Expression and Characterization of Human Transient Receptor Potential Melastatin 3 (hTRPM3)*

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Transient receptor potential (TRP) cation-selective channels are an emerging class of proteins that are involved in a variety of important biological functions including pain transduction, thermosensation, mechanoregulation, and vasorelaxation. Utilizing a bioinformatics approach, we have identified the full-length human TRPM3 (hTRPM3) as a member of the TRP family. The hTRPM3 gene is comprised of 24 exons and maps to human chromosome 9q-21.12. hTRPM3 is composed of 1555 amino acids and possesses the characteristic six-transmembrane domain of the TRP family. hTRPM3 is expressed primarily in kidney and, at lesser levels, in brain, testis, and spinal cord as demonstrated by quantitative RT-PCR and Northern blotting. In situ hybridization in human kidney demonstrated that hTRPM3 mRNA expression is predominantly found in the collecting tubular epithelium. Homologous expression of hTRPM3 in human embryonic kidney cells (HEK 293) showed that hTRPM3 is localized to the cell membrane. hTRPM3-expressing cells exhibited Ca2+ concentration-dependent Ca2+ entry. Depletion of intracellular Ca2+ stores by lowering extracellular Ca2+ concentration and treatment with the Ca2+-ATPase inhibitor thapsigargin or the muscarinic receptor agonist carbacol further augmented hTRPM3-mediated Ca2+ entry. The nonselective Ca2+ channel blocker, lanthanide gadolinium (Gd3+), partially inhibited hTRPM3-mediated Ca2+ entry. These results are consistent with the hypothesis that hTRPM3 mediates a Ca2+ entry pathway that apparently is distinct from the endogenous Ca2+ entry pathways present in HEK 293 cells.

Intracellular Ca2+ plays a pivotal role in various cellular functions such as protein secretion, cell differentiation, cell division, and apoptosis. Ca2+ entry into cells can be mediated via voltage-dependent Ca2+ channels, ligand-gated Ca2+ channels, nonselective cation channels, and the relatively less characterized class store-operated Ca2+ channels (SOC).1 SOC mediate Ca2+ entry from the extracellular space following Ca2+ release from the intracellular stores to generate sustained increases in intracellular Ca2+ concentration and replenish the internal Ca2+ stores. The molecular mechanism of SOC activation and the molecular identity of SOC remain elusive. Transient receptor potential (TRP) channels, an emerging class of the Ca2+-permeable cation channel superfamily, are probable candidates for SOC (for review see Ref. 1). Following the identification of the founding member of this family, dTRP, which is from a Drosophila mutant with abnormal visual signal transduction (2), mammalian homologues have been cloned and all of them contain a six-transmembrane domain followed by a TRP motif (XWFKXX). Based on homology, they are divided into three subfamilies: TRPC (canonical), TRPV (vanilloid), and TRPM (melastatin) (3). Members of the TRPM subfamily have unusually long cytoplasmic tails at both ends of the channel domain, and some of the family members have an enzyme domain in the C-terminal region. Despite their similarities of structure, TRPMs have different ion-conductive properties, activation mechanisms, and putative biological functions. TRPM1 is down-regulated in metastatic melanomas (4). TRPM2 is a Ca2+-permeable channel that contains an ADP-ribose pyrophosphatase domain and can be activated by ADP-ribose, NAD (5, 6), and changes in redox status (7). The TRPM2 gene is mapped to the chromosome region linked to bipolar affective disorder, nonsyndromic hereditary deafness, Knobloch syndrome, and holoprosencephaly (8). Two splice variants of TRPM4 have been described. TRPM4a is predominantly a Ca2+-permeable channel (9); whereas TRPM4b conducts monovalent cations upon activation by changes in intracellular Ca2+ (10). TRPM5 is associated with Beckwith-Wiedemann syndrome and a predisposition to neoplasias (11). TRPM7, another biofunctional protein, has kinase activity in addition to its ion channel activity. TRPM7 is regulated by Mg2+-ATP and/or inositol 1,4,5-trisphosphate and is required for cell viability (12–14). TRPM8 is up-regulated in prostate cancer and other malignancies (15). Recently, it has been shown to be a receptor that senses cold stimuli (16, 17).

