

The Role of Canonical Transient Receptor Potential 7 in B-cell Receptor-activated Channels*

Received for publication, July 13, 2005, and in revised form, August 10, 2005 Published, JBC Papers in Press, August 25, 2005, DOI 10.1074/jbc.M507606200

Jean-Philippe Lievreumont^{†1,2}, Takuro Numaga^{§1}, Guillermo Vazquez^{†1}, Loïc Lemonnier[‡], Yuji Hara[§], Emiko Mori[§], Mohamed Trebak[‡], Stephen E. Moss[¶], Gary S. Bird[‡], Yasuo Mori[§], and James W. Putney, Jr.^{†3}

From the [†]Department of Health and Human Services, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709, [§]Laboratory of Molecular Biology, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 615-8510, Japan, and [¶]Division of Cell Biology, Institute of Ophthalmology, University College London, Bath Street, London EC1V 9EL, United Kingdom

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. Transient receptor potential 7 (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 DT40 B-cells, which expresses both forms of Ca^{2+} entry. No difference in the store-operated current I_{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 γ 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 appeared to participate in the formation of ion channels that could be activated by phospholipase C-linked receptors. This represents the first demonstration of a physiological function for endogenous TRPC7 channels.

Transient receptor potential channels (TRPCs)⁴ 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 has provided evidence that TRPC7 could function as a store-operated channel (5). Finally, it has been demonstrated that the different findings from the two laboratories likely result from differences in expression conditions. When TRPC7 is tran-

siently expressed in HEK293 cells, it behaves as a diacylglycerol-activated channel; but when expressed stably, it appears 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 of DT40, a cell line previously shown to endogenously express both store-operated and non-store-operated pathways (7–9). Although knock out of TRPC7 from DT40 cells caused significant alterations in cellular Ca^{2+} homeostasis and signaling, it did not result in a diminution in whole-cell I_{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 oleyl acetyl glycerol (OAG) were purchased from Calbiochem, San Diego, CA. BAPTA was purchased from Calbiochem (La Jolla, CA). Methacholine was purchased from Sigma. Fura-2-acetoxymethyl ester (Fura-2) was from Molecular Probes, Inc.

Ca^{2+} and Ba^{2+} Measurements—The immortalized chicken B-lymphocyte cell line DT40 (RIKEN (Institute of Physical and Chemical Research) 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 Ca^{2+} indicator Fura-2, as previously described (10). The cells were then washed and bathed in HEPES-buffered physiological saline solution at room temperature at least 20 min before Ca^{2+} or Ba^{2+} measurements were made. In some experiments, a nominally Ca^{2+} -free medium was used, which was identical in composition, except for the omission of added CaCl_2 . For all cell lines, measurements of Fura-2 fluorescence ratios, indicative of changes in intracellular Ca^{2+} or Ba^{2+} , 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 were 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 Ba^{2+} entry following the addition of 10 mM Ba^{2+} 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 Instruments, Foster City, CA) and analyzed off-line using Origin 6 (Microcal) software. The extracellular solution (osmolarity 310 mosmol/liter) con-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ These authors contributed equally to this work.

² Present address: Neuro3d S.A., Parc d'Innovation, Ecole Supérieure de Biotechnologie de Strasbourg, Blvd. Sébastien Brandt, F-67412 Illkirch, France.

³ To whom correspondence should be addressed: NIEHS, National Institutes of Health, P.O. Box 12233, Research Triangle Park, NC 27709. Tel.: 919-541-1420; Fax: 919-541-1898; E-mail: putney@niehs.nih.gov.

⁴ The abbreviations used are: TRPC, transient receptor potential channel; GTP γ S, guanosine 5'-3-O-(thio)triphosphate; OAG, oleyl acetyl glycerol; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; Fura-2, Fura-2-acetoxymethyl ester; IP₃, inositol 1,4,5-trisphosphate; PAR2, protease-activated receptor 2; WT, wild-type; I_{crac} , Ca^{2+} release-activated Ca^{2+} current.

