Cloning, expression and subcellular localization of two novel splice variants of mouse transient receptor potential channel 2

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Transient receptor potential channels (TRPCs) are known as candidate molecular correlates of receptor-activated or store-operated calcium entry. While functional roles for most TRPCs have been suggested, the physiological relevance of TRPC2 remains obscure. Whereas human and bovine TRPC2 are candidate pseudogenes, full-length rodent TRPC2 transcripts have been reported. There is, however, considerable controversy concerning mRNA splicing, tissue distribution and the function of these proteins. We report the molecular cloning of two novel murine TRPC2 splice variants, mTRPC2α and mTRPC2β. mTRPC2α RNA is expressed at low levels in many tissues and cell systems, while mTRPC2β is exclusively and abundantly expressed in the vomeronasal organ (VNO). When expressed in human embryonic kidney (HEK)-293 cells, mTRPC2 did not enhance receptor- or store-activated calcium entry. In order to investigate the basis of such a functional defect, mTRPC2–green fluorescent protein fusion proteins were examined by confocal microscopy. Fusion proteins were retained in endomembranes when expressed in HEK-293 or other cells of epithelial or neuronal origin. Co-expression of TRPC2 with other TRPCs did not restore plasma-membrane trafficking. We conclude that TRPC2 may form functional channels in the cellular context of the VNO, but is unlikely to have a physiological function in other tissues.

Key words: calcium, protein trafficking, receptor-stimulated channel, store-operated channel, vomeronasal organ.

INTRODUCTION

Receptor-activated and store-operated cation channels contribute to the temporo-spatial pattern of the intracellular free calcium concentration ($[Ca^{2+}]_i$) in response to stimulation of plasma membrane receptors. *Drosophila melanogaster* transient receptor potential (TRP) [1] and transient receptor potential-like (TRPL) [2] channels were identified as the ancestors of a gene family of potential receptor-activated cation channels in mammals, the TRPC (transient receptor potential channel) family. Diptera express TRP channels mainly in the eye. The physiological role of these proteins is to translate the signal generated by a light-activated receptor/G-protein/phospholipase C (PLC) signal-transduction cascade into a depolarizing receptor potential [3]. In contrast, most mammalian TRPCs are widely distributed throughout the organism, with no obvious preference for sensory tissues. TRPCs are discussed as candidate molecular correlates of store- [4–7] or, more likely, receptor- and second-messenger-activated cation channels, which are activated by signalling cascades initiated by membrane receptors [8]. TRPC4 and TRPC5 form a subfamily within the TRPCs and were initially characterized as store-operated channels [5,6]. Recently, evidence for non-capacitative modes of activation was provided [9,10]. Another subfamily is made up of TRPC3, TRPC6 and TRPC7. Whereas it was proposed that these channels are regulated by a store-dependent mechanism by directly interacting with the Ins(1,4,5)P_3 receptor under low expression conditions [7], evidence is accumulating that demonstrates a store-independent receptor-mediated activation of this TRPC subfamily [11–13]. The membrane-confined lipid second messenger diacylglycerol has been shown to link PLC activity to the activation of TRPC3, TRPC6 and TRPC7 [14,15].

Tissue distribution, mode of activation, and the physiological role of TRPC2, however, have not yet been clarified. TRPC2 is a pseudogene in man [16] and a potential pseudogene coding for an N-terminally truncated protein in the bovine system [17]. Full-length transcripts were found in mouse [18] and rat [19]. Two splice variants of mouse TRPC2 mRNA (mTRPC2A and mTRPC2B), both of which are considerably longer than rat TRPC2 and any other member of the TRPC family, were reported to be mainly expressed in testis. Mouse TRPC2 has been proposed to augment capacitative calcium entry in COS-M6 cells [18]. In rat, TRPC2 mRNA was shown to be confined to the vomeronasal organ (VNO) [19], a chemosensory apparatus involved in pheromone perception.

In the present study we report the molecular cloning of two novel splice variants of mouse TRPC2, mTRPC2α and mTRPC2β. One of these mRNAs is specific for the VNO; the other is ubiquitously expressed, albeit in very low amounts. We provide evidence that mouse TRPC2 is not involved in receptor- or store-mediated cation entry in cells of epithelial and neuronal origin. We explain this defect by impaired trafficking of mTRPC2 to the plasma membrane in these cells.

EXPERIMENTAL

Molecular cloning

Mouse testis total RNA was reverse transcribed using an oligo-d(T) primer and SuperScript reverse transcriptase (Gibco BRL Life Technologies, Eggenstein, Germany). Using a degenerate

Abbreviations used: $[Ca^{2+}]_i$, intracellular free calcium concentration; (e)CFP, (enhanced) cyan fluorescent protein; (e)GFP, (enhanced) green fluorescent protein; HA, haemagglutinin; HEK, human embryonic kidney; $NMDG^+$, *N*-methyl-D-gluconate; PLC, phospholipase C; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TRP, transient receptor potential; TRPL, transient receptor potential-like; TRPC, TRP channel; VNO, vomeronasal organ.

