The Role of Canonical Transient Receptor Potential 7 (TRPC7) in B-cell Receptor-activated Channels

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Running Title: TRPC7 Function in B-cells

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Phospholipase C signaling stimulates Ca\(^{2+}\) entry across the plasma membrane through multiple mechanisms. Ca\(^{2+}\) store depletion stimulates store-operated Ca\(^{2+}\)-selective channels, or alternatively, other phospholipase C-dependent events activate Ca\(^{2+}\)-permeable non-selective cation channels. TRPC7 is a non-selective cation channel that can be activated by both mechanisms when ectopically expressed, but the regulation of native TRPC7 channels is not known. We knocked out TRPC7 in a DT-40 B-cells, which expresses both forms of Ca\(^{2+}\) entry. No difference in the store-operated current, \(I_{\text{crac}}\) was detected between TRPC7\(^{-/-}\) and wild-type cells. Wild type cells demonstrated non-store-operated cation entry and currents in response to activation of the B-cell receptor or protease-activated receptor 2, intracellular dialysis with GTP\(_\gamma\)S, or application of the synthetic diacylglycerol, oleyl-acetyl-glycerol. These responses were absent in TRPC7\(^{-/-}\) cells, but could be restored by transfection with human TRPC7. In conclusion, In B-lymphocytes, TRPC7 appears to participate in the formation of ion channels that can be activated by phospholipase C-linked receptors. This represents the first demonstration of a physiological function for endogenous TRPC7 channels.

TRPC channels are believed to function as multifunctional calcium-permeable cation channels (1). Depending on cell type, expression level, or expression environment, the channels can be activated through the phospholipase C pathway, or in some instances, by depletion of intracellular Ca\(^{2+}\) stores (1-3). The calcium-permeable cation channel, canonical transient receptor potential 7 (TRPC7) was originally shown to function as a phospholipase C-regulated channel,
presumably through production of diacylglycerol (4). However, a subsequent report provided evidence that TRPC7 could function as a store-operated channel (5). Finally it was demonstrated that the different findings from the two laboratories likely resulted from differences in expression conditions. When TRPC7 was transiently expressed in HEK293 cells, it behaved as a diacylglycerol-activated channel; but when expressed stably, it appeared to function as a capacitative or store-operated calcium entry channel (6). It is thus important to determine which, if either, of these two behaviors corresponds to the physiological function of TRPC7 when expressed endogenously. To date, there have been no studies of the physiological function of endogenously expressed TRPC7. Thus, in the current work, we utilized targeted homologous recombination to knock out TRPC7 in B-lymphocytes, DT40, a cell line previously shown to express endogenously both store-operated and non-store-operated pathways (7-9). Although knockout of TRPC7 from DT40 cells caused significant alterations in cellular Ca2+ homeostasis and signaling, it did not result in a diminution in whole-cell $I_{\text{crac}}$ current. Rather, in the avian B-cell line, TRPC7 appears to function as a phospholipase C-regulated, diacylglycerol-activated channel.

**Materials and Methods**

**Reagents**- Thapsigargin, methacholine and OAG were purchased from Calbiochem, San Diego, CA. 1,2-bis(2-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid (BAPTA) were purchased from Calbiochem (La Jolla, CA). Methacholine was purchased from Sigma (St Louis, MO, USA) Fura-2-acetoxymethylester was from Molecular Probes Inc.

$Ca^{2+}$ and $Ba^{2+}$ measurements- The immortalized chicken B-lymphocyte cell line, DT-40 (RIKEN Cell Bank number RCB1464), and a mutant version with the gene for TRPC7 disrupted (see below) were maintained in suspension culture and loaded with the Ca2+ indicator, Fura-2, as previously described (10). The cells were then washed and bathed in Heps buffered physiological saline solution at room temperature at least 20 min before Ca2+ or Ba2+ measurements were made. In some experiments, a nominally Ca2+-free medium was used, which was identical in composition except for the omission of added CaCl2. For all cell lines, measurements of Fura-2 fluorescence ratios, indicative of changes in intracellular Ca2+ or Ba2+, were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging Inc., Cincinnati, OH) as described previously (11). All experiments were conducted at room temperature and data are reported as the ratio of fluorescence due to excitation at 340 and 380 nm. Transmembrane divalent cation flux, either constitutive or stimulated was estimated by measuring the initial rate of Ba2+ entry following addition of 10 mM Ba2+, in fluorescence ratio units/min.

