Comparison of Human TRPC3 Channels in Receptor-activated and Store-operated Modes

Differential Sensitivity to Channel Blockers Suggests Fundamental Differences in Channel Composition

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Capacitative calcium entry or store-operated calcium entry in nonexcitable cells is a process whereby the activation of calcium influx across the plasma membrane is signaled by depletion of intracellular calcium stores. Transient receptor potential (TRP) proteins have been proposed as candidates for store-operated calcium channels. Human TRPC3 (hTRPC3), an extensively studied member of the TRP family, is activated through a phospholipase C-dependent mechanism, not by store depletion, when expressed in HEK293 cells. However, store depletion by thapsigargin is sufficient to activate hTRPC3 channels when expressed in DT40 avian B-lymphocytes. To gain further insights into the differences between hTRPC3 channels generated in these two expression systems and further understand the role of hTRPC3 in capacitative calcium entry, we examined the effect of two well characterized inhibitors of capacitative calcium entry, Gd$^{3+}$ and 2-aminoethoxydiphenyl borane (2APB). We confirmed that in both DT40 cells and HEK293 cells, 1 mM Gd$^{3+}$ or 30 μM 2APB completely blocked calcium entry due to receptor activation or store depletion. In HEK293 cells, 1 mM Gd$^{3+}$ did not block receptor-activated hTRPC3-mediated cation entry, whereas 2APB had a partial (~60%) inhibitory effect. Interestingly, store-operated hTRPC3-mediated cation entry in DT40 cells was also partially inhibited by 2APB, whereas 1 mM Gd$^{3+}$ completely blocked store-operated hTRPC3 activity in these cells. Furthermore, the sensitivity of store-operated hTRPC3 channels to Gd$^{3+}$ in DT40 cells was similar to the endogenous store-operated channels, with essentially 100% block of activity at concentrations as low as 0.1 μM. Finally, Gd$^{3+}$ has a rapid inhibitory effect when added to fully developed hTRPC3-mediated calcium entry, suggesting a direct action of Gd$^{3+}$ on hTRPC3 channels. The distinct action of these inhibitors on hTRPC3-mediated cation entry in these two cell types may result from their different modes of activation and may also reflect differences in basic channel structure.

Calcium signaling plays a central role in regulating many physiological processes such as muscle contraction, cellular proliferation and differentiation, neurotransmitter secretion, and apoptosis. In a variety of nonexcitable cells, calcium signaling is initiated through cell membrane receptors coupled to phospholipase C, resulting in production of inositol 1,4,5-trisphosphate (IP$_3$)1 (1). IP$_3$ releases intracellular Ca$^{2+}$ from the endoplasmic reticulum. This release of intracellular Ca$^{2+}$ triggers an influx of Ca$^{2+}$ across the plasma membrane, a process known as capacitative calcium entry (CCE) or store-operated calcium entry (2–5). The mechanism underlying the activation of plasma membrane calcium-permeable channels is not fully understood, but the initiating signal is the depletion of endoplasmic reticulum calcium content. Currents mediated by store-operated channels have been measured in various cell types (6). The best defined store-operated current to date is the Ca$^{2+}$ release-activated Ca$^{2+}$ current (I$_{\text{crac}}$), found predominantly in hematopoietic cells (6, 7). I$_{\text{crac}}$ measured in mast cells, Jurkat cells, and rat basophilic leukemia cells is both highly selective for Ca$^{2+}$ and completely blocked by low concentrations of lanthanides (1 μM Gd$^{3+}$). However, in many other cell types, store-operated currents with moderate divalent ion selectivity have been recorded (8, 9), as well as nonselective cation currents (10, 11).

