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Molecular determinant of sensing extracellular pH in classical transient receptor potential channel 5

Min Ji Kim [a], Jae-Pyo Jeon [a], Hyun Jin Kim [a], Byung Joo Kim [a], Young Mee Lee [b], Han Choe [c], Ju-Hong Jeon [a], Seon Jeong Kim [d], Insuk So [a,*]

[a] Center for Bio-Artificial Muscle and Department of Physiology, Seoul National University College of Medicine, 28 Yongon-Dong, Chongno-Gu, Seoul 110-799, Republic of Korea
[b] Department of Dermatology, Seoul National University College of Medicine, 28 Yongon-Dong, Chongno-Gu, Seoul 110-799, Republic of Korea
[c] Department of Physiology, University of Ulsan College of Medicine, Republic of Korea
[d] Center for Bio-Artificial Muscle, Hanyang University, Seoul 133-791, Republic of Korea

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Abstract

The classical transient receptor potential channel 5 (TRPC5) is a molecular candidate for nonselective cation channel (NSCC) activated by muscarinic receptor stimulation whereas extracellular pH inhibits or enhances NSCC activated by muscarinic receptor stimulation depending on extracellular cation compositions in native tissues. We investigated the effect of extracellular pH on TRPC5 and determined amino acid residues responsible for sensing extracellular pH. Extracellular acidosis inhibits TRPC5 with pK_a of 6.24. Under 50 mM intracellular HEPES buffer condition, extracellular acidosis inhibits TRPC5 with pK_a of 5.40. We changed titratable amino acids (C, D, E, H, K, R, Y) to nontitratable amino acids (A, N, Q, N, N, N, F) within pore region between transmembrane segments 5 and 6 in order to determine the residues sensing extracellular pH. Glutamate (at the position 543, 595, and 598), aspartate (at the position 548) and lysine (at the position 554) were responsible for sensing extracellular pH. The effect of extracellular pH in TRPC5 was also dependent on the composition of extracellular monovalent cations. In conclusion, TRPC5 is a molecular candidate for NSCC activated by muscarinic receptor stimulation, has glutamate amino acid residues responsible for sensing extracellular pH, and has a unique gating property depending on the composition of extracellular monovalent cations.

Keywords: Transient receptor potential channel; Extracellular pH; Nonselective cation channel; Muscarinic receptor

Extracellular pH modulates the pattern and amplitude of smooth muscle contractions associated with action potential, high K^+, or acetylcholine. Extracellular acidosis inhibits smooth muscle contraction by modulating transmembrane calcium influx, receptor sensitivity and actomyosin ATPase. Nonselective cation channel activated by muscarinic receptor stimulation is involved in calcium homeostasis by inducing membrane depolarization and calcium influx through channel itself in visceral smooth muscles [1]. Initially Inoue et al. showed that extracellular acidosis enhanced NSCC activated by muscarinic receptor stimulation [2]. In subsequent study, however, Zholos and Bolton argued that the effect of extracellular acidosis on NSCC depended on the compositions of extracellular monovalent cations [3]. Under symmetrical Cs condition (140 mM extracellular Cs and intracellular Cs), extracellular acidosis inhibited NSCC activated by muscarinic receptor stimulation, whereas extracellular acidosis increased NSCC activated by muscarinic receptor stimulation under the normal Tyrode condition [3].

Previously we showed that TRPC5 is a molecular candidate for NSCC activated by muscarinic receptor stimulation [4,5]. In the following studies, we showed that TRPC5 has two regulatory pathways as NSCC activated by muscarinic receptor stimulation in native tissues

* Corresponding author. Fax: +82 2 763 9667.
E-mail address: insuk@plaza.snu.ac.kr (I. So).

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[6–10]; Ca-diacylglycerol (DAG)-protein kinase C (PKC) negative feedback pathway [6,7,10; see also [11]] and Ca-calmodulin (CaM)-myosin light chain kinase (MLCK) positive pathway [8,9; see also [12]]. To further confirm the correlation of TRPC5 with NSCC activated by muscarinic receptor stimulation, we investigated the effect of extracellular pH on TRPC5 channels expressed in HEK cells. We found out that TRPC5 is a molecular candidate for NSCC activated by muscarinic receptor stimulation, has glutamate residues within the pore region responsible for extracellular pH sensing, and has a unique gating property depending on the composition of extracellular monovalent cations.

Materials and methods

Cell culture and transient transfection. Human embryonic kidney (HEK293) cells (ATCC, Manassas, VA) were maintained according to the supplier's recommendations. For transient transfection, cells were seeded in 12-well plates. The following day, 0.5 μg/well of pcDNA vector containing the cDNA for TRPC5-GFP was transfected into the cells using the transfection reagent FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's protocol. After 30–40 h, the cells were trypsinized and used for whole-cell recording.

Molecular biology. Plasmid containing human TRPC5 gene was given kindly by Dr. Shuji Kaneko in Japan. Point mutations in TRPC5 were introduced using the QuickChange site-directed mutagenesis kit (Stratagene) and appropriate primer sets. Sequences of the mutants were confirmed by DNA sequencing.