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The sequences of mTRPC2α and mTRPC2β have been submitted to GenBank under the accession numbers AF230802 and AF230803 respectively.

primer set (NSK-FW, 5′-TKGGYCCMYTRCAGATYTC-3′; NSK-BW, 5′-GMWCGAGCAAACTTCCATTC-3′), we amplified a 360-bp fragment of mTRPC2 coding for the C-terminal two putative transmembrane segments. The 3′-terminus was amplified by 3′- rapid amplification of cDNA ends (RACE) after reverse transcription with a 3′-anchor primer and two PCR amplification steps using nested forward primers (5′-CATCC-TGACTGCCTTCCTCTGTGG-3′ and 5′-TGCCATGATCAC-CAACTCCTTCCAG-3′) together with nested 3′-anchor primers. The 5′-terminus was cloned in two subsequent steps of 5′-RACE using a 5′-RACE kit (Gibco BRL Life Technologies). In the first step, reverse transcription was initiated with the primer m2RT1 (5′-TGGTGATCATGGCAATAAG-3′). The resulting first-strand cDNA was purified and elongated by a poly(dC) stretch using terminal deoxynucleotidyltransferase. Amplification of the RACE product was carried out in two subsequent steps using nested primer pairs with the specific reverse primers m2bw1 (5′-GAACTGAGGCATGTCCACCA-CTGT-3′) and m2bw2 (5′-TGCCGAACATGGTCCAAAAGA-GAAA-3′), and the two anchor primers provided by the supplier. For the second round of RACE, the reverse transcriptase (RT) primer was m2RT2 (5′-GGCAATCTCATACAGGTC-3′), the mTRPC2-specific reverse primers used for PCR amplification were m2bw3 (5′-GGCAAAGCGGGAGCCATCAATC-3′) and m2bw4 (5′-CACTGCTGGCTGGTTTGTGTCC-3′). Finally the entire coding sequence was amplified and verified by sequencing of multiple independent amplification products. mTRPC2 was inserted into the eukaryotic expression vector pcDNA3, and splice variant-specific 5′-termini obtained from different 5′-RACE reactions were attached to the core sequence using an internal *Nhe*I site. The 5′ non-coding region of mTRPC2α was removed. Amplification of the 5′ non-coding region of mTRPC2A was carried out using the primers m2bw4 and TRPC2A-fw (5′-GCCACCATGCTAATGTCCCGCACTGA-3′). Using sequence stretches taken from different amplification products, we reconstructed the N-terminus of mTRPC2A (GenBank accession number AF111108) using *Bsm*BI and a *Sal*I sites which were introduced by silent mutagenesis.

C-terminal green fluorescent protein (GFP) chimaeras were generated using a pcDNA3-based C-terminal enhanced GFP (eGFP) fusion vector. The stop codon and the last 7 amino acids of mTRPC2 were removed using *Bgl*II. The resulting product was inserted into the *Bam*H1-cut cloning cassette of the vector. The cDNA of hTRPC6 was eGFP-tagged in a similar manner. The last 9 amino acids were removed using *Nco*1, and eGFP was fused to the C-terminal end [8]. For immunocytochemistry of mTRPC2, the haemagglutinin (HA) tag [(M)YPYDVPDYA] was fused to either the N-terminus or the C-terminus of mTRPC2β using a PCR-based approach. The targeting-deficient hTRPC6 mutant used for co-targeting studies was generated by exchanging amino acids 383–435 for the corresponding residues (amino acids 310–362) of *Drosophila* TRP using a PCR-based approach. All constructs were confirmed by cDNA sequencing.

Northern blotting

A mouse multiple-tissue Northern blot (ClonTech Laboratories, Heidelberg, Germany) was probed with nucleotides 1101–2203 of mTRPC2 that were randomly labelled with [α-^{32}P]dATP (Megaprime Kit; Amersham, Braunschweig, Germany). The specific radioactivity of the probe was 3.6×10^9 c.p.m./μg. The blot was prehybridized in ExpressHyb solution (ClonTech Laboratories) at 68 °C for 1 h and hybridized for 1 h under the same conditions. The final out of three washing steps was carried out in 0.1 × SSC (where 1 × SSC corresponds to 0.15 M NaCl/0.015 M sodium citrate) and 0.1 % SDS at 55 °C for 15 min. The blot was exposed to a PhosphoImager plate (Fuji) for two weeks and was re-probed with a β-actin probe under identical hybridization conditions (specific radioactivity 3×10^8 c.p.m./μg; overnight exposure). An additional Northern blot was prepared by electrophoresis of 30 μg of total RNA in a denaturing gel followed by capillary transfer on to a nylon membrane (Schleicher & Schuell, Dassel, Germany), which was subsequently baked at 80 °C for 2 h. The blot was prehybridized at 65 °C for 2 h in 0.5 M Na_2HPO_4 and 3 % SDS, pH 7.0, followed by an overnight hybridization under the same conditions using a probe with a specific radioactivity of 2×10^9 c.p.m./μg prepared from the same template as described above. Final washing steps were the same as described above. Autoradiography was carried out for 18 h.

Cell culture and transfection

Human embryonic kidney (HEK)-293 cells were cultured in minimal essential medium with Earle's salts, supplemented with 10 % fetal bovine serum. For morphological studies, cells were allowed to adhere to glass coverslips. For transfections, we used 4 μl of FuGene6 transfection reagent (Boehringer Mannheim, Mannheim, Germany) and 3 μg of DNA per 20-mm dish. When no other fluorescent protein was transfected, we added 100 ng of either peGFP-C1 encoding enhanced GFP (ClonTech Laboratories) or peCFP-C1 (encoding enhanced cyan fluorescent protein; eCFP) to allow detection of transfected cells. When two populations of differently transfected cells were examined on the same coverslip, cells were washed twice in PBS, harvested, counted and re-plated 1 day before measurement. Equal numbers of cells from each population were mixed, seeded on the same glass coverslip and used for analysis the following day. Cells transfected with different plasmids could be distinguished by virtue of the spectral differences of co-expressed GFP or CFP.

