Identification of a single aspartate residue critical for both fast and slow calcium-dependent inactivation of the human TRPML1 channel

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Transient receptor potential mucolipin subfamily 1 (TRPML1) is a nonselective cation channel mainly located in late endosomes and lysosomes. Mutations of the gene encoding human TRPML1 can cause severe lysosomal diseases. The activity of TRPML1 is regulated by both Ca2+ and H+, which are important for its critical physiological functions in membrane trafficking, exocytosis, autophagy, and intracellular signal transduction. However, the molecular mechanism of its dual regulation by Ca2+ and H+ remains elusive. Here, using a mutant screening method in combination with a whole-cell patch clamp technique, we identified a key TRPML1 residue, Asp-472, responsible for both fast calcium-dependent inactivation (FCDI) and slow calcium-dependent inactivation (SCDI) as well as H+ regulation. We also found that, in acidic pH, H+ can significantly delay FCDI and abolish SCDI and thereby presumably facilitate the ion conductance of the human TRPML1 channel. In summary, we have identified a key residue critical for Ca2+-induced inhibition of TRPML1 channel currents and uncovered pH-dependent regulation of this channel, providing vital information regarding the detailed mechanism of action of human TRPML1.

Lysosomes, derived from membrane-enclosed compartments of late endosomes, play a critical role in a wide range of physiological functions (1). They can degrade macromolecules and participate in intracellular signal transduction as well as in membrane trafficking (2, 3). Consequently, lysosome dysfunction causes many diseases (4, 5).

The transient receptor potential mucolipin subfamily 1 (TRPML1) channel protein is an important regulator in many lysosome-dependent cellular physiological events (3, 6–10). It is widely expressed in every tissue in mammals (3). The gene encoding TRPML1 is MCOLN1, whose mutation causes a lysosomal storage disorder called mucolipidosis type IV (6), which is an autosomal recessive genetic disease with the typical features of psychomotor retardation, corneal opacities, retinal degeneration, strabismus, elevated blood gastrin levels, and achlorhydria (11–13).

Similar to other TRP2 channels, TRPML1 is a nonselective cation channel (7) with permeability to Na+, K+, Ca2+, and Fe2+ but not H+. It typically shows inwardly rectifying whole-cell or whole-lysosome currents (14, 15). TRPML1 is mainly located in the membranes of late endosome and lysosome (LEL) organelle compartments (16, 17). These LEL compartments are filled with high concentrations of ions, such as Ca2+ (7, 18). Another feature of both late endosomes and lysosomes is the low luminal pH established by vacuolar-type H+-ATPase (v-ATPase), resulting in a pH of ~5–6 in late endosomes and ~4–5 in lysosomes (2, 7, 19–21). Because of the special resident environment associated with both high Ca2+ and H+, TRPML1 channel activity is regulated by both Ca2+ and H+ (3, 6, 7, 9, 22). However, the molecular mechanism of TRPML1 channel regulation by Ca2+ and H+ remains elusive. In this study, using site-directed mutagenesis in combination with a whole-cell electrophysiological patch clamp technique, we identified a key aspartic acid residue that determines both FCDI and SCDI of the human TRPML1 channel in environments with different pH values.

Results

Ca2+ inhibits TRPML1 currents

TRPML1 is mainly located in LEL membranes (16, 17). Therefore, it is a challenge to perform direct LEL patch clamp experiments to record WT TRPML1 currents. We instead conducted whole-cell patch clamp experiments as described previously (14, 15). Briefly, an amino acid substitution (V432P) causes the TRPML1 channel to be widely expressed on cell plasma membranes. The acquisition of TRPML1-V432P currents was proven to show the same electrophysiological properties as that of WT TRPML1. For convenience, hereafter we call the TRPML1-V432P mutant TRPML1V432P (14, 15).

