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Expression of Functional Receptor-coupled TRPC3 Channels in DT40 Triple Receptor $InsP_3$ knockout Cells*

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The TRPC3 channel, an intensively studied member of the widely expressed transient receptor potential (TRP) family, is a Ca^{2+}-conducting channel activated in response to phospholipase C-coupled receptors. Despite scrutiny, the receptor-induced mechanism to activate TRPC3 channels remains unclear. Evidence indicates TRPC3 channels interact directly with intracellular inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) and that channel activation is mediated through coupling to $InsP_3Rs$. TRPC3 channels were expressed in DT40 chicken B lymphocytes in which all three $InsP_3R$ genes were deleted (DT40$InsP_3R$-k/o). Endogenous B-cell receptors (BCR) coupled through Syk kinase to phospholipase C-γ (PLC-γ) activated the expressed TRPC3 channels in both DT40w/t and DT40$InsP_3R$-k/o cells. The diacylglycerol (DAG) analogue 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) also activated TRPC3 channels independently of $InsP_3Rs$. BCR-induced TRPC3 activation was blocked by the PLC enzymic inhibitor, U-73122, and also blocked by wortmannin-induced PLC substrate depletion. Neither U-73122 nor wortmannin modified either OAG-induced TRPC3 activation or store-operated channel activation in DT40 cells. Cotransfection of cells with both G protein-coupled M5 muscarinic receptors and TRPC3 channels resulted in successful M5 coupling to open TRPC3 channels mediated by PLC-β. We conclude that TRPC3 channels are activated independently of $InsP_3Rs$ through DAG production resulting from receptor-mediated activation of either PLC-γ or PLC-β.

The TRP[1] channel family comprises a large group of channels mediating an array of signal and sensory transduction pathways (1). The proteins of the mammalian TRPC subfamily are the products of at least seven genes coding for cation channels that appear to be activated in response to PLC-coupled receptors (1–3). These channels are closely related 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 (4, 5). Particular interest surrounds the mammalian TRPC subfamily because these channels have been implicated as important mediators of Ca^{2+} entry (1, 3, 6). Reports indicate that they may function as "store-operated" channels (6–12) mediating the process of capacitative Ca^{2+} entry that is essential for longer term Ca^{2+} signals and replenishment of Ca^{2+} stores (13, 14). Of great interest has been the elucidation of a coupling mechanism for the TRPC3 channel involving intracellular $InsP_3Rs$ (8, 15). Thus, a direct functional communication between TRPC3 channels and $InsP_3Rs$ has been revealed from reconstitution studies (8, 15–17). Moreover, recent studies have revealed a physical interaction between the TRPC3 channel and the $InsP_3R$ (17, 18) and have mapped the interacting loci of the two proteins (19, 20). Interactions have also been described between other TRPC channels and $InsP_3Rs$ (21, 22). Considering these results, it was important to assess the function and coupling of TRPC3 channels expressed in the DT40 chicken B lymphocyte cell line in which all three $InsP_3R$ subtypes have been genetically eliminated.

EXPERIMENTAL PROCEDURES

Culture of Cells—Cells of both the wild-type DT40 chicken B cell line (DT40w/t) and triple $InsP_3R$-knockout cell line (DT40$InsP_3R$-k/o), were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine as described previously (23, 24).

Transfection of DT40 Cells—DT40 wild-type and $InsP_3R$-knockout cells cultured overnight in RPMI 1640 with 10% fetal bovine serum were scraped from plates and washed in reduced-serum OptiMEM (Life Technologies, Inc.) and then resuspended in OptiMEM at a final concentration of 10^7 cells/ml. 12 μg of each of the plasmids to be transfected (human M5 muscarinic receptor, human TRPC3, and/or EYFP, each within pcDNA3) were 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 the 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 then recovered in OptiMEM (no serum added) for 5 h and then resuspended in OptiMEM with 10% fetal bovine serum, applied to coverslips, and allowed to attach for 1.5 h before Ca^{2+} measurements were undertaken. The overall efficiency of transfection (EYFP-positive cells) was 20–30%. When cells were cotransfected with EYFP-pcDNA3 and either TRPC3-pcDNA3 or M5R-pcDNA3, ~30–50% of EYFP-positive cells showed TRPC3- or M5R-mediated Ca^{2+} responses, respectively. When both TRPC3 and M5R were cotransfected, ~10–20% of EYFP-expressing cells were positive for both responses.