Although CCE has been widely studied in many different cell types, the molecular identity of store-operated channels (SOCs) and the signal by which store emptying activates those channels remain uncertain. Two major hypotheses about the mechanism of CCE have been proposed. The first proposes the release of a putative calcium influx factor from the endoplasmic reticulum (12, 13). The second, the “conformational coupling” model, involves direct interaction of IP$_3$ receptors in the endoplasmic reticulum with SOCs in the plasma membrane (3, 14). Mammalian homologues of the Drosophila transient receptor potential (TRP) channel are candidates for store-operated calcium channels. Among the canonical TRP subfamily (designated TRPC1 through TRPC7), human TRPC3 (hTRPC3), first cloned by Zhu et al. (15), has been shown in many heterologous expression systems, including HEK293, to behave as a receptor-activated channel with constitutive activity that cannot be further increased by store depletion (15–18). Furthermore, Hofmann et al. (16) showed that TRPC3 and its structural relative TRPC6 can be activated by diacylglycerol, providing a possible mechanism of activation of these channels by phospholipase

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1 The abbreviations used are: IP$_3$, inositol 1,4,5-trisphosphate; IP$_3$R, IP$_3$ receptor; CCE, capacitative calcium entry; TRP, transient receptor potential; hTRPC3, human transient receptor potential gene product; SOC, store-operated channel; I$_{\text{crac}}$, calcium release-activated calcium current; GFP, green fluorescent protein; 2APB, 2-aminoethoxydiphenyl borane; FLIPR, fluorometric imaging plate reader; HBSS, Hapes-buffered physiological saline solution.
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C-linked receptors, independently of IP$_3$ and store depletion. On the other hand, despite the demonstrated absence of regulation of hTRPC3 by store emptying in these expression systems, a substantial body of evidence suggests that hTRPC3 activation involves interaction with IP$_3$ receptors (IP$_3$R$_s$) or ryanodine receptors consistent with the conformational coupling model (19–21).

This laboratory recently demonstrated that hTRPC3 is regulated by store depletion when transiently expressed in DT40 chicken B-lymphocytes (22). A substantial increase in Ba$^{2+}$ entry was observed in hTRPC3-DT40 cells after store depletion by thapsigargin. Moreover, the store depletion activity of hTRPC3 expressed in DT40 cells partly depended on the presence of IP$_3$R$_s$. Therefore, we proposed hTRPC3 as a candidate for store-operated, Ba$^{2+}$-permeable channels, perhaps similar to those observed in nonhematopoietic cells.

The differences between hTRPC3 channels generated in HEK293 cells versus those generated in DT40 cells are intriguing. Thus, we have undertaken a comparison of the sensitivity of hTRPC3 channels expressed in either HEK293 cells or DT40 cells, as well as their endogenous CCE channels, to the actions of two well characterized inhibitors of CCE. Gd$^{3+}$, at least when employed in the low micromolar range, is a relatively specific blocker of store-operated channels (23, 24) and is presumed to block through direct binding to sites in the channel pore. 2-Aminoethoxydiphenyl borane (2APB) appears to block store-operated channels through a direct action (25–29) but also appears capable of inhibiting conformationally coupled TRPC3 in HEK293 cells by virtue of an action on intracellular IP$_3$ receptors (17). Our findings show that receptor activation of hTRPC3-mediated cation entry in HEK293 cells is partially inhibited by 2APB, and the store-operated entry in DT40 cells is slightly reduced. This is consistent with the suspected involvement of IP$_3$ receptors in these two cell types. A surprising finding was that, in contrast to HEK293 cells, hTRPC3-mediated store-operated entry in DT40 cells is completely blocked by low concentrations of Gd$^{3+}$. This finding reveals a fundamental difference in the molecular structure of hTRPC3 channels in these two cell lines, and this difference seems likely to be related to the two distinct modes of activation.

**MATERIALS AND METHODS**

**Reagents—**Thapsigargin and methacholine were purchased from Calbiochem. 2APB was synthesized as described previously (30).