Using a bioinformatics approach, we have identified a member of the human TRPM subfamily that we have called hTRPM3, consistent with the unified TRP nomenclature (3). hTRPM3 contains long N and C termini, although it does not contain any additional enzymatic features. hTRPM3 mRNA is expressed primarily in kidney with lower levels in brain, testis, and spinal cord. When expressed in HEK 293 cells, hTRPM3 is co-localized with the plasma membrane and is capable of mediating Ca2+ entry. This hTRPM3-mediated Ca2+ conductance

The abbreviations used are: SOC, store-operated Ca2+ channel; TRP, transient receptor potential; HEK, human embryonic kidney; TG, thapsigargin; HMM, hidden Markov model; FLIPR, fluorometric imaging plate reader; CCh, carbacol; hemagglutinin; RT, reverse transcriptase; Gd3+, lanthanide gadolinium; HSH, hypomagnesemia with secondary hypocalcemia.
is partially lanthanide gadolinium (Gd\textsuperscript{3+})-sensitive and can be enhanced upon Ca\textsuperscript{2+} stores depletion or receptor activation.

**EXPERIMENTAL PROCEDURES**

**Bioinformatics Analysis**—The sequence homology search program BLAST and the gene-finding program GeneWise/Wise2 package (18, 19) were used to identify novel ion channel sequences in the TRP family. Individual known ion channel protein sequences in the TRP family were used as probes in a TBLASTN analysis versus the human genome sequence. A multiple sequence alignment of known TRP family members was also generated using the ClustalW alignment program in the software, Vector NTI 5.5. A hidden Markov model (HMM) specific for the TRP family was then constructed using the HMMER-BUILD program in HMMER2.2 package from the above multiple sequence alignment (20). This HMM model was then used to search the human genome sequence data base using the GeneWise program in GeneWise. Results from the TBLASTN and GeneWise searches were pooled. The high scoring hit segments from the data base search were extracted and searched back against nonredundant protein and patent sequence databases to determine novelty. For potential novel protein-encoding segments, the most similar protein sequence hits were used as templates to predict putative exons from the genomic sequence using the GeneWise program. From this analysis, exons encoding potential novel ion channel sequences were identified. To extend the 5' and 3' sequences of putative novel ion channel molecules, the genomic regions surrounding the matching exons from genomic contigs were analyzed using Genscan and Genesh programs to generate de novo exons (21). From these analyses, one of the predicted full-length protein coding sequences of novel human ion channel related genes was cloned for further study.

The phylogenetic tree for the TRPM subfamily was generated by the neighbor-join method using the GCG GrowTree program with Kimura distance correction method. Members of TRP family with their respective GenBank\textsuperscript{TM} accession numbers are as follows: hTRPM1 (NM_0002420), hTRPM2 (NM_003307), hTRPM4 (NM_176368), hTRPM5 (NM_014555), hTRPM6, hTRPM7 (XM_030709), and hTRPM8 (XM_030709), and hTRPM9 (NM_024500).

**Cloning of hTRPM3**—Using the predicted exon genomic sequence from BAC AL358786, primers were designed (forward: 5'-ATGTATGTGGGCAGTATCTTGTATACAAACG-3', reverse: 5'-CGCGAGTATCTTTTGATACAAAACCT-3'). The PCR data were converted into a relative assessment of the difference in transcript abundance by normalizing to the data obtained with the primers from cyclophilin, performing a parallel experiment using a primer pair from cyclophilin.

**Quantitative RT-PCR**—A PCR primer pair (forward: 5'-CGCGAGTATCTTGTATACAAACG-3', reverse: 5'-GGAGGACACCGGACATGTTGTTGTTGATCC-3') and used to amplify fragments from the human kidney Ready cDNA library (Clontech). The reaction mixture in 50 µl contains 5 µl of cDNA library, 0.5 µM each primer, 1.25 µM each dNTP, TaqPlus Precision buffer and 0.5 units of TaqPlus Precision polymerase buffer (Stratagene). The reaction was repeated for 30 cycles (94 °C for 45 s, 55 °C for 45 s, and 72 °C for 4 min). The amplified fragments were cloned into the sequencing vector pCR4 Blunt-TOPO (Invitrogen) for sequence analysis. The four sequences were then assembled to generate the full-length cDNA. For functional studies, the cDNA was fused in frame with an HA epitope at its C terminus and subcloned into the mammalian expression vector pcDNA3.1/Hygro (Invitrogen).