tained (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/liter) contained (in mM): cesium-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 Instruments). 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 Fig. 1. The chicken genomic TRPC7 DNA was obtained by PCR using pairs of primers: chTRPC7-P1, sense, 5'-TTGAAGTTGTAGCACACGCAGTTTC-3' and chTRPC7-P1FR, antisense, 5'-ACGACGCCACAAAGATGGACA-3'; chTRPC7-P1F, sense, 5'-TGTCATCTTTGTGGCGTCGT-3' and chTRPC7-P2, antisense, 5'-GGTGTGTTTATCATGGCTATTAGC, respectively. The targeting vector of TRPC7 was constructed by replacing the genomic sequence, which encodes the hydrophobic segment H-3–H-6 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 medium supplemented with 10% fetal bovine serum, 1% chicken serum, penicillin, streptomycin, and glutamine. The targeting vector was linearized and transfected sequentially into DT40 cells by electroporation (550 V, 25 microfarads). 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 (Fig. 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 reverse transcription-PCR using specific primers (chTRPC7-P1F and chTRPC7-P6, 5'-GATTACTCCATAGAGTACGTATCC-3'). The Random Primer DNA labeling kit, version 2 (Takara Shuzo) 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 enhanced yellow fluorescent protein (5 μg) as a 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 enhanced yellow fluorescent protein-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 analysis of variance with Tukey-Kramer multiple comparisons as post hoc tests. In some instances, in which multiple knock-out cells gave (in every cell) no response at all, we simply provided the mean and S.E. of the wild-type cells and cited the number of knock-out cells that failed to respond.

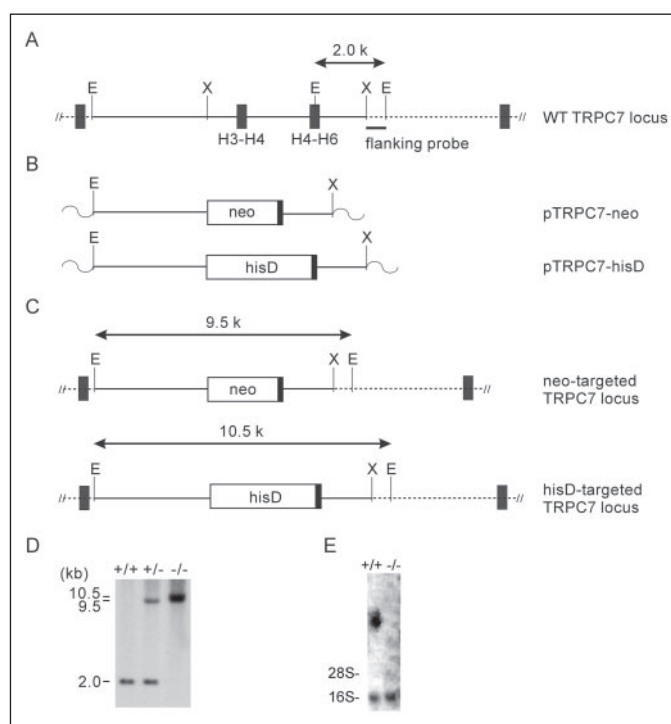


FIGURE 1. Targeted disruption of the TRPC7 gene in DT40 B-lymphocytes. Partial restriction map of chicken TRPC7 gene (A), targeting construct (B), and expected structure of the disrupted allele (C). D, Southern blot analysis of genomic DNAs from DT40 cells. Genomic DNAs were prepared from WT (+/+), *neo*-targeted (+/-), and *neo*/*hisD*-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 knock-out DT40 cells. The positions of ribosomal RNAs (16 and 28 S) are shown on the left.

RESULTS

Knock Out of TRPC7 in DT40 B-lymphocytes Alters Thapsigargin-activated $[Ca^{2+}]_i$ 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_{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 the TRPC7 message (Fig. 1) were generated, as described under "Materials and Methods." The lines were designated TRPC7^{-/-} clone 14.3 and TRPC7^{-/-} clone 14.304. Fig. 2 shows initial experiments examining store-operated or capacitative calcium entry in wild-type and TRPC7^{-/-} cells. Both knock-out lines differed from wild-type cells in two ways. First, both lines appeared to have substantially increased intracellular Ca^{2+} stores, based on the size of the response to thapsigargin in the absence of extracellular Ca^{2+} . Similar results were obtained using the Ca^{2+} ionophore ionomycin to discharge the stores (not shown). Second, the rate of Ba^{2+} 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, $n = 180$; analysis of variance, $p < 0.0001$; wild type versus clone 14.3, $p < 0.001$; wild type versus clone 14.304, $p < 0.001$; clone 14.3 versus clone 14.304, $p > 0.05$).