**Electrophysiology**- Macroscopic membrane ion currents were recorded using the patch-clamp technique in its whole-cell configuration. The currents were acquired using pCLAMP-9.2 (Axon Instr., USA) and analyzed offline using Origin 6 (Microcal, USA) software. The extracellular solution (osmolarity 310 mosmol/l) contained (in mM): NaCl, 145; KCl, 5; HEPES, 10; MgCl2, 1; CaCl2, 2 or 10, as indicated; pH, 7.3 (adjusted with NaOH). The intracellular pipette solution (osmolarity 290 mosmol/l) contained (in mM): Cs-Methanesulfonate, 145; BAPTA, 10; HEPES, 10; MgCl2, 1; CaCl2, 0 or 2.2; pH, 7.2 (adjusted with CsOH). Patch pipettes were fabricated from borosilicate glass capillaries (WPI, USA). The resistance of the pipettes varied between 3 and 5 MΩ. Necessary supplements were added directly to the respective solutions, in concentrations that would not significantly change the osmolarity. Changes in the external solutions were carried
out using a multibarrel puffing micropipette with common outflow that was positioned in close proximity to the cell under investigation. During the experiment, the cell was continuously superfused with the solution via puffing pipette to reduce possible artifacts related to the switch from static to moving solution and vice versa.

**Generation of TRPC7-deficient DT40 Cells** - The strategy is summarized in Figure 1. The chicken genomic TRPC7 DNA was obtained by PCR using pairs of primers chTRPC7-P1 (sense, 5'-ACGACGCCCAAAAGATGGACA-3') and chTRPC7-P1FR (antisense, 5'-ACGACGCCCAAAAGATGGACA-3'), chTRPC7-P1F (sense, 5'-TGTCCATCTTTGTGGCGTCGT-3') and chTRPC7-P2 (antisense, 5'-GGTGTTGTATCATGGCTATTAGC), respectively. The targeting vector of TRPC7 was constructed by replacing the genomic sequence, which encodes the hydrophobic segment H3 to H6, of chicken TRPC7, with a histidinol (hisD) or neomycin resistance gene (neo) cassette (12). The upstream 4.3-kb and downstream 1.6-kb genomic sequences of TRPC7 were used as a targeting vector. DT40 cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% chicken serum, penicillin, streptomycin, and glutamine. The targeting vector was linearized and transfected sequentially into DT40 cells by electroporation (550 V, 25 µF). After isolation of several clones in the presence of 1 mg/ml histidinol or 2 mg/ml G418, genomic DNAs were prepared and analyzed by Southern blot analysis using the 3'-flanking probe (Figure 1).

**Northern blot analysis** - RNA blot hybridization analysis was carried out using total RNA (30 µg) from wild type or TRPC7-/- cells. The probe used to detect TRPC7 RNA was amplified by RT-PCR using specific primers (chTRPC7-P1F and chTRPC7-P6: 5'-GATTACTCCATAGAGTACGTATCC-3'). Random Primer DNA Labeling Kit version 2 (Takara) was used to 32P label the probe. Hybridization was performed at 42°C in 50% formamide, 5× SSC, 50 mM sodium phosphate buffer (pH 7.0), 0.1% SDS, 5×Denhardt's solution, and 0.2 mg/ml sonicated herring sperm DNA, as described previously (13).

**Transient transfections** - Transient transfections of the wild-type and TRPC7-/- DT40 cells were carried out by electroporation essentially as described previously (14), with either the human isoform of TRPC7 (10 µg/ml) into pcDNA3 vector, or vector alone (pcDNA3, mock transfected cells) along with EYFP (5 µg) as marker for transfection. Cells were used for real-time fluorescence measurements 20–25 h post-transfection. In figures involving transient transfections, average traces from 6–10 EYFP-positive cells are shown for a single experiment, and these are representative of three independent experiments.

**Statistics** - When comparisons were made between two groups, we used Student’s t-test; when three groups were analyzed, we used ANOVA with Tukey-Kramer Multiple Comparisons as post hoc tests. In some instances, in which multiple knockout cells gave, in every cell, no response at all, we simply provide the mean and SEM of the wild type cells, and cite the number of knockout cells which failed to respond.

