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Regulation of Canonical Transient Receptor Potential (TRPC) Channel Function by Diacylglycerol and Protein Kinase C*

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The mechanism of receptor-induced activation of the ubiquitously expressed family of mammalian canonical transient receptor potential (TRPC) channels has been the focus of intense study. Primarily responding to phospholipase C (PLC)-coupled receptors, the channels are reported to receive modulatory input from diacylglycerol, endoplasmic reticulum inositol 1,4,5-trisphosphate receptors and Ca^{2+} stores. Analysis of TRPC5 channels transfected within DT40 B cells and deletion mutants thereof revealed efficient activation in response to PLC-β or PLC-γ activation, which was independent of inositol 1,4,5-trisphoshate receptors or the content of stores. In both HEK293 cells and DT40 cells, TRPC5 and TRPC3 channel responses to PLC activation were highly analogous, but only TRPC3 and not TRPC5 channels responded to the addition of the permeant diacylglycerol (DAG) analogue, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG). However, OAG application or elevated endogenous DAG, resulting from either DAG lipase or DAG kinase inhibition, completely prevented TRPC5 or TRPC4 activation. This inhibitory action of DAG on TRPC5 and TRPC4 channels was clearly mediated by protein kinase C (PKC), in distinction to the stimulatory action of DAG on TRPC3, which is established to be PKC-independent. PKC activation totally blocked TRPC3 channel activation in response to OAG, and the activation was restored by PKC-blockade. PKC inhibition resulted in decreased TRPC3 channel deactivation. Store-operated Ca^{2+} entry in response to PLC-coupled receptor activation was substantially reduced by OAG or DAG-lipase inhibition in a PKC-dependent manner. However, store-operated Ca^{2+} entry in response to the pump blocker, thapsigargin, was unaffected by PKC. The results reveal that each TRPC subtype is strongly inhibited by DAG-induced PKC activation, reflecting a likely universal feedback control on TRPCs, and that DAG-mediated PKC-independent activation of TRPC channels is highly subtype-specific. The profound yet distinct control by PKC and DAG of the activation of TRPC channel subtypes is likely the basis of a spectrum of regulatory phenotypes of expressed TRPC channels.

The superfamily of TRP[1] ion channels contains a large group of channels mediating an array of signal and sensory transduction pathways (1–4). Members of the TRPC subfamily of channels are ubiquitously expressed in vertebrate cells and are the products of at least seven genes coding for cation channels that appear to be activated primarily in response to PLC-coupled receptors (1, 2, 5, 6). TRPC channels are related closely in structure and function to the group of TRP channel proteins first identified in *Drosophila* that mediate the PLC-dependent light-induced current in retinal cells (2, 7, 8). Interest has focused on the vertebrate TRPC subfamily because these channels have been implicated as important mediators of Ca^{2+} entry (3–5, 9). Evidence indicates that they may function as "store-operated" channels (6, 10–16) mediating the process of capacitative Ca^{2+} entry, which is essential for longer term Ca^{2+} signals and replenishment of Ca^{2+} stores (6, 10, 17, 18). Reports on the coupling between TRPC channels and intracellular $InsP_3Rs$ (12, 19–24) have suggested that TRPC channels can receive information directly from Ca^{2+} stores. However, there is also considerable evidence that TRPC channels can function independently of stores (4–6, 8, 10). In studies utilizing the triple $InsP_3R$-deficient variant of the chicken B-cell line, DT40 (DT40 $InsP_3R^{-/-}$), we and others have determined that endogenous store-operated channels are observed to operate identically as in wild-type DT40 cells (DT40*wt*), indicating that the $InsP_3R$ is nonessential for endogenous store-operated channels (25–27). Moreover, we recently reported (28, 29) that TRPC3 channels expressed in the DT40 $InsP_3R^{-/-}$ line can be activated in response to PLC-coupled receptors and function identically as TRPC3 channels expressed in DT40 *wt* cells. Our analyses reveal that TRPC3 channels are activated in response to PLC-coupled receptors and are mimicked by the application of exogenous DAG (28). Another report using the same cells revealed that the expressed TRPC3 channels can reflect input from stores and $InsP_3Rs$ (30), and it appears that the conditions under which channels are expressed may alter their coupling phenotype (6). Nevertheless, under all conditions of expression, the TRPC3 channels are clearly activated by the application of DAG (28, 30). Certainly, these results are consistent with the earlier report from Hofmann *et al*. (31) indicating that members of the closely related subgroup of TRPC3, TRPC6, and TRPC7 channels can each be activated in response

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[1] The abbreviations and trivial names used are: TRP, transient receptor potential; SOC, store-operated channel; TRPC, canonical TRP; PLC, phospholipase C; PKC, protein kinase C; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; DAG, diacylglycerol; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; TG, thapsigargin; BCR, B-cell receptor; GPCR, G protein-coupled receptor; M5R, M5 muscarinic receptor; CCh, carbachol; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; eYFP, enhanced yellow fluorescent protein; PMA, phorbol 12-myristate 13-acetate; R59949, 3-[2-[4-(bis-(4-fluorophenyl)methylene]piperidin-1-yl)ethyl]-2, 3-dihydro-2-thioxo-4(1*H*)-quin-azolinone.

to DAG through a mechanism independent of PKC. Other members of the TRPC channel family appear to behave differently. Thus, the subgroup represented by the closely related TRPC4 and TRPC5 channel proteins are reported to respond to store depletion (11, 13, 15) and to have an essential requirement for $InsP_3R$ (32). Moreover, both TRPC4 and TRPC5 channels are reported to be unresponsive to the application of DAG (31). We therefore considered it important to investigate the role of store emptying and $InsP_3Rs$ in the activation of TRPC4 and TRPC5 channels utilizing the DT40 knockout cell lines and to assess how the activation of these channels in response to PLC-coupled receptors compares with the activation of TRPC3 channels. Our results indicate some important differences in the role of DAG as a mediator of TRPC channel activation and reveal that each TRPC subtype is strongly inhibited by DAG-induced PKC activation, reflecting a likely universal feedback control mechanism for TRPC channels.

EXPERIMENTAL PROCEDURES

Culture of Cells—The DT40 chicken B-cell lines, wild-type (DT40-*wt*), triple $InsP_3R$ knockout (DT40 *InsP$_3$R$^{-/-}$*), and the PLC-γ2 knockout (DT40 *PLCγ2$^{-/-}$*) cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine, as described previously (25, 25, 26, 29). HEK293 cells and T3-65 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and G-418 as described previously (20)

Transfection of Cells—All three DT40 cell types were cultured overnight in RPMI 1640 with 10% fetal bovine serum, harvested from plates by scraping, washed in reduced-serum Optimem (Invitrogen), and then resuspended in Optimem at a final concentration of 10^7 cells/ml. 12 μg of each of the plasmids to be transfected (containing either the human M5 muscarinic receptor, mouse TRPC5 channel, or human TRPC3 channel, each in the pcDNA3.1 vector) were taken with 5 μg of the marker DNA (eYFP) and added to 0.5-ml transfection cuvettes with an electrode gap of 0.4 cm followed by the addition of 0.5 ml of the cells in Optimem (10^7 cells/ml). After thorough mixing of cells and DNA, transfection was carried out using the Gene Pulser II electroporation system (Bio-Rad) at 350 mV, 960 microfarads, and infinite resistance. The cells were recovered in Optimem (no serum added) for 3 h and then resuspended in Optimem with 10% fetal bovine serum and applied to coverslips. Cells were allowed to attach for 3 h in the case of DT40 cells and overnight in the case of HEK293 cells before fura-2 measurements were undertaken. The overall efficiency of transfection (eYFP-positive cells) was 20–30% as detected during fluorescent imaging. The methods were similar to those described previously (28, 33, 34).

Imaging of Intracellular Calcium in Single Transfected Cells—Cells grown on coverslips after transfection were placed in Hepes-buffered Krebs medium (107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11.5 mM glucose, 0.1% bovine serum albumin, 20 mM Hepes-KOH, pH 7.4) and loaded with fura-2/acetoxymethylester (2 μM) for 25 min at 20 °C. Cells were washed, and dye was allowed to de-esterify for a minimum of 15 min at 20 °C. Approximately 95% of the dye was confined to the cytoplasm as determined by the signal remaining after saponin permeabilization (35, 36). Cells on coverslips were place in "cation-safe" medium free of sulfate and phosphate anions (107 mM NaCl, 7.2 mM KCl, 1.2 mM $MgCl_2$, 11.5 mM glucose, 20 mM Hepes-NaOH, pH 7.2) in the absence or presence of 1 mM $CaCl_2$, $SrCl_2$, or $BaCl_2$ as shown elsewhere (Ref. 28, figures therein). Ca^{2+} measurements in single transfected and groups of untransfected cells were made using an InCyt dual-wavelength fluorescence imaging system (Intracellular Imaging Inc.). Cotransfected eYFP served as the transfection marker and was detected at an excitation wavelength of 485 nm. Untransfected cells (not expressing eYFP) were identified from the same field and served as control cells. After cell identification, fluorescence emission at 505 nm was monitored with excitation at 340 and 380 nm; intracellular divalent cation measurements (either Ca^{2+}, Sr^{2+}, or Ba^{2+}) are shown as 340/380 nm ratios obtained from groups of single untransfected and transfected cells. The details of these divalent cation measurements were described previously (20, 25, 25, 28, 37). Resting Ca^{2+} levels in all of the DT40 cell lines were similar, ~100–130 nM. Resting Ca^{2+} levels in the HEK293 cells and T3-65 cells were 50–100 nM. All measurements shown are representative of a minimum of three and in most cases of a larger number of independent experiments.

