Receptor-activated Ca\textsuperscript{2+} Influx via Human Trp3 Stably Expressed in Human Embryonic Kidney (HEK)293 Cells

EVIDENCE FOR A NON-CAPACITATIVE Ca\textsuperscript{2+} ENTRY*

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Ca\textsuperscript{2+} release from its internal stores as a result of activation of phospholipase C is accompanied by Ca\textsuperscript{2+} influx from the extracellular space. Ca\textsuperscript{2+} influx channels may be formed of proteins homologous to Drosophila Trp. At least six non-allelic Trp genes are present in the mouse genome. Full-length human, bovine, mouse, and rat cDNAs for Trp1, 3, 4, 6 have been cloned. Expression of these genes in various mammalian cells has provided evidence that Trp proteins form plasma membrane Ca\textsuperscript{2+}-permeant channels that can be activated by an agonist that activates phospholipase C, by inositol 1,4,5-trisphosphate, and/or store depletion. We have stably expressed human Trp3 (hTrp3) in human embryonic kidney (HEK)293 cells. Measurement of intracellular Ca\textsuperscript{2+} concentrations in Fura2-loaded cells showed that cell lines expressing hTrp3 have significantly higher basal and agonist-stimulated influxes of Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, Ba\textsuperscript{2+}, and Sr\textsuperscript{2+} than control cells. The increase in Ca\textsuperscript{2+} entry attributable to the expression of hTrp3 obtained upon store depletion by thapsigargin was much lower than that obtained by stimulation with agonists acting via a G\textsubscript{i} coupled receptor. Addition of agonists to thapsigargin-treated Trp3 cells resulted in a further increase in the entry of divalent cations. The increased cation entry in Trp3 cells was blocked by high concentrations of SKF 96365, verapamil, La\textsuperscript{3+}, Ni\textsuperscript{2+}, and Gd\textsuperscript{3+}. The Trp3-mediated Ca\textsuperscript{2+} influx activated by agonists was inhibited by a phospholipase C inhibitor, U73122. We propose that expression of hTrp3 in these cells forms a nonselective cation channel that opens after the activation of phospholipase C but not after store depletion. In addition, a subpopulation of the expressed hTrp3 may form heteromultimeric channels with endogenous proteins that are sensitive to store depletion.

The activities of a large number of enzymes are regulated through changes of intracellular Ca\textsuperscript{2+} concentration (ICa\textsuperscript{2+}).

Under resting conditions, cells keep [Ca\textsuperscript{2+}]\textsubscript{i} at approximately 100 nm. A rise of the cytosolic Ca\textsuperscript{2+} triggers a cascade of Ca\textsuperscript{2+}-sensitive events that are both immediate, such as secretion, contraction, and mobilization of energy resources (e.g. glycogenolysis), and long term, such as changes in the transcription of many genes. Some of the Ca\textsuperscript{2+}-responsive transcription processes are known to cause proliferation and programmed cell death (1–3). In both nonexcitable and excitable cells, Ca\textsuperscript{2+} signaling pathways can be activated by a ligand binding to cell surface receptors that activate phospholipase C (PLC). These include receptors that activate heterotrimeric G proteins and receptors signaling through activation of protein tyrosine kinases. The activation of PLC leads to the production of inositol 1,4,5-trisphosphate (IP\textsubscript{3}), which binds to IP\textsubscript{3} receptors, a class of intracellular ligand-operated Ca\textsuperscript{2+} release channels. The opening of IP\textsubscript{3} receptors allows Ca\textsuperscript{2+} to exit from its internal storage pools, causing a rapid increase in [Ca\textsuperscript{2+}]\textsubscript{i}. The increased cytosolic Ca\textsuperscript{2+} level is reduced quickly by Ca\textsuperscript{2+} pumps located on both the endoplasmic reticulum and the plasma membrane, causing [Ca\textsuperscript{2+}]\textsubscript{i} to decrease. The Ca\textsuperscript{2+} signal is prolonged, however, by the opening of a set of plasma membrane Ca\textsuperscript{2+}-permeant channels that allow Ca\textsuperscript{2+} to enter cells from the extracellular space, where the concentration of Ca\textsuperscript{2+} is in the millimolar range. In many, and possibly all cells, the entering Ca\textsuperscript{2+} is taken up rapidly by a storage compartment from which it is re-released in the continued presence of the triggering extracellular signal. This may, although not necessarily, be accompanied by periodic oscillations of the [Ca\textsuperscript{2+}] and allows for regulation of cytosolic as well as membrane-associated functions of the affected cells (for reviews, see Refs. 1 and 4).

Putney (5, 6) coined the term capacitative Ca\textsuperscript{2+} entry (CCE) for Ca\textsuperscript{2+} entry that is activated upon stimulation of cells with agonists that promote Ca\textsuperscript{2+} release from internal stores. After the discovery that CCE can be stimulated by mere store depletion, as occurs after inhibition of sarcoplasmic endoplasmic reticulum Ca\textsuperscript{2+}-activated ATPases with thapsigargin (TG) (7, 8), channels that mediate this type of CCE have been referred to as store-operated channels. In most cells, Ca\textsuperscript{2+} entry after stimulation by agonists can be explained by activation of store-operated channels, but neither the mechanism of how the store depletion signal is transmitted to the plasma membrane nor the molecular nature of the plasma membrane channels mediating Ca\textsuperscript{2+} has been clearly identified. Moreover, although store-operated plasma membrane Ca\textsuperscript{2+}-permeant channel activities have been found in many cell types (9–13), channels regulated by other mechanisms, such as a G protein, IP\textsubscript{3}, inositol 1,3,4,5-tetrakisphosphate (IP\textsubscript{4}), Ca\textsuperscript{2+}, or ATP, seem to coexist with store-operated channels (14–17). The molecular basis of this diversity and the relation of store depletion-insen-
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Sensitive Ca\(^{2+}\)-permeant channels to store depletion-sensitive Ca\(^{2+}\)-permeant channels are unclear. However, it is likely that multiple Ca\(^{2+}\) influx channels are involved in Ca\(^{2+}\) entry initiated by the activation of PLC-linked receptors.

