Alternative Splicing Switches the Divalent Cation Selectivity of TRPM3 Channels*

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TRPM3 is a poorly understood member of the large family of transient receptor potential (TRP) ion channels. Here we describe five novel splice variants of TRPM3, TRPM3a–1–5. These variants are characterized by a previously unknown amino terminus of 61 residues. The differences between the five variants arise through splice events at three different sites. One of these splice sites might be located in the pore region of the channel as indicated by sequence alignment with other, better-characterized TRP channels. We selected two splice variants, TRPM3a1 and TRPM3a2, that differ only in this presumed pore region and analyzed their biophysical characteristics after heterologous expression in human embryonic kidney 293 cells. TRPM3a1 as well as TRPM3a2 induced a novel, outwardly rectifying cationic conductance that was tightly regulated by intracellular Mg2+. However, these two variants are highly different in their ionic selectivity. Whereas TRPM3a1-encoded channels are poorly permeable for divalent cations, TRPM3a2-encoded channels are well permeated by Ca2+ and Mg2+. Additionally, we found that currents through TRPM3a2 are blocked by extracellular monovalent cations, whereas currents through TRPM3a1 are not. These differences unambiguously show that TRPM3 proteins constitute a pore-forming channel subunit and localize the position of the ion-conducting pore within the TRPM3 protein. Although the ionic selectivity of ion channels has traditionally been regarded as rather constant for a given channel-encoding gene, our results show that alternative splicing can be a mechanism to produce channels with very different selectivity profiles.

The transient receptor potential (TRP) gene family comprises at least 28 mammalian genes divided into seven subfamilies (1, 2). Most of the encoded proteins exhibit common structural features such as six predicted transmembrane (TM) domains with a putative pore loop between TM5 and TM6 and the so-called TRP box after TM6 (1, 2). Although all members of this group have been reported to form cationic channels, their mechanisms of activation, their regulation, and their biological functions are remarkably diverse. They also display a large variety of different cation selectivities (1, 2). For example, TRPM4 and TRPM5 have been described as impermeable for divalent cations (3–5), whereas TRPV5 and TRPV6 appear to be exclusively permeable for Ca2+ (6, 7). The diversity of TRP channels is further increased by the fact that most members of the TRP gene family can give rise to several different transcripts due to alternative splicing (8). In a few cases, the functional consequences of these alternative splice events are now beginning to emerge. For example, missplicing of TRPM6 transcripts is associated with a hereditary disorder called hypomagnesemia with secondary hypocalcemia (9, 10), and an amino-terminal-truncated variant of TRPM4 appears to modulate Ca2+ oscillations after receptor stimulation in T lymphocytes (11).

However, up to now, the largest number of different splice variants for any TRP family member has been described for TRPM3 (12, 13). Lee et al. (12) reported six splice variants of human TRPM3, which they named TRPM3a–f. Their lengths range from 1544 to 1579 amino acid residues. Functional data were only reported for the TRPM3a splice variant. Heterologously expressed TRPM3a exhibits a constitutive, Ca2+ concentration-dependent Ca2+ entry that can be augmented by Ca2+ store depletion or by stimulation of muscarinic receptors (12). In addition, Grimm et al. (13) reported one further human variant that has a total of only 1325 residues because of its considerably shorter carboxyl terminus (13). On its amino terminal end, however, this variant, which we will refer to as TRPM31325 throughout this report, possesses an additional 153 amino acids completely missing in the other reported TRPM3 variants. As is the case with TRPM3a, TRPM31325 generates constitutively active, Ca2+-permeable channels when heterologously expressed in human embryonic kidney 293 (HEK-293) cells. The activity of TRPM31325 channels can reportedly be enhanced by hypo-osmotically induced cell swelling (13) and by d-erythro-sphingosine (14).

