Calcium-permeable Acid-sensing Ion Channel Is a Molecular Target of the Neurotoxic Metal Ion Lead*

Wei Wang†§1, Bo Duan†§1, Han Xu†§, Lin Xu‡, and Tian-Le Xu†§3

From the †Institute of Neuroscience and Key Laboratory of Neurobiology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, the §School of Life Sciences, University of Science and Technology of China, Hefei 230027, and the ¶Laboratory of Learning and Memory, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China

Acid-sensing ion channels (ASICs)1 are emerging as fundamental players in the regulation of neural plasticity and in pathological conditions. Here we showed that lead (Pb2+), a well known neurotoxic metal ion, reversibly and concentration-dependent inhibits ASIC currents in the acutely dissociated spinal dorsal horn and hippocampal CA1 neurons of rats. In vitro expression of ASIC sub-units in combination demonstrated that both ASIC1 and -3 sub-units were sensitive to Pb2+. Mechanistically, Pb2+ reduced the pH sensitivity of ASICs independent of membrane voltage change. Moreover, Pb2+ inhibited the ASIC-mediated membrane depolarization and the elevation of intracellular Ca2+ concentration. In addition, we compared the effect of Pb2+ with that of Ca2+ or amiloride to explore the possible interactions of Pb2+ and Ca2+ in regulating ASICs, and we found that Pb2+ inhibited ASIC currents independent of the amiloride/Ca2+ blockade. Because ASIC1b and -3 sub-units are mainly expressed in peripheral neurons, our data identified ASIC1a-containing Ca2+-permeable ASIC as a novel central target of Pb2+ action, which may contribute to Pb2+-neurotoxicity.

Acid-sensing ion channels (ASICs)4 belong to the members of voltage-insensitive, amiloride-sensitive epithelial Na+ channel/degenerin superfamily (1). To date, four genes encoding six ASIC subunit proteins, including ASIC1a, -1b, -2a, -2b, -3, and -4, have been identified. ASIC1b and -2b are splice variants of ASIC1a and -2a, respectively. Functional ASICs are thought to be tetramer assemblies of homomeric or heteromeric ASIC subunits (2). Studies of the structure of ASICs indicate that ASICs consist of two transmembrane domains (TM1 and TM2) and a large extracellular loop, and the pre-TM2 region is essential for Ca2+ permeability and the gating of this channel (3, 4). Some of the endogenous cations such as the divalent metal ions Ca2+, Zn2+, and Mg2+ (5–15) and the polyvalent cation spermine (5) have been shown to modify the gating and activity of specific ASICs.

Of all ASIC subunits, ASIC1a subunit renders ASICs a high Ca2+ permeability (16, 17). The unraveling of ASIC1a in synaptic function (18, 19) would be a plausible cellular mechanism responsible for a variety of physiological and pathological processes such as learning and memory (18–20), nociceptive transmission (15, 21), visceral sensation (22), and ischemic cell death (17). Therefore, it is reasonable to speculate that disturbance of ASIC1a function would impair the contribution of ASICs to synaptic function.

Lead (Pb2+) is a well known divalent metal ion because of its harm to the human body. It is widely accepted that Pb2+-induced impairment of synaptic function is one of the fundamental cellular mechanisms of Pb2+ neurotoxicity in the central nervous system (CNS) (23–25). Considering the importance of ASICs in synaptic physiology, we asked whether ASICs are novel molecular targets of Pb2+ action. Furthermore, because Ca2+ and Pb2+ are both divalent cations, we asked whether they share similar mechanisms in modulating ASICs. To answer these questions, we performed whole-cell patch clamp recordings in rat spinal dorsal horn (SDH) and hippocampal CA1 neurons as well as CHO cells transfected with five major ASIC subunits, 1a, 1b, 2a, 2b, and 3 in combination. We found that the ASIC1a, -1b, and -3 sub-units were sensitive to Pb2+. Because ASIC1b and -3 are mainly expressed in peripheral neurons, our study identifies ASIC1a subunit as a novel molecular target of Pb2+ in CNS neurons, which may contribute to Pb2+-neurotoxicity.

