Introduction

The acid-sensing ion channels (ASICs) are voltage-insensitive cation channels that belong to the degenerin/epithelial Na+ channel superfamily [1] and expressed primarily in neurons throughout the central and peripheral nervous system [2-4]. Besides protons, emerging evidence demonstrates that non-proton ligands such as heteromeric Texas coral snake toxin and agmatine can also activate the ASICs under normal pH condition [5, 6]. So far, at least seven ASIC subunits encoded by four genes (ASIC1 - ASIC4) have been cloned [4, 7, 8]. Each ASIC subunit has two hydrophobic transmembrane domains, a large extracellular loop with cysteine-rich residues, and short cytoplasmic N- and C-termini [3, 8]. The crystal structure of the chicken ASIC1a channels demonstrates that three individual ASIC subunits associate to form functional channels [9, 10].

The ACCN2 gene encodes two splice variants, ASIC1a and ASIC1b [2-4]. ASIC1a is expressed both in the brain and in peripheral sensory neurons [8, 11]. In the brain, ASIC1a contributes to synaptic plasticity [12-14] and plays important roles in multiple neurological diseases [15-20]. In the periphery, ASIC1a modulates pain sensation [21, 22]. Unlike ASIC1a, ASIC1b is primarily expressed in the periphery sensory neurons [23-25]; however, the physiological and pathological roles of ASIC1b are poorly defined [22, 26-28]. Recently, a Texas coral snake toxin, which directly activates both ASIC1a and ASIC1b, produces pain [5]. These results suggest that either ASIC1a or ASIC1b may contribute to nociception and implicate ASIC1 as a potential therapeutic target for pain.

To gain more insight into the physiological role of ASIC1b, we recently studied its modulation by zinc [29], which plays important roles in pain sensation [30]. Zinc exhibits an inhibitory effect on rat ASIC1b channels and cysteine149 located in the extracellular finger domain of rat ASIC1b subunit is responsible for zinc-induced inhibition [29]. Recently, Hoagland et al. [25] identified a human homolog of rodent ASIC1b [23, 24]. Rat and human ASIC1b show 95%
Zinc inhibits the hASIC1b channels

identity in amino acid sequence and shares similar electrophysiological and pharmacological properties. However, hASIC1b is calcium permeable and displays an acid-dependent sustained current [25]. Given the fact that zinc plays critical roles in physiological processes as well as pathological conditions [31-33], here we tested the zinc effect on hASIC1b channels by using whole-cell patch-clamp recording combined with site-directed mutagenesis in cultured CHO cells expressing hASIC1b subunit. Our results demonstrate that hASIC1b channels are sensitive target of zinc and cysteine 196 in the extracellular domain is responsible for zinc-mediated inhibition.

Materials and methods

Tissue culture and hASIC1b transient expression in Chinese hamster ovary cells

Tissue culture and transfection of Chinese hamster ovary (CHO) cells with various ASIC subunits were described in detail previously [34, 35]. Briefly, CHO cells were maintained in standard F12 medium (American Type Culture Collection, Manassas, VA) supplemented with 10% fetal bovine serum at 37°C in a CO2 incubator. Cells were split with trypsin-EDTA, plated on a 35-mm culture dish at 10 to 20% confluence, and allowed to recover for 1 day at 37°C. At ~70% confluence, cells were transiently transfected with pcDNA 3.1 expression vectors containing hASIC1b cDNA [25] and enhanced green fluorescent protein (eGFP) at a 3:2 molar ratio (Life Technologies, Carlsbad, CA) using X-tremeGENE HP DNA transfection reagent (Roche Diagnostics, Indianapolis, IN). Transfected cells were identified by expression of eGFP and used for electrophysiological recording 2-3 days after transfection. The cDNA of the hASIC1b clone was a gift from Dr. C. Askwith (The Ohio State University, Columbus, OH, USA).

Solutions and compounds

Standard extracellular fluid (ECF) contained (mM) 140 NaCl, 5.4 KCl, 2.0 CaCl2, 1.0 MgCl2, 20 HEPES, and 10 glucose (pH 7.4; 320 ~ 330 mOsm). For solutions with pH of 6.0 or lower, MES was used instead of HEPES for more reliable pH buffering [14, 29, 36]. The pipette solution contained (mM) 140 K-Gluconate, 10 HEPES, 11 EGTA, 2 TEA, 1 CaCl2, 2 MgCl2, and 4 K2ATP (pH 7.2 ~ 7.3; 290 ~ 300 mOsm). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO). A multi-barrel perfusion system (SF-77, Warner Instrument Co., CT) was employed to achieve a rapid exchange of extracellular solutions. For bath application of zinc, zinc was present in the ECF of both pH 7.4 and lower pH (e.g. 6.0). For solution containing different concentrations of extracellular calcium (e.g. 2, 5 or 10 mM), the same concentration of calcium was present in the ECF of both pH 7.4 and lower pH (e.g. 6.0). For co-application of zinc, zinc was only present in the ECF of lower pH (e.g. 6.0).

