The Role of Endogenous Human Trp4 in Regulating Carbachol-induced Calcium Oscillations in HEK-293 Cells*

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We utilized 2-aminoethoxydiphenyl borane, an agent that blocks store-operated Ca\(^{2+}\) entry, as well as an antisense approach to characterize endogenous Ca\(^{2+}\) entry pathways in HEK-293 cells. The thapsigargin- and carbachol-induced, but not the 1-oleolyl-2-acyetyl-sn-glycerol (OAG)-induced, entry was blocked by 2-aminoethoxydiphenyl borane. Both reverse transcriptase-PCR and Western blot analyses demonstrated endogenous expression for HTRP1, HTRP3, and HTRP4 and specific suppression of mRNA levels and Trp protein levels in cells stably expressing antisense constructs. Expression of HTRP4 antisense inhibited 35% of the carbachol (CCh)-stimulated Ba\(^{2+}\) entry and 46% of the OAG-stimulated Sr\(^{2+}\) entry but in contrast had no effect on the thapsigargin-stimulated Ba\(^{2+}\) or Sr\(^{2+}\) entry. HTRP3 antisense reduced, while HTRP1 antisense had no effect on, OAG-induced Sr\(^{2+}\) entry. Of greater importance, HTRP4 antisense expression, but not HTRP3 antisense expression, blocked the sustained Ca\(^{2+}\) oscillations produced by low doses of CCh (15 \(\mu M\)), arguing that receptor-stimulated rather than store-operated channels are involved in these sustained oscillations. HTRP4 antisense also inhibited 75% of the arachidonic acid-induced Ca\(^{2+}\) entry. In summary, these data suggest that HTRP4 proteins in HEK-293 cells, differing from HTRP3 and HTRP1 proteins, do not serve as functional subunits of store-operated channels but do function as subunits for CCh- and OAG-stimulated channels. Furthermore, evidence is provided for the first time for the involvement of a Trp isoform (HTRP4) in the formation of the channel responsible for both arachidonic acid-induced Ca\(^{2+}\) entry and the Ca\(^{2+}\) entry needed to sustain long term Ca\(^{2+}\) oscillations induced by low doses of carbachol.

It is widely accepted that Ca\(^{2+}\) serves as an essential signaling molecule, and for that reason, the level of intracellular calcium ([Ca\(^{2+}\)])\(_i\) is strictly controlled. This control extends beyond regulating static levels of Ca\(^{2+}\) to include regulation of Ca\(^{2+}\) levels in subregions of the cell and regulation of the frequency and amplitude of Ca\(^{2+}\) oscillations within the cell. This multifaceted regulation is required for countless cellular functions including muscle contraction, protein secretion, cell proliferation, and apoptosis (1). Mutations inducing drastic alterations in intracellular Ca\(^{2+}\) homeostasis are most likely not compatible with life (2).

In most cells, there are two sources of Ca\(^{2+}\) that can be tapped in order to modify resting Ca\(^{2+}\) levels, the intracellular Ca\(^{2+}\) stores and the extracellular space. Intracellularly, Ca\(^{2+}\) is released from the sarcoplasmic reticulum stores via two types of Ca\(^{2+}\) channels: inositol 1,4,5-trisphosphate receptors and ryanodine receptors. Cytosolic Ca\(^{2+}\) is taken up into the sarcoplasmic and endoplasmic reticulum by one of the three sarco- or endoplasmic reticulum Ca\(^{2+}\)-ATPase Ca\(^{2+}\)-pumps.

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1 The abbreviations used are: SOC, store-operated channel; SOCE, store-operated calcium entry; InsP\(_3\), inositol 1,4,5-trisphosphate; OAG, 1-oleolyl-2-acytel-sn-glycerol; 2-APB, 2-aminoethoxydiphenyl borane; RT, reverse transcriptase; HBSS, Hanks’ balanced salt solution; CCh, carbachol.

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highest levels in brain, heart, testis, and ovary; they are ~450 amino acids shorter than Drosophila Trp; they have six-transmembrane domain regions; and regions in the N-terminal domain have similarity to domains found in ankyrin and in the C terminus similar to dystrophin (11, 12). Although, when expressed in a heterologous cell system, the Drosophila Trp channel appears to be store-operated, there is considerable evidence to suggest that Trp is regulated by a different mechanism during the phototransduction process (13). A similar controversy has arisen from numerous studies where various mammalian Trp homologs have been overexpressed in various cell systems; results suggest that mammalian Trps either may (14–18) or may not (19–21) respond to store depletion. For example, if we focus on the overexpression studies for the Trp4 isoform, there are significant disagreements concerning whether the channel is regulated by store depletion. The initial reports on bovine Trp4 described it as a capacitative calcium channel, since its overexpression in HEK-293 cells (16) or Chinese hamster ovary cells (22) led to channel activity that could be stimulated by depletion of Ca\textsuperscript{2+} stores with thapsigargin. A role for rat Trp4 in capacitative calcium entry was suggested by the demonstration of a potentiated capacitative calcium entry-mediated chloride current in oocytes expressing rat Trp4 (23) and was further supported by the observation that expression of a Trp4 antisense significantly reduced calcium release activated channel-like currents in adrenal cells (24). However, two recent studies argue that Trp4 does not participate in forming store-operated calcium channels. The expression of murine Trp4 in HEK-293 cells was reported to have no effect on channel activity following depletion of intracellular calcium stores but was reported to enhance the channel activity in response to activation of G\textsubscript{i} coupled receptors or receptor tyrosine kinases (25). The expression of HTRP4 in HEK-293 cells was reported to have no effect on barium entry stimulated by calcium store depletion, supporting the findings for murine Trp4. However, in contrast to results for murine Trp4, the HTRP4 produced a constitutively active channel which was not stimulated by either phospholipase C-linked receptor activation or by OAG (26). Given these dramatically different results from previous Trp4 overexpression studies, there is a compelling need to utilize alternative approaches to help resolve the role of Trp4 in the regulation of capacitative calcium entry.

Similar discrepancies in findings between laboratories exist for Trp1 (14, 15, 27–29), and Trp3 (14, 20, 30–35). One can think of a number of theoretical reasons for overexpression studies to produce results that may not reflect the role of endogenous Trps in native Ca\textsuperscript{2+} channel activity. The endogenous channels may be heterotetramers, and the channels formed in the overexpression studies may be homotetramers due to their high expression level, or the expressed Trp may be unable to form the appropriate heterotetramer channels due to the lack of the appropriate Trp isoforms in the cell being tested. We have also published data on clone-to-clone variation within the HEK-293 cell population that points out the potential pitfalls of interpreting data based on studies with a small number of stable clones expressing Trps (36). To circumvent many of the problems of overexpression studies, we have used an antisense approach to investigate these questions.

In a previous study, we reported that HEK-293 cells express mRNA for HTRP1, HTRP3, HTRP4, and HTRP6, and evidence was presented that HTRP1 and HTRP3 are involved in mediating store-operated Ca\textsuperscript{2+} entry (32). In the present study, we attempt to determine whether multiple Ca\textsuperscript{2+} entry pathways are mediated by endogenous HTRPs in HEK-293 cells. We have used the inhibitor, 2-aminoethoxydiphenyl borane (2-APB), to analyze and compare Ca\textsuperscript{2+} entry induced by thapsigargin, carbobal, and OAG in HEK-293 cells. We also have generated antisense constructs for HTRP1, HTRP3, and HTRP4 and stably expressed them in HEK-293 cells. We provide evidence that in HEK-293 cells endogenous HTRP4 proteins do not participate in the formation of cation channels regulated by store depletion with thapsigargin but do participate in channel activity activated by CCh or OAG. More importantly, we provide evidence that HTRP4 proteins are involved in the formation of channels responsible for both the arachidonic acid-induced Ca\textsuperscript{2+} entry and the Ca\textsuperscript{2+} entry needed to sustain long term Ca\textsuperscript{2+} oscillations in HEK-293 cells in response to low doses of CCh.