Materials and Miscellaneous Procedures—Plasmids obtained were as follows: human TRPC3 cDNA from Craig Montell (Johns Hopkins University), mouse TRPC5 from Michael Schaeffer (Freie Universitaet, Berlin), eYFP cDNA from Clontech, and human M5 musarinic receptor cDNA from L. Birnbaumer (UCLA). 1-Oleoyl-2-acetyl-*sn*-glyecrol (OAG), RHC-80267, R59949, and GF-109203X were from Calbiochem. EGTA, carbachol, and PMA were from Sigma. Thapsigargin (TG) was from LC Services (Woburn, MA). Fura-2/acetoxymethylester was from Molecular Probes, Eugene, OR. Anti-chicken IgM (M4 clone) was from Southern Biotechnology Associates, Birmingham, AL. The DT40 cell lines were kindly supplied by Dr. Tomohiro Kurosaki (Kyoto, Japan). The T3-65 clone was a kind gift from Lutz Birnbaumer (UCLA).

RESULTS AND DISCUSSION

The DT40 chicken B-cell line has been useful for evaluating the mechanisms by which store-operated channels and TRPC channels are activated. The high rate of homologous recombination in these cells facilitates targeted disruption of certain genes or groups of genes (26, 38). Two such derivatives of these cells have been of particular use in assessing Ca^{2+} signaling mechanisms: the triple $InsP_3R$-knockout variant DT40 cell line (DT40-*InsP$_3$R$^{-/-}$*), in which all three $InsP_3R$ genes have been eliminated (26), and the PLC-γ2-knockout variant (DT40-*PLCγ2$^{-/-}$*), which is devoid of both PLC-γ1 and PLC-γ2 subtypes (34, 39). These cell lines have allowed us to assess the roles of $InsP_3Rs$ and PLC-γ in the activation and maintenance of both endogenous SOCs and also of over-expressed TRPC3 channels (25, 28, 29, 34). To gain more information on the activation of TRPC channels, we investigated the role of $InsP_3Rs$ and PLC-γ on the activation of TRPC5 channels in DT40 cells. As in earlier studies (25, 28, 29), we assessed the entry of Ba^{2+}, which does not enter through endogenous SOCs in DT40 cells because of their high Ca^{2+} selectivity. DT40 cells were transiently co-transfected with TRPC5 together with eYFP to identify transfected cells as described previously (28). The activation of TRPC5 channels in response to B-cell receptor cross-linking induced by the addition of 3 μg/ml anti-IgM was assessed in fura-2-loaded wild-type B cells in the absence of extracellular Ca^{2+} (Fig. 1*A*). Stimulation of the BCR complex results in a cascade of non-receptor tyrosine phosphorylation events leading to activation of the PLC-γ2 enzyme, which cleaves phosphatidylinositol 4,5-bisphosphate to the products $InsP_3$ and DAG (28). As shown in Fig. 1*A*, a substantial $InsP_3$-mediated release of stored Ca^{2+} is observed, which slowly declines over a 6-min period as stores are depleted. As described previously (25, 28), the subsequent addition of extracellular Ca^{2+} under this condition results in substantial store-operated Ca^{2+} entry in DT40 cells. However, the addition of Ba^{2+} does not result in entry (Fig. 1*A*), reflecting the high divalent cation specificity of the endogenous store-operated Ca^{2+} entry process in these cells. Indeed, in keeping with other cells of hematopoietic origin, recent evidence clearly identifies operation of the CRAC (Ca^{2+} release-activated Ca^{2+}) channel in DT40 cells (29, 40), a channel with remarkable selectivity for Ca^{2+}, that is virtually impermeable to other alkaline-earth cations including Sr^{2+} and Ba^{2+} (28, 41). In contrast to wild-type cells, the transfected cells showed clear Ba^{2+} entry, reflecting the function of the exogenously expressed TRPC5 channel (Fig. 1*A*).

Evidence has indicated that certain members of the TRPC channel family interact with and require the presence of $InsP_3Rs$ (6, 12, 19). Indeed, recent evidence indicates that there is an important functional requirement of $InsP_3Rs$ for the TRPC5 channel (32). Using DT40-*InsP$_3$R$^{-/-}$* cells transiently cotransfected with TRPC5 and eYFP (Fig. 1*B*), the addition of anti-IgM resulted in no release of Ca^{2+} in either transfected or untransfected cells. However, there was obvious cation entry following Ba^{2+} addition, but it was exclusively in the transfected cells. The lack of release is a clear reflection of the absence of $InsP_3Rs$. Indeed, an exhaustive search for the pres-

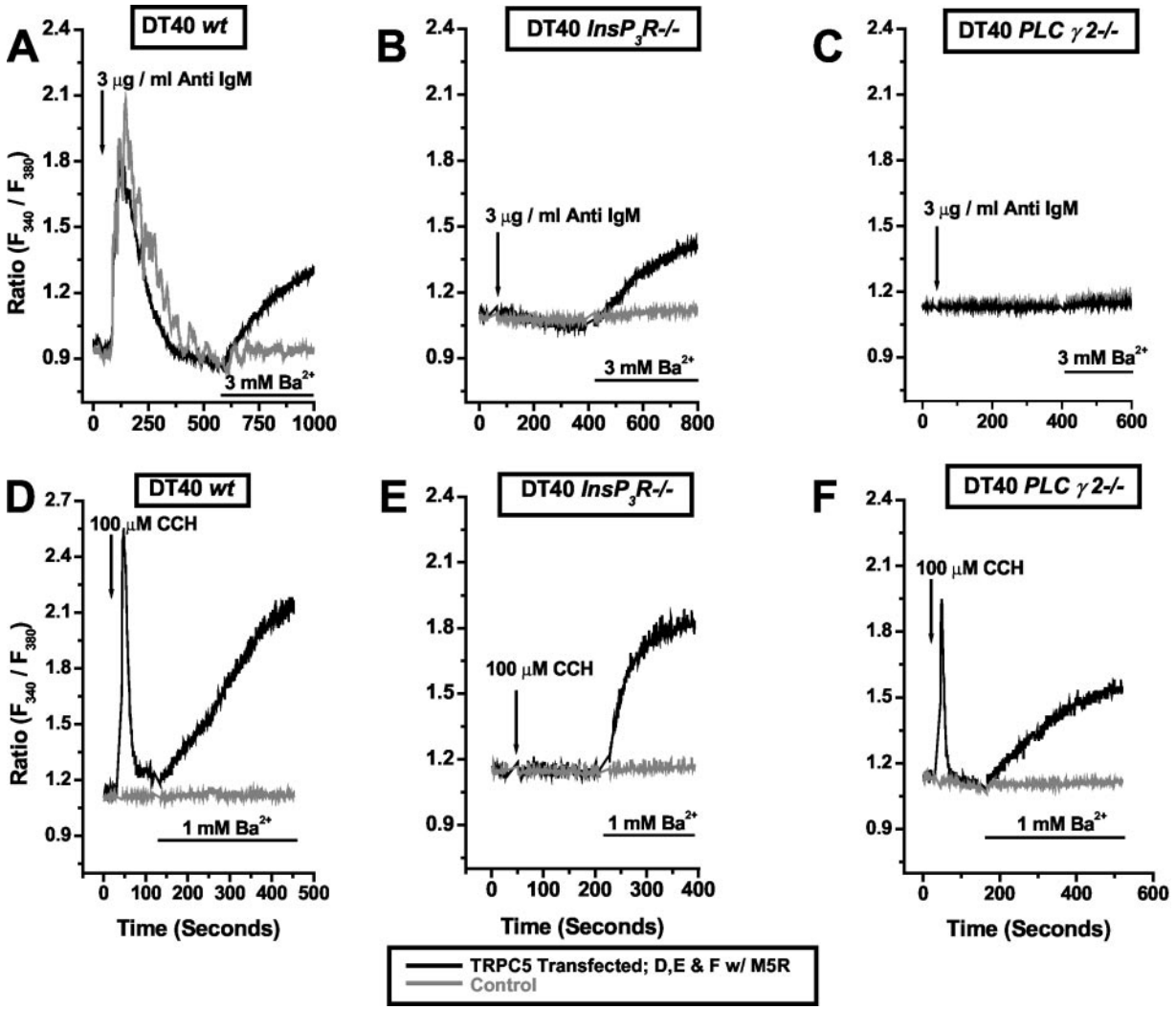

FIG. 1. **TRPC5 channels transfected into DT40 cells are activated in response to PLC-β- or PLC-γ-coupled receptors independently of $InsP_3Rs$.** Standard conditions included Ca^{2+}-free medium; *bars* indicate replacement of Ca^{2+}-free medium with medium containing Ba^{2+}. *A*, using DT40 *wt* cells cotransfected with TRPC5 and eYFP, BCR was activated by the addition of 3 μg/ml anti-IgM (*arrow*) leading to $InsP_3$-mediated Ca^{2+} release though PLC-γ2 activation. The subsequent addition of 3 mM Ba^{2+} (*bar*) caused entry of Ba^{2+} only in transfected cells (*black trace*) and not in untransfected cells (*gray trace*). *B*, same as in *A*, but in the DT40 triple $InsP_3R^{-/-}$ cells. *C*, same as in *A*, but in the DT40 $PLC\gamma2^{-/-}$ cells. *D*, using DT40 *wt* cells cotransfected with TRPC5, M5R, and eYFP, M5R was activated by the addition of 100 μM CCh (*arrow*) leading to rapid $InsP_3$-mediated Ca^{2+} release through PLC-β activation. The subsequent addition of 1 mM Ba^{2+} (*bar*) caused entry of Ba^{2+} only in the transfected cells (*black trace*) and not in the untransfected cells (*gray trace*). *E*, same as in *D*, but in the DT40 triple $InsP_3R^{-/-}$ cells. *F*, same as in *D*, but in the DT40 $PLC\gamma2^{-/-}$ cells.

ence of $InsP_3Rs$ by examining transcripts, full-length proteins or fragments thereof, $InsP_3$ binding activity, or physiological $InsP_3R$-mediated Ca^{2+} release in intact or permeabilized cells confirmed, in all cases, the absence of any $InsP_3Rs$ in these cells (25–28). The observed Ba^{2+} entry in transfected cells provides clear evidence that the over-expressed TRPC5 channel *can* function in the absence of $InsP_3Rs$. Moreover, the TRPC5 channel becomes activated without any prior depletion of Ca^{2+} stores and requires only PLC activation. This is a further important observation because there are a number of reports indicating the TRPC5 channels are store-dependent (11, 13). These results agree with others indicating that the TRPC5 channel displays a receptor-operated PLC-dependent phenotype and is activated independently of store depletion (42).