EXPERIMENTAL PROCEDURES

Acute Isolation of Rat CNS Neurons—The use and care of experimental animals were approved by the Anhui Health Department, China. Neurons from rat SDH or hippocampal CA1 region were mechanically dissociated as described previously (11, 15). In brief, 2-week-old Wistar rats were sacrificed by decapitation, and then a segment of lumbar (L4–S2) spinal cord or brain was quickly removed and immersed into a Transtar solution containing 0.2% papain at 37°C (26). After 30 min of incubation, suspend the tissue in the Transtar solution and assemble into a monolayer of tightly packed cells. The primary neurons are then plated on poly-D-lysine– and laminin–coated 35-mm tissue culture dishes (26) at a density of 5–10 000 cells per dish. After 4 to 7 days in vitro, the cultures are ready for experiment.

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**Transfection of CHO Cells**—All constructs were expressed in CHO cells. CHO cells were cultured at 37 °C in a humidified atmosphere of 5% CO$_2$ and 95% air. Cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 2 mM l-glutamine, 10% fetal bovine serum, and 100 units/ml penicillin/streptomycin (all from Invitrogen). Transient transfection of CHO cells was carried out using the conventional calcium phosphate method. Co-transfection with a green fluorescent protein expression vector, pEGFP-N1, was used to enable identification of transfected cells for patch clamping by monitoring green fluorescent protein fluorescence in some experiments. When more than one ASIC subunit was expressed, the plasmids were co-transfected in a ratio of 1:1. Electrophysiological recordings were performed 16–48 h after transfection. The GW1-CMV-ASIC1a and GW1-CMV-ASIC2a were generous gifts from Dr. Jian-hong Luo (Zhejiang University Faculty of Medicine, Hangzhou, China), and the two plasmids were subcloned into pEGFP-C3 that was digested with HindIII and XhoI for subsequent experiments. The pcDNA3-ASIC1b was kindly from Dr. Zhi-Gang Xiong (Legacy Clinical Research Center, Portland, OR). The pN1-x-ASIC2b was kindly provided by Dr. Philip K. Ahring (NeuroSearch A/S, Ballerup, Denmark), and ASIC3 was from Rainer Waldmann (Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France). The pEGFP-N1 was from Dr. Jian-hong Luo (Zhejiang University Faculty of Medicine, Hangzhou, China).

**Electrophysiology**—The electrophysiological recordings were performed in the conventional whole-cell patch clamp recording configuration under voltage clamp or current clamp conditions. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm (Narishige, Tokyo, Japan) on a two-stage puller (PP-830, Narishige Co. Ltd., Tokyo, Japan). The resistance between the recording electrode filled with pipette solution and the reference electrode was 4–6 megohms. Membrane currents were measured using a patch clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA), filtered at 1 kHz, sampled, and analyzed using a DigiData 1320A interface and a computer with the pCLAMP system (Version 8.0, Axon Instruments, Foster City, CA). The series resistance, estimated from optical cancellation of the capacity transient, was 10–30 megohms, and in most experiments, 70–90% series resistance was compensated. The membrane potential was voltage clamped at −50 mV throughout the experiments under voltage clamp conditions except when the current-voltage ($I$-$V$) relationships for ASIC currents were examined. All experiments were carried out at room temperature (22–25 °C).

