Lys$^{1110}$ of TRPM2 is critical for channel activation

Taek-Keun KIM*1, Joo Hyun NAM†1, Won-Gyun AHN*1, Nam-Ho KIM*, Hwa-Yong HAM*, Chang-Won HONG*, Ju-Suk NAM*, Jongho LEE*, Sung-Oh HUH*, Insuk SO‡, Sung Joon KIM*1, and Dong-Keun SONG*2

*Department of Pharmacology, Infectious Disease Medical Research Center, College of Medicine, Hallym University, Chuncheon 200-702, South Korea, †Department of Physiology, College of Medicine, Dongguk University, Gyeongju 780-714, South Korea, and ‡Department of Physiology, College of Medicine, Seoul National University, Seoul 110-799, South Korea

INTRODUCTION

Changes in free cytosolic calcium concentration ([Ca$^{2+}$]) represent one of the most important signalling events in a vast spectrum of cellular physiology and pathology, including muscle contraction, exocytosis, enzyme activation, gene regulation, differentiation, adhesion and apoptosis [1].

TRPM2 (transient receptor potential melastatin 2) is a non-selective Ca$^{2+}$-permeable cation channel [2]. It is highly expressed in a wide variety of cells such as neutrophils [3], monocytes [4], dendritic cells [5], lymphocytes [6], neuronal cells [7], microglia [8], cardiomyocytes [9], endothelial cells [10,11], pancreatic β-cells [12–14] and prostatic cancer cells [15]. The TRPM2 channel is specifically activated by intracellular ADPR (adenosine diphosphoribose) [16,17]. Additionally, NAADP (nicotinic acid adenine dinucleotide phosphate) [18], cADPR (cyclic ADPR) [19], 2′-O-acetyl-ADPR [20], H$_2$O$_2$ [21–23] and intracellular Ca$^{2+}$ [24–26] facilitate activation of TRPM2 channels. Furthermore, protons were found to negatively regulate TRPM2 channel activity [27–29]. Previously, we reported that TRPM2 channel activation is sensitive to intracellular Ca$^{2+}$ concentration [30]. However, the regulatory mechanism of TRPM2 channel activation by intracellular Ca$^{2+}$ is not yet known.

SLO-2, a member of a multigene family of high-conductance K$^+$ channels [31], is activated in an intracellular Cl$^-$-dependent manner [32]. A particular site in the tail, called the ‘chloride bowl’, containing a string of positively charged amino acid residues, has been shown to confer Cl$^-$ sensitivity in SLO-2 channels [32]. Thus we hypothesized that a positively charged amino acid residue-rich region in the TRPM2 channel might also confer sensitivity to Cl$^-$. We focused on the positively charged residue-rich region of residues 1104–1112, located between the TRP (transient receptor potential) domain and coiled-coil region (Figure 1A) in the membrane-proximal C-terminal region of TRPM2, which is well preserved among various vertebrate species (Figure 1B). By site-directed mutagenesis, we analysed the effect of point mutation of each positively charged residue in residues 1104–1112, located between the TRP (transient receptor potential) domain and coiled-coil region (Figure 1A) in the membrane-proximal C-terminal region of TRPM2, which is well preserved among various vertebrate species (Figure 1B). By site-directed mutagenesis, we analysed the effect of point mutation of each positively charged residue in residues 1104–1112 on TRPM2 channel activation induced by Cl$^-$ as well as ADPR and H$_2$O$_2$ in TRPM2-transfected HEK (human embryonic kidney)-293 cells. We found that, in contrast with our initial hypothesis, Lys$^{1110}$ is critical for TRPM2 channel activation in response not only to Cl$^-$, but also to ADPR and H$_2$O$_2$.

MATERIALS AND METHODS

Cell culture and transfection

HEK-293 cells were obtained from the KCLB (Korean Cell Line Bank) and cultured in DMEM (Dulbecco’s modified Eagle’s medium; Gibco) supplemented with 10% FBS (HyClone) and 1% penicillin/streptomycin (Gibco) at 37°C in a 5% CO$_2$ incubator. Cells were grown to 80% confluence 24 h before transfection and then transiently transfected with plasmids using replacing the lysine residue with a positively charged amino acid arginine (K1110R) displayed channel activity similar to wild-type TRPM2. Interestingly, in the K1107N/K1110N double-point mutant, the impaired function of the K1110N mutant in response to ADPR and H$_2$O$_2$, but not to Cl$^-$, was recovered. There were no changes in protein expression, membrane trafficking and oligomerization of the mutant channels. The extent of [Ca$^{2+}$], increase by H$_2$O$_2$ in HEK (human embryonic kidney)-293 cells expressing TRPM2 mutants was well correlated with the degree of susceptibility to H$_2$O$_2$-induced cell death. These results display the crucial role of a positively charged amino acid residue at position 1110 for TRPM2 channel activity.