In contrast, to most other cells that express the two known PLC-γ isoforms, PLC-γ and PLC-γ2, B cells are unusual in expressing only the PLC-γ2 isoform, which is activated in response to BCR cross-linking (34). Hence, knockout of the gene for PLC-γ2 in DT40 cells results in cells devoid of all PLC-γ enzyme activity (34, 39). As expected, anti-IgM induced no release of Ca^{2+} in the DT40-$PLC\text{-}\gamma2^{-/-}$ cells (Fig. 1*C*) because no $InsP_3$ was generated. Moreover, TRPC5 channels are clearly not activated in these cells. This reveals that even though the rest of the BCR-coupled complex is intact, BCR-induced TRPC5 activation requires the PLC-γ2 enzyme. We determined a similar PLC-γ2-requirement for BCR-induced activation of TRPC3 (data not shown).

Whereas the PLC-γ2-mediated activation of TRPC5 channels in response to BCR appears independent of store emptying and $InsP_3Rs$, we wished to determine whether the same held for TRPC5 activation in response to GPCRs coupled to PLC-β. Endogenous GPCRs in DT40 cells have not been detected, and yet GPCRs give robust Ca^{2+} responses when exogenously expressed in these cells (28). We transiently cotransfected the M5 muscarinic receptor, together with TRPC5 and the marker eYFP in the DT40-*wt* cells (Fig. 1*D*). The efficient coexpression of all three plasmids within single DT40 cells was recently documented (28). The rapid release of Ca^{2+} induced in response to CCh in the transfected DT40 cells reflects the fact that the

cells express the coupling machinery (G protein and PLC-β) required for $InsP_3$-mediated Ca^{2+} signal generation. Moreover, the addition of Ba^{2+} reveals that the TRPC5 channel is clearly activated in response to PLC-β activation. As shown in Fig. 1*F*, TRPC5 channels were activated in response to M5R stimulation in the DT40-*$InsP_3R^{-/-}$* cells revealing that, for the BCR, neither store emptying nor the presence of the $InsP_3R$ is required in the PLC-β-mediated activation of TRPC5. Lastly, the same triple transfection (M5R, TRPC5, and eYFP) was conducted on DT40-*$PLC\gamma2^{-/-}$* cells (Fig. 1*F*). Upon stimulation with CCh, the transfected cells exhibited clear Ca^{2+} release, which is absent in the untransfected cells; and subsequent Ba^{2+} addition resulted in robust entry only in the transfected cells, indicative of TRPC5 activation. In analogous triple transfection studies, we also observed that TRPC3 channels could be activated in the absence of PLC-γ2 (data not shown). Thus, it appears that overexpressed TRPC channels can be activated in the absence of PLC-γ. These results are interesting because it was recently revealed that GPCR-induced endogenous SOC activation in DT40 cells requires the PLC-γ2 protein (34), whereas TG-induced SOC activation was independent of PLC-γ2. One possibility is that TRPC channels are not the mediators of store-operated Ca^{2+} entry in response to receptors in DT40 cells. However, we must also consider that the overexpressed TRPC channels do not necessarily reflect the phenotype of endogenously expressed TRPC channels (6)

We also investigated the coupling of TRPC5 channels expressed in HEK293 cells, which endogenously express muscarinic receptors. TRPC5 function was measured using Sr^{2+} rather than Ba^{2+} because HEK293 cells display a constitutive permeability to Ba^{2+} (20). Also, because Sr^{2+} can be transported by SERCA and plasma membrane pumps, we could examine the reversal of TRPC5 more directly. As in DT40 cells, endogenous SOC-mediated entry is highly selective for Ca^{2+}. Thus, after CCh-induced store-depletion in wild-type HEK293 cells, no entry was observed upon addition of Sr^{2+} (Fig. 2*A*), whereas, under this same condition, robust entry of Ca^{2+} would be observed (20). In TRPC5-transfected HEK293 cells, Sr^{2+} addition resulted in substantial entry. Upon removal of CCh, there was a rapid decrease in Sr^{2+} indicating that maintenance of TRPC5-mediated divalent cation entry requires the continued activation of receptor and providing further evidence that the depletion of stores *per se* is not the trigger for activating TRPC5 channels. More direct evidence for this was provided by examining TRPC5 channel activity following the addition of TG, which causes complete and irreversible store depletion as a result of SERCA pump inhibition (35, 43). Whereas TG-induced store depletion results in substantial Ca^{2+} entry in HEK293 cells (20), no entry of Sr^{2+} was observed (Fig. 2*B*). With TRPC5-transfected cells, the addition of Sr^{2+} after TG-induced store release again resulted in no entry. The subsequent addition of CCh resulted in apparently normal Sr^{2+} entry through TRPC5 channels. Similar results on the activation of TRPC5 channels following TG-induced Ca^{2+} release were obtained using DT40 cells (data not shown). These data reveal yet more compellingly that the action of TRPC5 is independent of store emptying. Indeed, the complete emptying of stores with TG does not appear to have even a permissive action on the subsequent activation of TRPC5 channels. The results contrast with earlier reports suggesting that TRPC5 channels could become activated by store depletion (11, 13), however, it is possible that cell type and expression conditions are significant determinants in the function and coupling of over-expressed TRPC channels as discussed recently (6).

Thus far, our results indicate that in both DT40 and HEK293 cells the functional phenotype of the TRPC5 channel is almost

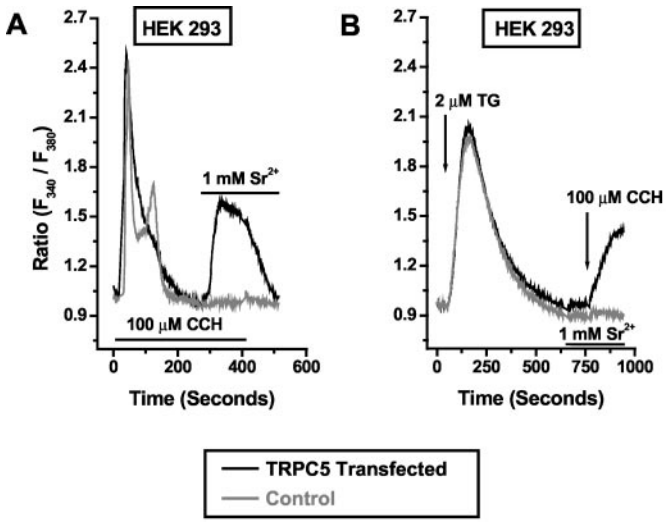

FIG. 2. **TRPC5 channels transfected into HEK293 cells are activated by the PLC-β-coupled muscarinic receptor but not by passive store depletion with the SERCA pump blocker, thapsigargin.** Standard conditions included Ca^{2+}-free medium; *bars* indicate either the addition of CCh or replacement of Ca^{2+}-free medium with medium containing Sr^{2+}. *A*, in HEK293 cells cotransfected with TRPC5 and eYFP, rapid $InsP_3$-mediated Ca^{2+} release was induced by the addition of 100 μM CCh (*bar*). The subsequent addition of 1 mM Sr^{2+} (*bar*) resulted in Sr^{2+} entry only in transfected cells (*black trace*) and not in untransfected cells (*gray trace*). Removal of CCh led to a rapid inactivation of the channel (*black trace*). *B*, in HEK293 cells cotransfected with TRPC5 and eYFP, passive and complete depletion of Ca^{2+} stores was induced by the addition of 2 μM TG (*arrow*). The addition of 1 mM Sr^{2+} (*bar*) did not lead to any entry of Sr^{2+}. The subsequent PLC-β activation due to the addition of 100 μM CCh (*arrow*) resulted in entry of Sr^{2+} via TRPC5 in the transfected (*black trace*) cells but not in the untransfected cells (*gray trace*).

identical to that of the TRPC3 channel (28). Both channels can be activated in response to GPCR-induced activation of PLC-β or receptor-induced tyrosine kinase-mediated activation of PLC-γ; and the activation of both channels does not require the presence of $InsP_3Rs$ or store depletion. Because PLC activation is required and because TRPC3 channels can be activated by exogenously applied DAG, we concluded that DAG is the mediator through which TRPC3 channels are stimulated in response to receptors (28). However, an interesting conundrum poses itself with respect to TRPC5, because it was shown earlier by Hofmann *et al.* (31) that DAG, in contrast to its stimulation of TRPC3 channels, does not activate TRPC5 channels. It was important therefore to ascertain whether a similar differential effect of DAG applied to the function of TRPC3 and TRPC5 channels in our systems or whether our expression conditions had somehow rendered the TRPC5 channel sensitive to DAG.