In this report we describe five novel TRPM3 splice variants that we cloned as full-length cDNA constructs from mouse brain. These variants are 1699–1721 amino acid residues long and are characterized by a novel amino terminal sequence of 61 residues not described so far. Interestingly, two of the novel variants, designated TRPM3a1 and TRPM3a2, differ only in a region between the fifth and sixth transmembrane domains where the pore-forming region of the channel is assumed. This region is 12 amino acids longer in TRPM3a1 compared with TRPM3a2; additionally, a proline is substituted by an alanine. The principal
finding of our study is that this alteration of the primary structure induces a large change in the ionic selectivity of the resulting ion channels. We thus identify alternative splicing as a mechanism to modify the selectivity of TRPM3 channels.

**EXPERIMENTAL PROCEDURES**

**Cloning of TRPM3 Variants**—Oligonucleotide primers A, 5′-GAG AGC TGA GCG CAG GCT G-3′, and Z, 5′-TCC TGC ACA CCC TGA GCC-3′, were used to amplify Trpm3 transcripts after oligo(dT)18 primed reverse transcription of mouse brain total RNA. For amplification we used the Long Expand PCR kit (Roche Applied Science) and the following conditions: addition of the enzymes at 62 °C followed by 2 min at 94 °C, 10 cycles (94 °C, 10 s; 62 °C, 30 s; 68 °C, 5 min), 25 cycles (94 °C, 10 s; 62 °C, 30 s; 68 °C, 5 min + 20 s/cycle) and 7 min at 68 °C. Sequencing both strands of cloned fragments identified five independent clones encoding TRPM3b1, three encoding TRPM3b2, and three encoding TRPM3b3. Regarding TRPM3b4 and TRPM3b5, one clone was found for each splice variant (Fig. 1C). We introduced the ribosome binding site ACC GCC ACC and a Myc tag in-frame immediately 5′ to the start codon of Trpm3a1 and Trpm3a2 cDNAs, which were subsequently cloned into pCAGGS-IRE-S-GFP (15) for transient dicistronic expression of TRPM3 together with the green fluorescent protein (GFP). HEK-293T cells were transfected with plasmids, and equivalently 200 ng cDNA, were transfected in water for 24 h after transfection to reduce cell density. For generation of clones stably expressing Myc-tagged TRPM3b1-1-EYFP or TRPM3b2-EYFP fusion proteins, transfected cells were kept for 5 weeks in 500 μM fura-2 AM for 30 min at room temperature, washed in Ringer solution containing (in mM) 138 NaCl, 5.4 KCl, 2 CaCl2, 2 MgCl2, 20 glucose, 10 HEKPS, pH 7.2 adjusted to 7.2 (hydroxide of main cation and HCl) and osmolality to 315–335 mosmol/kg (adjusted with H2O or glucose). A liquid junction potential of ~15 mV was corrected for.

Cells were maintained during the recordings at room temperature in Ringer solution. All extracellular solutions (Table I) were based on 10 mM HEKPS; pH was adjusted to 7.2 (hydroxide of main cation and HCl) and osmolality to 315–335 mosmol/kg (glucose or H2O). Solutions were applied for short duration by a custom-made local perfusion system whose outlet was placed at <200 μm from the recorded cell.

**Fluorescent Ca2+ Measurements**—[Ca2+]i measurements of HEK-293 cells using fura-2-AM were performed as described previously (18). In brief, HEK-293 cells and HEK cell clones stably expressing Myc-tagged TRPM3 were transfected with Alexa 647- or Alexa 546-labeled cDNAs. Cells were imaged using a custom-made epifluorescence microscope (Spinning Disk Confocal) with a 60× objective (Carl Zeiss, Jena, Germany). Cells were imaged using 488 nm excitation, and 505–550 nm emission. Images were acquired at a rate of 2 s/cycle. Data were acquired and analyzed using MetaMorph software (Molecular Devices, Camp Hill, PA). Data were expressed as the difference between the fluorescence intensity of the channel of interest and the fluorescence intensity of a background area.