**Cell Culture and Transfection—**HEK293 cells were obtained from ATCC and were transfected, using Superfect reagent (Qiagen) according to the vendor’s instructions, with pcDNA3 vector containing the green fluorescent protein (GFP) coding sequence added in frame to the C terminus of hTRPC3 (18). Cells stably expressing hTRPC3-GFP fusion protein were selected first by antibiotic resistance and second by GFP fluorescence by flow cytometry. Cells were grown under selection to 80% confluence, and 60,000 cells/well were plated and used 24 h after plating. Cells were loaded with either of two single visible wavelength indicators: for Ca$^{2+}$, Fluor-4 (4 μM Fluo-4/AM for 45 min at 37°C in complete Dulbecco’s modified Eagle’s medium) or Thapsigargin (4 μM Thapsigargin, 45 min at 37°C in complete Dulbecco’s modified Eagle’s medium) excited at 488 nm, and emission-selected by 510-nm bandpass filter, for Ba$^{2+}$, Calcium Green-1 (4 μM Calcium Green-1/AM for 120 min at room temperature in a Hepes-buffered physiological saline solution (HBSS; 140 mM NaCl, 4.7 mM KCl, 1 mM MgCl$_2$, 10 mM glucose, 10 mM HEPES pH 7.4) with 250 μM probenecid and 0.2% pluronic acid). In both cases, cells were washed twice in nominally Ca$^{2+}$-free i.e. nominally Ca$^{2+}$+-free medium, with the excitation wavelength at 488 nm, and emission wavelength at 520 nm.

**RESULTS**

**Effects of Gd$^{3+}$ and 2APB on Ca$^{2+}$ Entry in Wild-type and hTRPC3-transfected HEK293 Cells—**The generation of a stable population of hTRPC3-transfected HEK293 cells makes it practical to investigate Ca$^{2+}$ signaling mechanisms utilizing the real time fluorescence-imaging plate reader system, FLIPR. In these experiments, background controls (i.e. Ca$^{2+}$-additions in the absence of agonist or thapsigargin) were carried out in parallel for both wild-type and transfected cells. The background data are summarized in Fig. 1 and indicate that hTRPC3 produces a constitutive Ca$^{2+}$ entry, consistent with previous findings (15, 18). Interestingly, this entry was partly blocked by Gd$^{3+}$ and blocked to a greater extent by 2APB (see also Ref. 27). Fig. 2A shows the results of experiments examining the sensitivity to Gd$^{3+}$ and 2APB of Ca$^{2+}$ entry in response to either agonist (300 μM methacholine) or thapsigargin (2 μM) activation in wild-type and hTRPC3-transfected cells. The background data from experiments of Fig. 1 have been subtracted. The results in the wild-type cells are similar to those reported previously for this cell line (31); both Gd$^{3+}$ and 2APB caused essentially complete block of both agonist- and thapsigargin-induced Ca$^{2+}$ entry. For the case of the

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$^2$ F.-J. Braun, unpublished data.
hTRPC3 cells, somewhat unexpected results were obtained. With agonist activation, there was no significant effect of Gd$^{3+}$ on Ca$^{2+}$ entry, and 2APB reduced only the peak of the Ca$^{2+}$ entry response. Additionally, the hTRPC3 cells exhibited a thapsigargin-activated Ca$^{2+}$ entry that was partially resistant to inhibition by Gd$^{3+}$ or 2APB.$^3$

The results with agonist activation present something of a paradox; methacholine induces complete depletion of thapsigargin-sensitive Ca$^{2+}$ stores,$^3$ yet it does not appear to activate any Gd$^{3+}$-sensitive store-operated entry. Also, previous studies have indicated that the agonist activation of hTRPC3 is blocked by 2APB, presumably reflecting a role of IP$_3$ receptors (17). Finally, the results with thapsigargin could be interpreted to indicate that hTRPC3 forms Ca$^{2+}$-permeable, store-operated channels, despite previous conclusions that only noncapacitative channels are formed in HEK293 cells (15, 17, 18).