Key words: adenosine diphosphoribose (ADPR), chloride, hydrogen peroxide, positive charge, point mutation.

Abbreviations used: ADPR, adenosine diphosphoribose; AM, acetoxymethyl ester; DMEM, Dulbecco’s modified Eagle’s medium; HEK, human embryonic kidney; HRP, horseradish peroxidase; hTRPM2, human TRPM2; NMDG, N-methyl-D-glucamine; TRP, transient receptor potential; TRPM2, transient receptor potential melastatin 2; WT, wild-type.

1 These authors contributed equally to this work.

2 To whom correspondence should be addressed (e-mail dksong@hallym.ac.kr).
Figure 1  Lys1110 is critical for [Ca2+]i increase induced by intracellular injection of Cl− in HEK-293 cells

(A) Schematic drawing of the membrane topology and the positively charged amino acid-rich region (residues 1104–1112) of human TRPM2. (B) Alignment of amino acid sequences of TRPM2 from various vertebrates (ClustalW2, http://www.ebi.ac.uk/Tools/msa/clustalw2/). Indicated in bold are the positively charged amino acids of residues 1104–1112. (C–E) Five positively charged residues at position 1104–1112 were changed from lysine (K) to asparagine (N) or from arginine (R) to glutamine (Q) to produce a mutant with neutral residues. [Ca2+]i was measured in Fluo-3 AM-loaded transfected HEK-293 cells. Traces of Cl− injection-induced [Ca2+]i changes in HEK-293 cells expressing the vector, TRPM2 WT, quintuple-point (5 p) K1104N/R1105Q/K1107N/K1110N/K1112N, triple-point (3 p) K1104N/R1105Q/K1107N, double-point (2 p) K1110N/K1112N and single-point K1110N and K1112N mutant channels were shown (n = 21–34). KCl (150 mM) was intracellularly injected as indicated by the arrow. Inset: changes in [Ca2+]i were shown as area under curve (AUC) in arbitrary fluorescence units. The results are the means ± S.E.M. of more than three independent experiments. ***P < 0.001 compared with the respective TRPM2 WT channel.

X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science) according to the manufacturer’s instructions.

Molecular cloning

Site-directed mutagenesis of hTRPM2 (human TRPM2) cDNA in pCI-neo vector (Promega) was created using overlap extension PCR [33]. Two separate PCR fragments for each half of a final product were generated with mutagenesis primers (Supplementary Table S1 at http://www.biochemj.org/bj/455/bj4550319add.htm). The two products were mixed, and a second PCR was performed using the two outside primers. The final products were digested with AccI and subcloned into the AccI site of pCI-neo (hTRPM2). The full coding regions of all mutant constructs used in the present study were verified by DNA sequencing.

Calcium measurement

[Ca2+]i was measured using the fluorescent Ca2+ indicator Fluo-3 AM (acetoxymethyl ester) (Molecular Probes). HEK-293 cells were transiently transfected with TRPM2 WT (wild-type) or its mutants. Cells were loaded with Fluo-3 AM (2 μM) in Tyrode’s buffer [145 mM NaCl, 2.5 mM KCl, 1.2 mM MgCl2, 1.5 mM CaCl2, 10 mM glucose and 10 mM Hepes (pH was adjusted to 7.4 with 1 M NaOH)] for 1 h at 37°C. After washing with Tyrode’s buffer, Fluo-3 AM-loaded cells were resuspended in Tyrode’s buffer and plated on to 96-well plates. Traces of [Ca2+]i in Fluo-3 AM-loaded cells were measured with λex = 485 nm/λem = 525 nm using a Spectramax M2e fluorescence microplate reader (Molecular Devices). Fluorescent emission readings were recorded every 10 s. Changes in [Ca2+]i were expressed as the relative fluorescence intensity of Fluo-3 AM over baseline fluorescence intensity (F/F0).

Whole-cell recording

For electrophysiological recordings, HEK-293 cells were transfected with a plasmid containing hTRPM2 or mutated hTRPM2 by using Lipofectamine™ and Plus reagent (Life Technologies). Whole-cell currents were measured 1–2 days after transfection. Transfected cells expressing GFP were visually identified with a Nikon inverted microscope (Ti-U) equipped with a high-density mercury lamp light source for excitation of green fluorescence from GFP. The cells were transferred into a bath mounted on the stage of the inverted microscope. The bath (approximately 0.2 ml) was superfused at 5 ml/min, and voltage-clamp experiments were performed at room temperature (22–25°C). Patch pipettes with a free-tip resistance of approximately 3 MΩ were connected with the head stage of a patch-clamp amplifier (Axopatch 200B; Molecular Devices). The pCLAMP software v.10.3 and the Digidata 1440 system (Molecular Devices) were used to acquire data and apply command pulses. Whole-cell currents were recorded at a sampling rate of 10 kHz and were low-pass filtered at 5 kHz. Current traces were analysed using the Clampfit v.10.3 and Origin v.8.0 software (Microcal). The current amplitudes were normalized to the membrane area measured by electrical capacitance (pA/pF). The pipette solution for the whole-cell patch clamp contained 90 mM KCl, 40 mM potassium aspartate, 10 mM NaCl, 15 mM aspartic acid, 0.5 mM MgCl2, 0.2 mM EGTA and 10 mM Hepes, pH 7.2 (titrated with KOH). The bath solution contained 90 mM NaCl, 50 mM sodium aspartate, 3 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose and 10 mM Hepes, pH 7.4 (titrated with NaOH).
Microinjection