In Drosophila eyes, the light-induced current is carried by channels formed from at least two related photoreceptor-specific proteins, Trp and Trpl (Trp-like). Trpl was identified by molecular cloning as a protein missing from the transient receptor potential (trp) mutant (18). Trp was identified biochemically as a calmodulin-binding protein and was cloned by standard techniques revealing a structure that shares a high degree of homology with Trp (19). Because the phototransduction pathway in insects resembles the PLC/IP\(_3\) signaling pathway in mammalian cells, it was speculated that mammalian homologs of Trp may exist and form Ca\(^{2+}\) influx channels. In support of this, expression of Trpl in Sf9 cells led to development of an agonist-stimulated non-selective Ca\(^{2+}\)-permeable ion conductance, and that of Trp led to the development of a TG-stimulated ion conductance (20–22). However, neither Trp-formed channels nor Trpl-formed channels displayed the ion permeation properties of endogenous insect CCE channels and, while forming channels, Trpl was not activated by TG treatment. More recently, work from the laboratories of Minke (23) and Montell (24) showed that Trp and Trpl can form homomultimeric ion channels with properties that differ from those of their parental molecules, suggesting that native voltage-independent Ca\(^{2+}\) influx channels in insects are likely to be formed of more than one type of subunit, i.e., to be heteromultimers.

Several mammalian sequences homologous to the Drosophila Trps have been identified from the data base of expressed sequence tags and by reverse transcriptase-polymerase chain reaction using degenerate oligonucleotide primers based on the Drosophila sequences (25–30). In the mouse we found the existence of at least six non-allelic Trp genes that can be divided into four major types based on primary amino acid sequence similarity (26, 31). Full-length cDNAs for Trp1, 3, 4, and 6 from human, murine, rat and bovine sources have been reported (25–27, 29–32). Functional expression of human Trp1, Trp3, bovine Trp4, or mouse Trp6 in COS, Chinese hamster ovary, or human embryonic kidney (HEK)293 cells results in enhancement of either agonist-stimulated Ca\(^{2+}\) entry or of IP\(_3\)- or TG-stimulated inward currents that are at least in part carried by Ca\(^{2+}\) (26, 29, 31, 32). More importantly, agonist-stimulated Ca\(^{2+}\) influx in murine L cells was blocked by transfection of murine Trp cDNA fragments in the antisense direction (26, 31), showing that mammalian Trp proteins are involved in agonist-stimulated Ca\(^{2+}\) influx.

For the present work we developed stable HEK293 cell lines expressing human Trp3 (hTrp3) and studied their Ca\(^{2+}\) influx properties. We report that the expression of hTrp3 caused an increase of [Ca\(^{2+}\)]\(_i\), under basal and agonist-stimulated conditions. The influx pathway formed by hTrp3 permeates Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) equally well, whereas the pathways intrinsic to HEK293 cells seem to be more Ca\(^{2+}\) selective. The influx due to hTrp3 can be blocked by SKF 96365, a known CCE blocker, or by verapamil, a nonspecific blocker for L-type voltage-sensitive Ca\(^{2+}\) channels. Although treatment with TG caused a small increase of Ca\(^{2+}\) influx over the basal in cell lines expressing hTrp3, a large portion of the influx pathway due to hTrp3 is not

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2 The nomenclature of mammalian Trps is used according to Zhu et al. (26). Thus, the human sequence that appeared in Wes et al. (27) and Zitt et al. (32) is hTrp1; the partial murine sequence published by Petersen et al. (28) and the full-length bovine and rat sequences reported by Philipp et al. (29) and Funayama et al. (30), respectively, are Trp4.
from the culture dish in 1 ml of ice-cold Dulbecco’s phosphate-buffered saline containing 1 mM EDTA and 0.1 mM phenylmethanesulfonyl fluoride, and pelleted by centrifugation at 5,000 rpm in a microcentrifuge at 4 °C for 5 min. Cells were then resuspended in 0.5 ml of RIPA buffer (150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 10 mM Tris-Cl, pH 8.0) containing 0.1 mM phenylmethanesulfonyl fluoride, 1 µg/ml soybean trypsin inhibitor, and 0.5 µg/ml leupeptin. Immunoprecipitation was performed using the anti-HA monoclonal antibody (12CA5) as described by Innamorati et al. (35). For glycosidase treatment, cell lysate was incubated with peptide-N-glycosidase F (2 units/ml) or endoglycosidase H (10 milliunits/ml) at room temperature for 1 h before antibody (12CA5) was added. The immunoprecipitated proteins were eluted in 80 µl of 2 × Laemmli buffer (i.e., 62.5 mM Tris-Cl, 1% SDS, 10% glycerol, 10% β-mercaptoethanol, pH 6.8) and separated by SDS-polyacrylamide gel electrophoresis in 9% acrylamide gels at 40 mA for 2 h. The gels were stained with Coomassie Blue to visualize the molecular markers, destained, and then dried for autoradiography.

Measurement of [Ca^{2+}]_{i}—Changes of [Ca^{2+}]_{i}, in individual cells were monitored by loading cells with Fura2 by fluorescence videomaging microscopy using an Attolaser Digital Imaging and Photometry attachment of a Carl Zeiss Axiovert inverted microscope as described before (26).

