG analogues, the involvement of DAG in channel activation following GPCR stimulation remains unclear. In fact, there is little direct evidence in the literature that receptor-initiated generation of DAG activates TRPC3, TRPC6, or TRPC7 channels or that the effects of DAG can fully explain channel activation following receptor stimulation. In the present study, the activation of human TRPC6 currents was examined in stably expressing HEK cells. The TRPC6 currents recorded had characteristics essentially identical to those previously reported for human TRPC6 expressed in CHO-K1 cells (14) and for mouse TRPC6 expressed in HEK cells (16). The currents, which were not constitutively active, could be activated by stimulation of GPCRs, exhibited distinctive outward and inward rectification properties, and were blocked by appropriate concentrations of
La\(^{3+}\) and SK&F 96365. TRPC6 was not activated by dialysis of the cell with Ins(1,4,5)P\(_3\), but could be activated by exogenous application of the membrane-permeant DAG analog, OAG. However, activation by OAG was slow when compared with the activation by CCh. A number of groups have reported activation of TRPC6 currents by OAG, but it is difficult to compare activation rates or magnitudes because of differences in channel expression levels. Inoue et al. (16) showed that mouse TRPC6 currents in HEK cells could be activated by 100 \(\mu\)M OAG. The inward current activation was slow (0.02 pA pF\(^{-1}\) s\(^{-1}\), estimated from their Fig. 1) relative to receptor agonists and reached a current density that was only 50% of that observed with either CCh or ATP. Hofmann et al. (14) reported the effect of several DAG analogues on single human TRPC6 channels recorded in inside-out membrane patches, but did not report the effect of OAG on whole cell currents. Surprisingly, the effects at the single channel level were rather transient, lasting only 1–2 min in the continuous presence of DAG. Delmas et al. (24) reported that exogenous OAG could rapidly activate mouse TRPC6 currents when over-expressed in rat superior cervical ganglion cells. The I-V relationship for this current however, was linear from −120 to +20 mV. Thus, TRPC6 may have different properties in this expression system. Whole cell currents from heterologously expressed rat TRPC6B splice variant in response to 100 \(\mu\)M OAG have been reported by Jung et al. (17). Although current activation rate appeared to be fast (10 pA s\(^{-1}\), estimated from Fig. 1 in Ref. 17), cell-to-cell variability and current densities achieved by OAG relative to that observed with receptor agonists were not reported. Lastly, Basora et al. (18) report that 100 \(\mu\)M OAG activates inward mouse TRPC6 currents expressed in HEK cells, and the rate of current activation was slow (2.7 pA s\(^{-1}\), estimated from Fig. 2 in Ref. 18). In the present study, 100 \(\mu\)M OAG activated inward TRPC6 currents at a rate of 1.5 pA s\(^{-1}\).
(Fig. 4) or 0.03 pA pF−1 s−1. These activation rates are similar to those reported by Inoue et al. (16), and Basora et al. (18), but appear to be slower than those reported by Jung et al. (17) and Delmas et al. (24). Interestingly, TRPC6 currents activated by OAG and submaximal concentrations of CCh continue to increase with time, often without reaching a clear maximum over the time course examined (generally 2–3 min). A recent study by Cayouette et al. (27) showed that CCh stimulates translocation of TRPC6 channels into the plasma membrane presumably via an exocytotic mechanism. Thus, the slow increase in currents observed in the present study may reflect a time-dependent insertion of channels, which then become active during continuous receptor stimulation.