The diminished rate of Ba^{2+} entry seen in the thapsigargin-treated TRPC7^{-/-} cells is indicative of a diminished store-operated entry and

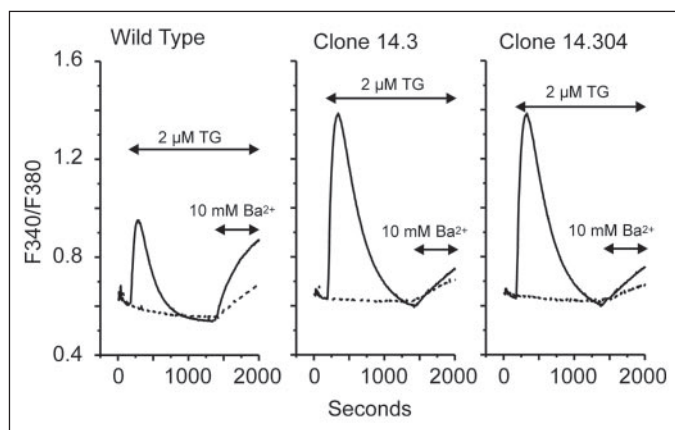


FIGURE 2. Thapsigargin-induced Ba^{2+} entry is reduced in TRPC7 knock-out DT40 B-lymphocytes. Wild-type TRPC7^{-/-} DT40 cells (clones 14.3 and 14.304) were incubated in the absence of added Ca^{2+} . $2 \mu M$ thapsigargin (TG) followed by $10 mM Ba^{2+}$ was added where indicated (solid line). The constitutive Ba^{2+} entry was also determined in all three cell lines (dotted line). Ba^{2+} entry measurements were performed with single cells attached to a coverslip, as described under "Material and Methods." The trace presented is the average of 2–3 coverslips with at least 50 cells attached/coverslip from one experiment performed in triplicate.

thus might imply a role for TRPC7 in this pathway. However, we also considered the possibility that the enlarged thapsigargin-sensitive Ca^{2+} pool might retain Ca^{2+} for a longer time, resulting in less pool depletion and thus less activation of Ba^{2+} entry (see, for example, Ref. 22). In experiments extending the time of Ba^{2+} addition by 15 min (not shown), the effect of TRPC7 knock out on the rate of Ba^{2+} 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 I_{crac} (13, 23) using whole-cell patch clamp. With this technique, Ca^{2+} stores can be rapidly depleted by a combination of IP_3 and a Ca^{2+} chelator, BAPTA, in the patch pipette. As shown in Fig. 3, I_{crac} activation in TRPC7^{-/-} cells did not differ significantly from that in wild-type cells. Note, however, that as reported by others, the magnitude of I_{crac} is rather small in these cells (13, 15, 16), such that a small, partial inhibitory effect of TRPC7 knock out 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 Ca^{2+} entry, which is known to occur in this cell line (8, 9). In these experiments, we examined agonist activation of Ba^{2+} entry in the presence of $500 nM Gd^{3+}$, which is known to block completely the store-operated channels in DT40 cells (24). Fig. 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 Ca^{2+} , DT40 cells exhibit irregular oscillations that dissipate gradually. The addition of $10 mM Ba^{2+}$, in the presence of $500 nM Gd^{3+}$, reveals the presence of a Ba^{2+} -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 because of the increased capacity of intracellular Ca^{2+} stores (see Fig. 2). However, the addition of $10 mM Ba^{2+}$ 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 Ba^{2+} entry was largely absent in these cells.