**Primary SDH and Hippocampal Neuronal Cultures**—SDH neurons from 15-day-old embryonic Sprague-Dawley rats or hippocampal neurons from 18-day-old embryonic Sprague-Dawley rats were isolated by a standard enzyme treatment protocol. Briefly, SDH or hippocampal rats were dissociated in Ca$^{2+}$-free saline with sucrose (20 mM) and plated (1–2 × 10$^5$ cell/ml) on poly-d-lysine (Sigma)-coated cover glasses. The neurons were grown in Dulbecco’s modified Eagle’s medium with l-glutamine plus 10% fetal bovine serum and 10% F-12 nutrient mixture (Invitrogen). After 1 day, Neurobasal medium (1.5 ml, Invitrogen) with 2% B27 (Invitrogen) was replaced every 3–4 days. Treatment with 5-fluoro-2’-deoxyuridine (20 μg/ml, Sigma) on the 4th day after plating was used to block cell division of non-neuronal cells, which helped to stabilize the cell population. The cultures were maintained at 37 °C in a 5% CO$_2$-humidified atmosphere. Neurons were used for Ca$^{2+}$ imaging 9–14 days after plating.

**Ca$^{2+}$ Imaging**—Cultured SDH or hippocampal neurons grown on 8 × 8-mm glass cover slips were washed three times with phosphate-buffered saline and incubated with 1 μM fura-2-acetoxyethyl ester for 20 min at 37 °C, washed three times, and incubated in standard extracellular solution for 30 min. Coverslips were transferred to a perfusion chamber on an inverted microscope (Nikon TE2000-E, Japan). Experiments were performed by using a 40× UV fluor oil-immersion objective lens, and images were recorded by a cooled CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). To block potential Ca$^{2+}$ entry through voltage-gated Ca$^{2+}$ channels or glutamate receptors, 10 μM nifedipine, 20 μM 2-amino-5-phosphonopentanoic acid, and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione were added to the bath solution. The fluorescence excitation source was a 75-watt xenon arc lamp. Ratio images were acquired by using alternating excitation wavelengths (340/380 nm) with a monochromator (Till Polychrome IV, Munich, Germany), and fura-2 fluorescence was detected at emission wavelength of 510 nm. Digitized images were acquired and analyzed in a personal computer controlled by SimplePCI (Compix Inc.). Ratio images (340/380 nm) were analyzed by averaging pixel ratio values in circumscribed regions of cells in the field of view. The values were exported to Origin 7.0 for further analysis.

**Solutions and Chemicals**—The ionic composition of the incubation solution was as follows (mM): 124 NaCl, 24 NaHCO$_3$, 5 KCl, 1.2 KH$_2$PO$_4$, 2.4 CaCl$_2$, 1.3 MgSO$_4$, 10 glucose, aerated with 95% O$_2$ and 5% CO$_2$ to a final pH of 7.4. The standard extracellular solution contained the following (mM): 150 NaCl, 5 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 glucose, buffered to various pH values with either 10 mM HEPES (pH 6.0–7.4) or 10 mM MES (pH 6.0–7.4). The osmolarity of all external solutions was adjusted to 325–330 mosmol liter$^{-1}$ with sucrose. The patch pipette solution for whole-cell patch clamp recording was as follows (mM): 120 KCl, 30 NaCl, 0.5 CaCl$_2$, 1 MgCl$_2$, 5 EGTA, 2 MgATP, and 10 HEPES; pH was adjusted to 7.2 with Tris base. The osmolarity of the pipette solution was adjusted to 280–300 mosmol liter$^{-1}$ with sucrose. The extracellular pH was adjusted to different values by the addition of 1 N NaOH or 1 N HCl and was routinely checked before and during experiments. When the $I$-$V$ relationships for ASIC currents were examined, 300 mM tetradoxotidone and 100 μM CdCl$_2$ were added to the extracellular solutions, and KCl was replaced with equimolar CsCl in the pipette solution. Changes in divalent ion content of the bathing solution were kept iso-osmotic.

All chemicals were purchased from Sigma. Lead acetate was dissolved in distilled water at a concentration of 20 mM to make stock. The stock solutions were diluted in the extracellular solutions just before each experiment to avoid precipitation. The solution containing a high concentration of Ca$^{2+}$ was obtained in the same way. Drugs were applied using a rapid application technique termed the “Y-tube” method, which allowed a complete exchange of external solution surrounding a neuron within 20 ms. (15).