Consistent with previous reports, TRPC6 currents were not acutely activated by the phorbol ester PMA, suggesting that the effect of OAG was independent of PKC. However, we also found that activation of TRPC6 currents by OAG was unaffected by pretreatment with PMA, a result not previously reported. In sharp contrast, pretreatment of the cells with PMA essentially eliminated CCh-induced activation of TRPC6 currents. Using Ba2+ influx in fura-2 assays as the indicator of channel activity, Zhang and Saffen (25) showed that OAG activates rat TRPC6A overexpressed in COS cells, but not TRPC6B, a splice variant lacking a 54-amino acid segment in the N-terminal cytoplasmic domain. Interestingly, both TRPC6A and TRPC6B were activated by CCh. This led to speculation that the channels could be activated by both DAG-dependent and DAG-independent mechanisms. In contrast, Jung et al. (17) report that 100 μM OAG activates the same TRPC6B isomorph when membrane currents were recorded in T-Rex-293 cells. The reason for this difference is unknown. It may reflect the cell type used for channel expression or the method used to estimate channel activity. In this regard, a recent study by Rosker et al. (28) suggests that the interpretation of fura-2 experiments may be more complicated than previously thought. These investigators showed that Ca2+ influx in response to receptor stimulation in TRPC3-expressing HEK cells actually occurs via the Na+/Ca2+ exchanger operating in reverse mode. They propose that Na+ entry via TRPC3 in the absence of extracellular Ca2+ produces a significant alteration in the Na+ gradient, which upon readdition of Ca2+ to the bath, fuels Ca2+ entry via the exchanger. It is well established that the Na+/Ca2+ exchanger will also transport Ba2+ and Sr2+ (29–31). Furthermore, PKC appears to be part of the Na+/Ca2+ exchanger macromolecular complex (32) and exchange activity may be activated (33) or inhibited (34) by a PKC-dependent phosphorylation event. The fura-2 experiments are further complicated by the fact that the Na+/Ca2+ exchanger is regulated by PIP2 (35, 36). Thus, PIP2 hydrolysis following receptor stimulation could have a pronounced impact on divalent cation influx in TRPC6-expressing cells via changes in Na+/Ca2+ exchange activity. In this regard, the HEK cells used in the present study appear to have a very low level of Na+/Ca2+ exchange activity, since replacement of extracellular Na+ with NMDG+ had little or no effect on outward current (Fig. 1).

The mechanism by which PMA inhibits CCh-induced activation of TRPC6 currents is unknown. It is possible that PMA attenuates receptor-mediated activation of PLC. In this regard, Zhang and Saffen (25) showed a slight reduction in CCh-induced Ca2+ release in PMA-treated TRPC6A-expressing COS cells relative to control. Likewise, the CCh-induced release of Ca2+ from internal stores of DT40 cells expressing TRPC5 was attenuated by the presence of OAG or prevented by PMA (19). Alternatively, PMA may affect the putative DAG-independent coupling mechanism between PLC and the channel, or activation of PKC may cause direct TRPC6 channel desensitization as suggested for TRPC3 and TRPC5 (19). A number
of consensus PKC phosphorylation sites are present in the putative cytoplasmic regions of the TRPC6 protein, including several that are conserved in all of the TRPC channel proteins.

The membrane potential assay for TRPC6 described in this study was developed based on the assay conditions used in the electrophysiological studies in order to specifically measure the TRPC6 response. The sensitivity of pharmacological blockade of TRPC6 in the FLIPR assay by putative cation blockers such as La3+/H11001 and SK&F 96365 in this novel functional cellular assay is similar to that described electrophysiologically (Ref. 16 and this study), which suggests that this assay format can be used for the evaluation of potential TRPC6 channel blockers. Furthermore, the ability of the assay to run in, at minimum a 96-well format, means that it has potential use as a high-throughput screening platform for the identification of novel modulators of TRPC6 channels. Using the potential-sensitive dye assay and FLIPR we showed that the CCh-induced depolarization via activation of TRPC6 occurred in a concentration-dependent fashion, as expected for an ion channel activated by GPCRs. Likewise, CCh produced a concentration-dependent increase in whole cell TRPC6 currents. However, both profiles were steep with calculated Hill coefficients of −2–4. Hill coefficients greater than one generally suggest cooperativity. If indeed, channel activation reflects a second messenger-mediated response (e.g. activation by DAG), the steep concentration-response relationship might indicate multiple cooperative binding sites. In this regard, TRPC channels are thought to be tetrameric in structure. Perhaps each subunit has a single lipid binding domain, which, through an allosteric mechanism, gives rise to cooperative channel activation upon binding of DAG. Alternatively, the steep concentration-response curve may reflect spare receptors. For example, if the channels are activated in a membrane-delimited manner by G protein subunits, agonist occupation of only a small percentage of GPCR may be sufficient to obtain full channel activation. In this scenario, agonist may bind to receptor with a Hill coefficient of 1, but full channel activation may occur at only 15% receptor occupancy. This would have two effects; an increase in slope and a shift to the left of the concentration-response curve.