**EXPERIMENTAL PROCEDURES**

Materials—Fura-2-free acid, fura-2/AM, and pluronic F-127 were purchased from Molecular Probes, Inc. (Eugene, OR); thapsigargin was from LC Laboratory; G418 was from Mediatech; Nusieve GTG-agarose was from FMC BioProducts; Hanks’ balanced salt solution (HBSS), Ca\textsuperscript{2+}-free, Mg\textsuperscript{2+}-free, HCO\textsubscript{3}-free HBSS and Dulbecco’s modified Eagle’s medium, penicillin/streptomycin, L-glutamine, and trypsin-EDTA were from Invitrogen; Chelex-100 was from Bio-Rad; arachidonic acid was from Biomol; and carbobal, OAG, and 2-APB, along with other chemicals, were purchased from Sigma.

Isolation of cDNA-Encoding Fragments of Trps by RT-PCR—The poly(A)-RNA was isolated from HEK-293 cells using guanidinium thiocyanate extraction followed by an oligo(dT) binding method (QuickPrep Micro mRNA Purification Kit; Amersham Biosciences). The extracted mRNA was then treated with DNase (Invitrogen). The first strand cDNA was reverse transcribed using an oligo(dT) primer, and it was then amplified directly using PCR (SuperScript Preamplification System; Invitrogen). Sequences of primers to amplify specific regions of HTRP1, HTRP3, and HTRP4 as well as human \( \beta \) actin are described in Table I. Sequence similarity analysis was performed using Seqlab software by Genetics Computer Group. We used the inner primers to amplify fragments of endogenous HTRP cDNA in normal HEK-293 cells for the productions of the sense and antisense constructs and the outer primers to amplify HTRP cDNA fragments in HEK-293 cells in which sense or antisense constructs were stably expressed. The hot start PCR was performed with Taq polymerase; the initial denaturation was at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 30 s. A final extension was performed at 72 °C for 3 min. The PCR conditions were optimized to 30 cycles of annealing at 55 °C for each of HTRP1 and HTRP3 and to 35 cycles of annealing at 53 °C for endogenous HTRP4, using the inner primers. For the outer primers, the PCR was cycled 30 times, and the annealing was at 60 °C for all HTRP isoforms. For the experiments to test for mRNA expression for the \( \alpha \) and \( \beta \) isoforms of HTRP4, the PCR was cycled 30 times, and the annealing was performed at 52.5 °C. The PCR mixture (48 μl) consisted of ~22% of first stand cDNA as a template, 1 μl of 100 pm solution of each primer, 5 μl of 25 mM Mg\textsubscript{2+}, 1 μl of 10 mM dNTP mix, and 2.5 units of Taq DNA polymerase (Invitrogen). For a positive control and to determine whether equal amounts of mRNA were used for each condition, we used a human \( \beta \)-actin primer, and a negative control without RT was performed alongside all experimental samples. The cDNA fragments of HTRP1 (369 base pairs), HTRP3 (323 base pairs), and HTRP4 (412 base pairs) from RT-PCRs were separated by electrophoresis in a 3% GTG-agarose gel. The corresponding bands were cut out of gels, extracted (QIAEX II Gel Extraction 150), and subcloned into an eukaryotic TA cloning vector pCR3.1 that accepts products in both the forward and reverse directions (eukaryotic TA cloning kit, bidirectional; Invitrogen). The clones were selected randomly, and the cDNAs were purified (Qiagen Plasmid Maxi Kit 25) and sequenced. The antisense constructs encoding HTRP1, HTRP3, and HTRP4 were expressed in HEK-293 cells, whereas the corresponding short sense constructs were expressed for the controls.

Cell Culture—HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. Cells were grown in an incubator at 37 °C with humidified 5% CO\textsubscript{2} and 95% air.

Stable Transfection of Antisense HTRP1, HTRP3, and HTRP4 Constructs—HEK-293 cells were transfected with sense or antisense constructs using the Ca\textsuperscript{2+} phosphate method. G418-resistant transfectants were selected using 400 μg/ml G418. In total, three antisense cell lines (HTRP1AS, HTRP3AS, and HTRP4AS) and three sense cell lines (HTRP1S, HTRP3S, and HTRP4S) were established. For each cell line...
expressing sense or antisense constructs, all of the surviving clones (~200) were pooled together to generate a cell line stably expressing one of the Trp constructs. This was done in order to avoid the problems inherent with using small numbers of selected clones to compare the effects of gene transfection. In a previous publication, we reported significant after-thromboxane receptor characterization of HTRP antibodies (Alomone Laboratories, Jerusalem, Israel). We tested various cell line buffers, sample heating temperatures, support membranes, wash buffers, and antibody incubation times on samples of total cell protein, cell membrane protein, and immunoprecipitated tagged Trp protein prepared from HEK-293 cells overexpressing Trp proteins. Initial Western blot experiments were performed with anti-tag antibodies, whereas subsequent experiments compared results with anti-Trp antibodies with those obtained with anti-tag antibodies. Conditions were considered optimum when we could see clear distinct bands of the appropriate molecular weight on Western blots of Trp-expressing cells and the intensity of these bands was in great excess of corresponding bands in the lane for control HEK-293 cells. Since control samples of HEK-293 cells often did not show endogenous Trp proteins, we scaled up the procedure in order to routinely observe endogenous Trp proteins. We went to a large gel format (16 x 16 cm) so that we could load more protein, and we included a protein precipitation and wash step in order to reduce the amount of contaminants associated with loading a larger protein sample onto the gels. The precipitation and wash step was found to greatly reduce the background of the Western blots. Optimum blocking and wash conditions for Westerns with HTRP4 antibodies were slightly different from those for HTRP1 and HTRP3 antibodies (see below).

Cells were lysed in modified radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 2 mM EDTA). Protease Arrest (Geno Technology, Inc.) was added to prevent proteolysis. A quantitative precipitation of all of the proteins in the total cell extract was done with PAGE-Perfect (Geno Technology), and the protein precipitate was then washed to remove agents that would interfere with running high quality gels. Precipitated protein pellets were mixed with 2 x Laemmli buffer (plus 100 mM dithiothreitol) and heated for 30 min at 70 °C, and sample pairs (antisense cells versus control cells) containing equal amounts of protein (200 µg for HTRP1 and HTRP3 and 400 µg for HTRP4) were loaded on a regular size gel (16 x 16 cm, 7.5% SDS-PAGE). Electrophoresis was performed overnight, and then proteins were electrottered onto Immobilon membranes (Millipore Corp.). For blots with anti-HTRP1 and anti-HTRP3, the membranes were blocked with 5% milk solution in TBS-T (0.1% Tween 20) (for HTRP4 Western blots, the Tween 20 was omitted from the TBS solution) for 1 h and were then incubated with primary antibodies raised against the intended protein (Alomone Laboratories) overnight at room temperature. The antibodies were diluted 1:200 in the blocking solution. Membranes were washed four times for 15 min each with TBS-T (for Trp Western blots, three times for 15 min each with TBS and one time for 15 min with TBS-T), incubated for 30 min at room temperature with secondary anti-rabbit antibody (1:10000 in 5% milk/TBS-T (TBS for HTRP4 Western blots) were washed under the same conditions, and developed with SuperSignal Chemiluminescent Substrate (Pierce).