We transiently transfected the HEK293 cells with TRPC5 or TRPC3 and analyzed channel activation in response to both CCh-mediated PLC-β activation and treatment with OAG, the cell-permeant analogue of DAG (20, 31). It is clear from the data in Fig. 3*A* that TRPC5-mediated Sr^{2+} entry was activated in response to CCh-stimulation. However, after cessation of entry following removal of Sr^{2+} and CCh, the subsequent addition of 100 μM OAG with Sr^{2+} resulted in no entry. On the other hand, in exactly analogous experiments on TRPC3-transfected cells (Fig. 3*B*), the final addition of OAG caused a robust entry of Sr^{2+} (Fig. 3*B*). It should be noted that for both TRPC3 and TRPC5, the channels were transiently transfected under identical conditions; we did not utilize the stably TRPC3-transfected HEK293 T3-65 line used in earlier studies (20, 44) because there was no equivalent line stably expressing TRPC5 channels. Using TRPC5-transfected DT40 cells, the addition of

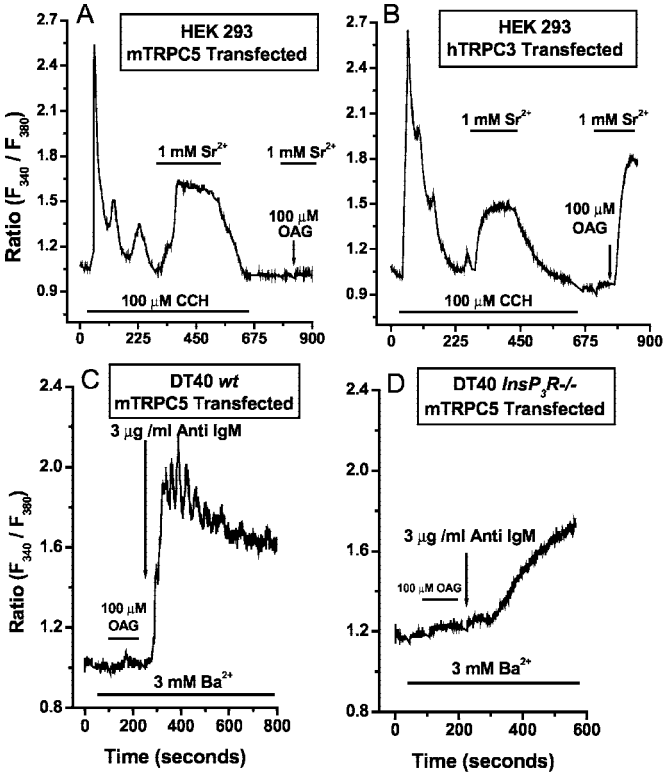

FIG. 3. **OAG activates TRPC3 channels but not TRPC5 channels in both HEK293 and DT40 cells.** Standard conditions included Ca^{2+}-free medium; *bars* indicate replacement of Ca^{2+}-free medium with medium containing Sr^{2+} or Ba^{2+}. *A*, in HEK293 cells cotransfected with TRPC5 and eYFP, rapid $InsP_3$-mediated Ca^{2+} release was induced by the addition of 100 μM CCh (*bar*). The addition of 1 mM Sr^{2+} (*bar*) caused the entry of Sr^{2+} via activated TRPC5. The subsequent addition of 100 μM OAG (*arrow*) did not activate the channel. *B*, in HEK293 cells cotransfected with TRPC3 and eYFP, rapid $InsP_3$-mediated Ca^{2+} release was induced by the addition of 100 μM CCh (*bar*). The addition of 1 mM Sr^{2+} (*bar*) caused the entry of Sr^{2+} via activated TRPC3. The subsequent addition of 100 μM OAG also caused activation of the channel leading to Sr^{2+} entry. *C*, in DT40 *wt* cells cotransfected with TRPC5 and eYFP, the addition of 3 mM Ba^{2+} (*bar*) did not lead to any constitutive entry via TRPC5. 100 μM OAG (*bar*) added in the presence of Ba^{2+} did not activate TRPC5. The subsequent addition of 3 μg/ml anti-IgM caused $InsP_3$-mediated Ca^{2+} release due to activation of PLC-γ and Ba^{2+} entry due to TRPC5 activation. *D*, same as in *C*, but in the DT40 $InsP_3R^{-/-}$ cells.

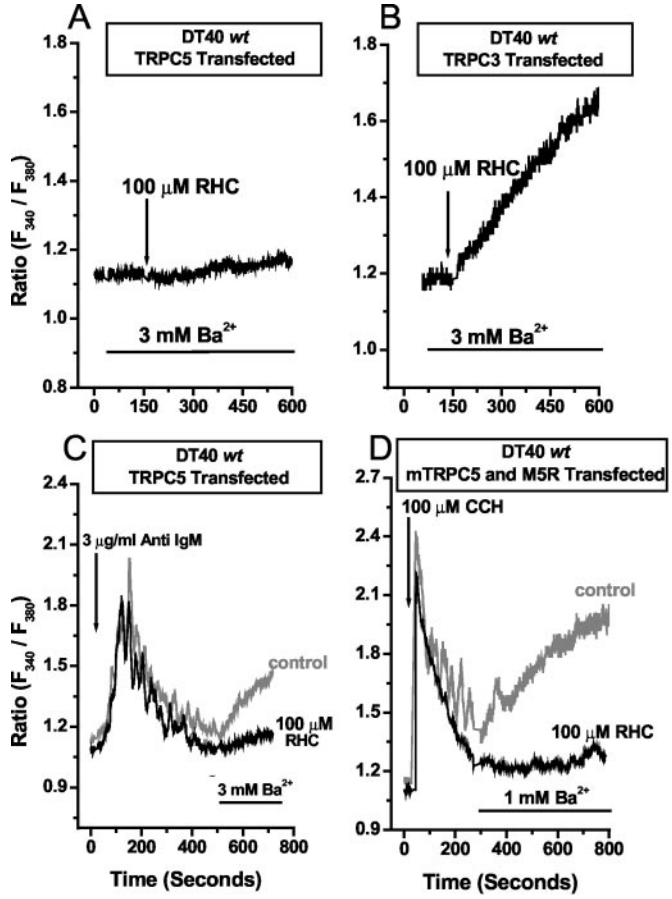

FIG. 4. **Inhibition of DAG lipase causes activation of TRPC3 channels but inhibits TRPC5 channels.** Standard conditions included Ca^{2+}-free medium; *bars* indicate replacement of Ca^{2+}-free medium with medium containing Ba^{2+}. *A*, in DT40 *wt* cells cotransfected with TRPC5 and eYFP, the addition of 3 mM Ba^{2+} (*bar*) did not cause constitutive entry via TRPC5. The subsequent addition of 100 μM RHC-80267 (*RHC*; *arrow*) did not activate TRPC5. *B*, in DT40 *wt* cells cotransfected with TRPC3 and eYFP, the addition of 3 mM Ba^{2+} (*bar*) did not result in constitutive entry via TRPC3. However, the subsequent addition of 100 μM RHC-80267 (*arrow*) activated TRPC3 and led to a rapid entry of Ba^{2+}. *C*, in DT40 *wt* cells cotransfected with TRPC5 and eYFP, the addition of 3 μg/ml anti-IgM led to $InsP_3$-mediated Ca^{2+} release and subsequent TRPC5-mediated Ba^{2+} entry upon addition of 3 mM Ba^{2+} (*bar*; *gray trace*). In the presence of 100 μM RHC-80267 (*black trace*), $InsP_3$-mediated Ca^{2+} release was intact, but TRPC5-mediated Ba^{2+} entry was absent. *D*, using DT40 *wt* cells cotransfected with TRPC5, M5R, and eYFP, the addition of 100 μMCCh led to rapid $InsP_3$-mediated Ca^{2+} release and subsequent TRPC5-mediated Ba^{2+} entry upon the addition of 1 mM Ba^{2+} (*bar*) (*gray trace*). In the presence of 100 μM RHC-80267 (*black trace*), $InsP_3$-mediated Ca^{2+} release was intact, but TRPC5-mediated Ba^{2+} entry was absent.

OAG in the presence of Ba^{2+}induced no entry, even though subsequent BCR cross-linking by anti-IgM induced a substantial entry of Ba^{2+} (Fig. 3*C*). In this experiment, however, the increased F_{340}/F_{380} ratiometric signal also has a substantial component from the BCR-induced release of stores. Therefore, we undertook the same experiment using the DT40 triple $InsP_3R^{-/-}$ cells (Fig. 3*D*). In this case, the Ca^{2+} store release component is eliminated, and although OAG again had no effect on Ba^{2+} entry, the BCR cross-linking results in TRPC5-mediated Ba^{2+} entry. A lag of ~1 min before the start of Ba^{2+} entry was observed consistently. This appears to reflect the slow BCR-induced activation of PLC-γ2. Thus, when the experiment was undertaken on cells cotransfected with M5R and TRPC5, there was little lag in the activation of TRPC5 following CCh addition (data not shown).