Microinjection was performed as described previously [6]. HEK-293 cells transfected with TRPM2 WT or mutants were cultured on a 35-mm-diameter glass bottom culture dish before experiments. Fluoro-3-loaded transfected HEK-293 cells were placed on to a heated microscope stage. KCl (150 mM) or ADPR (10 mM) was dissolved in intracellular injection buffer (27 mM K₂HPO₄, 8 mM Na₂HPO₄, and 26 mM KH₂PO₄, pH 7.2). Injection was performed with a Femtotet microinjector. InjectMan N12 micromanipulator (Eppendorf) and finely pulled glass capillaries with a tip inner diameter of 0.5 mm Femto tip II (Eppendorf). The systems were run in the semiautomatic mode with the following instrumental setup conditions: pipette angle 45°, injection pressure 30 hPa, injection time 0.1 s and velocity of the pipette 700 mm/s. Images were acquired using a Zeiss LMS 510 laser scanning confocal microscope. Fluoro-3 fluorescence emission readings were recorded every 0.5 s.

Western blotting analysis

HEK-293 cells were transiently transfected with TRPM2 WT or mutants (as described above). At 24 h after transfection, cells were washed with chilled PBS and lysed in 0.5 ml of lysis buffer containing 150 mM NaCl, 25 mM Tris/HCl, 1 mM EDTA, 5% (v/v) glycerol, 1% (v/v) Nonidet P40 and Complete™ protease inhibitor cocktail (Roche Applied Science). The lysates were incubated on ice for 30 min and centrifuged at 16 000 × g for 30 min. The supernatant was then transferred to a new tube. Cell lysates were separated on an 8% polyacrylamide gel and were transferred to PVDF membranes (Millipore). The membrane was blocked with 5% (w/v) non-fat dried skimmed milk powder in TBST (20 mM Tris/HCl, pH 7.5, 150 mM NaCl and 1% Tween 20) for 1 h and then incubated with anti-TRPM2 antibody (1:500 dilution; Bethyl Laboratories) overnight at 4°C. Blots were then washed with TBST and incubated with HRP-conjugated anti-(rabbit IgG) antibody (1:10 000 dilution; Jackson ImmunoResearch). ECL was used for detection of signal. Images were obtained by using LAS-4000 Imaging System (Fujifilm Life Science).

Surface biotinylation assay

HEK-293 cells transfected with TRPM2 WT or mutants in 60-mm-diameter dishes were washed with ice-cold PBS (pH 8.0), and incubated with 1 mg/ml sulfo-NHS-SS-biotin (N-hydroxysulfosuccinimide ester of biotin with an ethyl-1,3-dithiopropionate spacer) (Pierce) for 30 min at 4°C. Biotinylation was terminated by washing with ice-cold PBS containing 50 mM glycine. Cells were lysed in lysis buffer containing 150 mM NaCl, 25 mM Tris/HCl, 1 mM EDTA, 5% (v/v) glycerol, 1% (v/v) Nonidet P40 and protease and phosphatase inhibitor cocktail (Roche Applied Science) for 30 min at 4°C, and then centrifuged at 16 000 × g for 30 min. The supernatant was incubated with NeutrAvidin agarose beads (Pierce) for 18 h at 4°C. Biotin-NeutrAvidin complexes were collected, washed, resuspended in 2× Laemmli buffer, and incubated at 95°C for 5 min. Proteins were resolved by SDS/PAGE and Western blotting analysis.

Immunocytochemistry

Cells were fixed with 2% (w/v) paraformaldehyde for 15 min at room temperature. Cells were then permeabilized using 0.05% Triton X-100 for 10 min at room temperature. After a 1 h blocking period in PBS containing 3% BSA, the cells were incubated for 1 h with a rabbit anti-TRPM2 antibody (1:200 dilution) in blocking buffer. After 1 h of incubation at room temperature, cells were washed three times in PBS and were incubated for 1 h with the Alexa Fluor® 488 goat anti-(rabbit IgG) antibody (1:500 dilution). After three washes in PBS, the cells were mounted using mounting medium. Visual inspection and recording of images were performed using Axio Observer A1 fluorescence microscope (Carl Zeiss).