Assessment of $[Ca^{2+}]_i$ and Mn^{2+} influx

Measurements of $[Ca^{2+}]_i$ were performed as described previously [20]. Briefly, cells were loaded in Hepes-buffered saline (HBS: 140 mM NaCl, 6 mM KCl, 1.25 mM $MgCl_2$, 1.25 mM $CaCl_2$, 10 mM Hepes, 5 mM glucose and 0.1 % BSA, pH 7.4) with fura 2 acetoxymethyl ester (5 μM) at 37 °C for 30 min. After an additional 15-min incubation at room temperature, cells were rinsed with HBS and used for experiments within 2 h. Experiments were carried out using a Polychrome II monochromator and an IMAGO CCD camera (Till Photonics, Martinsried, Germany) coupled to an inverted Zeiss Axiovert 100 microscope. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. [21]. In Mn^{2+}-quench experiments, fluorescence signals at the isosbestic point (358 nm in our system) were expressed as the percentage of the initial value. Each experiment was performed at least three times with similar results. When statistical analysis was intended, the two populations of cells were assayed on the same coverslip in order to minimize systematic bias. Data sets were examined for similar variance and normal distribution (F-test; David, Pearson and Stephens test), and wherever feasible, statistical significance was determined using Student's *t* test (Figure 4d; m2β and m2A). In all other instances, an unpaired Wilcoxon test was applied.

Electrophysiology

Whole-cell patch clamp experiments were carried out essentially as described previously [14]. The external solutions were E1 (120 mM NaCl, 5 mM CsCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM Hepes, pH 7.4) and E2 (120 mM *N*-methyl-

D-glucamine chloride, 10 mM glucose and 10 mM Hepes, pH 7.4), the pipette solution contained 120 mM CsCl, 1 mM $MgCl_2$, 4.4 mM $CaCl_2$, 10 mM EGTA and 10 mM Hepes, pH 7.4. All solutions were adjusted to 290–310 mosmol with mannitol. The series resistance ranged between 5 and 10 MΩ and was not compensated. All records were filtered at 1 kHz using a 4-pole Bessel filter and were sampled at 3.3 kHz. The holding potential was −60 mV with voltage ramps from −80 to 80 mV being applied at 5-s intervals.

Immunocytochemistry

HEK-293 cells expressing either wild-type hTRPC6 or mTRPC2β cDNA constructs that were N-terminally (HA-mTRPC2) or C-terminally (mTRPC2-HA) fused with the HA tag were fixed in PBS containing 4% (v/v) formaldehyde. Cells were permeabilized in PBS containing 0.1% Triton X-100 (PBST), and were blocked for 1 h in Dulbecco's modified essential medium supplemented with 10% (v/v) fetal bovine serum. For the detection of hTRPC6, a rabbit polyclonal antiserum raised against the C-terminal peptide VGHNKQPSIRSSEDFHLNSFNNPP (amino acids 829–852 of hTRPC6) was used at a 1:1000 dilution. In the case of mTRPC2, 10 μg/ml of a mouse monoclonal antibody against the HA tag (Sigma, 12CA5) was used. The secondary antibody used was either a FITC-conjugated anti-rabbit-IgG or a FITC-conjugated anti-mouse IgG (Sigma), respectively. Each antibody was incubated for 1 h at 37 °C, followed by a 30-min washing step. All incubations were carried out in PBST. After staining, cells were analysed using confocal microscopy.

Immunofluorescent samples or living cells grown and transfected on glass coverslips were mounted on to the stage of a LSM 510 laser scanning microscope (Zeiss). For the detection of eGFP or FITC fluorescence, the sample was excited by the 488 nm line of an argon laser applied through a 488-nm beam-splitter. Emitted light was filtered through a 505-nm long-pass filter. Pinhole settings were adjusted to allow sections thinner than 0.8 μm to be used.

RESULTS

Cloning of mTRPC2

Using degenerate PCR primers, derived from the sequences of human TRPC1 and TRPC3, a 360 bp cDNA fragment was amplified from mouse testis RNA and subsequently elongated by RACE in both directions. The transcript with the longest open reading frame in testis, designated mTRPC2α (Figure 1), encoded an 886-amino-acid-protein which contained all the structural

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mTRPC2α                                                  MPQPNWTEIVNKKL
mTRPC2β                                                MDPLSPQPNWTEIVNKKL
rTRPC2                                                 MDPLS--PNWTEIVNKKL
mTRPC2A  250 aa ...CGESPPPQPASPASLSSSESVLRRHHVALTPVPLVPKPQPNWTEIVNKKL

mTRPC2α  KFPPTLLRAIQEGQLGLVQQLLESSSDASGAGPGGPLRNVEESEDRSWREALNLAIRLGH
mTRPC2β  KFPPTLLRAIQEGQLGLVQQLLESSSDASGAGPGGPLRNVEESEDRSWREALNLAIRLGH
rTRPC2   KFPPTLLRAIQEGQLGLVQQLLESGSDPSGAGPGGPLRNVEESEDRSWREALNLAIRLGH
mTRPC2A  KFPPTLLRAIQEGQLGLVQQLLESSSDASGAGPGGPLRNVEESEDRSWREALNLAIRLGH

mTRPC2α  EVITDVLLANVKFDFRQIHEALLVAVDTNQPAVVRRLLARLEREKGRKVDTKSFSLA...
mTRPC2β  EVITDVLLANVKFDFRQIHEALLVAVDTNQPAVVRRLLARLEREKGRKVDTKSFSLA...
rTRPC2   EVITDVLLANVKFDFRQIHEALLVAVDTNQPAVVRRLL-RLEREKGRKVDTKSFSLA...
mTRPC2A  EVITDVLLANVKFDFRQIHEALLVAVDTNQPAVVRRLLARLEREKGRKVDTKSFSLA...
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Figure 1 Differential splicing of mTRPC2