Ca2+ Imaging—(Ca2+)-concentration was measured in cells loaded with the fluorescent indicator fura-2. Transfected cells were plated onto 25-mm coverslips 1 day before the experiment. On the next morning, cells were washed twice with a HEPES-buffered HBSS (HHBSS); loaded for 30 min with 5 µM fura-2/AM that was dissolved in HHBSS supplemented with 1 mg/ml bovine serum albumin, 0.025% pluronic F127; and then unloaded in HBSS for another 30 min. The coverslips were mounted as the bottom of a chamber that was placed on the stage of a Nikon Diaphot inverted epifluorescence microscope equipped with a Zeiss Fluor x10 (or x20 for single cell analysis) objectives. Cells in the chamber were perfused via an eight-channel syringe system (Anspec). A sucking pipette maintained a constant volume of solution (~0.5 ml) in the chamber.

An InCyt IM2™ fluorescence imaging system (dual wavelength fluorescence imaging system; Intracellular Imaging Inc., Cincinnati, OH) was used to measure [Ca2+]i, during the experiment. Excitation light from a xenon light source was alternately passed through 340 and 380 nm narrow pass filters mounted in a Sutter filter wheel (Lambda 10-C). The 510 nm emissions were captured by a cooled CCD camera (Cohu 4915). The images were transmitted to a computer (Intel Pentium Pro 500-MHz based PC) and processed with the imaging software InCyt IM2™. [Ca2+]i, was calculated by measuring the ratio of the two emission intensities for excitation at 340 and 380 nm. Calcium standard solutions, which were prepared with fura-2 potassium salt, were used to create a graph of fluorescence ratio (F340/F380) as a function of Ca2+ concentration (in mM). This graph was then used to convert fluorescence ratios in an experiment to calcium concentrations.

In experiments in which Ba2+- and Sr2+- influx were measured, the data are reported as the 340/380 ratio (R340/380), since the Ba2+ and Sr2+ calibration curves for fura-2 differ from the calibration curve for Ca2+.

During most experiments, an average response of ~800 cells from a single field on each coverslip was represented as one trace. The level of store-operated Ca2+-entry in the cells expressing Trp sense or antisense constructs was obtained by subtracting the slope of the Ba2+ leak (before stimulation) from the slope of Ba2+-influx (after stimulation) for each coverslip. In Ca2+ oscillation experiments, responses of single cells were reported.

Nominally Ca2+-free HBSS was prepared by stirring Ca2+-free, Mg2+-free, and HCO3−-free HBSS with Chelex-100 beads. After filtering out the Chelex-100 beads, MgCl2 was added to a final concentration of 1 mM.

### RESULTS

**Effects of 2-APB on Ca2+ Signaling Induced by Thapsigargin, Carbachol, and OAG**—The membrane-permeant inhibitor of the Inp3 receptor (37), 2-APB, has been utilized in several recent studies to address the question of whether a direct interaction occurs between the inositol 1,4,5-trisphosphate receptor and overexpressed HTRP channels. However, recent reports suggest that 2-APB may be directly inhibiting the store-operated channels (38, 39). Given the lack of a well-defined mechanism of action, we set out simply to determine whether 2-APB could help distinguish between the various endogenous Ca2+-entry pathways in our HEK-293 cells.

To investigate thapsigargin-stimulated Ca2+-entry, cells in HBSS were switched to a Ca2+-free HBSS medium and allowed to establish a new base line, and then the cells were treated with thapsigargin (1 µM). As expected, in the absence of Ca2+, thapsigargin induced a transient Ca2+-peak that reflects the depletion of intracellular stores. The level of [Ca2+]i, subsequently declined, suggesting the removal of [Ca2+]i, from the cell by the plasma membrane Ca2+-ATPase. After [Ca2+]i, returned to basal levels, Ba2+ (5 mM) was added into Ca2+-free medium, and the initial slope of Ba2+-entry was taken to indicate the level of store-operated Ca2+ entry (SOCE) (Fig. 1A). In unstimulated cells in Ca2+-free medium, 2-APB (100 µM) slightly elevated Ca2+ above basal levels (Fig. 1B), possibly through its inhibitory effect on Ca2+-ATPase in the endoplasmic reticulum (37). As reported for cells overexpressing HTRP3 (40), we observed that 2-APB produced a concentration-dependent inhibition of thapsigargin-stimulated Ba2+-entry (Fig. 1B). At 75 µM 2-APB, there was an ~50% reduction (n = 3) of Ba2+-entry, whereas at 100 µM 2-APB, a higher level of inhibition (75%) was observed (n = 3).

We used a similar protocol to investigate the effect of 2-APB on carbachol-induced Ca2+-entry. In the absence of Ca2+, carbachol (100 µM) induced a large, transient increase in [Ca2+]i, (Fig. 1C), indicating that basal release was stimulated by intracellular stores (following InsP3 receptor activation. As the cytosolic Ca2+ concentration (in nM). This graph was then used to convert fluorescence ratios in an experiment to calcium concentrations.

In experiments in which Ba2+- and Sr2+- influx were measured, the data are reported as the 340/380 ratio (R340/380), since the Ba2+ and Sr2+ calibration curves for fura-2 differ from the calibration curve for Ca2+.

During most experiments, an average response of ~800 cells from a single field on each coverslip was represented as one trace. The level of store-operated Ca2+-entry in the cells expressing Trp sense or antisense constructs was obtained by subtracting the slope of the Ba2+- leak (before stimulation) from the slope of Ba2+-influx (after stimulation) for each coverslip. In Ca2+ oscillation experiments, responses of single cells were reported.

Nominally Ca2+-free HBSS was prepared by stirring Ca2+-free, Mg2+-free, and HCO3−-free HBSS with Chelex-100 beads. After filtering out the Chelex-100 beads, MgCl2 was added to a final concentration of 1 mM.
Inhibitory effect of 2-APB on Ca^{2+} entry induced by thapsigargin, CCh, or OAG. In the absence of Ca^{2+}, fura-2-loaded HEK-293 cells were treated with 1 μM thapsigargin (A and B) or 100 μM CCh (C and D). The SOCE or CCh-stimulated entry was assessed by the re-addition of BaCl2 (Ba^{2+}; 5 mM) in Ca^{2+}-free medium following store depletion with either thapsigargin or CCh. A, the SOCE induced by thapsigargin in control HEK-293 cells; B, the inhibitory effect of 2-APB (100 μM) on SOCE. C, the CCh-stimulated Ba^{2+} entry in control HEK-293 cells; D, the CCh-stimulated Ba^{2+} entry in 2-APB-treated cells. 2-APB (100 μM) was added at 3 min prior to carbacbol addition. To monitor OAG effects (E and F), SrCl2 (Sr^{2+}; 5 mM) was added in Ca^{2+}-free medium. Stimulated Sr^{2+} entry was initiated by the addition of OAG (100 μM) into the chamber immediately after solution flow was stopped. 2-APB was added 3 min before OAG stimulation. Shown is the OAG-stimulated Sr^{2+} entry in control cells (E) and in cells treated with 100 μM 2-APB (F). Each trace represents one coverslip (~800 cells). Three coverslips (n = 3) were done for each condition, and similar results were observed. The basal [Ca^{2+}], was ~50 nM, and the peak of Ca^{2+} following thapsigargin stimulation was ~350 nM. The peak of Ca^{2+} following CCh stimulation was ~850 nM.