**RESULTS**

**Identification and Cloning of TRPM3 Variants from Mouse Brain**—Screening public databases we identified two partial human TRPM3 cDNA clones, AL136545 and XM_0136545 (Fig. 1C). Both clones derive from a single gene locus on human chromosome 9. The corresponding mouse gene shows a highly similar organization with 28 exons and spans more than 850 kb on mouse chromosome 19b (NCBI gene number 226025, Fig. 1A). Primers A and Z (Fig. 1A) were deduced from the mouse genomic sequence. Their sequences are located 5′ and 3′ to stop codons flanking an open reading frame with a predicted translation start that is also present in the human cDNA of clone AL136545. Using this primer combination, ~5.2-kbp cDNA fragments from mouse brain total RNA were amplified by reverse transcription PCR and subcloned (Fig. 1B). Similar products were amplified from mouse eye (data not shown). Each clone contained the complete coding sequence of mouse Trpm3. We identified five different splice variants, designated TRPM3a1, TRPM3a2, TRPM3b1, TRPM3b3, and TRPM3b5 (Fig. 1C). Their amino acid sequences comprise 1699 up to 1721 amino acids, which are encoded by exons 1, 3–7, and 9–28. The predicted exons 2 and 8 (NCBI gene number 226025) are not present in the cDNA of these TRPM3 variants from mouse, but 18 amino acid residues encoded by the corresponding exon 8 have been detected in the recently described human kTRPM3 variant (12). Exon 2 encodes a 59-amino acid sequence that corresponds to the amino terminal part of the human TRPM3var1 variant (13) but is absent in the a–f variants of hTRPM3. Exon 1 codes for a completely novel amino terminal sequence that is not present in any of the already described human TRPM3 variants. Among 13 independent mouse cDNA clones, we could not find a single one that contained exon 1 together with 2 (Fig. 1C), suggesting that these exons are...
FIG. 1. Identification of TRPM3 variants from mouse brain. A, schematic diagram of the mouse Trpm3 gene, comprising 28 exons. Location of primers A and Z used to amplify the cDNA by reverse transcription PCR and stop codons in-frame to the translation start are indicated. B, reverse transcription PCR amplification of 5.2-kbp fragments using primers A and Z. C, schematic presentation of TRPM3 with transmembrane domains 1–6, coiled coil region (cc), and TRP homology domain (Trp). Novel mouse TRPM3 protein variants shown as thick black lines are compared with the human variants hTRPM3a–f (12) and hTRPM3c (13). The numbers of amino acid residues of each variant are indicated in parentheses. D, putative pore regions of TRPM3α1 and TRPM3α2 compared with the corresponding mouse sequences of TRPM6 (accession number NP_700466), TRPM7 (accession number NP_067425), TRPV5 (accession number IC7795), and TRPV6 (accession number CAD62684). The 12 additional amino acid residues present in TRPM3α1 are indicated. Identical residues are boxed in black, conserved in gray. An aspartate residue that determines Ca\(^{2+}\) permeation of the TRPV5/TRPV6 pore is marked by an asterisk. Residues proposed to build the selectivity filter of TRPV6 are underlined (29).

expressed in a mutually exclusive fashion. This could be explained either by tightly regulated posttranscriptional processing or by expression of these variants from alternative promoters.

Starting from residue 156, however, mouse TRPM3 proteins show ~97% amino acid sequence identity to the human variants. Therein, mouse TRPM3α1 corresponds to human TRPM3c, mTRPM3α2 to hTRPM3a, mTRPM3α3 to hTRPM3b, mTRPM3α4 to hTRPM3e and mTRPM3α5 to hTRPM3d (Fig. 1C). The close correspondence between the respective mouse and human transcripts indicates that not only the amino acid sequence but also the splice events are highly conserved between mouse and human. Such strong conservation is highly suggestive of functional importance of TRPM3 proteins generally but also of their regulation and modification by alternative splicing.