**In Situ Hybridization**—Human Kidney was collected and received from the National Disease Research Interchange (Philadelphia, PA) according to Institutional Review Board-approved protocol. Tissue sections were embedded in Tissue Tek\textsuperscript{®} O.C.T. compound (Sakura Finetek USA, Inc.) and snap-frozen by immersion in 2-methylbutane cooled in dry ice and subsequently stored at -70 °C. The sections were examined by a pathologist to ascertain the normality of the tissue before performing the following experiments.

Templates for hTRPM3 cRNA probes were derived from a 678-bp hTRPM3 fragment cloned in a pCR-BluntII-TOPO vector (Invitrogen) using the primer pair: (forward: 5'-CAGCTGGAAACCTTATCGG-3', reverse: 5'-TGGGAGGTTGGTGTAGCTCGAAAG-3'). The template for positive control cRNA human lysozyme probe was derived from a 638-bp cDNA expression sequence tag (Incyte Genomics). GenBank\textsuperscript{TM} accession number A58808.1. Super35-Labeled riboprobes were synthesized via in vitro transcription utilizing the Riboprobe\textsuperscript{®} Combination System (Promega) where T7 and Sp6 RNA polymerase yielded sense and antisense probes, respectively, for hTRPM3, whereas T7 and T3 RNA polymerases yielded antisense and sense probes, respectively, for human lysozyme. Cryostat tissue sections cut at 10 µm and fixed in 4.0% formalin were used for in situ hybridization as described previously (23). Tissue sections were acetylated, dehydrated in a graded ethanol series, immersed in chloroform, alcohol-rinsed, and air-dried, and then hybridized with sense and antisense 35S-labeled RNA probes (1.5 x 10⁶ cpm/ml) for 16–20 h at 60 °C. Following hybridization, slides were rinsed in 4 x SSC/50% formamide and 4 x SSC, treated with RNase A (20 µg/ml, Invitrogen) at 37°C, washed through increasing stringent conditions in 0.1 x SSC/1% SDS, dehydrated, air-dried, and then coated with NTB-2 emulsion (Eastman Kodak Co.). Slides were placed in a dark box with desiccant at 4°C and developed after a 1- and 4-week exposure. Sections were stained with hematoxylin and eosin and coverslipped. Expression signals were detected by dark phase microscopy. Cellular phenotype identification was by bright field microscopy. The results have been confirmed in the kidney from three different donors and from nonhuman primate.

**Mammalian Cell Expression and Immunofluorescence Staining of hTRPM3**—HEK293 cells were cultured in Dulbecco's modified Eagle medium containing 10% heat-inactivated fetal bovine serum and grown on poly-L-lysine-coated glass coverslips. The cells were transiently transfected with hTRPM3-HA with FuGENE 6 (Roche Applied Sciences). 48 h later, cells were stained in culture media with the membrane probe Vybrant 228 CM-Dil (5µM/ml; Molecular Probes) at 37 °C for 5 min and 4 °C for 15 min. After washing with phosphate-buffered saline, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.1% Triton X-100, blocked in phosphate-buffered saline containing 5% fetal bovine serum and 5% normal goat serum, and stained with 10 µM Cy3-conjugated anti-HA high affinity antibody (3F10, Roche Applied Sciences) and 4',6-diamidino-2-phenylindole (0.5 µg/ml, Molecular Probes). Immunostained cell cultures were examined using a laser-scanning confocal microscope (ZEISS LSM510), a ×63 oil immersion objective, and appropriate filter sets. Images shown are of a single optical section ~1-µm thick.

