An optimized recording method to characterize biophysical and pharmacological properties of acid-sensing ion channel

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Abstract: Objective To re-confirm and characterize the biophysical and pharmacological properties of endogenously expressed human acid-sensing ion channel 1a (hASIC1a) current in HEK293 cells with a modified perfusion methods. **Methods** With cell floating method, which is separating the cultured cell from coverslip and putting the cell in front of perfusion tubing, whole cell patch clamp technique was used to record hASIC1a currents evoked by low pH external solution. **Results** Using cell floating method, the amplitude of hASIC1a currents activated by pH 5.0 in HEK293 cells is twice as large as that by the conventional method where the cells remain attached to coverslip. The time to reach peak at two different recording conditions is (21±5) ms and (270±25) ms, respectively. Inactivation time constants are (496±23) ms and (2284±120) ms, respectively. The cell floating method significantly increases the amiloride potency of block on hASIC1a [IC_{50} is (3.4±1.1) μ mol/L and (2.4±0.9) μ mol/L, respectively]. Both recording methods have similar pH activation EC_{50} (6.6±0.6, 6.6±0.7, respectively). **Conclusion** ASICs channel activation requires fast exchange of extracellular solution with the different pH values. With cell floating method, the presence of hASIC1a current was re-confirmed and the biophysical and pharmacological properties of hASIC1a channel in HEK293 cells was precisely characterized. This method could be used to study all ASICs and other ligand-gated channels that require fast extracellular solution exchange.

Keywords: acid-sensing ion channel; patch-clamp recording; pH

1 Introduction

Acid-sensing ion channels (ASICs) that can be activated by external protons belong to a supergene family, which, for humans, encompass ASIC1a, ASIC2a, ASIC3, and ASIC4. ASICs are predominantly expressed in the mammalian nervous system. Strong evidence for the physiological function of these channels has come from recent knockout experiments.

It is now clear that ASICs play an important role in touch, pain sense, acidity sense and memory process; in addition, ASICs also participate in some pathological reactions. They can be modulated by neuropeptides, temperature, metal ions, ischemia, *etc.*, thus integrating each signal of surroundings of cell to exercise their functions^[1-4].

ASIC1a, one of ASIC subunits, seems to play an important role in the physiology and pathophysiology of the central nervous system^[5-7], and contributes to the most abundant ASICs in the brain: homomeric ASIC1a and heteromeric ASIC1a/2a. The knockout of *asic1* leads to deficits in spatial memory and learned fear, suggesting a contribution to higher brain functions. Recent study also showed that ASIC1a in periphery neurons contributes to inflammatory pain and hyperalgesia^[8-10].

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HEK293 cells, a commonly used cell line for the expression and characterization of many ion channels, endogenously express hASIC1a channels^[11] so it is a convenient and economic system to study ASIC channels. Activation of ASIC channels requires rapid extracellular pH exchange. A variety of techniques for rapidly switching bathing solutions are adopted in the study of ASIC channels^[8,12-15]. In all these existing techniques, the cells remain attached to the recording chamber causing dead space around the cell, and only half the cells surface is exposed to the new solution, so it takes a much longer time to exchange the solution surrounding the cell. This will significantly underestimate the biophysical and pharmacological properties of the channels. In this paper, we precisely study the biophysical and pharmacological properties of ASIC1a channels using a commercially available fast switch solution exchange system and cell floating method. This method provides us a useful tool to study not only ASIC channels but also other ligand-gated channels.

2 Materials and methods

2.1 Electrophysiology Whole-cell patch-clamp recordings were performed at room temperature (20–24 °C) in voltage-clamp mode using a Multiclamp 700B amplifier (Axon Instruments, Foster City, CA, USA) controlled by the pClamp10 software suite and output was digitized with a Digidata 1440A converter (Axon instruments).