Expression Pattern of TRPM3—We subsequently analyzed the expression pattern of the mouse Trpm3 gene by Northern blot and by in situ hybridization (Fig. 2). We found Trpm3 expression in brain and eye with transcripts of ~1.8–2.6, ~3.7, ~5.8, ~7.6, ~9.4, ~12, and >15 kb (Fig. 2A). In agreement with previous results (13), we could not detect Trpm3 transcripts in mouse kidney by Northern blot (data not shown). In situ hybridization experiments showed Trpm3 transcripts in several regions of the mouse brain such as the dentate gyrus, the intermediate lateral septal nuclei, the intragrusus, and the tenia tecta (Fig. 2, C and D). Strongest Trpm3 expression was found in the epithelial cells of choroid plexus (Fig. 2, B–D) where transcripts could readily be detected in 20 μg of total RNA (Fig. 2A).

TRPM3α1 and TRPM3α2 Form Cation Channels Regulated by Intracellular Mg\(^{2+}\)—Alternative splicing within exon 24 of both the mouse and the human TRPM3 gene leads to the presence of 12 additional amino acid residues and the additional replacement of an alanine by a proline residue (Fig. 1D) (12). Interestingly, this domain, which is present in mTRPM3α1 and hTRPM3c (12) but absent in all other variants, lies between the presumed fifth and sixth transmembrane domains (Fig. 1C). In analogy to the topologically similar members of the TRPV subfamily (19), it is likely that this part of the protein contributes to the pore-forming region of the channel (Fig. 1D). The variants TRPM3α1 and TRPM3α2 differ only in this region (Fig. 1, C and D). We set out to test whether this change in the primary sequence altered the biophysical characteristics of the resulting ion channels. To this end, we expressed TRPM3α variants in HEK-293 cells, which do not express TRPM3 endogenously (Fig. 2A).

Starting with TRPM3α1, we noticed that transfected cells, but not control cells, exhibit a constitutively active, outwardly rectifying current. This current was already visible directly after establishing the whole cell configuration (Fig. 3, A and B). Replacing the extracellular cations by the impermeant cation N-methyl-d-glucamine (NMDG\(^{–}\)) shifted the reversal potential from −9.5 ± 0.5 to −66.2 ± 8 mV (n = 5), indicating that the current was predominately carried by cations (Fig. 3A). When we used pipette solutions without Mg\(^{2+}\), TRPM3α1-induced currents increased strongly within the first minutes of whole cell recording (Fig. 3, B and C). Conversely, when we increased the free Mg\(^{2+}\) concentration in the recording pipette to 9 mM (Fig. 3, C and D), TRPM3α1-induced currents vanished rapidly. Using 0.9 mM free Mg\(^{2+}\) in the recording pipette, currents of intermediate size were observed (Fig. 3D). These data indicate that TRPM3α1 is regulated by physiological concentrations of free intracellular Mg\(^{2+}\), which typically is found to be in the order of 0.5–1 mM (20).

We next turned to TRPM3α2 and asked whether this variant also forms a functional channel. In extracellular Ringer solu-
tion, TRPM3α2 currents were small and increased only slightly during prolonged recording (Fig. 4A). However, when we again replaced all extracellular cations with NMDG⁺, we observed large outward currents, which disappeared as soon as standard conditions were re-established (Fig. 4, A and B). Such currents were not observed in control cells measured under identical conditions. The absence of detectable inward currents under these conditions indicates that, similar to TRPM3α1, the TRPM3α2-induced conductance is mainly permeable to cations. We then tested whether intracellular Mg²⁺ also regulates TRPM3α2 channels. Very similar to our results with TRPM3α1, we found large currents in the absence of intracellular Mg²⁺ but no detectable currents with 7 mM free Mg²⁺ in the pipette solution (Fig. 4C). Therefore, block by intracellular Mg²⁺ appears to be a common property of the two splice variants.

