Proton Conductivity through the Human TRPM7 Channel and Its Molecular Determinants

TRPM7 is a divalent cation-permeable channel that is ubiquitously expressed. Recently, mouse TRPM7 has been shown to be sensitive to, and even permeable to, protons when heterologously expressed. Here we have demonstrated that human TRPM7 expressed either heterologously or endogenously also exhibits proton conductivity. The gene silencing of TRPM7 by small interfering RNA suppressed H⁺ currents in human cervical epithelial HeLa cells. In HEK293T cells transfected with human TRPM7, the inward proton conductance was suppressed by extracellular Mg²⁺ or Ca²⁺ with IC₅₀ values of 0.5 and 1.9 mM, respectively. Anomalous mole fraction behavior of H⁺ currents in the presence of Mg²⁺ or Ca²⁺ indicated that these divalent cations compete with protons for binding sites. Systematic mutation of negatively charged amino acid residues within the putative pore-forming region of human TRPM7 into the neutral amino acid alanine was tested. E1047A resulted in non-functional channels, and D1054A abolished proton conductance, whereas E1052A and D1059A only partially reduced proton conductivity. Thus, it is concluded that Asp-1054 is an essential determinant of the proton conductivity, whereas Glu-1047 might be required for channel formation, and the remaining negatively charged amino acids in the pore region (Glu-1052 and Asp-1059) may play a facilitating role in the proton conductivity of human TRPM7. It is suggested that proton conductivity of endogenous human TRPM7 plays a role in physiologically/pathologically acidic situations.

TRPM7 is a cation channel that belongs to the melastatin-related transient receptor potential (TRP)³ ion channel subfamily (1–4). TRPM7 is endogenously expressed in a wide variety of tissues including brain and hematopoietic tissues (5) as well as kidney and heart (3, 6, 7). The TRPM7 cation channel supports multiple cellular and physiological functions, including cellular Mg²⁺ homeostasis (8, 9), cell viability and growth (9–12), anoxic neuronal cell death (13), synaptic transmission (14), cell adhesion (15, 16), and intestinal pacemaking (17).

High proton concentrations in the extracellular milieu are generated under physiological conditions in several tissues such as gastric epithelia (18), skin (19, 20), and cervico-vaginal epithelia (21, 22) as well as under pathophysiological conditions such as ischemia and inflammation, in which the pH may fall below 6 in many tissues (23–27). Protons have been reported to modulate the activity of a variety of ion channels (28, 29). Thus, the study of the effects of protons on the multifunctional, ubiquitously expressed TRPM7 channel is of importance physiologically. Recently, Jiang et al. (30) demonstrated that extracellular protons enhance inward cation currents through the mouse TRPM7 channel and even permeate the channel when it was heterologously expressed in HEK293 cells. However, it has not yet been determined whether human TRPM7, which exhibits 94% amino acid sequence identity with mouse TRPM7, can conduct protons not only in heterologously expressed cells but also in endogenously expressed cells. Thus, in the present study proton conductivity of the human TRPM7 channel was investigated in HEK293T cells transfected with human TRPM7 and human cervical cancer HeLa cells, which endogenously express TRPM7 (31).

Recently, there has been accumulating evidence that transmembrane domains other than the putative pore (P or H5) region of voltage-gated ion channels serve as proton conduction pathways. Starace and Bezanilla (32) showed that the fourth transmembrane segment (S4) in the Shaker K⁺ channel, the voltage-sensing domain, behaves as a proton pore when the first arginine of S4 is replaced with a histidine. Sasaki et al. (33) and Ramsey et al. (34) demonstrated that a four-transmembrane domain protein with similarity to the S1-S4 regions of voltage-gated ion channels is the molecule corresponding to a voltage-gated proton channel; this molecule, however, lacks a classical pore domain. Thus, there is a possibility that some transmembrane domain (in particular S4), and not the P region, of TRPM7 is responsible for proton permeation. On the other hand, Li et al. (35) have recently shown that amino acid residues Glu-1047 and Glu-1052 in the putative pore region of mouse TRPM7 have an essential role in enhancing the effects of protons on inward current responses. This sensitivity to protons is due to competition of the protons with the divalent cations Mg²⁺ and Ca²⁺, which block the channel, for their binding sites (35); it is, therefore, hypothesized that the putative pore region (the P region) is involved in the conduction of protons through TRPM7. In this study we have tested this possibility by observ-
TRPM7 Conducts Protons

ing the effects of mutating negatively charged amino acid residues within the putative pore-forming region of human TRPM7 into the neutral amino acid alanine.