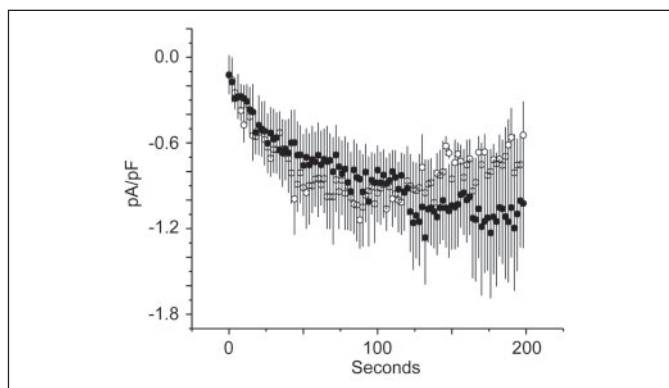


FIGURE 3. I_{crac} is not significantly affected by knock out of TRPC7. Average time course of I_{crac} development at $-150 mV$ in wild-type (solid circles, $n = 10$) and TRPC7^{-/-} (open circles, $n = 4$) DT40 cells. The bath solution contained $10 mM Ca^{2+}$, whereas the pipette solution contained $100 \mu M IP_3$, $6 mM Mg^{2+}$, and no Ca^{2+} . The results are presented as means \pm S.E.

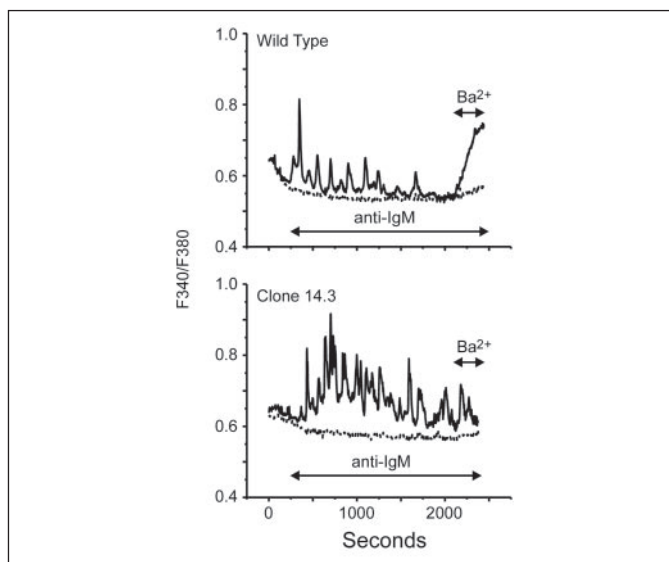


FIGURE 4. B cell receptor stimulation activates a non-store-operated, Gd^{3+} -insensitive cation entry pathway in wild-type but not in TRPC7^{-/-} DT40 cells. Fura-2-loaded wild-type (top) or TRPC7^{-/-} (bottom) DT40 cells were maintained in a nominally Ca^{2+} -free medium containing $0.5 \mu M Gd^{3+}$ (to block store-operated channels) and then exposed to anti-IgM ($2 \mu g/ml$) to induce B cell receptor-dependent intracellular Ca^{2+} mobilization. When indicated, Ba^{2+} ($10 mM$) 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.

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 Fig. 5, treatment of DT40 cells with $200 nM$ trypsin, which activates PAR2s, 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 ($\sim 55\text{--}60\%$) or to PAR2 ($\sim 55\text{--}70\%$), we activated G-proteins directly by including GTP γS in the patch pipette solution, while examining whole cell currents in the whole-cell mode. Fol-