Changes of [Ca^{2+}]_{i} in cell populations were measured using an Aminco-Bowman Series 2 luminescence spectrophorometer (SLM Instruments, Inc.). Briefly, cells grown to confluence in 15-cm dishes were transfected as described in a 50-ml capped polypropylene tube. After a 20 min centrifugation at 500 × g and removal of the supernatant, the cell pellets were resuspended at room temperature in an extracellular solution (ECS) composed of 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 10 mM glucose, 0.1% bovine serum albumin, 15 mM Hepes, pH 7.4. Cells were incubated in ECS supplemented with 5 µM Fura2/AM and 0.05% Pluronic F-127 at 37 °C for 40 min. Cells were then washed once and resuspended in ECS at 2 × 10^6 cells/ml. Aliquots of 2 ml were kept in the dark at room temperature until use. Before each measurement, cells were washed twice in ECS or twice in ECS to which no CaCl2 was added if a Ca^{2+}-free/Ca^{2+} readdition protocol was used. 2 ml of cell suspension was transferred to a quartz cuvette and maintained at 32 °C under continuous stirring. Changes in intracellular Fura2 fluorescence intensity were measured by alternating excitation at 340 and 380 nm at 3-s intervals and detecting emission at 510 nm. Autofluorescence of the cells at 340 and 380 nm was determined from unloaded cells of an equivalent cell density and subtracted from values obtained for the Fura2-loaded cells. Drugs were added by a small aliquot (~20 µl) of concentrated stocks (dissolved either in water or dimethyl sulfoxide) to achieve the final concentrations. At the end of each recording, 0.1% Triton X-100 was added to the cells to determine the maximal fluorescence ratio (R_{max}). This was followed by an addition of 20 µM carbachol (CCh) to determine the minimal fluorescence ratio (R_{min}). [Ca^{2+}]_{i}, was calculated according to Grynkiewicz et al. (36).

In most experiments, Ca^{2+} entry under the basal or stimulated conditions was measured by a Ca^{2+}-free/Ca^{2+} readdition protocol, in which cells were incubated in a nominally Ca^{2+}-free ECS stimulated or not by the addition of an agonist or a store depletion drug. The stimulation normally caused a transient [Ca^{2+}], increase due to the release of Ca^{2+} from intracellular Ca^{2+} stores. After allowing the first [Ca^{2+}], peak to decrease to a steady-state level (normally 3–7 min), CaCl2 was added to give a final concentration of 1.8 mM. Typically, this led to a second [Ca^{2+}], increase. This extracellular Ca^{2+}-dependent [Ca^{2+}], increase is assumed to be caused by Ca^{2+} influx.

Measurements of influxes for Sr^{2+}, Ba^{2+}, and Mn^{2+}—A Ca^{2+} free addition cation protocol, analogous to the Ca^{2+}-free/Ca^{2+} readdition protocol in which Sr^{2+} or Ba^{2+} was added during the cation readdition phase, was used to study the entry of Sr^{2+} and Ba^{2+} into cells. The extent of influx of these divalent cations is expressed in the figures as changes in the ratio of 340 to 380 nm fluorescence without the estimation of their intracellular concentrations.

In a similar manner, MnCl2 was added to a final concentration of 50 µM to cells incubated in a nominally Ca^{2+}-free ECS under either unstimulated or stimulated conditions. Fluorescence quenching kinetics were studied using the Fura2 isosbestic excitation wavelength at 360 nm and recording emitted fluorescence at 510 nm. At the end of each recording, 0.1% Triton X-100 was added to cells to determine the maximal quenching of Fura2 by Mn^{2+}. Results of fluorescence quenching are expressed (in percent) as the amount of fluorescence decreased from the initial value (before the addition of Mn^{2+}) divided by the maximal loss after the addition of Triton X-100.
mature glycosylated form (band 2), as shown by its sensitivity to the endoglycosidase H treatment (see Fig. 7 in Ref. 31). This is not surprising since transiently transfected COS cells are known to synthesize a large quantity of the exogenous proteins of which only a fraction is processed to mature cell surface form (37).

The maturation process of hTrp3 can be followed in a pulse-chase experiment, as shown in Fig. 2B. After 15 min of labeling with a mixture of [35S]Met and [35S]Cys, the high mannose glycosylated hTrp3 was the major form. At 30 min, the more mature forms of glycosylated hTrp3 started to appear. At 60 and 120 min, the intensities of all of the bands increased proportionally compared with that at 30 min. The haze of radioactivity became more evident. When cells were labeled for 15 min and then the labeled [35S] was chased with culture medium containing unlabeled amino acids, the intensities of both the non-glycosylated and the high mannose-containing form of hTrp3 decreased as the chase time increased (Fig. 2C). Bands 3 and 4 and the haze remained after 3 h of chase. The proportions of bands 3 and 4 and the haze remained constant for up to 8 h (not shown).

**Basal and Stimulated Ca2+ Influx in hTrp3-expressing HEK Cells**—Ca2+ influx activities in the T3-9 and T3-65 cells under basal or stimulated conditions were compared with that of control cells by studying extracellular Ca2+-dependent [Ca2+]i changes in Fura2-loaded cells suspended at a density of 2 × 106 cells/ml. For the most part, we show results obtained from T3-9 and C1 cells. Similar results were obtained with T3-65 cells. C1 and C2 cells did not differ in their way of handling [Ca2+]i. Fig. 3 shows traces of [Ca2+]i changes during the course of typical experiments in which Trp3 (upper traces) or control (lower traces) cells were either not stimulated (panel A) or stimulated with 200 µM CCh (panel B) or 0.2 µM TG (panel C). Ca2+ influx was studied using the Ca2+-free/Ca2+ readout protocol described under “Experimental Procedures.” CCh was used to activate the PLC pathway via an endogenous muscarinic receptor found in the HEK293 cells. Fig. 4A shows the abundance of the muscarinic receptor sites in each cell line determined according to Liao et al. (38). Similar results were obtained by stimulating cells with 100 µM ATP, which activates an endogenous purinergic receptor. To study Ca2+ influx induced by store depletion, we used 0.2 µM TG to block the endoplasmic reticulum Ca2+-ATPase, which passively depletes the internal store without increasing the production of IP3 (4). Fig. 4B summarizes results obtained for two Trp3 cells (T3-9 and T3-65) and two control cells (C1 and C2).