One possible explanation for these unexpected findings is that they arise from complex mechanisms of Ca$^{2+}$ regulation known to occur in most cell types. In the case of responses to agonist, it is possible that activation of plasma membrane pumps by Ca$^{2+}$ and feedback inhibition of the store-operated channels by Ca$^{2+}$ limit the extent of the Ca$^{2+}$ rise when both capacitative and noncapacitative mechanisms operate simultaneously. The transient rise in [Ca$^{2+}$]$\text{_{i}}$ in thapsigargin-treated hTRPC3 cells could reflect store-operated channels; on the other hand, this could as easily reflect the constitutive activity of hTRPC3 channels, exaggerated by the inability (due to thapsigargin) of intracellular endoplasmic reticulum to efficiently buffer entry through these channels.

To avoid complications of alterations in Ca$^{2+}$ transport and Ca$^{2+}$ regulation of channels, we next carried out experiments utilizing Ba$^{2+}$ as a surrogate for Ca$^{2+}$. The use of Ba$^{2+}$ avoids many of these problems, since it generally does not activate Ca$^{2+}$-sensitive regulatory sites and is a poor substrate for Ca$^{2+}$ transport mechanisms (32–35). Fig. 3 illustrates Ba$^{2+}$ (1 mM) entry data obtained with FLIPR analysis of both wild-type and hTRPC3 HEK293 cells. As Ba$^{2+}$ accumulates in the cell, complex and unknown mechanisms may limit its maximum level of accumulation. Thus, rather than utilizing the maximum value for Ba$^{2+}$ entry, we calculated the initial rates of Ba$^{2+}$ entry with the different modes of activation and in the two cell types (summarized in Fig. 4).

As seen in the Ca$^{2+}$ experiments, the basal rate of Ba$^{2+}$ entry was significantly greater in hTRPC3 cells than in wild-type cells (Fig. 4). This statistically significant difference was seen also in the presence of Gd$^{3+}$, in the presence of 2APB, or both ($p < 0.05$). When the Gd$^{3+}$-insensitive Ba$^{2+}$ entry, which should contain the contribution of hTRPC3, was subtracted from the basal rates, the remaining Gd$^{3+}$-sensitive component was not significantly different in the two cell lines (rates: control, 5.9 ± 0.4 cps$^2$; TRPC3, 5.7 ± 0.5 cps$^2$). Thus, the increase in basal entry appears to result from constitutive

$^3$ The size of the Ca$^{2+}$ release transient appears smaller in the hTRPC3 cells than in wild type, causing us to consider the possibility that hTRPC3 causes some depletion of Ca$^{2+}$ pools. However, further investigation leads us to conclude that the difference in the magnitude of the apparent [Ca$^{2+}$]$_i$ signals in wild-type and TRPC3-transfected cells results from different loading or quantitative behavior of Fluo-4 in the two cell lines. For example, attempts to measure $F_{\text{max}}$ by use of high concentrations of an ionophore gave a smaller maximum signal in the TRPC3 cells than in the wild-type cells. Additionally, the thapsigargin-induced Ca$^{2+}$ entry appears less in the TRPC3 cells, but when Ba$^{2+}$ is used (see Fig. 4), this difference is not seen (control rate, 11.5 ± 0.6; TRPC3-transfected rate, 13.3 ± 2.7). To resolve this issue more quantitatively, we carried out some additional experiments using an imaging system with which we could use a ratioing dye (Fura-2) that could be calibrated. With this technique, the peak [Ca$^{2+}$]$_i$, values following thapsigargin addition to the different cell lines were not statistically different: wild-type, 258 ± 32 nm; TRPC3, 272 ± 33 nm (n = 3). Thus, there does not appear to be any significant pool depletion associated with expression of TRPC3.

$^3$ M. Trebak, G. St. J. Bird, R. R. McKay, and J. W. Putney, Jr., unpublished observation.
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![Graph showing effect of Gd<sup>3+</sup> and 2APB on capacitative calcium entry in DT40 cells.](http://www.jbc.org/)

**Fig. 3.** Effect of methacholine (MeCh) on influx of Ba<sup>2+</sup> in wild-type (left) and hTRPC3-transfected (right) HEK293 cells. Cells were incubated in the absence of added Ca<sup>2+</sup> and 300 μM methacholine (MeCh) and 1 mM Ba<sup>2+</sup> were added where indicated. Dotted line, control (no methacholine); solid line, 300 μM methacholine. Curves are averages of seven (wild type) and eight (hTRPC3-transfected) independent experiments, each involving four wells per condition, carried out utilizing the FLIPR<sup>®</sup> real time plate scanner.