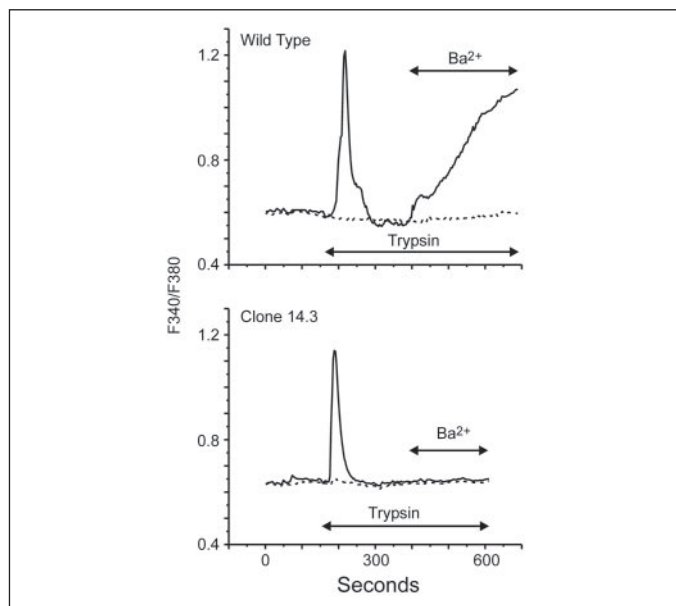


FIGURE 5. Stimulation of an endogenous G-protein-coupled receptor results in activation of the non-store-operated, Gd^{3+} -insensitive cation entry pathway in wild-type but not in $TRPC7^{-/-}$ DT40 cells. Fura-2-loaded wild-type (top) or $TRPC7^{-/-}$ (bottom) DT40 cells were maintained in a nominally Ca^{2+} -free medium containing $0.5 \mu M$ Gd^{3+} (to block store-operated channels) and then exposed to trypsin (200 nM) to induce PAR2-dependent intracellular Ca^{2+} mobilization. When indicated, Ba^{2+} (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.

lowing break-in with a pipette solution containing $300 \mu M$ GTP γ S, a current developed that reversed near 0 mV and was slightly outwardly rectifying (Fig. 6, A and B; maximum currents were: outward, 14.7 ± 1.2 picoamperes/picofarads; inward, -8.7 ± 0.9 picoamperes/picofarads, $n = 7$). This behavior is reminiscent of the behavior of ectopically transfected TRPC7 (4). The current was largely absent in $TRPC7^{-/-}$ cells (Fig. 6A, $n = 6$).

In its non-store-operated mode, TRPC7 can also be activated by synthetic diacylglycerols (4, 6). However, the addition of the synthetic diacylglycerol OAG failed to activate any significant Ba^{2+} entry in wild-type or $TRPC7^{-/-}$ cells (Fig. 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 γ S activation. Thus, we utilized a combination of two protein kinase C inhibitors, Gö6976 (1 μM) and calphostin C (0.5 μM), and under these conditions, OAG activated significant Ba^{2+} entry in wild-type DT40 cells (Fig. 7, bottom) and activated a current similar to that seen with GTP γ S (Fig. 6C). The OAG-induced Ba^{2+} entry was significantly reduced in $TRPC7^{-/-}$ cells (Fig. 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 cytomegalovirus promoter (14). Transient expression of human TRPC7 resulted in a 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-

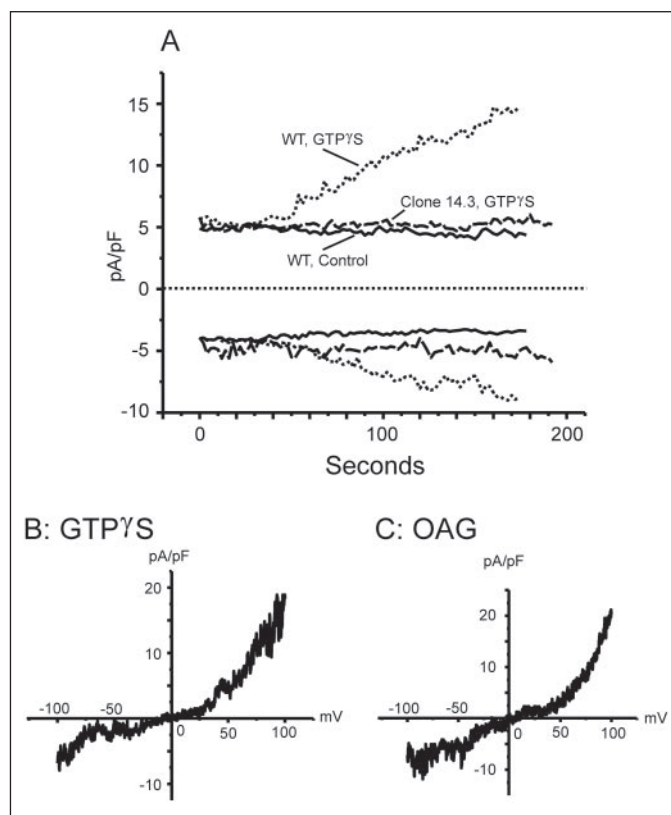


FIGURE 6. Stimulation of endogenous G-proteins results in development of a non-selective cation current in wild-type but not in $TRPC7^{-/-}$ DT40 cells. A, average time courses of whole-cell current development in wild-type (solid line and dotted line) and $TRPC7^{-/-}$ (dashed line) DT40 cells at -100 and $+100$ mV when breaking in with or without (WT cells only) GTP γ S (300 μM) in the patch pipette. For wild-type cells, $n = 7$ and $n = 5$, for experiments with and without GTP γ S, respectively; for $TRPC7^{-/-}$ cells, $n = 6$ under both conditions (controls without GTP γ S for $TRPC7^{-/-}$ are not shown). The intracellular pipette solution contained (in mM): 145 cesium-methanesulfonate; 10 BAPTA; 10 HEPES; 1 $MgCl_2$; 2 $MgATP$; 3 $CaCl_2$ (~ 80 nM free Ca^{2+}). B and C, leak-subtracted current voltage relationship for the GTP γ 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 Fig. 7).