**Results**

**Knockout of TRPC7 in DT40 B-lymphocytes alters thapsigargin-activated [Ca2+]i signals** - Initially, we sought to determine whether TRPC7 might play a role in either store-operated or non-store-operated calcium entry in a cell in which both modes of entry occur. We chose the avian pre-B-cell line, DT40, because these cells have been shown to express the archetypical store-operated current, \( I_{\text{crac}} \) (13;15;16), as well as a
less well characterized non-store-operated pathway (8;9), and because of the relative ease of targeting genes in these cells for disruption by homologous recombination (7;17;18). Two independent DT40 cell lines null for TRPC7 message (Figure 1) were generated, as described in Materials and Methods. The lines were designated TRPC7−/− clone 14.3 and TRPC7−/− clone 14.304. Figure 2 shows initial experiments examining store-operated or capacitative calcium entry in wild-type and TRPC7−/− cells. Both knockout lines differed from wild type cells in two ways. First, both lines appeared to have substantially increased intracellular Ca2+ stores, based on the size of the response to thapsigargin in the absence of extracellular Ca2+. Similar results were obtained using the Ca2+ ionophore ionomycin to discharge the stores (not shown). Second, the rate of Ba2+ entry in response to store depletion by thapsigargin (a measure of store-operated divalent cation entry, (6;19-21)) appeared to be significantly depressed in both lines (wild type, 0.063 ± 0.009 ratio units/min, n=193; clone 14.3, 0.009 ± 0.002 ratio units/min, n=205; clone 14.304, 0.023 ± 0.004 ratio units/min, n=180; ANOVA, P<0.0001; wild type vs. clone 14.3, P<0.001; wild type vs. clone 14.304, P<0.001; clone 14.3 vs. clone 14.304, P>0.05).

The diminished rate of Ba2+ entry seen in the thapsigargin-treated TRPC7−/− cells is indicative of a diminished store-operated entry and thus might imply a role for TRPC7 in this pathway. However, we also considered the possibility that the enlarged thapsigargin-sensitive Ca2+ pool might retain Ca2+ for a longer time, resulting in less pool depletion and thus less activation of Ba2+ entry (see, for example, (22)). In experiments extending the time of Ba2+ addition by 15 minutes (not shown) the effect of TRPC7 knockout on the rate of Ba2+ entry was diminished, although still statistically significant (wild type, 0.063 ± 0.008 ratio units/min, n=197; clone 14.3, 0.040 ± 0.006 ratio units/min, n=205; P=0.0213). In addition, in fluorescence experiments, membrane potential is not controlled and a partially inhibited response could result from alterations in the driving force for entry. Thus, we next turned to analysis of the store-operated current, Icrac (13;23), by using whole-cell patch-clamp. With this technique, Ca2+ stores can be rapidly depleted by a combination of IP3 and a Ca2+ chelator, BAPTA, in the patch pipette. As shown in Figure 3, Icrac activation in TRPC7−/− cells did not differ significantly from that in wild-type cells. Note, however, that as reported by others the magnitude of Icrac is rather small in these cells (13;15;16), such that a small, partial inhibitory effect of TRPC7 knockout would not likely be detected. However, this would contrast sharply with the almost complete loss of a non-store-operated pathway, documented below.

Non-store-operated entry in DT40 wild type and TRPC7−/− cells. We next considered that TRPC7−/− cells might be deficient in non-store-operated Ca2+ entry, which is known to occur in this cell line (8;9). In these experiments, we examined agonist-activation of Ba2+ entry in the presence of 500 nM Gd3+, which is known to block completely the store-operated channels in DT40 cells (24). Figure 4 shows responses to activation of the endogenous B-cell receptors with an anti-IgM. As reported previously, in response to B-cell receptor activation in the absence of extracellular Ca2+, DT40 cells exhibit irregular oscillations that dissipate gradually. Addition of 10 mM Ba2+, in the presence of 500 nM Gd3+, reveals the presence of a Ba2+-permeable pathway activated through the B-cell receptor (0.051 ± 0.006 ratio units/min, n=161). In TRPC7−/− cells, the oscillations were larger, more frequent, and more persistent presumably due to the increased capacity of intracellular Ca2+ stores (see Figure 2). However, addition of 10 mM Ba2+ did not result in a detectable elevation in basal fluorescence ratio in any of the TRPC7−/− cells (n=183), indicating that the B-cell receptor dependent Ba2+ entry was largely absent in these cells.
The irregular pattern of Ca\(^{2+}\) oscillations following B-cell receptor activation makes quantitative analysis of the Ba\(^{2+}\) entry difficult. Thus, we also examined signaling through a G-protein-activated pathway, the protease-activated receptor 2 (PAR2), known to be expressed in this cell line (25). As shown in Figure 5, treatment of DT40 cells with 200 nM trypsin, which activates PAR2 receptors, resulted in release of intracellular stores and Gd\(^{3+}\)-insensitive entry of Ba\(^{2+}\). This Ba\(^{2+}\) entry was absent in TRPC7\(^{-/-}\) cells (n=136). Interestingly, the release of Ca\(^{2+}\) by trypsin did not appear to be augmented in the TRPC7\(^{-/-}\) cells; this may indicate that this receptor type recruits a very specific endoplasmic reticulum Ca\(^{2+}\) pool.