**Fig. 4.** Effect of Gd<sup>3+</sup> (3 μM) and 2APB (30 μM) and both inhibitors in combination on methacholine- and thapsigargin-activated influx of Ba<sup>2+</sup> in wild-type (top) and hTRPC3-transfected (bottom) HEK293 cells. Cells were incubated in the absence of added Ca<sup>2+</sup> and 300 μM methacholine (MeCh, black bars) or 2 μM thapsigargin (TG, hatched bars) followed by 1 mM Ba<sup>2+</sup> was added following the protocol shown in Fig. 3. Initial rates of Ba<sup>2+</sup> entry (fluorescence increase upon the addition of Ba<sup>2+</sup>) were calculated. Presented are means ± S.E. from seven (wild-type) and eight (hTRPC3-transfected) independent experiments, each involving four wells per condition.

Activity of hTRPC3 rather than from a small activation of a store-operated pathway.

In wild-type HEK293 cells, both methacholine and thapsigargin activated Ba<sup>2+</sup> entry and to approximately the same extent, as expected if both agents are acting through store depletion. This entry was essentially completely blocked by either Gd<sup>3+</sup> or 2APB, as expected from previous findings (31) and from the results in Fig. 2. In hTRPC3 cells, Ba<sup>2+</sup> entry in response to methacholine was clearly greater than in the wild-type cells, but the response to thapsigargin was not significantly different. As in the wild-type cells, the response to thapsigargin was completely blocked by either Gd<sup>3+</sup> or 2APB. The response to methacholine in hTRPC3 cells was partially blocked by Gd<sup>3+</sup>, consistent with a co-activation of both capacitative and noncapacitative (hTRPC3-mediated) pathways. While 2APB did not produce a complete block of the agonist response, it produced a statistically greater effect than did Gd<sup>3+</sup> and was able to further reduce entry in cells blocked by Gd<sup>3+</sup>. This is consistent with the view that Gd<sup>3+</sup> blocks the capacitative component of the entry, whereas 2APB blocks the capacitative component, and at least partially inhibits activation of hTRPC3. Interestingly, the failure of 2APB to completely block the hTRPC3 response does not appear to result from an inadequate concentration (30 μM in this experiment), since concentration-effect studies revealed no additional inhibitory effect with concentrations as high as 100 μM (data not shown).

Thus, the data with Ca<sup>2+</sup> involve a number of potential artifacts; the Ba<sup>2+</sup> data indicate that the apparent effect of thapsigargin in activating entry of Ca<sup>2+</sup> through hTRPC3 channels results from thapsigargin-induced reduction of intracellular Ca<sup>2+</sup> buffering by endoplasmic reticulum rather than an actual store-dependent regulation of hTRPC3. It is likely that a similar explanation underlies findings of altered basal Ca<sup>2+</sup> kinetics in hTRPC3 cells in a previous study (15). Likewise, the failure of Gd<sup>3+</sup> to inhibit the Ca<sup>2+</sup> entry in hTRPC3 cells is not borne out by the Ba<sup>2+</sup> data and thus probably results from nonlinear buffering of Ca<sup>2+</sup> or perhaps Ca<sup>2+</sup>-mediated cross-talk between SOCs and hTRPC3 channels.