type cells; in the TRPC7-transfected $TRPC7^{-/-}$ cells, OAG could activate entry even in the absence of protein kinase C (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 (Fig. 8, A and C). In the presence of PKC inhibitors, the wild-type response was slightly but not significantly potentiated (Fig. 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 (Fig. 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

Knock out of TRPC7 from DT40 B-lymphocytes produced three apparent phenotypes: an increased size of the Ca^{2+} 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

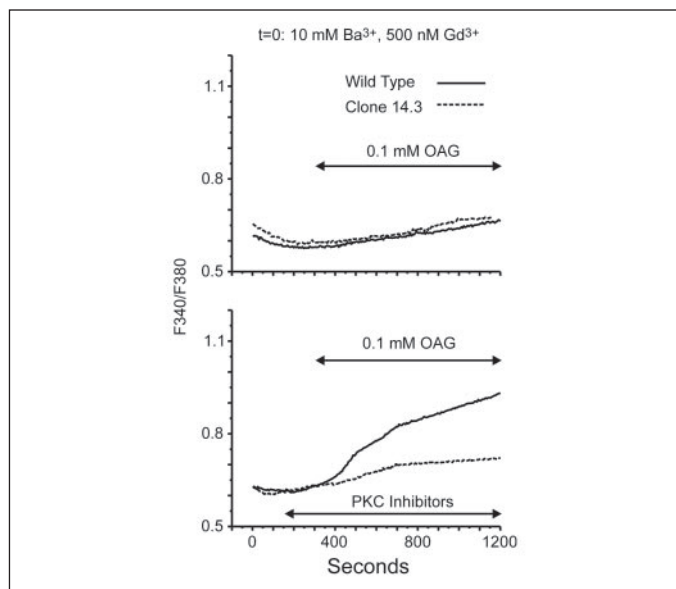


FIGURE 7. Inhibition of PKC reveals the existence of an endogenous diacylglycerol-sensitive, non-store-operated and Gd^{3+} -insensitive cation entry pathway in wild-type but not in $TRPC7^{-/-}$ DT40 cells. *Top*, Fura-2-loaded wild-type (solid line) or $TRPC7^{-/-}$ (dotted line) DT40 cells were maintained in a nominally Ca^{2+} -free medium containing $0.5 \mu M Gd^{3+}$ and $10 mM Ba^{2+}$ and then exposed to the membrane-permeant diacylglycerol analog OAG at a concentration of $100 \mu M$. *Bottom*, as described above, but the PKC inhibitors Gö6976 ($1 \mu M$) and calphostin C ($0.5 \mu M$) were added 3 min before challenging the cells with OAG. Shown are average traces representative of three independent experiments (in the top panel, $n = 166$ for WT; $n = 167$ for $TRPC7KO$ cells; in the bottom panel, $n = 170$ for WT; $n = 183$ for $TRPC7KO$ cells).

