Cloning and expression of the human transient receptor potential 4 (TRP4) gene: localization and functional expression of human TRP4 and TRP3

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Mammalian homologues of the *Drosophila* transient receptor potential (TRP) protein have been proposed to function as ion channels, and in some cases as store-operated or capacitative calcium entry channels. However, for each of the mammalian TRP proteins, different laboratories have reported distinct modes of cellular regulation. In the present study we describe the cloning and functional expression of the human form of TRP4 (hTRP4), and compare its activity with another well studied protein, hTRP3. When hTRP4 was transiently expressed in human embryonic kidney (HEK)-293 cells, basal bivalent cation permeability (barium) was increased. Whole-cell patch-clamp studies of hTRP4 expressed in Chinese hamster ovary cells revealed a constitutively active non-selective cation current which probably underlies the increased bivalent cation entry. Barium entry into hTRP4-transfected HEK-293 cells was not further increased by phospholipase C (PLC)-linked receptor activation, by intracellular calcium store depletion with thapsigargin, or by a synthetic diacylglycerol, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG). In contrast, transient expression of hTRP3 resulted in a bivalent cation influx that was markedly increased by PLC-linked receptor activation and by OAG, but not by thapsigargin. Despite the apparent differences in regulation of these two putative channel proteins, green fluorescent protein fusions of both molecules localized similarly to the plasma-membrane, notably in discrete punctate regions suggestive of specialized signalling complexes. Our findings indicate that while both hTRP4 and hTRP3 can apparently function as cation channels, their putative roles as components of capacitative calcium entry channels are not readily demonstrable by examining their behaviour when exogenously expressed in cells.

Key words: capacitative calcium entry, calcium channels, HEK-293 cells, patch-clamp, non-selective cation current.

INTRODUCTION

In mammalian cells, the mechanism by which phospholipase C (PLC) activation activates plasma-membrane channels is poorly understood, but is thought to involve signals from depleted internal calcium stores, a process known as capacitative calcium entry [1]. A number of recent studies have suggested a role for human homologues of the *Drosophila* transient receptor potential (TRP) channel as capacitative calcium entry channels [2–9]. However, expression studies have yielded inconsistent results with regard to the regulation of TRP channels by calcium store depletion. Zitt et al. [10] found that, when transiently overexpressed in Chinese hamster ovary (CHO) cells, TRP3 acts as a constitutively active, non-selective cation channel that is not activated by store depletion. Li et al. [11] reported that TRP3 functions as a PLC-activated channel in the brain. When stably expressed in human embryonic kidney (HEK)-293 cells, Zhu et al. [12] concluded that TRP3 forms a non-selective cation channel, which is activated downstream of PLC-linked receptor activation, but not by store depletion. However, these authors also speculate that there may exist a subpopulation of TRP3-containing channels that are responsive to store depletion. Indeed Kiselyov et al. [13] demonstrated that TRP3 single channels were activated by store depletion and that this process involved a functional coupling of TRP3 with the Ins(1,4,5)P_3 receptor [14]. However, Ma et al. [15], utilizing the same TRP3-expressing cell line used by Kiselyov et al. [13] concluded that TRP3 activation involves interaction with an Ins(1,4,5)P_3 receptor, but found no evidence for its activation by Ca^{2+} store depletion. Further evidence that TRP3 can be activated independently of store depletion comes from Hofmann et al. [16] who reported that TRP3, and the highly similar TRP6, can be activated by 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), a diacylglycerol analogue. OAG was also demonstrated to activate TRP7 [17], the most recent TRP family member identified, and a member which is most similar to TRP3 and TRP6.

TRP4 is of particular interest, because work from two groups has provided strong evidence that TRP4 and the structurally similar TRP5 are activated by store depletion. A bovine TRP4 was shown to be activated in response to store-depletion by both thapsigargin and Ins(1,4,5)P_3 when expressed in HEK-293 cells [5]. Similarly, the rat TRP4 homologue, when expressed in *Xenopus* oocytes, enhanced the thapsigargin-induced calcium entry, as assessed by monitoring calcium-activated chloride currents [18]. In addition, rabbit TRP5, which is structurally similar to TRP4, has been reported to be activated by store depletion [19]. These results make TRP4 a compelling candidate as one of the molecular entities responsible for the physiological

Abbreviations used: BAC, bacterial artificial chromosome; CHO, Chinese hamster ovary; DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; FISH, fluorescence *in situ* hybridization; HA, haemagglutinin; HEK, human embryonic kidney; HRP, horseradish peroxidase; NMDG, *N*-methyl-D-glucamine; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PDZ, an acronym of the three proteins PSD-95, Dlg and ZO-1; PLC, phospholipase C; RT-PCR, reverse transcriptase PCR; TRP, transient receptor potential; hTRP, human TRP; TRPL, transient receptor potential-like.

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process of store-operated calcium entry. However, another laboratory has reported that mouse TRP5 is a receptor-operated not a store-operated channel [20]. Schaefer et al. [21] have reported that not only mouse TRP5, but also mouse TRP4, functions as a receptor-operated channel. The authors conclude that for both of these channels activation occurs independently of store depletion.

At present, no unambiguous picture exists of the physiological mechanism of activation of any TRP isoform. The present study assesses and compares the functions of two members of the TRP family, one from the TRP4/5 subfamily and the other from the TRP3/6/7 subfamily. We have focused on human TRP4 (hTRP4), the cloning and characterization of which is presented here, and hTRP3. We assess activation of these channels by thapsigargin, the PLC-linked agonist, methacholine, and OAG. Our results for hTRP3 support the view that it functions as an Ins(1,4,5)P_3- and diacylglycerol-gated channel, but not as a store-operated channel, at least when transiently expressed. However, our data showing that, in HEK-293 cells, hTRP4 functions as a constitutively active channel, which cannot be activated by either store depletion or receptor activation poses conflicts with the results of other groups.

The disparities between our results and those in the literature may imply a more complex regulation of TRP channel function than initially anticipated. Possible complicating factors include interactions with other signalling proteins, such as multimerization with other TRP family members to form channels with altered properties. As well, spatial localization of the channels and their assembly into macromolecular signalling complexes are likely to be important for proper channel function. In the present study we report confocal localization studies of hTRP3 and hTRP4, which hint at this latter possibility.

EXPERIMENTAL

Isolation and sequencing of hTRP4

A partial hTRP4 cDNA was obtained by reverse transcriptase-PCR (RT-PCR) from HEK-293 cells using degenerate oligonucleotides designed against bCCE1 [5]. A human kidney cDNA library (Gibco BRL) was screened using the Genetrapper Positive Selection System (Gibco BRL). An SDS/PAGE-purified primer complimentary to a partial sequence of hTRP4, 5′-CTA CCT GAT AGC TCC CAA AAG CCC-3′ (nucleotides 1038–1061) was biotinylated and hybridized to 5 μg of single-stranded phagemid DNA from the library. Hybridized DNA was captured by incubating the mixture with streptavidin-coated magnetic beads. The captured single-stranded DNA was repaired to make it double-stranded and then transformed by electroporation into electrocompetent *Escherichia coli* strain DH10B. Twenty-four clones were picked and analysed by Southern blotting. The clone with the largest insert was sequenced and shown to contain the entire full-length hTRP4 gene, and is the clone presented in this paper. Sequencing was performed using a dRhodamine Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems) and an automated sequencer. Sequence analysis was performed using the Genetics Computer Group suite of sequence analysis software.