Cell viability assay

Cell viability assay was assessed by the Trypan Blue exclusion method. Briefly, HEK-293 cells transfected with TRPM2 WT or mutants were seeded on to 12-well plates at a density of 2.5 × 10⁴ cells per well. H₂O₂ (1 mM) was added to DMEM for the indicated periods, and then cells were immediately stained with 0.4% Trypan Blue for 5 min at room temperature. Viability of the cells was estimated as the percentage of the ratio of the number of unstained, viable cells to the total number of stained and unstained cells.

Blue native PAGE

Blue native gel electrophoresis was performed using the Bis-Tris NativePAGE system (Invitrogen) according to the manufacturer’s instructions. Briefly, HEK-293 cells were transfected for 24 h, washed with ice-cold PBS, and pelleted by centrifugation at 1200 rev./min for 5 min at 4°C. Cell pellets were washed twice with ice-cold PBS, followed by resuspension in 2% digitonin native lysis buffer [50 mM BisTris, 50 mM NaCl, 10% (w/v) glycerol, 0.001% Ponceau S, 2% digitonin, Complete™ protease inhibitor cocktail (EDTA-free), pH 7.2]. Cell lysates were incubated on ice for 30 min, and then insoluble cell debris was pelleted by centrifugation at 13 000 rev./min for 20 min at 4°C. Lysates were quantified for total protein using the RC DC™ protein assay (Bio-Rad Laboratories), equalized for total protein, and separated by NativePAGE using the Novex Bis-Tris gel (4–16%) system according to the manufacturer’s instructions (Invitrogen). Native gels were soaked in 0.1% SDS for 10 min before transfer to PVDF membranes (Millipore) using the iBlot® device (Invitrogen) with program P3 and conventional Western blotting analysis. Antibodies used were anti-TRPM2 (Santa Cruz Biotechnology) and HRP-conjugated anti-(goat IgG) (Jackson ImmunoResearch Laboratories). Blocking peptide was from Santa Cruz Biotechnology.

Immunoprecipitation

Cells were washed with ice-cold PBS and lysed in ice-cold immunoprecipitation lysis buffer (25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40 and 5% glycerol) and Complete™ protease inhibitor cocktail (Roche Applied Science). Lysates were clarified by centrifugation at 16 000 × g for 20 min. The lysates were pre-cleared for 2 h at 4°C with Protein A/G PLUS–agarose (Santa Cruz Biotechnology). The lysates without Protein A/G PLUS–agarose were collected and were incubated with anti-TRPM2 antibody (Bethyl Laboratories) overnight at 4°C. The immunocomplexes were incubated with Protein A/G PLUS–agarose beads (20 μl of 50% bead slurry) with gentle rocking for 2 h at 4°C. Immunoprecipitates were washed three times with immunoprecipitation lysis buffer and resuspended in 2× SDS sample buffer. The protein samples were separated by SDS/PAGE (8% gels).
Figure 2  Lys\(^{1110}\) mutants had no defects in protein expression, membrane localization and oligomerization

(A) Immunofluorescence staining of HEK-293 cells expressing vector, TRPM2 WT and K1110N mutant with anti-TRPM2 antibody. (B) Western blot analysis of whole-cell lysates and biotin-labelled membrane proteins from HEK-293 cells expressing vector, TRPM2 WT and K1110N mutant with anti-TRPM2 antibody. (C) Blue native PAGE analysis of HEK-293 cells expressing vector, TRPM2 WT, K1110E and K1110R mutants with anti-TRPM2 antibody and a blocking peptide. Molecular masses in kDa are indicated.

Silver staining

Silver staining was performed as described previously [34]. Briefly, after electrophoresis, the gel was fixed in 40% ethanol and 10% acetic acid for at least 1 h. It was then washed for 20 min with 30% ethanol in double-distilled water and additionally for 20 min with double-distilled water to remove the remaining acid. The gel was sensitized by 1 min incubation in 0.02% sodium thiosulfate, and it was then rinsed with two changes of double-distilled water for 20 s each. After rinsing, the gel was submerged in chilled 0.2% silver nitrate (with 0.02% formalin) solution and incubated for 20 min at 4 °C. After incubation, the silver nitrate was discarded, and the gel was rinsed twice with double-distilled water for 1 min and then developed in 3% sodium carbonate (with 0.05% formalin) solution with intensive shaking. After the desired intensity of staining was achieved, the development was terminated with 5% glycine.

Statistical analysis

Data were analysed with Graphpad Prism 5.0, using either a two-tailed Student’s \(t\) test or ANOVA. Bonferroni test was used for post-hoc comparison. All data was presented as means ± S.E.M. \(P < 0.05\) was considered statistically significant.