Our results have revealed the following: first, human TRPM7, expressed either heterologously or endogenously, exhibits proton conductivity. Second, divalent cations interfere with the conduction of protons through TRPM7. Third, in the putative pore region, the negatively charged amino acid residue Asp-1054 is essential for proton conductivity. This finding shows for the first time that the pore region per se provides the pathway for proton conduction in TRPM7. Our results also suggest that Glu-1047 is required for channel formation and the remaining negatively charged amino acid residues Glu-1052 and Asp-1059 may facilitate proton conduction.

EXPERIMENTAL PROCEDURES

Cell Culture—Human cervix HeLa and human embryonic kidney HEK293T cells were grown as monolayers in minimum essential medium and Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% fetal bovine serum, 40 units/ml penicillin G, and 100 μg/ml streptomycin under a 95% air, 5% CO₂ atmosphere at 37 °C. For electrophysiological experiments, cells were detached from a plastic substrate and cultured in suspension with agitation for 15–300 min.

Recombinant Expression and Small Interfering RNA Transfection—Recombinant expression was performed as previously described (36). HEK293T cells in 6-well plates were transfected 24 h after plating with pIRE2-EGFP vector (Clontech, Mountain View, CA) for mock-transfected (HEK/Mock) cells or pIRE2-EGFP-TRPM7 for TRPM7-transfected (HEK/ TRPM7) cells. pIRE2-EGFP-TRPM7 was prepared by inserting TRPM7 from pCI-neo-TRPM7 (a gift from Y. Mori) into pIRE2-EGFP. The mutations were introduced into hTRPM7 by using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Mutations were confirmed by sequencing the entire insert region. Lipofectamine 2000 (Invitrogen) was used for transfections according to the manufacturer’s instructions. All experiments using these HEK/Mock and HEK/TRPM7 cells were performed between 36–48 h after transfection.

HeLa cells were transfected with small interfering RNA (siRNA) as previously reported (31). Mock siRNA and TRPM7 siRNA, which were both Alexa 488-conjugated, were purchased from Qiagen (Chatsworth, CA). The mock siRNA had a scrambled, nonsilencing sequence. For experiments, successfully transfected cells were selected by their Alexa 488 fluorescence. Suppression of molecular expression of TRPM7 by the siRNA treatment in HeLa cells was confirmed by reverse transcription-PCR and immunoblot analyses in our previous study (31).

Patch Clamp Experiments—Whole-cell recordings were performed at room temperature (22–26 °C). Pipettes were pulled from borosilicate glass capillaries with a micropipette puller (P-97; Sutter Instruments, Novato, CA). The electrode had a resistance of around 5 megaohms for whole-cell recordings when filled with pipette solution. Currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Current signals were filtered at 5 kHz using a 4-pole Bessel filter and digitized at 20 kHz. pClamp software (Version 9.0.2; Axon Instruments) was used for command pulse control, data acquisition, and analysis. Series resistance (<5 megaohms) was compensated (to 70–80%) to minimize voltage errors. The reversal potential was measured by ramp pulses from −100 mV to +100 mV at a speed of 1 mV/ms from a holding potential of 0 mV. To observe the voltage dependence of the current profile, step pulses were applied from a holding potential of 0 mV to test potentials (50 ms duration) up to +100 in 20-mV increments or 0 to −200 mV in 20-mV decrements.

The intracellular (pipette) solution (300 mosmol/kg of H₂O) contained 100 mM NMDG-aspartate, 1 mM EGTA, 1 mM EDTA, 100 mM HEPES, and 0.5 mM HCl (pH 7.4). The control bath solution (320 mosmol/kg of H₂O) contained 100 mM NMDG-aspartate, 1 mM EDTA, 100 mM HEPES or mM MES, and 30 mM mannitol (pH 2.0–8.0). To observe the effects of Mg²⁺ and/or Ca²⁺, varying concentrations (up to 10 mM) of MgSO₄ and/or calcium gluconate were added to the control bath solution while maintaining a constant osmolality by reducing the concentration of mannitol. The free Mg²⁺ or Ca²⁺ concentration was calculated with CaBuf software (provided by Dr. G. Droogmans, KUL, Belgium).

Chemicals—EDTA, EGTA, and dimethyl sulfoxide were purchased from Dojinido (Kumamoto, Japan). All other reagents were obtained from Sigma Aldrich.