Northern blot analysis and rapid scan analysis

A human Multiple Tissue Northern Blot (ClonTech Laboratories, Palo Alto, CA, U.S.A.) was probed using a cDNA probe derived from nucleotides 2125–2836 of the hTRP4 coding sequence. The probe was labelled using Gibcos RadPrime random primer labelling system. Blots were prehybridized for 3 h at 68 °C in ExpressHyb Solution (ClonTech). Hybridization was performed by adding 1.5×10^6 c.p.m./ml of probe to fresh hybridization solution and hybridizing to the blot for 18 h at 68 °C. The filters were washed and exposed to a PhosphorImager screen to obtain an image. As a control for loading and RNA integrity, the blot was stripped and reprobed using a human β-actin probe.

Using PCR primers, we screened a Rapid Scan Gene Expression Panel (OriGene Technologies Inc., Rockville, MD, U.S.A.). The panel consists of cDNA from 24 different human tissues arrayed on a 96-well plate. The cDNA concentrations used covered a three-log dilution and were normalized against β-actin. A pair of PCR oligonucleotide primers (forward primer, nucleotides 2429–2453; reverse primer, nucleotides 2728–2704), corresponding to a region near the 3′-end of the hTRP4 coding sequence, was used to amplify a 300 bp fragment. The PCR reactions were run on an agarose gel and a Southern blot was performed to detect the PCR product. Hybridization to the Southern blot was detected and quantified on a PhosphorImager.

Fluorescence *in situ* hybridization (FISH) analysis of hTRP4

FISH was performed to determine the chromosomal location of the hTRP4 gene. A human bacterial artificial chromosome (BAC) library was screened to obtain a genomic clone containing the hTRP4 gene (Genome Systems, Inc., St. Louis, MO, U.S.A.). The probe was labelled with digoxigenin-dUTP by nick translation and hybridized to normal metaphase human chromosomes in a solution of 50% (v/v) formamide/10% (w/v) dextran sulphate/2 × SSC (where 1 × SSC corresponds to 0.15 M NaCl/0.015 M sodium citrate). Hybridization was detected using fluorescein-labelled anti-digoxigenin antibodies and the chromosomes were counterstained using 4,6-diamidino-2-phenylindole (DAPI). Preliminary hybridization assigned hTRP4 to human chromosome 13. For labelling of the centromere, a biotinylated chromosome 13 centromere-specific probe was used, followed by detection with Texas Red avidin.

RT-PCR analysis of TRP transcripts in HEK-293 cells

Total RNA was extracted from HEK-293 cells using the Trizol reagent (Life Technologies Inc., Rockville, MD, U.S.A.). After DNase I treatment, 5 μg of total RNA was reverse-transcribed into first-strand cDNA using random and oligo-dT primers according to the SUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis instructions (Life Technologies, Inc). Aliquots of the cDNA products were then used as templates for PCR amplification with primers specific for hTRP1 (forward primer, CTT CCT CTC CAT CCT CTT CC; reverse primer, GTT TCT GAC ACC CGT AGT CC; 273 bp fragment), hTRP3 (forward primer, ATG CTG CTT TTA CCA CTG TAG; reverse primer, TGA GTT AGA CTG AGT GAA GAG; 449 bp fragment), hTRP4 (forward primer, TTG CCT CTG AAA GAC ATA ACA TAA G; reverse primer, CTA CTA ACA CAC ATT GTT CAC TGA G; 300 bp fragment), hTRP5 (forward primer, TGC ATT GCT CTA TGC CAT ACG CAA G; reverse primer, CCT CTG AAC TAG ACA CAC ACT CCA C; 266 bp fragment) or hTRP6 (forward primer, CTG AGC TGT TCC AGG GCC AT; reverse primer, CTC TTG ATT TGG TTC CAT G; 428 bp fragment). 'No template' controls were run for all experiments. PCR products were then run on a 1% agarose gel stained with ethidium bromide. The identity of the PCR products was verified, after purification from the PCR sample (NucleoTrap PCR purification kit; CloneTech Laboratories), by both forward and reverse sequencing.

Green fluorescent protein (GFP) and epitope tagged constructs

An haemagglutinin (HA)-hTRP4 construct was made by the addition, through PCR, of an HA epitope (YPYDVPDYA) to the N-terminus of the hTRP4 gene. A GFP–hTRP4 fusion protein plasmid was subsequently constructed by fusing the open reading frame of the GFP to the N-terminus of HA-hTRP4. A hTRP3–GFP fusion protein was made by adding the GFP coding sequence in frame to the C-terminus of hTRP3. The hTRP3 clone was obtained from Dr Lutz Birnbaumer (Department of Anesthesiology, University of California, Los Angeles, CA, U.S.A.).

Cell culture and transfection methods

HEK-293 cells were obtained from A.T.C.C. (Rockville, MD, U.S.A.). HEK-293 transfections were performed using Superfect Reagent (Qiagen) according to the manufacturer's instructions. CHO-K1 cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). For CHO-K1 cells, the TransFast™ transfection reagent (Promega) was used for transfections. For fura 2 experiments, cells were co-transfected with either the pcDNA3 vector or the gene of interest and the construct pEGFP-C1. For electrophysiology experiments, cells were transfected with the pEGFP vector (control), or with the GFP–hTRP4 fusion construct. GFP expression was used as a marker for transfection. Cells were assayed 16–72 h post-transfection.

Immunoblots

Immunoblots were performed on total cell lysates run on denaturing SDS/PAGE using the Novex Tris-Glycine gel system. Gels were electrotransferred on to nitrocellulose membranes. The primary antibody used was the mouse monoclonal anti-HA antibody clone 12A5 (Boehringer Mannheim) followed by a goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce). Antibody–antigen complexes were detected using SuperSignal luminescent HRP substrate (Pierce).

Immunocytochemistry and confocal microscopy

Fluorescent images of GFP fusion proteins in HEK-293 cells were obtained with a Zeiss LSM 410 confocal microscope (Carl Zeiss Inc., Thornwood, NY, U.S.A.) using an argon/krypton laser and excitation at 488 nm through a 100× objective lens. The optical thickness of the confocal images was estimated to be 0.8 μm. For triple-labelling experiments, DAPI was used to stain the nuclei of fixed HEK-293 cells, and immunocytochemistry was performed using an antibody to the β1-integrin subunit (Immunotech, Beckman Coulter Inc., Fullerton, CA, U.S.A.) to stain the plasma membrane. Briefly, cells were fixed in 2% formaldehyde/0.1% glutaraldehyde in PBS. Cells were incubated with a 1:20 dilution of antibody in a solution of 3% (w/v) BSA/2% (v/v) goat serum in PBS for 60 min at 37 °C. After washing, cells were incubated with rhodamine-conjugated secondary antibody diluted 1:10 or 1:50 in the same solution for 30 min at room temperature. Rhodamine staining was detected by excitation at 568 nm using the argon/krypton laser. DAPI staining was detected using a UV laser, exciting at 351 nm.