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/AM (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

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[1] The abbreviations used are: TRP, transient receptor potential; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; fura-2/AM, fura-2 acetoxymethylester; PLC, phospholipase C; DAG, diacylglycerol; EYFP, enhanced yellow fluorescent protein; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol, BCR, B cell receptor;, GPCR, G protein-coupled receptor; M5R, M5 muscarininc receptor; SOC, store-operated channel; w/t, wild type; k/o, knockout.

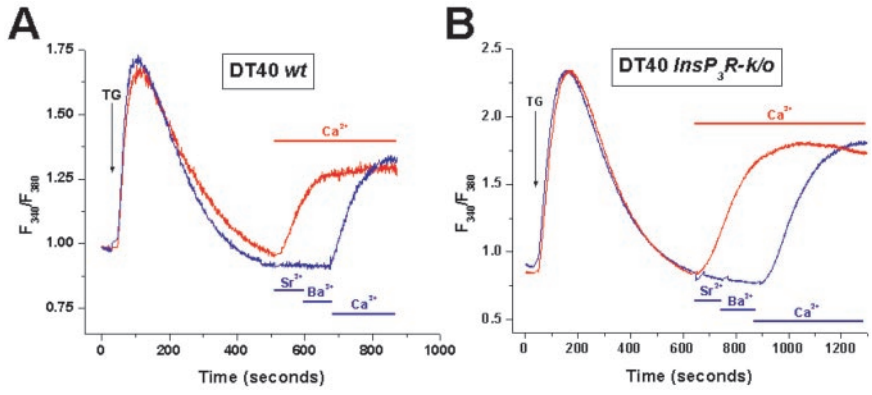

FIG. 1. **Store-operated channels in DT40w/t and triple $InsP_3R$-knockout DT40 cells are highly selective for Ca^{2+}.** Cytosolic Ca^{2+} was measured in fura-2-loaded DT40w/t cells (*A*) or DT40InsP_3R-k/o cells (*B*) as described under "Experimental Procedures." Standard conditions included Ca^{2+}-free external medium; *bars* indicate times of replacement of medium with media containing 1 mM Ca^{2+}, Sr^{2+}, or Ba^{2+}. 1 μM thapsigargin (*TG*) was added as indicated (*arrow*).

saponin permeabilization (25). 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 in the figures. Ca^{2+} measurements in single transfected and groups of untransfected cells were made using an InCyt dual wavelength fluorescence imaging system (Intracellular Imaging Inc.). Transfected EYFP served as the transfection marker and was detected at excitation wavelength 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 (Ca^{2+}, Sr^{2+}, or Ba^{2+}) are shown as 340/380 nm ratios obtained from groups of 10–12 untransfected and single transfected cells. Details of these divalent cation measurements were described previously (24, 26, 27). Resting Ca^{2+} levels in the two DT40 cell lines were similar, ~100–130 nM. All measurements shown are representative of a minimum of three, and in most cases, a larger number of independent experiments.

Materials and Miscellaneous Procedures—Plasmids were from the following sources: hTRPC3 cDNA from C. Montell (Johns Hopkins); EYFP cDNA from CLONETECH; and human M5 musarinic receptor cDNA from L. Birnbaumer (UCLA). OAG, U-73122, and wortmannin, were from Calbiochem. EGTA and carbachol were from Sigma. Thapsigargin was from LC Services (Woburn, MA). Fura-2/AM was from Molecular Probes (Eugene, OR). Anti-chicken IgM (supernatant, M-4 clone) was from Southern Biotechnology Associates (Birmingham, AL). The DT40 cell line was kindly supplied by Dr. Tomohiro Kurosaki, Kyoto, Japan.