It has been reported that the whole-cell currents of TRPML1V432P are inhibited by Ca2+ (15). To verify this result, we

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2 The abbreviations used are: TRP, transient receptor potential; LEL, late endosome and lysosome; FCDI, fast calcium-dependent inactivation; SCDI, slow calcium-dependent inactivation; HEK, human embryonic kidney; CDI, calcium-dependent inactivation; NMDG, N-methyl-D-glucamine; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid.
recorded TRPML1\textsuperscript{Va} currents in the presence or absence of Ca\textsuperscript{2+} under weakly basic conditions (pH 7.4). Our results showed that Ca\textsuperscript{2+}/H11001 inhibited the monovalent cation currents of the TRPML1 channel with an IC\textsubscript{50} of 0.7 mM (Fig. 1, A and B, and Fig. S1A). It was proposed that three aspartic acid residues, Asp-111, Asp-114, and Asp-115, located between transmembrane 1 and transmembrane 2, might be responsible for Ca\textsuperscript{2+}/H11001-induced inhibition of TRPML1 currents (22). Therefore, we generated the TRPML1\textsuperscript{Va} (3DQ) mutant, in which Asp-111, Asp-114, and Asp-115 were mutated to glutamines, to record the whole-cell currents before and after addition of 2 mM Ca\textsuperscript{2+}/H11001. Our results showed that Ca\textsuperscript{2+}/H11001-induced inhibition still occurred in cells expressing the TRPML1\textsuperscript{Va} (3DQ) mutant (Fig. 1C and Fig. S1B), indicating that other candidate residues are crucial for Ca\textsuperscript{2+}/H11001-induced inhibition of the TRPML1 channel.

Asp-472 is responsible for Ca\textsuperscript{2+}/H11001-induced inhibition of the TRPML1 channel

TRP channel members generally share sequence conservation in their transmembrane domains (12, 23). Thus, we explored whether residues vital for Ca\textsuperscript{2+}/H11001-induced inhibition were located in the TRPML1 pore region. Sequence alignment of the pore-forming regions from several TRP channel members, including human TRPML1, human TRPP2, human TRPV1, human TRPA1, and rat TRPV1 (for which structural information is available) (24–30), showed that this region is highly conserved (Fig. 2A). Therefore, we performed whole-cell patch clamp experiments before and after addition of 2 mM Ca\textsuperscript{2+} in HEK293T cells expressing each human TRPML1\textsuperscript{Va} mutant (single amino acid mutation from the asparagine (Asp-472) to glutamine). Our results showed that Ca\textsuperscript{2+}/H11001-induced inhibition still occurred in cells expressing each mutant, indicating that Asp-472 is crucial for Ca\textsuperscript{2+}/H11001-induced inhibition of the TRPML1 channel.
Mechanism of Ca$^{2+}$ inhibition of TRPML1

A

MCLN1_Human 462 ———— LFSLLINGDMVTFAAM—QAQQGRSS
TRPP2_Human 628 ———— TRQECI—FTQFRILGINDPAELA
TRPA1_Human 905 ———— I—IQTFSMLGQINVRESEFLPYLRNELAH
TRPV1_Human 616 WRPACRPDPSSYNLSLSTCLELFKFT1GMGDLFETENYDF
TRPV1_Rat 616 CRGSACKP—NSYNLSLSTCLELFKFT1GMGDLFETENYDF

B

TRPML1$^{\alpha}$ (N469L) (4)

Current (pA)

2 mM Ca$^{2+}$ (pH 7.4) 0 Ca$^{2+}$ (pH 7.4)

Normalized currents

Time (s)

20 40 60 80

C

TRPML1$^{\alpha}$ (G470A) (5)

Normalized currents

Time (s)

20 40 60 80

D

TRPML1$^{\alpha}$ (D471Q) (6)

Normalized currents

Time (s)

20 40 60 80

E

TRPML1$^{\alpha}$ (D472Q) (9)

Normalized currents

Time (s)

20 40 60 80

F

TRPML1$^{\alpha}$ (M473A) (5)

Normalized currents

Time (s)

20 40 60 80

G

TRPML1$^{\alpha}$ (F474L) (5)

Normalized currents

Time (s)

20 40 60 80

H

TRPML1$^{\alpha}$ (V475A) (6)

Normalized currents

Time (s)

20 40 60 80

I

TRPML1$^{\alpha}$ (T476A) (5)

Normalized currents

Time (s)

