Canonical transient receptor potential TRPC7 can function as both a receptor- and store-operated channel in HEK-293 cells

Jean-Philippe Lièvremont, Gary St. J. Bird, and James W. Putney, Jr.

Laboratory of Signal Transduction, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, North Carolina 27709

Submitted 19 July 2004; accepted in final form 13 August 2004

Lièvremont, Jean-Philippe, Gary St. J. Bird, and James W. Putney, Jr. Canonical transient receptor potential TRPC7 can function as both receptor- and store-operated channel in HEK-293 cells. Am J Physiol Cell Physiol 287: C1709–C1716, 2004. First published September 1, 2004; doi:10.1152/ajpcell.00350.2004.—Previous studies on the activation mechanism of canonical transient receptor potential (TRPC) channels have often produced conflicting conclusions. All seven have been shown to be activated by phospholipase C (PLC)-coupled receptors, but TRPC1, TRPC2, TRPC3, TRPC4, TRPC5, and TRPC7 have also been proposed to function as store-operated channels.1 In the case of TRPC3, the expression environment and the expression level appear to determine the mode of regulation. Evidence of a close structural relative of TRPC3, TRPC7, has been presented that this channel is activated by receptor activation or by store depletion. On the basis of previous findings for TRPC3, we reasoned that subtle differences in structure or expression conditions might account for the apparent distinct gating mechanisms of TRPC7. To reexamine the mode of activation of TRPC7, we stably and transiently transfected human embryonic kidney (HEK)-293 cells with cDNA encoding for human TRPC7. We examined the ability of the expressed channels to activate these channels. Our findings demonstrate that when transiently expressed in HEK-293 cells, TRPC7 forms channels that are activated by PLC-stimulating agonists, but not by Ca2+ store depletion. However, when stably expressed in HEK-293 cells, TRPC7 can be activated by either Ca2+ store depletion or PLC activation. To our knowledge, this is the first demonstration of a channel protein that can be activated by both receptor- and store-operated modes in the same cell. In addition, the results reconcile the apparently conflicting findings of other laboratories regarding TRPC7 regulation.

calcium signaling; nonselective cation channels

CALCIUM SIGNALING PLAYS A KEY ROLE in the regulation of many cellular functions, such as contraction, fertilization, neurotransmitter secretion, cell proliferation, cell death, cell differentiation, and gene transcription. In many different cell types, agonist activation of phospholipase C (PLC)-linked receptors initiates breakdown of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) at the plasma membrane (1). IP3 diffuses into the cytoplasm and binds to its receptor in the endoplasmic reticulum, inducing release of Ca2+ from intracellular stores (1). Depletion of the stores then activates Ca2+ entry at the plasma membrane, a process known as capacitative Ca2+ entry or store-operated Ca2+ entry (25, 26). This Ca2+ entry allows refilling of the stores and full development of the physiological response. Despite intensive investigation, the mechanism of activation of capacitative Ca2+ entry is still poorly understood, and the molecular components of store-operated channels have not been identified clearly.

Speculation regarding the molecular nature of store-operated channels arose from studies of signaling events and molecular components involved in phototransduction in Drosophila melanogaster. These investigations led to identification and cloning of the transient receptor potential (trp) gene (5, 21). The trp gene encodes for TRP protein, which is a component of the Ca2+-permeable, nonselective cation channels underlying sustained Ca2+ entry upon light activation of PLC (7, 18). Seven mammalian homologs with structural and functional similarities to Drosophila TRP have been identified and cloned (3, 22–24, 35, 41, 43, 45, 47). They constitute the “classical” or “canonical” TRP family (designated TRPC1–TRPC7), which is one family among the larger TRP superfamily of cation channels (2, 8, 10, 19, 20, 28, 32, 40, 46). The TRPC family can be divided into four different subfamilies based on structural and functional characteristics: TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5. TRPC4 and TRPC5 share ~65% homology based on amino acid sequence, whereas members of the TRPC3/6/7 subfamily have even higher amino acid identity, 70–80%, with TRPC3 and TRPC7 being slightly more similar to each other than to TRPC6. In many cell types, TRPC have been proposed to encode for components of native, nonselective cation channels activated by a mechanism dependent on receptor-mediated activation of PLC (20, 38, 46). Since their initial discovery, the activation mechanisms of members of the TRPC3/6/7 subfamily have been investigated in several different cell lines and using various experimental approaches, leading to conflicting evidence regarding their activation mechanism. For example, the majority of studies have concluded that TRPC3 is activated by agonist receptors linked to PLC, but not by store depletion. However, when transiently expressed in chicken B lymphocyte DT40 cells, TRPC3 was shown to behave as a store-operated channel (31, 36, 37). In this instance, it was found that a very low expression level of TRPC3, rather than a different cell environment, favored its behavior as a store-operated channel (37).