A further question we considered was whether the lack of effect of OAG on TRPC5 channels might reflect some divergence in the function of the permeant DAG analogue from the function of authentic, endogenously generated DAG. Endogenous DAG undergoes continual turnover through the combined actions of DAG kinase and DAG lipase, and the latter can be effectively eliminated by the DAG lipase inhibitor, RHC-80267, resulting in a rapid elevation in the steady-state level of endogenous DAG sufficient to activate TRPC3 channels (20, 31). We examined the action of RHC-80267 on DT40-*wt* cells transiently transfected with TRPC5 and found that it had no effect on Ba^{2+} entry (Fig. 4*A*), whereas it clearly activated Ba^{2+} entry in TRPC3-transfected DT40-*wt* cells (Fig. 4*B*), confirming previous observations on DAG-activation of this channel (20). Thus, despite the many similarities in function of TRPC3 and TRPC5 channels, it appears the TRPC5 channel differs in being insensitive to either exogenously added or endogenously generated DAG. With these observations in mind, we sought to evaluate whether DAG was playing a role in agonist-mediated activation of TRPC5, that is, whether agonist-mediated activation of TRPC5 was also independent of elevated levels of DAG. Therefore, we undertook experiments to assess whether increasing levels of DAG with RHC-80267 would have any per-

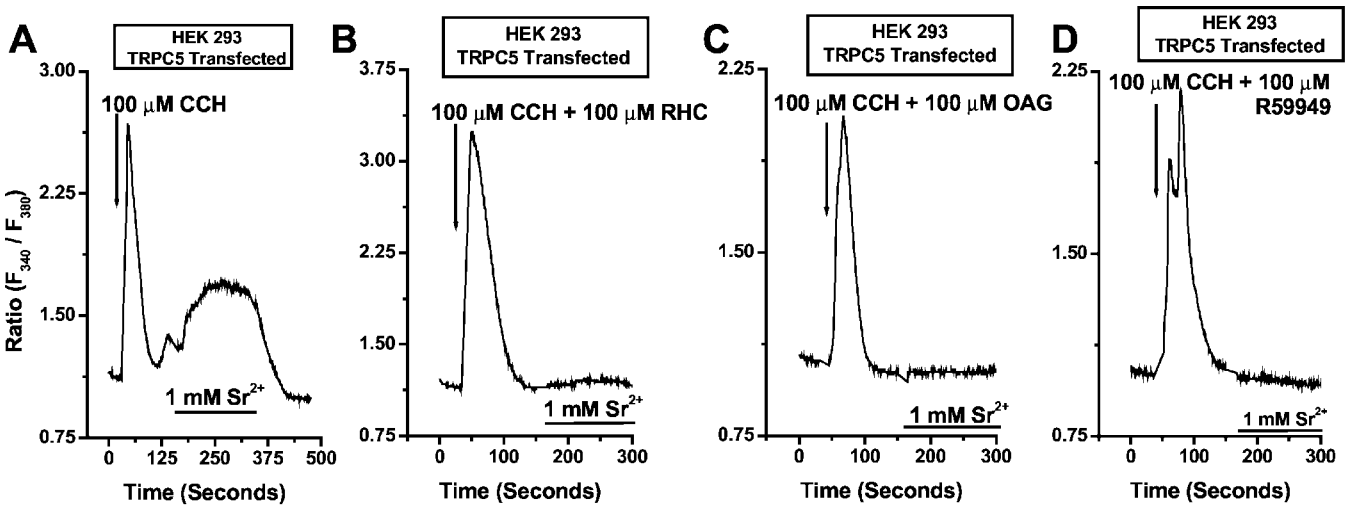

FIG. 5. **TRPC5 channels are turned off in response to an increase in DAG resulting from inhibition of DAG lipase or DAG kinase or application of exogenous OAG.** In HEK293 cells cotransfected with TRPC5 and eYFP, standard conditions included Ca^{2+}-free medium; *bars* indicate replacement of Ca^{2+}-free medium with medium containing Sr^{2+}. *A*, the addition of 100 μM CCh (*arrow*) led to a rapid $InsP_3$-mediated Ca^{2+} release. The subsequent addition of 1 mM Sr^{2+} (*bar*) led to entry of Sr^{2+} through TRPC5. *B*, same as in *A*, but with 100 μM RHC-80267 added with CCh (*arrow*). *C*, same as in *A*, but with 100 μM OAG added with CCh (*arrow*). *D*, same as in *A*, but with 100 μM R59949 added with CCh (*arrow*).

missive effect on receptor-induced TRPC5 activation. To our surprise we found that RHC-80267 completely blocked TRPC5 activation. Thus, as shown in Fig. 4*C*, using TRPC5-transfected DT40 cells, the activation of Ba^{2+} entry in response to BCR cross-linking was abolished in the presence of RHC-80267. Likewise, the DAG lipase blocker completely prevented TRPC5 activation in response to CCh in DT40 cells cotransfected with TRPC5 and M5R (Fig. 4*D*). Therefore, it appears that RHC-80267-mediated elevation of DAG blocks activation of TRPC5, whereas it activates TRPC3.

This inhibition of TRPC5 induced by the DAG lipase inhibitor could have resulted from either an elevation in the levels of basal DAG or a decrease in the levels of the products of DAG lipase, monoacylglycerol and arachidonic acid. It is also possible that the RHC-80267 molecule itself could have had a specific action on the TRPC5 channel, a property distinguishing it from TRPC3. We therefore assessed the actions of modifying DAG on TRPC5 channels by examining the effects of exogenous OAG added directly to the cells and by modifying the function of the DAG kinase. We also extended the analysis by examining these actions within the HEK293 cells. As compared with the activation of Sr^{2+} entry through TRPC5 channels in response to CCh-induced stimulation of endogenous muscarinic receptors (Fig. 5*A*), the presence of RHC-80267 added together with CCh completely abolished the activation of TRPC5 channels (Fig. 5*B*), confirming the results obtained using DT40 cells. These results further confirm that the release of stored Ca^{2+} is unaffected by the DAG lipase inhibitor, indicating that its action is not to alter the function or production of $InsP_3$ and hence not to modify the activation of PLC. Interestingly, the application of 100 μM OAG together with CCh also completely prevented the activation of the TRPC5 channel (Fig. 5*C*), consistent with the conclusion that DAG itself mediates the inhibitory action on TRPC5 activation. We sought to further this proposition by examining the modification of endogenous DAG levels by altering the function of DAG kinase, which actively converts DAG to phosphatidic acid. The agent R59949 is an effective and specific inhibitor of Ca^{2+}-activated DAG-kinase (45), the catalytic function of which is very effective in reducing the levels of DAG resulting from receptor-induced PLC activation (45). Significantly, the addition of 100 μM R59949 together with CCh completely blocked the TRPC5 channel activation. (Fig. 5*D*). This provides another independent verification that an increased level of DAG results in the deactivation of TRPC5 channel activity. It should be noted that, unlike the action of the DAG lipase inhibitor, the application of OAG or the DAG kinase blocker does significantly reduce the receptor-induced store emptying (by ~30%; see Fig. 5, *C* and *D*). However, in contrast, the effect on TRPC5 is absolute.

The results provide compelling evidence that an elevation of endogenous DAG or exogenous addition of its analogue, OAG, lead to a complete inhibition of the TRPC5 channel. We observed essentially the same inhibitory action of OAG on the TRPC4 channel, which is a close structural and functional relative of the TRPC5 channel (2). The results suggest that for the TRPC5 channel the action of DAG could represent a modification of the stimulatory action observed on TRPC3 channels (28, 31). Indeed, it was possible that combinations of stimulatory and inhibitory subunits within the likely heterotetrameric structure of TRPC channels might be important determinants of receptor activation. Because there is compelling evidence from Hofmann *et al.* (31) that the action of DAG on the closely related group of TRPC3, TRPC6, and TRPC7 channels is independent of PKC, we considered it crucial to ascertain whether the novel inhibitory action on TRPC5 and TRPC4 channels we describe here, was similarly PKC-independent.

To evaluate a role for PKC on the inhibitory action of DAG on TRPC5 channels, we utilized the aminoalkyl bisindolylmaleimide GF-109203X, which is recognized as a highly selective and potent inhibitor of multiple PKC subtypes (46). We examined the action of this PKC-modifier on DT40-*wt* cells cotransfected with the M5R and TRPC5 channel, determining its effect on the actions of the DAG lipase and DAG kinase inhibitors and exogenously added OAG. As shown in Fig. 6*A*, the activation of Ba^{2+} entry through TRPC5 channels in response to CCh was blocked by the DAG lipase inhibitor RHC-80267. Importantly, when GF-109203X was present with the DAG lipase inhibitor, TRPC5 activation was exactly as it was without inhibitors. Thus, the PKC blocker prevented the inhibition of TRPC5 channels resulting from DAG lipase inhibition. We next assessed the effect of the PKC blocker on the action of directly added OAG (Fig. 6*B*). The results clearly indicated that the inhibitory action of OAG was also prevented by the simultaneous presence of GF-109203X. Lastly, TRPC5 channel inhibition by the DAG kinase blocker, R59949, was also reversed by the PKC inhibitor (Fig. 6*C*). There are two conclusions to be drawn