RESULTS AND DISCUSSION

The DT40 chicken B lymphocyte cell line has been a useful tool for generating gene knockouts, including knockouts of the three genes coding for $InsP_3Rs$. The loci of all three $InsP_3R$ subtypes normally expressed in DT-40 wild-type cells were disrupted sequentially by the targeted introduction of mutations of the alleles of each $InsP_3R$-subtype by homologous recombination (23). The resulting triple $InsP_3R$ gene-knockout cells (DT40InsP_3R-k/o) have no detectable transcripts or translation products from $InsP_3R$ genes, are devoid of any functional $InsP_3R$ activity, and contain no detectable $InsP_3$-sensitive Ca^{2+} stores (23, 24, 28). We recently reported that Ca^{2+} entry could be stimulated after store emptying in the DT40InsP_3R-k/o cells (24), confirming earlier observations of Sugawara *et al.* (23). Before assessing the function of TRP channels in these cells, we sought to determine the selectivity of cation entry activated by store depletion in DT40 cells. Thus, one particular hallmark for SOCs is their extreme cation selectivity, permitting the passage of Ca^{2+} but not other even closely related alkaline-earth ions such as Sr^{2+} and Ba^{2+} (26, 29). Using cation-safe conditions in which anions other than Cl^- were eliminated from the external medium (see "Experimental Procedures"), the characteristic selectivity of SOCs was clearly apparent in the DT40 cells. Thus, as shown in Fig. 1*A*, after emptying stores in the absence of external Ca^{2+} using the SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase) pump blocker, thapsigargin, the addition of Ca^{2+} resulted in SOC-mediated Ca^{2+} entry, whereas no entry of Sr^{2+} or Ba^{2+} could be observed (Fig. 1*A*). The entry of divalent cations in response to store emptying in the DT40InsP_3R-k/o cells revealed the same ion selectivity (Fig. 1*B*). We may therefore conclude that the presence of $InsP_3Rs$ is not required for operation of authentic cation-specific store-operated entry channels.

The importance of determining this cation selectivity of SOCs in the DT40 cells has yet greater significance in studies here to assess the expression and function of TRPC3 channels in the DT40 cell lines. Many members of the TRPC or "classical" TRP subfamily of channels, including TRPC3 channels, are activated in response to PLC-coupled receptors (1). Whether store emptying has any input into the activation of TRPC channels is controversial, with evidence found both in favor (7–12) and against (3, 5, 26, 30–34) such a role. Despite this question, the nature of the coupling process between receptors and TRPC channels remains unknown. There is considerable evidence that TRPC channels, particularly the TRPC3 channel, are activated as a result of specific interactions with intracellular $InsP_3Rs$ (8, 15, 17–20). Thus, the DT40 cells provide an important means for testing this hypothesis. The DT40 cell line retains an αIgM isotype B cell receptor (BCR), which through activation of the Syk and Lyn nonreceptor tyrosine kinases results in tyrosine phosphorylation and activation of PLC-γ (35). Using DT40w/t cells we could not detect any Ca^{2+} signals in response to a wide selection of G protein-coupled receptor agonists (including serotonin, bradykinin, and purinergic and muscarinic agonists), indicating no Ca^{2+} mobilization in these cells mediated through activation of endogenous GPCRs coupled to PLC-β. The response of DT40w/t cells to BCR activation by the M-4 clone of anti-chicken IgM (anti-IgM) is shown in Fig. 2*A*. In the absence of external Ca^{2+}, the cells displayed a series of rapidly generated Ca^{2+} spikes representing repeated Ca^{2+} release from and re-uptake into stores. These spikes slowly subsided after ~5 min, at which time stores remained in a mostly depleted state. No entry of Sr^{2+} or Ba^{2+} could be detected, but Ca^{2+} addition resulted in entry through SOCs albeit smaller than that seen with thapsigargin because of incomplete store emptying. In stark contrast, the DT40 InsP_3R-k/o cells were entirely devoid of the Ca^{2+} release response to anti-IgM, and there was no subsequent activation of SOCs (Fig. 2*B*). DT40 cells were transiently cotransfected with separate pcDNA3 vectors containing genes coding for the marker EYFP and the human TRPC3 channel. The successful transfection of functional TRPC3 channels in DT40 cells was dependent on transfection conditions, and successful transfection was effected as described under "Experimental Procedures." Analysis of transfected DT40w/t cells revealed that after the anti-IgM-induced BCR-mediated Ca^{2+} response subsided, the addition of Ba^{2+} resulted in a rapid and substantial entry into cells (Fig. 2*C; red trace*). This clearly reflects functional expression of