Statistical Analysis—Data are presented as the means ± S.E. of n observations. Statistical differences of the data were evaluated by the paired or unpaired Student’s t test and were considered significant at p < 0.05.

RESULTS

Proton Conductance of Human TRPM7 Heterologously Expressed in HEK293T Cells—When whole-cell patch clamp was performed using NMDG-aspartate-rich intracellular (pipette) and extracellular (bath) solutions free of divalent cations, small currents were recorded at pH 7.4 (−2.8 ± 0.8 pA/picofarads (pF) at −100 mV, n = 10) and pH 4.0 (−10.2 ± 1.8 pA/pF at −100 mV, n = 10) in HEK/Mock cells. In HEK/TRPM7 cells at pH 7.4, only small currents, like those recorded in HEK/Mock cells, could be observed in response to positive voltage step pulses, but sizable inward currents were observed at negative potentials ≤−60 mV as shown in Fig. 1. The inward currents exhibited time-dependent activation (Fig. 1A, upper left panel) and inward rectification (Fig. 1B, triangles). When extracellular pH was decreased to 4.0, inward (but not outward) currents recorded in HEK/TRPM7 cells prominently increased. The inward currents again exhibited inward rectification (Fig. 1B, circles) and activation kinetics (Fig. 1A, upper right panel), although inactivation followed rapid activation at large negative potentials (≤−100 mV). Because the extracellular and intracellular solutions contained only ions of large size such as NMDG⁺, aspartate−, HEPES, and MES, it is likely that these inward currents are carried mainly by H⁺. In fact, the reversal potential (Erev) shifted to positive potentials when extracellular pH decreased from 7.4 to 4.0 (Fig. 1B). In contrast, the reversal potential changed little (by −0.1 ± 0.3 mV, n = 6) when 50 mM NMDG-aspartate was replaced with 100 mM mannitol in the extracellular solution at pH 4.0. The slope of changes in Erev...
The peak amplitude of the inward proton currents recorded at −100 mV increased with decreasing extracellular pH (Fig. 2A). The half-maximal pH value was 3.9. For a given pH value, the slope conductance increased with hyperpolarization (Fig. 2B). At pH 4.0, the voltage for half-maximal conductance was estimated to be −75.9 mV by fitting the G-V curve to the Boltzmann equation. Activation kinetics of the inward proton currents recorded at large negative potentials also exhibited voltage and pH dependence. The activation time constant (τ) estimated by single exponential fits decreased with decreasing extracellular pH (Fig. 2C).

From the data shown in Figs. 1 and 2, it is concluded that human TRPM7 exhibits voltage-dependent proton currents under divalent cation-free conditions and that these currents are substantially augmented by low pH.

**Competitive Inhibition of Human TRPM7 Proton Currents by Divalent Cations**—When 10 mM Mg^{2+} was added to the extracellular solution, inward currents recorded upon application of voltage steps from 0 mV to −100 mV in 20-mV decrements at pH 4.0 were prominently suppressed, as shown in Fig. 3A (top and middle traces). Similar suppression was also observed upon the addition of 10 mM Ca^{2+} (Fig. 3B). Inhibition of TRPM7 proton currents by both divalent cations was concentration-dependent (bottom panels). The half-maximal inhibitory concentration (IC_{50}) was 0.5 mM for Mg^{2+} and 1.9 mM for Ca^{2+}.

Concentration-dependent inhibition by extracellular Mg^{2+} or Ca^{2+} was also observed for proton currents recorded at −200 mV under control pH conditions (pH 7.4), as shown in Fig. 4. A and B). However, divalent cation-induced inhibition took place at much lower concentrations with IC_{50} values of around 8.8 ± 0.1 and 3.7 ± 0.1 μM for Mg^{2+} and Ca^{2+}, respectively. Minimal current was observed when the divalent cations were applied at a concentration of 0.1 mM. However, further addition of divalent cations to micromolar concentrations was found to increase the inward currents at −200 mV; this is presumably due to the inflow of divalent cations per se because TRPM7 is known to be permeable to Mg^{2+} and Ca^{2+} (10, 37). Such anomalous mole fraction behavior indicates that divalent cations compete with protons for binding and permeation sites.