We examined the ability of the G-protein pathway to activate membrane currents likely responsible for the Gd\(^{3+}\)-insensitive Ba\(^{2+}\) entry that appears to depend on TRPC7. Because not all cells respond to either B-cell receptor ligation (~55-60 %) or to PAR2 (~55-70 %), we activated G-proteins directly by including GTP\(_{\gamma}\)S in the patch pipet solution, while examining whole cell currents in the whole-cell mode. Following break-in with a pipet solution containing 300 \(\mu\)M GTP\(_{\gamma}\)S, a current developed that reversed near 0 mV, and was slightly outwardly rectifying (Figure 6A and 6B; maximum currents were: outward, 14.7 ± 1.2 pA/pF; inward, -8.7 ± 0.9 pA/pF, n=7). This behavior is reminiscent of the behavior of ectopically transfected TRPC7 (4). The current was largely absent in TRPC7\(^{-/-}\) cells (Figure 6A, n=6).

In its non-store-operated mode, TRPC7 can also be activated by synthetic diacylglycerols (4;6). However, addition of the synthetic diacylglycerol, oleyl acetyl glycerol (OAG) failed to activated any significant Ba\(^{2+}\) entry in wild type or TRPC7\(^{-/-}\) cells (Figure 7, top). TRPC7 is known to be potently inhibited by protein kinase C (4) which would also be activated by OAG, perhaps to a greater extent than with agonist or GTP\(_{\gamma}\)S activation. Thus, we utilized a combination of two protein kinase C inhibitors, Gö6976 (1 \(\mu\)M) and calphostin C (0.5 \(\mu\)M), and under these conditions, OAG activated significant Ba\(^{2+}\) entry in wild type DT40 cells (Figure 7, bottom) and activated a current similar to that seen with GTP\(_{\gamma}\)S (Figure 6C). The OAG-induced Ba\(^{2+}\) entry was significantly reduced in TRPC7\(^{-/-}\) cells (Figure 7, bottom) (wild type, 0.048 ± 0.005 ratio units/min, n=170; clone 14.3, 0.012 ± 0.005, n=183; P<0.0001).

We next sought to rescue the effects of TRPC7 deletion by transiently transfecting TRPC7\(^{-/-}\) cells with a plasmid encoding for human TRPC7 (6). Initially, we used a rather high concentration of the pcDNA3 plasmid (100 \(\mu\)g/ml) because of the low expression levels generally obtained with the CMV promoter (14). Transient expression of human TRPC7 resulted in Ba\(^{2+}\) entry that could be activated by either a receptor agonist or OAG, but did not reverse the increased capacity of intracellular Ca\(^{2+}\) stores (not shown). The appearance of receptor- and OAG-activated entry following transient overexpression of TRPC7 is perhaps not surprising, given previous reports that overexpression of the close structural relative, TRPC3, leads to similar behavior (14;26). The OAG-activated entry seen in TRPC7\(^{-/-}\) cells transiently transfected with TRPC7 differed from OAG-activated entry in wild-type cells; in the TRPC7-transfected TRPC7\(^{-/-}\) cells, OAG could activate entry even in the absence of PKC inhibitors (not shown). Thus, we next transfected TRPC7\(^{-/-}\) cells with a lower concentration of TRPC7 encoding plasmid (10 \(\mu\)g/ml); in our previous work, this concentration of TRPC3 plasmid did not produce any additional receptor- or OAG activated entry (14;24). With this apparently lower level of expression, neither wild type nor TRPC7\(^{-/-}\) cells responded to OAG in the absence of PKC inhibitors, whether transfected with TRPC7 or not (Figure 8A, C). In the presence of PKC inhibitors, the wild type response was slightly but not significantly potentiated (Figure 8B; mock transfected, 0.050 ± 0.005 ratio units/min,
n=59; TRPC7 transfected, 0.053 ± 0.004 ratio units/min, n=65; P=0.64); more significantly, in the TRPC7-/- cells, the response to OAG was restored by transfection with human TRPC7 (Figure 8D; mock transfected, 0.017 ± 0.006 ratio units/min, n=51; TRPC7 transfected, 0.049 ± 0.007 ratio units/min, n=77; P=0.016).