20 40 60 80

J

TRPML1$^{\alpha}$ (F477L) (7)

Normalized currents

Time (s)

20 40 60 80
469) close to the selective filter to the phenylalanine (Phe-477) above the selective filter) at pH 7.4. The mutants N469L, G470A, D471Q, D472Q, M473A, F474L, V475A, T476A, and F477L were generated using a site-direct mutagenesis methodology. As shown in Fig. 2, all mutants showed Ca\(^{2+}\)-induced inhibition properties after addition of Ca\(^{2+}\), except for the TRPML1Va (D472Q) mutant, which displayed significantly increased whole-cell currents (Fig. 2E and Fig. S2D). Then we examined the dose-dependent effects of Ca\(^{2+}\) on TRPML1Va (D472Q) mutant currents, showing significant current potentiation in a Ca\(^{2+}\) concentration-dependent manner (Fig. 3A). This result is in sharp contrast with Ca\(^{2+}\)-induced inhibition of the TRPML1Va currents. The negative charge of Asp-472 is important for Ca\(^{2+}\)-induced inhibition of the TRPML1 channel. To elucidate the underlying mechanism, we examined the charge effect of Asp-472 on Ca\(^{2+}\)-induced inhibition of the TRPML1 channel. We first mutated aspartic acid to glutamic acid (Asp-472E), glutamine (Asp-472Q), and asparagine (Asp-472A) in the TRPML1Va channel. We then examined the charge effect of Asp-472 on Ca\(^{2+}\)-induced inhibition of the TRPML1Va currents (Fig. 1A). We further utilized NMDG/Ca\(^{2+}\)-containing solutions to test concentration-dependent effects, which also showed an obvious current potentiation phenomenon (Fig. 3B). Consequently, Asp-472 may play a key role in Ca\(^{2+}\)-induced inhibition of TRPML1Va currents.

**The negative charge of Asp-472 is important for Ca\(^{2+}\)-induced inhibition**

To elucidate the underlying mechanism, we examined the charge effect of Asp-472 on Ca\(^{2+}\)-induced inhibition of the TRPML1Va channel. We first mutated aspartic acid to glutamic acid (Asp-472E), glutamine (Asp-472Q), and asparagine (Asp-472A) in the TRPML1Va channel. We then examined the charge effect of Asp-472 on Ca\(^{2+}\)-induced inhibition of the TRPML1Va currents (Fig. 1A). We further utilized NMDG/Ca\(^{2+}\)-containing solutions to test concentration-dependent effects, which also showed an obvious current potentiation phenomenon (Fig. 3B). Consequently, Asp-472 may play a key role in Ca\(^{2+}\)-induced inhibition of TRPML1Va currents.

**Figure 2. The Asp-472 residue is important for Ca\(^{2+}\)-induced inhibition of the TRPML1 channel.**

A, amino acid sequence alignment of pore regions from human TRPML1, human TRP2, human TRPA1, human TRPV1, and rat TRPV1. MCLN1 represents TRPML1 for clarity. Highly conserved residues are colored red. B–J, current–voltage plots and corresponding time courses for normalized currents of the TRPML1Va (N469L), TRPML1Va (G470A), TRPML1Va (D471Q), TRPML1Va (D472Q), TRPML1Va (M473A), TRPML1Va (F474L), TRPML1Va (V475A), TRPML1Va (T476A), and TRPML1Va (F477L) mutants.
acid at residue 472 to maintain the same negative charge properties. Similar to TRPML1Va, addition of 2 mM Ca\(^{2+}\) dramatically decreased the current amplitude of TRPML1Va (D472E) (Fig. 3C and Fig. S3A). At the same time, we monitored extracellular Ca\(^{2+}\) entry using Fura-2 dye. Our results showed that TRPML1Va (D472E) mediated the movement of extracellular Ca\(^{2+}\) into cells, in line with the Ca\(^{2+}\) conductance of TRPML1Va (Fig. 3F). We then mutated aspartic acid to lysine at residue 472 to reverse the negative charge with the positive charge. In the presence of 0 and 2 mM Ca\(^{2+}\), the whole-cell currents were negligible (Fig. 3D and Fig. S3B), and neither allowed extracellular Ca\(^{2+}\) entry (Fig. 3F), indicating that the D472K mutant lost its cation conductance. Finally, we mutated aspartic acid to alanine, which has a small and hydrophobic side chain. Surprisingly, 2 mM Ca\(^{2+}\) largely increased the current amplitude of TRPML1Va (D472A) (Fig. 3E and Fig. S3C), and this mutant also induced extracellular Ca\(^{2+}\) entry (Fig. 3F). Notably, despite the opposite effects of adding Ca\(^{2+}\) to the two mutants TRPML1Va (D472A) and TRPML1Va (D472E), the nearly identical elevations in their \(F_{340}/F_{380}\) ratio may be attributed to the similar current levels upon 2 mM Ca\(^{2+}\) addition (Fig. 3, C and E, left panels). Thus, the negative charge of Asp-472 plays an essential role in mediating both the monovalent ion conductance and Ca\(^{2+}\)-induced inhibition of the TRPML1 currents.

