Gain-of-function Mutation in TRPML3 Causes the Mouse Varitint-Waddler Phenotype*  

TRPML3 is a member of the TRPML subfamily of the transient receptor potential cation channel superfamily. The TRPML3(A419P) mutation causes a severe form, whereas the TRPML3(I362T/A419P) mutation results in a milder form of the varitint-waddler phenotype. The channel properties of TRPML3 and how the mutations cause each phenotype are not known. In this study, we report the first channel properties of TRPML3 as a strongly inward rectifying cation channel with a novel regulation by extracytosolic Ca²⁺. Preincubating the extracytosolic face of TRPML3 in Na⁺-free medium is required for channel activation, but then the channel slowly inactivates. The A419P mutation locks the channel in an open unregulated state. Similar gain of function was observed with the A419G mutation, which, like A419P, is expected to destabilize the α-helical fifth transmembrane domain of TRPML3. The I362T mutation results in an inactive channel, but the channel properties of TRPML3(I362T/A419P) are similar to those of TRPML3(A419P). However, the surface expression and current density of TRPML3(I362T/A419P) are lower than those of TRPML3(A419P). The A419P mutation also affects channel glycosylation and causes massive cell death. These findings show that the varitint-waddler phenotype is due to a gain of function of TRPML3(A419P) that is reduced by the TRPML3(I362T/A419P) mutant, resulting in a milder phenotype.

EXPERIMENTAL PROCEDURES

Plasmid Construction, Mutagenesis, and Reagents—Human TRPML3 was amplified from human placenta and cloned into the pEGFP-C1 and p3XFLAG-CMV-7.1 vectors. Mutations were introduced with the QuikChange kit.

Cell Culture, Transfection, and Western Blotting—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were transfected with Lipofectamine 2000 and used 24–48 h post-transfection. Cell extracts were prepared by sonication in homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 5 mM MgCl₂, and one protease inhibitor tablet (Roche Applied Science). Microsomal pellets were extracted with 1% Triton X-100, treated with PNGase F or Endo H, and immunoblotted with anti-GFP antibodies.

Surface Biotinylation Assay—Cells were washed with PBS and incubated in 1 mg/ml sulfo-L-cysteine in PBS for 30 min on ice, and free biotin was quenched with 100 mM glycine in PBS. The cells were washed with PBS, and lysates were prepared as described above. The lysates were centrifuged; protein was equalized; and 12.5% avidin beads were added. After a 2-h incubation at 4 °C, the beads were collected and washed three times with 0.5% Triton X-100 in PBS, and the proteins were Western-blotted with anti-FLAG antibodies.

Current Recordings—The whole cell current was recorded at room temperature with an Axopatch 200B amplifier and filtered at 5 kHz. Data were acquired with pCLAMP 9.0 and DigiData 1322. Currents were measured by application of 400-ms ramps from −100 to +100 mV every 5 s from a holding potential of 0 mV or by holding the membrane potential at −100 mV. Results were analyzed with pCLAMP 9.0 and Origin software. The pipette solution contained 140 mM KCl, 1 mM HEPES, 10 mM NaCl, 0.5 mM MgCl₂, 2 mM CaCl₂, 0.5 mM EGTA, and 10 mM glucose.

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2. The abbreviations used are: PNGase F, peptide-N-glycosidase F; Endo H, endoglycosidase H; GFP, green fluorescent protein; PBS, phosphate-buffered saline; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid; NMDG, N-methyl-D-glucamine; ER, endoplasmic reticulum; EGF, enhanced green fluorescent protein; WT, wild-type.
1.13 mM MgCl₂, 5 mM MgATP, and 10 mM BAPTA or 10 mM EGTA (pH 7.3) with KOH. When required, KCl was replaced with NaCl or NMDG-Cl. The bath solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES (pH 7.4) with NaOH. The Na⁺/H⁺-free solution contained 140 mM NMDG-Cl, 0.2 mM EGTA, and 10 mM HEPES (pH 7.4) with HCl.