**Measurements of Changes in Intracellular Ca\textsuperscript{2+}**—The cytoplasmic Ca\textsuperscript{2+} indicator Fluo-4-AM (Molecular Probes) and a fluorometric imaging plate reader (FLIPRTM, Molecular Devices) instrument were used to detect changes in intracellular Ca\textsuperscript{2+} concentration. The hTRPM3-transfected cells were seeded on poly-L-lysine-coated 96-well plates at a density of 75,000 cells/well and used 24 h after transfecting with FuGENE 6 (Roche Applied Sciences). Cells were loaded with 4 µM Fluo-4-AM at 37 °C for 30 min in a nominally Ca\textsuperscript{2+}-free or 1 mM CaCl\textsubscript{2} buffer containing (in mM): 140 NaCl, 4.7 KCl, 10 MgCl\textsubscript{2}, 100 HEPES, 10 glucose, and 2.5 Probenecid (Sigma), pH 7.4. Extracellular Fluo-4-AM, was removed, and cells were maintained in either Ca\textsuperscript{2+}-free buffer or containing 1 mM Ca\textsuperscript{2+} at room temperature prior to the experiments, which were conducted within 30 s after dye removal. Fluo-4-1 was excited by an argon laser, and emitted light was selected using a 510–570 nm band pass filter. Base-line intracellular fluorescence was established during the initial 50 s of the FLIPR read, and then 1, 5, or 10 mM Ca\textsuperscript{2+} was added to each well and subsequent changes in the intracellular Ca\textsuperscript{2+} were monitored for 8 min. For store-depletion or receptor activation studies, 2 mM thapsigargin (Tg) or 50 µM carbachol (CCl) respectively, was added to Fluo-4-loaded cells in Ca\textsuperscript{2+}-free buffer before adding 2 mM Ca\textsuperscript{2+} on FLIPR. For pharmacology studies, 100 µM GdCl\textsubscript{3} was added to Fluo-4-loaded cells in 0 or 1 mM Ca\textsuperscript{2+} buffer as described in figure legends prior to the start of the FLIPR recordings. Experiments were carried out at room temperature.

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\textsuperscript{N. Lee, unpublished data.}
hTRPM3 Is a Novel Member of the TRP Channel Family—

Human BAC AL358786 was found to cover a partial open reading frame of a novel TRP family gene based on the BLASTTN and GENEWISDB searches. TRPM1, the most similar protein for this novel TRP gene, was used as a template to predict additional exons from the same genomic sequence using the GENEWISDB program. To extend the 5′ and 3′ sequences of the TRP gene, the genomic regions surrounding the matching exons in genomic sequence NT_008306 were analyzed using GENSCAN and GENEWISDB programs to generate de novo exons (21, 22). A full-length TRP family gene was discovered using these analyses. This computational prediction was then confirmed experimentally. A cDNA was isolated from a human kidney library by using the putative exon sequences as PCR templates. We refer to this gene as hTRPM3 (3). The C-terminal sequence is nearly identical to a previously reported cDNA fragment KIAA1616 (24) and later denoted as TRPM3 (3). As compared with KIAA1616, our sequence contains 566 additional amino acids at the N terminus of KIAA1616 (Fig. 1B). With the following experimental evidence, we believe that we have the full-length functional hTRPM3.

hTRPM3 is predicted to be 1555 amino acids long and is comprised of the following characteristic features of a TRP channel: six transmembrane domains; an ion transport signature domain (amino acids 748–959); a TRP signature motif (XWKFXR) located downstream of the sixth transmembrane region; and a coiled-coil domain located further downstream of the TRP signature domain (Fig. 1A). However, unlike some of the TRPM family members including TRPM2 (5, 6), TRPM7 (12–14), and TRPM6,2 hTRPM3 does not contain an enzyme domain in the C-terminal cytoplasmic region.

hTRPM3 is most similar to hTRPM1 with 57% identity and 67% similarity (Fig. 1, A and C). Greater homology (over 80% identity) was observed at the N terminus (between amino acids 1 and 1219). There is a 58 amino acid gap in the hTRPM3 sequences as shown in the alignment of hTRPM3 with hTRPM1. GENEWISDB was used to look for possible exons at the corresponding genomic DNA region, and none was found. There are good splice junctions around the sequence gap, and the exon forced out by GENEWISDB has no homology to TRPM1. Therefore, it is unlikely that any coding sequence was missed within that region.

hTRPM3 Gene Is Located at 9q-21.12—An analysis of the genomic sequence of hTRPM3 (Fig. 1B) showed that the coding region spans 311 kb and is comprised of 24 exons. hTRPM3 gene is located between the two genomic markers, D9S1874 and D9S1807, and its chromosomal localization is 9q-21.12.