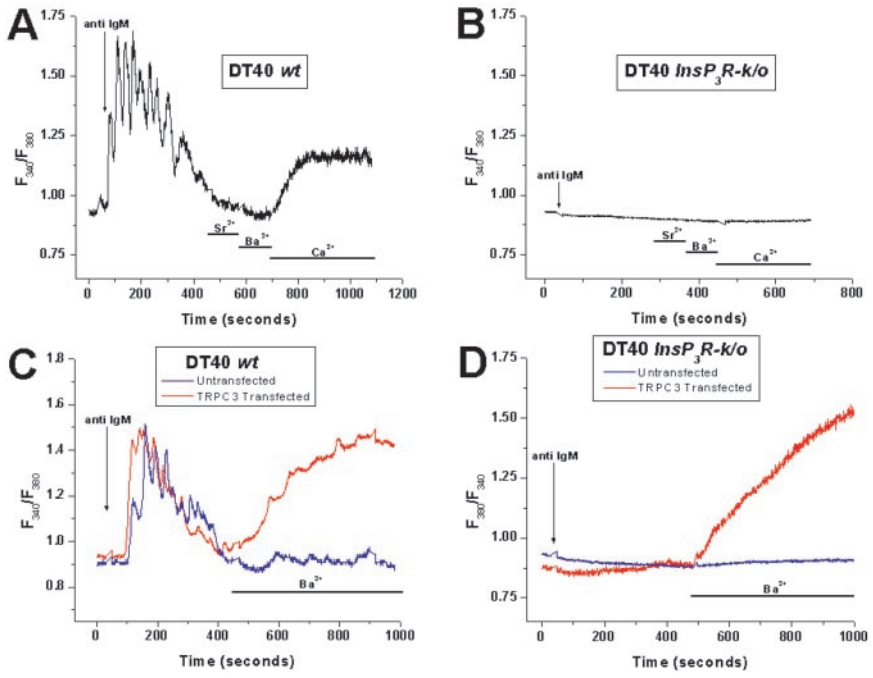

FIG. 2. **TRPC3 channels transfected into both DT40w/t and DT40InsP_3R-k/o cells are activated in response to BCR-mediated stimulation of PLC-γ.** Cytosolic Ca^{2+} was measured as in Fig. 1. Standard conditions included Ca^{2+}-free medium; *bars* indicate times of replacement with media containing 1 mM Ca^{2+}, Sr^{2+}, or Ba^{2+}. *A*, DT40w/t cells. BCR was stimulated with 3 μg/ml anti-IgM (*arrow*) leading to $InsP_3$-mediated Ca^{2+} release and SOC-mediated entry only upon addition of Ca^{2+} but not Sr^{2+} or Ba^{2+}. *B*, same as in *A* but in DT40InsP_3R-k/o cells. *C*, DT40w/t cells transfected with TRPC3 and EYFP (*red trace*). TRPC3 channels were activated by the addition of 3 μg/ml anti-IgM (*arrow*) followed by the addition of medium containing 1 mM Ba^{2+} (*bar*). *D*, same as in *C* but in DT40InsP_3R-k/o cells. All traces are the means of between 3 and 10 single cells within a field. *Red traces* are from EYFP-positive cells, and *blue traces* are from EYFP-negative (untransfected) cells from the same field.

TRPC3 channels, which, in distinction from SOCs, have much lower cation selectivity (26). Although Ba^{2+} responses were observed in only 30–50% of all EYFP-positive DT40 cells, virtually all brightly fluorescent EYFP-positive cells showed Ba^{2+} responses. The magnitude of the Ba^{2+} response varied between individual cells likely reflecting variation in the degree of TRPC3 expression. Ba^{2+} responses were never observed in untransfected (EYFP-negative; *blue trace*, Fig. 2*C*) cells imaged simultaneously within the same field as transfected cells. Most significantly, the TRPC3 channel was clearly functional when expressed in the DT40InsP_3R-k/o cells. As shown in Fig. 2*D*, even though there was no Ca^{2+} release response to anti-IgM in the k/o cells, the addition of Ba^{2+} to the TRPC3-transfected cells resulted in clear Ba^{2+} entry. Again, no untransfected cells displayed any Ba^{2+} entry, and from repeated experimentation there was no significant difference in the size or rapidity of activation of TRPC3-mediated responses in DT40w/t as opposed to DT40InsP_3R-k/o cells. The only difference was a persistent slight fluctuation in w/t cells because of persistent, albeit reduced, Ca^{2+} release spikes.