Fura 2 fluorescence measurements

Cells were loaded with 1 μM fura 2 acetoxymethyl ester for 30 min at room temperature in normal growth medium. The cells were then washed and incubated in HPSS [20 mM Hepes (pH 7.4), 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 11.1 mM glucose] for 15 min. Cells were washed and incubated in HPSS, without added calcium, before fluorescent measurements were made. Where indicated, 10 mM $BaCl_2$ was included in the medium. The fluorescent measurements were made either on a CCD (charge-coupled-device) camera-based imaging system or a photomultiplier-based system. The imaging system consisted of a Zeiss Axiovert 35 microscope attached to a CCD camera. Data collection and analysis were performed using the MetaFluor software program (Universal Imaging Corp., West Chester, PA, U.S.A.). Data from individual GFP-positive cells in the field of view were collected. The photomultiplier system consisted of a Nikon Diaphot microscope attached to a Deltascan D101 fluorescent light source equipped with a light path chopper and dual excitation monochromators (Photon Technologies Inc., Monmouth Junction, NJ, U.S.A.). Emission intensity was monitored by a photomultiplier tube. Before plating for assay on the photomultiplier system, cells were subjected to flow cytometric analysis to select GFP-positive cells. Cells were treated with trypsin and applied to a FACSVantage flow cytometer equipped with Cell Quest software (Beckton–Dickinson, San Jose, CA, U.S.A.), and their fluorescence was assayed using an excitation wavelength of 488 nm and an emission wavelength of 530 nm. GFP-positive cells were collected and plated on to glass coverslips for assay on the photomultiplier system. Fields of 5–40 GFP-positive cells were assayed. With either system, cells were alternately illuminated with 340 nm and 380 nm light and the emission at 510 nm was monitored. A rise in the ratio of emission outputs from excitation at 340 nm compared with that at 380 nm indicated a rise in intracellular calcium or barium concentration. Data were calibrated using calcium standards and are presented as rates of change in apparent calcium concentration. All experiments were conducted at room temperature (19–23 °C).

Electrophysiology

Currents in CHO-K1 cells showing the fluorescence of GFP were measured with the patch-clamp technique in the whole-cell mode [22]. The solution in the pipette contained 10 mM Hepes (pH 7.4), 110 mM Cs-aspartate, 20 mM CsCl, 20 mM tetraethylammonium chloride, 2 mM $MgCl_2$, 10 mM EGTA and 0.3 mM ATP. The standard bath solution contained 10 mM Hepes (pH 7.4), 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$ and 10 mM glucose. The solution labelled '$NMDG + Ca^{2+}$' in Figure 8(B) contained 150 mM *N*-methyl-D-glucamine (NMDG) and 5 mM calcium gluconate instead of NaCl. The 'NMDG' solution in Figure 8(B) contained 150 mM NMDG instead of NaCl. The standard holding potential was −60 mV. All experiments were performed at room temperature (19–23 °C). The significance of the results was determined using the Mann–Whitney (rank sum) test.

RESULTS

Cloning of hTRP4

Degenerate oligonucleotides based on the bovine TRP4 sequence [5] were used to amplify a fragment of hTRP4 from HEK-293 cells. An oligonucleotide matching the human fragment was used to screen a human kidney library utilizing the Genetrapper (Gibco BRL) liquid hybridization positive selection system. A 3.5 kb full-length hTRP4 clone was isolated (GenBank® accession number AF175406). It consists of a 231 bp 5′ untranslated region, which contains an in-frame stop codon just upstream of the putative ATG start codon, a coding region of 2934 nt, a

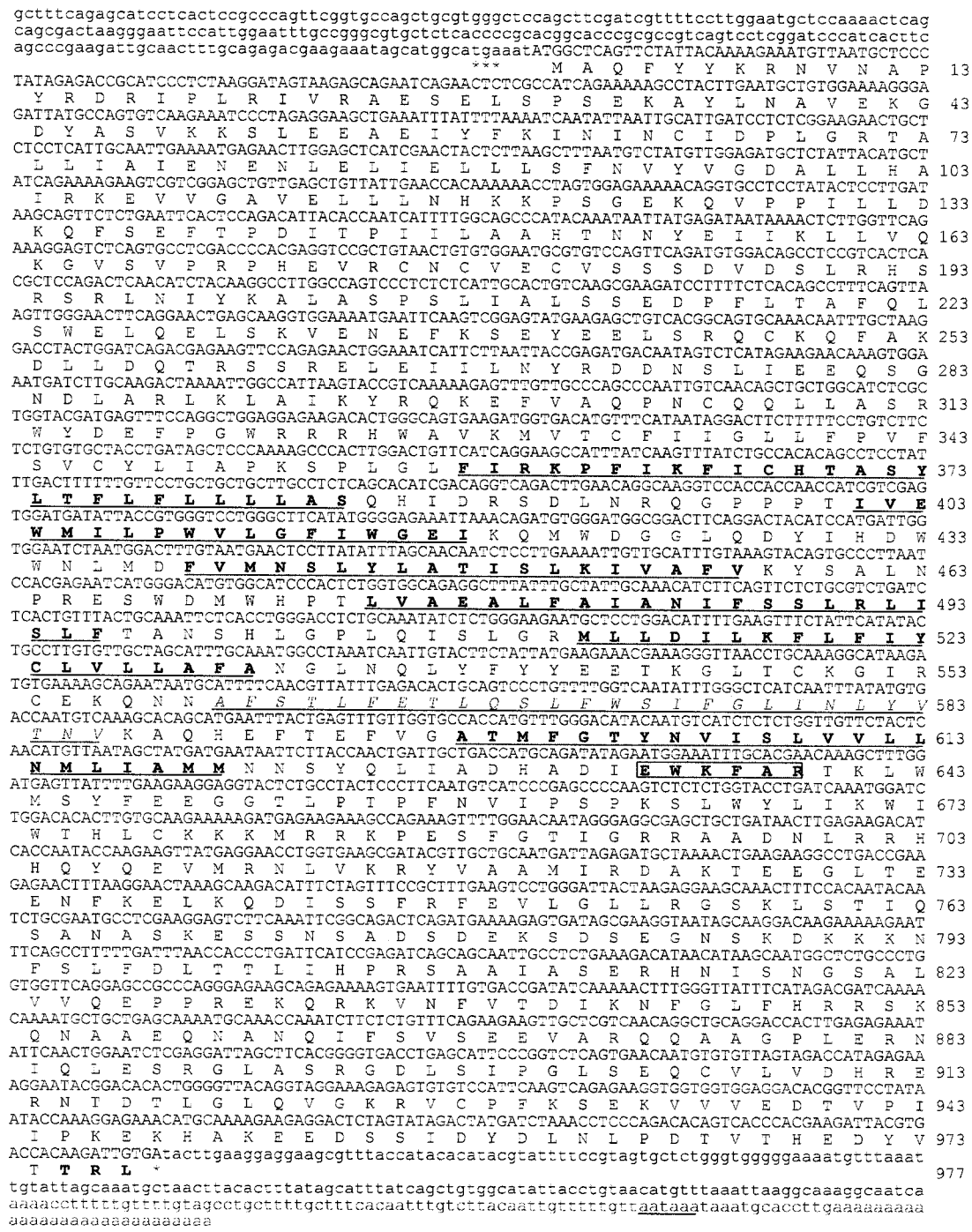

Figure 1 Sequence of full-length hTRP4 cDNA

The nucleotide sequence of the full-length cDNA of the hTRP4 gene along with the deduced amino acid sequence of the open reading frame are illustrated. The 3.4 kb cDNA, isolated from a human brain library, encodes a 977-amino-acid protein. Nucleotides are numbered starting at +1 for the A of the ATG codon encoding the initiator methionine. An in-frame upstream stop codon in the 5′ untranslated region is indicated by three asterisks and the polyadenylation signal in the 3′ untranslated region is underlined. Amino acids encoding six putative transmembrane (TM) regions are underlined and are in bold. The putative channel pore region between TM5 and TM6 is underlined and italicized. Boxed and in bold is a six-amino-acid motif after TM6, which is absolutely conserved in all members of the TRP family. A putative PDZ domain binding region at the extreme C-terminus is indicated in bold.