Electrophysiological recording in CHO cells

Whole-cell patch-clamp recordings were conducted as described previously [14, 29, 35]. Patch electrodes, whose resistance ranged from 4 to 6 MΩ when filled with intracellular solution, were constructed from thin-walled borosilicated glass (1.5 mm diameter, WPI, Sarasota, FL) on a two-stage puller (PC-10, Narishige, Tokyo, Japan). Whole-cell currents were triggered by a drop in pH from 7.4 to various levels at a holding potential of -60 mV and recorded using Axopatch 200B amplifiers (Axon CNS, Molecular Devices, Foster City, CA). Data were collected at 2 kHz and digitized at 5 Hz using Digidata 1440 DAC units (Axon CNS, Molecular Devices, Foster City, CA). The on-line acquisition was done using pCLAMP software (Version 10.2, Axon CNS, Molecular Devices, Foster City, CA).

In general, hASIC1b channels were activated by a drop in pH from 7.4 to target levels every 2 min to allow for a complete recovery of the channels from desensitization. During each experiment, a voltage step of ~10 mV from the holding potential (~60 mV unless specified otherwise) was applied periodically to monitor the cell capacitance and the access resistance. Recordings in which either the access resistance or the capacitance changed by more than 10% during the experiment were excluded from data analysis.

hASIC1b construct

Human ASIC1b construct in mammalian expression vector (pcDNA 3.1) was kindly provided by Dr. Candice C. Askwith (The Ohio State University, Columbus, OH, USA). Site-directed mutagenesis was performed as described previously [14, 29, 34, 35], using the Quick-Change Site-Directed Mutagenesis system (Stratagene,
Zinc inhibits the hASIC1b channels

La Jolla, CA) in accordance with the manufacturer’s protocol. The primers were obtained from Integrated DNA Technologies (Coralville, IA). Mutations were confirmed by sequencing.

Data analysis and statistics

All data were analyzed using Clampfit 10.2 software (Axon CNS, Molecular Devices, Foster City, CA). For half-maximum inhibitory concentration (IC50) curves of zinc, pH 6.0-triggered hASIC1b currents with different concentrations of zinc treatment (1, 3, 10, 30, 100, and 300 µM) were normalized to a pH 6.0-triggered hASIC1b current without zinc treatment. The sustained component of hASIC1b current was measured at 5 seconds time point following pH drop (7 seconds duration). Normalized values were fitted to the Hill equation to obtain IC50 values and Hill coefficients. Statistical analyses were carried out using SigmaPlot software (Version10.0.1, Systat). Significant differences between mean values from each experimental group were tested using the Student’s t-test for two groups and one-way analysis of variance (ANOVA) for multiple comparisons. Differences were considered significant if \( p < 0.05 \).

Results

Co-application of zinc don’t affect hASIC1b currents

To test whether co-application of zinc with pH drop has any effect on hASIC1b currents, eEGF positive CHO cells expressing hASIC1b subunit were identified and hASIC1b currents were recorded by a drop (7 seconds in duration) in pH from 7.4 to 6.0 under whole-cell voltage clamp configuration. As shown in Figure 1A, co-application of zinc, at concentrations between 1 to 300 µM, did not affect hASIC1b currents both in peak amplitude (Figure 1B) and sustained component (Figure 1C). These data suggest that zinc doesn’t bind to the channel pores to affect hASIC1b activity when they are in the open state.

Pre-application of zinc concentration-dependently suppresses the peak amplitude, but not the sustained component of hASIC1b currents

We next examined the concentration-response relationship on hASIC1b currents by bath application of zinc for 2 minutes before decreasing the pH. As shown in Figure 2A, pre-application of 1 or 3 µM zinc revealed slight decreases in peak amplitude of hASIC1b currents, but these changes were not significant (Figure 2B and 2C). Pre-application of zinc, at concentrations of 10, 30, 100 or 300 µM, however, profoundly and concentration-dependently suppressed peak amplitude, but not sustained component of the hASIC1b currents (Figure 2B and 2C). At
Zinc inhibits the hASIC1b channels

10, 30, 100 and 300 µM, the peak amplitude of hASIC1b currents were reduced by 27%, 46%, 69% and 82%, respectively. The inhibitory effect of zinc on the peak amplitude of hASIC1b currents was rapidly reversed after washout (Figure 1A). The concentration-inhibition curve for zinc is shown in Figure 2B, with an IC50 value of 36.45 ± 1.52 µM and a Hill coefficient of 1.0 ± 0.01. These data indicate that the peak amplitude, but not the sustained component of the hASIC1b current is inhibited by pre-application of zinc in a concentration-dependent manner; and that zinc appears to interact with hASIC1b channels in the closed state.