The extracellular solution contained (in mmol/L): NaCl, 130; KCl, 5; MgCl₂, 1; glucose, 10; 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 10; adjusted to pH 8.0, 7.5, 7.0, 6.5, 6.0, 5.5, 5.0 by the addition of either NaOH or HCl. When pH was lower than 6.5, HEPES was replaced by MES buffer. The glass pipettes were pulled from glass capillary (1B200-4 standard borosilicate glass tubing, WPI). Electrodes (2–6 M Ω) were filled with (in mmol/L) CsCl, 140; MgCl₂, 4; EGTA, 10; HEPES, 10; adjusted to pH 7.3 with CsOH.

2.2 Extracellular solution delivery Drug application and changes in extracellular pH were performed using a commercially available automated fast solution exchange system (Warner Instruments, SF-77B perfusion fast step system), which can perform individual solution changes in 20 ms about and was interfaced with our acquisition software so as to generate precisely timed alterations in extracellular solution. The recording chamber containing the cells had a volume of

370 μ L and was continuously perfused with the external buffer (pH 8.0). The bottom of the theta-tube output was sanded flat to decrease its wall thickness and thereby minimize the distance between the solutions emerging from the barrels and the cell on the bottom of the cell chamber. The switcher was positioned above the cell bath so that the output end of the theta tube was within the microscope viewing field and nearly touching the glass bottom of the bath. A suitable cell laying on the bottom of the bath was positioned 400–600 μ m in front of the appropriate barrel using the x,y-positioning of the microscope stage. Control (pH 8.0) and test solutions (pH 7.0–5.0) flow continuously through adjacent stimulus delivery tubes. Rapid switching is achieved by using the presetable pClamp recording protocol to start patch clamp experiment which also sends a signal to the controller of the solution exchange system to trigger the rapid lateral movement of the double barrel theta tube resulting in a fast change of solution surrounding the cell from pH 8.0 to various low pH values.

Conventional recording method (cells attached to glass coverslip): HEK293 cells were plated onto 10 mm glass coverslip in 100 mm plastic culture dish for 24 h with 50% to 70% confluence. The coverslip that contained HEK293 cells was placed on bottom of recording chamber before the experiment.

Cell floating method: HEK293 cells were plated onto 100 mm plastic culture dish without coverslip for 24 h with 50% to 70% confluence. Cells were trypsinized and suspended in culture media. A few drops of cell solution were dropped into the recording chamber and the cells will settle to the bottom of recording chamber in a few minutes. After a gigaseal cell attachment was made, rupture of the membrane patch under the suction pipette and establishment of a stable whole cell configuration, slowly move up pipette that sealed with cell by motorized manipulator. In most case, the cell will move up with pipette and float in the bathing solution (if cells are hard to detach from the bottom of recording chamber, you can move the pipette left and right using a micromanipulator to loosen the cells from the bottom of recording chamber). hASIC1a currents can be recorded by using the rapid switching method described previously.

2.3 Chemicals Amiloride, HEPES, MES and EGTA were purchased from Sigma (USA). Cell culture materials came from GIBCO (Life Technologies, Rockville, MD).

2.4 Analysis Concentration-response data were fitted with

a logistic function according to the equation $I = I_{\max} / (1 + EC_{50}/[L]^n)$, where $I_{\max}$ is the maximal response, EC_{50} is the concentration of agonist required for half-minimal effect, $[L]$ is the concentration of agonist, and n is the Hill coefficient. All data are presented as the mean $\pm$ SEM. Data were analyzed for statistical significance unpaired Student's *t*-test. The statistical significance was indicated as $P < 0.05$.