246 bp 3′ untranslated region, and a long polyadenylated tail (Figure 1). Conceptual translation of hTRP4 yields a 977-amino-acid protein with a calculated molecular mass of 112 kDa. hTRP4 exhibits 97.1 %, 97.1 % and 91.3 % amino acid identity over its entire length to bovine TRP4, mouse TRP4 and rat TRP4 respectively. Assignment of the pore region and membrane spanning regions (Figure 2B) was based on hydrophobicity analysis [23] of hTRP4 (Figure 2A) and the topology of hTRP3 proposed by Vannier et al. [24], although the accuracy of this model for TRP4 was not examined in the present study. Of

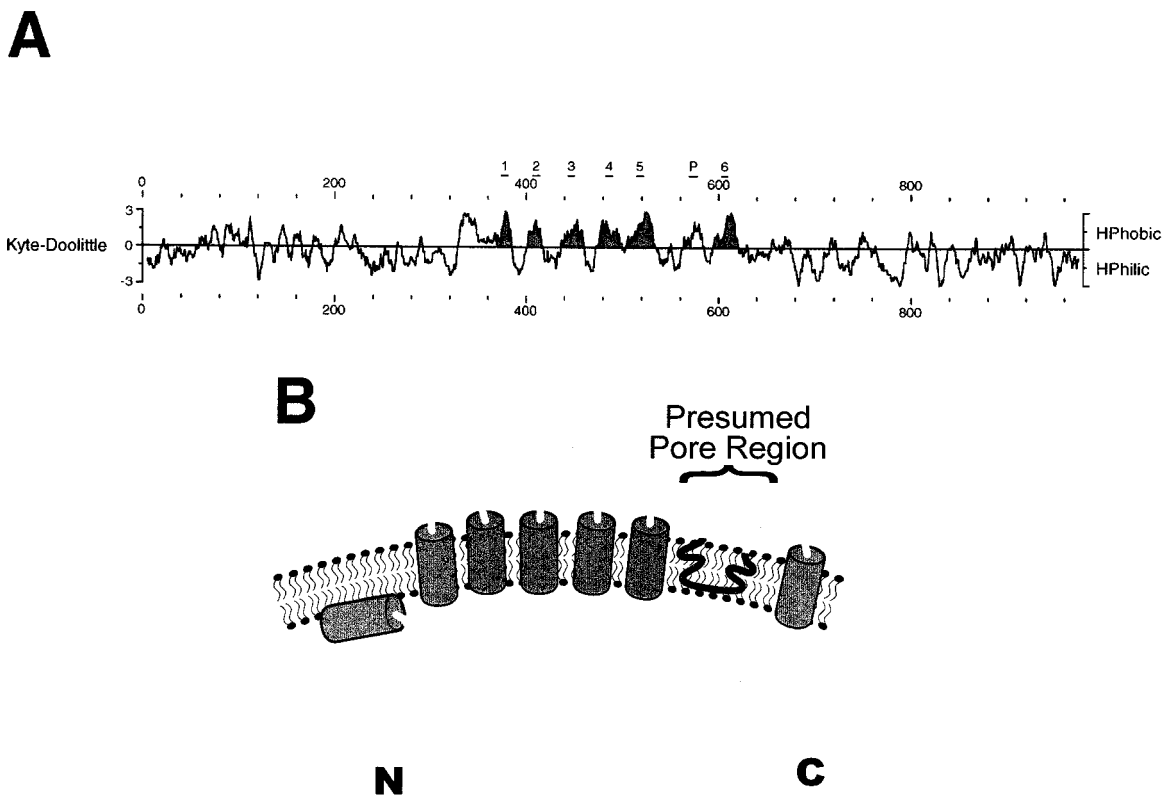

Figure 2 Putative structure of hTRP4

(**A**) Kyte–Doolittle hydrophobicity analysis of hTRP4. Putative transmembrane domains (1–6) are shaded. The putative pore region is also indicated (P). (**B**) Proposed structure and topology of the hTRP4 protein.

particular interest is the presence of a putative PDZ (an acronym of the three proteins PSD-95, Dlg and ZO-1) domain binding region at the extreme C-terminus of the protein (Figure 1A), which is present in all TRP4 and TRP5 sequences known except for rat TRP4. The majority of the divergence between these species occurs in the putative C-terminal cytoplasmic tail, a region, which in the case of *Drosophila* TRP and transient receptor potential-like (TRPL), has been implicated in the regulation of channel activity [25].

Tissue distribution of hTRP4

Northern blot data (Figure 3) revealed the existence of three transcripts of hTRP4 of sizes 4.0 kb, 4.3 kb and 7.4 kb. All three transcripts were expressed in the heart. In the pancreas the two smaller bands were seen, while the 7.4 kb band appeared at the limit of detection. Only the 4.0 and 7.4 kb transcripts were detected in placenta at high levels, and in the brain and kidney in much lower abundance. No expression was detected by Northern blot analysis in lung, liver or skeletal-muscle.

As is often the case for ion channels, the hTRP4 message was expressed at low abundance, giving a generally weak signal. To assess more rigorously tissue-specific and developmental expression of this gene, a more sensitive PCR-based assay was applied. A human Rapid Scan Gene Expression Panel (OriGene Technologies) was screened using PCR, and the resulting products were detected by Southern blot analysis and quantified on a PhosphorImager (Figure 4). Twenty-four tissues of adult and foetal origin were screened over a three-log range of cDNA input concentrations. At the highest cDNA concentration ($100\times$), strong signals were obtained in several tissues including adrenal gland, prostate, heart and pancreas. hTRP4 was detected to a lesser extent in brain, kidney and placenta, among other tissues. Little or no detectable signal was obtained from liver, colon, lung, muscle and salivary gland. At the lowest cDNA concentration ($1\times$) an easily detectable signal was obtained in prostate, adrenal gland, heart, foetal brain and pancreas, indicating that these tissues express the highest levels of hTRP4. There is generally very good agreement between these data and the Northern blot data. However, while Northern blot results indicated high levels of expression of 4.0 and 7.4 kb messages in placenta, our Rapid-Scan data showed little hTRP4 expression in this tissue. Recently, splice variant forms of hTRP4 have been reported (GenBank® accession numbers AF063823, AF063824 and AF063825). While our Northern BLOT hybridization probe would be expected to detect all known forms of TRP4, our Rapid-Scan PCR primers would only amplify the long form presented herein and not the three splice variant forms which exist. Thus significant levels of expression of one or more of these splice variant forms in placenta would account for the apparent discrepancy.

Chromosomal localization of hTRP4

FISH was performed to determine the chromosomal localization of the hTRP4 gene. A fluorescein-labelled genomic BAC clone containing the hTRP4 gene (see the Materials and methods section) was hybridized to spreads of human chromosomes, in

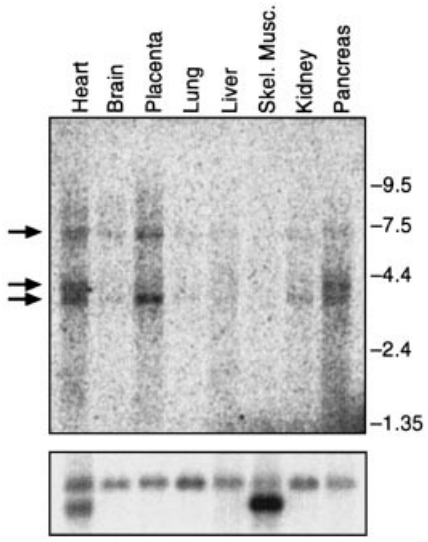

Figure 3 Northern blot analysis of hTRP4

Top panel: a human multiple tissue Northern blot was probed with a ^{32}P-labelled probe derived from the 3′ region of the hTRP4 coding sequence. Arrows indicate the position of the 7.4, 4.3 and 4.0 kb transcripts. Bottom panel: the blot was stripped and re-probed with a β-actin probe as a control for loading.