RESULTS

TRPM2 Lys\(^{1110}\) is critical for [Ca\(^{2+}\)]\(i\) increase induced by intracellular injection of Cl\(^-\) in HEK-293 cells

We searched for a positively charged amino acid-rich region in human TRPM2 channel. As shown in Figure 1(A), the region of residues 1104–1112 located between the TRP domain and the coiled-coil region in the C-terminal region of hTRPM2 has five positively charged amino acid residues out of nine residues, which were well preserved among various species (Figure 1B).

Next, we investigated the functional role of these five positively charged residues by site-directed mutagenesis. We first replaced all of the five positively charged residues (Lys\(^{1104}\), Arg\(^{1105}\), Lys\(^{1107}\), Lys\(^{1110}\) and Lys\(^{1112}\)) in this region with neutral residues (changing lysine to asparagine, and arginine to glutamine) (K1104N/R1105Q/K1107N/K1110N/K1112N). [Ca\(^{2+}\)]\(i\) changes induced by intracellular injection of Cl\(^-\) were monitored in HEK-293 cells expressing TRPM2 WT or mutant channels. The five-point neutral residue mutant showed a marked reduction in Cl\(^-\)-induced [Ca\(^{2+}\)]\(i\) response (Figure 1C). Next, we examined whether the first three-point neutral residue mutant (K1104N/R1105Q/K1107N) and the last two-point neutral residue mutant (K1110N/K1112N) also show the impaired response to Cl\(^-\). Intriguingly, the last two-point neutral residue mutant (K1110N/K1112N), but not the first three-point neutral residue mutant (K1104N/R1105Q/K1107N), failed to respond to Cl\(^-\) (Figure 1D). We next examined the effects of each single mutation (K1110N or K1112N) on the Cl\(^-\)-induced changes in [Ca\(^{2+}\)]\(i\). Interestingly, K1110N mutant, but not K1112N, showed a markedly reduced [Ca\(^{2+}\)]\(i\) increase in response to Cl\(^-\) (Figure 1E). Altogether, these results indicate that Lys\(^{1110}\) is important for TRPM2 channel activation induced by intracellular injection of Cl\(^-\).

Lys\(^{1110}\) mutants had no defects in protein expression, plasma membrane localization and oligomerization

Loss of channel function as a result of mutation could be attributed to defects in protein expression or membrane trafficking. To investigate these possibilities, we performed immunofluorescence staining. Immunofluorescence for TRPM2 WT and K1110N mutant was similar, suggesting essentially similar protein expression (Figure 2A), which was also confirmed by Western blot analysis (Figure 2B, left-hand panel). Low levels of endogenous TRPM2 expression in HEK-293 cells have been reported previously [35,36]. In addition, we also performed
surface biotin-labelling experiments. As shown in Figure 2(B) (right-hand panel), there was no noticeable difference in surface expression levels between TRPM2 WT and the K1110N mutant. Subunit interaction is the prerequisite for assembly of the TRPM2 channel [37]. Thus we checked whether TRPM2 oligomerization was altered in TRPM2 mutants by analysing with Blue native PAGE (Figure 2C). TRPM2 WT, K1110E and K1110R mutants displayed the same expression level of tetrameric structure. In the presence of a blocking peptide, the bands completely disappeared, indicating the specificity. Collectively, these results suggest that the channel mutation (K1110N, K1110E and K1110R) did not significantly alter protein expression, membrane localization or subunit oligomerization, excluding these possibilities as the cause of the loss of channel activity.

Positively charged residue at position 1110 is essential for TRPM2 channel activity

Next, we tried to replace Lys^1110 (a positively charged residue) with either a negatively charged residue (glutamic acid) or another positively charged residue (arginine). Replacement of Lys^1110 with the negatively charged amino acid glutamic acid generated a mutant (K1110E) that failed to induce [Ca^{2+}]_i, increase by KCl injection, as in the case with the neutral amino acid asparagine (K1110N) (Figure 3A). However, replacing lysine with another positively charged amino acid, arginine (K1110R), displayed channel activity roughly similar, although significantly lower, to TRPM2 WT in response to Cl^- injection (Figure 3A). H_2O_2 [21–23] and ADPR [16,17] are major activators of TRPM2 channel opening. Therefore we asked whether these mutants display similar [Ca^{2+}]_i response patterns when stimulated with H_2O_2 or ADPR. Remarkably, H_2O_2 (Figure 3B) and intracellular injection of ADPR (Figure 3C) failed to induce [Ca^{2+}]_i changes in HEK-293 cells transfected with K1110N and K1110E mutants. However, the K1110R mutant displayed channel activity similar, although significantly higher, to TRPM2 WT (Figures 3B and 3C). Taken together, these data indicate that a positive charge at residue 1110 is very important for TRPM2 channel activation in response not only to Cl^-, but also to H_2O_2 and ADPR (Figure 3). It was also noted that K1110E (mutant with a negatively charged residue) displayed a significantly more depressed [Ca^{2+}]_i change than K1110N (mutant with a neutral residue) in response to all three stimuli (Figure 3).