**Fig. 5.** Effects of Gd<sup>3+</sup> and 2APB on capacitative calcium entry in DT40 cells. The cells were loaded with Fura-2, and single-cell calcium measurements were performed as described under “Materials and Methods.” Experiments were initiated in Ca<sup>2+</sup>-free medium, and 2 μM thapsigargin and 1.8 mM Ca<sup>2+</sup> were added where indicated. Ca<sup>2+</sup> entry induced by 2 μM thapsigargin in wild-type DT40 cells was essentially blocked when 1 μM Gd<sup>3+</sup> or 30 μM 2APB were added. Data are representative of three independent experiments.

**Gd<sup>3+</sup> and 2APB Inhibit Capacitative Calcium Entry in DT40 Cells**—In Fig. 5, typical Fura-2 single cell experiments with wild-type DT40 cells are shown. Thapsigargin was added to the cells initially in a nominally Ca<sup>2+</sup>-free solution. After the Ca<sup>2+</sup> release phase, Gd<sup>3+</sup> or 2APB was added a few minutes before restoring Ca<sup>2+</sup> to a concentration of 1.5 mM in the external medium. Similar to findings in HEK293 cells, the endogenous CCE in DT40 cells was essentially completely blocked by either Gd<sup>3+</sup> or 2APB (Fig. 5).

**Store-operated hTRPC3-mediated Cation Entry in DT40 Cells Is Inhibited by Gd<sup>3+</sup> and Partially Inhibited by 2APB**—We reported previously that hTRPC3 behaves as a store-operated Ba<sup>2+</sup>-permeable channel when transiently expressed in the avian B cell line DT40 (22). Unlike the HEK293 expression system, wild-type DT40 cells do not exhibit thapsigargin-activated Ba<sup>2+</sup> entry (22). Also, when hTRPC3 is ex-
pressed in DT40 cells, no constitutive permeability is detected with either Ca\(^{2+}\) or Ba\(^{2+}\) (22). However, store depletion with thapsigargin induces a significant Ba\(^{2+}\) entry that is observed in neither wild type nor mock-transfected cells. We have not as yet succeeded in producing stable, hTRPC3-expressing DT40 cells, and thus we have utilized transient transfection as described in our earlier report (22). Ba\(^{2+}\) entry in response to thapsigargin was assessed in single enhanced yellow fluorescent protein-positive cells using Fura-2 imaging as indicated under “Materials and Methods.”

We then investigated the concentration dependence of the inhibitory actions of Gd\(^{3+}\) on store-operated hTRPC3-mediated barium entry and on endogenous capacitative calcium entry. Fig. 7 shows that these two store-operated cation entries show similar sensitivity to Gd\(^{3+}\) inhibition, with a nearly complete block (90% or greater) at a concentration of 0.1 \(\mu\)M.

Statistical analysis of a number of experiments with 2APB revealed that this reagent caused a small but statistically significant inhibition (~25%) of store-operated Ba\(^{2+}\) entry (Fig. 8). A similar degree of inhibition was observed with 75 and 100 \(\mu\)M 2APB, indicating that this is the maximal degree of inhibition obtainable with 2APB.

Although store depletion clearly activates a Ba\(^{2+}\)-permeable channel in hTRPC3-DT40 cells, it is not clear how significant such a channel might be as a mechanism for Ca\(^{2+}\) signaling. The ability of 2APB to block endogenous CCE completely inhibited hTRPC3-mediated barium entry in response to thapsigargin in DT40 cells, whereas 30 \(\mu\)M 2APB had minimal effect (Fig. 6, but see Fig. 8 and discussion below).

DISCUSSION

Transient expression of hTRPC3 in DT40 B lymphocytes generates a divergent cation-permeable channel that is activated by store depletion (22). Data from a number of laboratories including our own have shown that hTRPC3 transiently or stably expressed in HEK293 cells behaves as a receptor-operated cation channel that is not activated by store depletion (15, 17, 18). We thus investigated the actions of previously characterized inhibitors of store-operated channels, comparing the behavior of hTRPC3 expressed in DT40 cells with hTRPC3 expressed in HEK293 cells.