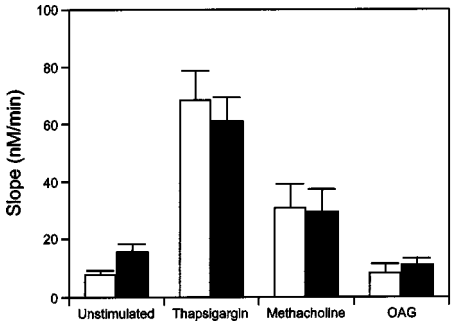

Figure 5 Barium influx in hTRP4-transfected HEK-293 cells

Barium influx was measured in fura 2-loaded HEK-293 cells transfected with either vector only (white bars) or hTRP4 cDNA (black bars). Influx was assessed in the absence of any stimulation or after stimulation of the cells with thapsigargin, methacholine or OAG. Data are presented as the initial rates of change of apparent calcium concentration. Data are shown as means ± S.E.M., and represent between three and six replicates for stimulated data and between 12 and 15 experiments for the unstimulated data (each replicate being a composite of multiple cells on a single coverslip).

order to map accurately the location of hTRP4. Initial experiments indicated that the gene was present as a single locus in the genome on chromosome 13. Further double-labelling experiments using a labelled chromosome 13 centromere-specific probe indicated that hTRP4 localized to the 13q13.1–13.2 region of the chromosome. No known disease locus currently co-localizes with this gene and in fact no member of the TRP gene family has as yet been firmly associated with any known human disease.

RT-PCR detection of TRP transcripts in HEK-293 cells

Prior to evaluation of the behaviour of hTRP4 and hTRP3 expressed in HEK-293 cells, we determined which of several TRP mRNAs are naturally expressed in these cells. RT-PCR experiments were performed several times with cDNA prepared from different preparations of total HEK-293 RNA as template and primer sets specific for each of five hTRP transcripts. hTRP1, hTRP3, hTRP4 and hTRP6 mRNAs were detected, whereas the mRNA of hTRP5 was either not detected or, in some experiments, was barely detectable. With the primer sets used in the present study, hTRP5 mRNA was readily detected in human brain, a tissue in which TRP5 has been recently shown to be abundantly expressed [19,26]. Our findings regarding the mRNA distribution of the hTRP isoforms confirm previous data obtained in HEK-293 cells [27–29].

Expression of hTRP4 and hTRP3

To assess the activity of hTRP3 and hTRP4 we employed a transient transfection assay. Either hTRP3 or hTRP4 was transfected into HEK-293 cells along with a construct encoding GFP as a marker for transfection. Preliminary experiments examining co-transfection of GFP and a blue fluorescent protein indicated a co-transfection approaching 100 % with this technique. Barium entry was assessed in GFP-positive cells either in

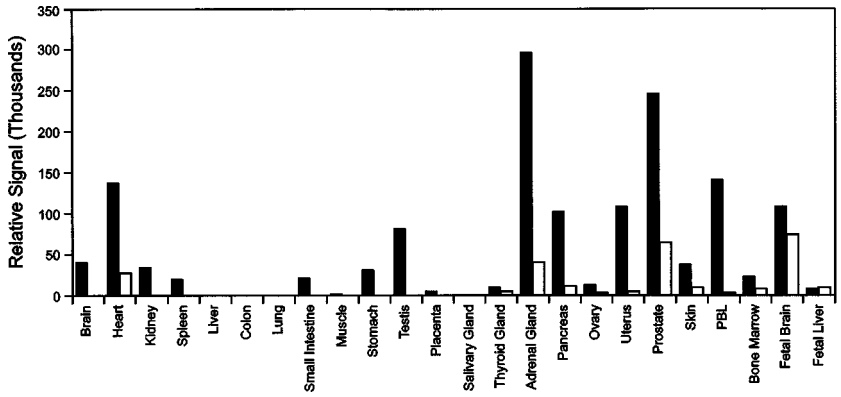

Figure 4 RT-PCR analysis of hTRP4 tissue-specific expression

PCR was used to screen a Rapid-Scan Panel of human cDNA from various tissues (OriGene Technologies) utilizing hTRP4-specific oligonucleotide primers. The plot shows the hybridization signal obtained when relative concentrations of 1 × (white bars) and 100 × (black bars) cDNA were used as templates in the PCR reaction.

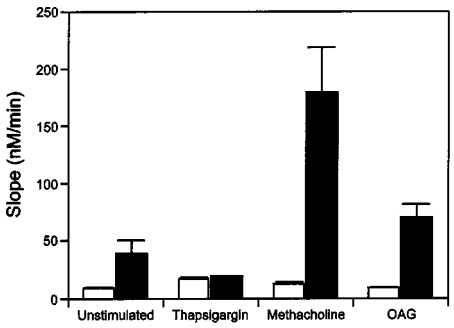

Figure 6 Barium influx in hTRP3-transfected HEK-293 cells

Barium influx was measured in fura 2-loaded HEK-293 cells transfected with either vector only (white bars) or hTRP3 cDNA (black bars). Influx was assessed in the absence of any stimulation or after stimulation of the cells with thapsigargin, methacholine or OAG. Data are presented as the initial rates of change of apparent calcium concentration. Data are shown as means ± S.E.M., and represent three to five replicates (each replicate being a composite of multiple cells on a single coverslip) for all data sets.

the unstimulated state, or after stimulation with thapsigargin, methacholine or OAG. Rises in intracellular calcium due to activation of calcium influx reflect a composite of the influx mechanisms mediated by plasma-membrane channels as well as the efflux and sequestering mechanisms mediated by plasma-membrane calcium ATPases and endoplasmic reticulum calcium ATPases respectively. As barium is a poor substrate for these ATPases [30], but is readily passed by most calcium permeant channels, including both TRP3 [12] and TRP4 [5], we used barium as a surrogate ion for calcium.

Transient expression of hTRP4 in HEK-293 cells led to an approximate doubling of basal barium permeability (15.54 ± 3.02 nM/min for hTRP4-expressing cells compared with 7.74 ± 1.32 nM/min for control cells; see Figure 5). This is presumably due to constitutive, unregulated activity of these channels when they are transiently overexpressed. This result has been seen in similar experiments with other members of the TRP family when expressed either transiently (see Figure 6) or stably [6,10,12,31]. We next assessed the ability of various agents to activate hTRP4. When challenged with the store-depleting agent thapsigargin, we saw a stimulation of barium influx into both control and hTRP4-transfected cells, presumably due to activation of endogenous store-operated channels. However, the extent of stimulation was similar in both cell types (Figure 5). Thus the presence of exogenous hTRP4 did not augment the store-operated influx in these cells. To investigate whether hTRP4 was receptor-operated, the PLC-linked muscarinic receptor agonist methacholine was employed. Methacholine elicited an increase in barium influx, but there was no significant difference between control and hTRP4 cells (Figure 5). Taken together these results are unexpected given the results obtained for bovine, rat and mouse TRP4. This finding represents the first TRP4 isoform that fails to be activated by either store depletion or receptor activation. When OAG, which has been shown to activate some members of the TRP family, was applied, we observed no OAG-stimulated hTRP4 activity. However, unlike the case for thapsigargin or methacholine, we saw no evidence of an endogenous OAG-activated channel. Barium influx after OAG stimulation was similar to the basal barium influx rates, indicating that such an activity was not normally present in these cells (Figure 5).