We next sought to assess the effect of 2-APB on OAG-induced Ca^{2+} entry. Since previous studies investigating OAG activation of overexpressed, human Trp proteins monitored Sr^{2+} entry, we measured Sr^{2+} entry in the presence of 100 μM OAG and the presence or absence of 2-APB (100 μM). As noted for the Ca^{2+} data in Fig. 1B, there was a dose-dependent effect of 2-APB on basal Sr^{2+} levels (Fig. 1F). However, 2-APB, regardless of dose used, had no effect on the large slope of OAG-induced Sr^{2+} entry (Fig. 1, E and F). The data suggest that the cation entry pathway activated by OAG is distinct from cation entry pathways activated either by store depletion with thapsigargin or by CCh stimulation of HEK-293 cells.

**Level of Expression of mRNA for Trp Homologs in HTRP1AS, HTRP3AS, and HTRP4AS Cells**—Our previous studies indicated that HEK-293 cells express mRNA for HTRP1, HTRP3, and HTRP4. To evaluate the role of HTRP1, HTRP3, and HTRP4 in mediating thapsigargin-, CCh-, and OAG-stimulated Ca^{2+} entry, we measured short antisense cDNA constructs specific for HTRP1, HTRP3, and HTRP4 that were stably transfected into HEK-293 cells. This allowed us to generate three cell lines, i.e. HTRP1AS, HTRP3AS, and HTRP4AS. Stable transfection of short sense cDNA constructs (300-400 nucleotides) for each of these Trp homologs was used as a control (i.e. HTRP1S, HTRP3S, and HTRP4S). As we described previously, we mixed all of the surviving clones (~200) to generate heterogeneous populations of transfected cells for each cell line; therefore, the large cell-to-cell variations of SOCE we previously reported in the parent HEK-293 population should have no impact on our interpretations (36).

We extracted poly(A) RNAs from all cell lines mentioned above, reverse transcribed first strand cDNAs, and then specifically amplified the appropriate cDNAs via PCR using primers specific for regions just outside the sequence of the sense and antisense constructs (outer primers, Table I). This was done so as not to amplify the expressed sense and antisense constructs along with the regions from the endogenous mRNA. As shown in Fig. 2, expression of HTRP1, HTRP3, and HTRP4 mRNA was detected in HTRP1S, HTRP3S, and HTRP4S cells, respectively. Since a previous report has shown alternative splice variants of HTRP4 (41), we used a different set of primers to determine whether HTRP4 mRNA expressed was for the α or the β isoform. We detected both isoforms, with the α isoform being the more abundant species (the β isoform was about 20% of the level of the α isoform; data not shown). In addition, the data in Fig. 2 show that the expression of the mRNA for the specific Trp homolog was significantly reduced in HTRP1S, HTRP3S, and HTRP4S cells in which antisense cDNA constructs were stably expressed.

**Level of Expression of Trp Proteins in HTRP1AS, HTRP3AS, and HTRP4AS Cells**—To confirm that antisense expression was reducing the protein levels as well as the mRNA levels of the various endogenous Trps, we performed Western blot analysis of all of the cells utilized. Although we were ultimately successful in observing endogenous Trp proteins on Western blots of HEK-293 cells utilizing the Alomone anti-Trp antibodies, this occurred only after an extensive characterization procedure for the anti-Trp antibodies (see “Experimental Procedures”). Initial experiments gave high background, multiband blots where the differences in the appropriate molecular weight band was barely discernible between lanes for control and Trp-overexpressing cells. However, after optimization of the
techniques, we were able to see single, clear, strong bands at the appropriate molecular weight for endogenous HTRP1, HTRP3, and HTRP4. The Western blot data in Fig. 3 demonstrate that HTRP1, HTRP3, and HTRP4 are endogenously expressed in HEK-293 cells and that the expression of antisense constructs specifically reduces individual HTRP protein levels. As seen in Fig. 3C, the introduction of HTRP1 antisense results in a reduction of HTRP1 protein to a value that is 36 ± 2.8% of the control protein level (significantly different from control value, p < 0.001, n = 3), while having no effect on either HTRP3 or HTRP4 protein levels. The expression of antisense to HTRP3 results in a reduction of HTRP3 protein to a value that is 43.0 ± 6.0% of the control protein level (significantly different from control value, p < 0.005, n = 4), while having no effect on HTRP1 and HTRP4 protein levels (Fig. 3B). Finally, the expression of HTRP4 antisense results in a reduction of HTRP4 protein to a value that is 31 ± 14% of the control protein level (significantly different from control value, p < 0.02, n = 3), while having no effect on HTRP1 and HTRP3 protein levels (Fig. 3A). Therefore, the antisense constructs demonstrate the specificity of action required to analyze the involvement of individual Trp isoforms in various endogenous Ca2+ entry pathways.

Role of HTRP4 in Thapsigargin-stimulated, CCh-stimulated, and OAG-stimulated Ca2+ Entry—To investigate the functional importance of HTRP4 in regulating Ca2+ influx, we examined entry in HTRP4AS cells, compared with HTRP4S cells, in response to thapsigargin, carbachol, and OAG. We monitored either Ba2+ or Sr2+ entry prior to stimulation and then again after stimulation. We summarized these results in Fig. 4. In the left panels, we show traces from two representative coverslips (HTRP4AS versus HTRP4S), each with the response averaged over ∼800 cells in the microscope field. In the study of thapsigargin- or carbachol-induced Ca2+ entry pathways, we measured levels of Ba2+ (or Sr2+) before (basal leak) and after (total influx) stimulation. We then determined the stimulated influx by subtracting the basal leak from the total influx. In the study of the OAG-induced entry pathway, we simply measured the slope of Sr2+ entry in the presence of OAG in HTRP4AS versus HTRP4S cells, since no basal Sr2+ entry was observed. In the right panel, we show the mean values (with error bars showing the S.E.) of the stimulated Ba2+ or Sr2+ uptakes plotted as percentages of its control. In this case, we determined the mean value of final Ba2+ (or Sr2+) influx for a series of coverslips for each cell type, and we determine whether the difference between HTRP4AS and HTRP4S cells was statistically significant by Student’s t test.

Fig. 4A shows both the basal Ba2+ leak (before store depletion) and the total Ba2+ influx following store depletion with thapsigargin (1 μM). When one compares the responses in HTRP4AS and HTRP4S cell lines, there is little difference in the initial transient Ca2+ peak (Fig. 4, A and C), suggesting that the suppression of HTRP4 expression does not significantly alter the size of the internal Ca2+ stores. Furthermore, there is no difference in thapsigargin-stimulated Ba2+ entry (slope: HTRP4AS, 0.69 ± 0.07, n = 15; HTRP4AS, 0.67 ± 0.07, n = 15; p > 0.8), suggesting that HTRP4 plays no major role in regulation of SOCE (Fig. 4B).

We also used Sr2+ to monitor SOCE. Interestingly, Sr2+ leak was undetectable in both HTRP4AS and HTRP4S cells (Fig. 4C). Furthermore, the Sr2+ influx following store depletion was dramatically enhanced, independent of cell lines (Fig. 4C). As shown in Fig. 4D, there was no difference in Sr2+ entry in HTRP4AS compared with HTRP4S cells (slope: HTRP4AS, 0.62 ± 0.02, n = 12; HTRP4S, 0.64 ± 0.02, n = 12; p > 0.50). The Ba2+ and Sr2+ data both agree that HTRP4 proteins are not involved in the Ca2+ entry pathway activated by calcium store depletion with thapsigargin.