As has become almost commonplace in the TRPC field, the two studies reported to date, performed by two independent laboratories, regarding the mechanism of activation of TRPC7 have also led to conflicting conclusions. Okada et al. (23) first

1 Although PLC activation inevitably leads to activation of store-operated channels, in this report when we refer to PLC-activated channels, we mean those channels that are specifically activated by PLC independently of store depletion.
cloned TRPC7 and investigated its mode of activation. They transiently expressed the murine ortholog of TRPC7 in human embryonic kidney (HEK)-293 cells and reported that it behaved as a nonselective cation channel activated via DAG after receptor-mediated activation of PLC. However, they also reported that the channel was clearly not activated by store depletion. Subsequently, Riccio et al. (29) identified and cloned human TRPC7. They stably overexpressed the protein in HEK-293 cells and reported that TRPC7 behaves as a store-operated channel. However, their study included no assessment of TRPC7’s constitutive activity (i.e., activity in the absence of apparent stimulation), which can sometimes create the appearance of regulation by store depletion (31, 32). In attempting to reconcile these discrepant findings in addition to the technical issues, two notable differences in the two studies must be considered. First, the sequence of TRPC7 cloned by Riccio et al. (29) was essentially identical to that obtained by Okada et al. (23), except that a leucine at position 111 (L111) was replaced by a proline (P111). Second, the study of Riccio et al. was performed by stably transfecting TRPC7 into HEK-293 cells, whereas Okada et al. used transient transfection. On the basis of previous experience with TRPC3 in our laboratory (37), we reasoned that subtle differences in structural or expression conditions might account for the apparent distinct gating mechanism of TRPC7.

Therefore, to reexamine the mode of activation of TRPC7, we stably as well as transiently transfected HEK-293 cells with either wild-type (wt)-TRPC7 (L111-TRPC7) or L111P-TRPC7. We then examined the ability of methacholine, an agonist known to stimulate PLC-linked muscarinic receptors, and thapsigargin, an intracellular Ca$^{2+}$ store-depleting agent, to activate TRPC7 channels. Our findings demonstrate that TRPC7 can function as both a receptor- and a store-operated channel when ectopically expressed in HEK-293 cells. In this regard, TRPC7 differs from TRPC3 and TRPC6, which, in HEK-293 cells, can be activated only through PLC activation and not by passive store depletion. To our knowledge, this study provides the first demonstration that a channel protein can be activated by both receptor- and store-operated modes in the same cell. In addition, the results reconcile the apparently conflicting findings of other laboratories with regard to TRPC7 regulation.

MATERIALS AND METHODS

Reagents. Thapsigargin was purchased from Alexis Biochemicals (San Diego, CA), and methacholine was obtained from Sigma (St. Louis, MO).

Cell culture and transfection. HEK-293 cells stably expressing TRPC3-green fluorescent protein (GFP) fusion protein were described previously (17). Transient and stable transfections of wild-type HEK-293 cells (wt-HEK-293) obtained from American Type Culture Collection (Manassas, VA) were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Wt-HEK-293 cells were transfected with either pcDNA3 vector containing the coding sequence for human TRPC6 [kindly provided by Dr. T. Gudermann, Institut für Pharmakologie und Toxikologie, Fachbereich Medizin, Philipps-Universität Marburg, Marburg, Germany (9)]. Other cells were transfected with pcDNA3.1(−) expression vector encoding for either wild-type human TRPC7 [wt-TRPC7 (leucine at position 111)], jointly supplied by Christine Murphy and Adrian Wolstenholm of University of Bath, Bath, UK, and John Westwick of Novartis, Horsham, UK, or L111P-TRPC7 (proline at position 111; see below).