**Data Analysis**—Clampfit software was used for data analysis. The continuous theoretical curves for concentration-response relationships for ASIC currents in the presence or absence of Pb$^{2+}$ were obtained according to a modified Michaelis-Menten equation by the method of least squares (the Newton-Raphson method) after normalizing the amplitude of the response shown in Equation 1,

$$I = I_{\text{max}} C^h/(C^h + EC_{50}^h)$$  \hspace{1cm} (Eq. 1)

where $I$ is the normalized value of the current; $I_{\text{max}}$ is the maximal response; $C$ is the drug concentration; $EC_{50}^h$ is the concentration that induced the half-maximal response; and $h$ is the apparent Hill coefficient. The curve for the effect of Pb$^{2+}$ or amiloride on ASIC currents was fitted to the following Equation 2,

$$I = I_{\text{max}} (IC_{50})^h/(C^h + IC_{50})$$  \hspace{1cm} (Eq. 2)
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RESULTS

Inhibition of ASIC-mediated Currents by Pb^{2+} in Acutely Dissociated SDH and Hippocampal CA1 Neurons—The role of ASICs in synaptic function has been characterized in CNS neurons (18, 19). Our previous studies indicate that the acid-induced currents in acutely dissociated SDH (15) and hippocampal CA1 (11) neurons are mediated by ASICs. Therefore, we examined the effects of Pb^{2+} on ASIC-mediated currents in these neurons under whole-cell voltage clamp configuration. After recording a stable control in ASIC current, we pre-perfused the neurons with 10 μM Pb^{2+} for 15 s, and then the ASICs were activated again by acidic solutions in the presence of 10 μM Pb^{2+}. Pb^{2+} reversibly reduced the amplitude of the ASIC currents induced by pH 6.0 extracellular solution in both SDH (Fig. 1A) and CA1 (Fig. 1B) neurons at a holding potential (V_h) of ~−50 mV. Application of Pb^{2+} alone did not evoke any detectable current. The Pb^{2+}-induced inhibition of the ASIC currents was concentration-dependent. In SDH neurons, the IC_{50} and Hill coefficients (Equation 2) of the inhibition-response curves were 8.7 μM and 1.2, respectively (n = 10); and in CA1 neurons, the two values were 7.1 μM and 1.2, respectively (n = 10). However, Pb^{2+} (10 μM) did not exert any apparent effects on the decay time constant (τ, Equation 3) of the currents in either SDH neurons (without Pb^{2+}, τ = 1.1 ± 0.1 s, and with Pb^{2+}, τ = 1.3 ± 0.1 s, n = 10) or CA1 neurons (without Pb^{2+}, τ = 1.2 ± 0.1 s, and with Pb^{2+}, τ = 1.3 ± 0.1 s, n = 6).

ASIC1 and -3 Subunits Were Sensitive to Pb^{2+}—To study the subunit responsible for the Pb^{2+}-induced inhibition, we expressed five major ASIC subunits 1a, 1b, 2a, 2b, and 3 in CHO cells. ASIC currents were elicited by extracellular acidic solutions in these cells at a holding potential of ~−50 mV. We employed a moderate acidic solution of pH 6.0 to evoke ASIC currents mediated by homomeric ASIC1a-, 1b-, or heteromeric ASIC2a-containing channels, and an acidic solution of pH 5.0 was applied to activate homomeric ASIC2a- or heteromeric ASIC2a-containing channels or homomeric ASIC3 channels. As in neurons, after control ASIC currents became stable, we pre-perfused the CHO cells with Pb^{2+} for 15 s, and then the ASIC currents were induced in the presence of Pb^{2+} (Fig. 2A). We found that Pb^{2+} significantly reduced the amplitude of the ASIC currents mediated by homomeric 1a and 1b and heteromeric 1a + 2a and 1a + 2b channels in concentration-dependent manners with the IC_{50} and Hill coefficient of 3.7 μM and 2.2, 1.5 μM and 1.8, 4.9 μM and 2.3, 2.8 μM and 1.5, respectively (n = 5–7) (Fig. 2). Similar to the effect on CNS neurons, Pb^{2+}-induced inhibition in CHO cells was reversible after the removal of Pb^{2+}. In addition, Pb^{2+} did not affect the kinetics of ASIC currents in CHO cells (data not shown). In contrast, Pb^{2+} exerted no significant inhibitory effects on the ASIC currents mediated by homomeric 2a and heteromeric 2a + 2b channels at the concentrations ranging from 0.1 to 100 μM (n = 4–5). Pb^{2+} exerted no significant inhibition on homomeric ASIC3 channel at concentrations ranging from 0.1 to 10 μM, but decreased its current amplitude at a high concentration of 100 μM (36.1 ± 5.3% of the control, n = 5, p < 0.01, Student’s paired t test), suggesting the existence of low affinity site(s) for Pb^{2+} on ASIC3 subunit.