When equimolar Mg^{2+} and Ca^{2+} were added together, similar effects on proton currents recorded at −200 mV under acidic conditions (pH 5.5 or 4.75) were observed, as shown in Fig. 4C. At low concentrations, these divalent cations inhibited proton currents with an IC_{50} of around 0.1 mM each. The minimum currents at pH 5.5 or 4.75 were observed at concentrations of around 0.1 and 1 mM, respectively, of these divalent cations (Mg^{2+} plus Ca^{2+}). The data shown in Figs. 3 and 4 clearly indicate that proton conduction through human TRPM7 is sensitive to divalent cations.
TRPM7 Conducts Protons

TRPM7 is blocked by Mg\(^{2+}\) and Ca\(^{2+}\) and that this is due to its competition with the protons for binding and permeation sites within the pore region.

Determinant Amino Acid Residues for Proton Conduction through Human TRPM7—The half-maximal pH value (pH 3.9; Fig. 2A) is close to the \(pK_a\) value of free glutamate (4.3) and aspartate (3.7). Also, in the putative pore region of TRPM7, the only negatively charged amino acid residues are two glutamates and two aspartates (38), as seen in Fig. 5B. Thus, it is likely that one or more of these four residues serve as sites that determine the proton conductivity of human TRPM7. Therefore, we next examined the effects of sequentially neutralizing these negatively charged residues (Glu-1047, Glu-1052, Asp-1054, Asp-1059) on the proton conductance.

Western blot analysis showed molecular expression of not only wild type but also the E1047A, E1052A, D1054A, D1054E, and D1059A mutants in the membrane fraction derived from transfected HEK293T cells (supplemental Fig. 1). By confocal microscopy it was found that a large part of the immunoreactivity to a polyclonal antibody to TRPM7 was localized in the peripheral regions of the cell, presumably the plasma membrane, in HEK293T cells transfected with wild-type TRPM7 or any of these mutants (supplemental Fig. 2).

The neutralized mutant D1054A, confirmed to be molecularly expressed, abolished proton currents measured at pH 4.0, as shown in Fig. 5, A and B. On the other hand, the negatively charged mutant D1054E exhibited proton currents as large as those of wild-type TRPM7 (WT). Therefore, it is concluded that the Asp-1054 residue serves as a crucial determinant of the proton conductivity of human TRPM7.

The neutralized E1047A mutant failed to express any currents (Fig. 5B) despite the fact that there was clear molecular expression of the protein. Furthermore, we were unable to detect functional expression of another neutralized mutant, E1047Q, or of the negatively charged mutant E1047D (data not shown). These results indicate that Glu-1047 in the human TRPM7 protein is required for channel formation or for maintenance of pore integrity.

Neutralization of Glu-1052 or Asp-1059 resulted in protein with functional expression but a significantly reduced proton conductance, as summarized in Fig. 5B. This reduced functional expression may be partly due to the less marked molecular expression of E1052A and D1059A in the membrane fraction compared with wild-type TRPM7 (supplemental Fig. 1). However, molecular expression of these mutants in the plasma membrane region looked to be similar to that of wild-type TRPM7 (supplemental Fig. 2). Furthermore, both the E1052A and D1059A mutants were found to exhibit reduced sensitivity to protons with decreasing a half-maximal pH value by around 1.5 (data not shown). Thus, it is possible that Glu-1052 and Asp-1059 are involved in facilitating the conduction of protons through the channel.

Proton Conductance of Human TRPM7 Endogenously Expressed in HeLa Cells—To explore whether human TRPM7 can function endogenously as a proton-conducting channel, whole-cell patch clamp studies were performed in human epithelial HeLa cells, which express TRPM7 (31), using divalent cation-free, NMDG-aspartate-rich solutions. The inward currents markedly increased when extracellular pH was decreased from 7.4 to 4.0, as shown in Fig. 6A. When extracellular pH was decreased, the \(E_{rev}\) value shifted to positive potentials; plotting \(\Delta E_{rev}\) against extracellular pH gave a slope of 42.1 mV/pH (Fig. 6B).

When TRPM7 expression in HeLa cells was suppressed by using
siRNA specific for TRPM7, as in our previous study (31), the inward currents recorded at both pH 7.4 and 4.0 were largely abolished (Fig. 6, C and D). In contrast, mock siRNA failed to affect the inward currents (Fig. 6D). These data clearly indicate that human TRPM7 endogenously expressed in HeLa cells serves as a proton-conducting pathway under acidic conditions.