To generate stable TRPC6- and TRPC7-expressing cells, the transfected cells were grown for 4 wk under continuous selection with 500 µg/ml of genetin (Invitrogen, Carlsbad, CA) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine (complete DMEM) in a humidified 95% air-5% CO2 incubator. TRPC6- and TRPC7-transfected cell populations were then tested for the ability of methacholine, an activator of PLC-linked muscarinic receptors, to activate the channels in the presence of 10 µM Gd$^{3+}$ (to isolate TRPC responses; see below) by Ca$^{2+}$ measurements performed on single cells (see RESULTS). Between 80 and 90% of TRPC6- and TRPC7-transfected cells showed receptor-mediated Ca$^{2+}$ entry insensitive to Gd$^{3+}$, a percentage of responding cells not significantly different from that observed for the TRPC3-GFP-expressing cell population (data not shown; see Ref. 17). For transient expression, HEK-293 cells were transiently transfected with pcDNA3.1(−) vector containing the sequence encoding for either wt-TRPC7 or the point mutant L111P-TRPC7, in which leucine at position 111 was replaced by proline, along with pEYFP-C1 vector (Clontech, Palo Alto, CA) as a marker for transfection. Cells were assayed 18–30 h posttransfection.

Mutagenesis. A point mutation [single base change at position 332 (T to C), leading to a proline instead of a leucine at position 111] was introduced in the wt-TRPC7 coding sequence cloned into pcDNA3.1(−) vector using specific oligonucleotides and a single site-directed mutagenesis kit (Qiagen, Valencia, CA). The presence of the mutated nucleotide was verified by performing DNA sequencing.

Measurement of intracellular Ca$^{2+}$. For wild-type and stable TRPC3-, TRPC6-, and TRPC7-expressing HEK-293 cells and transient wt-TRPC7- and L111P-TRPC7-expressing HEK-293 cells, Ca$^{2+}$ and Ba$^{2+}$ measurements were performed on single cells attached to coverslips. The coverslips were mounted in a Teflon chamber and incubated at 37°C for 30 min in complete DMEM containing 2 µM fura-2 AM (Molecular Probes, Eugene, OR). Cells were then washed and bathed in a nominally Ca$^{2+}$-free HEPES-buffered saline solution (HBSS) for at least 10 min before Ca$^{2+}$ and Ba$^{2+}$ measurements were performed. The composition of the nominally Ca$^{2+}$-free HBSS was (in mM) 140 NaCl, 4.7 KCl, 2.4 CaCl$2$, 10 MgCl$2$, 10 glucose, and 10 HEPES, pH 7.4, adjusted with NaOH. In some experiments, the NaCl of the HBSS was substituted with an equimolar quantity of KCl. To detect divalent cation entry, 2 mM Ca$^{2+}$ or Ba$^{2+}$ was added to the medium. For experiments performed under depolarizing conditions, the Ba$^{2+}$ concentration was raised to 5 mM and then to 10 mM. For experiments in which cells were transiently transfected with TRPC, 40–50 single cells were selected on the basis of enhanced yellow fluorescent protein (EYFP) expression, with fluorescence detected when excited at 488 nm and emission wavelength observed at 520 nm. In transiently and stably transfected cells, measurements of intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]i) changes as well as Ba$^{2+}$ measurements with fura-2 were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2; Intracellular Imaging, Cincinnati, OH) as described previously (30). For each experiment, with the exception of the one shown in Fig. 7, background fluorescence values at 340- and 380-nm excitation were obtained at the end of the experiment by addition of 4 µM ionomycin plus 20 mM Mn$^{2+}$. These values were subtracted from individual values at each wavelength and at each time point of the experiment, and resulting background-corrected values were used to calculate fluorescence intensity ratios due to excitation at 340 and 380 nm. Because the signals in some instances derived from a mixture of Ca$^{2+}$ and Ba$^{2+}$, these ratio values...
were not processed further to obtain estimates of [Ca$$^{2+}$$]. All experiments were conducted at room temperature.