To evaluate whether HTRP4 plays a role in receptor-stimulated Ca2+ entry, we measured Ba2+ influx before and after carbachol (100 μM) stimulation of HTRP4AS cells, using HTRP4S cells as a control. As shown in Fig. 4E, in the absence of Ca2+, carbachol induced a large, transient increase in [Ca2+]i, followed by a decline to basal levels. Stable transfection of antisense constructs for HTRP4 had no measurable effect on the size of the CCh-stimulated Ca2+ release; however, it did cause a dramatic decrease in final Ba2+ influx (Fig. 4E). As shown in Fig. 4F, the expression of HTRP4 antisense resulted in a 35% inhibition of Ba2+ entry, which was a statistically significant reduction (slope: HTRP4AS, 1.19 ± 0.15, n = 36; HTRP4AS, 0.77 ± 0.10, n = 36; p = 0.03).

To evaluate whether HTRP4 plays a role in OAG-stimulated Ca2+ entry, we measured Sr2+ influx before and during stimulation with OAG (100 μM) in HTRP4AS cells, using HTRP4S cells as a control. As observed in Fig. 4G, Sr2+ leak was undetectable in both HTRP4AS and HTRP4S cells. However, OAG-induced Sr2+ entry was dramatically reduced in HTRP4AS compared with HTRP4S. The expression of HTRP4 antisense resulted in a 46% inhibition of OAG-stimulated Sr2+ entry, a statistically significant reduction (Fig. 4H) (slope: HTRP4AS, 2.24 ± 0.23, n = 28; HTRP4AS, 1.20 ± 0.19, n = 28; p = 0.001).

In summary, HTRP4 does not seem to play a role in regulation of SOCE. However, it does play a role in carbachol-, and OAG-induced Ca2+ entry.

**OAG-induced Calcium Signaling in HEK-293 Cells Stably Transfected with HTRP1AS, HTRP3AS, and HTRP4AS—** In a previous study, we showed that stable expression of HTRP3 antisense in HEK-293 cells resulted in a 32% inhibition of

### Table I

| Genes          | Accession number | Primer sequence | Location |
|----------------|------------------|-----------------|----------|
| HTRP1          | U31110           | 5'-GAT TTG GGA AAA TTT CTT GGG ATG 7'-3' | 2341-2365 |
|                | Reverse (inner)  | 5'-TTT GTC TCC ATG ATT TGC TAT CA-3' | 2687-2709 |
|                | Reverse (outer)  | 5'-ATA GCA TAT TTA GAA GTC CCA AAG C-3' | 3074-3098 |
| HTRP3          | U47050           | 5'-GAC ATA TCC AAG TCC ATG ATC TCT-3' | 1858-1911 |
|                | Reverse (inner)  | 5'-ACA TCA TGG TCA TCC TCA ATT TC-3' | 2188-2210 |
|                | Reverse (outer)  | 5'-CAA CAT TTA TCC AGC ACC TAC TAT-3' | 3088-3111 |
| HTRP4          | NM016179         | 5'-CTG ACC ATA ATC CTG GGA AGA-3' | 1744-1764 |
|                | Reverse (inner)  | 5'-GCT TGG TCC GTG CAA ATT TCC-3' | 2135-2155 |
|                | Reverse (outer)  | 5'-CTT CAC CAG GTC CTC CAT AAC-3' | 2536-2576 |
| HTRP4 α vs. β  | AF063822         | 5'-CAT CAC CAA TAC CAA GAA GTT A-3' | 2342-2363 |
|                | Reverse          | 5'-CGT ATT CCT TCT TCT ATG GTC T-3' | 2982-2983 |
| Humanβ-actin   | J00068           | 5'-GCC ACC CGG CAA ACT AGA CAC-3' | 79-102 |
|                | Reverse          | 5'-CCA GCT GGT GAT GAT GCC CTG CT-3' | 324-346 |

*Sequences of inner primers and outer primers for detection of HTRPs*
HTRP3S cells were used as the corresponding controls. Sr2
dependent to support the sustained Ca2+
response to CCh (sustained for at least 1 h) are strictly depend-
lent on the presence of extracellular Ca2++. While initial theories
suggested that the Ca2+ entry required for sustained Ca2+
oscillations was mediated via store-operated channels, very
recent data suggest that the Ca2+ entry is via non-store-operated
channels (42, 44). Since we could reduce Ca2+ entry via
either store-operated or non-store-operated pathways by ex-
pressing either HTRP3 antisense or HTRP4 antisense, we in-
vestigated what effect the expressions of these antisense con-
structs have on cell Ca2+ oscillations.

Since we observed above that HTRP4 does not play a role in
SOCE but does play a role in CCh-stimulated Ca2+ entry, it
seemed possible that HTRP4 is involved in mediating the Ca2+
entry required to support the sustained Ca2+ oscillations in
CCh. To test this hypothesis, we monitored CCh-induced Ca2+
oscillations in individual cells for both the HTRP4S and
HTRP4AS populations of cells. The data in Fig. 6 (left panel),
show 10 representative single cell responses recorded from one
HTRP4S coverslip. Cells were stimulated with 15 μM CCh in a
medium containing 1.8 mM Ca2+. These cells exhibited repeti-
tive peaks over the 10-min time period shown. In Fig. 6 (right
panel), we show 10 representative individual cell responses
from one HTRP4AS coverslip. Note that there are dramatically

FIG. 2. RT-PCR Detection of expression of mRNA in control
and antisense-expressing cells. Antisense cDNA constructs for
HTRP1 (HTRP1AS), HTRP3 (HTRP3AS), and HTRP4 (HTRP4AS) were
stably transfected into HEK-293 cells, whereas short sense cDNA
constructs (~300 nucleotides) for HTRP1 (HTRP1S), HTRP3 (HTRP3S),
and HTRP4 (HTRP4S) were used as controls. For each cell line, poly(A)
RNA was extracted, treated with DNase, and reverse transcribed with
oligo(dT)12-18 primers and Superscript™ II reverse transcriptase (In-
vitrogen). The first strand cDNA was then amplified with PCR using
the forward and outer reverse primers that are specific for each Trp
isoform (Table I) to amplify the various Trp isoform cDNA.

FIG. 3. Western blot analysis of HTRP1, HTRP3, and HTRP4
protein levels in control and antisense-expressing cells. Total
cell lysates from HTRP4S, HTRP4AS, HTRP3S, HTRP3AS, HTRP1S,
and HTRP1AS cells were obtained using procedures described under
“Experimental Procedures.” Total protein (200 or 400 μg) was separated by
7.5% SDS-PAGE and transferred to Immobilon membrane. Poly-
clonal anti-human Trp4, Trp3, or Trp1 antibody and horseradish per-
oxidase-labeled goat anti-rabbit immunoglobulin were used as primary
and secondary antibodies, respectively. The signals were detected by
ECL using standard protocols. The results shown are representative of
at least three independent experiments.

SOCE. Transient transfection of an HTRP1 antisense construct
in HTRP3S cells led to a higher level of inhibition (55%) of
store-operated Ca2+ entry (32). To evaluate whether HTRP1 or
HTRP3 also play a role in mediating OAG-induced Ca2+
signaling, we measured Sr2+ influx induced by OAG (100 μM) in
HEK-293 cells expressing antisense cDNA constructs for
HTRP1 (HTRP1AS) or HTRP3 (HTRP3AS). HTRP1S or
HTRP3S cells were used as the corresponding controls. Sr2+
leak was undetectable in any of these experiments. Sr2+ influx
induced by OAG was dramatically reduced in HTRP3AS com-
pared with HTRP3S. The magnitude of the effect was a 52% reduction, which is statistically significant (Fig. 5) (slope:
HTRP3S, 2.46 ± 0.18, n = 14; HTRP3AS, 1.17 ± 0.22, n = 11;
p = 0.0002). In contrast, there was no difference in OAG-
duced Sr2+ influx between HTRP1AS and HTRP1S cells (Fig.
5) (slope: HTRP1S, 2.12 ± 0.36, n = 10; HTRP1AS, 2.20 ± 0.31,
n = 11, p > 0.8). The data suggest that HTRP3 and HTRP4, but
not HTRP1, play an important role in OAG-induced Sr2+ entry
(Fig. 5).