We identified five more splice variants from a human kidney cDNA library using the primers designed from the predicted coding sequence of hTRPM3 gene (Fig. 1B). A comparison with the genomic DNA sequence shows that the exon boundaries of all of the splice variants obey the gt-ag rule of the splice donor-donor-acceptor sites. We designate the splice variants as “TRPM a–f” according to their relative abundance, subject to the ratios of products formed from the PCR amplification. The following experiments were all performed using the “a” form.

hTRPM3 Is Expressed Selectively in Human Kidney—Fig. 2A illustrates the relative expression level of hTRPM3 among various human mRNA tissue sources by Northern analysis using a 645-bp hTRPM3-specific probe (Fig. 1A, corresponding to the region between two arrowheads). The transcripts of ~8 kb corresponding to hTRPM3 are expressed predominately in kidney tissue. The hTRPM3 polypeptide was also expressed at lesser levels in the brain and testis. Consistent with the identification of several other splice variants, multiple species of hTRPM3 transcripts were also detected in the Northern blot.

A similar expression pattern was observed by an independent method, quantitative RT-PCR. As shown in Fig. 2B, transcripts corresponding to hTRPM3 expressed predominately in kidney tissue and, at lesser levels, in brain, testis, and spinal cord. On an extended panel of human tissue RNAs, it was demonstrated that within brain subregions, the highest levels of expression were found in the cerebellum, choroid plexus, the locus coeruleus, the posterior hypothalamus, and the substantia nigra (data not shown).

hTRPM3 mRNA expression in human kidney was further analyzed by in situ hybridization. hTRPM3 was localized to the cytoplasm of collecting tubular epithelium in the medulla, medullary rays, and periglomerular regions (Fig. 2C, i and v). Tubules in the medulla exhibited the most intense expression. Other tubular epithelia, e.g. proximal convoluted tubular epithelium, exhibited minimal expression. Expression patterns were compared with hTRMP3 sense mRNA-labeled human kidney sections as negative controls (Fig. 2C, iii and vi) and to human lysozyme antisense mRNA-labeled human kidney sections as positive controls (data not shown).

An analysis of hTRPM3 expression has also been made in mRNA isolated from various tumors and control tissues. Renal tumors showed a significant decrease (average of ~80% lower) in hTRPM3 steady-state mRNA levels in the tumors compared with their matched normal kidney controls. Similarly, in testicular cancers, lower steady-state mRNA levels were also observed (data not shown). These data suggest that a loss of hTRPM3 expression might play a role in tumorigenesis.

Overexpressed hTRPM3 Can Be Detected at the Plasma Membrane in HEK 293 Cells—The complete open reading frame of hTRPM3 with a C-terminal HA tag was transiently transfected into HEK 293 cells to analyze the biological function. The expression of full-length protein was assessed with the immunoblot using an anti-HA antibody and detected as the expected size of ~170 kDa (data not shown).

The cellular localization of HA-tagged hTRPM3 was detected using a fluorescein-conjugated anti-HA antibody and a laser-scanning confocal microscope. Anti-HA staining was found to be associated with the membrane marker CM-DiI, indicating hTRPM3 protein in or near the plasmalemmal compartment of transfected cells (Fig. 2D). Plasmalemmal localization is consistent with the function of the TRP family as Ca2+-permeable membrane protein. hTRPM3 was also observed in intracellular compartments, possibly resulting from overexpression in this heterologous expression system as observed with other ion channels (25).

hTRPM3 Mediates Ca2+ Entry—To assess the functional role of hTRPM3, we tested for Ca2+ permeability, a property common to most TRP channels. Cells transiently transfected with vector or hTRPM3 were loaded with the cytoplasmic Ca2+-indicator, Fluo-4. Intracellular Ca2+ was monitored using a FLIPR that measures real-time intracellular fluorescence changes. Initially, cells were maintained in 1 mM Ca2+ solution, which is in the normal range of physiological conditions. After measuring base-line intracellular Ca2+ upon FLIPR addition of 1, 3, or 10 mM CaCl2 to the media resulted in a concentration-dependent increase in intracellular Ca2+ in hTRPM3-expressing cells (Fig. 3A, right panel). In contrast, vector-transfected cells showed minimal Ca2+ entry under the same experimental conditions (Fig. 3A, left panel). Non-transfected cells were indistinguishable from vector-transfected cells (data not shown). These results indicate that hTRPM3 is capable of mediating Ca2+ entry.