Whole-cell patch-clamp experiment. Isolated cells were transferred to a small chamber on the stage of an inverted microscope (IX50, Olympus, Japan), and were constantly perfused with PSS at a rate of 2–3 ml/min. A glass microelectrode with a resistance of 2–5 MΩ was used to make a gigaohm seal. The conventional whole-cell patch-clamp technique was adopted to hold the membrane potential at −60 mV using an Axopatch 200B patch-clamp amplifier (Axon Instrument, USA). For data acquisition and the application of command pulses, pCLAMP software v.9.2 and Digidata 1322A (Axon Instruments) were used. Data were filtered at 5 kHz and displayed on a computer monitor. Data were analyzed using pCLAMP and Origin software (Microcal origin v.6.0, USA).

Solutions and drugs. Physiological salt solution (PSS, in mM) contained NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, and HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]) 10, and pH was adjusted to 7.4 using NaOH. Cs-rich external solution was made by replacing NaCl and KCl with equimolar CsCl. In Fig. 4, Physiological salt solution contained 140 mM NaCl and 5 mM CsCl instead of 135 mM NaCl and 5 mM KCl. HEPES (6.4–8.9) or MES (4.4–5.9) was used as a pH buffer. The pipette solution (in mM) contained CsCl 140, HEPES 10, Tris–GTP (Tris–Guanosine 5′-triphospate) 0.2, EGTA [Guanosine 5′-*O*-(3-thiotriphosphate)] 0.5, Mg–ATP (Adenosine 5′-triphosphate) 3, and its pH was adjusted to 7.3 with CsOH.

Statistics. All data are expressed as means ± SEM. Statistical significance was determined using the Student's unpaired *t*-tests. *P* values of <0.05 were considered statistically significant. The number of cell recordings is represented by *n*.

Results

The effect of extracellular pH on TRPC5

Whole-cell currents were recorded using patch-clamp techniques. Intracellular GTPγS (0.2 mM) induced an inward current at a holding potential of −60 mV. In the beginning, whole-cell currents were recorded under the condition of PSS (140 mM $[Na^+]_o$) and $[Cs^+]_i$. In order to obtain current–voltage (*I–V*) relationship, we applied a ramp pulse from +100 mV to −100 mV for 500 ms. After changing the external solution from PSS to 140 mM $[Cs^+]_o$ solution, currents increased because extracellular Cs enhanced channel numbers, open probability and single channel conductance of TRPC5 channels [13]. Whole-cell current under the condition of 140 mM $[Cs^+]_o$ and $[Cs^+]_i$ showed a typical doubly rectifying current–voltage relationship (Fig. 1A). The current amplitude at −60 mV was 406 ± 89 p*A*/p*F* ($n = 14$). We used results only obtained from cells showing the typical *I–V* relationship of TRPC5 for the subsequent studies. When extracellular pH was decreased, the currents were inhibited with pK_a of 6.24 ($n = 20$) as in native tissues (Fig. 1A and also see [2,3]). When the extracellular pH decreased, outwardly rectifying currents were activated (Supplementary Fig. 1; see also [14]). Lambert and Oberwinkler [14] showed that the current is a proton activated, outwardly rectifying anion current, and inhibited by anion channel blocker, like 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), rapidly and potently (IC_{50}: 2.9 μM). Thus, we compared the amplitude of the currents under the different pH conditions at a potential of −60 mV to get dose-response curve. Extracellular acidosis decreased the inward and outward currents of TRPC5 channels (Fig. 1A). Even when 50 mM HEPES was used for buffering intracellular hydrogen ions, extracellular acidosis inhibited TRPC5 currents with pK_a of 5.40 ($n = 7$). These results give evidence that TRPC5 is a molecular candidate for NSCC activated by muscarinic receptor stimulation.

The effect of extracellular pH on TRPC5 mutants

To identify the amino acids responsible for proton sensing, we mutated each of the 20 titratable amino acids in the pore region of TRPC5 to a closely related nontitratable residue (Fig. 2A; see also [15]) and investigated the sensitivity of each of the mutants to inhibition by extracellular acidosis. The current amplitude of each mutant was smaller than that of wild type except E549Q (368 ± 58 p*A*/p*F*, $n = 6$, $p > 0.05$) and K560N (273 ± 137 p*A*/p*F*, $n = 4$, $p > 0.05$) (Fig. 2B). Y542F, C553A, or C558A mutant was not expressed in HEK cells. The current amplitude of E559Q (39.1 ± 8.2 p*A*/p*F*, $n = 5$, $p < 0.05$), K591N (72.4 p*A*/p*F*), or H594N (15.6 ± 5.6 p*A*/p*F*, $n = 3$, $p < 0.05$) was too small to be investigated for the effect of extracellular proton on TRPC5 currents although they were expressed in HEK cells and showed typical doubly rectifying *I–V* relationship (Supplementary Fig. 2). The remained 14 mutants also showed typical doubly rectifying *I–V* relationship, and the response to extracellular proton was classified into three categories; sensitivity to extracellular proton similar to WT, dual response to extracellular proton, and almost no or decreased sensitivity to extracellular proton. We could not find out any correlation between the level of expression and pH sensitivity of each mutant (Fig. 2).