3 Results

3.1 Comparison of endogenous ASIC1a currents in HEK293 cells

Before whole-cell patch-clamp recording, cells were bathed in external solution with pH 8.0. With cell attached method, cell membrane potentials were held at -40 mV for 5 s. Then, the extracellular solutions with the different pH (pH 7.5-5.0) were applied for 1 s, respectively. No current was observed with the application of an extracellular solution at pH 7.0 or higher than 7.0 (pH 7.5). Inward hASIC1a currents were

first recorded by applying an acidic solution at pH 6.5 (Fig. 1A) and were gradually enhanced with decreasing pH value from 6.5 to 5.0. The peak currents reached the maximum at pH 5.5. The inward current had a rapid activation phase followed by a slow desensitization phase at pH 6.5 and lower solution. The average current amplitude evoked by pH 5.0 was (650 ± 12) pA (Tab. 1). It takes an average (270 ± 5) ms for fast inward phase to reach peak ($n = 13$). The desensitization phase could be fitted by a single exponential function with a time constant, τ , of (2584 ± 250) ms ($n = 13$). We also investigated the proton sensitivity of this current by measuring the response generated by rapid shift to a range of proton concentrations between pH 7.5 and pH 5.0. The resulting pH-response curves showed a pH EC_{50} of 6.6 ± 0.3 for proton activation of the hASIC1a ($n = 13$).

On the contrary, in the cell floating method, the peak current amplitude is twice as large as that in the cell attached

Fig. 1 pH dependence of hASIC1a with cell attached to the bottom of recording chamber. **A**, pH dependence of the hASIC1a current. Proton-activated currents were induced by decreasing the extracellular pH values rapidly from 8.0 to 5.0. **B**, pH-current response curve derived from **A**. The pH required for half-maximal activation was pH 6.6. Proton-induced membrane currents were recorded from HEK293 cells using the whole-cell recording technique. Membrane potential was held at -40 mV.

Fig. 2 pH dependence of hASIC1a with cell floating method. **A**, Representative current traces of hASIC1a from HEK293 cell at different external pH values in cell floating method. Bathing pH value is 8.0. Membrane potential was held at -40 mV for 5 s and test pH values were applied to cells for 500 ms. **B**, pH-current response curve derived from **A** and has a half maximal activation pH of 6.6.

Tab. 1 Comparison of cell floating and cell attached methods

	Current amplitude (pA)	Time to reach peak (ms)	Desensitization time constant (ms)	Time to return to baseline (ms)	pH EC ₅₀	Amiloride IC ₅₀ (μ mol/L)
Cell floating	1230 $\pm$ 40 <i>n</i> = 12	21 $\pm$ 5 <i>n</i> = 12	496 $\pm$ 23 <i>n</i> = 12	22 $\pm$ 7 <i>n</i> = 12	6.6 $\pm$ 0.6 <i>n</i> = 12	2.43 $\pm$ 0.9 <i>n</i> = 12
Cell attached	650 $\pm$ 12 ** <i>n</i> = 13	270 $\pm$ 25 ** <i>n</i> = 13	2584 $\pm$ 120 ** <i>n</i> = 13	265 $\pm$ 31 ** <i>n</i> = 13	6.6 $\pm$ 0.7 <i>n</i> = 13	3.4 $\pm$ 1.1 * <i>n</i> = 13

P* < 0.05, *P* < 0.01

recording method [(1230 $\pm$ 40) pA, Fig. 2A and Fig. 3] and the activation time to reach peak is much quicker [(21 $\pm$ 5) ms, Fig. 2A and Fig. 3). The desensitization phase is also fitted by a single exponential function with time constant, τ , of (496 $\pm$ 23) ms. The proton sensitivity (pH EC₅₀) is 6.6 $\pm$ 0.6 (Fig. 2B).

3.2 Effect of amiloride on hASIC1a channel Amiloride can idiosyncratically block ASICs channels and has a different effect on each ASIC subunit. The result of this experiment shows that amiloride can dose-dependently inhibit the hASIC1a currents, and have an IC₅₀ of (3.4 $\pm$ 1.1) μ mol/L in cell-attached method (Fig. 4), and (2.4 $\pm$ 0.9) μ mol/L in floating methods (Fig. 5). These results indicate that the perfusion rate may also affect the drug potency on this channel.