RESULTS AND DISCUSSION

TRPML3 Is an Inward Rectifying Cation Channel Regulated by Extracellular Ions—The predicted structure of TRPML3 and its putative N-glycosylation sites are shown in Fig. 1a. Native TRPML3 is localized in intracellular vesicles and in the plasma membrane of hair cells (4). However, a recent study reported that TRPML3 tagged at its C terminus with yellow fluorescent protein is targeted to the endoplasmic reticulum (ER) (6). This may be because TRPML channels have targeting motifs in their C termini (7). Therefore, we tagged TRPML3 at its N terminus with EGFP. Biotinylation assay showed that EGFP-TRPML3 was targeted to the plasma membrane and thus was used in all experiments reported below. The current was recorded either by ramps or by holding the membrane potential at −100 mV (see “Experimental Procedures”).

Notably, Fig. 1b shows that TRPML3 is not active before exposing the extracytosolic face of the channel to Na⁺-free solution. However, when the cells were briefly incubated in Na⁺-free bath solution and then Na⁺ was re-added, a large, strongly inward rectifying current was observed (Fig. 1b). The current then slowly inactivated. It was necessary to incubate the cells in Na⁺-free medium because preincubation with 20 mM external Na⁺ (Na⁺o) was not sufficient to activate the channel (data not shown). Fig. 1c shows the I/V relationship of TRPML3. Fig. 1d shows that inhibition by cations and channel inactivation are specific to extracytosolic cations. Thus, replacing pipette K⁺ with NMGD⁺ did not activate the current, did not overcome the need to expose the cells to Na⁺-free solution, and did not prevent the spontaneous channel inactivation. Finally, Fig. 1e shows that after each inactivation, the current can be fully reactivated by re-exposing the cells to Na⁺-free medium. Although the rate and extent of spontaneous current inactivation were somewhat variable, Fig. 1 shows that TRPML3 channel activity is specifically inhibited by extracytosolic Na⁺.

TRPML3(A419P) Is Locked in an Open Unregulated State—To understand how the A419P mutation leads to the varitint-waddler

FIGURE 1. Channel properties of TRPML3. a, the model shows TRPML3 with the predicted glycosylation sites and the positions of Ile(n)2 and Ala(n)9. b–e, the whole cell current was measured in human embryonic kidney cells transfected with GFP-TRPML3. The pipette solution contained 140 mM K⁺ (b, c, and e) or 140 mM NMGD⁺ (d) and 10 mM EGTA (b–d) or 10 mM BAPTA (e). The cells were alternately perfused with bath solution containing either 140 mM Na⁺ (black bars) or 140 mM NMDG⁺ (0 Na⁺; gray bars). The current was measured by applying 400-ms ramps from −100 to +100 mV every 5 s and is plotted at −100 mV (b, d, and e) and +100 mV (e).
phenotype, we analyzed the function and expression of TRPML3(A419P). Fig. 2 (a–c) shows that TRPML3(A419P) is constitutively active and lost the regulation by Na\(^{+}\)/H\(^{+}\). Thus, the TRPML3(A419P) current is observed without preincubation in Na\(^{+}\)/H\(^{+}\)-free medium; incubation in Na\(^{+}\)/H\(^{+}\)-free medium does not increase channel activity; and the current does spontaneously inactivate. The same behavior was observed by recording the TRPML3(A419P) current at a holding potential of \(-100\) mV. Fig. 2c shows that no current is observed in cells expressing TRPML3 upon clamping the membrane potential at \(-100\) mV and then incubated in Na\(^{+}\)-free medium (gray bar) and subsequently in Na\(^{+}\)-containing medium. Note that stepping the membrane potential from \(-100\) mV in cells expressing TRPML3(A419P) resulted in a large current. The response of TRPML3(A419G) to stepping the membrane potential to \(-100\) mV is shown in f.