RESULTS

The purpose of this study was to reexamine the mode of activation of wt-TRPC7 and L111P-TRPC7 when the proteins were either stably or transiently expressed in the same cell type, i.e., HEK-293 cells, and when Ca$$^{2+}$$ signaling was analyzed under conditions of receptor- and store-operated signaling. Two different TRPC7 cell populations were generated by stably transfecting HEK-293 cells with expression vectors carrying either wt-TRPC7 or L111P-TRPC7 cDNA under the control of the same promoter. We then examined the ability of methacholine, an agonist for PLC-linked muscarinic receptors and thapsigargin, an intracellular Ca$$^{2+}$$ store-depleting agent, to activate the channels. As controls, we used a TRPC6-expressing cell line generated in parallel by stably transfecting HEK-293 cells with expression vector containing cDNA encoding for TRPC6 under the control of the same promoter as well as a population of TRPC3-expressing HEK-293 cells previously characterized (17).

TRPC3, TRPC6, and TRPC7 form channels activated by receptor-regulated PLC. As demonstrated by Trebak et al. (31), when Ca$$^{2+}$$ is used to assess channel activation, misleading results can sometimes be obtained. For example, the inability of the endoplasmic reticulum to buffer Ca$$^{2+}$$ entering through the channels (due to irreversible block of sarcoendoplasmic reticulum Ca$$^{2+}$$-ATPase produced by thapsigargin) can exaggerate constitutive Ca$$^{2+}$$ entry occurring through ion channels expressed in living cells, leading to the erroneous conclusion that these channels are activated upon store depletion. Likely this explains the small apparent store-operated Ca$$^{2+}$$ entry in TRPC3-expressing HEK-293 cells reported by Zhu et al. (44) and by Kwan et al. (14) and could contribute to the apparent store-operated behavior of TRPC7 reported by Riccio et al. (29). To examine more quantitatively the mode of activation of TRPC3, TRPC6, and TRPC7, and to avoid complications caused by altered Ca$$^{2+}$$ buffering, we used Ba$$^{2+}$$ as a surrogate for Ca$$^{2+}$$ (4, 13, 31, 33, 34). Furthermore, Ba$$^{2+}$$ measurements were performed in the presence of 10 $$\mu$$M Gd$$^{3+}$$. Methacholine (MeCh; 300 $$\mu$$M) (solid line) followed by Ba$$^{2+}$$ (2 mM) was added where indicated. The constitutive Ba$$^{2+}$$ entry was determined in parallel for each cell line (dotted line). Bar graphs of Ba$$^{2+}$$ entry measurements were performed with single cells attached to coverslips as described in MATERIALS AND METHODS. The trace presented is the average of 3 coverslips with at least 50 cells/cover slip from 1 experiment performed in triplicate.

TRPC3, TRPC6, and TRPC7 proteins form cation channels activated by PLC-coupled agonist, results consistent with those of previous reports (9, 11, 23).

Effect of store depletion on TRPC3/6/7. Figure 2 summarizes the results of our experiments examining the effect of store depletion with thapsigargin on members of the subfamily TRPC3/6/7. As shown in Fig. 1, TRPC3- and TRPC7-expressing cells again exhibited similar constitutive Ba$$^{2+}$$ entry, while wild-type and TRPC6-expressing HEK-293 cells showed no detectable constitutive Ba$$^{2+}$$ entry. Consistent with previous observations (31), Ba$$^{2+}$$ entry induced by store depletion with thapsigargin was absent in the presence of 10 $$\mu$$M Gd$$^{3+}$ in wt-HEK-293 cells. In TRPC3- and TRPC6-expressing HEK-293 cells, rates of Ba$$^{2+}$$ entry in the presence or in the absence of thapsigargin were not significantly different. However, TRPC7-expressing cells exhibited a substantial thapsigargin-activated Ba$$^{2+}$$ entry in the presence of 10 $$\mu$$M Gd$$^{3+}$.