**Zinc inhibition of hASIC1b is independent of pH activation and steady-state desensitization**

To determine whether inhibition of hASIC1b channels by bath application of zinc is pH-dependent, we produced pH concentration-response curves before and after bath (pre- plus co-) application of 50 µM zinc. Bath application of 50 µM zinc inhibited hASIC1b currents induced by pH drops from 7.4 to 6.5, 6.0, 5.0, 4.0, and 3.0 to 42.9 ± 2.7%; 45.1 ± 2.8%, 45.5 ± 2.3%, 48.1 ± 2.6%, and 49.0 ± 3.1% of the control value, respectively (Figure 3A and 3B). The degree of zinc to inhibit hASIC1b currents induced by drops in pH from 7.4 to 6.0 did not differ significantly from the level of inhibition observed with other pH values (p > 0.05; ANOVA). These results suggest that the degree of inhibition of hASIC1b currents by bath application of zinc does not depend on the pH-dependent activation of the hASIC1b channels.

To further explore the mechanism underlying the inhibition of the hASIC1b current by bath application of zinc, we determined the effect of 50 µM zinc on steady-state desensitization of hASIC1b. CHO cells expressing hASIC1b subunit were incubated in extracellular solutions at various conditioning pH values between 7.8 and 6.8 for ~6 min before the hASIC1b currents were activated by a drop in pH to 5.0. As shown in Figure 3C and 3D, bath application of 50 µM zinc inhibited hASIC1b currents induced by pH drops from different conditioning values to 5.0. The capacity of zinc to inhibit hASIC1b currents triggered by drops in pH to 5.0 from different conditioning values did not differ significantly (p>0.05; ANOVA). These results demonstrate that the degree of inhibition of hASIC1b currents by bath application of zinc is independent of the steady-state desensitization of the hASIC1b channels. Taken together, these data suggest that a noncompetitive mechanism is responsible for zinc inhibition.
Zinc inhibits the hASIC1b channels

To determine whether zinc-induced inhibition of hASIC1b currents is affected by changes in the concentration of extracellular Ca\(^{2+}\), increasing the extracellular Ca\(^{2+}\) concentration from 2 to 5 or 10 mM (in the ECF of both pH 7.4 and 6.0) had an inhibitory effect on hASIC1b currents, as shown in Figure 4A. However, the capacity of bath application of 50 µM zinc to inhibit hASIC1b currents triggered by a drop in pH from 7.4 to 6.0 was not significantly affected by increasing the extracellular Ca\(^{2+}\) concentration from 2 to 5 or 10 mM (Figure 4B; \(p > 0.05\)). The percentile of inhibition on hASIC1b currents by bath application of 50 µM zinc was 39.7 ± 4.5%, 44.7 ± 3.5%, and 46.1 ± 3.6% in the presence of 2.0, 5.0 and 10 mM Ca\(^{2+}\), respectively (Figure 4B). The failure of extracellular Ca\(^{2+}\), at concentrations between 2.0 and 10 mM, to significantly impact zinc-mediated inhibition of hASIC1b currents, in conjunction with the capacity of Ca\(^{2+}\) to inhibit hASIC1b currents, suggests that zinc and calcium inhibit hASIC1b by binding to distinct sites on the hASIC1b subunit, and that they do not compete with one another for the binding sites.

*Zinc-mediated inhibition is via extracellular domain of hASIC1b subunit*

To determine whether zinc blocks hASIC1b by binding to extracellular or intracellular domains, we included zinc at a concentration of 50 µM in the recording pipette before testing the effect of...
Zinc inhibits the hASIC1b channels

Zinc-mediated inhibition involves cysteine 196, but not cysteine 309, in the extracellular domain of the hASIC1b subunit

Recently, we found that cysteine 149 located in the extracellular finger domain of rat ASIC1b subunit is critical for zinc inhibition [29]. Cysteine 149 in rat ASIC1b is comparable to cysteine 196 in hASIC1b. Cysteine 309, another cysteine residue in extracellular domain of hASIC1b subunits, was randomly chosen as a control. Cysteine, as a non-charged residue, was replaced with alanine (A), which is also non-charged residue. Site-directed mutagenesis studies were performed to identify whether these residues are responsible for zinc inhibition. Two mutants (hASIC1b-C196A and hASIC1b-C309A) were generated, in which alanine was substituted for each of the two cysteines listed above. Similar to wild-type hASIC1b, hASIC1b-C309A currents were inhibited by bath application of zinc in a concentration-dependent manner in CHO cells expressing hASIC1b-C309A mutants (Figure 6A). A detailed concentration-response curve is presented in Figure 6C, and the IC50 was determined to be 31.4 ± 1.3 µM. In cells expressing hASIC1b-C196A mutants, however, hASIC1b-C196A currents were not significantly suppressed by zinc application at any of the concentrations tested (Figure 6B, 6D). Taken together, these data suggest that C196, but not C309, at the extracellular domain of the hASIC1b subunit is critical for zinc-mediated inhibition.