Pb^{2+} Prevented Neuronal Ca^{2+} Elevation Mediated by ASIC Activation—The ASIC1a subunit, which renders ASICs a high Ca^{2+} permeability (16), is abundantly expressed in SDH (15) and hippocampal (14, 18, 20, 26) neurons, whereas the splice variants ASIC1b and ASIC3 subunits are expressed in peripheral neurons (27, 28). Thus we hypothesized that Pb^{2+} affects ASIC-mediated Ca^{2+} entry by specifically inhibiting ASIC1a channels in CNS neurons. To test the hypothesis, we performed Ca^{2+} imaging experiments in cultured SDH and hippocampal neurons. We observed an elevation of [Ca^{2+}] when extracellular acidic solution was applied to these cultured neurons (Fig. 3A). This acid-induced elevation of [Ca^{2+}] was pH-dependent and was reversibly blocked by 100 μM amiloride (data not shown). Pb^{2+} (10 μM) reversibly reduced the ASIC-mediated elevation of [Ca^{2+}] in cultured SDH (59.7 ± 6.4% of control, n = 6) or hippocampal neurons (64.3 ± 11.2% of control, n = 6) (Fig. 3B). In addition, no significant difference of Pb^{2+} inhibition was observed between hippocampal and SDH neurons (Fig. 3B). Because the blockers of ionic glutamate receptors and voltage-gated Ca^{2+} channels were added into the bath solution to prevent potential Ca^{2+} entry through these receptors/channels (see “Experimental Procedures”), we can attribute the Pb^{2+}-induced reduction in [Ca^{2+}] to Pb^{2+}-induced inhibition of neuronal ASIC1a-containing channels.

Pb^{2+} Inhibited ASIC Currents Independent of Membrane Voltage—In the subsequent experiments, we investigated further the mechanisms of...
Pb\(^{2+}\)-induced inhibition of ASIC currents in acutely dissociated SDH neurons, in which homomeric ASIC1a channel-mediated currents were dominant (15). Next, we determined whether alternation of membrane potential affects the inhibitory effect of Pb\(^{2+}\). We changed the \(V_T\), the test potential, of the tested neurons from -60 to +60 mV in the absence or presence of 10 \(\mu\)M Pb\(^{2+}\) (Fig. 4A), and we plotted the current-voltage curve (Fig. 4B). The reversal potential of the ASIC currents was not altered by Pb\(^{2+}\) (without Pb\(^{2+}\), 33.5 ± 3.7 mV and with Pb\(^{2+}\), 37.0 ± 4.9 mV; \(n = 7\)), suggesting that Pb\(^{2+}\) inhibited ASIC function without changing its ion selectivity. Moreover, the Pb\(^{2+}\)-induced inhibition was independent of \(V_T\) (Fig. 4C), suggesting that the action site(s) of Pb\(^{2+}\) are not within the channel pore where Pb\(^{2+}\) would experience the membrane electric field.