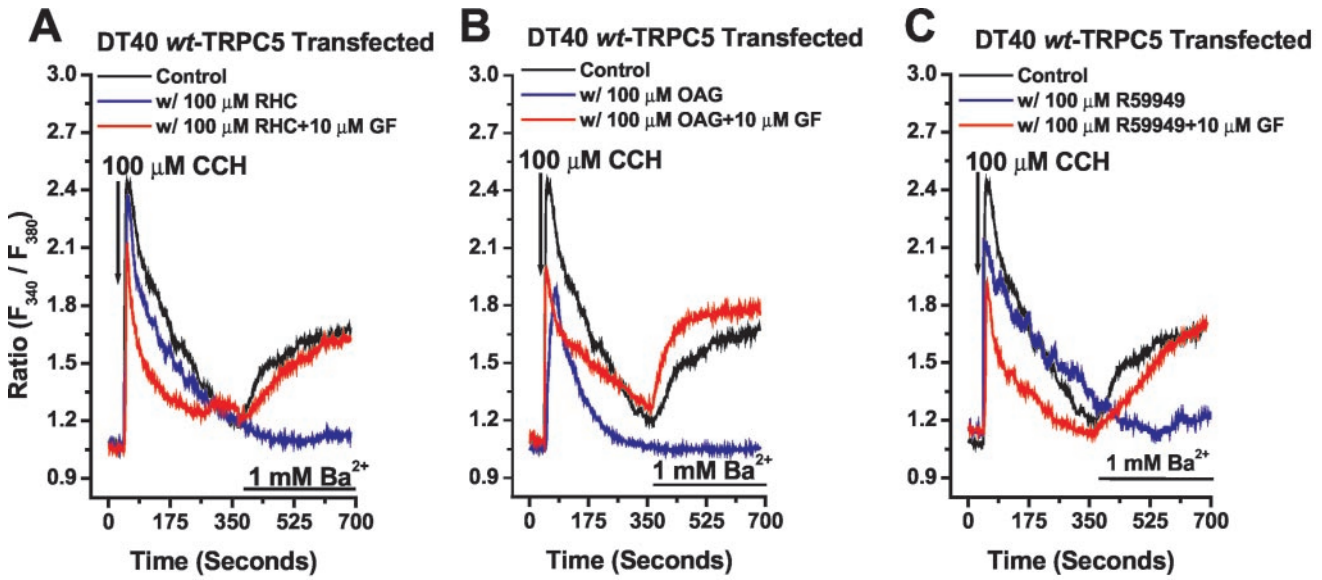

FIG. 6. **DAG-mediated inhibition of TRPC5 channels is dependent on PKC.** The experiments used DT40 *wt* cells cotransfected with TRPC5, M5R, and eYFP. Standard conditions included Ca^{2+}-free medium; *bars* indicate replacement of Ca^{2+}-free medium with medium containing Ba^{2+}. *A*, the addition of 100 μM CCh (*arrow*) caused a rapid $InsP_3$-mediated Ca^{2+} release. The subsequent addition of 1 mM Ba^{2+} caused TRPC5-mediated Ba^{2+} entry in the control cells (*black trace*). When the same trace was performed with 100 μM RHC-80267 added with CCh (*arrow*; *blue trace*), TRPC5-mediated Ba^{2+} entry was absent without affecting CCh-mediated Ca^{2+} release. When both 10 μM GF-109203X (*GF*) and 100 μM RHC-80267 were added with CCh (*arrow*; *red trace*), TRPC5-mediated Ba^{2+} entry was recovered. *B*, the addition of 100 μM Cch (*arrow*) caused rapid $InsP_3$-mediated Ca^{2+} release. The subsequent addition of 1 mM Ba^{2+} caused TRPC5-mediated Ba^{2+} entry in the control cells (*black trace*). When the same trace was performed with 100 μM OAG added with CCh (*arrow*) (*blue trace*), TRPC5-mediated Ba^{2+} entry was absent without affecting CCh-mediated Ca^{2+} release. When both 10 μM GF-109203X and 100 μM OAG were added with CCh (*arrow*; *red trace*), TRPC5-mediated Ba^{2+} entry was recovered. *C*, the addition of 100 μM CCh (*arrow*) caused rapid $InsP_3$-mediated Ca^{2+} release. The subsequent addition of 1 mM Ba^{2+} caused TRPC5-mediated Ba^{2+} entry in control cells (*black trace*). When 100 μM R59949 was added with CCh (*arrow*; *blue trace*), TRPC5-mediated Ba^{2+} entry was absent without affecting CCh-mediated Ca^{2+} release. When both 10 μM GF-109203X and 100 μM R59949 were added with CCh (*arrow*; *red trace*), TRPC5-mediated Ba^{2+} entry was recovered.

from these results. First, the induction of DAG by three different means into DT40 cells has inhibitory effects on TRPC5 channel activation, exactly as observed in HEK293 cells. Second, and more significantly, the results provide rather compelling evidence that the effects of each of these different means of inducing increased DAG levels can be reversed by the inhibition of PKC. The fact that we have a "return" of function induced by inhibition of PKC provides evidence that the function of the TRPC5 channel *per se* is not directly modified by any of the agents used. Instead, the results indicate that PKC has an important modulatory role in the receptor-induced coupling process that leads to TRPC5 channel activation.

We also analyzed the effects of PKC-induced modification of TRPC5 channels in the DT40 triple $InsP_3R^{-/-}$ cells. We observed (data not shown) that the DAG-induced inhibition of TRPC5 channels (by either inhibition of DAG lipase or the addition of OAG) is prevented by PKC inhibition in the same way as in wild-type cells. These observations exclude the possibility that the effects of DAG and PKC on TRPC5 channels are mediated through $InsP_3$Rs. Also, each of the effects of DAG modification and PKC inhibition were the same on the function of expressed TRPC4 channels (data not shown).

Our next question was whether other members of the TRPC channel family might be similarly PKC-regulated. We turned our attention to the TRPC3 channel, which we have studied in detail (20, 28). Obviously, we needed to examine a means of activating PKC that was independent of DAG, which is clearly an activator of the TRPC3 channel (28, 31). We therefore utilized the powerful PKC-activator PMA, which causes pronounced PKC-mediated phosphorylation of targets at nanomolar levels (47). Using the stably TRPC3-transfected HEK293 T3-65 cell line used in earlier studies (20), we found that OAG-mediated activation of TRPC3 is totally abolished by a 5-min pretreatment with 1 μM PMA (Fig. 7*A*). This inhibition of TRPC3 activity was completely reversed when cells were pretreated with PMA together with 10 μM GF-109203X (Fig. 7*A*). This finding provides compelling evidence that the TRPC3 channel is also PKC-modulated. Thus, it appears that DAG induces a potentially crucial bimodal regulation of TRPC3 channels. Moreover, closer examination of the data in Fig. 7*A* reveals that whereas TRPC3 channel activity following OAG addition is transient (the activity deactivates in the continued presence of 100 μM OAG), in the presence of the PKC inhibitor, this deactivation is clearly retarded. In contrast, the *rate* of OAG-induced activation of TRPC3 is identical in the presence or absence of GF-109203X. In other words, it appears that DAG rapidly activates TRPC3 prior to a slower PKC-mediated deactivation of the channel

Thus far we have addressed the function and regulation of exogenously expressed TRPCs. Although controversial, much recent work provides evidence that endogenous store-operated Ca^{2+} channels involve the function of TRPC channels (6). Because the action of PKC may be a useful and hitherto unrecognized signature of TRPC channel function, we examined the effects of PKC modification on endogenous store-operated Ca^{2+} entry. The data in Fig. 7*B* reveal that the potent PKC activator PMA had no effect on the rate or duration of Ca^{2+} entry induced in HEK293 cells in response to complete emptying of stores induced by TG. However, an interesting finding was that the Ca^{2+} entry induced in response to activation of the endogenous muscarinic receptor was prevented by almost 70% in the presence of the DAG lipase inhibitor RHC-80267, whereas Ca^{2+} release from stores was unaffected (Fig. 7*C*). In this case, the more potent direct PKC activator, PMA, completely prevented receptor-induced store emptying (likely a result of direct actions on PLC) and hence could not be used to examine effects on entry. However, the inhibitory action of RHC-80267 on receptor-induced Ca^{2+} entry was mimicked precisely by the