Discussion

Zinc has been implicated as a key endogenous molecule that plays a critical role in physiological as well as pathological conditions [31-33]. More recently, zinc has been reported to reduce pain in animal models [30, 37]. Because of its potential importance in diseases, the effect of zinc in voltage- and ligand-gated ion channels has been extensively studied [38-40]. For example, four residues of extracellular N-terminal domain of the NR2A subunit control high-affinity Zn2+ binding to NMDA receptors [41]. Consistent with this finding, Nozaki et al. recently found that zinc alleviates pain behavior through high-affinity binding to the NMDA receptor NR2A subunit by using NR2A-H128S knock-in mice [30]. Moreover, zinc also inhibits voltage-gated K+ channel in olfactory neurons [42]. Here, we...
Zinc inhibits the hASIC1b channels

showed that zinc inhibits hASIC1b through modulating cysteine 196 in the extracellular domain of the channel. Collectively, these data demonstrate that zinc affects a wide variety of ion channels. The effect of zinc on physiology or pathology may depend upon the exact paradigm.

Finding zinc regulates ASIC1 is interesting, because both ASIC1a and ASIC1b are expressed in sensory neurons that receive nociceptive input [23, 24, 43]. More importantly, ASIC1 channels have been implicated in pain sensation [5, 23]. For example, Psalmotoxin 1, a peptide extracted from the South American tarantula Psalmopoeus cambridgei, has profound analgesic properties against thermal, mechanical, chemical, inflammatory and neuropathic pain in rodents. It exerts its action by blocking ASIC1a, suggesting that ASIC1a channels contribute to pain modulation [44]. More recently, Bohlen et al. [14] found that injection MitTX, a venom isolated from Texas coral snake, into the hindpaw of wild-type mice produces robust pain-related behavior, which are reduced by deleting the ASIC1 but not ASIC3 gene. Further, in CHO cells expressing homomeric ASIC1a and ASIC1b subunits, MitTX can trigger robust inward currents in both ASIC1a and ASIC1b channels under normal pH value (7.4). These findings suggest that predominant ASIC1 channels contribute to coral snake toxin-evoked nociception [5]. Taken together, all these results suggest that ASIC1 plays a critical role in pain sensation. It will be interesting to test whether zinc, a potent inhibitor of ASIC1 channels, can combat pain induced by MitTX or other paradigms.

Unlike rodent ASIC1b, hASIC1b has significant calcium permeability and exhibits a small sustained current in response to a drop in pH [25]. The physiological significance of the sustained current is unclear but has been implicated in persistent pain [45, 46]. Besides hASIC1b, ASIC3 also displays a sustained component [7, 46]. However, unlike hASIC1b, zinc inhibits both peak and sustained component of rat ASIC3 channels [47]. These results suggest that mechanisms regulating zinc susceptibility are different between ASIC1b and ASIC3.

Although further study are needed to understand how zinc has differential effects on the acute and sustained components, our data suggest that zinc inhibits hASIC1b currents in the closed state because pre-application, but not co-application of zinc inhibits ASIC1b current. In addition, zinc-mediated inhibition of hASIC1b channels is independent of pH activation, steady-state desensitization and extracellular Ca2+, suggesting noncompetitive mechanisms.

The effects of zinc on ASICs in native neurons are complex due to the fact that these channels may consist of combinations of different ASIC subunit, and the fact that zinc has differential effects on different ASIC subunits [48, 49]. For example, zinc potentiates homomeric ASIC2a
Zinc inhibits the hASIC1b channels

and heteromeric ASIC1a/2a, but inhibits homomeric ASIC1b and ASIC3 channels with low-affinity [29, 47, 50]; while zinc inhibits homomeric ASIC1a and heteromeric ASIC1a/2a channels with high-affinity [51]. Since ASICs can function as homomeric and heteromeric channels, these data suggest that the effect of zinc on acid-activated currents depends upon subunit combination [51]. In addition, heteromerization may also regulate the spatial location of ASIC channels [52], which is another important variable that can influence the functional outcome of heteromeric channels. These results indicate that the effect of zinc on ASICs is complex, and is determined by the exact stoichiometry of the channel.

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Address correspondence to: Dr. Xiang-Ping Chu, Department of Basic Medical Science, University of Missouri-Kansas City School of Medicine, 2411 Holmes Street, Kansas City, MO 64108, USA Tel: +1-816-235-2248; Fax: +1-816-235-6517; E-mail: chux@umkc.edu

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Zinc inhibits the hASIC1b channels

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