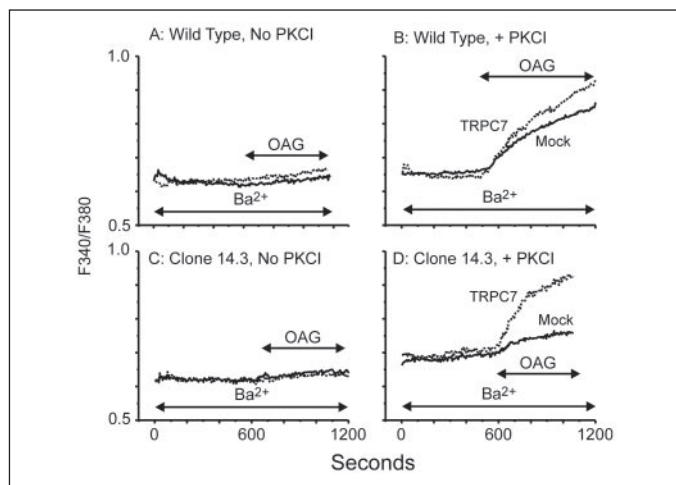


FIGURE 8. Transient expression of human TRPC7 in $TRPC7^{-/-}$ DT40 cells rescues the diacylglycerol-sensitive cation entry pathway. Wild-type (A and B) or $TRPC7^{-/-}$ (C and D) DT40 cells were transfected with either $10 \mu g/ml$ TRPC7 in pcDNA3 or vector alone (Mock), along with a construct encoding enhanced yellow fluorescent protein as the transfection marker. Following loading with Fura-2, cells were maintained in a nominally Ca^{2+} -free medium containing $0.5 \mu M Gd^{3+}$ and $10 mM Ba^{2+}$ and then exposed to the membrane-permeant diacylglycerol analog OAG at a concentration of $100 \mu M$ (A and B). C and D, same as described for A and B, but the PKC inhibitors Gö6976 ($1 \mu M$) and calphostin C ($0.5 \mu M$) were added 3 min before challenging the cells with OAG. Shown are average traces of the indicated number of enhanced yellow fluorescent protein-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.

DT40 B-cells. The apparent decrease in store-operated entry appears to be secondary to the larger Ca^{2+} stores and the greater difficulty in completely depleting these stores with the sarco(endo)plasmic reticulum Ca^{2+} ATPase inhibitor, thapsigargin. Thus, when stores were more

rapidly depleted with a combination of IP_3 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). Although the simplest interpretation of our data is that TRPC7 does not play an obligatory role in forming the Ca^{2+} release-activated Ca^{2+} channel in DT40 B-cells, it remains a possibility that, following knock out of the gene, other TRPC 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 Ca^{2+} stores might indicate a role for TRPC7 in maintaining endoplasmic reticulum Ca^{2+} homeostasis, perhaps by functioning as either a constitutively active or regulated Ca^{2+} -permeable channel. This would not be the first example of a TRPC capable of gating Ca^{2+} fluxes in endoplasmic reticulum as well as in the plasma membrane (28, 29). It is also not the first example of TRPC knock out affecting endoplasmic reticulum Ca^{2+} signaling. Mori *et al.* (13) found that knock out of TRPC1 resulted in reduced sensitivity of endoplasmic reticulum stores to IP_3 -induced Ca^{2+} release. However, in that case, the thapsigargin-sensitive stores appeared unchanged. In the current study, the effects of TRPC7 knock out on calcium stores was not reversed by transient restoration of TRPC7. This may indicate that the effects on Ca^{2+} 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 Ba^{2+} 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 IP_3 receptors. However, the precise role of IP_3 receptors in this pathway is controversial. In the present study, we demonstrated for the first time that (i) this entry appears to involve non-selective cation channels similar to TRPCs, (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 Ba^{2+} entry and currents seen under the various conditions of B-cell receptor activation, PAR2 activation, activation by intracellular application of GTP γ 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.

Acknowledgments—The human TRPC7 plasmid was jointly supplied by Christine Murphy and Adrian Wolstenholm of the University of Bath and John Westwick of Novartis, Horsham, UK. Drs. David Miller and David Armstrong read the manuscript and provided helpful comments.

REFERENCES

- Putney, J. W., Jr. (2004) *Trends Cell Biol.* **14**, 282–286
- Vazquez, G., Wedel, B. J., Aziz, O., Trebak, M., and Putney, J. W., Jr. (2004) *Biochim.*