**Asp-472 plays key role in both FCDI and SCDI of Ca\(^{2+}\) inhibition**

To further verify that Asp-472 is important for Ca\(^{2+}\)-induced inhibition, we employed NMDG\(^+\)/Ca\(^{2+}\) solution with Ca\(^{2+}\) as the only permeant ion. First, the whole-cell currents were recorded as the control experiment in cells expressing WT TRPML1 before and after addition of 2 mM Ca\(^{2+}\). Fairly small Ca\(^{2+}\) currents were observed after addition of Ca\(^{2+}\) (Fig. 4A and Fig. S4A). Then the currents of the TRPML1Va (D472Q) and TRPML1Va (D472A) mutants were acquired in the absence and presence of 2 mM Ca\(^{2+}\), both showing much larger Ca\(^{2+}\) currents than the control experiment (Fig. 4, B and C, and Fig. S4, B and C). We also added a Ca\(^{2+}\) chelator (10 mM EGTA or 10 mM BAPTA) to the intracellular pipette solution to record the whole-cell currents of the TRPML1Va (D472Q) mutant after addition of 2 mM Ca\(^{2+}\). The resulting currents are similar to those without a Ca\(^{2+}\) chelator (Fig. 4, D and E, and Fig. S4, D and E), indicating that the Ca\(^{2+}\) current potentiation of the TRPML1Va (D472Q) mutant is not regulated by intracellular Ca\(^{2+}\).

Ca\(^{2+}\) channels, such as store-operated calcium channels (31, 32) and voltage-gated calcium channels (33), can be modulated by Ca\(^{2+}\) to cause inactivation called calcium-dependent inactivation (CDI). CDI comprises Ca\(^{2+}\)-dependent fast inactivation (FCDI) and Ca\(^{2+}\)-dependent slow inactivation (SCDI), which have distinct spatial and temporal mechanisms; the former at the millisecond level and the latter at the second level (34, 35). However, for the TRPML1 channel, CDI and SCDI processes have not yet been described. We therefore further explored the CDI of the TRPML1Va channel. To verify that monovalent cations cannot induce the CDI phenomenon in the TRPML1Va channel, the currents were first recorded with the 0 Ca\(^{2+}\) solution, showing no FCDI-like currents (Fig. S4F). Then obvious FCDI was observed after replacement of the 0 Ca\(^{2+}\) solution with 2 mM Ca\(^{2+}\) solution (Fig. S4G). Ba\(^{2+}\) caused few FCDI currents (Fig. S4F). Therefore, Ca\(^{2+}\) indeed induces the classic CDI of the TRPML1Va channel.