Based on the rather substantial information that the DT40InsP_3R-k/o cells are devoid of $InsP_3$Rs (23, 24, 28), we may conclude that the $InsP_3$R does not play an obligatory role in the coupling between BCRs and the activation of TRPC3 channels. Under the conditions we describe here for TRPC3 expression in DT40 cells, the functional coupling of TRPC3 channels was not modified by store emptying. Thus, we show here that TRPC3 channels can be activated by PLC-coupled receptors under conditions in which there is no concomitant store emptying. The question arising is how receptor-induced PLC activation mediates TRPC3 channel activation. That is, how does receptor-mediated PLC activation couple to open TRPC3 channels if neither the action of $InsP_3$ to empty stores nor the physical presence of the $InsP_3$ are required? The most likely possibilities are that coupling is mediated either by the other PLC-derived product, diacylglycerol, or perhaps by a direct physical coupling role of the PLC enzyme itself as was recently suggested by Broad *et al.* (28) for the activation of SOCs. An action of DAG derivatives on TRP channels has been reported previously (26, 34), and we therefore examined their actions on TRPC3 channels in the DT40 cells. The membrane-permeant derivative of DAG, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) was a powerful activator of TRPC3 channels transfected in both DT40w/t cells (Fig. 3*A*) and in the DT40InsP_3R-k/o cells (Fig. 3*B*). The action of OAG in both cell types was clearly through TRPC3 channels because no untransfected cells in the same imaged fields showed any response to OAG (Fig. 3, *A* and *B, blue traces*). The extent of TRPC3 activation with 100 μM OAG varied between cells depending on channel expression but, averaged from many experiments, TRPC3 activity was not significantly different between the two cell lines. We may conclude, therefore, that this action of OAG is also entirely independent of the $InsP_3$R. The question of the dependence of TRP channels on PLC activity was addressed by determining the effects of blocking PLC activity with the PLC inhibitor U-73122 (36). As shown in Fig. 3*C* (*blue trace*), pretreatment of TRPC3-transfected DT40w/t cells with 20 μM U-73122 completely prevented anti-IgM-induced Ca^{2+} release. It also completely prevented activation of TRPC3 channels. However, the U-73122 blockade of coupling between receptors and TRPC3 channels was overcome by the subsequent addition of OAG, which stimulated TRPC3 channels the same as untreated cells. The actions of U-73122 on responses in TRPC3-transfected DT40InsP_3R-k/o cells were the same (Fig. 3*C, red trace*; the difference seen in the extent of OAG activation of TRPC3 channels in the w/t and k/o cells was not consistent and reflects only the degree of TRPC3 expression). These results are consistent with the view that BCR-induced TRPC3 activation is mediated by PLC-induced DAG production and is independent of the $InsP_3$R. This provides another important distinction from the activation process for SOCs. Thus, using DT40-w/t cells, the complete inhibition of PLC using U-73122 did not alter thapsigargin-induced Ca^{2+} release or the subsequent activation of SOCs (Fig. 3*D*). This result is distinct from the U-73122-induced blockade of SOCs reported by Broad *et al.* (28), which suggested that PLC activation was a requirement for SOC activation in mouse lacrimal cells and Rat Basophilic Leukemia cells. The distinction between the PLC requirement for SOCs and TRPC3 channels described here is particularly compelling because the function of both channels was compared directly within a single cell type.