N-terminal amino acid sequences of mTRPC2α, mTRPC2β, rTRPC2 and mTRPC2A were aligned using the Clustal algorithm.

hallmarks and the putative membrane topology [22] of the TRPC family: an intracellular N-terminus with ankyrin repeats, eight hydrophobic segments that constitute six transmembrane segments and a pore loop, and a putative intracellular C-terminus. It shared less than 20–30% similarity with other known TRPC proteins, including *Drosophila* TRP and TRPL, and 12% similarity with the rat vanilloid receptor rVR-1. When this work was in preparation, two splice variants of mTRPC2 were reported, mTRPC2A and mTRPC2B (GenBank accession numbers AF111108 and AF111107; see Figure 1). These sequences share the entire sequence with mTRPC2α except the first residue. The N-termini, however, are 286 (mTRPC2A) or 186 (mTRPC2B) amino acids longer than mTRPC2α, and show no obvious sequence similarity to other members of the TRPC family in these regions (Figure 1). The N-terminus of mTRPC2B corresponds to mTRPC2A with the exception of the translation start site, which is shifted in the 3′ direction due to an intervening putative exonic fragment [18]. Using oligonucleotides specific for the 5′-terminus of mTRPC2A, RT-PCR was performed with mouse testis and cerebellum RNA as templates. PCR products of the expected length were subcloned and sequenced. Two out of four amplification products from testis and three out of four clones from cerebellum corresponded to mTRPC2α in terms of the translation start site, the remaining clones containing even shorter reading frames. The mTRPC2α amplification products differed from each other in the composition of the long 5′ non-coding regions, whose nucleotide sequences contained large parts of mTRPC2A and mTRPC2B interrupted by highly variable intervening segments. Because we were unable to amplify the N-termini of mTRPC2A and mTRPC2B, we engineered the mTRPC2A N-terminus by combination of three different overlapping parts taken from the 5′ non-coding regions of the PCR products.

Comparison of the cDNA sequences of mTRPC2α with the corresponding parts of mTRPC2A and mTRPC2B disclosed eight nucleotide sustitutions, six of which resulted in amino acid replacements (K342R, F347S, R367Q, Y488C, A511T and P875L). An alignment of our clone with the highly homologous amino acid sequence of rat TRPC2 (96.1% similarity) confirmed the mTRPC2α sequence in all instances. Additional RACE analysis was performed with RNA prepared from mouse VNO. In this tissue, an additional mTRPC2 transcript with a different translation start codon, named mTRPC2β, was isolated. The open reading frame of mTRPC2β is 4 amino acids longer than that of mTRPC2α (Figure 1).

Tissue distribution of mTRPC2

mTRPC2 was amplified from testis, cerebellum and forebrain by RT-PCR. In testis, heart and brain, only faint signals in the high-molecular-mass range (approx. 7000 bp) were detected by Northern blotting (Figure 2). In an additional Northern-blotting experiment using 30 μg of total RNA from different tissues, a strong and sharp band of approximately 3000 bp was detected exclusively in the VNO (Figure 2).

We performed 5′-terminal RACE with testis, brain, heart and VNO total RNA. Whereas we amplified the 5′-terminus of mTRPC2β with a very short 5′ non-coding region exclusively from VNO, the expression of mTRPC2α could be confirmed in the other tissues. Open reading frames longer than mTRPC2α could not be detected.

Functional expression of mTRPC2

Expression plasmids of mTRPC2A, mTRPC2α and mTRPC2β were prepared and transiently co-expressed with fluorescent

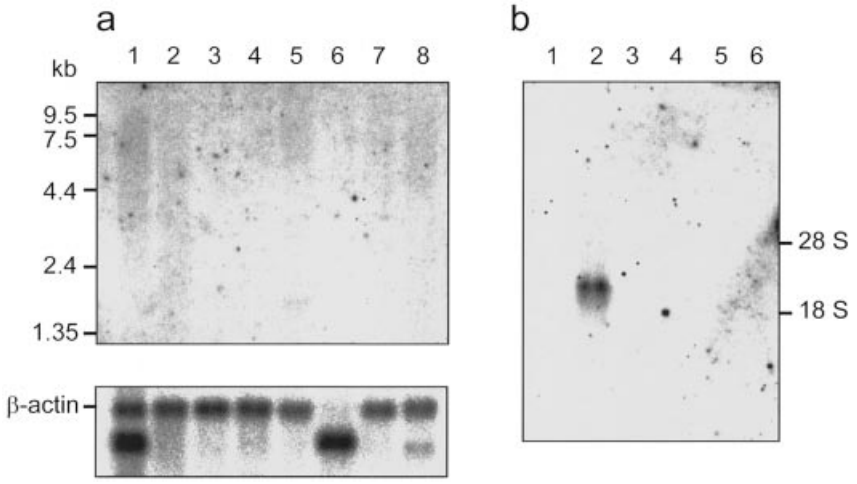

Figure 2 Tissue distribution of mTRPC2 mRNA

(**a**) A commercially available mouse multiple-tissue Northern blot was hybridized with a probe derived from the mTRPC2 coding sequence under highly stringent conditions and subjected to autoradiography for 14 days. In the lower panel the same blot was re-probed with a human β-actin probe. Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. (**b**) A 30 μg portion of total RNA from each tissue was blotted and examined using a similar mTRPC2 probe. Lane 1, testis; lane 2, VNO; lane 3, heart; lane 4, cerebellum; lane 5, forebrain; lane 6, kidney.