When we evaluated the effects of overexpression of another well-studied TRP family member, hTRP3, we were able to obtain activation of hTRP3 channels (Figure 6). In agreement with previously published data on stably expressed hTRP3 [12], we observed a significantly increased basal rate of barium influx in hTRP3-transfected cells as compared with control cells. In response to stimulation with methacholine, the hTRP3 cells exhibited a very high barium influx compared with control cells (Figure 6). This increase was much greater than that observed in the unstimulated state, indicating that only a minor portion of this difference was due to the higher basal influx in hTRP3 cells. Upon thapsigargin stimulation, barium influx in the hTRP3 cells did not differ significantly from that in control cells. hTRP3 cells were also activated by OAG, but not to the same extent observed with a maximal dose of agonist (Figure 6). We conclude that, in this transient expression assay, hTRP3 does not function as a store-operated channel, but can be activated by receptor activation and by OAG.

While our functional expression results for hTRP3 were in agreement with published data from some laboratories, our hTRP4 data differed significantly from other published work on this channel. Thus we wished to confirm that as a result of transient transfection of hTRP4 cDNA, significant quantities of full-length hTRP4 protein were expressed. Unfortunately, to date our attempts to generate peptide-directed hTRP3 or hTRP4 antibodies have been unsuccessful. Thus we performed an immunoblot on cells transfected with either vector DNA as a control, or a construct encoding HA-hTRP4. The overexpressed hTRP4 protein, of the predicted mass (approx. 115 kDa), was readily detected in cells 48 h after transfection by using an anti-HA antibody (results not shown).

Localization of hTRP4 and hTRP3 proteins

Correct functioning of an ion channel protein is not only a function of expression of adequate quantities of the protein, but is crucially dependent upon its correct localization. In order to assess subcellular localization and targeting of hTRP4 *in vivo*, we performed confocal fluorescence microscopy on cells transiently transfected with a GFP–hTRP4 fusion protein. Localization of hTRP4 was compared with that of a hTRP3–GFP fusion protein. Confocal images were taken 48–72 h post-transfection, a time-frame consistent with that used in both the hTRP4 and hTRP3 functional assays (Figure 5 and 6) and the HA-hTRP4 immunoblot. Figure 7(A) shows a confocal image of a HEK-293 cell transiently transfected with the GFP–hTRP4 fusion protein. The protein was mainly localized at or near the plasma-membrane, consistent with hTRP4 having a role as an ion channel. There was also considerable staining at an internal site in a region where the Golgi apparatus is expected to be located. This internal cellular staining pattern was similar to that obtained when HEK-293 cells were transiently transfected with a Golgi-targeted yellow fluoescent protein (results not shown). Of note is that the staining at the plasma membrane was not uniform, but appeared in a punctate pattern. In addition to the apparent plasma-membrane staining, there was punctate staining in the cytoplasm as well. Figure 7(B) shows a similar confocal image for a cell transiently transfected with a hTRP3–GFP fusion construct. The pattern of staining was similar to that obtained with hTRP4.

To demonstrate that a significant portion of the punctate TRP staining occurred at or near the plasma membrane, triple-labelling confocal experiments were performed. Cells transiently transfected with either GFP–hTRP4 (Figure 7C) or hTRP3–GFP (Figure 7D) were labelled with DAPI to stain the nucleus (blue) and were immunocytochemically stained with an anti-β1 integrin antibody as a marker for the plasma-membrane. Figures 7(C)

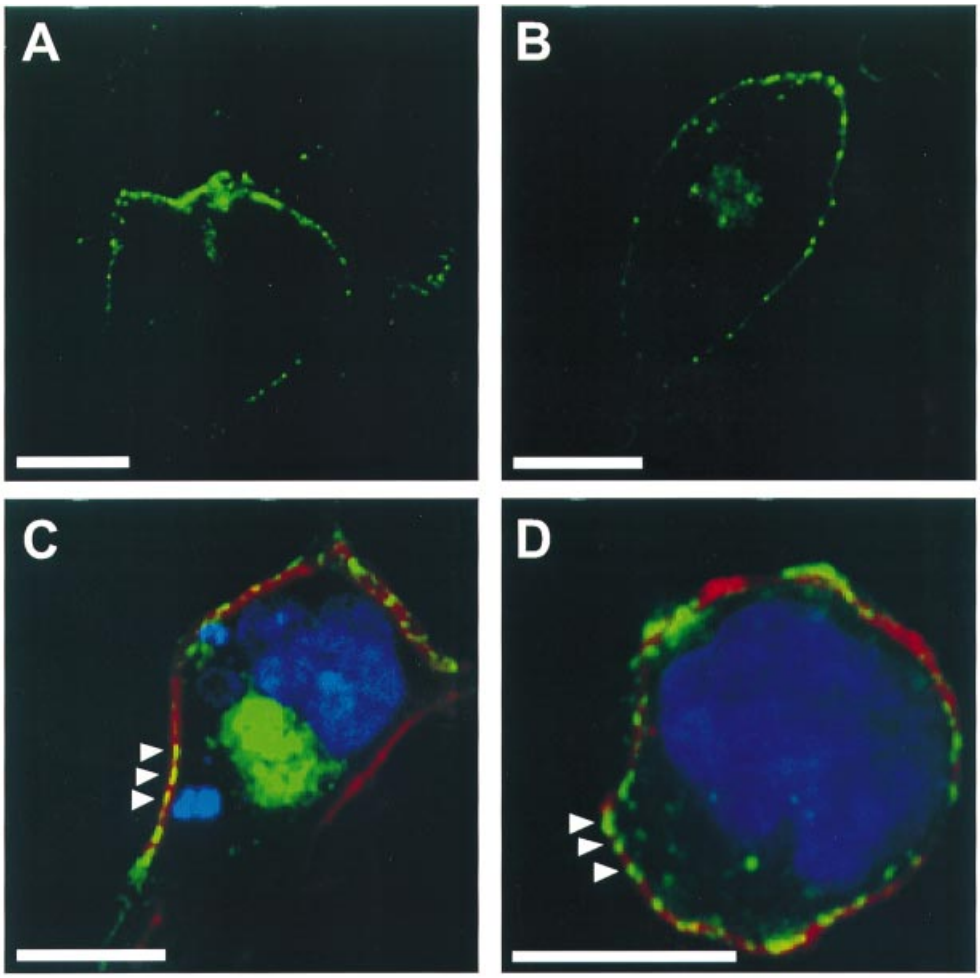

Figure 7 Localization of GFP–hTRP4 and hTRP3–GFP fusion proteins

Confocal microscopy was performed on HEK-293 cells transiently transfected with (**A**) a hTRP3–GFP fusion protein construct or (**B**) a GFP–hTRP4 fusion protein construct. (**C** and **D**) Triple-labelling of the cells with DAPI to stain the nucleus (blue), immunocytochemistry with antibody to β-integrin as a marker for the plasma-membrane (red), and either (**C**) hTRP3–GFP or (**D**) GFP–hTRP4 (green). Co-localization of hTRP3 or hTRP4 and β-integrin appears yellow. Bar = 10 μm.

and 7(D) show considerable co-localization of both hTRP4 and hTRP3 with β-integrin, indicating that a significant portion of the TRP expression is at the plasma-membrane. For both TRPs however, additional internal staining could be seen as a punctate staining at regions in the cytoplasm underneath the plasma membrane.

Whole-cell patch-clamp analysis of hTRP4 currents in CHO cells

The characteristics of the constitutive activity of hTRP4 were further investigated by using the whole-cell patch-clamp technique. The small constitutive activity observed in HEK-293 cells did not result in a reproducibly discernable increase in basal whole-cell current (results not shown). However, in CHO cells transfected with the GFP–hTRP4 fusion construct, substantial constitutively active whole-cell currents were observed. Control cells exhibited cation current densities between 0 and 2 pA/pF (mean $\pm$ S.E.M. was -0.5 ± 0.2 pA/pF; see Figure 8A). In contrast, 14 out of 16 hTRP4 cells had currents that exceeded the control range, with a median of -4.5 pA/pF and reaching -18.5 pA/pF in one cell (mean $\pm$ S.E.M. was -6.5 ± 1.5 pA/pF; $P < 0.0001$). The currents were present immediately after obtaining the whole-cell configuration (Figure 8B) and remained at a stable plateau for 1–5 min. They had an almost linear current–voltage relation (Figure 8C), with a reversal potential close to 0 mV (range from $+3$ to -10 mV). Similar results were obtained when current measurements were made on cells transfected with hTRP4 that was not fused to GFP, but was co-transfected with GFP (four out of seven GFP-positive cells showed currents; results not shown).