Block by intracellular Mg²⁺, however, is not a unique feature of TRPM3 channels but has also been described for the related ion channels TRPM6 (21) and TRPM7 (22). Channels encoded by TRPM7 or TRPM6 do not show such a voltage-dependent facilitation upon depolarization to values higher than +60 mV (Fig. 3E, arrows), similar to those described for TRPM4, TRPM5, and TRPM8 (4, 5, 24). Channels encoded by TRPM7 or TRPM6 do not show such a voltage-dependent facilitation (21, 25). Also, when we exposed TRPM3α2-expressing cells to an extracellular solution containing only NMDG⁺ (to evoke the large outward currents), a voltage-dependent facilitation of the current could be observed (Fig. 4D, arrows). Therefore, TRPM3α1 and TRPM3α2 channels show a unique combination of biophysical properties that are not found in any other member of the TRPM channel family.

Splicing Changes the Ion Selectivity of TRPM3 Channels—To determine the ionic selectivity of the TRPM3α1 and TRPM3α2 splice variants, we measured TRPM3-dependent currents under bi-ionic conditions and analyzed the reversal potential (Fig. 5). Fig. 5B shows that the reversal potential is
significantly more positive in cells expressing TRPM3α2 compared with TRPM3α1-expressing cells when Ca\(^{2+}\) and NMDG\(^{+}\) are the only cations present in the extracellular solution. Similar results were obtained with Mg\(^{2+}\) instead of Ca\(^{2+}\). An estimation of the relative permeability ratios utilizing the Goldman-Hodgkin-Katz formalism (26) showed that TRPM3α2 channels are at least 10 times more permeable for Ca\(^{2+}\) (Fig. 5C) and at least 100 times more permeable for Mg\(^{2+}\) than TRPM3α1 channels (Fig. 5D). This large difference in ion permeation properties establishes that the ion-conducting pore of TRPM3 channels is affected by the sequence differences between the two splice variants α1 and α2 (Fig. 1C). It therefore provides evidence that this region of the protein is part of the ion-conducting pore of TRPM3. Furthermore, this finding proves conclusively that the observed currents after overexpressing TRPM3 proteins are mediated by these proteins and are not the result of up-regulated, endogenous conductances.

Divalent Inward Currents through TRPM3α2—The higher selectivity for divalent cations of the TRPM3α2 variant does not necessarily imply that this variant is capable of conducting sizeable divalent inward currents. However, when we applied high extracellular concentrations of either Ca\(^{2+}\) or Mg\(^{2+}\) (Fig. 6), TRPM3α2-expressing cells conducted inward currents of up to 1 nA (at −80 mV, Fig. 6). Such currents were seen neither in
TRPM3\(\alpha1\)-expressing cells nor in control cells. These data directly demonstrate that, in sharp contrast to TRPM3\(\alpha1\), TRPM3\(\alpha2\)-encoded channels not only have a rather high selectivity for divalent cations but are also capable of conducting sizeable divalent currents.

**TRPM3 Channel Variants Are Differentially Regulated by Extracellular Cations**—Comparison of TRPM3\(\alpha1\) and TRPM3\(\alpha2\) outward currents obtained in extracellular Ringer solution and a solution containing NMDG\(^+\) as the only cation implied that outward currents through TRPM3\(\alpha2\) are inhibited by cations in the Ringer solution (Figs. 3A and 4B). We were intrigued by the fact that this block affected only outward currents through TRPM3\(\alpha2\) but not through TRPM3\(\alpha1\). We therefore investigated the nature of this inhibition using solutions that contained only one permeable cation in addition to NMDG\(^+\). We found that Ca\(^{2+}\) blocks outward currents through TRPM3\(\alpha2\) as well as through TRPM3\(\alpha1\) dose dependently (Figs. 5A and 7B). Similar results were obtained for Mg\(^{2+}\) (Fig. 7B). On the other hand, the monovalent cations Na\(^+\) and K\(^+\) quite strongly reduced TRPM3\(\alpha2\) outward currents, whereas neither of them affected currents through TRPM3\(\alpha1\) (Fig. 7, A and B).