proteins in HEK-293 cells as described in the Experimental section. Human TRPC6 (GenBank accession number AF080394) [14] was used as a positive control for a receptor-activated channel. Stimulation of an endogenously expressed $G\alpha_{q/11}$-coupling muscarinic receptor with 100 μM carbachol (results not shown) or the guinea-pig H_1 histamine receptor with 100 μM histamine resulted in a $[Ca^{2+}]_i$ transient which was potentiated in hTRPC6-expressing cells as compared with control cells or mTRPC2β-expressing cells (Figure 3a). Concomitantly, hTRPC6-expressing cells displayed a slight basal and a considerable receptor-stimulated Mn^{2+} influx. In cells expressing mTRPC2β, the receptor-stimulated $[Ca^{2+}]_i$ transient, as well as

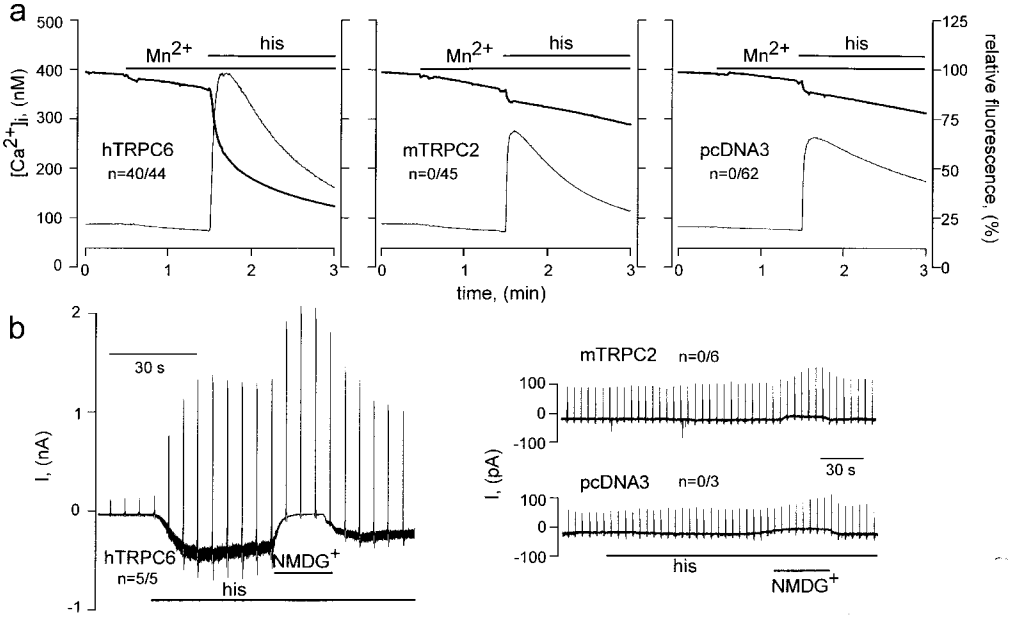

Figure 3 Receptor-mediated cation entry in HEK-293 cells expressing mTRPC2 or hTRPC6

(**a**) Fura 2-loaded cells expressing either hTRPC6, mTRPC2β or the empty pcDNA3 vector were stimulated with 100 μM histamine in the presence of 1 mM $MnCl_2$ in the bath solution as indicated by application bars. The time course of $[Ca^{2+}]_i$ (thin line, left-hand axis) and of the relative fura 2 fluorescence recorded at the isosbestic point (thicker line, right-hand axis) are superimposed as ensemble averages of all cells from one representative experiment. The fractions indicate the number of individual cells of the entire population that responded to histamine (his) application with a detectable Mn^{2+} quench. In (**b**) one representative whole-cell current recording from a hTRPC6, mTRPC2β or control cell is depicted. Histamine application as well as the exchange of extracellular cations for $NMDG^+$ are indicated. Similar to (**a**), the number of responsive cells is indicated as the fraction of all cells tested. The regular spikes represent voltage ramps applied at 5-s intervals.

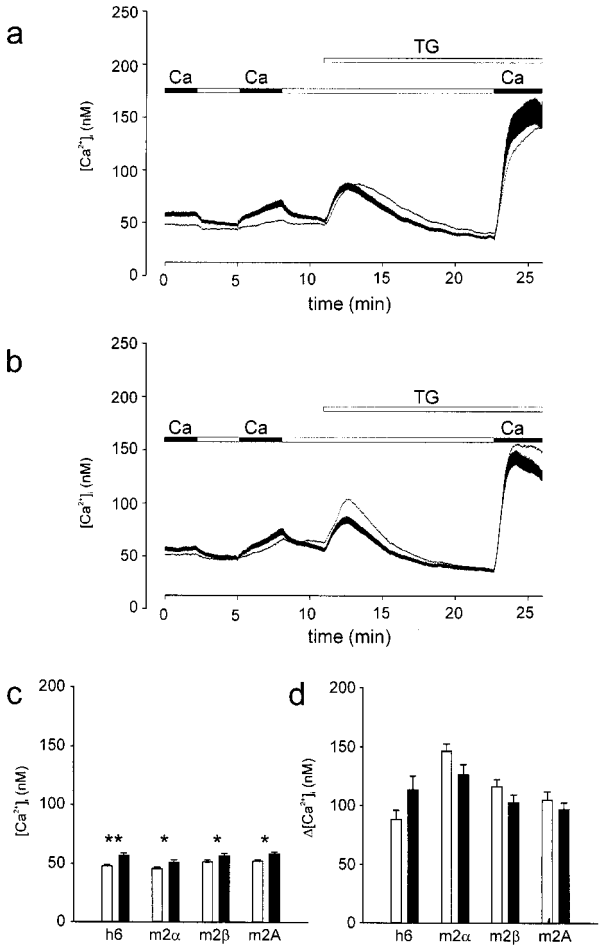

Figure 4 Store-operated calcium influx in HEK-293 cells expressing mTRPC2 or hTRPC6