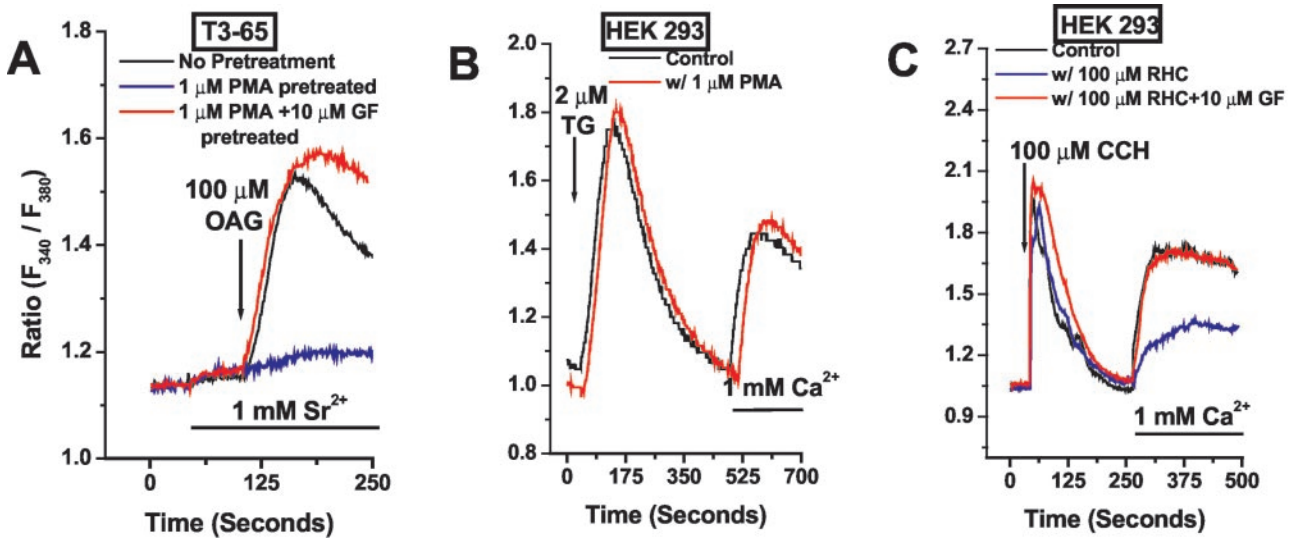

FIG. 7. **PKC activation inhibits both OAG-mediated TRPC3 channel activation and the development of endogenous GPCR-mediated Ca^{2+} entry without affecting Ca^{2+} entry due to TG-mediated store depletion.** Standard conditions included Ca^{2+} free medium; *bars* indicate replacement of Ca^{2+}-free medium with medium containing either Sr^{2+} or Ca^{2+}. *A*, in the TRPC3 stably expressing T3-65 clone of HEK293 cells, the addition of 1 mM Sr^{2+} (*bar*) led to minimal constitutive entry via TRPC3. The subsequent addition of 100 μM OAG (*arrow*) in control cells (*black trace*) led toTRPC3 activation and Sr^{2+} entry. A 5-min pretreatment with 1 μM PMA completely prevented OAG-mediated TRPC3 activation (*blue trace*). A 5-min pretreatment with 1 μM PMA and 10 μM GF-109203X rescued the effect of PMA on OAG-mediated TRPC3 activation and led to substantial Sr^{2+} entry. *B*, in HEK293 cells, passive store depletion with 2 μM TG (*arrow*) led to the activation of store-operated Ca^{2+} entry upon the readdition of 1 mM Ca^{2+} (*bar*; *black trace*). Pretreatment with 1 μM PMA did not affect either TG-mediated Ca^{2+} release or subsequent Ca^{2+} entry (*red trace*). *C*, in HEK293 cells, the addition of 100 μM CCh (*arrow*) led to rapid $InsP_3$-mediated Ca^{2+} release in control cells (*black trace*). The subsequent addition of 1 mM Ca^{2+} (*bar*) resulted in Ca^{2+} entry due to GPCR-mediated Ca^{2+} release. When 100 μM RHC-80267 was added with CCh (*arrow*), $InsP_3$-mediated Ca^{2+} release was the same size as in control cells, but subsequent Ca^{2+} entry was reduced by about 60% (*blue trace*). When both 100 μM RHC-80267 and 10 μM GF-109203X were added with CCh (*arrow*), both $InsP_3$-mediated Ca^{2+} release and subsequent Ca^{2+} entry were the same as in control cells (*red trace*).

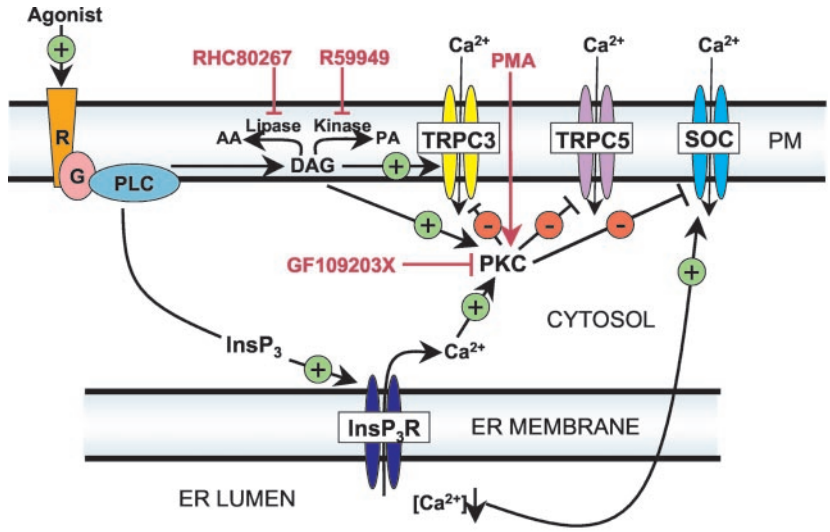

FIG. 8. **Diagram summarizing the modifications and actions of DAG and PKC on the activation of TRPC channels and SOCs.** The established and potentially significant stimulatory and inhibitory regulatory pathways are indicated by *green* "*plus*" *signs* and *red* "*minus*" *signs*, respectively. The actions of pharmacological modifiers are shown in *red*. Details of these pathways are given in the text.

addition of exogenous OAG (not shown). Significantly, the inhibitory action of DAG lipase blockade on Ca^{2+} entry in response to CCh was reversed completely by the PKC blocker GF-109203X (Fig. 7*C*). Likewise, the inhibitory action of OAG was completely reversed by the PKC blocker (data not shown). These results provide evidence for a potential link between the endogenous entry of Ca^{2+} induced by a receptor and the activity of exogenously expressed TRPC channels. However, in contrast to the complete inhibition of TRPC channels by PKC, the partial effect on receptor-induced endogenous Ca^{2+} entry may reflect the heterogeneity of channel subtypes involved in this process. It is interesting to reflect on the difference in the effects of PKC on TG-induced as opposed to receptor-induced Ca^{2+} entry. Thus, it was revealed recently that there are significant differences between the receptor-induced activation of SOCs as opposed to SOC activation resulting from depletion of stores through nonphysiological SERCA pump blockade or ionophore (34). Our results may be a further reflection of this difference.

A summary of the modifications of DAG and PKC used in these studies and the resulting effects on Ca^{2+} entry channels is given in Fig. 8. Overall, our results indicate that three members of the TRPC family of channels, TRPC3, TRPC4, and TRPC5, are each negatively regulated by PKC. The TRPC4 and TRPC5 channels form a structurally closely related subgroup (2). The TRPC3 channel is part of a structurally distinct subgroup of closely related channels including TRPC6 and TRPC7 (2, 48). This latter subgroup is distinguished functionally by

being activated by DAG through a non-PKC mechanism (31). Although we have not examined the actions of PKC on TRPC6 and TRPC7, given their structural and functional similarity to TRPC3 it would be surprising if they were distinct with respect to the PKC effects. Therefore, the actions of PKC on TRPC channels from different subgroups may signify a universal and important component in the feedback regulation of TRPC channels following PLC-dependent activation. Somewhat enigmatically, although we may have shed light on a potentially important turn-off mechanism for TRPC channels, the mediation of the turning on of TRPC channels is still a mystery. Thus, whereas TRPC channels seem to be activated universally by receptor-induced PLC activation, only the TRPC3/TRPC6/TRPC7 subgroup appears to respond to DAG (49). What accounts for the activation of the TRPC4/TRPC5 channels, which are unresponsive to DAG? Certainly, a large body of evidence has pointed to the other PLC product, $InsP_3$, functioning through $InsP_3Rs$ to activate TRPC channels (6, 12, 19–24). Indeed, although there are conflicting reports, evidence suggests that $InsP_3Rs$ can exert a direct conformational coupling role in the activation of TRPC channels in addition to activation resulting from store depletion (reviewed in Ref. 6). However, we reveal here that receptors, either G protein-coupled through PLC-β or tyrosine kinase-coupled through PLC-γ2, can activate TRPC5 channels in DT40 cells devoid of any $InsP_3Rs$, a conclusion mirroring activation of TRPC3 channels (28). The enigma of TRPC activation extends to the prototypic *Drosophila* TRP channel, which also is dependent on receptor-induced PLC activation, even though PLC products have no obvious mediating action (2, 7). Closely resembling vertebrate TRPC channels, the *Drosophila* TRP channel exists in a functional complex containing photoreceptor, PLC, PKC, and calmodulin, held within the PDZ (PSD-95/DLG/ZO-1) domain-containing INAD (inactivation after depolarization) scaffold protein (2, 7). Indeed there is evidence that the PKC within this complex directly phosphorylates and inhibits the TRP channel in a negative feedback loop controlling phototransduction (50, 51). In vertebrate systems, TRPC channels may be organized within similar regulatory complexes via PDZ domain-containing proteins such as NHERF (Na(+)/H(+)-exchanger regulatory factor), which is shown to interact with and organize TRPC4 and TRPC5 channels and PLC-β isoforms (52). It is also well known that PKC-mediated inhibition of receptor-induced PLC provides an important feedback loop mediated by DAG and Ca^{2+} on the PLC enzyme (53–56).

Notable in the current studies is that induction of DAG by DAG lipase or DAG kinase activation, or the application of OAG, fully inhibits TRPC5 channel activation through a PKC-dependent mechanism but only slightly reduces receptor-induced PLC activation, revealed by the $InsP_3$-mediated release of Ca^{2+} from stores. In contrast, application of the potent PKC activator PMA prevents both PLC and TRPC activation. Thus, it may be that the PKC affecting TRPC channels is not the same as that which controls PLC. Indeed, it is possible that a subpopulation of PKC, perhaps one that is closely associated with the TRPC-containing complex, is highly responsive to changes in DAG that are induced within the membrane. This subpopulation may contrast with a more globally distributed subfraction of PKC exerting actions on PLC, which is less sensitive to membrane DAG changes but nevertheless highly activated by PMA. The function of a closely coupled PKC moiety within a local TRPC channel complex that is highly sensitive to local DAG levels provides an intriguing control process for the entry channels. Indeed, the control of the assembly of the complex with respect to the relative amounts of PKC in combination with TRPC channel subunits that are DAG-sensitive (such as TRPC3) or -insensitive (such as TRPC5) may provide functional channel assemblies that have profoundly different levels of responsiveness to receptor activation.