The Role of HTRP3 and HTRP4 in Ca2+ Entry-dependent,
CCh-induced Ca2+ Oscillations—Two recent studies (42, 43)
indicate that CCh, at low doses, can initiate long lasting Ca2+
oscillations in HEK-293 cells. Sustained oscillations of Ca2+
entry, reactions were performed without reverse trans-
scriptase (No RT; last two lanes). The results shown are representative of
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at least three independent experiments.
fewer repetitive Ca\(^{2+}\) spikes in HTRP4AS cells in comparison with HTRP4S cells. In Fig. 7, we show the statistical analysis of the responses recorded from over 300 individual cells of each type. It is clear that many more HTRP4S cells than HTRP4AS cells undergo in excess of three Ca\(^{2+}\) oscillations, the number of damped oscillations seen in normal HEK-293 cells in a Ca\(^{2+}\)-free medium (data not shown). However, it is also clear that the amplitude of the first spike is similar in the two cell lines, indicating that the difference is not in the initial intracellular Ca\(^{2+}\) pool content but rather in the inability of the HTRP4AS cells to continue to refill the internal Ca\(^{2+}\) stores after the initial depletion.

To determine whether the effects observed with HTRP4 antisense were specific and therefore supported a role for Human Trp4 in Calcium Oscillations.

**FIG. 4.** Role of HTRP4 in thapsigargin-, CCh-, and OAG-stimulated Ba\(^{2+}\) or Sr\(^{2+}\) entry. HTRP4 antisense cDNA constructs were stably transfected into HEK-293 cells, generating a HTRP4AS cell line. Stable transfection of HTRP4 sense cDNA constructs (~300 nucleotides) into HEK-293 cells (i.e. HTRP4S cells) was used as a control. The left panel shows the time course of representative experiments during which levels of Ba\(^{2+}\) (or Sr\(^{2+}\)) before (basal leak) and after (total influx) agonist stimulation were measured. SOCE was determined by subtracting the basal leak from the total influx following store depletion with 1 \(\mu M\) thapsigargin (A and C). CCh-stimulated entry was determined by subtracting basal Ba\(^{2+}\) leak from total Ba\(^{2+}\) influx following stimulation with 100 \(\mu M\) carbachol (E). OAG-stimulated entry was monitored by measuring Sr\(^{2+}\) entry following stimulation with 100 \(\mu M\) OAG (G). Each trace represents a single coverslip with the response averaged over ~800 HTRP4S cells (thick line) or HTRP4AS cells (thin line). The right panel shows the statistical analysis of the data, with the mean value of the leak-subtracted Ba\(^{2+}\) (or Sr\(^{2+}\)) entry (for \(n\) coverslips) plotted as a percentage of control. Statistical differences between values in HTRP4AS cells and HTRP4S cells were analyzed by Student’s \(t\) test.

**FIG. 5.** OAG-induced Sr\(^{2+}\) entry in HEK-293 cells stably transfected with HTRP1AS, HTRP3AS, or HTRP4AS. Sr\(^{2+}\) influx induced by OAG (100 \(\mu M\)) was measured in HTRP1AS, HTRP3AS, and HTRP4AS cells in comparison with their corresponding controls HTRP1S, HTRP3S, and HTRP4S. Sr\(^{2+}\) leak was undetectable in any of these experiments. The mean value of the Sr\(^{2+}\) entry (for \(n\) coverslips) is plotted as a percentage of control. Statistical differences between values in control and antisense-expressing cells were analyzed by Student’s \(t\) test.

**FIG. 6.** The role of HTRP4 in the sustained Ca\(^{2+}\) oscillations induced by low doses of CCh. Ca\(^{2+}\) responses to CCh (15 \(\mu M\)) were recorded in individual HTRP4AS or HTRP4S cells over a period of 10 min in Ca\(^{2+}\)-containing HBSS. Traces for 10 representative cells from a single HTRP4S coverslip (left panel) and 10 representative cells from a single HTRP4AS coverslip (right panel) are shown. Sampling rate was 90 image pairs/min.

**FIG. 7.** Frequency plot for CCh-induced Ca\(^{2+}\) oscillations in HTRP4AS versus HTRP4S cells. The number of HTRP4S cells (upper panel) or HTRP4AS cells (lower panel) responding with a given number of Ca\(^{2+}\) spikes within a 10-min period is plotted versus that number of Ca\(^{2+}\) spikes. The data summarizes the results in 311 HTRP4S cells and 309 HTRP4AS cells.
HTRP4 in maintaining the ongoing Ca\(^{2+}\) oscillations, we determined whether the expression of an HTRP3 antisense construct, which reduces store-operated Ca\(^{2+}\) entry in HEK-293 cells (32), would affect the Ca\(^{2+}\) oscillations induced by low doses of CCh. The data in Fig. 8 (left panel), show 10 representative single cell responses recorded from one HTRP3S coverslip. Cells were stimulated with 15 \(\mu\)M CCh in a medium containing 1.8 mM Ca\(^{2+}\). These cells exhibited repetitive peaks over the 10-min time period shown. In Fig. 8 (right panel), we show 10 representative individual cell responses from one HTRP3AS coverslip. Note that there are no obvious differences in the number of Ca\(^{2+}\) spikes in HTRP3AS cells compared with HTRP3S cells. In Fig. 9, we show the statistical analysis of the responses recorded from over 300 individual cells of each type. It is clear that HTRP3S and HTRP3AS cells undergo a similar number of Ca\(^{2+}\) oscillations, arguing that reduction of store-operated Ca\(^{2+}\) entry has no effect on the sustained Ca\(^{2+}\) oscillations.

The Role of HTRP3 and HTRP4 in Arachidonic Acid-stimulated Ca\(^{2+}\) Entry—Since a recent report suggested that the Ca\(^{2+}\) entry pathway required for sustained Ca\(^{2+}\) oscillations is an arachidonic acid-activated channel (43), we sought to determine whether expression of HTRP4 antisense had any impact on the arachidonic acid-stimulated Ca\(^{2+}\) entry. The data in Fig. 10A are representative traces that show that the addition of arachidonic acid to previously unstimulated HTRP4S cells produces a dramatic rise in Ca\(^{2+}\) levels, while a similar addition to HTRP4AS cells produced much less of a response. The data in Fig. 10B summarize our results and demonstrate that expression of HTRP4 antisense results in a 75% reduction in the level of arachidonic acid-stimulated Ca\(^{2+}\) entry (slope: HTRP4S, 0.21 ± 0.03, \(n = 19\); HTRP4AS, 0.05 ± 0.01, \(n = 20\); \(p < 0.0005\)). To determine whether this was a specific effect of HTRP4 antisense, we expressed HTRP3 antisense and determined the effect on arachidonic acid stimulation of Ca\(^{2+}\) entry. As seen in Fig. 10, C and D, there is no significant effect of expressing HTRP3 antisense.
isozymes in HEK-293 cells (46). Although we did provide Northern blot evidence for endogenous expression of HTRP3 (32) and another publication provided Western blot evidence for endogenous expression of HTRP1 protein (47) in HEK-293 cells, there is not extensive data in the literature to confirm the RT-PCR evidence of endogenous expression of Trp isoforms in HEK-293 cells. Thus, our Western blot data in Fig. 3 represents important evidence for the endogenous expression of HTRP1, HTRP3, and HTRP4 proteins in HEK-293 cells. However, based on our RT-PCR and Western blot data, it would appear that HTRP1 and HTRP3 proteins are more abundant than HTRP4 proteins. In the PCR analysis, cDNA fragments for HTRP1 and HTRP3 were visible after 30 PCR cycles, whereas the cDNA fragment for HTRP4 was visible only after 35 cycles, suggesting that less mRNA for HTRP4 is present. Likewise, for the Western blot experiments, we loaded the gels with twice as much protein and exposed the films for much longer time periods for the HTRP4 blots than for the HTRP1 and HTRP3 blots.