We next investigated whether K1110N and K1110E mutants affect TRPM2 channel activity. Whole-cell patch-clamp recordings were performed to compare ADPR-induced TRPM2 currents in HEK-293 cells that expressed TRPM2 WT or K1110N and K1110E mutants. Channels were stimulated with 30 μM ADPR applied to the cytosolic side by diffusion from the patch pipette. Inward currents induced by TRPM2 WT gradually developed during diffusion of ADPR into the cells and reached peak amplitudes (Figure 4A). These inward currents were completely abolished when we replaced the external cation (Na^+ and K^+) with the large impermeable cation NMDG (N-methyl-D-glucamine). However, ADPR-induced currents were markedly reduced in the case of K1110N and K1110E mutants (Figures 4B and 4C). The peak amplitudes of inward currents after obtaining the whole-cell configuration have been summarized in a histogram, which also shows that these mutants cannot generate ADPR-induced cation currents (Figure 4D). These data indicate that a positive charge at residue 1110 is very important for TRPM2 channel activity in response to ADPR, confirming results obtained with [Ca^{2+}]_i measurement.

**Figure 3** A positively charged residue at Lys^{1110} is essential for TRPM2 channel activity

Traces of [Ca^{2+}]_i increase in Fluo-3 AM-loaded HEK-293 cells expressing TRPM2 WT, K1110N, K1110E and K1110R mutants in response to Cl^- injection (A). H_2O_2 (B) and ADPR injection (C) were shown. [Ca^{2+}]_i fluorescence was expressed as arbitrary units or the relative intensity over baseline fluorescence intensity (F/F0). The arrow indicates the time point when KCl (150 mM) and ADPR (10 mM) were injected, or H_2O_2 (1 mM) was added. The histogram shows the area under curve (AUC) of [Ca^{2+}]_i fluorescence. The results are the means ± S.E.M. of three independent experiments. **P < 0.001 compared with TRPM2 WT channel; ++P < 0.01, +++P < 0.001.

K1107N/K1110N double-point mutant recovered from impaired channel activity of K1110N in response to H_2O_2 and ADPR

Next, we examined whether H_2O_2- and ADPR-induced [Ca^{2+}]_i changes were also inhibited in the five-point neutral residue mutant (K1104N/R1105Q/K1107N/K1109N/K1112N). Surprisingly, H_2O_2- and ADPR-induced [Ca^{2+}]_i changes in the five-point neutral residue mutant were similar to those of TRPM2 WT channel (Figures 5A and 5B). These results suggest that an additional single mutation among K1104N, R1105Q, K1107N and K1112N can rescue the K1110N mutant from the functional impairment in response to H_2O_2 and ADPR. We next examined whether functional recovery occurs in HEK-293 cells transfected with K1104N/K1110N, R1105Q/K1110N, K1107N/K1110N and K1109N/K1112N double-point mutant channels, in terms of its [Ca^{2+}]_i response to stimulation with H_2O_2 or ADPR. Remarkably, the K1107N/K1110N double-point mutant channel, but not others, showed recovery in H_2O_2- and ADPR-induced [Ca^{2+}]_i increase (Figures 5C and 5D). Taken together, these results show...
Figure 4  Impairment of ADPR-induced whole cell currents in K1110N and K1110E mutants

Whole-cell recordings from HEK-293 cells expressing TRPM2 WT and K1110N and K1110E mutants in the presence of ADPR (30 μM; n = 11–19). (A–C) Representative current traces after obtaining the whole-cell configuration in TRPM2 WT and in K1110N and K1110E mutants. ADPR (30 μM) was included in the pipette solution. At the end of each experiment, the normal bath solution (140 mM Na⁺ and 3 mM K⁺) was changed to a solution with NMDG as the main cation (143 mM) in order to confirm the ADPR-induced TRPM2 currents. Cells constantly maintained their membrane potentials at −60 mV. Note that the ADPR-induced inward currents were significantly reduced in K1110N and K1110E mutants (B and C). (D) Comparison of the peak amplitudes of TRPM2 currents measured at −60 mV in TRPM2 WT and in K1110N and K1110E mutants. The results are the means ± S.E.M. (***P < 0.001).

Figure 5  K1107N/K1110N double mutant recovered from impaired channel activity in response to H2O2 and ADPR

[Ca²⁺], was measured in Fluo-3 AM-loaded HEK-293 cells transfected with vector, TRPM2 WT and K1104N/R1105Q/K1107N/K1110N/K1112N mutants (A and B) or with vector, TRPM2 WT, K1107N, K1110N and K1110N/K1112N mutants (C and D). (A and C) H₂O₂ (1 mM)-induced [Ca²⁺] changes. (B and D) [Ca²⁺] changes induced by intracellular injection of ADPR (10 mM). The arrow denotes the time point when H₂O₂ (1 mM) was added (A and C) or ADPR (10 mM) was injected (B and D). Inset: changes in [Ca²⁺], were shown as the area under curve (AUC) of arbitrary fluorescence units. The results are the means ± S.E.M. for more than three independent experiments. ***P < 0.001 compared with the respective TRPM2 WT channel; +++ P < 0.001.
that an additional K1107N mutation can rescue the K1110N mutant from the functional impairment in response to H2O2 and ADPR. Furthermore, these results suggest the differential modes of TRPM2 channel activation by Cl− and H2O2/ADPR.