It could be argued that by interacting directly with the PLC

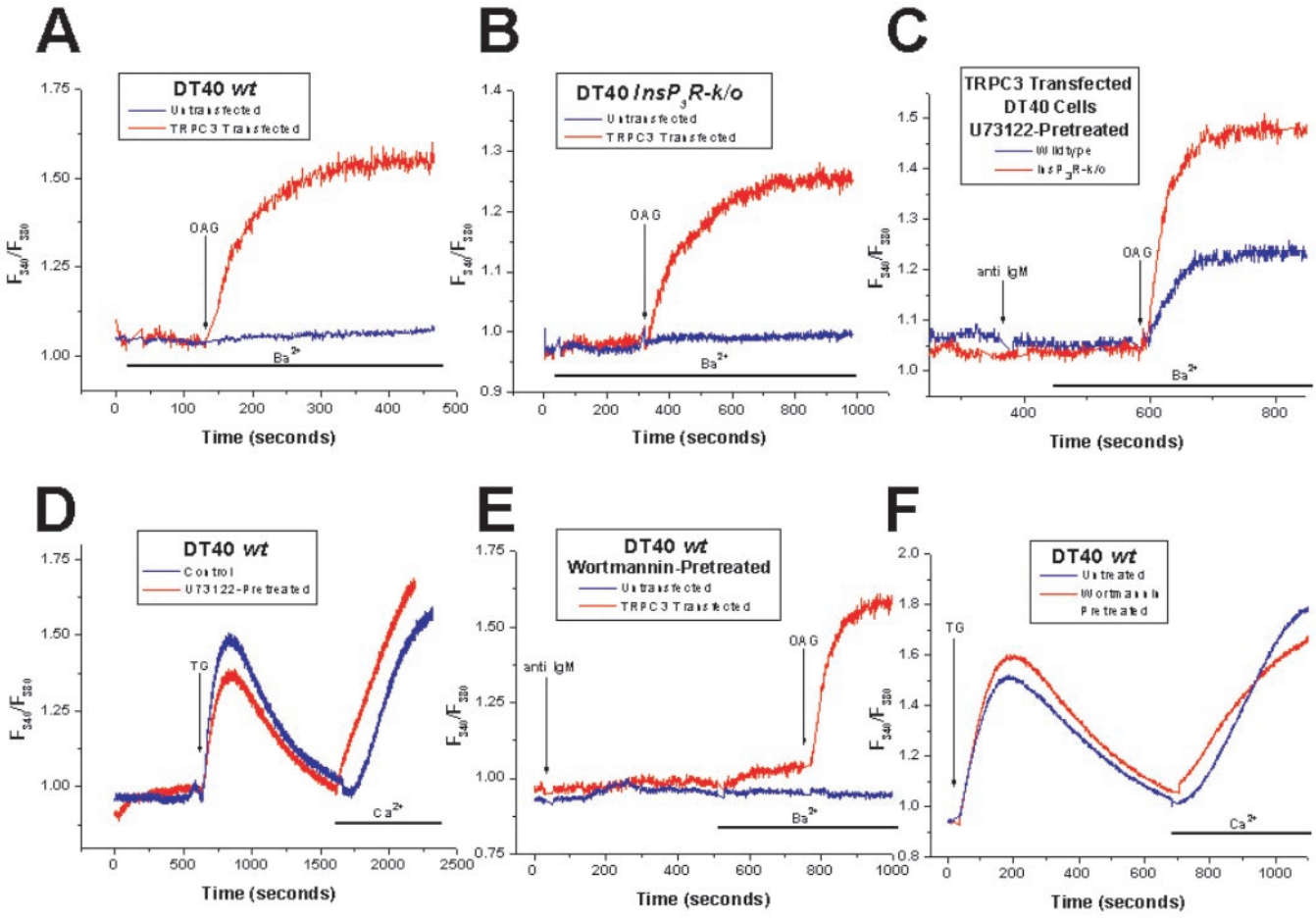

FIG. 3. **TRPC3 channels transfected in DT40w/t and DT40InsP$_3$R-k/o cells are stimulated by 1-oleoyl-2-acetyl-*sn*-glycerol; TRPC3 channel activation through BCR but not OAG is blocked by U-73122 or wortmannin.** Standard conditions included Ca^{2+}-free medium; *bars* indicate replacement with medium containing 1 mM Ba^{2+}. *A*, DT40w/t cells transfected with TRPC3 and EYFP (*red trace*). TRPC3 channels were activated by the addition of 100 μM OAG (*arrow*) in the presence of 1 mM Ba^{2+} (*bar*); untransfected wild-type DT40 cells in the same field are shown in the *blue trace*. *B*, same as in *A* but with DT40InsP$_3$R-k/o cells. *C*, DT40w/t (*blue trace*) and DT40InsP$_3$R-k/o cells (*red trace*) cells were pretreated with 15 μM U-73122 in for 5 min in Ca^{2+}-free medium. TRPC3 activation was evaluated by adding 1 μg/ml anti-IgM (*arrow*) followed by the addition of 1 mM Ba^{2+} (*bar*) and later 100 μM OAG (*arrow*). *D*, Ca^{2+} mobilization after the addition of 1 μM thapsigargin (*TG*, *arrow*) and SOC-mediated Ca^{2+} entry after the addition of 1 mM external Ca^{2+} (*bar*) in control (*blue trace*) and 15 μM U-73122-pretreated (5 min) DT40w/t cells (*red trace*). *E*, DT40 cells transfected with TRPC3 and EYFP (*red trace*) and pretreated with 10 μM wortmannin for 30 min in external medium containing 1 mM Ca^{2+}; TRPC3 activation was evaluated after the addition of 1 μg/ml anti-IgM (*arrow*) followed by the addition of 1 mM Ba^{2+} (*bar*) and then 100 μM OAG (*arrow*). Untransfected cells in the same field are shown in the *blue trace*. *F*, Ca^{2+} mobilization after the addition of 1 μM thapsigargin (*TG*, *arrow*) and SOC-mediated Ca^{2+} entry after addition of 1 mM external Ca^{2+} (*bar*) in control (*blue trace*) or 10 μM wortmannin-pretreated (30 min in 1 mM Ca^{2+}-containing medium) (*red trace*) DT40w/t cells.