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REFERENCES

1. Montell, C., Birnbaumer, L., and Flockerzi, V. (2002) *Cell* **108,** 595–598
2. Montell, C. (2001) *Science's STKE*
3. Clapham, D. E., Runnels, L. W., and Strubing, C. (2001) *Nat. Rev. Neurosci.* **2,** 387–396
4. Zitt, C., Halaszovich, C. R., and Luckhoff, A. (2002) *Prog. Neurobiol.* **66,** 243–264
5. Hofmann, T., Schaefer, M., Schultz, G., and Gudermann, T. (2000) *J. Mol. Med.* **78,** 14–25
6. Venkatachalam, K., van Rossum, D. B., Patterson, R. L., Ma, H. T., and Gill, D. L. (2002) *Nat. Cell Biol.* **4,** E263–E272
7. Montell, C. (1999) *Annu. Rev. Cell Dev. Biol.* **15,** 231–268
8. Harteneck, C., Plant, T. D., and Schultz, G. (2000) *Trends. Neurosci.* **23,** 159–166
9. Birnbaumer, L., Zhu, X., Jiang, M., Boulay, G., Peyton, M., Vannier, B., Brown, D., Platano, D., Sadeghi, H., Stefani, E., and Birnbaumer, M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 15195–15202
10. Putney, J. W., Jr., Broad, L. M., Braun, F. J., Lievremont, J. P., and Bird, G. S. (2001) *J. Cell Sci.* **114,** 2223–2229
11. Philipp, S., Cavalié, A., Freichel, M., Wissenbach, U., Zimmer, S., Trost, C., Marquart, A., Murakami, M., and Flockerzi, V. (1996) *EMBO J.* **15,** 6166–6171
12. Kiselyov, K. I., Xu, X., Mohayeva, G., Kuo, T., Pessah, I. N., Mignery, G. A., Zhu, X., Birnbaumer, L., and Muallem, S. (1998) *Nature* **396,** 478–482
13. Philipp, S., Hambrecht, J., Braslavski, L., Schroth, G., Freichel, M., Murakami, M., Cavalie, A., and Flockerzi, V. (1998) *EMBO J.* **17,** 4274–4282
14. Vannier, B., Peyton, M., Boulay, G., Brown, D., Qin, N., Jiang, M., Zhu, X., and Birnbaumer, L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2060–2064
15. Philipp, S., Trost, C., Warnat, J., Rautmann, J., Himmerkus, N., Schroth, G., Kretz, O., Nastainczyk, W., Cavalie, A., Hoth, M., and Flockerzi, V. (2000) *J. Biol. Chem.* **275,** 23965–23972
16. Liu, X., Wang, W., Singh, B. B., Lockwich, T., Jadlowiec, J., O'Connell, B., Wellner, R., Zhu, M. X., and Ambudkar, I. S. (2000) *J. Biol. Chem.* **275,** 3403–3411
17. Putney, J. W. (1997) *Capacitative Calcium Entry*, Springer, New York
18. Putney, J. W., and Ribeiro, C. M. (2000) *Cell Mol. Life Sci.* **57,** 1272–1286
19. Kiselyov, K. I., Mignery, G. A., Zhu, M. X., and Muallem, S. (1999) *Mol. Cell* **4,** 423–429
20. Ma, H.-T., Patterson, R. L., van Rossum, D. B., Birnbaumer, L., Mikoshiba, K., and Gill, D. L. (2000) *Science* **287,** 1647–1651
21. Boulay, G., Brown, D. M., Qin, N., Jiang, M., Dietrich, A., Zhu, M. X., Chen, Z., Birnbaumer, M., Mikoshiba, K., and Birnbaumer, L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14955–14960
22. Birnbaumer, L., Boulay, G., Brown, D., Jiang, M., Dietrich, A., Mikoshiba, K., Zhu, X., and Qin, N. (2000) *Recent Prog. Horm. Res.* **55,** 127–161
23. Zhang, Z., Tang, J., Tikunova, S., Johnson, J. D., Chen, Z., Qin, N., Dietrich, A., Stefani, E., Birnbaumer, L., and Zhu, M. X. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 3168–3173
24. Tang, J., Lin, Y., Zhang, Z., Tikunova, S., Birnbaumer, L., and Zhu, M. X. (2001) *J. Biol. Chem.* **276,** 21303–21310
25. Ma, H.-T., Venkatachalam, K., Li, H. S., Montell, C., Kurosaki, T., Patterson, R. L., and Gill, D. L. (2001) *J. Biol. Chem.* **276,** 18888–18896
26. Sugawara, H., Kurosaki, M., Takata, M., and Kurosaki, T. (1997) *EMBO J.* **16,** 3078–3088
27. Broad, L. M., Braun, F. J., Lievremont, J. P., Bird, G. S., Kurosaki, T., and Putney, J. W., Jr. (2001) *J. Biol. Chem.* **276,** 15945–15952
28. Venkatachalam, K., Ma, H. T., Ford, D. L., and Gill, D. L. (2001) *J. Biol. Chem.* **276,** 33980–33985
29. Ma, H. T., Venkatachalam, K., Parys, J. B., and Gill, D. L. (2002) *J. Biol. Chem.* **277,** 6915–6922
30. Vazquez, G., Lievremont, J. P., St J. Bird, G., and Putney, J. W., Jr. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 11777–11782
31. Hofmann, T., Obukhov, A. G., Schaefer, M., Harteneck, C., Gudermann, T., and Schultz, G. (1999) *Nature* **397,** 259–263
32. Kanki, H., Kinoshita, M., Akaike, A., Satoh, M., Mori, Y., and Kaneko, S. (2001) *Mol. Pharmacol.* **60,** 989–998
33. Patterson, R. L., van Rossum, D. B., and Gill, D. L. (1999) *Cell* **98,** 487–499
34. Patterson, R. L., van Rossum, D. B., Ford, D. L., Hurt, K. J., Bae, S. S., Suh, P. G., Kurosaki, T., Snyder, S. H., and Gill, D. L. (2002) *Cell* **111,** 529–541
35. Waldron, R. T., Short, A. D., and Gill, D. L. (1995) *J. Biol. Chem.* **270,** 11955–11961
36. Short, A. D., Klein, M. G., Schneider, M. F., and Gill, D. L. (1993) *J. Biol. Chem.* **268,** 25887–25893
37. van Rossum, D. B., Patterson, R. L., Ma, H.-T., and Gill, D. L. (2000) *J. Biol. Chem.* **275,** 28562–28568
38. Winding, P., and Berchtold, M. W. (2001) *J. Immunol. Methods* **249,** 1–16
39. Takata, M., Homma, Y., and Kurosaki, T. (1995) *J. Exp. Med.* **182,** 907–914
40. Prakriya, M., and Lewis, R. S. (2001) *J. Physiol.* **536,** 3–19
41. Parekh, A. B., and Penner, R. (1997) *Physiol. Rev.* **77,** 901–930
42. Schaefer, M., Plant, T. D., Obukhov, A. G., Hofmann, T., Gudermann, T., and Schultz, G. (2000) *J. Biol. Chem.* **275,** 17517–17526

43. Waldron, R. T., Short, A. D., and Gill, D. L. (1997) *J. Biol. Chem.* **272,** 6440–6447
44. Zhu, X., Jiang, M., and Birnbaumer, L. (1998) *J. Biol. Chem.* **273,** 133–142
45. Jiang, Y., Sakane, F., Kanoh, H., and Walsh, J. P. (2000) *Biochem. Pharmacol.* **59,** 763–772
46. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., and Loriolle, F. (1991) *J. Biol. Chem.* **266,** 15771–15781
47. Quest, A. F., Ghosh, S., Xie, W. Q., and Bell, R. M. (1997) *Adv. Exp. Med. Biol.* **400A,** 297–303
48. Montell, C., Birnbaumer, L., Flockerzi, V., Bindels, R. J., Bruford, E. A., Caterina, M. J., Clapham, D. E., Harteneck, C., Heller, S., Julius, D., Kojima, I., Mori, Y., Penner, R., Prawitt, D., Scharenberg, A. M., Schultz, G., Shimizu, N., and Zhu, M. X. (2002) *Mol. Cell* **9,** 229–231
49. Zakim, D. (1996) *Proc. Soc. Exp. Biol. Med.* **212,** 5–14
50. Liu, M., Parker, L. L., Wadzinski, B. E., and Shieh, B. H. (2000) *J. Biol. Chem.* **275,** 12194–12199
51. Huber, A., Sander, P., Bahner, M., and Paulsen, R. (1998) *FEBS Lett.* **425,** 317–322
52. Tang, Y., Tang, J., Chen, Z., Trost, C., Flockerzi, V., Li, M., Ramesh, V., and Zhu, M. X. (2000) *J. Biol. Chem.* **275,** 37559–37564
53. Ryu, S. H., Kim, U. H., Wahl, M. I., Brown, A. B., Carpenter, G., Huang, K. P., and Rhee, S. G. (1990) *J. Biol. Chem.* **265,** 17941–17945
54. Yue, C., Ku, C. Y., Liu, M., Simon, M. I., and Sanborn, B. M. (2000) *J. Biol. Chem.* **275,** 30220–30225
55. Filtz, T. M., Cunningham, M. L., Stanig, K. J., Paterson, A., and Harden, T. K. (1999) *Biochem. J.* **338,** 257–264
56. Litosch, I. (1997) *Biochem. J.* **326,** 701–707