The average initial rates of Ba$$^{2+}$$ entry for TRPC3 and TRPC7 are summarized in Fig. 3. TRPC3 was activated by agonist, but not by store depletion, while TRPC7 was activated by store depletion and to an even greater extent by methacholine. The distinct behaviors of TRPC3 and TRPC7 argue against secondary effects such as changes in membrane potential underlying the apparent store-operated behavior. In addition, we also examined the ability of thapsigargin to activate TRPC7 in TRPC7-expressing HEK-293 cells whose plasma membranes were completely depolarized by equimolar substitution of K$$^{+}$ extracellular Na$$^{+}$$ and whose membrane potentials would thus be clamped at the equilibrium potential for K$$^{+}$ (Fig. 4). The reduction in driving force reduced both constitutive and thapsigargin-activated Ba$$^{2+}$$ entry; nonetheless, Ba$$^{2+}$$ entry was still increased by store depletion. The
Finding that thapsigargin-activated Ba$^{2+}$ entry in TRPC7-expressing cells is diminished but not abolished by membrane depolarization is consistent with the conclusion that TRPC7 forms Gd$^{3+}$-insensitive divalent cation permeable channels activated by depletion of intracellular Ca$^{2+}$ stores.

The data in Fig. 3 indicate that agonist activation causes greater activation of TRPC7 than does thapsigargin, despite the fact that thapsigargin completely empties the intracellular Ca$^{2+}$ stores to which the agonist has access. Because >90% of cells responded to either thapsigargin or to agonist, this would suggest that in the TRPC7-expressing cells, both store-operated and agonist-activated activities coexist. Figure 5 shows that when Ca$^{2+}$ is used instead of Ba$^{2+}$, thapsigargin activates Ca$^{2+}$ entry, and subsequent addition of agonist causes an

Fig. 2. Effect of thapsigargin (TG) on Ba$^{2+}$ entry in wild-type and stable TRPC3-, TRPC6-, and TRPC7-transfected HEK-293 cells. WT-HEK-293 cells stably transfected with either empty pcDNA3 (A) or pcDNA3 vector carrying the cDNA encoding for TRPC3 (B), TRPC6 (C), or TRPC7 (D) were incubated in the absence of added Ca$^{2+}$ and the continual presence of 10 μM Gd$^{3+}$. TG (2 μM) followed by Ba$^{2+}$ (2 mM) was added where indicated (solid line). The passive leak of Ba$^{2+}$, as in Fig. 1, was determined in parallel for each cell line (dotted line). Ba$^{2+}$ entry measurements were performed with single cells attached to coverslips as described in MATERIALS AND METHODS. The trace shown is the average of several coverslips with at least 50 cells/coverslip from 1 experiment performed at least in triplicate.

Fig. 3. Ba$^{2+}$ entry in response to methacholine (MC) and passive store depletion using TG. TRPC3 and TRPC7 stably transfected HEK-293 cells (TRPC3 and TRPC7, respectively). TRPC3- and TRPC7-expressing HEK-293 cells were incubated in the absence of added Ca$^{2+}$ and the continual presence of 10 μM Gd$^{3+}$. The data represent initial rates of Ba$^{2+}$ entry and are summarized results from multiple experiments depicted in Figs. 1 and 2. C, constitutive entry; MC, entry in response to 300 μM methacholine; TG, entry in response to 2 μM thapsigargin. Data are means ± SE from 3 independent experiments performed with TRPC3- and TRPC7-expressing cells.

Fig. 4. Effect of membrane depolarization on TG-induced Ba$^{2+}$ entry in wild-type and stable TRPC7-transfected HEK-293 cells. Wild-type and TRPC7-expressing HEK-293 cells were incubated in the absence of added Ca$^{2+}$ and the continual presence of 10 μM Gd$^{3+}$. The plasma membrane of these cells was completely depolarized by equimolar substitution of K$^+$ for extracellular Na$^+$. TG (2 μM) followed by 5 and 10 mM Ba$^{2+}$ were added where indicated (arrows, solid line). The constitutive Ba$^{2+}$ entry was determined in TRPC7-expressing cells (dotted line). Note that under conditions for measuring Ba$^{2+}$ entry, no passive leak of Ba$^{2+}$ was detected in WT-HEK-293 cells (shaded line). Ba$^{2+}$ entry measurements were performed by single-cell digital imaging as described in MATERIALS AND METHODS. The trace shown represents the average of 3 coverslips with at least 50 cells/coverslip from 1 experiment performed at least in triplicate.