enzyme, U-73122 might prevent a "coupling" role of the PLC enzyme as well as blocking the PLC enzymic activity. We therefore investigated the role of depleting the PLC substrate PtdIns 4,5-bisphosphate by using wortmannin, which at 10 μM inhibits PtdIns 4-kinase (28, 37). The effect of wortmannin pretreatment of TRPC3-transfected DT40w/t cells was to block anti-IgM-mediated store release and TRPC3-mediated Ba^{2+} entry, but OAG could still induce Ba^{2+} entry (Fig. 3*E*). Thus, the action of wortmannin is the same as U-73122 and would indicate that the role of PLC in stimulating TRPC3 channels is through formation of products, most likely DAG. We also examined the effects of the same wortmannin pretreatment on SOC-mediated Ca^{2+} entry and found no effect (Fig. 3*F*), which appears to contrast with the results of Broad *et al.* (28), a fact we cannot explain. Certainly, we observe a stark difference between the effects of PLC blockade on TRPC3 activation as opposed to SOC activation.

One last significant question remained on receptor-induced coupling to activate TRPC3 channels. Our results so far on TRPC3 activation had been confined to responses through the BCR, which is mediated via nonreceptor tyrosine kinase activation of the PLC-γ enzyme (35). The important question to answer was whether InsP$_3$Rs play a role in TRPC3 channel activation in response to G protein-coupled receptors, which are coupled to PLC-β activation. Such a determination derives special significance because the reported role of InsP$_3$Rs in TRP activation has been determined from studies on GPCR-induced responses, in particular those mediated by muscarinic receptors (8). As described above, the DT40 cells are curiously devoid of endogenously expressed GPCRs mediating Ca^{2+} responses through PLC-β. Therefore, we assessed functional coupling in DT40 cells transiently expressing plasmids coding for both the human M5 muscarinic receptor and the human TRPC3 channel (in addition to the EYFP marker plasmid). Initially we determined whether transfection with the M5R alone could couple through endogenous PLC-β activity in DT40 cells to give Ca^{2+} signals. As shown in Fig. 4*A* for M5R-transfected DT40w/t cells, application of the muscarinic agonist carbachol induced a substantial release of Ca^{2+} from stores, whereas no untransfected cells in the same fields showed any response. Indeed, the release of Ca^{2+} from stores was sufficient to give good activation of SOCs. Thus, the addition of Ca^{2+} but