TRPM3\(\alpha2\) thus is inhibited by all cations tested on the extracellular side. It is important to note, however, that at their respective physiological concentrations, none of the cations blocked outward currents through TRPM3\(\alpha2\) completely. Nevertheless, these results imply that TRPM3 channel activity is tightly regulated by the concentration of extracellular cations. While TRPM3\(\alpha1\) channels are only sensitive to divalent cations, TRPM3\(\alpha2\) channels are sensitive to all extracellular cations.

**Expression of TRPM3 Variants Increases Resting Ca\(^{2+}\) Levels**—We compared intracellular Ca\(^{2+}\) concentrations in untransfected control cells to HEK-293 cells stably expressing either TRPM3\(\alpha1\) or TRPM3\(\alpha2\) at similar levels (Fig. 8A). In Ringer solution containing 2 mM Ca\(^{2+}\), TRPM3\(\alpha2\)-expressing cells showed significantly (p < 0.001) increased steady state Ca\(^{2+}\) levels of 145 ± 2.6 nM compared with TRPM3\(\alpha1\)-expressing cells (114 ± 2.3 nM) and control cells (84 ± 1.6 nM) (Fig. 8B). When we exposed the cells to an extracellular solution devoid of Ca\(^{2+}\) (containing 2 mM EGTA), intracellular Ca\(^{2+}\) levels dropped to 70–80 nM, irrespective of the expression of TRPM3 channels (Fig. 8B). Upon re-addition of 2 mM extracellular Ca\(^{2+}\), TRPM3\(\alpha2\)-expressing cells showed an overshooting rise of the intracellular Ca\(^{2+}\) concentration (Fig. 8B). Such an overshoot was seen neither in TRPM3\(\alpha1\)-expressing cells nor in control cells (Fig. 8B). These data indicate that TRPM3\(\alpha2\)-expressing cells possess an increased permeability for Ca\(^{2+}\) ions under physiological extracellular ionic conditions that likely causes the larger basal intracellular Ca\(^{2+}\) levels. On the other hand, TRPM3\(\alpha1\)-expressing cells seem to have a reduced permeability for Ca\(^{2+}\) (compared with TRPM3\(\alpha2\)-expressing cells) as witnessed by the reduced Ca\(^{2+}\) influx after extracellular Ca\(^{2+}\) re-addition and the lower basal intracellular Ca\(^{2+}\) concentration. These observations agree with our electrophysiologic experiments in which we showed that the concentration of extracellular cations influences the resting intracellular Ca\(^{2+}\) concentration. For instance, we found that the outward currents through TRPM3\(\alpha2\) are highly sensitive to extracellular Na\(^+\) and K\(^+\), whereas those through TRPM3\(\alpha1\) are only sensitive to divalent cations.