(**a**) Fura 2-loaded hTRPC6-expressing cells (mean ± S.E.M. of $n = 37$ cells; solid line) and control cells (mean of $n = 52$ cells; dotted line) were subjected to changes in extracellular Ca^{2+} concentration between 0 and 2 mM as indicated, before and after treatment with 2 μM thapsigargin (TG). The time course of $[Ca^{2+}]_i$ was monitored at 2-s intervals. (**b**) A similar experiment was performed with mTRPC2β-expressing cells ($n = 46$) and control cells ($n = 58$). In (**c**) and (**d**) the effects of the expression of either hTRPC6 (h6) or three different splice variants of mTRPC2 (m2α, m2β and m2A) on basal $[Ca^{2+}]_i$ (**c**) and the increases in $[Ca^{2+}]_i$ after application of 2 mM Ca^{2+} subsequent to thapsigargin pretreatment within the same experiment (**d**) are summarized. Each pair of bars represents the mean ± S.E.M. of control cells (open bar) and TRPC-expressing cells (black bar) from one out of three similar experiments. Significant differences are indicated (*, $P < 0.05$; **, $P < 0.001$).

basal and receptor-stimulated Mn^{2+} influx, did not exceed control levels. Identical results were obtained when either mTRPC2α or mTRPC2A was used (results not shown). In whole-cell patch-clamp recordings (Figure 4b), histamine application evoked a transient noisy inward current at a holding potential of −60 mV in five out of five hTRPC6-expressing cells tested. These currents displayed a typical dually rectifying current–voltage relationship and their inward component could be completely blocked by exchange of all external cations against *N*-methyl-D-glucamine ($NMDG^+$), as described previously [14]. In contrast, currents elicited by histamine could not be detected in control cells or in cells expressing mTRPC2β. Furthermore, no increase in the membrane noise level was observed. Taken together, these data strongly indicated that hTRPC6, but not mTRPC2 was capable of promoting bivalent or univalent cation entry subsequent to receptor activation.

In order to investigate further a possible contribution of mTRPC2 to capacitative calcium entry, we examined the calcium influx into mTRPC2-expressing HEK-293 cells after passive store depletion by pretreatment with 2 μM thapsigargin as depicted in Figure 4. After determination of basal $[Ca^{2+}]_i$, subsequent wash-out of extracellular calcium was achieved by superfusing cells with a calcium-free, 2 mM EGTA-containing solution. Before and after thapsigargin treatment, extracellular calcium was increased to 2 mM (Figures 4a and 4b). Figures 4(c) and 4(d) summarize the results obtained with hTRPC6-, mTRPC2α-, mTRPC2β- and mTRPC2A-expressing cells. A considerable elevation of basal $[Ca^{2+}]_i$ was detected in hTRPC6-expressing cells (56.8 ± 2.38 nM versus 49.9 ± 1.75 nM in control cells, $n = 25$), whereas cells expressing mTRPC2 splice variants displayed statistically significant but rather minor elevations of basal $[Ca^{2+}]_i$ (Figure 4c). After thapsigargin treatment, no significant changes of recalcification transients were observed in any of the four cell populations (Figure 4d).

Subcellular localization of mTRPC2

In order to clarify whether the obvious lack of function of mTRPC2 might be attributable to impaired translation or protein trafficking, we fused the C-terminus of hTRPC6 and each of the mTRPC2 splice variants with eGFP. The resulting cDNAs were expressed in HEK-293 cells and analysed by confocal laser scanning fluorescence microscopy as described in the Experimental section.

hTRPC6–GFP expression resulted in a sharp fluorescent line in the region of the plasma membrane and additional intracellular fluorescence that was excluded from the nuclear area and most probably represented nascent protein at the endoplasmic reticulum and Golgi apparatus (Figure 5e). hTRPC6–GFP was functionally active as a receptor- and diacylglycerol-activated channel (results not shown).

In contrast, none of the three mTRPC2 GFP-tagged splice variants was detectable at the plasma membrane. They rather accumulated in the nuclear envelope and perinuclear granular and reticular compartments presumably representing endoplasmic reticulum and Golgi apparatus (Figures 5a–5c). In cells of either neuronal (NG 108-15, Figure 5d; N1E-115 and PC-12, results not shown) or epithelial (CaCo-2, results not shown) origin, we noted a similar subcellular distribution of mTRPC2. The subcellular localization of hTRPC6 and mTRPC2β observed with eGFP chimaeras was confirmed by immunocytochemistry of HEK-293 cells expressing either wild-type hTRPC6 or mTRPC2β bearing an HA tag appended to the N- or the C-terminus (Figures 5f–5h).