To further address the mechanism of hTRPM3-mediated Ca2+ entry, Ca2+ addition experiments were performed on
**Fig. 1. Human TRPM3.**

**A**, sequence comparisons of human TRPM3 with TRPM1 (hTRPM1). Amino acid residues are numbered on the left. Residues within hTRPM3 identical to hTRPM1 are indicated by blanks, and gaps are indicated by dashes. The transmembrane (TM) domains are indicated by lines, the TRP signature motif (XWKFX) is indicated by asterisks, and the coiled-coil domain is indicated by dashed lines. The corresponding amino acid sequence of the probe used for the Northern blot is denoted as the region between the two arrowheads.

**B**, genomic organization of hTRPM3 gene, its five additional splice variants b–f and KIAA1616. Differences within b–f are boxed. The coding exons are shown as boxes with the exon number as indicated. hTRPM3b gene contains an extra exon 11. hTRPM3c gene contains an additional 12 amino acid at exon 20. hTRPM3d gene is missing exon 13. hTRPM3e gene contains an extra exon 11 and is missing exon 13. hTRPM3f gene contains an extra exon 4. **C**, phylogenetic tree showing the evolutionary relationship among the human TRPM subfamily. Phylogram branch lengths are proportional to distance from common ancestor. The scale represents the number of amino acid substitutions per 100 residues.
transfected cells incubated (~30 min) in a nominally Ca²⁺-free solution. Previous studies have shown that lowering extracellular Ca²⁺ concentration below physiological levels can deplete intracellular Ca²⁺ stores in many cell types including HEK 293 (26). Incubating vector-transfected HEK 293 cells in a nominally Ca²⁺-free solution gave rise to Ca²⁺ entry that was dependent on the concentration of Ca²⁺ added subsequently to the buffer, indicating that Ca²⁺ entry was mediated through endogenous SOCs in HEK 293 cells (Fig. 3B, left panel). In hTRPM3-transfected cells, the Ca²⁺ transients triggered by a similar Ca²⁺ treatment were much larger (Fig. 3B, right panel). In addition, Ca²⁺ entry observed in hTRPM3-transfected cells incubated in Ca²⁺-free media was greater than in 1 mM Ca²⁺ media (compared with Fig. 3A, right panel), indicating that hTRPM3-mediated Ca²⁺ entry was potentiated by store depletion.

The store-operated mechanism of hTRPM3-mediated Ca²⁺ entry was tested further by passively depleting Ca²⁺ stores with TG, an inhibitor of microsomal Ca²⁺-ATPase whose normal function is to pump ions from the cytosol back into the stores. The addition of 2 μM TG equivalently depleted Ca²⁺ stores in hTRPM3- and vector-transfected cells (Fig. 3C). Following store depletion with TG, the addition of Ca²⁺ to the buffer induced a much larger Ca²⁺ entry in hTRPM3 cells compared with the vector control cells (Fig. 3C, ~14% increase measured at peak selected from t = 600–660 s; hTRPM3, 19550 ± 226; vector, 17213 ± 413, n = 12). Receptor-mediated Ca²⁺ entry was also more pronounced in...
hTRPM3-transfected cells. Carbachol (CCh) can activate an endogenous muscarinic receptor and trigger inositol 1,4,5-trisphosphate production, leading to the activation of SOCs in HEK 293 cells (27). The addition of 50 μM CCh caused a transient and rapid intracellular Ca\(^{2+}\) increase in both hTRPM3- and vector-transfected cells (Fig. 3D). After the receptor activation with CCh, the addition of Ca\(^{2+}\) to the buffer induced a much larger influx of Ca\(^{2+}\) into hTRPM3 cells as compared with vector control cells (Fig. 3D, ~40% increase measured at peak selected from t = 660–720 s; hTRPM3, 11067 ± 218; vector, 7879 ± 248; n = 12). These results show that after store depletion with TG or receptor activation with CCh, hTRPM3-transfected cells exhibit an increased Ca\(^{2+}\) influx when compared with control cells.