Contrary to a number of earlier published results on exogenously expressed Trp4 (16, 22–24), we were not able to detect a role for endogenous HTRP4 in channel activation by store depletion with thapsigargin (Fig. 4, A–D). Also, our results run counter to those described in a paper that was published during the preparation of this manuscript, describing studies on vascular endothelial cells from Trp4 knockout mice (48). In that report, a reduction of store-operated Ca2+ entry was observed in the absence of murine Trp4. On the other hand, our results, using the antisense approach, agree with at least part of the conclusions of two recent overexpression studies, namely that murine Trp4 (25) and HTRP4 (26) do not contribute to store-operated channel activity. In addition, a paper that became available in electronic form during the revision our manuscript argues that neither the a nor β splice variant of human Trp4 is regulated by depletion of internal calcium stores (49).

Although we could see no evidence for HTRP4 involvement in capacitative calcium entry, we were able to detect a significant effect of HTRP4 antisense expression on channel activation by the muscarinic receptor. It is likely that endogenous HTRP4 functions as a component of receptor-mediated calcium entry channels (Fig. 4, E and F). Thus, our results agree with overexpression studies showing that murine Trp4 is regulated by agonist stimulation (25), but they disagree with the report that overexpressed HTRP4 is constitutively activated and not regulated by agonists (26). In terms of cation selectivity, we see a nonselective permeability for Ba2+ and Sr2+ (Fig. 4, A–D). This finding agrees with results from human Trp4 (26) and mouse Trp4 (25) but not bovine Trp4 (22). Subtle differences between HTRP4 and Trp4 from other species may affect, at least partially, its contribution to channel properties.

We also observed that HTRP4 was involved in the pathway responsible for OAG stimulation of Sr2+ entry (Fig. 4, G and H). Since phospholipase C activation by carbachol should produce an elevation of diacylglycerol, one might expect the Ca2+ entry pathway activated by OAG to be the same pathway that is activated following CCh stimulation. Consistent with this hypothesis are the findings that both the OAG-induced and the CCh-stimulated Ca2+ entry pathways are at least partially dependent on the presence of HTRP4. We observed that expression of HTRP4 antisense can reduce the CCh-stimulated Ca2+ entry by 35% and can reduce the OAG-stimulated Ca2+ entry pathway by 46%. However, based on the effects of 2-APB on these two pathways, it seems unlikely that CCh stimulates the OAG-inducible pathway in HEK-293 cells. We see that most of the CCh-stimulated entry pathway(s) is inhibited by 100 μM 2-APB, while none of the OAG-induced entry pathway is inhib-
ited by this compound. Clearly, 2-APB can block the capacitative portion of the CCh-stimulated entry, based on the ability of 2-APB to block the thapsigargin-stimulated Ba2+ entry (Fig. 1). However, it is also clear that the noncapacitative component of Ca2+ entry stimulated by CCh is sensitive both to HTRP4 expression levels and to the presence of 2-APB. These data strongly suggest that the physiological levels of diacylglycerol produced by CCh stimulation of cells is not sufficient to activate the Ca2+ entry pathway activated by pharmacological levels of OAG. While one could certainly argue that high concentrations of diacylglycerol near the membrane could be produced following the activation of phospholipase C, the ability of 2-APB to block most of the CCh-stimulated Ca2+ entry, while having no effect on the OAG-stimulated entry pathway, indicates that this is not the case.

Our data demonstrating that Ba2+ entry due to store depletion with thapsigargin is inhibited by 2-APB (Fig. 1) is, on the surface, consistent with the notion that 2-APB blocks SOCE via its interaction with the InsP3 receptor (40). Original reports showed that 2-APB inhibits agonist-induced Ca2+ release in platelets, neutrophils, and aorta, probably via inhibition of InsP3 receptors, type 1 or type 3. While it does not affect InsP3 binding to its receptor, it does partly inhibit Ca2+ uptake into the store, like thapsigargin, at least at high doses (100 μM) (37). In recent studies, it was reported that 2-APB completely blocks Ca2+ entry induced by different store-depleting agents, and the results were observed in several cell lines, including HEK-293 cells (40, 42). 2-APB was found to block Ca2+ entry mediated by overexpressed HTRP3 channels if the channels are activated through phospholipase C-coupled receptors but not by diacylglycerol. The results were interpreted to argue for a direct interaction between the InsP3 receptor and HTRP3 (40). In our 2-APB studies, we found that there is a striking difference between the short and long term effects of 2-APB on InsP3 signaling. While a 3-min incubation with 2-APB had essentially no effect on the CCh-induced Ca2+ release (Fig. 1D), longer incubation times dramatically reduced the CCh-stimulated release of store Ca2+ (data not shown). Surprisingly, the 3-min exposure to 2-APB, which had no measurable effect on CCh-induced Ca2+ release, dramatically inhibited CCh-induced Ba2+ entry (Fig. 1D). Therefore, the inhibitory effect of 2-APB on SOCE is not correlated with the ability to reduce the initial depletion of the Ca2+ stores. This suggests that 2-APB may be directly blocking plasma membrane Ca2+ channels, a finding that is consistent with recent observations that 2-APB blocks thapsigargin-induced Ca2+ entry, even in cells lacking functional InsP3 receptors (38, 39).

Cyclical processes are part of the essence of life, and oscillations in cell calcium are one example of such cyclical processes, one that seems to be fundamental to the growth and differentiation of many cell types. Cytosolic calcium oscillations can be induced by a variety of agonists. Although the underlying mechanisms remain unclear, several models have been proposed, with InsP3 being the center of attention (4, 50–52). In the current study, we set up to record oscillations induced by carbachol (15 μM) in fura-2-loaded HEK-293 cells. We observed that the addition of CCh in the continued presence of external Ca2+ led to a sustained oscillatory pattern that is truncated shortly after the removal of extracellular Ca2+ (data not shown). We set out to investigate the role of HTRP4 in mediating the Ca2+ entry that is necessary for this sustained oscillatory pattern. We found that in cells expressing an antisense construct for HTRP4 (Fig. 6, right panel), the oscillations induced by CCh were more similar to the damped oscillations seen in nominally Ca2+-free medium than those seen in the presence of Ca2+ (Fig. 6, left panel). In Fig. 7, we plot the number of cells versus the number of oscillations that occur in a cell over a 10-min period. It is clear that most of the cells expressing HTRP4 antisense are clustered in the leftmost portion of the bar graph, indicating that they are unable to sustain oscillations over a long period of time. This appears to be a specific effect of the HTRP4 antisense, since the expression of the HTRP3 antisense construct had no effect on Ca2+ oscillations (Figs. 8 and 9). The determination that HTRP4 is involved in regulating agonist-induced Ca2+ oscillations is a very important one. Although much of the work in the Ca2+ signaling field focuses on the plateau phase of the Ca2+ response following high dose agonist addition, it is likely that under physiological conditions, cells see agonist at much lower doses. Thus, the Ca2+ oscillations seen in response to 15 μM CCh are likely to be physiologically more important than the plateau response seen at 100 μM CCh. Thus, the determination that HTRP4 is involved in supporting Ca2+ oscillations is an extremely important one.