- Biophys. Acta* **1742**, 21–36
3. Venkatachalam, K., van Rossum, D. B., Patterson, R. L., Ma, H.-T., and Gill, D. L. (2002) *Nat. Cell Biol.* **4**, E263–E272
 4. Okada, T., Inoue, R., Yamazaki, K., Maeda, A., Kurosaki, T., Yamakuni, T., Tanaka, I., Shimizu, S., Ikenaka, K., Imoto, K., and Mori, Y. (1999) *J. Biol. Chem.* **274**, 27359–27370
 5. Riccio, A., Mattei, C., Kelsell, R. E., Medhurst, A. D., Calver, A. R., Randall, A. D., Davis, J. B., Benham, C. D., and Pangalos, M. N. (2002) *J. Biol. Chem.* **277**, 12302–12309
 6. Lievreumont, J. P., Bird, G. S., and Putney, J. W., Jr. (2004) *Am. J. Physiol.* **287**, C1709–C1716
 7. Sugawara, H., Kurosaki, M., Takata, M., and Kurosaki, T. (1997) *EMBO J.* **16**, 3078–3088
 8. Vazquez, G., Wedel, B. J., Bird, G. St. J., Joseph, S. K., and Putney, J. W., Jr. (2002) *EMBO J.* **21**, 4531–4538
 9. 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
 10. Broad, L. M., Braun, F.-J., Lièvreumont, J.-P., Bird, G. St. J., Kurosaki, T., and Putney, J. W., Jr. (2001) *J. Biol. Chem.* **276**, 15945–15952
 11. Trebak, M., Bird, G. St. J., McKay, R. R., Birnbaumer, L., and Putney, J. W., Jr. (2003) *J. Biol. Chem.* **278**, 16244–16252
 12. Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H., and Kurosaki, T. (1994) *EMBO J.* **13**, 1341–1349
 13. Mori, Y., Wakamori, M., Miyakawa, T., Hermosura, M., Hara, Y., Nishida, M., Hirose, K., Mizushima, A., Kurosaki, M., Mori, E., Gotoh, K., Okada, T., Fleig, A., Penner, R., Iino, M., and Kurosaki, T. (2002) *J. Exp. Med.* **195**, 673–681
 14. Vazquez, G., Wedel, B. J., Trebak, M., Bird, G. St. J., and Putney, J. W., Jr. (2003) *J. Biol. Chem.* **278**, 21649–21654
 15. Prakriya, M., and Lewis, R. S. (2001) *J. Physiol. (Lond.)* **536**, 3–19
 16. Kiselyov, K., Shin, D. M., Shcheynikov, N., Kurosaki, M., and Muallem, S. (2001) *Biochem. J.* **360**, 17–22
 17. Buerstedde, J.-M., and Takeda, S. (1991) *Cell* **67**, 179–188
 18. Takata, M., Homma, Y., and Kurosaki, T. (1995) *J. Exp. Med.* **182**, 907–914
 19. Kwan, C. Y., and Putney, J. W., Jr. (1990) *J. Biol. Chem.* **265**, 678–684
 20. Byron, K. L., and Taylor, C. W. (1995) *J. Physiol. (Lond.)* **485**, 455–468
 21. Broad, L. M., Powis, D. A., and Taylor, C. W. (1996) *Biochem. J.* **316**, 759–764
 22. Leissring, M. A., Akbari, Y., Fanger, C. M., Cahalan, M. D., Mattson, M. P., and LaFerla, F. M. (2000) *J. Cell Biol.* **149**, 793–797
 23. Hoth, M., and Penner, R. (1992) *Nature* **355**, 353–355
 24. Trebak, M., Bird, G. St. J., McKay, R. R., and Putney, J. W., Jr. (2002) *J. Biol. Chem.* **277**, 21617–21623
 25. Morita, T., Tanimura, A., Nezu, A., Kurosaki, T., and Tojyo, Y. (2004) *Biochem. J.* **382**, 793–801
 26. Venkatachalam, K., Ma, H.-T., Ford, D. L., and Gill, D. L. (2001) *J. Biol. Chem.* **276**, 33980–33985
 27. Trepakova, E. S., Gericke, M., Hirakawa, Y., Weisbrod, R. M., Cohen, R. A., and Bolotina, V. M. (2001) *J. Biol. Chem.* **276**, 7782–7790
 28. Turner, H., Fleig, A., Stokes, A., Kinet, J. P., and Penner, R. (2003) *Biochem. J.* **371**, 341–350
 29. Wisnoskey, B. J., Sinkins, W. G., and Schilling, W. P. (2003) *Biochem. J.* **372**, 517–528
 30. van Rossum, D. B., Patterson, R. L., Kiselyov, K., Boehning, D., Barrow, R. K., Gill, D. L., and Snyder, S. H. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 2323–2327
 31. Grafton, G., and Thwaite, L. (2001) *Immunology* **104**, 119–126