**hTRPM3-mediated Ca\(^{2+}\) Entry Can Be Partially Blocked by Gd\(^{3+}\).**—The Gd\(^{3+}\) is a nonselective Ca\(^{2+}\)-permeable channel blocker that inhibits most known TRP channels. The effects of 100 μM Gd\(^{3+}\) on Ca\(^{2+}\) permeability were tested in vector- and hTRPM3-transfected cells. The minimal Ca\(^{2+}\) influx observed upon the addition of 10 mM Ca\(^{2+}\) to the buffer was 1470 ± 140 to 58 ± 8 in vector-transfected cells from 6000 ± 322 to 2080 ± 199 (n = 12). Fluorescence values were measured 150 s after adding 10 mM Ca\(^{2+}\), and percent blockade was calculated as 1 − (F\(_{\text{hTRPM3}}\) − F\(_{\text{Vector}}\))/F\(_{\text{Vector}}\). The effects of Gd\(^{3+}\) on hTRPM3-mediated Ca\(^{2+}\) entry induced by 10 mM Ca\(^{2+}\) in the presence of TG or CCh were also examined. Cells were incubated in nominally Ca\(^{2+}\)-free medium for TG and CCh experiments. Gd\(^{3+}\) inhibited Ca\(^{2+}\) entry by 51% after depletion of intracellular stores with TG (Fig. 4B). Gd\(^{3+}\) reduced peak fluorescence after 10 mM Ca\(^{2+}\) addition in vector-transfected cells from 26,444 ± 2410 to 1316 ± 60, and in hTRPM3-transfected cells from 37,676 ± 2425 to 6783 ± 250 (Fig. 4, C and D, respectively, n = 12). Gd\(^{3+}\) inhibited Ca\(^{2+}\) entry by 72% after depletion of intracellular stores with CCh. Gd\(^{3+}\) reduced peak Ca\(^{2+}\) fluorescence in vector-transfected cells from 9327 ± 466 to 453 ± 15, and in hTRPM3-transfected cells from 14,747 ± 988 to 1975 ± 79 (Fig. 4, E and F, respectively, n = 12). These results show that under identical conditions, the endogenous Ca\(^{2+}\) entry pathway was strongly blocked by the application of 100 μM Gd\(^{3+}\), whereas the hTRPM3-mediated pathway was partially blocked (53%) (Fig. 4, A and B). Stimulation of Ca\(^{2+}\) entry in hTRPM3-transfected cells in the presence of TG or CCh was also partially blocked by 100 μM Gd\(^{3+}\). These results are consistent with the hypothesis that hTRPM3 mediates a

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**FIG. 3.** hTRPM3-mediated Ca\(^{2+}\) entry can be triggered by store depletion. A and B, hTRPM3 mediates concentration-dependent permeability to Ca\(^{2+}\). Transfected HEK 293 cells loaded with Fluo-4 were incubated in 1 mM Ca\(^{2+}\) solution (A) or a nominally Ca\(^{2+}\)-free solution (B) and 0, 1, 3, and 10 mM Ca\(^{2+}\) solutions were added to cells as indicated by arrows. Left panel, vector-transfected control cells. Right panel, hTRPM3-transfected cells. C and D, Ca\(^{2+}\) entry was induced upon store depletion (C) and receptor activation (D) in the hTRPM3-expressing cells. 2 μM TG (C) or 50 μM CCh (D) was first added to cells in the absence of external Ca\(^{2+}\) (first arrow). After 9 min, 2 mM Ca\(^{2+}\) solution was added to the bath solution (second arrow). Black, vector-transfected cells. Red or pink, hTRPM3-expressing cells. Representative traces from one of three independent experiments are shown. Each trace is the mean of 12 wells/condition.
Ca\(^{2+}\) entry pathway that apparently is distinct from the endogenous Ca\(^{2+}\) entry pathways present in HEK 293 cells.