**DISCUSSION**

Protons are known to modulate the activity of channels from a wide variety of channel superfamilies (28, 29); these channels include the acid-sensitive ion channels ASIC (39, 40) and TASK (41, 42). Extracellular acidification inhibits the channel activity of TRPV5 (43) and TRPM5 (44), whereas it enhances the currents of TRPV1 (45, 46) and the inward currents of TRPM7 (30, 35) and TRPM6 (35). Also, protons are known to permeate some channel types. Voltage-gated proton-selective channels have been reported to be involved in epithelial secretion, muscle contraction, metabolic modulation, fertilization, and phagocyte burst respiration (47). Recently, a four-transmembrane domain protein lacking a putative pore domain has been identified as the molecule corresponding to this particular type of proton channel (33, 34). A member of the TRP channel superfamily, TRPML1, has been identified as a lysosomal proton channel (48). Under acidic conditions, a proton-conductive pathway is also provided by voltage-gated Na<sup>+</sup> channels in the absence of Na<sup>+</sup>/H<sup>+</sup> (49) and amiloride-sensitive Na<sup>+</sup>/H<sup>+</sup> channels (50). Evidence for proton conduction through heterologously expressed mouse TRPM7 has also been presented by Jiang et al. (30). The present study demonstrated proton conductivity of not only heterologously expressed, but also endogenously expressed, human TRPM7. The TRPM7-mediated conduction of protons was activated by hyperpolarization and exhibited inward rectification, in contrast to the proton current through the voltage-gated proton channel, which was activated by depolarization and was outwardly rectifying (47).
pathway (34). In contrast, it was shown in the present study that a negatively charged amino acid residue in the P region of human TRPM7, Asp-1054, is essential for its proton conductivity. Here we also showed that Glu-1052 and Asp-1059 facilitate proton conduction but that Glu-1047 may be required for formation of the human TRPM7 channel. The results from a study on the mouse TRPM7 channel by Li et al. (35) are distinct from our findings on human TRPM7. First, in that study the E1047Q mutant was functional, exhibiting monovalent cation currents but lacking Ca$^{2+}$ and Mg$^{2+}$ permeation and proton sensitivity, whereas in the present study the E1047A, E1047Q, and E1047D mutants resulted in non-functional channels. Second, the D1054A mutation did not produce a significant change, but in the present study the E1047A, E1047Q, and E1047D mutations resulted in functional channels. Here we also showed that Glu-1052 and Asp-1059 facilitate proton conduction as well, so it is likely that the same mutations result in different effects on the mouse and human TRPM7 channel activities. In this regard it must be noted that five of 61 amino acid residues in the S5-S6 region of human and mouse TRPM7 are different, although the amino acid sequences of the putative pore-forming regions were identical to each other.

The present study also demonstrated that the proton conductance of human TRPM7 is competitively inhibited by Ca$^{2+}$ and Mg$^{2+}$. Under neutral pH conditions proton currents were inhibited by micromolar concentrations of Ca$^{2+}$ or Mg$^{2+}$. It was found that at an acidic pH (5.5 or 4.75), however, submillimolar concentrations of these divalent cations were required to inhibit the proton current (see Fig. 4C). Application of these divalent cations at 0.1–1 mm could not abolish the TRPM7-mediated proton current completely. The proton conductance of this ubiquitously expressed member of the TRP channel family may have a function when extracellular pH falls below 5.5 even in the presence of divalent cations at physiologically relevant concentrations. Because a cervico-vaginal pH between 4.0 and 5.0 is considered normal for women with active menstrual cycles (21, 22), the TRPM7 proton conductance, which was shown to be expressed in human cervical HeLa cells in the present study, may be constitutively active in vaginal or cervical epithelial cells in vivo. Similarly high proton concentrations were found in tumor tissues (to pH 5.5), in cardiac ischemia (to pH 5.7), and in the brain during ischemia, seizures, and hyperglycemia (below pH 6) (23–25, 27, 51, 52). Also, local acidosis (to pH 5.4), which influences immune cell function (53), is known to be associated with the inflammatory loci; the acidosis results from increased lactate production through the glycolytic activity of infiltrated neutrophils and from accumulation of short-chain fatty acids due to bacterial metabolism (54). Thus, it is possible that TRPM7-mediated proton influx contributes to pain and other symptoms as well as to the modulation of immune cell function in these pathological conditions.

Acknowledgments—We are grateful to E. L. Lee for discussion and reading the manuscript, to K. Shigemoto and M. Ohara for technical assistance, and to T. Okayasu for secretarial assistance. We thank Y. Fujiwara for discussion. We also thank Y. Mori for providing the human TRPM7 cDNA and S. Kiyonaka for helping with detection of protein expression.

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