Although in a nominally Ca2+-free buffer, basal [Ca2+]i in Trp3 cells is not significantly different from that of control cells; when the cells are maintained in a normal physiological solution containing 1.8 mM Ca2+, the resting [Ca2+]i in Trp3 cells is 40–70% higher than that in control cells (Fig. 4B). Addition of 1.8 mM Ca2+ to the extracellular medium of cells incubated in the Ca2+-free solution causes a 75–130 nM increase of [Ca2+]i, in the Trp3 cells (Figs. 3A and 4C). In control cells, the increase was less than 20 nM, of which a fraction could represent titration of Fura2 leakage or a small proportion of leaking cells in the whole population. Therefore, under non-stimulated conditions, control HEK293 cells exhibit very low Ca2+ influx, whereas Ca2+ influx channels in the Trp3 cells appear to have a higher spontaneous activity.

In control cells maintained in a nominally Ca2+-free solution, CCh caused a rapid and transient increase of [Ca2+]i, because of the release from internal Ca2+ stores. Readdition of 1.8 mM Ca2+ to the medium causes [Ca2+]i to increase about 120 nM. In TG-treated cells, readdition of Ca2+ causes a larger increase in [Ca2+]i (up to 240 nM). In the Trp3 cells, the [Ca2+]i, following Ca2+ readout after CCh or TG treatment is higher than under basal conditions, increasing by about 230 and 270 nM for CCh and TG, respectively (Fig. 4C).

A low cytosolic Ca2+ level is maintained by Ca2+ pumps...
Ca\textsuperscript{2+} added to the ECS to a final concentration of 1.8 mM. Data are maximal levels of [Ca\textsuperscript{2+}] \text{free} from the number of experiments indicated in parentheses from the number of experiments indicated in parentheses. Panel C, Ca\textsuperscript{2+} readdition elicited [Ca\textsuperscript{2+}], increases in control and Trp3 cells at basal or stimulated by agonist or TG. Cells were incubated in a nominally Ca\textsuperscript{2+}-free solution unstimulated (open bars) or stimulated with 200 \mu M CCh (filled bars) or 0.2 \mu M TG (shaded bars). Then Ca\textsuperscript{2+} was added to the ECS to a final concentration of 1.8 mM. Data are maximal [Ca\textsuperscript{2+}], reached within 1 min of Ca\textsuperscript{2+} addition minus the [Ca\textsuperscript{2+}], before Ca\textsuperscript{2+} addition from each experiment, averages \pm S.E. of results located on plasma membrane and endoplasmic reticulum, as well as other Ca\textsuperscript{2+}-buffering systems inside cells, which actively extrude Ca\textsuperscript{2+} out of the cell or into its intracellular stores. Opening of the Ca\textsuperscript{2+} influx channels results in a rapid rise of [Ca\textsuperscript{2+}], which in turn causes an increase in extrusion of Ca\textsuperscript{2+} mediated by the Ca\textsuperscript{2+}-pumps. Therefore, at any given time, [Ca\textsuperscript{2+}], is a result of the dynamic interplay of Ca\textsuperscript{2+}-pumps, intracellular Ca\textsuperscript{2+} channels (e.g. IP\textsubscript{3} receptors), and plasma membrane Ca\textsuperscript{2+} influx channels. Upon readdition of Ca\textsuperscript{2+} to the extracellular medium, three influx activities could contribute positively to the increase of [Ca\textsuperscript{2+}]. In CCh- or TG-stimulated Trp3 cells. The first is the basal activity mediated by Trp3. The second is influx intrinsic to the HEK cells stimulated by CCh or TG. The third is a Trp3-mediated influx stimulated by the same drugs. Because a significant fraction of Ca\textsuperscript{2+} entering from external space is removed by the Ca\textsuperscript{2+}-pumps, the net increase of [Ca\textsuperscript{2+}], will not be the sum of influx arising from the three activities. Therefore, from the experiments shown in Figs. 3 and 4, it is difficult to conclude whether stimulation by CCh or TG increases a Trp3-mediated Ca\textsuperscript{2+} influx pathway or if the stimulated increase in [Ca\textsuperscript{2+}], observed in the Trp3 cells results merely from the opening of Ca\textsuperscript{2+} influx channels endogenous to the HEK cells. More difficult is to see whether store depletion causes more Ca\textsuperscript{2+} influx in Trp3 cells than in control cells because the increase in [Ca\textsuperscript{2+}], in the two Trp3 cell lines is not significantly higher than that in C2 cells (Fig. 4C). To test whether any stimulated Ca\textsuperscript{2+} influx is caused by the expression of hTrp3, we selectively blocked the endogenous Ca\textsuperscript{2+} influx with 10 \mu M Gd\textsuperscript{3+}. As shown in Fig. 5, A, B, and E, in the presence of 10 \mu M Gd\textsuperscript{3+}, the endogenous Ca\textsuperscript{2+} influx activated by CCh and TG decreased >85% in control cells. In contrast, addition of 10 \mu M Gd\textsuperscript{3+} to the Trp3 cells reduced <10% of the basal Ca\textsuperscript{2+} influx (Fig. 5E), about 15% of Ca\textsuperscript{2+} influx stimulated by CCh (Fig. 5, C and E), and 55–60% stimulated by TG (Fig. 5, D and E). Assuming that the Trp3-mediated influx pathway is insensitive to 10 \mu M Gd\textsuperscript{3+}, we conclude that for Trp3 cells treated with CCh, a significant proportion of Ca\textsuperscript{2+} influx is mediated by Trp3, whereas in cells treated with TG, most of the Ca\textsuperscript{2+} influx is carried by the endogenous CCE channels.
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then [Ca\(^{2+}\)]\(_i\), gradually decreased. At 1 min after the addition of Ca\(^{2+}\), the increase in [Ca\(^{2+}\)]\(_i\) in TG-stimulated cells was equal to that in non-stimulated cells. Then [Ca\(^{2+}\)]\(_i\) in the TG-stimulated cells continued to drop, whereas that in non-stimulated cells stayed constant. Fig. 6A, comparison of Ca\(^{2+}\) influx under basal (open symbols) and TG-stimulated conditions (filled symbols) in Trp3 (T3-9 at upper left and T3-65 at upper right) and control cells (C1 at lower left and C2 at lower right). Cells were incubated in a nominally Ca\(^{2+}\)-free solution unstimulated or stimulated with 0.2 \(\mu\)M TG. GdCl\(_3\) was added to a final concentration of 10 \(\mu\)M to cells 2 min before the addition of 1.8 mM Ca\(^{2+}\) to the external solution. Data are from the same experiments shown in Fig. 5E and averages \(\pm\) S.E. of [Ca\(^{2+}\)]\(_i\), obtained at 3-s intervals minus the [Ca\(^{2+}\)]\(_i\) before Ca\(^{2+}\) addition. Panel B, Gd\(^{3+}\)-resistant CCE for Trp3 (circles) and control (squares) was obtained by subtracting the averages of [Ca\(^{2+}\)]\(_i\) increase in TG-stimulated cells (filled symbols in panel A) from that in unstimulated cells (open symbols in panel A).