Susceptibility of Lys1110 mutant-transfected HEK-293 cells to H2O2-induced cell death

TRPM2-mediated Ca2+ influx is responsible for H2O2-induced cell death [7,38], including HEK-293 cells transfected with TRPM2 [22]. Therefore we examined the susceptibility of Lys1110 mutant-transfected HEK-293 cells to H2O2-induced cell death. HEK-293 cells were transfected with vector, TRPM2 WT, K1110N, K1110E or K1110R. After 24 h, the cells were treated with 1 mM H2O2 for 0, 0.5, 3 or 6 h. Cell viability was then assessed by Trypan Blue exclusion. As shown in Figure 6, K1110N, but not K1110R, significantly inhibited the susceptibility to H2O2-induced cell death, compared with TRPM2 WT. Furthermore, in K1110E-transfected cells, H2O2-induced cell death was completely blocked, displaying a good correlation between susceptibility to cell death (Figure 6A) and calcium increase (Figure 3B) in response to H2O2.

TRPM2 was reported previously to directly interact with other proteins. The TRPM2 N-terminus has a calmodulin-binding IQ-like motif, which plays a role in modulating channel activation [36]. Interaction of TRPM2 channel with PTPL1 (protein tyrosine phosphatase-L1) [39] and EFHC1 [40] has also been reported. Thus we examined whether TRPM2 mutants have an impairment of binding with interacting proteins. Immunoprecipitation with anti-TRPM2 antibody with a subsequent separation and silver staining showed that there was no discernible change between TRPM2 WT and mutants regarding binding proteins either under unstimulated or H2O2-stimulated conditions (Figures 6B and 6C).

DISCUSSION

In the present study, we identified by point mutation a single lysine residue of TRPM2 (Lys1110) that crucially participates in the activation of the TRPM2 channel, which is located in the membrane-proximal C-terminal region. This region between the TRP domain and coiled-coil region (Figure 1A) has not received any special attention thus far. Previously, an important role of some amino acid residues in the pore region (Glu960, Gln981, Asp987, Glu1022 [41], Gln992, His995 [42], Cys996, Cys1008 [43] and Pro1018 [44]) for TRPM2 channel function has been reported. Outside the pore region, virtually nothing is known about the requirement of specific amino acid residues for TRPM2 channel activity, except Ile658 in the N-terminal coiled-coil domain [45]. To our knowledge, this is the first report on the important role of single amino acid residue located in the C-terminal region for TRPM2 channel activity.

However, Lys1110 was critical not only for Cl−-induced TRPM2 channel activation, but also for H2O2- and ADPR-induced activation (Figures 3 and 4). Thus, in contrast with our initial hypothesis that residues 1104–1112 may play a ‘chloride bowl’-like role, our results indicate that Lys1110 is involved in the TRPM2 channel activation in response to a wide range of stimuli (Figures 1, 3 and 4). Replacement of lysine with another positively charged amino acid, arginine (K1110R), but not neutral asparagine (K1110N) or the negatively charged amino acid glutamate (K1110E), largely maintained channel function (Figure 3), indicating the.
requirement of a positively charged amino acid residue at position 1110. This result further indicates that the possible requirement of any modification of lysine residue (Lys1110) for TRPM2 channel activation does not need to be considered.

Interestingly, the K1107N/K1110N double-point mutant as well as the five-point mutant (K1104N/R1105Q/K1107N/K1110N/K1112N) rescued the K1101N mutant from impaired channel activity in response to H2O2 and ADPR (Figure 5) but not to Cl⁻ (Figure 1C). Furthermore, compared with TRPM2 WT, the K1110R mutant displayed a significantly greater response to H2O2/ADPR (Figures 3B and 3C), whereas it showed a significantly lesser response to Cl⁻ (Figure 3A). Taken together, these results suggest that Cl⁻ has a TRPM2 activation mechanism, which is different from that of H2O2/ADPR.

Under physiological conditions, PtdIns(4,5)P₂ is negatively charged, allowing it to interact electrostatically with positively charged residues on membrane proteins. Residues 1104–1112 of TRPM2 were proposed to belong to a ‘proximal binding region’, which was supposed to potentially interact with PtdIns(4,5)P₂ and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology [grant number 2012-0000312].