DISCUSSION

We have identified the full-length hTRPM3 cDNA as a member of the TRP channel family. hTRPM3 is predominantly expressed in human kidney and, at lesser levels, in brain, testis, and spinal cord. In the kidney, hTRPM3 is localized in the collecting tubular epithelium as demonstrated by in situ hybridization. We have also shown that when expressed in HEK 293 cells, hTRPM3 is capable of mediating Ca\(^{2+}\) entry and is partially inhibited by Gd\(^{3+}\). hTRPM3-mediated Ca\(^{2+}\) entry is distinct from Ca\(^{2+}\) entry in vector-transfected cells, which is strongly blocked by Gd\(^{3+}\). Moreover, Ca\(^{2+}\) entry via hTRPM3 was enhanced after depletion of Ca\(^{2+}\) stores with TG or CCh.

hTRPM3 is the first member of the TRPM subfamily that has been shown to be modulated by store depletion. It is of interest to note that some members of the TRPC and TRPV subfamilies also are modulated by store depletion (for review see Ref. 1). The known TRPM subfamily members exhibit diverse channel characteristics. TRPM1 and TRPM4a are Ca\(^{2+}\)-permeable channels, but it is unclear whether they can be stimulated by store depletion (9). Distinct from TRPM4a, TRPM4b is activated directly by changes in intracellular Ca\(^{2+}\) without significant permeation of Ca\(^{2+}\) (10). TRPM2 is activated by ADP-ribose, NAD, and changes in redox status (5–7). TRPM7 is regulated by Mg\(^{2+}\)-ATP and/or inositol 1,4,5-trisphosphate (12–14). TRPM8 is activated by cold temperatures and cooling agents (16, 17). Therefore, in conjunction with a restricted tissue expression not observed with any other family members, hTRPM3 may have a unique biological function in humans.

We have shown that hTRPM3-mediated Ca\(^{2+}\) entry can be stimulated by lowering extracellular Ca\(^{2+}\) concentration (Fig. 3B), by passively depleting Ca\(^{2+}\) stores with TG treatment (Fig. 3C), and by treatment with CCh that activates G protein-coupled receptors (Fig. 3D). The store depletion-induced Ca\(^{2+}\) entry was significantly stimulated by adding 1 mM Ca\(^{2+}\) to the media in hTRPM3-expressing cells (Fig. 3B, right panel), whereas the addition of 1 mM Ca\(^{2+}\) to the media triggered only minimal hTRPM3 activity in cells of which intracellular Ca\(^{2+}\) stores were not depleted (compared with Fig. 3A, right panel). These results show that Ca\(^{2+}\) entry in hTRPM3-transfected cells was stimulated by store depletion, unlike some TRPC subfamily members such as TRPC3 and TRPC6, which show receptor-mediated activation independent of the store depletion (for review see Ref. 1).

The hTRPM3 gene maps to chromosome 9q21.12 between the two markers, D9S1874 and D9S1807. Diseases that have been linked to this region include amyotrophic lateral sclerosis with frontotemporal dementia, early-onset pulmonary cataract, familial hemophagocytic lymphohistiocytosis, infantile nephrophophathy, and hypomagnesemia with secondary hypocalcemia (ESH). Given its selective expression in kidney, hTRPM3 could be considered a candidate gene for HSH, be-
cause the phenotype is a renal insufficiency. The chromosomal location of hTRPM3 is 600 kb downstream of an Xq.9 translocation breakpoint interval described for one patient with HSH (28). Recently, two groups (29, 30) reported that a new member of the TRPM subfamily, TRPM6, expressed in both intestinal tissues and kidney, is associated with HSH. Indeed, human TRPM3 is 4 million bp 5′ to TRPM6 on 9q-21. Although TRPM6 has been implicated directly in HSH, this may not preclude the involvement of hTRPM3.

The kidney plays a major role in Ca\(^{2+}\) homeostasis. hTRPM3 could be involved in Ca\(^{2+}\) absorption directly because of its Ca\(^{2+}\) permeability. Indeed, in situ hybridization analysis demonstrated that hTRPM3 is predominantly present in the collecting tubule, which has frequently been implicated in active transcellular Ca\(^{2+}\) reabsorption for review see Ref. 31). Alternatively, hTRPM3 may function as a SOC that regulates parathyroid hormone-related peptide through their respective G protein-coupled receptors and downstream SOCs (for review see Ref. 32). Future experiments using antisense RNA or knock-out mice may help define the functional role of hTRPM3 in the kidney.

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