Divalent Cation Selectivity of Influx Channels in Control and the Trp3 HEK Cells—We compared the influx of Ba\(^{2+}\) and Sr\(^{2+}\) through the Trp3-mediated pathway in Trp3 cells and the endogenous pathway in control cells. Increasing concentrations of both Sr\(^{2+}\) and Ba\(^{2+}\) have been shown to produce a shift in Fura2 excitation wavelength spectrum similar to that produced by Ca\(^{2+}\), but with somewhat higher dissociation constants (39, 40). Therefore, increases in the fluorescence ratio of Fura2 obtained at excitation wavelengths of 340 and 380 nm reflect increases in intracellular concentrations of Ba\(^{2+}\) or Sr\(^{2+}\). Control or Trp3 cells were incubated in nominally Ca\(^{2+}\)-free solutions either unstimulated or stimulated by CCh before 1.8 mM Sr\(^{2+}\) or Ba\(^{2+}\) was added into the ECS substituting for Ca\(^{2+}\). Fig. 7, A–D, shows that the rates of Ba\(^{2+}\) and Sr\(^{2+}\) influx into Trp3 cells are much higher than into control cells. Because Ba\(^{2+}\) is a poor substrate for Ca\(^{2+}\) pumps responsible for extrusion from the cytosol (39), a declining phase of intracellular [Ba\(^{2+}\)]\(_i\) is normally not seen, as is the case with Ca\(^{2+}\) and Sr\(^{2+}\) (Fig. 7, C and D). In CCh-stimulated control cells, the initial rate of fluorescence ratio increase with Ca\(^{2+}\) (2.0 units/min) is at least two times faster than the rates with Sr\(^{2+}\) (0.6 units/min) and Ba\(^{2+}\) (0.6 units/min). In contrast, the rates of fluorescence ratio increase in Trp3 cells are very similar regardless of which divalent cation was added (5.5 for Ca\(^{2+}\), 6.8 for Sr\(^{2+}\), and 6.2 for Ba\(^{2+}\), in units/min). Therefore, in HEK293 cells, the endogenous influx pathway activated by CCh seems to be more selective for Ca\(^{2+}\) than Ba\(^{2+}\) and Sr\(^{2+}\), whereas the Trp3-mediated influx is not selective for these cations.

Mn\(^{2+}\) can enter cells through certain types of Ca\(^{2+}\) influx channels and quenches Fura2 fluorescence (41, 42). The addition of 50 \(\mu\)M MnCl\(_2\) to cells suspended in a nominally Ca\(^{2+}\)-free solution causes a slow but constant decrease of fluorescence, indicative of Mn\(^{2+}\) entry into the cells under basal conditions.
The rate of Mn\(^{2+}\) entry into Trp3 cells was faster than into control cells. In CCh-stimulated cells, Mn\(^{2+}\) entry was increased in both the control and the Trp3 cells (Fig. 7F).

Effect of Ca\(^{2+}\) Channel Blockers—Although not specific, SKF 96365 has been shown to block agonist-stimulated Ca\(^{2+}\) influx in many cells (43). Fig. 8A shows that at 25 \(\mu M\), SKF 96365 inhibits completely the basal Ca\(^{2+}\) influx of Trp3 cells. In CCh-treated cells, the drug inhibits Ca\(^{2+}\) influx in both Trp3 and control cells. However, SKF 96365 seems to inhibit the Trp3-mediated influx more effectively than the endogenous influx. The residual Ca\(^{2+}\) influx left in the Trp3 cells is comparable to that seen in control cells. The IC\(_{50}\) for SKF 96365 was about 5 \(\mu M\) as estimated from measuring the CCh-stimulated Ca\(^{2+}\) influx in the Trp3 cells (Fig. 8B). Verapamil, a blocker of L-type voltage-gated Ca\(^{2+}\) channels, not known to affect CCE or agonist-stimulated Ca\(^{2+}\) entry in general, also prevents divalent cation entry via the Trp3-mediated pathway with an IC\(_{50}\) of about 4 \(\mu M\) (Fig. 8C). Both the basal and the stimulated cation influx in Trp3 cells can be blocked by high concentrations of Ni\(^{2+}\) (6 \(\mu M\)), La\(^{3+}\) (150 \(\mu M\)), and Gd\(^{3+}\) (200 \(\mu M\)).