REFERENCES

AUTHOR CONTRIBUTION
Taek-Keun Kim, Joo Hyun Nam and Won-Gyun Ahn executed the experiments and analysed the data. Nam-Ho Kim, Hwa-Yong Ham, Chang-Won Hong, Ju-Suk Nam, Jongho Lee, Sung-Oh Huh, Insuk So and Sung Joon Kim contributed to experimental design, data analysis and supply of critical materials. Taek-Keun Kim, Joo Hyun Nam and Dong-Keun Song wrote the paper. Dong-Keun Song conceived and supervised the experiments.

ACKNOWLEDGEMENTS
We thank Dr Masamitsu Iino (The University of Tokyo), Dr Sung-Chan Kim (Hallym University) and Dr Tal-Soo Ha (Daegu University) for valuable comments on the study.

FUNDING
This study was supported by the Hallym University Specialization Fund [grant number HRF-S-41] and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology [grant number 2012-0000312].
42 Zou, J., Yang, W., Beech, D. J. and Jiang, L.-H. (2011) A residue in the TRPM2 channel outer pore is crucial in determining species-dependent sensitivity to extracellular acidic pH. Pflugers Arch. 462, 293–302
# SUPPLEMENTARY ONLINE DATA

## Lys\(^{1110}\) of TRPM2 is critical for channel activation

Taek-Keun KIM\(^*\), Joo Hyun NAM\(^†\), Won-Gyun AHN\(^*\), Nam-Ho KIM\(^*\), Hwa-Yong HAM\(^*\), Chang-Won HONG\(^*\), Ju-Suk NAM\(^*\), Jongho LEE\(^*\), Sung-Oh HUH\(^*\), Insuk SO\(^\S\), Sung Joon KIM\(^\S\) and Dong-Keun SONG\(^*\)\(^2\)

\(^*\)Department of Pharmacology, Infectious Disease Medical Research Center, College of Medicine, Hallym University, Chuncheon 200-702, South Korea, \(^†\)Department of Physiology, College of Medicine, Dongguk University, Gyeongju 780-714, South Korea, and \(^\S\)Department of Physiology, College of Medicine, Seoul National University, Seoul 110-799, South Korea

---

### Table S1 Reverse transcription primer sequences for TRPM2 mutants

<table>
<thead>
<tr>
<th>Primer</th>
<th>5′→3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPM2-F</td>
<td>AAGCGGATGATGAAGGACGT</td>
</tr>
<tr>
<td>TRPM2-R</td>
<td>CCTCTTCGCTATACGCCAG</td>
</tr>
<tr>
<td>TRPM2 K1104N/R1105Q/K1107N-F</td>
<td>GCCAACCAGCACAACACCAGCTCAA</td>
</tr>
<tr>
<td>TRPM2 K1104N/R1105Q/K1107N-R</td>
<td>TGGACTGGTGTGCTGTTGTCG</td>
</tr>
<tr>
<td>TRPM2 K1110N/K1112N-F</td>
<td>CAGCTCAACAAAACACCTGAGAA</td>
</tr>
<tr>
<td>TRPM2 K1110N/K1112N-R</td>
<td>TTCCCAAGCTGTTGCTGAGCTG</td>
</tr>
<tr>
<td>TRPM2 K1110N-F</td>
<td>CAGCTCAAGAAACAAGCAGAGAA</td>
</tr>
<tr>
<td>TRPM2 K1110N-R</td>
<td>TTCCCAAGCTGTTGCTGAGCTG</td>
</tr>
<tr>
<td>TRPM2 K1112N-F</td>
<td>CAGCTCAAGAAACACCTGAGAA</td>
</tr>
<tr>
<td>TRPM2 K1112N-R</td>
<td>TTCCCAAGCTGTTGCTGAGCTG</td>
</tr>
<tr>
<td>TRPM2 K1107N-F</td>
<td>GCCAAGAGGCAACAACAGAGCTCAA</td>
</tr>
<tr>
<td>TRPM2 K1107N-R</td>
<td>TGGACTGGTGTGCTGTTGTCG</td>
</tr>
<tr>
<td>TRPM2 K1110R-F</td>
<td>CAGCTCAAGAAACACCTGAGAGA</td>
</tr>
<tr>
<td>TRPM2 K1110R-R</td>
<td>TTCCCAAGCTGTTGCTGAGCTG</td>
</tr>
<tr>
<td>TRPM2 K1110E-F</td>
<td>CAGCTCAAGAAACACAGAGAGA</td>
</tr>
<tr>
<td>TRPM2 K1110E-R</td>
<td>TTCCCAAGCTGTTGCTGAGCTG</td>
</tr>
</tbody>
</table>

---

Received 27 February 2013/12 August 2013; accepted 19 August 2013
Published as BJ Immediate Publication 19 August 2013, doi:10.1042/BJ20130303

\(^1\) These authors contributed equally to this work.

\(^2\) To whom correspondence should be addressed (e-mail dksong@hallym.ac.kr).