Fig. 5. Dual regulation of TRPC7 channels in TRPC7 stably transfected cells. TRPC7-expressing cells (stably transfected) were incubated in the absence of added Ca$^{2+}$ and the continual presence of 10 μM Gd$^{3+}$. TG (2 μM) followed by Ca$^{2+}$ (2.0 mM) added where indicated (arrows, solid line). The constitutive Ca$^{2+}$ entry was determined in TRPC7-expressing cells (dotted line). In both cases, after Ca$^{2+}$ entry was determined in TRPC7-expressing cells (dotted line), MeCh (2 mM) was added. Ca$^{2+}$ entry measurements were performed using single-cell digital imaging as described in MATERIALS AND METHODS. The trace shown represents the average of 2–4 coverslips with at least 50 cells/coverslip from 1 experiment performed at least in triplicate.
additional increase in Ca\(^{2+}\) entry showing that in individual cells, TRPC7 can be activated by store depletion and to an even greater extent by agonist.

Recently, Riccio et al. (29) cloned human TRPC7, the sequence of which was essentially identical to the murine ortholog of TRPC7 obtained by Okada et al. (23), except that proline at position 111 (P111) replaced leucine (L111) found in all other known sequences of members of the TRPC3/6/7 subfamily. When Riccio et al. stably expressed the protein in HEK-293 cells, the channel appeared to be activated by store depletion. In this study, the human TRPC7 clone used to generate the TRPC7-expressing HEK-293 cell line has leucine at position 111. Thus we introduced a point mutation into wt-TRPC7 by single site-directed mutagenesis, resulting in proline instead of leucine at position 111, and we generated a HEK-293 cell line stably expressing L111P-TRPC7. We then examined the effect of store depletion induced by thapsigargin in L111P-TRPC7-expressing cells. As depicted in Fig. 6, L111P-TRPC7-expressing cells exhibited a thapsigargin-induced Ba\(^{2+}\) entry insensitive to 10 \(\mu\)M Gd\(^{3+}\), showing that L111P-TRPC7 formed channels that were activated by store depletion when stably expressed in HEK-293 cells, a result consistent with the conclusions of Riccio et al. (29).

In their study, Okada et al. (23) found that the murine ortholog of TRPC7, whose primary sequence has a leucine at position 111, formed channels that were activated by PLC-coupled receptor upon agonist stimulation, but not by store depletion. A fundamental difference between the study of Okada et al. and the two studies in which TRPC7 was store operated [our present study and Riccio et al. (29)] is that Okada et al. transiently expressed TRPC7 in HEK-293 cells. Thus we next transiently cotransfected HEK-293 cells with vectors carrying either wt-TRPC7 or L111P-TRPC7, along with EYPFP, to identify cells expressing TRPC7 (for both proteins, 97% of EYPFP-positive cells exhibited Ba\(^{2+}\) entry insensitive to Gd\(^{3+}\) upon agonist activation; see below). Figure 7 summarizes the results of experiments examining activation of both wt- and L111P-TRPC7 proteins by store depletion using Ba\(^{2+}\) as a surrogate for Ca\(^{2+}\). Consistent with previous observations, both agonist- and thapsigargin-induced Ba\(^{2+}\) entry in wt-HEK-293 cells were completely blocked by Gd\(^{3+}\) (15, 31). Neither wt-TRPC7- nor L111P-TRPC7-expressing cells exhibited thapsigargin-activated Ba\(^{2+}\) entry in the presence of Gd\(^{3+}\), whereas subsequent addition of agonist consistently activated Ba\(^{2+}\) entry. These results demonstrate that neither wt-TRPC7 nor L111P-TRPC7 behaved as store-operated channels when transiently expressed in HEK-293 cells. Taken together, these data suggest that TRPC7 forms ion channels that are activated not only by stimulation of PLC-coupled receptors but also by passive store depletion. At least for HEK-293 cells, the store-operated mode of activation is observed only when the cells stably express TRPC7.