Fig. 3 Comparison of kinetics of hASIC1a current in two different recording conditions. Red trace recorded was from cell floating and black trace recorded from cell attached to recording chamber.

Fig. 4 A, Effect of amiloride on hASIC1a current in cell attached method. External solution of pH 5.0 was applied to HEK293 cells to evoke the currents. B, Dose-response curve.

Fig. 5 A, Effect of Amiloride on hASIC1a current in cell floating method. External solution of pH 5.0 was applied to HEK293 cells to trigger the currents. B, Dose-response curve.

4 Discussion

ASICs, belonging to the larger family known as degenerin/epithelial Na⁺ (DEG/ENaC) channel^[1], can be activated by external protons. ASICs are important for certain sensory modalities (mechanoreception and nociception) at the periphery and important for learning and memory in the brain. Activation of ASICs requires rapid change of external solution from pH 7.4 or higher to pH 6.5 or lower. Two main types of fast switching systems are available for use: First, producing a complete replacement of the bath solution; Second, making regional changes in the bath solution. Almost all experiments (mammalian cells) for ASICs channel study were using regional change in the bath solution by home-made or commercially available fast switching systems. Even with the fastest commercial switching system, the ASICs channel response to rapid acidic solution change was still slow, which may be due to the diffusion delays in the space between the cells and the bottom of the bath.

In the present study, we re-confirmed the presence of hASIC1a channel and compared the biophysical and pharmacological characterizations of human ASIC1a endogenously expressed in HEK293 cells by using two different perfusion methods. With the conventional perfusion method, the peak current amplitude that is activated by pH 5.0, activation time to reach peak, desensitization time constant, pH activation EC₅₀ and sensitivity to amiloride are very similar to those in previous report^[11], while with cell floating method, the peak current amplitude that is activated by pH 5.0 is twice as large as that in the cell attached method. Cell floating method also significantly shortens the time to reach peak and the decay time of the current. Only the pH EC₅₀ remains unchanged in both perfusion methods. The steep pH dependence of hASIC1a channels suggests that these channels can function as a rapid on/off switch in response to subtle changes in extracellular pH under normal and pathological conditions.

In the screening study of compounds, it is very important to precisely measure the potency of agonists (or antagonists) on specific channels and to determine the structure-activity relationship. Perfusion rate (local solution change) can affect the potency on ion channels, especially on ligand-gated channels that require fast solution change. For this purpose, the floating method will have more advan-

tage over the conventional perfusion technique. This floating method not only can be used in the cell line but also in neurons. With the fast switching systems, ASICs channels and other ligand-gated channels that require fast external exchange to activate can be readily studied. Furthermore, separating the cell from the bottom of recording chamber and floating the cell in front of the perfusion tubing can optimize the recording condition and characterize the biophysical and pharmacological properties of ASICs and other similar channels more precisely.

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References:

- [1] Waldmann R, Lazdunski M. H⁺-gated cation channels: neuronal acid sensors in the NaC/DEG family of ion channels. *Curr Opin Neurobiol* 1998, 8: 418-424.
- [2] Wemmie JA, Chen J, Askwith CC, Hruska-Hageman AM, Price MP, Nolan BC, *et al.* The acid-activated ion channel ASIC contribution to synaptic plasticity, learning, and memory. *Neuron* 2002, 34: 463-477.
- [3] Price MP, Lewin GR, McIlwrath SL, Cheng C, Xie J, Heppenstall PA, *et al.* The mammalian sodium channel BNC1 is required for normal touch sensation. *Nature* 2000, 407: 1007-1011.
- [4] Price MP, McIlwrath SL, Xie J, Cheng C, Qiao J, Tarr DE, *et al.* The DRASIC cation channel contribute to the detection of cutaneous touch and acid stimuli in mice. *Neuron* 2001, 32: 1071-1083.
- [5] Xiong ZG, Zhu XM, Chu XP, Minami M, Hey J, Wei WL, *et al.* Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. *Cell* 2004, 118: 687-698.
- [6] Yermolaieva O, Leonard AS, Schnizler MK, Abboud FM, Welsh MJ. Extracellular acidosis increases neuronal cell calcium by activating acid-sensing ion channel 1a. *Proc Natl Acad Sci USA* 2004, 101: 6752-6757.
- [7] Pignataro G, Simon RP, Xiong ZG. Prolonged activation of ASIC1a and the time window for neuroprotection in cerebral ischaemia. *Brain* 2007, 130: 151-158.
- [8] Wu LJ, Duan B, Mei YD, Gao J, Chen JG, Zhuo M, Characterization of Acid-sensing Ion Channels in Dorsal Horn. *J Biol Chem* 2004, 279: 43716-43724.
- [9] Duan B, Wu LJ, Yu YQ, Ding Y, Jing L, Xu L, *et al.* Upregulation of acid-sensing ion channel ASIC1a in spinal dorsal horn neurons