**Table 1: Ionic composition of extracellular solutions**

| Name of solution | NaCl | KCl | CsCl | NMDG | CaCl\(_2\) | MgCl\(_2\) | Glucose |
|------------------|------|-----|------|------|----------|----------|---------|
| Ringer           | 145  | 10  | 0    | 2    | 2        | 10       | 10      |
| NMDG             | 0    | 0   | 0    | 10   | 2        | 10       | 10      |
| 1 Ca             | 145  | 0   | 0    | 1    | 0        | 10       | 10      |
| 10 Ca            | 145  | 0   | 0    | 10   | 0        | 10       | 10      |
| 65 Ca            | 73   | 0   | 0    | 65   | 0        | 10       | 10      |
| 120 Ca           | 0    | 0   | 0    | 120  | 0        | 10       | 10      |
| 1 Mg             | 145  | 0   | 0    | 1    | 0        | 10       | 10      |
| 10 Mg            | 145  | 0   | 0    | 10   | 0        | 10       | 10      |
| 65 Mg            | 73   | 0   | 0    | 65   | 0        | 10       | 10      |
| 120 Mg           | 0    | 0   | 0    | 120  | 0        | 10       | 10      |
| 0 Na             | 145  | 0   | 0    | 0    | 0        | 10       | 10      |
| 10 Na            | 145  | 0   | 0    | 0    | 0        | 10       | 10      |
| 73 Na            | 73   | 0   | 0    | 73   | 0        | 10       | 10      |
| 145 Na           | 145  | 0   | 0    | 0    | 0        | 10       | 10      |
| 0 K              | 0    | 0   | 0    | 0    | 0        | 10       | 10      |
| 10 K             | 145  | 0   | 0    | 145  | 0        | 10       | 10      |
| 73 K             | 73   | 2   | 0    | 73   | 0        | 10       | 10      |
| 145 K            | 145  | 2   | 0    | 145  | 0        | 10       | 10      |
In this report, we have described five novel splice variants expressed in mouse brain and eye tissues that originate from the Trpm3 gene. Presently, four regions of modifications within the protein are known, and, additionally, variations of the length and sequence of the amino- and carboxyl-terminal regions have been described (Refs. 12 and 13 and this report). The Trpm3 gene therefore potentially encodes for a plethora of different proteins, 12 of which have up to now been verified experimentally. Here, we have focused on splice variants that differ only in a domain predicted to contribute to the ion-conducting pore.

**Alternative Splicing Switches the Ion Selectivity of TRPM3 Channels**—The selectivity of ion channels is thought to be determined by the geometry and charge distribution of the selectivity filter, usually envisioned as the narrowest part of the channel pore (26). Typically, all members of an ion channel family, such as voltage-gated Na⁺, K⁺, or Ca²⁺ channels, share common ion selectivities. The TRP family of ion channels is already somewhat unusual in this respect as it encompasses members with quite diverging cationic selectivity profiles (Refs. 12 and 13). In vivo, such a change in ionic selectivity must be expected to have considerable consequences for the function of the channel and the cellular ionic environment.
physiology of the cell that expresses it.

Previously, it has been shown that subtle manipulations of key amino acid residues can substantially influence the divalent selectivity of ion channels. Voltage-gated Na\(^+\) channels that are transformed to conduct Ca\(^{2+}\) ions by exchanging a lysine and/or an alanine to a glutamate residue are an extreme example (27). However, these changes in the primary amino acid sequence have been introduced artificially, whereas the variations in selectivity we describe for TRP3M have evolved naturally and occur in vivo. Although alternative splicing is increasingly recognized as a potent mechanism to dynamically modify ion channel properties (28), it has not yet been implicated in directly modifying the selectivity of a channel. Interestingly, Trpm1, the closest relative of Trpm3, also encodes splice variants that differ in the pore-forming region (8) and might therefore represent a further example for such a mechanism.

Editing of RNA is another mechanism of posttranscriptional modification occurring in vivo. Deamidation of a selected adenosine in primary transcripts of non-NMDA ionotropic glutamate receptors leads to the substitution of a glutamine by an arginine within the channel pore. This has been shown to reduce the Ca\(^{2+}\) selectivity of those channels with important consequences for their physiology (27).

**Locating the Ion-conducting Pore in TRPM Channels**—The switch of ionic selectivity in TRP3M variants is brought about by removing a short stretch of 12 amino acid residues and exchanging 1 further residue within the linker domain between the presumed fifth and sixth transmembrane regions (Fig. 1B). The differences in ion selectivity seen for the TRP3M splice variants strongly indicate that this linker domain constitutes the pore of TRP3M. Although this domain could already be suspected to be the ion-conducting pore, due to direct evidence obtained for TRPV1, TRPV4, TRPV5, and TRPV6 channels (19), this prediction has not been confirmed up to now for any member of the TRP subfamily.