Pb\(^{2+}\) Reduced pH Sensitivity of ASICs through a Competitive Mechanism—Next, we examined whether the Pb\(^{2+}\)-induced inhibition of ASICs was modified by extracellular H\(^{+}\). As illustrated in Fig. 5A, Pb\(^{2+}\) (10 \(\mu\)M) shifted the activation curve of ASIC currents rightward and reduced the pH value for the half-maximal activation (pH\(_{50}\)) from pH 6.4 to pH 5.8, suggesting a reduced pH sensitivity of ASICs in the presence of Pb\(^{2+}\). Furthermore, Pb\(^{2+}\) did not significantly alter the Hill coefficient (1.1 without Pb\(^{2+}\); 1.3 with Pb\(^{2+}\), \(n = 5–7\), Equation 1) or the maximal or minimal pH values of the activation curve, indicating a competitive mechanism.

Previous studies have shown that Pb\(^{2+}\) stimulates intracellular protein kinases such as protein kinase C and protein kinase A (23, 29, 30), which may be associated with ASIC activity (31–33). To examine whether intracellular signaling messengers are involved in mediating Pb\(^{2+}\) inhibition of ASIC currents, we studied the effects of 10 \(\mu\)M Pb\(^{2+}\) at various pretreatment durations. As shown in Fig. 5B, alternation of Pb\(^{2+}\) pretreatment duration from 15 to 120 s did not affect its inhibitory potency (\(n = 8\); \(p > 0.05\), ANOVA). In addition, when loaded into neurons via the patch electrode, staurosporine (10 \(\mu\)M), a nonselective protein kinases inhibitor, or BAPTA (10 mM), the Ca\(^{2+}\) chelator, did not alter the inhibition of ASIC currents by 10 \(\mu\)M Pb\(^{2+}\) (Fig. 5C). These results suggest that intracellular signaling messengers are not involved in the Pb\(^{2+}\)-induced inhibition of ASIC currents.

Distinctions between Pb\(^{2+}\) and Ca\(^{2+}\) Modulation of ASICs—To date, a series of studies on the interaction of Ca\(^{2+}\), the physiologically relevant divalent metal ion, with ASICs has been made (5, 9–15). Because Pb\(^{2+}\) binds to a protein that usually contains Ca\(^{2+}\) binding domain(s) (23), we co-applied these two divalent cations to assess their possible interactions in mediating ASIC regulation. We employed three different modes of Ca\(^{2+}\) or Pb\(^{2+}\) application to differentiate the effects of the two divalent cations. In co-application protocol (protocol a), neurons were co-applied with external Ca\(^{2+}\) or Pb\(^{2+}\) and pH 6.0 solution; in sequential application protocol (protocol b), neurons were applied with pH 6.0 solution alone immediately after 15 s of perfusion of Ca\(^{2+}\) or Pb\(^{2+}\); in pretreatment protocol (protocol c), neurons were pretreated with Ca\(^{2+}\) or Pb\(^{2+}\) for 15 s and then applied with Ca\(^{2+}\) or Pb\(^{2+}\) and acidic solution.
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FIGURE 3. Pb²⁺ reduced ASIC-mediated elevation of [Ca²⁺], in cultured SDH and hippocampal neurons. A, representative 340/380 nm ratio showing the changes in [Ca²⁺]i, induced by pH 5.0 solution in the absence or presence of 10 µM Pb²⁺. The reduction of [Ca²⁺]i elevation was completely recovered after washout of Pb²⁺. B, statistical data illustrating the inhibition of ASIC-mediated elevation of [Ca²⁺]i by 10 µM Pb²⁺ in the SDH and hippocampal neurons, respectively. Each column represents the average response of six neurons. n.s. means no significant difference (p > 0.05; ANOVA).