In investigations of hTRPC3 regulation in HEK293 cells, we discovered potential ambiguities and artifacts arising from measurements of Ca\(^{2+}\) entry. These are probably attributable to complex and nonlinear actions of Ca\(^{2+}\) transport, buffering, and negative feedback mechanisms. These problems were revealed and also alleviated by use of Ba\(^{2+}\), a surrogate for Ca\(^{2+}\), which passes through many types of Ca\(^{2+}\) channels but which is a poor substrate for Ca\(^{2+}\) pumps and a poor activator of Ca\(^{2+}\) feedback processes (32). Thus, expression of hTRPC3 results in an apparent entry of Ca\(^{2+}\) following depletion of stores with thapsigargin. However, despite the fact that TRPC3 channels
are readily Ba\(^{2+}\)-permeable, no such entry of Ba\(^{2+}\) was observed. The simplest explanation of this discrepancy is that the apparent entry of Ca\(^{2+}\) was a manifestation of constitutive activity of hTRPC3, amplified by thapsigargin’s abrogation of intracellular buffering by the endoplasmic reticulum. Thus, caution should be used in studies involving expression of Ca\(^{2+}\) channels in which some channels become constitutively active. A similar problem occurred in studies of the action of inhibitors. Thus, Gd\(^{3+}\) appeared incapable of affecting agonist-activated Ca\(^{2+}\) entry in HEK293 cells, yet when Ba\(^{2+}\) entry was examined, about half of the activated entry was blocked. The latter result is consistent with the expectation that the agonist, by virtue of its ability to deplete endoplasmic reticulum Ca\(^{2+}\) stores, should activate a Gd\(^{3+}\)-sensitive store-operated entry. The apparent failure of Gd\(^{3+}\) to inhibit Ca\(^{2+}\) entry under the same circumstances may result from a nonlinear activation of plasma membrane Ca\(^{2+}\) extrusion such that a limiting level of [Ca\(^{2+}\)], is set. It is also possible that Ca\(^{2+}\) entering through hTRPC3 channels specifically suppresses the endogenous store-operated channels, which are known to be highly sensitive to inhibition by Ca\(^{2+}\). Regardless of the explanation, the use of Ba\(^{2+}\) as a surrogate for Ca\(^{2+}\) in HEK293 allows detection of both modes of entry.

In both cell types, transfection with hTRPC3 resulted in the expression of channels with properties clearly distinct from the endogenous channels. Thus, it is likely that hTRPC3 is at least a constituent of the newly formed channels in both instances. However, a significant finding in this study is that hTRPC3-dependent store operated cation entry in DT40 cells is blocked by Gd\(^{3+}\), whereas in HEK293 cells it is completely insensitive to this lanthanide. Lanthanides are generally believed to block channels by binding to sites in the channel pore (36, 37). Therefore, the molecular composition of the hTRPC3 channels in DT40 cells is probably different from hTRPC3 channels previously generated in other expression systems, including HEK293 cells. When hTRPC3 was overexpressed in different mammalian cell lines (15, 16, 38), it behaved as a receptor-operated nonsel ective cation channel that was not activated by store depletion. Specifically, hTRPC3 expressed in HEK293 cells was insensitive to inhibition by 10 \(\mu\)M Gd\(^{3+}\) (15), and Ohki et al. (39) succeeded in inhibiting TRPC3 expressed in Xenopus oocytes only when very high concentrations of Gd\(^{3+}\) (2 mM) were used. These data are consistent with our findings on hTRPC3 expressed in HEK293 cells.

Store-operated hTRPC3-mediated Ba\(^{2+}\) entry in DT40 cells shows a sensitivity to Gd\(^{3+}\) that is similar to the endogenous store-operated Ca\(^{2+}\) entry, with nearly complete block at concentrations as low as 0.1 \(\mu\)M (Fig. 7). In addition, Gd\(^{3+}\) inhibits fully established hTRPC3 activity (Fig. 9), suggesting that as for SOCs, Gd\(^{3+}\) acts directly on the hTRPC3 channels themselves. We previously speculated that a low expression level of hTRPC3 in DT40 cells is related to its regulation by store depletion (22). It is thus possible that the association of hTRPC3 with a limited quantity of endogenous components of CCE in the DT40 expression system determines the mode of coupling of hTRPC3 channels as well as the sensitivity to low concentrations of Gd\(^{3+}\). On the other hand, a high hTRPC3 expression level in HEK293 cells may result in homotetrameric hTRPC3 channels that would be insensitive to activation by store depletion and also insensitive to inhibition by low concentrations of lanthanides.