PLC-dependent and Store Depletion-insensitive Cation Influx in Trp3 Cells—To explore further the activation mechanism of Trp3-mediated Ca\(^{2+}\) influx, we tested whether depleting internal stores with TG would prevent agonist-stimulated [Ca\(^{2+}\)]\(_i\) increase. In control cells maintained in a nominally Ca\(^{2+}\)-free solution, the addition of 200 \(\mu M\) CCh after incubating cells with 200 mM TG for 6 min caused no significant [Ca\(^{2+}\)]\(_i\) increase (not shown). In the Trp3 cells, a small rise in [Ca\(^{2+}\)]\(_i\), (27 ± 6 nM net increase at the peak, \(n = 4\)) was induced by CCh after cells had been incubated with TG for 6 min. However, when the cells were treated with TG in a medium containing 1.8 mM Ca\(^{2+}\) and the endogenous influx pathway was blocked by 10 \(\mu M\) Gd\(^{3+}\), the addition of CCh caused a transient increase of [Ca\(^{2+}\)]\(_i\) of 105 ± 8 nM (\(n = 11\)) in Trp3 cells (Fig. 9A). A similar increase of [Ca\(^{2+}\)]\(_i\) was not seen in control cells. In the experiment shown in Fig. 9B, the endogenous Mn\(^{2+}\) influx stimulated by either CCh or TG was blocked by 10 \(\mu M\) Gd\(^{3+}\). Addition of CCh to Trp3 cells pretreated with TG caused an increase in the rate of Mn\(^{2+}\) entry, which was not seen in control cells (right panel). The rate of Mn\(^{2+}\) entry under these conditions (TG followed by CCh) was not significantly different from the rate of entry observed in Trp3 cells treated only with CCh (left panel). In similar experiments, Ba\(^{2+}\) or Sr\(^{2+}\) was added to the TG-treated cells. Stimulation by CCh in the presence of extracellular Ba\(^{2+}\) or Sr\(^{2+}\) caused additional influx of these cations in Trp3 cells (Fig. 9, C and D) but not in control cells. These results suggest that in HEK cells expressing hTrp3, not only does receptor stimulation activate the influx of divalent cations better than store depletion, but signals from store depletion do not prevent or occlude the Trp3-mediated influx activated via receptor stimulation. Most of the influx activity that resulted from overexpression of hTrp3 alone in the HEK293 cells is thus activated by a mechanism other than store depletion.

The signaling cascade initiated by an agonist includes the activation of its receptor, of a G protein, of PLC, and thus the production of IP\(_3\) and the release of Ca\(^{2+}\) from its internal stores. Although store depletion does not appear to be the cause for agonist-activated Ca\(^{2+}\) influx through the Trp3-mediated pathway, activation of PLC seems to be necessary. When 15 \(\mu M\) U73122, an inhibitor of PLC (44), was used, not only Ca\(^{2+}\) mobilization (not shown) but also Ca\(^{2+}\) and Ba\(^{2+}\) influx stimulated by CCh was blocked (Fig. 10). Basal Ca\(^{2+}\) influx in the Trp3 cells was little affected by the treatment of U73122. Therefore, either the production of IP\(_3\) or the active form of the PLC is required for the activation of the agonist-stimulated Trp3-mediated influx pathway.

DISCUSSION

Calcium mobilization from internal stores and subsequent Ca\(^{2+}\) entry from extracellular space are the two major components of Ca\(^{2+}\) signaling following activation of PLC by cell surface receptors. It has long been established that Ca\(^{2+}\) is released from its internal stores via IP\(_3\) receptors in response to the increase of IP\(_3\) production (1). Only recently has evidence accumulated showing that Ca\(^{2+}\) influx can occur via plasma membrane channels formed of Trp homologs (26, 29, 32). The two Drosophila photoreceptor Trp proteins have different functional features. DTrp was found to form a Ca\(^{2+}\) influx channel activated either by IP\(_3\) or by store depletion with TG, when expressed in insect S/9 cells (22), Xenopus oocytes (23, 28), or 293T cells (24). On the other hand, DTrp forms a non-selective cation channel that is activated by stimulation of G\(_i\)-coupled receptors but is not sensitive to store depletion (20, 21, 24). In addition, DTrpl also displayed significant basal activity (20). By comparing the amino acid sequences of the mammalian Trp...
**Fig. 9.** Influx of divalent cations through the hTrp3-mediated pathway can be activated in cells previously depleted of internal Ca$^{2+}$ stores. Fura2-loaded HEK293 cells expressing hTrp3 (T3-9) or control cells (C1) were treated with 0.2 μM TG either in a solution containing 1.8 mM Ca$^{2+}$ (panel A) or in a nominally Ca$^{2+}$-free solution (panels B–D). Panel A, in the presence of 1.8 mM extracellular Ca$^{2+}$, the endogenous Ca$^{2+}$ influx was blocked by the addition of 10 μM Gd$^{3+}$. Stimulation by 200 μM CCh caused a transient increase of [Ca$^{2+}$], only in the Trp3 cells. Panel B, Mn$^{2+}$ entry in the presence of 10 μM Gd$^{3+}$ recorded by Fura2 fluorescence quenching using the Fura2 isosbestic excitation wavelength at 360 nm and emission at 510 nm. Panel B, left, 50 μM MnCl$_2$ was added as indicated 3 min after the cells had been incubated with 200 μM CCh in a nominally Ca$^{2+}$-free solution. Under these conditions, CCh only increased the rate of Mn$^{2+}$ entry over the basal (not shown) rate in the Trp3 cells but not the control cells. Panel B, right, 50 μM MnCl$_2$ was added as indicated 3 min after cells had been incubated with 0.2 μM TG. 200 μM CCh was added 4 min later as indicated. An increase of Mn$^{2+}$ entry was seen in the Trp3 cells. In panels C and D, a similar protocol was used. Ba$^{2+}$ (panel C) or Sr$^{2+}$ (panel D) was added to TG-treated cells to a final concentration of 1.8 mM. CCh (200 μM) was added 4 min later. The increase of Fura2 fluorescence ratio in response to CCh seen in Trp3 cells was caused by the entry of Ba$^{2+}$ or Sr$^{2+}$.