- contributes to inflammatory pain hypersensitivity. *J Neurosci* 2007, 27: 11139-11148.
- [10] Mazzuca M., Heurteaux C, Alloui A, Diochot S, Baron A, Voilley N, *et al.* A tarantula peptide against pain via ASIC1a channels and opioid mechanisms. *Nat Neurosci* 2007, 10: 943-945.
- [11] Gunthorpe MJ, Smith GD, Davis JB, Randall AD. Characterisation of a human acid-sensing ion channel (hASIC1a) endogenously expressed in HEK293 cells. *Pflugers Arch* 2001, 442: 668-674.
- [12] Chen CC, England S, Akopian AN, Wood JN. A sensory neuron-specific, proton-gated ion channel. *Proc Natl Acad Sci USA* 1998, 95: 10240-10245.
- [13] Baron A, Waldmann R, Lazdunski M. ASIC-like, proton-activated currents in rat hippocampal neurons. *J Physiol* 2002, 539: 485-494.
- [14] Bässler EL, Ngo-Anh TJ, Geisler HS, Ruppersberg JP, Gründer S. Molecular and functional characterization of acid-sensing ion channel (ASIC) 1b. *J Bio Chem* 2001, 276: 33782-33787.
- [15] Spitzer KW, Bridge JH. A simple device for rapidly exchanging solution surrounding a single cardiac cell. *Am J Physiol* 1989, 256: C441-C447.

一种改良的精确分析酸敏感性离子通道特性的优化记录方法

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摘要: 目的 用一种改良的灌流方法研究和再次证实HEK 293细胞内源性酸敏感性离子通道的生物物理和药理学特性。**方法** 使用全细胞膜片钳技术, 将记录细胞与玻片分离, 并使细胞位于输液管前, 呈漂浮状态, 以记录在低pH值外液中, HEK293细胞的酸敏感性1a型离子通道的电流。**结果** 使用细胞漂浮方法, pH 5.0的细胞外液诱发的酸敏感性1a型离子通道电流是传统细胞附着法诱发的两倍。在两种不同的方法下, 通道电流达到峰值的时间分别是(21±5) ms和(270±25) ms, 失活的时间常数分别是(496±23) ms和(2284±120) ms。细胞漂流法也明显增强amiloride对酸敏感性1a型离子通道的阻断效能。两种方法具有相似的pH激活的EC₅₀ (分别为6.6±0.6, 6.6±0.7)。**结论** 酸敏感性1a型离子通道的激活需要细胞外液快速的交换。通过细胞漂浮的方法, 我们再次证明了酸敏感性1a型离子通道电流的存在, 更精确地分析了HEK 293细胞内源性酸敏感型离子通道的生物物理和药理学特性。这一改良的方法能够用于研究所有的酸敏感型离子通道和需要快速细胞外液交换的配体门控通道。

关键词: 酸敏感性1a型离子通道; 膜片钳记录; pH值