As discussed above, there was a differential effect of PKC activation by PMA on OAG- and CCh-induced TRPC6 currents; PMA had no significant effect on OAG-activated currents, whereas PMA essentially eliminated regulation by receptor stimulation. This suggested that the mechanism of activation of OAG and receptor stimulation may be different and independent, however this does not seem to be the case. To test this hypothesis, the effects of submaximal concentrations of agonists were examined to evaluate possible additivity. Interestingly, the inward and outward current responses following the sequential addition of both agents were 2–4-fold greater than the sum of either agent alone. The synergistic effect appeared to be independent of the addition order and was seen with concentrations of CCh below threshold (i.e. 10–100 nM). Synergy between OAG and receptor stimulation may provide important clues to the actual mechanism of activation of these channels. One possibility is that hydrolysis of PIP2 per se, i.e. reduction in channel-associated PIP2, is the critical event in

![FIG. 7. Summary of current responses following the sequential application of CCh and OAG to TRPC6-expressing cells. Experiments were performed as described in the legend to Fig. 6 using different concentrations of CCh and OAG. Mean ± S.E. outward (A–C) and inward (A'–C') values for current activation rate recorded before (pre), in the presence of CCh alone, OAG alone, or the simultaneous presence of both agents at the concentrations indicated below each bar. The bar labeled sum indicates the sum of the means for the two agonists alone. The number in parentheses indicates the number of individual cells tested under each condition.](image-url)
channel activation. A number of channels and transporters are now thought to be regulated by PIP$_2$ binding (35) as are several members of the TRP channel family including TRPV1 (37), TRPM7 (38), and Drosophila TRP and TRPL (39, 40). We recently showed that TRPL single channels recorded in excised inside-out membrane patches could be activated by specific hydrolysis of PIP$_2$ by exogenous application of PLC-β and reversibly inhibited by application of PIP$_2$, but not by PI, PS, PC, or PE (39). Furthermore, we provided evidence that neither PIP$_2$ hydrolysis nor DAG generation alone were sufficient to explain activation of TRP by receptor stimulation and speculated that DAG and enzymatic cleavage of PIP$_2$ may act in a synergistic fashion to fully activate the channel. Another possibility is that elevated [Ca$^{2+}$], within a restricted microdomain may provide a mechanism of priming or sensitizing the channels to a subsequent large increase in DAG. In a recent review article, Hardie (40) suggests that such a mechanism may be responsible for the explosive positive feedback observed during channel activation in the microvilli of the Drosophila photoreceptor cell. Likewise, the synergistic effects of OAG and receptor stimulation on TRPC6 channels observed in the present study may explain the steep concentration-response relationship noted above. Lastly, in a recent report Cayouette et al. (27) have shown that TRPC6 channels are inserted into the membrane, presumably via an exocytotic mechanism, in response to receptor stimulation by CCH. The concentration of agonist required however, was less than that needed for activation of channel current. Thus, DAG production may facilitate membrane fusion events and increase channel density in the plasma membrane, which in turn would enhance subsequent current responses to receptor agonists. Irrespective of the exact mechanism of synergy, the results of the present study clearly show that activation of TRPC6 by receptor stimulation is complex, and although DAG plays at least a modulatory role, other events downstream of PLC appear to be critical to full channel activation.

Acknowledgments—We thank Dr. A. J. Wolstenholme (Department of Biology and Biochemistry, University of Bath, Bath, UK) for the gift of the human TRPC6 cDNA clone, Dr. Pamela Tranter for technical assistance with the KCl FLIPR experiments, and Dr. Anne Dodge (Novartis Respiratory Research Centre, Horsham, UK) for her expert review of the article.

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