While recent publications from the Shuttleworth laboratory have suggested that the Ca2+ entry pathway required for sustained Ca2+ oscillations is regulated by arachidonic acid, this hypothesis is based on the use of pharmacological inhibitors (43), which can always raise questions of drug specificity. When we observed that expression of HTRP4 antisense produced a block of the CCh-induced Ca2+ oscillations, we recognized that this provided an opportunity to test the arachidonic acid hypothesis utilizing a molecular approach. We first determined that the addition of arachidonic acid to control cells (HTRP4S cells) produced a significant level of Ca2+ entry (Fig. 10A). While the dose of arachidonic acid used in Fig. 10 is somewhat higher than those used to define the arachidonic acid-regulated channel (ARC) (53), we observed that the exact dose for activating Ca2+ entry depended on the batch of arachidonic acid received from the supplier. With some batches, we did see activation with doses in the range of 5–10 μM, while with other batches, it took 30–40 μM to stimulate robust levels of Ca2+ entry. The variability is probably due to the known instability of arachidonic acid in solution. However, no matter which batch was used, we would first define a window of doses to use by titrating the amount that would stimulate Ca2+ entry without giving a rise in Ca2+ concentration when added to cells in a Ca2+-free environment.

We next determined whether expression of HTRP4 antisense would block the arachidonic acid-stimulated Ca2+ entry pathway. The data in Fig. 10, A and B, illustrates that HTRP4AS expression blocks ~75% of the arachidonic acid-stimulated Ca2+ entry. This appears to be a specific effect of HTRP4 antisense, since the expression of HTRP3 antisense has no significant effect on the arachidonic acid-stimulated Ca2+ entry (Fig. 10, C and D). Therefore, this finding provides the first evidence for the involvement of one of the Trp proteins in the arachidonic acid-regulated Ca2+ channel. It also supports the hypothesis that the arachidonic acid-regulated pathway is the one that provides the Ca2+ influx necessary for the maintenance of sustained Ca2+ oscillations.

Among all mammalian Trp homologs, HTRP3 has been the most extensively studied. Early studies showed that expression of full-length cDNA encoding HTRP3 increased SOCE (14), and expression of a partial cDNA fragment of HTRP3 in the antisense orientation significantly reduced SOCE (32), following store depletion with thapsigargin. Additional studies suggested that HTRP3 might also be activated by a conformational coupling mechanism by interaction with InsP3 receptor (30, 40, 54). Other recent studies show that overexpressed HTRP3 can be activated by application of OAG (26, 45, 55). For comparison, human Trp1 has been shown to be a nonselective cation chan-
nknown, which, when expressed in mammalian cells, is activated by store depletion by thapsigargin as well as inositol 1,4,5-trisphosphate (15). Our recent studies indicated that expression of HTPR1 antisense in combination with HTPR3 antisense produces a further reduction in thapsigargin-stimulated Ca\(^{2+}\) entry in addition to that seen with expression of HTPR3 alone (32). When HTPR1 is expressed in S9 insect cells, it may (15) or may not (27) be sensitive to depletion of internal Ca\(^{2+}\) stores. In addition, overexpression of HTPR1 leads to channels that are sensitive to stimulation by carbachol (14) but insensitive to OAG (45, 56). The conclusion from the present study is that HTPR3 proteins as well as HTPR4 proteins play a role in forming the endogenous channels, which are activated by OAG. In contrast, endogenous HTPR1 proteins appear to play no role in the activation of channels by OAG. As shown in Fig. 5, in HEK-293 cells expressing antisense cDNA constructs for HTPR3 or HTPR4, Sr\(^{2+}\) entry induced by OAG was dramatically reduced. In contrast, Sr\(^{2+}\) entry induced by OAG was unaffected in cells expressing antisense cDNA for HTPR1. This provides useful evidence that OAG is indeed acting on the HTPR3 and HTPR4 but not HTPR1 channels.

We should point out that these results differ from earlier results, in that OAG stimulates Sr\(^{2+}\) entry via endogenous channels in our HEK-293 cells, while in other studies OAG had no effect on parental HEK-293 cells but did stimulate Sr\(^{2+}\) entry in cells overexpressing HTPR3 (26, 40). This difference in results is probably due to the variation of characteristics of cultured lines grown in different laboratories. It is widely known for cell lines such as PC12 cells that there can be dramatic differences in cultures carried for a number of years in different laboratories. Different laboratories utilize serum from different sources, and other more subtle differences in tissue culture procedure also exist. Since our previous work demonstrates dramatic clone-to-clone variation in levels of SOCE within our HEK-293 cell population, it is not difficult to imagine that culture conditions in one laboratory might offer a competitive advantage for growth of some clones over others, thereby leading to high endogenous levels of SOCE in HEK-293 populations in one laboratory versus low endogenous levels of SOCE in HEK-293 populations in another laboratory. Thus, while we see substantial SOCE via endogenous channels, some laboratories see very little endogenous SOCE. The cell lines with the low endogenous SOCE have been popular for electrophysiological studies of the overexpression of Trp proteins, since there is a low basal current, and an increment in current due to expression of Trp can be easily observed. One possible drawback to the use of cell lines with low endogenous currents might exist if the transfected Trp needs to form heterotetramers with another Trp isoform to show the correct channel characteristics. Cell lines having vanishingly small endogenous currents may not express the full complement of endogenous Trps leading to the study of homotetramers that may have very different properties from endogenous channels that may normally be assembled from heterogeneous subunits. In addition to these problems, we published a recent report that cautions about the study of low numbers of stable clones expressing Trps (36). That paper estimates the probability that clone-to-clone variation in the population of HEK-293 cells contributes to the misinterpretation of data in clones overexpressing Trp isoforms. In light of these potential problems, we decided that a strategy that reduces the level of endogenous Trps would be a more desirable way to study the functional role of individual Trp isoforms.

In summary, our results define some interesting similarities and differences in endogenous Ca\(^{2+}\) entry pathways activated by thapsigargin, CCh, or OAG in HEK-293 cells. The endogenous Ca\(^{2+}\) entry pathways activated by store depletion or by carbachol can be inhibited by 2-APB, while the endogenous Ca\(^{2+}\) entry pathway stimulated by OAG is not inhibited by 2-APB. The Ca\(^{2+}\) entry pathway activated by store depletion does not require HTPR4 as a channel subunit. On the other hand, the Ca\(^{2+}\) entry pathways activated by either carbachol or OAG do require HTPR4 expression. The CCh- and OAG-stimulated, HTPR4-dependent Ca\(^{2+}\) entry pathways appear not to be one and the same, since 2-APB inhibits most of the CCh-stimulated pathway but none of the OAG-stimulated pathway. HTPR3 proteins appear to be more widely used than HTPR4 proteins, since they are required in both the store-operated channels and the OAG-stimulated channels. In contrast, HTPR1 appears to be a functional subunit of endogenous store-operated channels but not OAG-stimulated channels. And of greater physiological importance, HTPR4 plays a role in both the Ca\(^{2+}\) entry pathway activated by arachidonic acid and the Ca\(^{2+}\) entry pathway required to sustain Ca\(^{2+}\) oscillations induced by low doses of CCh.

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The Role of Endogenous Human Trp4 in Regulating Carbachol-induced Calcium Oscillations in HEK-293 Cells

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