To further investigate the CDI of the TRPML1Va channel, NMDG/Ca\(^{2+}\) solution was used. The current trace of TRPML1Va was first recorded in the NMDG with 2 mM Ca\(^{2+}\) solution, showing a small Ca\(^{2+}\) current with the classic FCDI phenomenon, with two time constants, including the fast \(\tau\) value of 1.29 ± 0.14 ms (n = 7) and the slow \(\tau\) value of 12.73 ± 1.51 ms (n = 7) (Fig. 4F). TRPML1Va also showed a decreased current amplitude, by 18.8% ± 3.8% (n = 7), from the start to the end position (Fig. 4F). Interestingly, under the same conditions, the mutant TRPML1Va (D472Q) not only presented a much larger current amplitude than TRPML1Va but also dramatically abolished the CDI, showing an increased current amplitude, by 2.8% ± 0.7% (n = 6), from the start to the end position (Fig. 4F). This was also true for the mutant TRPML1Va (D472A), which showed an increased current amplitude, by 12.1% ± 2% (n = 8), from the start to end position (Fig. 4G). In the presence of 10 mM BAPTA in the pipette solution, FCDI abrogation by mutant TRPML1Va (D472Q) was not affected, showing an increased current amplitude, by 4.5% ± 0.4% (n = 4), from the start to end position, suggesting that abrogation was not attributed to the intracellular Ca\(^{2+}\) increase (Fig. 4H). It has been noted that the time constants of TRPML1Va (D472Q), TRPML1Va (D472Q) with 10 mM BAPTA, and TRPML1Va (D472A) were not calculated because their FCDI current traces were best fit with the linear function. The SCDI process of TRPML1Va was also studied with elapsed time through one repetitive depolarized potential. As illustrated in Fig. 4I, the current amplitude of the TRPML1Va channel showed a time-dependent decay with a small time constant (−9 s) in the presence of 2 mM Ca\(^{2+}\). However, the time-dependent decay curve of the current amplitude of the TRPML1Va (D472Q) mutant was significantly right-shifted, showing a much larger time constant than TRPML1Va (Fig. 4I). Addition of 10 mM EGTA or BAPTA to the pipette solution had no obvious effect on the decay time constant of the SCDI of the TRPML1Va (D472Q) mutant (Fig. 4I). These results suggest that Asp-472 is a key site to modulate both FCDI and SCDI of the TRPML1 channel.

**H\(^{+}\) alleviates Ca\(^{2+}\)-induced inhibition**

Late endosomes and lysosomes are acidic compartments filled with high concentrations of H\(^{+}\) (7, 20). Therefore, we investigated the effect of pH on the Ca\(^{2+}\)-concentration-dependent inhibition of the TRPML1Va channel. First, the effect of Ca\(^{2+}\)-induced inhibition was alleviated 5-fold with an IC\(_{50}\) value of 3.33 mM Ca\(^{2+}\) at pH 4.6 compared with that at pH 7.4 (Fig. 1A). Second, in the absence of Ca\(^{2+}\), the whole-cell currents of TRPML1Va were similar at pH 7.4 and pH 4.6 (Fig. 5A and Fig. S5A), indicating that H\(^{+}\) did not potentiate the monovalent cation conductance of TRPML1Va. In contrast, in the presence of 2 mM Ca\(^{2+}\), the whole-cell currents of TRPML1Va largely increased as the solution pH changed from 7.4 to 4.6 (Fig. 5B and Fig. S5B), indicating that H\(^{+}\) alleviated the Ca\(^{2+}\)-induced inhibition associated with TRPML1Va currents. To
Figure 4. Ca$^{2+}$ modulates both FCDI and SCDI in the TRPML1 channel through residue Asp-472. 

A–C, at pH 7.4, current–voltage plots of TRPML1 Va, TRPML1 Va (D472Q), and TRPML1 Va (D472A) with extracellular NMDG/2 mM Ca$^{2+}$ recording solution. 

D and E, at pH 7.4, current–voltage plots of TRPML1 Va (D472Q) with NMDG/2 mM Ca$^{2+}$ recording solution in the presence of 10 mM EGTA or 10 mM BAPTA pipette solutions. 

F and G, at pH 7.4, FCDI current traces of TRPML1 Va, TRPML1 Va (D472Q), and TRPML1 Va (D472A), with NMDG/2 mM Ca$^{2+}$ recording solution induced by a −100-mV step potential. 

H, at pH 7.4, FCDI current traces of TRPML1 Va and TRPML1 Va (D472Q) with NMDG/2 mM Ca$^{2+}$ recording solution induced by a −100-mV step potential in the presence of 10 mM BAPTA pipette solution. 

