Research Paper

Molecular determinants of sensitivity and conductivity of human TRPM7 to Mg\(^{2+}\) and Ca\(^{2+}\)

Tomohiro Numata\(^{1}\) and Yasunobu Okada\(^{*}\)

Department of Cell Physiology; National Institute for Physiological Sciences; Okazaki, Aichi Japan

\(^{1}\)Current Address: Department of Synthetic Chemistry and Biological Chemistry; Graduate School of Engineering; Kyoto University; Kyoto, Japan

Key words: TRPM7, Ca\(^{2+}\), Mg\(^{2+}\), proton, selectivity filter

It is known that extracellular Mg\(^{2+}\) and Ca\(^{2+}\) can permeate TRPM7 and at the same time block the permeation by monovalent cations. In the present study, we examined the molecular basis for the conductivity and sensitivity of human TRPM7 to these divalent cations. Extracellular acidification to pH 4.0 markedly reduced the blocking effects of Mg\(^{2+}\) and Ca\(^{2+}\) on the Cs\(^{+}\) currents, decreasing their binding affinities: their IC\(_{50}\) values increased 510- and 447-fold, respectively. We examined the effects of neutralizing each of four negatively charged amino acid residues, Glu-1047, Glu-1052, Asp-1054 and Asp-1059, within the putative pore-forming region of human TRPM7. Mutating Glu-1047 to alanine (E1047A) resulted in non-functional channels, whereas mutating any of the other residues resulted in functionally expressed channels. Cs\(^{+}\) currents through D1054A and E1052A were less sensitive to block by divalent cations; the IC\(_{50}\) values were increased 5.5- and 447-fold, respectively, for Mg\(^{2+}\) and 10.5- and 6.7-fold, respectively, for Ca\(^{2+}\). D1059A also had a significant reduction, though less marked compared to the reductions seen for D1054A and E1052A, in sensitivity to Mg\(^{2+}\) (1.7-fold) and Ca\(^{2+}\) (3.9-fold). The D1054A mutation largely abolished inward currents conveyed by Mg\(^{2+}\) and Ca\(^{2+}\). In the E1052A and D1059A mutants, inward Mg\(^{2+}\) and Ca\(^{2+}\) currents were sizable but significantly diminished. Thus, it is concluded that in human TRPM7, (1) both Asp-1054 and Glu-1052, which are located near the narrowest portion in the pore’s selectivity filter, may provide the binding sites for Mg\(^{2+}\) and Glu-1052, which are located near the narrowest portion in the pore’s selectivity filter, may provide the binding sites for Mg\(^{2+}\) and Ca\(^{2+}\) conductivity, and (3) Glu-1052 and Asp-1059 facilitate the conduction of divalent cations.

Introduction

TRPM7 is a ubiquitously distributed member of the transient receptor potential melastatin (TRPM) subfamily\(^{1,3}\) and has been recently shown to be a mechanosensitive cation channel.\(^{4,5}\) Although extracellular divalent cations such as Mg\(^{2+}\) and Ca\(^{2+}\) are known to block TRPM7 permeability to monovalent cations, they themselves can permeate TRPM7.\(^{6-9}\) The aim of the present study was to identify the amino acid residues responsible for the sensitivity and conductivity of human TRPM7 to Mg\(^{2+}\) and Ca\(^{2+}\).

Recently, Li et al.,\(^{10}\) identified E1047 as the residue that confers divalent cation permeability on mouse TRPM7. However, it is not known whether this residue does so in human TRPM7 as well. Mouse and human TRPM7 are different in five of 62 amino acid residues in the S5–S6 region, although the amino acid sequences of the putative pore-forming regions are identical to each other (Fig. 1). Thus, there is a possibility that the same mutations in the mouse and human TRPM7 channels could have different effects on the respective channel activities. Our recent study\(^{11}\) demonstrated, in fact, that the D1054A mutation abolished proton conduction through human TRPM7, although the same mutation was reported to produce no change in the proton sensitivity of mouse TRPM7.\(^{10}\) Thus, in this study, mutation of negatively charged amino acid residues within the putative pore-forming region of human TRPM7 into the neutral amino acid alanine was tested for effects on the channel’s sensitivity and conductivity to Mg\(^{2+}\) and Ca\(^{2+}\).