**Fig. 10.** CCh-activated influx of Ca$^{2+}$ (upper panel) and Ba$^{2+}$ (lower panel) via hTrp3 is prevented by a phospholipase C inhibitor, U73122. HEKTrp3-9 cells were used. U73122 was added to the ECS to a final concentration of 15 μM 3 min before the addition of 0.2 μM TG. Experiments were performed as described in Fig. 9, A and C. Note that the store depletion-insensitive influx activated by CCh as seen in Fig. 9, A and C, is blocked by the PLC inhibitor.

proteins with that of *Drosophila* Trp or Trpl, it is difficult to predict whether any of the mammalian Trps would functionally resemble DTrp or DTrpl. A recent report by Philipp et al. (29) showed that bovine Trp4 transiently expressed in HEK293 cells forms a channel that is relatively more selective for Ca$^{2+}$ than for monovalent cations and is activated to the same extent by either IP$_3$ or TG, suggesting that Trp4 may functionally resemble the DTrp. Zitt et al. (32) also reported that hTrp1 expressed in Chinese hamster ovary cells can be activated by store depletion treatment, although the channel formed in this case is non-selective. Thus, hTrp1 seems to resemble partly both DTrp and DTrpl. Experiments to be reported elsewhere indicate that the stable expression of hTrp3 in the HEK293 cells gives rise to a novel Ca$^{2+}$-permeant cation influx current that is spontaneously active, non-selective, and can be further stimulated by activation of G$_i$-coupled receptors. In the present experiments, the majority of the increased Ca$^{2+}$ influx due to hTrp3 is neither activated by store depletion alone nor occluded by store depletion with TG. Thus, hTrp3 functionally resembles *Drosophila* Trpl.

Ca$^{2+}$ influx mediated by channels insensitive to store depletion have been shown to coexist with the store depletion-activated pathway in many systems. Orrenius and colleagues (42, 46, 47) reported that in cells such as hepatocytes, T lymphocytes, adrenal glomerulosa cells, fibroblasts, platelets, and anterior pituitary cells, Ca$^{2+}$ influx activated by emptying the agonist-sensitive stores could not completely mimic the influx activated by receptor agonists. More importantly, the store-insensitive cation influx in hepatocytes was less selective for Ca$^{2+}$ than influx activated by TG. Clementi et al. (48) also reported that stimulation of PC12 cells with carbachol activated two Ca$^{2+}$ influx responses, of which one was store-dependent and the other was directly dependent on receptor activation. Work by Montero et al. (49) showed that in differ-

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$^{3}$ R. S. Hurst, X. Zhu, G. Boulay, E. Stefani, and L. Birnbaumer, submitted for publication.
entiated HL60 cells, N-formyl-leucyl-phenylalanine activated an additional Ca\(^{2+}\) influx after the internal Ca\(^{2+}\) store had been completely emptied by TG. In electrophysiological studies, multiple types of Ca\(^{2+}\)-conducting currents bearing distinct characteristics are often observed by investigators using carefully designed protocols. In mast cells, Ca\(^{2+}\) enters through both a Ca\(^{2+}\) release-activated current (I\(_{\text{CRAC}}\)) and a 50-picosiemem channel that is activated more directly by receptor/G protein coupling (16, 50). In A431 epithelial cells, up to four different types of Ca\(^{2+}\)-permeant channels could be involved in Ca\(^{2+}\) influx following the activation of G protein (shown by the action of GTP\(\gamma S\)) or perfusion of IP\(_3\) (14). In addition, IP\(_3\) was found to activate plasma membrane channels in B lymphocytes (51) and substance P receptor-transfected Chinese hamster ovary cells (52). Moreover, IP\(_3\) may activate the same channel that is sensitive to store depletion. In inside-out patches of vascular endothelial cells, IP\(_3\) was shown to modulate the activity of a Ca\(^{2+}\) influx channel which is indistinguishable from that activated by treatment with 2',5'-ditt(ert-butyl)1,4-benzo-hydroquinone, i.e. by store depletion (17). Thus, under physiological conditions, it is likely that after receptor activation, Ca\(^{2+}\) enters through multiple Ca\(^{2+}\)-permeant channels. The relative contribution of each influx pathway is likely to differ depending on the cell type and probably the type and the degree of the stimulation. An estimate has been made for rat mast cells in which, under physiological conditions, the amount of Ca\(^{2+}\) conducted by I\(_{\text{CRAC}}\) is three times of that carried by the 50-picosiemem channel (16, 53).

The activation mechanism of agonist-stimulated Ca\(^{2+}\) influx via hTrp3 is unclear. Controversy exists with respect to the mechanism of activation of the Drosophila Trp1 expressed in the S/9 cells. Dong et al. (54) reported that DTTrp1 is activated by intracellular perfusion with IP\(_3\), whereas Obukhov et al. (55) found that in excised patches, DTTrp1 is activated by the active forms of the \(\alpha\) subunits of the G protein \(G_{11}\) and \(G_{0}\), but not by IP\(_3\). The fact that agonist-stimulated store-insensitive Ca\(^{2+}\) influx via hTrp3 can be prevented by an inhibitor of PLC suggests that the channel formed by hTrp3 is activated by a factor downstream of PLC activation, and upstream of store depletion. Since our experiments were carried out under conditions in which store depletion occurred either before or upon the addition of the agonist, we cannot rule out the possibility that Ca\(^{2+}\) mobilization is also a necessary step for agonist-induced Ca\(^{2+}\) influx through Trp3. If that is the case, stimulation of hTrp3 would require both store depletion and the generation of this other factor downstream of PLC activation. The likely candidates for this factor are IP\(_3\) and its derivatives, for instance IP\(_4\). According to a so-called conformational coupling model (56), it is also possible that the channel is activated through direct interaction between the channel and the activated IP\(_3\) receptor. Less likely, although not impossible, is that the channels could be stimulated by the activated PLC itself. A more recent study by Zitt et al. (45) showed that hTrp3 may be activated by Ca\(^{2+}\). However, treatment with TG also induces an increase in [Ca\(^{2+}\)]. This increase does not seem to activate Ca\(^{2+}\) influx mediated by Trp3 as well as that stimulated by receptor activation, suggesting that factors more than just intracellular Ca\(^{2+}\) are involved in activating Trp3. The 50-picosiemem channel found in mast cells is not activated by IP\(_3\) but instead requires the activation of receptor/G protein system (16). Thus, whether hTrp3 expressed in the HEK293 cells forms a channel that resembles any of the Ca\(^{2+}\) influx channels found in native tissues or isolated primary cells remains to be elucidated.