To investigate whether mTRPC2 trafficking from intracellular membranes to the plasma membrane might be a stimulated rather than a constitutive process, mTRPC2–GFP-expressing cells were stimulated with 100 μM carbachol or 100 ng/ml epidermal growth factor. For epidermal growth factor experiments, cells were starved in serum-free medium for at least 12 h prior to the experiments. Confocal sections were scanned after 1 min, 5 min and 2 h following stimulation at room temperature in HBS. These protocols did not entail any detectable changes in the mTRPC2 fluorescence pattern (results not shown).

We further reasoned that mTRPC2 might be incorrectly folded due to an inherent lack of chaperones in the cell system. Several chemicals are used to restore plasma-membrane trafficking of misfolded membrane proteins, so-called 'chemical chaperones'.

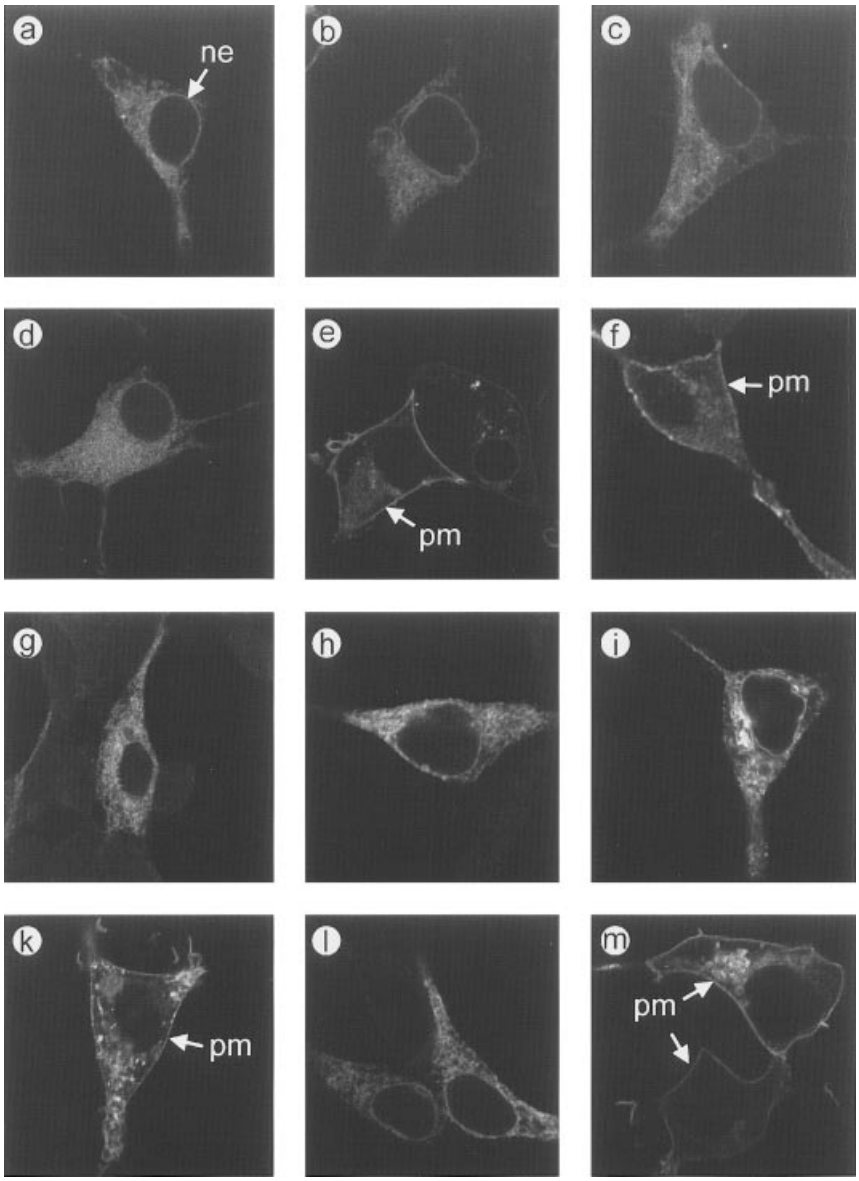

Figure 5 Subcellular distribution of mTRPC2 splice variants

Localization of the plasma membrane (pm) and nuclear envelope (ne) are indicated by white arrows. (**a**–**c**) Fluorescence pattern of (**a**) mTRPC2α, (**b**) mTRPC2β and (**c**) mTRPC2A each C-terminally tagged with eGFP in HEK-293 cells. (**d**) Localization of mTRPC2b–eGFP in a NG 108-15 neuroblastoma/glioma cell. (**e**) Localization of hTRPC6–eGFP in HEK-293 cells. (**f**–**h**) Immunocytochemistry of hTRPC6 (**f**), HA-mTRPC2β (**g**) and mTRPC2β-HA (**h**) expressed in HEK-293 cells. (**i** and **k**) Fluorescence pattern of a GFP-tagged trafficking-deficient hTRPC6 mutant without (**i**) and with wild-type hTRPC6 co-expressed (**k**). (**l**) Subcellular localization of mTRPC2β–GFP co-transfected with hTRPC1, hTRPC3, mTRPC4B, mTRPC5 and hTRPC6 cDNAs. (**m**) hTRPC6–eGFP co-expressed with a 5-fold excess of mTRPC2β cDNA.

We treated mTRPC2-expressing HEK-293 cells with either 5% (v/v) DMSO or 5% (v/v) glycerol. None of these approaches affected the cellular fluorescence pattern of mTRPC2. For several proteins, a lowering of the cell-culture temperature was shown to rescue plasma-membrane trafficking [23]. Culturing mTRPC2-expressing HEK-293 cells at 22 °C for 36 h, however, had no detectable effect on mTRPC2 trafficking (results not shown).