Results and Discussion

Effects of neutralizing mutations on monovalent cation currents observed in the presence of divalent cations. Human TRPM7 currents observed under ramp clamp in Cs\(^{+}\)-rich conditions and at pH 7.4 exhibited strong outward rectification when extracellular divalent cations were present (Fig. 2A, top: WT). The neutralized E1047A mutant failed to express any currents (Fig. 2A, bottom: NF), a result similar to that seen when examining proton currents through human TRPM7.\(^{11}\) However, this result contrasts with the previous observation made using mouse TRPM7; with mouse TRPM7, functional expression was seen with E1047A.\(^{10}\)

Neutralization of Asp-1054 by replacing with alanine significantly increased inward Cs\(^{+}\) currents (Fig. 2A, top and bottom), although the level of molecular expression of D1054A was not more marked than that of WT.\(^{11}\) Neutralizing replacement of Asp-1054 with asparagine similarly augmented the inward Cs\(^{+}\) currents (up to -81.1 ± 7.9 pA/pF; n = 18). In contrast, the negatively charged mutant D1054E did not affect the inward Cs\(^{+}\) conductance (Fig. 2A,
Enhancement of the inward conductance was also observed in the E1052A and D1059A mutants (Fig. 2A, bottom).

When extracellular pH was lowered to 4.0, the inward Ca\textsuperscript{2+} current through human TRPM7 was prominently increased, even with divalent cations present (Fig. 2B, top and bottom: WT), as observed for mouse TRPM7.\textsuperscript{5,10} Proton-induced enhancement of the inward Ca\textsuperscript{2+} conductance was largely abolished by the D1054A mutation (Fig. 2B, top and bottom) and significantly diminished by the E1052A mutation (Fig. 2B, bottom).

It is known that the outward rectification of TRPM7 channels is caused, at least in part, by blockage by extracellular Mg\textsuperscript{2+} and Ca\textsuperscript{2+}; furthermore, protons relieve the channel block by Mg\textsuperscript{2+} and Ca\textsuperscript{2+} by competing with the divalent cations for binding within the pore region.\textsuperscript{6,10,11} Therefore, the data shown in Figure 2 suggest that Glu-1052, Asp-1054 and Asp-1059 are directly or indirectly involved in the binding of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} within the pore region of human TRPM7.

Amino acid residues that determine sensitivity to divalent cations. To more directly test the possibility that the residues are involved in binding Mg\textsuperscript{2+} and Ca\textsuperscript{2+}, the effects of extracellular Mg\textsuperscript{2+} and Ca\textsuperscript{2+} were examined in the absence of Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, respectively. Inward Ca\textsuperscript{2+} currents passing through human TRPM7 were suppressed by extracellular Mg\textsuperscript{2+} in a concentration-dependent manner at both pH 7.4 and 4.0, as shown in Figure 3A. When extracellular pH was lowered from 7.4 to 4.0, there was a 510-fold reduction in the Mg\textsuperscript{2+} sensitivity calculated from currents at -100 mV; the IC\textsubscript{50} value increased from 5.1 μM to 2.6 mM (Fig. 3B). These IC\textsubscript{50} values are in good agreement with those calculated from currents at -120 mM in mouse TRPM7 at pH 7.4 (5.4 μM: Jiang et al.,\textsuperscript{6} 3.6 μM: Li et al.\textsuperscript{10}) and at pH 4.0 (5.9 mM: Jiang et al.\textsuperscript{6})

Extracellular Ca\textsuperscript{2+} had blocking effects that were essentially the same as those for extracellular Mg\textsuperscript{2+} at both pH 7.4 and 4.0, as shown in Figure 4A and B. The IC\textsubscript{50} values for inhibition by Ca\textsuperscript{2+} calculated from currents at -100 mV were 7.6 μM at pH 7.4 and 3.4 mM at pH 4.0; these values are in accord with those determined from currents at -120 mV in mouse TRPM7 at pH 7.4 (47 μM: Jiang et al.,\textsuperscript{6} 4.1 μM: Li et al.\textsuperscript{10}) and at pH 4.0 (5.6 mM: Jiang et al.\textsuperscript{6})

Neutralizing mutations of Glu-1052 and Asp-1054 markedly reduced the binding affinity of divalent cations. The IC\textsubscript{50} values for Mg\textsuperscript{2+} and Ca\textsuperscript{2+} were increased 3.9- and 6.7-fold, respectively, for E1052A and 5.5- and 10.5-fold, respectively, for D1054A, as summarized in Figures 3C and 4C. The effect of neutralization of Glu-1052 is in good agreement with the effect previously observed for the same mutation in mouse TRPM7.\textsuperscript{10} However, the present data for the D1054A mutant of human TRPM7 are at variance with observations made for the D1054A mutant of mouse TRPM7, which was not altered with respect to divalent cation sensitivity.\textsuperscript{10} The D1059A mutation of human TRPM7 also significantly diminished the binding affinity of Mg\textsuperscript{2+} and Ca\textsuperscript{2+}, though not nearly as much as the E1052A and D1054A mutations (Figs. 3C and 4C).