Proline introduces a kink or a break in \(\alpha\)-helical transmembrane domains because its amide cannot form hydrogen bonds and because its side chain interferes with helix formation (8). The A419P mutation may therefore lock TRPML3 in an open state by destabilizing its \(\alpha\)-helical fifth transmembrane domain, preventing the channel from closing. Alternatively, the A419P mutation may prevent channel inactivation by extracytosolic ions, resulting in a spontaneously active channel. To test the effect of \(\alpha\)-helix destabilization, we tested whether mutating Ala\(^{419}\) to Gly can reproduce the effect of the A419P mutation. We also tested the effect of mutating Ala\(^{419}\) to Val, which only weakly disrupts \(\alpha\)-helices (9). TRPML3(A419V) retains only \(\sim 20\%\) spontaneous activity and was activated by preincubation in Na\(^{+}\)-free solution (data not shown). On the other hand, to the extent tested here, the channel properties of TRPML3(A419G) are the same as those of TRPML3(A419P). Fig. 2 (d–f) shows that TRPML3(A419G) is spontaneously active, that incubation in Na\(^{+}\)-free medium does not further increase channel activity, and that the TRPML3(A419G) current shows minimal inactivation.
The results in Fig. 2 suggest that destabilization of the fifth transmembrane domain of TRPML3 locks the channel in an open state. This can occur by two independent mechanisms. Disruption of the \( \alpha \)-helical structure may prevent the conformational change that switches the channel between the open and close states. Alternatively, the fifth transmembrane domain may communicate with the regulatory extracytosolic site that inactivates the channel in the presence of \( \text{Na}^+ \). Destabilization of the fifth transmembrane domain may disrupt this communication, preventing channel inactivation. Future studies to determining the mechanism by which extracytosolic ions regulate TRPML3 activity should help distinguish between the two mechanisms. Nevertheless, the findings in Fig. 2 indicate that the varitint-waddler phenotype is due to a gain-of-function mutation in TRPML3. These findings also explain why the heterozygous wild-type (WT)/A419P mouse displays the varitint-waddler phenotype. The constitutively active channel is likely to disrupt a key cellular function(s) that results in disruption of multiple cellular activities because homozygous A419P/A419P results in embryonic lethality.

**TRPML3(I362T) Is Not Active, but TRPML3(I362T/A419P) Is Locked in an Open Unregulated State.**—The presence of the A419P and I362T mutations in the same allele results in a milder varitint-waddler phenotype (5). In an attempt to understand this phenotype, we analyzed the function of TRPML3(I362T) and TRPML3(I362T/A419P). Fig. 3a shows that TRPML3(I362T) is not active and cannot be activated by incubation in \( \text{Na}^+ \)-free solution. Yet, surprisingly, Fig. 3 (b and c) shows that TRPML3(I362T/A419P) is active and that the channel properties of TRPML3(I362T/A419P) are similar to those of TRPML3(A419P). However, Fig. 3d shows that the current density of TRPML3(I362T/A419P) is significantly lower than that of TRPML3(A419P). The biotinylation assay in Fig. 3f shows that although the I362T mutation alone had no effect on the surface expression of TRPML3, it reduced the surface expression of TRPML3(I362T/A419P) relative to that of TRPML3(A419P). The means \( \pm \) S.E. of three experiments are plotted in f. The blots in e and f were probed with anti-FLAG antibodies.