Because of some homology between the last four putative transmembrane segments of Trp and those of the subdomains of voltage-gated Ca\(^{2+}\) and Na\(^+\) channels, it has been proposed that a Trp-based channel may be a tetramer (19). Based on our finding that the mammalian genome contains at least six Trp genes, we speculated that a channel assembled by Trps can be either homotetrameric or heterotetrameric and that this could be a mechanism to create functionally diverse Ca\(^{2+}\)-permeant channels, including store depletion-activated, store depletion-insensitive, and channels activated by both store depletion and independently by agonists (31). In the stable HEK293 cell lines, a homotetrameric hTrp3 may have become the predominant influx channel formed because of overexpression of this protein. However, it may not represent any of the native Ca\(^{2+}\) influx channels present in this or other cell types. Because the cDNA for hTrp3 was isolated from HEK293 cells (26), we believe that there is endogenous hTrp3 protein in these cells. However, we do not know whether channels formed by hTrp3 alone are present in the native HEK293 cells because a component of Ca\(^{2+}\) influx resembling that expressed in the Trp3 cell lines is either missing or too small to be detected in the control cells. One possibility is that the endogenous hTrp3 in the HEK293 cells plays a very minor role in Ca\(^{2+}\) influx in response to agonist stimulation. The other possibility is that hTrp3 and other endogenous Trp proteins form heterotetrameric channels that are responsible for agonist and store depletion-activated Ca\(^{2+}\) entry in these cells. Evidence for the formation of a heteromultimeric Trp-based Ca\(^{2+}\) influx channel was recently obtained by Gillo et al. (23) who observed that coexpression of Drosophila Trp and Trpl in Xenopus oocytes leads to the appearance of a channel with an ion selectivity and La\(^{3+}\) sensitivity different from those seen in oocytes expressing either protein alone. Interestingly, the new channel is activated to the same extent either by IP\(_3\) or by TG, even though one of its components, DTTrpl, is not sensitive to store depletion. More recently, Montell and colleagues (24) demonstrated that the N termini of DTTrp and DTTrpl interact with each other both in vitro and in vivo. Coexpression of the two proteins in 293T cells gave rise to a store depletion-sensitive cation influx channel that had features from both DTTrp and DTTrpl. Interestingly, cells expressing both DTTrp and DTTrpl did not have an increased basal inward current as found in cells expressing DTTrpl alone. The authors proposed that DTTrp in Drosophila photoreceptors does not form homomultimers by itself, and its spontaneous activity is prevented by forming heteromultimeric channels with DTTrp or other Trp-related proteins. A similar conclusion may be drawn for hTrp3 since it behaves very similarly to DTTrpl when expressed alone. Because Drosophila Trp proteins can form store-operated heteromultimeric channels even if only one subunit is capable of detecting the signal from store depletion, it is possible that the exogenously expressed hTrp3 in the stably transfected cell lines also forms heteromultimers in combination with the endogenous Trp proteins, of which some can be activated by store depletion. This would explain the small but significant Gd\(^{3+}\)-resistant TG-stimulated Ca\(^{2+}\) influx in Trp3 cells (Fig. 6C). The smaller TG-stimulated increase of Ca\(^{2+}\) influx than the CCh-stimulated increase found in transiently transfected COS cells (26) can also be explained this way. On the other hand, the contribution of hTrp3-containing heteromultimeric channels to TG-stimulated Ca\(^{2+}\) influx could be more significant than that being revealed by the protocol shown in Fig. 6 if these channels are more sensitive to Gd\(^{3+}\) than the homomultimeric channel formed by hTrp3. This is even more so when the new channels formed cannot be distinguished from the native Ca\(^{2+}\) influx channels present in the HEK293 cells by Ca\(^{2+}\) channel blockers. Therefore, although our results show that overexpression of hTrp3 in HEK293 cells gives rise to a cation entry pathway insensitive
to regulation by a store-operated manner, we cannot at this point rule out the possibility that Trp3 is involved in forming native store-operated channels in HEK293 and other cells. Other Trp-unrelated proteins may also participate in the formation of the Ca\(^{2+}\) influx channels. It is yet to be firmly established that Trps are the true or the only subunits of store- or agonist-stimulated Ca\(^{2+}\) entry channels, as this will require purification of the channel complexes followed by reconstitution into either vesicles or planar lipid bilayers and analysis of channel activity. Further, even if they were channel subunits, other auxiliary proteins are expected to be required for the formation the native channel(s).

In conclusion, Ca\(^{2+}\) influx following the activation of the PLC/IP\(_3\) signaling pathway is an important phenomenon. Channels with different properties have been described in various cell types (for review, see Refs. 3 and 53). These include the store-operated channels that differ both in conductance and ion selectivity. These also include channels that do not appear to be store-operated. The presence of six Trp homologs in mammals and the possibility that they form heteromultimeric channels provide a plausible explanation for the tissue- and cell-specific behavior of Ca\(^{2+}\) influx pathways (and channels) found in different systems.

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