Even under conditions in which glutamate (pK\textsubscript{a} ~4.3) and aspartate (pK\textsubscript{a} ~3.7) were half-neutralized at pH 4.0, all the neutralizing mutations further increased the IC\textsubscript{50} for Mg\textsuperscript{2+} (Fig. 3D), and two of them (E1052A and D1059A) increased the IC\textsubscript{50} for Ca\textsuperscript{2+} (Fig. 4D). From these results, it appears that Glu-1052 and Asp-1054 are the major molecular determinants of sensitivity of human TRPM7 to Mg\textsuperscript{2+} and Ca\textsuperscript{2+}.

Figure 1. The sequences of 62 amino acid residues in the S5–S6 region of human TRPM7 (hTRPM7) and mouse TRPM7 (mTRPM7). The putative pore-forming region and the selectivity filter are designated by thin and thick bars, respectively. Asterisks denote the negatively charged amino acids mutated in this study. The amino acid residues that are different between hTRPM7 and mTRPM7 are indicated in italic.

Figure 2. Effects of mutating negatively charged amino acid residues in the putative pore-forming region of human TRPM7 on monovalent cation currents passing through the channel at pH 7.4 (A) and pH 4.0 (B). (Top) Representative I-V relationships for currents through WT TRPM7 and the D1054A mutant observed under ramp clamp. (Bottom) Average densities of inward currents through WT TRPM7 and its mutants recorded at -100 mV. Each column represents the mean ± s.e.m. (n = 6–16). NF, non-functional. *, Significantly different from the WT value.
extracellular Mg\textsuperscript{2+} and Ca\textsuperscript{2+}, providing binding sites for these ions; Asp-1059, on the other hand, may facilitate access of the divalent cations to these binding sites.

**Amino acid residues that determine conductivity of divalent cations.** Since it is known that Mg\textsuperscript{2+} and Ca\textsuperscript{2+} are permeant blockers of TRPM7,\textsuperscript{6,10,11,13} we next examined the effects of the neutralizing mutations on the conductivity of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} through human TRPM7. In these experiments, 10 mM MgSO\textsubscript{4} or Ca-gluconate was added to an NMDG-aspartate-rich extracellular solution. As summarized in Figure 5, the negatively charged D1054E mutant failed to affect the Mg\textsuperscript{2+} and Ca\textsuperscript{2+} conductances. In contrast, the D1054A mutant largely abolished both the inward currents conveyed by Mg\textsuperscript{2+} (A) and those conveyed by Ca\textsuperscript{2+} (B), although sizable molecular expression of D1054A was detected by immunoblotting and immunostaining in our preceding study.\textsuperscript{11} These observations contrast with the observations, for mouse TRPM7, that Mg\textsuperscript{2+} and Ca\textsuperscript{2+} currents were abolished by E1047Q but not D1054A.\textsuperscript{10} In human TRPM7, the E1052A and D1059A mutations significantly reduced the Mg\textsuperscript{2+} and Ca\textsuperscript{2+} conductances, though less markedly compared to the D1054A mutation (Fig. 5). These data indicate that Asp-1054 is an essential determinant of the conductivity of divalent cations through human TRPM7, whereas Glu-1052 and Asp-1059 may play a facilitating role in conducting these ions through the channel. It is possible that this effect of Asp-1054 on the divalent cation conductivity is linked to increased selectivity to Mg\textsuperscript{2+} or Ca\textsuperscript{2+}. In fact, our preliminary observations made under bi-ionic conditions showed that the values of PMg/PCs and PCa/PCs for WT (1.76 ± 0.47 and 1.45 ± 0.43 (n = 5), respectively) are significantly larger than those for the neutralizing D1054A mutant (0.23 ± 0.03 and 0.21 ± 0.03 (n = 14), respectively) but are indistinguishable from the negatively charged D1054E mutant (Numata T and Okada Y, unpublished data).

**Materials and Methods**

**Mutation and expression of human TRPM7.** Substitutions of negatively charged amino acid residues within the putative pore-forming region of human TRPM7,\textsuperscript{12} (Fig. 1, asterisks) were made using the Quik-ChangeIIXL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The mutations were confirmed by sequencing the entire insert region.