I, at pH 7.4, normalized SCDI current curves of TRPML1 Va and TRPML1 Va (D472Q) with NMDG/2 mM Ca$^{2+}$ recording solution with or without 10 mM EGTA or 10 mM BAPTA in pipette solution (left panel) and statistical analysis of decay time constant for SCDI (right panel). The curve was fit with Origin 9.0 software. ***, p < 0.001; NS, no statistical significance.
further verify whether the negative charge of Asp-472 is involved in the H⁺/H₁₁₀₀₁ regulation of TRPML1 currents, we used the TRPML1Va mutant (D472Q). In the presence of 0 and 2 mM Ca²⁺/H₁₁₀₀₁, the whole-cell currents of the mutant TRPML1 Va (D472Q) similarly decreased as the pH decreased from 7.4 to 4.6 (Fig. 5, C and D, and S5, C and D), indicating that the residue Asp-472 indeed significantly contributes to H⁺ regulation of the TRPML1 channel.

We then investigated the reciprocal regulation of both CDI and H⁺/H₁₁₀₀₁. As illustrated in Fig. 5E, the FCDI at the physiological pH 4.6 was significantly slowed compared with the pH 7.4 condition. At pH 4.6, TRPML1Va showed only one time constant value for the fast τ of 2.19 ± 0.39 ms (n = 4), and the current amplitude decreased by 10.7% ± 1.1% (n = 4) (Fig. 5E). We further compared SCDI processes at pH 7.4 and pH 4.6. Surprisingly, SCDI was completely abolished at pH 4.6 (Fig. 5F). In summary, H⁺ can alleviate the Ca²⁺-induced inhibition of the TRPML1 channel.

Discussion

TRPML1 is mainly located in late endosomes and lysosomes, whose compartments are filled with high concentrations of...
Ca\(^{2+}\) and H\(^+\) (7, 20). Its special dwelling environment determines the regulation of its functional activity by both Ca\(^{2+}\) and H\(^+\) (14, 15, 22). In this study, we identified a key residue, Asp-472, that is important for Ca\(^{2+}\)-induced inhibition of TRPML1 channel currents. We showed that the TRPML1\(^{Va}\) (D472E) mutant had similar ion channel properties as WT TRPML1\(^{Va}\). However, the TRPML1\(^{Va}\) (D472K) mutant, which included a positively charged amino acid substitution, completely abolished the channel conductance of TRPML1. The neutral amino acid substitution mutant TRPML1\(^{Va}\) (D472A) abrogated Ca\(^{2+}\)-induced inhibition of the TRPML1 channel activity but retained the cation conductance of TRPML1.

Calcium-dependent inactivation is an important negative feedback regulation mechanism to prevent excessive Ca\(^{2+}\) entry into the cytoplasm. FCDI occurs rapidly after Ca\(^{2+}\) influx, whereas SCID occurs upon slow global Ca\(^{2+}\) rise (34, 36, 37). Generally, there are different regulators to manage FCDI and SCDI. Among these regulating factors, Ca\(^{2+}\)-bound calmodulin is the most widely reported regulator (33, 35, 38–42). However, the TRPML1 channel showed unconventional behavior in both FCDI and SCDI regulation. The single residue Asp-472 itself can regulate both the FCDI and SCDI of the TRPML1 channel. Ca\(^{2+}\)-dependent calmodulin regulation seemed not to be involved in the regulation of the TRPML1 channel because the presence or absence of the Ca\(^{2+}\) chelator BAPTA caused no significant effects on both FCDI and SCDI. The FCDI induced by Ca\(^{2+}\) can be well-explained by the special localization of Asp-472 near the selectivity filter (24), parallel to the spatial and temporal mechanism of FCDI occurrence (34, 36, 43). Strikingly, Ca\(^{2+}\) also facilitated the SCDI process through residue Asp-472 because the mutant TRPML1\(^{Va}\) (D472Q) significantly prolonged the decay time constant of SCDI. Importantly, under physiological pH 4.6 conditions, we showed that Asp-472 also played a critical role in the H\(^+\) alleviation of the Ca\(^{2+}\)-induced inhibition of TRPML1 channel currents. The D472Q mutant fully abrogated the H\(^+\) attenuation effects on Ca\(^{2+}\)-induced inhibition. Further studies showed that H\(^+\) was able to not only significantly delay FCDI but also fully abolish SCDI. This unusual regulation of CDI can be further emphasized compared with other TRP channels adopting the Ca\(^{2+}\)-bound calmodulin regulation approach (44–47). This is probably an important molecular regulation mechanism for TRPML1-dependent cellular physiological events. Because lysosomes and late endosomes are filled with highly concentrated Ca\(^{2+}\) and H\(^+\) (7, 20), the opposite regulation between Ca\(^{2+}\) and H\(^+\) is required to synergistically manage TRPML1 Ca\(^{2+}\) release. These results together indicated that the residue Asp-472 is dispensable for both TRPML1 ion conductance and its regulation by Ca\(^{2+}\) and H\(^+\).