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**FIGURE 3. Effect of the I362T and A419P mutations on TRPML3 expression and function.** The protocol and conditions of Fig. 1 were used to measure the current of TRPML3(I362T) (a) or TRPML3(I362T/A419P) (b and c). The current amplitude of TRPML3, TRPML3(A419P), and TRPML3(I362T/A419P) is shown in d. pF, picofarad. In e, extract was prepared from cells expressing TRPML3 (ML3) or the mutants, and glycosylation was analyzed by digestion with PNGase F and Endo H. The Endo H-sensitive species are bounded by *thin white lines*, and the Endo H-resistant portions of TRPML3(A419P) and TRPML3(I362T/A419P) are bounded by *thick white lines*. Note the altered glycosylation of the A419P mutants and their partial Endo H resistance. The results from analysis of the surface expression by biotinylation of TRPML3 and the indicated mutants are shown in f. The bars to the right show the reduction in surface expression of TRPML3(I362T/A419P) relative to that of TRPML3(A419P). To determine the change in surface expression due to the I362T mutation, the surface expression of TRPML3(A419P) and TRPML3(I362T/A419P) was determined relative to that of TRPML3(I362T). The surface expression of TRPML3(A419P) was averaged and set as 1, and the surface expression of TRPML3(I362T/A419P) was determined relative to that of TRPML3(I362T). The means \( \pm \) S.E. of three experiments are plotted in f. The blots in e and f were probed with anti-FLAG antibodies.
is retained in the ER (6). However, analysis of TRPML3 glycosylation revealed that, as expected, PNGase F generated the non-glycosylated protein. Significantly, the majority of WT-TRPML3 was not sensitive to Endo H, indicating that TRPML3 must have exited the ER and was modified in the trans-Golgi. This is in agreement with the finding that native TRPML3 is expressed in vesicular compartments and the plasma membrane (4). In the previous work, TRPML3 was tagged at its C terminus (6), which contains important targeting sequences (7), likely resulting in mistargeting of TRPML3 to the ER.

The A419P mutants migrated faster compared with WT-TRPML3 and to a position slightly above the Endo H-sensitive form of WT-TRPML3 (Fig. 3e, bounded by thin white lines). Treatment with PNGase F generated the non-glycosylated proteins. Treatment with Endo H showed that a portion of the A419P mutants are Endo H-resistant (bounded by thick white lines), indicating that at least a portion of these mutants exited the ER. This was further verified by a biotinylation assay, which showed that TRPML3 and the mutants trafficked to the plasma membrane (Fig. 3f). Despite the lower expression level of the mutants, the rate of success in recording TRPML3 current in cells that survived expression of the mutants was the same as that for WT-TRPML3. In the same set of experiments, current was observed in 11/15, 19/20, and 13/14 cells expressing WT-TRPML3, TRPML3(A419P), and TRPML3(I362T/A419P), respectively. These findings suggest that the A419P mutation modifies glycosylation of TRPML3, but it does not prevent its targeting to the plasma membrane and perhaps other organelles.

The A419P Mutation Affects Cell Survival—A cellular effect of the A419P mutation became apparent when we noticed that expression of the A419P and I362T/A419P mutants resulted in massive cell death, which was manifested as a large number of floating cells in the culture. A more quantitative estimate of cell death was obtained by collecting all the cells from the culture (attached and floating) and blotting for expression of TRPML3 in the same amount of protein extract. The level of the A419P mutants was <10% that of WT-TRPML3 (data not shown). To reduce cell death, the cells were grown in medium containing 0.3 mM Ca\(^{2+}\). Fig. 3 (d and e) shows that even in reduced medium Ca\(^{2+}\), the expression of the A419P mutants was only ~23 ± 5% that of WT-TRPML3. The massive cell killing is, of course, an exaggerated effect of the overexpression of the constitutively active A419P mutants. Nevertheless, a lower expression level of the native A419P mutants may kill the cells at a slower rate but should be toxic and kill all cells that express TRPML3. A preliminary reverse transcription-PCR survey showed that all tissues express TRPML3 mRNA and that high levels are found in epithelial tissues and the brain. It is possible that the higher expression of TRPML3 in the inner ear, keratinocytes, and the brain accounts for the higher susceptibility of these tissues to the A419P mutation.

In summary, in this work, we have shown that the varitint-waddler phenotype is caused by the gain-of-function mutation TRPML3(A419P), which is toxic and kills cells. The milder phenotype of TRPML3(I362T/A419P) can be explained by the reduced surface expression and thus lower current density of this mutant.

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