Some recent studies have provided evidence for the conformational coupling model for SOC activation (19, 20). According to this model, IP\(_3\) receptors in the endoplasmic reticulum can sense Ca\(^{2+}\) depletion from the stores and convey a signal to SOCs in the plasma membrane via protein-protein interactions (3, 14). More recently, Ma et al. (17) provided data that were interpreted as supporting this conformational coupling model by use of 2APB, believed to act as a membrane-permeant IP\(_3\) receptor antagonist. These authors showed that 2APB prevented both store-induced SOC activation and receptor-coupled hTRPC3 activation when hTRPC3 was expressed in HEK293 cells and concluded that IP\(_3\)s were essential for both SOC and hTRPC3 channel activation. However, in rat basophilic leukemia cells, 2APB blocked \(I_{\text{Ca}}\) current completely when applied from the outside of the cells but inhibited only partially when applied from the cytoplasm, suggesting that 2APB may be acting as a direct blocker of SOC rather than as an IP\(_3\) receptor antagonist (40). Kukkonen et al. (41) confirmed these findings with whole-cell patch clamp experiments in the same cells and reported that \(I_{\text{Ca}}\) activity was rapidly blocked by extracellular 2APB, whereas intracellular 2APB was less effective. Furthermore, Broad et al. (25) showed that 2APB abolishes CCE induced by thapsigargin even in DT40 cells deficient for all isoforms of IP\(_3\) receptor, consistent with a direct action of 2APB on the SOC channels themselves.

On the other hand, Ma et al. (17) showed that 2APB blocks TRPC3 channels when activated by a phospholipase C-linked agonist but not when activated with diacylglycerol. Thus, it is likely that the inhibitory effect of 2APB on hTRPC3 channels is not a direct effect but rather results from an action on IP\(_3\)Rs. We found that in our stable hTRPC3-expressing HEK293 cells, 2APB partially blocks receptor-induced hTRPC3 activity (~60% inhibition). We cannot definitively explain the quantitative difference between our results and those of Ma et al. (17). However, we note that our data are statistical averages obtained using cell populations of our own independently generated TRPC3-expressing cell line in a high through put fluorescence system whereby hTRPC3 activity was assessed after stimulation with a maximal concentration of a cholinergic agonist. We also showed that 2APB even at high concentrations has a slight effect on store-operated hTRPC3 in DT40 cells (on average ~25% inhibition) that was statistically significant. In a previous study, we reported that store-dependent activation of TRPC3 channels in DT40 cells is about 50% diminished in a cell line lacking IP\(_3\)s, indicating that about half of the expressed channels depend on IP\(_3\)s for activation (22). Hence, the observed degree of inhibition is about what one would expect given that only half of the channels interact with IP\(_3\).
receptors and that, based on the results in HEK293 cells, we expect those channels to be only partially inhibited.

In conclusion, store-operated hTRPC3 channels in DT40 cells show similar sensitivity to low concentrations of Gd$^{3+}$ as the endogenous SOCs. The correlation between store-dependent regulation of hTRPC3 and their inhibition by low concentrations of lanthanides is consistent with our supposition that hTRPC3 forms a component of SOCs by combining with unknown components in DT40 that also convey Gd$^{3+}$ sensitivity. We also suggest that TRPC3 may be a constituent of a store-operated channel in nonhematopoietic cells in which store-operated channels are less Ca$^{2+}$-selective. Clearly, further studies are needed to elucidate the contribution of hTRPC3 in this important pathway known as capacitative calcium entry.

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