Compared with the presumed pore regions of other members of the TRP family, the pore loop of TRP3M is considerably longer by 8 (TRP3M\(\alpha\)2) and 20 (TRP3M\(\alpha\)1) additional amino acid residues (Fig. 1C). The domains that build the proposed selectivity filter of the Ca\(^{2+}\)-selective TRPV5/6 channels (29) are conserved in TRP3M proteins. The splicing within the TRP3M channel pore introduces additional, positively charged amino acid residues into this domain. This might decrease the Ca\(^{2+}\) permeability of TRP3M\(\alpha\)1 compared with TRP3M\(\alpha\)2, perhaps simply because of increased electrostatic repulsion. In line with this reasoning, in AMPA and kainate receptors the aforementioned replacement of a glutamate by a positively charged arginine residue by RNA editing reduces the Ca\(^{2+}\) selectivity of those channels as well (30). Conversely, artificially introducing negatively charged glutamate residues in the pore of Na\(^{+}\) channels increases their divalent selectivity (27).

**Block of TRP3M Channels by Intra- and Extracellular Cations**—Our data show that both TRP3M\(\alpha\)1 and TRP3M\(\alpha\)2 are regulated by physiological concentrations of intracellular Mg\(^{2+}\), similar to related members of the TRP family such as TRPM6 and TRPM7 (21, 22). Previously, the short human variant TRP3M\(\alpha\)25 has been reported to mediate increased calcium entry when the osmolality of the extracellular solution was shifted from 300 to 200 mosmol/kg (13), a stimulus that induces considerable swelling of the cells. Conceivably, the Mg\(^{2+}\) dependence that we observed in whole cell patch clamp experiments offers a mechanistic explanation of the sensitivity to changes in osmolality. Because the intracellular cation concentration and the cell volume are mutually and inversely related, regulation of TRP3M activity by intracellular Mg\(^{2+}\) might explain why TRP3M shows increased or reduced activity in hypotonic and hypertonic solutions, respectively.

Our experiments were performed in isotonic solutions. Under these conditions we found that TRP3M currents are also tightly regulated by extracellular cations. However, only TRP3M\(\alpha\)2 channel activity was influenced by the extracellular concentration of monovalent cations, whereas both TRP3M variants were inhibited by high extracellular divalent concentrations. Block by extracellular Na\(^{+}\) is a highly uncommon feature of ion channels, but not entirely unprecedented. Inward rectifier (31) and, especially, HERG (32) potassium channels have also been shown to be inhibited by extracellular Na\(^{+}\).

Although the block of TRP3M\(\alpha\)2 by extracellular Na\(^{+}\) concentrations in the physiological range seemed to be severe in electrophysiological recordings (Fig. 7), it does not appear to be complete. The intracellular Ca\(^{2+}\) concentration in TRP3M\(\alpha\)2-overexpressing cells was elevated (Fig. 8), indicating that, also under physiological extracellular conditions, Ca\(^{2+}\) can enter the cell through TRP3M\(\alpha\)2 channels at a rate too low to be detectable in electrophysiological recordings but measurable in Ca\(^{2+}\) imaging experiments.

**Functional Role of TRP3M**—We found that TRP3M is strongly expressed in the choroid plexus (Fig. 2). This tissue in the ventricles of the brain is responsible for the formation and the regulation of cerebrospinal fluid. It has been demonstrated that the concentrations of ions such as Ca\(^{2+}\) in cerebrospinal fluid are carefully regulated and are independent of variations in the plasma concentrations of these ions (33). The high expression in the choroid plexus might indicate involvement of TRP3M proteins in the production of cerebrospinal fluid or the regulation of its ionic composition (Fig. 2A). At present we do not know which of the various TRP3M variants are present in the choroid plexus. However, the high selectivity for divalent cations of TRP3M\(\alpha\)2 channels makes this variant a good candidate to play a role in the regulation of divalent cation concentration in cerebrospinal fluid.

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