The results discussed to this point indicate that when stably expressed in HEK-293 cells, TRPC7 forms both store-operated

---

**Fig. 6.** L111P-TRPC7 forms store-operated channels when stably expressed in HEK-293 cells. HEK-293 cells were stably transfected with pcDNA3.1(−) vectors containing a mutant variant of TRPC7 in which leucine at position 111 was replaced by proline (L111P-TRPC7). The L111P-TRPC7-transfected HEK-293 cells produced responses to agonist in the presence of Gd\(^{3+}\) as in Fig. 1, was determined in parallel for L111P-TRPC7-expressing cells. Ba\(^{2+}\) entry measurements were performed with single cells attached to coverslips as described in MATERIALS AND METHODS. The trace shown represents the average of 2–4 coverslips with at least 50 cells/coverslip from 1 experiment performed in triplicate.

**Fig. 7.** Neither wt-TRPC7 nor L111P-TRPC7 forms store-operated channels when transiently expressed in HEK-293 cells. Wt-HEK-293 cells were transiently cotransfected with pcDNA3.1(−) vectors containing either the wt-TRPC7 construct or a mutant variant in which leucine at position 111 was replaced by proline (L111P-TRPC7) along with the pEYFP-C1 construct to identify transfected cells. Both TG and agonist-induced Ba\(^{2+}\) entry were sequentially measured in single cells attached to coverslips as described in MATERIALS AND METHODS. Cells were incubated in the absence of added Ca\(^{2+}\) and in the continual presence of 10 \(\mu\)M Gd\(^{3+}\) (to inhibit endogenous capacitive Ca\(^{2+}\) entry; top) followed by sequential addition of TG (2 \(\mu\)M), Ba\(^{2+}\) (2 mM), and methacholine (300 \(\mu\)M) where indicated. The traces shown represent averages of 3 coverslips with at least 40 enhanced yellow fluorescent protein (EYFP)-positive cells/coverslip assessed on the same day.
and receptor-operated channels. This finding, to our knowledge, is without precedent. The question then arises as to whether there exist separate populations of channels subject to these distinct modes of regulation. The results summarized in Fig. 8 indicate that this is unlikely to be the case. In the experiment shown in Fig. 8, TRPC7 channels were first activated by a maximal concentration of the synthetic analog of DAG, 1-oleoyl-2-acetyl-sn-glycerol (OAG). Subsequently, methacholine was added, which, in addition to activating PLC, would also cause depletion of Ca\(^{2+}\) stores. However, this depletion of Ca\(^{2+}\) stores did not appear to increase the steady-state Ca\(^{2+}\) entry. Thus, once the channels had been activated by OAG, there did not appear to be an additional population of channels that could then be activated by store depletion. Note that the results shown in Fig. 5 indicate that activation by PLC and DAG caused a greater Ca\(^{2+}\) entry than that seen with store depletion. This could mean either that there are some channels activated by DAG that are not coupled to store depletion or that all channels can be activated by either means, but to a greater extent by DAG.

**DISCUSSION**

TRPC7 shares considerable structural similarity with the two other members of the TRPC3/6/7 subfamily. In most studies, all three share the ability to be activated through a PLC-dependent mechanism, most likely involving DAG (32). Of the three, TRPC7 has been the least studied, probably because it was the last of the three to be discovered (23). Nonetheless, two different laboratories have investigated the mode of activation of TRPC7 in the same cell line, i.e., HEK-293 cells. Okada et al. (23) reported that TRPC7 behaved as a store-operated channel when stably expressed in HEK-293 cells. Okada et al. (23) reported that TRPC7 behaved as a store-operated channel, unaffected by store depletion, while Riccio et al. (29) subsequently reported that it was a store-operated channel.

Close examination of the two previous studies reveals two fundamental differences. First, the clone obtained by Riccio et al. carries a single base change compared with other clones from human (this study) and other species, resulting in a proline at position 111 instead of a leucine. Riccio et al. (29) speculated that this single amino acid substitution might be responsible for producing the store-operated phenotype. A second difference is that Riccio et al. used stably transfected HEK-293 cells, while Okada et al. (23) used transient transfection. Our findings indicate that it is the latter that likely explains the different results. We found no difference in the behavior of TRPC7 proteins with proline or leucine at position 111. Both were clearly store operated when stably expressed but only agonist activated when transiently expressed. Thus the current study reconciles the previous conflicting conclusions regarding the activation mechanism of TRPC7 (23, 29).