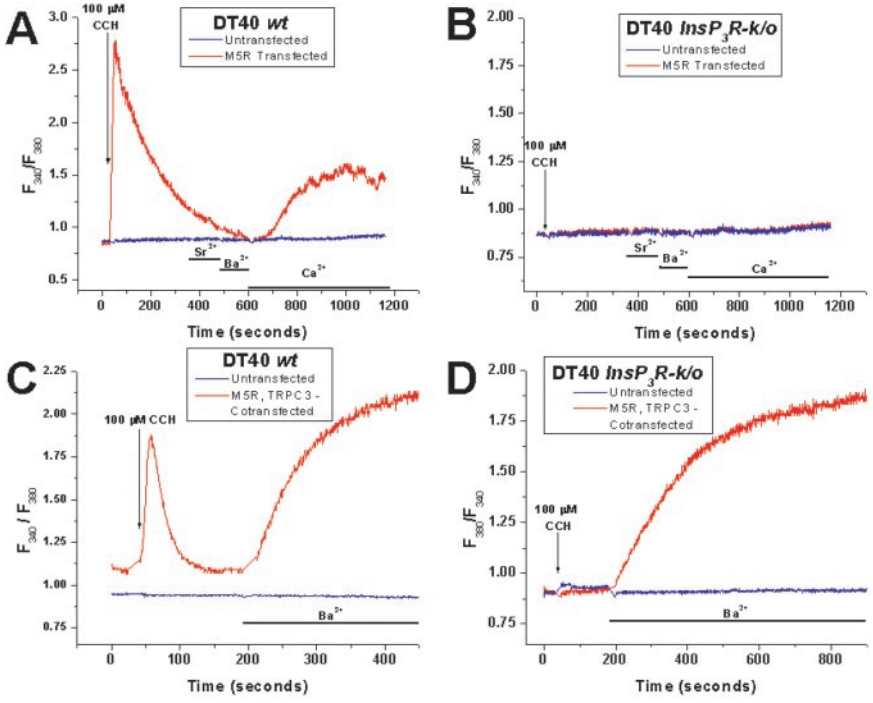

FIG. 4. **Cotransfection of M5 muscarinic receptors and TRPC3 channels results in PLC-β-mediated TRPC3 channel activation in both DT40w/t and DT40InsP_3R-k/o cells.** Standard conditions included Ca^{2+}-free external medium; *bars* indicate times of replacement with medium containing 1 mM Ba^{2+}. *A*, DT40w/t cells transfected with the M5R and EYFP (*red trace*); Ca^{2+} release was activated with 100 μM carbachol (*CCH*, *arrow*) followed by the addition of media containing 1 mM Sr^{2+}, 1 Ba^{2+}, or 1 Ca^{2+} (*bars*). Untransfected (EYFP-negative) DT40w/t cells in the same field are shown in the *blue trace*. *B*, same as in *A* but in DT40InsP_3R-k/o cells. *C*, DT40w/t cells transfected with M5R, EYFP, and TRPC3 (*red trace*). TRPC3 channels were activated by 100 μM carbachol (*CCH*, *arrow*) followed by medium containing 1 mM Ba^{2+} (*bar*). Untransfected DT40w/t cells in the same field are shown in the *blue trace*. *D*, same as in *C* but in DT40InsP_3R-k/o cells.

not Sr^{2+} or Ba^{2+} gave rise to a substantial Ca^{2+}-specific entry into M5 receptor-transfected but not untransfected cells (Fig. 4*A*). These results clearly reflect the expression of M5 receptors functionally coupled to endogenous PLC-β. As expected, the transfection of M5 receptor into DT40InsP_3R-k/o cells resulted in no release or activation of Ca^{2+} entry (Fig. 4*B*). Using DT40w/t cells co-transfected with M5R and TRPC3 channel plasmids, there was a clear and profound activation of Ba^{2+} entry through the expressed TRPC3 channel. (Fig. 4*C*). Most significantly, similar cotransfection in the DT40InsP_3R-k/o cells resulted in almost identical profound activation of TRPC3 channels in response to muscarinic receptor stimulation (Fig. 4*D*)

Overall, our results using DT40 cells provide a rather clear picture of the mode of activation of TRPC3 channels in response to receptor-stimulated PLC-γ or PLC-β enzymes. TRPC3 activation appears independent of InsP_3Rs and, at least under the conditions of expression described here, independent of stores. We cannot rule out the possibility that InsP_3 produced from PLC could mediate an action on TRPC3 channels, but it would not be through the known InsP_3Rs. However, because DAG derivatives can directly activate TRPC3 channels, it is reasonable to assume that DAG is the physiological mediator. Our studies (not shown) reveal that the actions of OAG and receptor-activated PLC on TRPC3 channels are non-additive, consistent with the view that DAG from PLC activation is sufficient to cause TRPC3 activation. Considering the substantial evidence indicating a direct interaction between TRPC3 channels and InsP_3Rs (8, 15–20), it is possible that this interaction plays a regulatory rather than an obligatory function in TRPC3 activation. It is also possible that the InsP_3R gene deletions in DT40InsP_3R-k/o cells were incomplete and that a partial gene product corresponding to an InsP_3R fragment could mediate TRPC3 activation; however, our results indicate that full-length InsP_3Rs are not required to activate TRPC3 channels. Recent studies implicate ryanodine receptors as possible mediators of TRPC3 channels and SOCs (38), and we should consider it possible that endogenous ryanodine receptors could fulfill a coupling role in the absence of InsP_3Rs in DT40 cells. Finally, whereas our results reveal some significant differences between the operation of TRPC3 channels and SOCs, we should also consider that both TRPs and SOCs, as well as InsP_3Rs, share the common feature of being modified by 2-aminoethoxydiphenyl borate (24, 26, 27, 39). Because this agent is selective in modifying these and not other channels (24), we consider it likely that they share some common feature, perhaps a regulatory element, which may be important in their function and/or coupling.

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