Discussion

Knockout of TRPC7 from DT40 B-lymphocytes produced three apparent phenotypes: an increased size of the Ca2+ stores, a diminished store-operated entry, and a diminished receptor- and diacylglycerol-activated entry. Of the three, only the latter, diacylglycerol-activated entry can be confidently attributed to the function of TRPC7 in native DT40 B-cells. The apparent decrease in store-operated entry appears to be secondary to the larger Ca2+ stores, and the greater difficulty in completely depleting these stores with the SERCA inhibitor, thapsigargin. Thus, when stores were more rapidly depleted with a combination of IP3 and calcium chelator, the development of the store-operated current, \( I_{crac} \), was not significantly different in wild type and TRPC7-/- cells. It has previously been argued that TRPC channels can function in some environments as either store-operated or non-store-operated channels (1), and when ectopically expressed in HEK293 cells, TRPC7 is clearly capable of functioning in both of these modes (6). While the simplest interpretation of our data is that TRPC7 does not play an obligatory role in forming the CRAC channel in DT40 B-cells, it remains a possibility that following knockout of the gene, other TRP channel subunits may increase and assume this role. In addition, it is also conceivable that TRPC7 may function as a store-operated channel in other cell types, for example in instances when the store-operated channels have conduction properties more akin to TRPCs (27).

The increased size of intracellular Ca2+ stores might indicate a role for TRPC7 in maintaining endoplasmic reticulum Ca2+ homeostasis, perhaps by functioning as either a constitutively active, or regulated Ca2+-permeable channel. This would not be the first example of a TRP channel capable of gating Ca2+ fluxes in endoplasmic reticulum as well as in the plasma membrane (28;29). It is also not the first example of TRPC knockout affecting endoplasmic reticulum Ca2+ signaling. Mori et al. (13) found that knockout of TRPC1 resulted in reduced sensitivity of endoplasmic reticulum stores to IP3-induced Ca2+ release. However, in that case the thapsigargin-sensitive stores appeared unchanged. In the current study, the effects of TRPC7 knockout on calcium stores was not reversed by transient restoration of TRPC7. This may indicate that the effects on Ca2+ stores are more complex than simply loss of TRPC7, and may involve other longer term compensatory changes.

On the other hand, the disappearance of a receptor- and diacylglycerol-activated Ba2+ entry and cation current was restored by transient transfection with a plasmid encoding for human TRPC7. Such an entry in DT40 cells has been reported in previous studies (8;9;30), and appears to depend in some manner on IP3 receptors. However, the precise role of IP3 receptors in this pathway is controversial. In the present study, we demonstrate for the first time that (i) this entry appears to involve non-selective cation channels similar to TRPC channels, (ii) this entry and current can be activated by exogenously applied diacylglycerols, and (iii) this entry and current are almost completely lost in the absence of TRPC7. The latter observation ties together the Ba2+ entry and currents seen under the various conditions of B-cell receptor activation, PAR2 receptor activation, activation by intracellular application of GTP\(\gamma\)S, and activation by OAG, as all of these responses were lost in the TRPC7-/- cells. This represents to our knowledge the first demonstration of a
physiological function of endogenously expressed TRPC7.

The broader role of non-store-operated TRPC7 channels in B-lymphocyte function cannot be determined at present. However, there is ample evidence for such channels in both T- and B-lymphocytes (31). The identity of a candidate channel subunit participating specifically in this pathway should open the way for more detailed analyses of the physiological role of receptor regulated TRPC channels in B-lymphocytes and other non-excitable cells.