To our knowledge, this report describes the first demonstration of a channel protein being regulated by both receptor- and store-operated modes in the same cells. What might be the basis for a dual activation mechanism of a channel? In nonexcitable cells, receptor-mediated activation of PLC results not only in the production of both IP\(_3\) and DAG but also in the depletion of the intracellular stores. While TRPC3 and TRPC6 activation has been shown to be dependent strictly on DAG formed as a result of PLC activation and not on store depletion (9, 12, 17, 31, 39, 42, 44), the data presented in this study suggest that either store depletion or DAG production can activate TRPC7 when stably expressed in HEK-293 cells. In TRPC7-expressing cells, receptor-mediated activation of PLC resulted in a greater activation of TRPC7 than when activated by passive store depletion using thapsigargin (Fig. 3). However, agonist activation, which involves depletion of Ca\(^{2+}\) stores, did not increase entry beyond that obtained with OAG (Fig. 8), an agent that activates by acting as a DAG but without depleting stores. This would indicate that there are not separate populations of TRPC7 channels activated by different mechanisms, but rather a single population of channels that can be activated by store depletion or to a greater extent by DAG.

Taking into account the high degree of amino acid identity among members of the TRPC3/6/7 subfamily, one might speculate that this difference in sensitivity to store depletion presumably reflects subtle functional differences among the amino acid sequences of TRPC3, TRPC6, and TRPC7. Such differences may permit TRPC7 to associate with other proteins in proper stoichiometric arrangements to function as a store-operated channel when stably expressed in HEK-293 cells.

This report clearly demonstrates that in the HEK-293 cell environment, both wt-TRPC7 and L111P-TRPC7 were activated by store depletion when stably expressed in these cells, while when the two proteins were transiently expressed in the same HEK-293 cell environment, neither wt-TRPC7 nor L111P-TRPC7 behaved as store-operated channels. This suggests that the mode of expression can influence the behavior of a channel protein. Such a conclusion is not without precedent. For example, the majority of studies of TRPC3 have concluded that TRPC3 is activated by agonist receptors linked to PLC but not by store depletion in HEK-293 cells (16, 17, 30, 31, 44).

Nevertheless, in a recent report, Vazquez et al. (37) demon-
strated that when expressed in the avian B-cell line DT40, TRPC3 could behave as either a store-operated channel or a PLC-activated channel, depending on the level of expression. Although we have not investigated the levels of TRPC7 expression under these two transfection conditions, experience with other TRPC indicates that with the powerful cytomegalovirus promoter used, expression levels are generally similar. In addition, there are a number of important differences between the current findings with TRPC7 and earlier findings with TRPC3 (37). First, at low expression levels, TRPC3 produced only store-operated channels, and at high expression levels, it led to only DAG-activated channels, while for TRPC7, the stable cells, both behaviors were observed. Also, the store-operated TRPC3 channels were blocked by Gd3+, while for TRPC7, clearly they are not. Thus we speculate that for TRPC7, stable transfection permits slow upregulation of other cellular components necessary to couple TRPC7 to store depletion. It is intriguing that the structurally similar TRPC3 does not show this phenomenon, and a comparison of changes in gene expression in cells stably expressing these two proteins may provide clues to the activation mechanism of store-operated channels in general.

In conclusion, the present results show that in HEK-293 cells, TRPC7 is activated both by receptor-mediated activation of PLC upon agonist stimulation and by store depletion. This behavior contrasts with that of TRPC3 and TRPC6, which, in our hands, formed only receptor-activated channels in this environment. These findings emphasize the importance of the mode of expression of channel proteins in studies aimed at elucidating their activation mechanism.

ACKNOWLEDGMENTS

We thank Dr. G. Vazquez for insightful discussions. Drs. Anant Parekh and Jerrel Yakel read the manuscript and provided helpful comments.

REFERENCES