Based on these results, we propose a possible molecular model for the regulation of the TRPML1 channel current by Ca\(^{2+}\) and H\(^+\) (Fig. 5G). Under weakly basic conditions (pH 7.4), Asp-472 is deprotonated and able to effectively aggregate Ca\(^{2+}\) to form a positively charged microdomain, causing obvious CDI of the TRPML1 channel, thereby inducing Ca\(^{2+}\) inhibition of conductance. Additionally, the positively charged microdomain may also interfere with the access of monovalent cations as well as Ca\(^{2+}\) to the selective filter because of repulsive inter-

### Experimental procedures

#### Plasmids

The human TRPML1 gene (accession number NM_020533) was synthesized by GeneWiz and cloned into the pcDNA-EGFP vector in the BamH1 and Xho1 restriction sites using transcription PCR. The mutants, including TRPML1\(^{Va}\), TRPML1\(^{Va}\) (D472E), TRPML1\(^{Va}\) (D472K), TRPML1\(^{Va}\) (D472A), TRPML1\(^{Va}\) (D111Q, D114Q, D115Q), TRPML1\(^{Va}\) (N469L), TRPML1\(^{Va}\) (G470A), TRPML1\(^{Va}\) (D471Q), TRPML1\(^{Va}\) (D472Q), TRPML1\(^{Va}\) (M473A), TRPML1\(^{Va}\) (F474L), TRPML1\(^{Va}\) (V475A), TRPML1\(^{Va}\) (T476A), and TRPML1\(^{Va}\) (F477L), were generated with the corresponding mutation primers listed in Table S1.

#### Cell culture and transfection

HEK293T cells were grown in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (HyClone). HEK293T cells were cultured at 37 °C with 5% CO\(_2\). All plasmids were transfected into HEK293T cells using polyethyleneimine.