HEK293T cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics, as previously described.\textsuperscript{4} The cells were transiently transfected with wild-type (WT) human TRPM7 or the mutants, as previously described.\textsuperscript{5} Molecular expression of these mutants in the plasmalemmal region was confirmed by observing immunofluorescence with confocal microscopy, as previously described.\textsuperscript{11}

**Whole-cell patch-clamp recordings.** Whole-cell currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Union City, CA) from cells that had been detached from a plastic substrate and cultured in suspension for 15 to 240 min at room temperature (22–26°C). Currents were digitized at 20 kHz and filtered at 5 kHz. Pulling of patch electrodes (~5 MΩ), compensation of series resistance and control of command pulses were performed, as previously described.\textsuperscript{11}

**Solutions.** To record monovalent cation currents, Cs-aspartate-rich solutions were used. The intracellular (pipette) solution contained (in mM): 100 Cs-aspartate, 0.1 CsCl, 1 EGTA, 1 EDTA, 90 mannitol and 10 HEPES (pH 7.4 adjusted with CsOH). The
**Acknowledgements**

This work was supported by Grants-in-Aid for Scientific Research from MEXT and JSPS. We thank E.L. Lee for reading the manuscript, to K. Shigemoto for technical assistance, and to T. Okayasu for secretarial assistance.

**References**

1. Clapham DE. TRP channels as cellular sensors. Nature 2003; 426:517-24.
2. Harteneck C, Plant TD, Schultz G. From worm to man: three subfamilies of TRP channels. Trends Neurosci 2000; 23:159-66.
3. Montell C. Physiology, phylogeny and functions of the TRP superfamily of cation channels. Sci STKE 2001; 2001:1.
4. Numata T, Shimizu T, Okada Y. TRPM7 is a stretch- and swelling-activated cation channel involved in volume regulation in human epithelial cells. Am J Physiol Cell Physiol 2007; 292:460-7.
5. Numata T, Shimizu T, Okada Y. Direct mechano-stress sensitivity of TRPM7 channel. Cell Physiol Biochem 2007; 19:1-8.
6. Jiang J, Li M, Yue L. Potentiation of TRPM7 inward currents by protons. J Gen Physiol 2005; 126:137-50.
7. Monteilh-Zoller MK, Hermosura MC, Nadler MJS, Scharenberg AM, Penner R, Fleig A. TRPM7 provides an ion channel mechanism for cellular entry of trace metal ions. J Gen Physiol 2003; 121:49-60.
8. Nadler MJ, Hermosura MC, Inabe K, Perraud AL, Zhu Q, Stokes AJ, Kurovski T, Kiner JP, Penner R, Scharenberg AM, Fleig A. LTRPC7 is a Mg, ATP-regulated divalent cation channel required for cell viability. Nature 2001; 411:590-5.
9. Runnels LW, Yue L, Clapham DE. TRP-PLIK, a bifunctional protein with kinase and ion channel activities. Science 2001; 291:1043-7.
10. Li M, Du J, Jiang J, Ratzan W, Su LT, Runnels LW, Yue L. Molecular determinants of Mg2+ and Ca2+ permeability and pH sensitivity in TRPM6 and TRPM7. J Biol Chem 2007; 282:25817-30.
11. Numata T, Okada Y. Proton conductivity through the human TRPM7 channel and its molecular determinants. J Biol Chem 2008; 283:15097-103.
12. Owsianik G, Talavera K, Voets T, Nilius B. Permeation and selectivity of TRP channels. Annu Rev Physiol 2006; 68:685-717.
13. Kirschbaum HH, Kosak JA, Cahalan MD. Polyvalent cations as permeant probes of MIC and TRPM7 pores. Biophys J 2003; 84:2293-305.

**Conclusion**

To identify the molecular determinants responsible for the sensitivity and conductivity of human TRPM7 to Mg2+ and Ca2+, negatively charged amino acid residues within the 15 amino acid-containing putative pore-forming region (Fig. 1) were systematically mutated to the neutral amino acid alanine. When transfected into HEK293T cells, E1047A never exhibited functional channel activity, whereas E1052A, D1054A and D1059A did exhibit activity, as monovalent cation channels. However, the three functional mutants were blocked by extracellular Mg2+ and Ca2+ to a lesser degree than the wild-type. Because neutralizing Glu-1052 or Asp-1054 was most effective in reducing the sensitivity to divalent cations, it appears that these two residues provide the binding sites for Mg2+ and Ca2+.

The conductivity of Mg2+ and Ca2+ was also affected by these neutralization mutations. D1054A largely abolished the divalent cation conductance, whereas the negatively charged D1054E mutant exhibited Mg2+ and Ca2+ currents as large as those of the wild-type. On the other hand, the E1052A and D1059A mutants exhibited sizable but significantly reduced divalent cation conductivity compared to the wild-type. It is therefore concluded that Asp-1054 is an essential determinant of the Mg2+ and Ca2+ conductivity through human TRPM7, whereas Glu-1052 and Asp-1059 may play a facilitating role in conducting these divalent cations through the channel. Also, it appears that Glu-1047 is required for channel formation.