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Reference List


Figure Legends

Figure 1. Targeted disruption of the TRPC7 gene in DT40 B lymphocytes. Partial restriction map of chicken TRPC7 gene (A), targeting construct (B), and expected structure of the disrupted allele (C). (D) Southern blot analysis of genomic DNAs from DT40 cells. Genomic DNAs were prepared from WT (+/+), neo-targeted (+/-), and neo/his-targeted (-/-) clones, digested with EcoRV, and hybridized with a 3'-flanking probe. The restriction endonuclease cleavage site of EcoRV and XhoI is abbreviated as E and X, respectively. (E) Northern blot analysis of WT or TRPC7 KO DT40 cells. The positions of ribosomal RNAs (18S and 28S) are shown on the left.

Figure 2. Thapsigargin-induced Ba<sup>2+</sup> entry is reduced in TRPC7-knockout DT40 B-lymphocytes. Wild type TRPC7<sup>+/+</sup> DT40 cells (clone 14.3 and clone 14.304) were incubated in the absence of added Ca<sup>2+</sup>. 2 µM thapsigargin (TG) followed by 10 mM Ba<sup>2+</sup> was added where indicated (solid line). The constitutive Ba<sup>2+</sup> entry was also determined in all three cell lines (dotted line). Ba<sup>2+</sup> entry measurements were performed with single cells attached to coverslip as described in Material and Methods. The trace presented is the average of 2-3 coverslips with at least 50 cells attached per coverslip from one experiment performed in triplicate.

Figure 3. I<sub>crac</sub> is not significantly affected by knockout of TRPC7. Average time course of I<sub>crac</sub> development at -150 mV in wild-type (solid circles, n=10) and TRPC7<sup>−/−</sup> (open circles, n=4) DT40 cells. The bath solution contained 10 mM Ca<sup>2+</sup> while the pipette solution contained 100 µM IP3, 6 mM Mg<sup>2+</sup> and no Ca<sup>2+</sup>. The results are presented as means ± s.e.m.

Figure 4. BCR stimulation activates a non-store-operated, Gd<sup>3+</sup>-insensitive cation entry pathway in wild-type but not in TRPC7<sup>−/−</sup> DT40 cells. Fura-2-loaded wild type (top) or TRPC7<sup>−/−</sup> (bottom) DT40 cells were maintained in a nominally Ca<sup>2+</sup>-free medium containing 0.5 µM Gd<sup>3+</sup> (to block store-operated channels) and then exposed to anti-IgM (2 µg/ml) to induce BCR-dependent intracellular Ca<sup>2+</sup> mobilization. When indicated, Ba<sup>2+</sup> (10 mM) was added to the bath to reveal activation of receptor-activated cation channels. Shown are average traces from at least 50 cells, representative of three independent experiments.

Figure 5. Stimulation of an endogenous G-protein coupled receptor results in activation of the non-store-operated, Gd<sup>3+</sup>-insensitive cation entry pathway in wild-type but not in TRPC7<sup>−/−</sup> DT40 cells. Fura-2-loaded wild type (top) or TRPC7<sup>−/−</sup> (bottom) DT40 cells were maintained in a nominally Ca<sup>2+</sup>-free medium containing 0.5 µM Gd<sup>3+</sup> (to block store-operated channels) and then exposed to trypsin (200 nM) to induce protease-activated-receptor-2 (PAR2)-dependent intracellular Ca<sup>2+</sup> mobilization. When indicated, Ba<sup>2+</sup> (10 mM) was added to the bath to reveal activation of receptor-activated cation channels. Shown are average traces from at least 50 cells, representative of three independent experiments.
**Figure 6.** Stimulation of endogenous G-proteins results in development of a non-selective cation current in wild-type but not in TRPC7<sup>-/-</sup> DT40 cells.  
A: Average time courses of whole-cell current development in wild-type (solid line, dotted line) and TRPC7<sup>-/-</sup> (dashed line) DT40 cells at -100 and +100 mV when breaking in with or without (WT cells only) GTP<sub>γ</sub>S (300 µM) in the patch pipette. For wild-type cells, n=7 and n=5, for experiments with and without GTP<sub>γ</sub>S, respectively; for TRPC7<sup>-/-</sup> cells, n=6 under both conditions (controls without GTP<sub>γ</sub>S for TRPC7<sup>-/-</sup> are not shown). The intracellular pipette solution contained (in mM): 145 cesium methanesulfonate; 10 BAPTA; 10 HEPES; 1 MgCl<sub>2</sub>; 2 MgATP; 3 CaCl<sub>2</sub> (~80 nM free Ca<sup>2+</sup>).  
B and C: Leak-subtracted current voltage relationship for the GTP<sub>γ</sub>S-activated (B) and OAG-activated (C) current. In C, current was activated by inclusion of 10 µM OAG in the patch pipette in the presence of inhibitors of protein kinase C (see Legend to Figure 7).