Inward currents were inhibited by substitution of Na^+ in the bath with NMDG (see Figure 8B). NMDG left the outward current unchanged, indicating that it acted as an impermeant ion that did not pass through the pore and did not block efflux of ions (Figure 8C, trace 1). Addition of Ca^{2+} (5 mM) to an NMDG bath produced a small inward current (Figure 8B). From the difference of the reversal potentials in a bath with 140 mM Na^+ and in a bath with 5 mM Ca^{2+}, a permeability ratio, p^{Na}/p^{Ca}, of 0.9 was calculated. From the reversal potential in the standard bath solution a permeability ratio of 0.8 was calculated. Addition of 10 μM Gd^{3+} to the bath completely inhibited hTRP4 currents ($n = 4$; results not shown). Outward and inward currents were

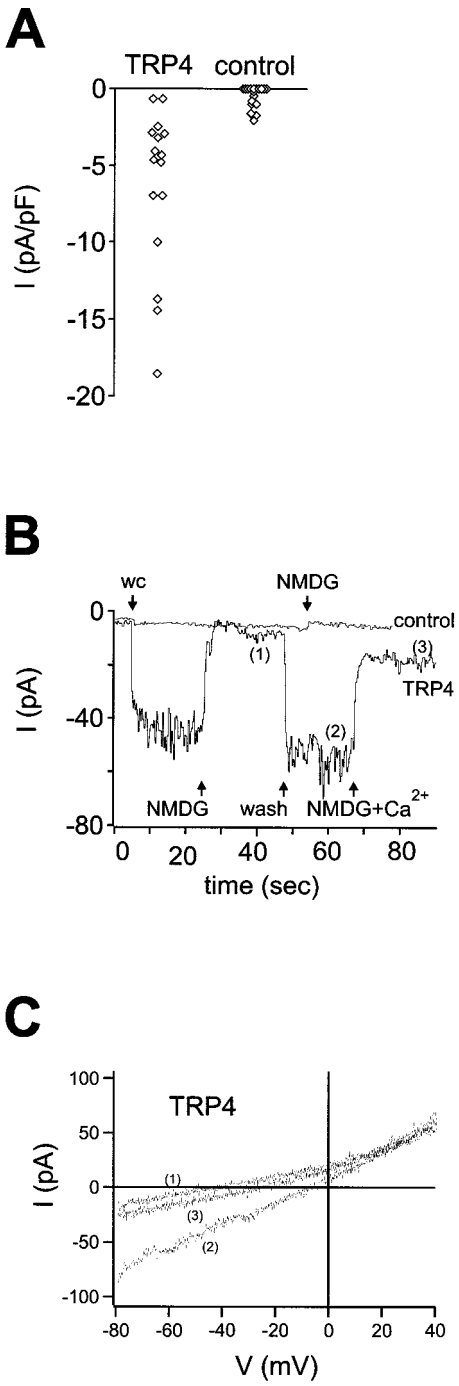

Figure 8 hTRP4-associated whole-cell currents in CHO cells

(**A**) Effects of expression of hTRP4 on cation currents in CHO cells. Currents are expressed as the NMDG-inhibitable part of inward currents at a holding potential of −60 mV, and are normalized to the cell capacitance. Data on the left represent currents in cells transfected with GFP–hTRP4; data on the right are from control cells (transfected with pEGFP). (**B**) Effects of expression of hTRP4 on Na^+ and Ca^{2+} currents (original current traces from one control cell and one cell transfected with GFP–hTRP4). Currents were recorded after obtaining the whole-cell (wc) configuration at −60 mV. The cells were first exposed to a bath containing 140 mM Na^+ and 1.2 mM Ca^{2+} and then to a bath in which Na^+ was substituted with NMDG. The hTRP4-expressing cell was further exposed to a bath with NMDG and 5 mM Ca^{2+} (but no Na^+). Numbers in parentheses indicate the time at which the current–voltage relations shown in (**C**) were recorded. (**C**) Current–voltage relation of Na^+ and Ca^{2+} currents in a GFP–hTRP4-expressing cell. The relations were obtained during the experiment shown in (**B**) by application of voltage ramps in the presence of three different bath solutions: (1) 150 mM NMDG and 1.2 mM Ca^{2+}; (2) 140 mM Na^+; (3) 150 mM NMDG and 5 mM Ca^{2+}.

equally affected, and this block was completely reversible after wash out.

DISCUSSION

In the present study we present the full-length cloning, localization and expression of the human homologue of the TRP4 gene. We have also assessed the activity of expressed hTRP4, as well as that of another, well-studied member of the TRP family, hTRP3.

The predicted hTRP4 protein has essentially the same overall structure as that predicted for other TRP family members, including six putative transmembrane segments, and cytoplasmic N- and C-termini. The protein exhibits extremely high sequence similarity to previously cloned rat, bovine and mouse TRP4 homologues. Because of this striking similarity, we investigated whether hTRP4 is functionally similar to any of these isoforms, which have been found to be either store depletion activated calcium permeant channels or receptor-operated non-selective cation channels. Contrary to published findings with TRP4 from other species, we were unable to detect either activity. We were, however, able to detect a constitutive activity of hTRP4 both by fura 2 fluorescence measurements and by electrophysiology. Such an activity has been revealed for other TRPs, including hTRP3, whose activation is also examined in the present study. We have confirmed that the expression of a constitutive but unregulated hTRP4 channel is associated with the production of a full-length protein product, which appears to be properly localized to the plasma membrane in a manner similar to the regulated hTRP3 protein. Electrophysiological studies indicate that hTRP4 functions as a constitutively active non-selective cation channel.

Most investigators have found that TRP4 and TRP5 appear to be activated by similar mechanisms. However, the differential expression of these proteins suggests that they probably carry out distinct functions. Our results show that hTRP4 is highly expressed in tissues such as the placenta, heart and pancreas, and is most highly expressed in the adrenal gland and prostate. Bovine TRP4 was also found to be highly expressed in the adrenal gland. In contrast, mouse TRP5 and rabbit TRP5 have been found to be almost exclusively expressed in the brain [19,20]. Our data indicate that hTRP4 is expressed in adult brain only at low levels. Interestingly, we find that one of the tissues in which hTRP4 is most abundantly expressed is foetal brain. Thus there may be a developmental switch that occurs where brain-specific hTRP4 expression is down-regulated. It may be that the role played by hTRP4 in the foetal brain is assumed by TRP5 in the adult brain.

Two groups have found TRP4 or TRP5 proteins to function as store-operated channels [5,18,19], and two other groups have found them to be receptor-operated [20,21]. The HEK-293 cells used in the present study clearly express hTRP4 mRNA (our findings and [27,29]), and it is likely that the endogenous hTRP4 functions as a component of the store-operated calcium entry channel or of other ion-channel complexes. However, transiently expressed hTRP4 was found to be constitutively active, and we were unable to stimulate further activity by either store depletion or receptor activation. The mouse TRP4, which was shown to be receptor activated by Schaefer et al. [21], was a splice variant form of TRP4, which spliced out a region of the C-terminus. This region is present in the bovine TRP4, which Philipp et al. [5] found to be store operated, and this sequence is present in the hTRP4 described in the current study. While it is possible that this splicing event may be the source of the differences seen between bovine and mouse TRP4, it does not account for those

observed between human and bovine TRP4. The failure of exogenously expressed TRP proteins to mimic endogenous ion channel behaviours appears to be a common finding with members of the TRP family, as discussed below for hTRP3.