We tested the hypothesis that mTRPC2 trafficking might depend on the presence of other TRPC family members. Performing RT-PCR on VNO RNA, we could amplify mTRPC1, mTRPC2, mTRPC4 and mTRPC6 cDNA. We observed that a trafficking-incompetent GFP-tagged hTRPC6 mutant (see the Experimental section) could be efficiently rescued when co-expressed with wild-type hTRPC6 (Figures 5i and 5k). These findings indicate that TRPCs can assemble at the level of intracellular compartments and that mTRPC2 might be trafficked to the plasma membrane only when additional TRPCs are available as possible heteromultimerization partners. In order to verify this hypothesis, mTRPC2–GFP was co-expressed with hTRPC1, hTRPC3, mTRPC4, mTRPC5, hTRPC6, TRP, TRPL or a cDNA mixture of TRPC1–6. The pattern of cellular mTRPC2 fluorescence, however, remained unchanged in any instance, thus excluding a role of these proteins for mTRPC2 co-targeting (Figure 5l). Similarly, plasma-membrane targeting of GFP-tagged mTRPC4 and hTRPC6 (Figure 5m) was not impaired by co-transfection of 5-fold excessive amounts of cDNA

coding for untagged mTRPC2. Heteromultimerization of TRPC family channels has been discussed [24], but in most cases direct experimental evidence is missing. Our data indicate that mTRPC2 is targeted independently of other known TRPCs.

DISCUSSION

We cloned the cDNA of mouse TRPC2 and found it to be expressed as two distinct splice variants. Like rat TRPC2 [19], both murine splice variants, mTRPC2α and mTRPC2β, are similar to other TRPCs in length (886 and 890 amino acids respectively; Figure 1), and contain all of the structural characteristics of the TRPC family. Our cDNAs differ from other published clones [18] in the length of the open reading frame and in six amino acid residues. A comparison with other known TRPC2 genes [17,19] corroborates our sequence data.

We could amplify mTRPC2α transcripts from several tissues and cell lines, including testis, brain and heart. mTRPC2A or mTRPC2B, however, could not be amplified. mTRPC2β transcripts were exclusively found in the VNO. Northern-blotting experiments showed specific expression of mTRPC2 in the VNO, but only negligible amounts of mRNA in testis. As mTRPC2β is the predominant, probably exclusive, mTRPC2 transcript in the VNO, it appears to represent the physiologically relevant mTRPC2 splice variant.

We expressed the cDNA of mTRPC2 in several cell lines, including HEK-293 cells. Because systematic errors due to cell-culture conditions, dye loading and drug application may considerably bias results, we decided to perform statistical analysis exclusively on different cell populations cultured and measured on the same coverslip. Applying fluorimetric techniques, we could not observe appreciable changes in bivalent cation entry after stimulation of a G-protein-coupled receptor or depletion of calcium stores. In whole-cell patch-clamp experiments, no receptor-activated currents were detected. Thus it appears unlikely that mTRPC2 contributes to store-operated or receptor-activated calcium entry in this cell system. These results differ from data by others who reported an increase in store-operated cation entry due to mTRPC2A and mTRPC2B expression in a heterologous expression system (COS-M6) [18].

In order to explain the lack of function observed in our experiments, we generated C-terminal fusion proteins with eGFP. By means of trafficking studies, we demonstrated an impaired subcellular targeting of the mTRPC2 protein, which is in agreement with its functional defect. We provide evidence that misfolding of the channel protein is probably not causative of mTRPC2 mistargeting, because mTRPC2 could not be targeted to the plasma membrane by treatment with 'chemical chaperones' or by culturing the cells at lower temperatures.

mTRPC2 was not found to be translocated to the plasma membrane by stimulation of a $G\alpha_{q/11}$-coupled receptor or a receptor tyrosine kinase, thus arguing against a secretion-like mode of activation as was recently reported for store-operated cation channels [25–27] and the vanilloid-receptor-like channel VRL-1 [28].

Finally, it was not possible to restore mTRPC2 trafficking by co-expression of other known TRPCs, and TRPC2 exerted no dominant negative effect on the trafficking of such channels. These results strongly argue against heteromultimerization of mTRPC2 with other currently known TRPCs.

Collectively, our data do not support the hypothesis that mTRPC2 might be a molecular correlate for store-operated calcium entry in heterologous expression systems. The present results may suggest that mTRPC2 either does not encode a plasma-membrane channel at all, but instead fulfils an as yet unknown function within endomembrane compartments or, more likely, functionally depends on additional cellular components which are so far unidentified. In analogy to *Drosophila* TRP [29,30], a scaffolding protein such as InaD or different proteins may be required to ensure correct mTRPC2 trafficking. In light of the data obtained by Liman et al. [19], who were able to localize rat TRPC2 in the microvillar plasma membrane of the sensory segment of VNO receptor neurons, TRPC2 in both mouse and rat is likely to represent a sensory transduction channel similar to *Drosophila* TRP [3,31,32], with a specific role only in the VNO. Thus future research will have to evaluate the function of TRPC2 within the context of VNO receptor cells. Because pheromone receptor signalling [33–35] activates G_{i2} or G_o-type G-proteins in the VNO [36], which in turn activate PLCβ [37], and because cyclic-nucleotide-gated channels were shown to play no obvious role in mammalian VNO signalling [38], the involvement of TRPC2 in the encoding of VNO receptor potentials represents an attractive hypothesis.

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