**Figure 7.** Inhibition of C kinase (PKC) reveals the existence of an endogenous diacylglycerol-sensitive, non-store-operated and Gd<sup>3+</sup>-insensitive, cation entry pathway in wild-type but not in TRPC7<sup>-/-</sup> DT40 cells.  
Top: Fura-2-loaded wild type (solid line) or TRPC7<sup>-/-</sup> (dotted line) DT40 cells were maintained in a nominally Ca<sup>2+</sup>-free medium containing 0.5 µM Gd<sup>3+</sup> and 10 mM Ba<sup>2+</sup>, and then exposed to 100 µM of the membrane-permeant diacylglycerol analog OAG. Bottom: As above, but the PKC inhibitors Gö6976 (1 µM) and calphostin C (0.5 µM) were added 3 min before challenging the cells with OAG. Shown are average traces, representative of three independent experiments (in A, n=166 for WT; n=167 for TRPC7KO cells; in B, n=170 for WT; n=183 for TRPC7KO cells).

**Figure 8.** Transient expression of human TRPC7 in TRPC7<sup>-/-</sup> DT40 cells rescues the diacylglycerol-sensitive cation entry pathway.  
Wild-type (A and B) or TRPC7<sup>-/-</sup> (C and D) DT40 cells were transfected with either 10 µg/ml TRPC7 in pcDNA3 or vector alone (Mock), along with a construct encoding EYFP as transfection marker. Following loading with Fura-2, cells were maintained in a nominally Ca<sup>2+</sup>-free medium containing 0.5 µM Gd<sup>3+</sup> and 10 mM Ba<sup>2+</sup>, and then exposed to 100 µM of the membrane-permeant diacylglycerol analog OAG (A and B). C and D: as in A and B, but the PKC inhibitors Gö6976 (1 µM) and calphostin C (0.5 µM) were added 3 min before challenging the cells with OAG. Shown are average traces of the indicated number of EYFP-positive cells (see below), and are representative of three independent experiments. In A, n=6 for both mock and TRPC7 transfected cells; in B, n=10 and n=8 for mock and TRPC7 transfected cells, respectively; in C, n=6 and n=7 for mock and TRPC7 transfected cells, respectively; in D, n=7 and n=6 for mock and TRPC7 transfected cells, respectively.
Figure 2

Wild Type

Clone 14.3

Clone 14.304

F340/F380

2 µM TG

10 mM Ba\(^{2+}\)

Seconds

0 1000 2000

0 1000 2000

0 1000 2000
Figure 5

Wild Type

Clone 14.3

F340/F380

0 300 600

Seconds

Ba\textsuperscript{2+}

Trypsin

Ba\textsuperscript{2+}

Trypsin

0.4 0.8 1.2

0.4 0.8 1.2

Wild Type

Clone 14.3
Figure 6

A

WT, GTP\textsuperscript{YS}

Clone 14.3, GTP\textsuperscript{YS}

WT, Control

Seconds

\begin{itemize}
\item B: GTP\textsuperscript{YS}
\item C: OAG
\end{itemize}
Figure 7

t=0: 10 mM Ba^{3+}, 500 nM Gd^{3+}

Wild Type
Clone 14.3

0.1 mM OAG

PKC Inhibitors

F340/F380

0.5 1.1

0 400 800 1200

Seconds
Figure 8

A: Wild Type, No PKCI

B: Wild Type, + PKCI

C: Clone 14.3, No PKCI

D: Clone 14.3, + PKCI

F340/F380

Ba$^{2+}$

OAG

TRPC7

Mock

0 600 1200

Seconds
The role of canonical transient receptor potential 7 (TRPC7) in B-cell receptor-activated channels
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