Immunoblotting experiments indicated that a protein of the expected length is expressed after transient transfection. Furthermore, we are confident that the hTRP4 protein expressed is capable of forming a channel, because we observed constitutive barium influx and a constitutive current in hTRP4-expressing cells. In addition, our ability to reproduce findings of others with hTRP3 (see below) serves as a positive control for the expression protocol. It is unlikely that we have obtained a mutant form of hTRP4 due to errors in library construction, because another full-length hTRP4 clone was obtained independently by another group (GenBank® accession number AF063822) which is 100 % identical with our clone. While hTRP4 is highly identical with TRP4 from the other species, it is possible that even a single amino acid change could affect its regulation or function, possibly by affecting its ability to interact with other proteins involved in the same signalling pathway.

When hTRP3 was overexpressed in a manner similar to hTRP4, we were able to detect several activities. hTRP3 contributed to a constitutive barium influx, indicating a degree of unregulated activity of that channel. We were also able to activate that channel further by application of the PLC-linked agonist methacholine or by application of OAG. However, treatment with thapsigargin, to deplete the intracellular calcium stores, did not activate hTRP3. This finding is consistent with some studies on cells stably transfected with hTRP3 [12,15]. It is also consistent with the conclusion that when exogenously expressed, TRP3 functions as an Ins(1,4,5)P_3-gated channel. As pointed out previously [12,27,29], and confirmed in the current work, HEK-293 cells express hTRP3 mRNA. Yet the endogenous store-operated channels and channels resulting from the overexpressed hTRP3 protein apparently do not behave in an identical manner. However, two reports from the same laboratory, also utilizing HEK-293 cells and this same construct, reported single channels attributable to hTRP3 that could be activated by store depletion as well [13,14]. The reason for this discrepancy is not known. It is possible that the number of hTRP3 channels functionally coupled to store depletion is relatively small, such that in assays of total cell current or flux, it is not statistically recognizable. Nonetheless, as pointed out previously [32], the failure of overexpressed hTRP3 to respond to store depletion may simply reflect an inability of the signalling complex to associate appropriately with endogenous PLC. This would then render the pathway dependent on receptor activation of PLC. In support of this interpretation, a recent report has shown that down-regulation of the hTRP3 message in HEK-293 cells by an antisense strategy diminished the activity of store-operated channels [29].

The constitutive cation influx we observed in hTRP4 cells in fura 2 experiments was further characterized electrophysiologically. In hTRP4-transfected cells, we observed non-selective cation currents with a reversal potential close to 0 mV in the presence of a Cl^- equilibrium potential of -31 mV. The inward component of this current was inhibited when Na^+ in the bath was substituted with the large impermeant cation NMDG. Under our experimental conditions, the current was carried mainly by Na^+ in the inward direction and by Cs^+ in the outward direction. In the absence of Na^+, hTRP4 was seen to pass Ca^{2+}. The ion selectivity of hTRP4 agrees more closely with that found for mouse TRP4 by Schaefer et al. [21], than that of bovine TRP4 [5], which was found to be highly calcium selective. Interestingly, the constitutive current density we observed for hTRP4 is similar to that reported for the structurally similar mouse TRP5 by Schaefer et al. [21], but in the latter study no constitutive activity was observed for mouse TRP4. In addition, Schaefer et al. [21] reported a rather unusual doubly rectifying current–voltage relationship for mouse TRP4 and TRP5, while in the present study a nearly linear relationship was observed.

To assess the localization of the putative ion-channel proteins, confocal microscopy on cells expressing GFP-tagged hTRPs was performed. Transfection with the hTRP3–GFP construct conferred upon the cells an OAG-activated barium influx (results not shown), indicating that hTRP3–GFP forms a functional channel, which can be activated similarly to the native channel. Our data suggest that hTRP4 and GFP–hTRP4 also form functional channels and, consistent with that idea, GFP-tagged hTRP3 and hTRP4 were localized similarly within the cell. In addition to their localization at the plasma membrane, both proteins were found in a punctate pattern in the cytoplasm, reminiscent of vesicularization. Whether this localization of TRPs is of physiological importance is unknown. It has been postulated that transport of vesicles containing store-operated channels to the cell surface after stimulation is a potential mechanism of activation [33].

The punctate localization pattern of the TRP proteins at the plasma-membrane may suggest a clustering of the channel molecules, as is achieved by caveolae or by scaffolding molecules, such as those containing multiple PDZ domains. This is consistent with the previous suggestion that hTRP3 may be associated with Ins(1,4,5)P_3 receptors and possibly with PLC in structured plasma-membrane domains [32]. Both caveolae and PDZ domain proteins function to cluster signalling molecules in a spatially confined region of the cell, in order to make signalling processes more rapid and efficient. Caveolae are compartments known to contain many different molecules involved in PLC mediated signalling, including plasma-membrane calcium pumps [34], heterotrimeric G-proteins [35], PtdIns(4,5)P_2 [36] and the Ins(1,4,5)P_3 receptor [37]. PDZ-domain-containing proteins serve as molecular scaffolds, binding multiple proteins involved in signalling processes. In *Drosophila* phototransduction, a multiple PDZ-domain-containing protein, INAD, functions to scaffold signalling molecules such as rhodopsin, PLC, protein kinase C and the TRP and TRPL channels [38–40]. In *inaD* mutants, visual signalling processes are disrupted, highlighting the importance of proper localization for function [41]. The punctate staining we observed may represent clustering of the TRP channel with caveolin, a PDZ-domain-containing protein, or other as yet unidentified molecules. Both hTRP3 and hTRP4 possess putative caveolin binding domains ($\Phi X \Phi XXXX \Phi$ or $\Phi XXXX \Phi XX \Phi$, where X = any amino acid and Φ = F,W or Y [42]), and hTRP4 contains a putative PDZ domain binding region at its C-terminus. It is interesting to note that other signalling proteins, such as the β2-adrenergic receptor, have been found to be enriched in caveolae [43] and also to have a functional PDZ domain binding region at the C-terminus [44].

We present evidence in the current study that hTRP4 functions differently from its highly similar mouse, rat and bovine homologues. Subtle differences between hTRP4 and the TRP4 from other species may affect its activity by altering its inherent channel properties or by affecting its interactions with other proteins. TRP channels have been shown to be able to associate with like molecules [45], other members of the TRP family [45], and channels outside of the TRP family, such as PKD2 [46]. TRP channels are thought to function as tetramers and as such, a variety of potential configurations of this complex are possible. As has been seen with other multi-subunit channel complexes, the composition of the complex can vastly alter the channel

properties [47,48]. Molecular differences which alter the oligomerization of TRP proteins into functional channels may drastically alter the observed properties of those channels. The subunit composition of the channels formed may also be affected by the level of overexpression of the TRP in question, as well as by the complement of TRP channels expressed endogenously in the host system. Our current findings do not provide for a means to evaluate these various possibilities, and we cannot definitively ascertain which of the various results, if any, represents the true behaviour of these channel molecules in their native environments. Given the disparate results obtained by overexpression of TRPs, we conclude that the physiological modes of regulation of TRP proteins may not always be revealed from their behaviour following exogenous overexpression. Rather, strategies which rely on the knockout of an endogenous TRP function would seem more likely to yield useful and interpretable findings, hopefully revealing the true physiological functions of these newly discovered channel proteins.

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