#### Whole-cell recordings and data analysis

The recording pipettes were pulled from borosilicate glass using a P-97 glass microelectrode puller (Sutter Instrument) and polished with an MF-830 (Narishige). The pipettes had a resistance of 3–5 megohms after being filled with the internal recording solution containing 120 mM cesium methanesulfonate, 4 mM NaCl, 2 mM MgCl\(_2\), 2 mM Na\(_2\)-ATP, 10 mM EGTA, and 20 mM HEPES (pH adjusted to 7.2 with CsOH). This internal solution was used for all non-NMDG solution recordings. After establishment of the whole-cell configuration, the currents were recorded using an Axopatch 700B amplifier (Molecular Devices) and digitized using a Digidata 1550A (Molecular Devices). The voltage protocol included 50-ms voltage steps to −100 mV from a holding potential of 0 mV, followed by a voltage ramp increasing from −100 to +100 mV in 50 ms with a frequency of 0.5 Hz. A 100-ms step potential from 0 mV to
−100 mV was applied for investigation of the FCDI process. All currents were sampled at 10 kHz and filtered at 2 kHz by the low-pass filter. Current recordings were acquired through pClamp software (Molecular Devices). The standard extracellular 0 Ca\(^{2+}\) recording solution contained 153 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 10 mM glucose, and 20 mM HEPES (pH 7.4 adjusted with NaOH). The standard extracellular Ca\(^{2+}\)-containing recording solutions were made in different concentrations by diluting the 1 mM CaCl\(_2\) stock solution in the standard extracellular 0 Ca\(^{2+}\) recording solution. The pH 4.6 extracellular 0 Ca\(^{2+}\) recording solution contained 153 mM sodium gluconate, 5 mM KCl, 1 mM MgCl\(_2\), 10 mM MES, 10 mM HEPES, and 10 mM glucose (pH 4.6 adjusted with HCl). The pH 4.6 extracellular Ca\(^{2+}\)-containing recording solutions were made in different concentrations by diluting the 1 mM CaCl\(_2\) stock solution in the pH 4.6 extracellular 0 Ca\(^{2+}\) recording solution. The pH 7.4 extracellular NMDG/0 Ca\(^{2+}\) recording solution contained 160 mM NMDG, 20 mM HEPES, and 10 mM glucose (pH 7.4 adjusted with HCl). The pH 7.4 extracellular NMDG/Ca\(^{2+}\)-containing recording solutions were made in different concentrations by diluting the 1 mM CaCl\(_2\) stock solution in the pH 7.4 extracellular NMDG/0 Ca\(^{2+}\) recording solution. The pH 4.6 extracellular NMDG/0 Ca\(^{2+}\) recording solution contained 160 mM NMDG, 10 mM HEPES, 10 mM MES, and 10 mM glucose (pH 4.6 adjusted with HCl). The pH 4.6 extracellular Ca\(^{2+}\)-containing recording solutions were made in different concentrations through diluting the 1 mM CaCl\(_2\) stock solution in the pH 4.6 extracellular NMDG/0 Ca\(^{2+}\) recording solution. To eliminate the obvious external currents recorded with the above internal pipette solution, we applied the following pipette solution containing 160 mM NMDG, 20 mM HEPES, and 10 mM glucose (pH 7.2 adjusted with HCl) for all NMDG-containing recording solutions. The pH 4.6 extracellular NMDG/0 Ca\(^{2+}\) recording solution contained 10 mM EGTA or BAPTA for the EGTA- or BAPTA-containing pipette solutions (pH 7.2 adjusted with HCl). The solution exchange was performed using a peristaltic pump and was accomplished within several seconds. Data were analyzed using pClamp and Origin software (OriginLab Corp.). All experiments were conducted at room temperature. The method for the normalization of the recorded currents was as follows. The very beginning current density was set as −1.0, the current densities during the measurement were displayed as the ratio of the very beginning current density to obtain the normalized current densities, and these densities were plotted as a function of the recording time. The time constants of FCDI were obtained through the nonlinear function fit (ExpDec 1 or ExpDec 2) in Origin 9.0 software. Statistical analysis was executed in SPSS Statistics 20 (Statistical Product and Service Solutions, IBM Corp.) by one-way analysis of variance. \(p < 0.05\) represents statistical significance. Data points represent the mean ± S.E.

**Intracellular Ca\(^{2+}\) measurements**

HEK293T cells expressing the TRPML1\(^{VA}\), TRPML1\(^{VA}\) (D472E), TRPML1\(^{VA}\) (D472K), and TRPML1\(^{VA}\) (D472A) mutants were plated on glass-bottom dishes coated with poly-L-lysine (Sigma-Alrich). Twenty-four hours after transfection, the cells were loaded with 5 \(\mu\)M Fura-2/AM (Invitrogen) in the standard extracellular 0 Ca\(^{2+}\) recording solution (pH 7.4) at room temperature for 30 min. After loading, the cells were transferred to Fura-2/AM-free solution for 30 min. Fluorescence imaging was undertaken at room temperature using a Leica DMi6000B microscope with the LAS software before and after addition of 2 mM Ca\(^{2+}\). Consecutive excitation occurred at 340 and 380 nm every 2 s, and the emission was collected at 510 nm. The intracellular Ca\(^{2+}\) concentration change is shown as the 340/380 ratio. Data points are shown as the mean ± S.E.

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