FIGURE 4. Effect of altered membrane potential on the modulation of ASIC currents in acutely dissociated SDH neurons by Pb²⁺ or Ca²⁺. A, sample recordings of ASIC currents elicited by extracellular pH 6.0 solution in the absence or presence of 10 µM Pb²⁺ in a representative neuron voltage clamped at various Vh values. B, scatter graph showing the relative inhibition of the peak currents recorded at various Vh values in the presence of 10 µM Pb²⁺ or 10 mM Ca²⁺. Pb²⁺-induced inhibition of ASIC currents did not depend on Vh values (n = 7; p > 0.05, ANOVA). The inhibition of ASIC currents by 10 mM Ca²⁺ was slightly decreased under more negative Vh values.

together. As shown in Fig. 6, A and C, different sequences of Ca²⁺ (10 mM) application produced different effects on the ASIC currents. Most notably, application of Ca²⁺ immediately before but not during application of pH 6.0 solution resulted in a significant enhancement of ASIC currents. In contrast, when three different protocols of drug application were employed, Pb²⁺ always produced an inhibitory effect on ASIC currents (Fig. 6, B and C). These findings suggest that Pb²⁺ modulates ASICs in a manner different from that of Ca²⁺.

Noncompetitive Interaction of Pb²⁺ and Amiloride with ASICs—Recently, it has been shown that Ca²⁺ competes with amiloride, a known open channel blocker of ASICs, suggesting a Ca²⁺-blocking site on ASIC1a at the entrance of the ion pore (13). Because Pb²⁺ inhibits ASICs at open state (Fig. 6B-a), co-application of Pb²⁺ and amiloride during the activation of ASICs was employed to assess whether Pb²⁺ competes with amiloride in modulating ASIC currents (Fig. 7A). In the absence of Pb²⁺, amiloride concentration-dependently blocked the ASIC currents in SDH neurons with an IC₅₀ and Hill coefficient of 18.0 µM and 0.7, respectively (Fig. 7B). In the presence of 6 µM Pb²⁺, the IC₅₀ and Hill coefficients of inhibition-response curves for the ASIC currents by amiloride were 11.6 µM and 0.8, respectively; and in the presence of 60 µM Pb²⁺, the IC₅₀ and Hill coefficients for the inhibition-response curves were 8.3 µM and 0.8, respectively (n = 3–7). Compared with the maximal value of the amiloride inhibition-response curve, the maximal responses in the presence of 60 µM and 6 µM Pb²⁺ were decreased approximately by 30 and 10%, respectively (n = 5). These data suggest that Pb²⁺ produces inhibition independent of the amiloride blockade of ASIC1a.

Pb²⁺ Decreased Acidosis-induced Membrane Depolarization—Our previous study demonstrated that activation of ASICs in SDH neurons induces membrane depolarization, which may contribute to SDH synaptic transmission (15). Because Pb²⁺ exerts inhibitory effects on ASICs, we examined whether Pb²⁺ has an impact on acidosis-induced membrane excitation. Membrane potential was recorded in current clamp mode with no current injection. The acutely dissociated SDH neurons had a resting potential of −51.3 ± 1.8 mV (n = 8). A slight decrease of extracellular pH, which is in the range locally reached by pH fluctuations due to normal neuronal activity (34, 35), was applied to induce membrane depolarization. As shown in Fig. 8, extracellular acidic solution of pH 6.5 induced a change in membrane potential of 37.0 ± 2.9 mV (n = 6). This acidosis-induced depolarization was reversibly inhibited by 100 µM amiloride (change in membrane potential of 14.2 ± 3.2 mV). In the same neurons, 10 µM Pb²⁺ reversibly reduced the change in membrane potential to 20.8 ± 4.1 mV. Moderate acidic solu-
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FIGURE 5. Inhibition of ASIC currents by Pb²⁺ in acutely dissociated SDH neurons was pH-dependent but was independent of intracellular signal messengers. A, left, representative traces from an SDH neuron illustrating the effect of 10 μM Pb²⁺ on ASIC currents induced by extracellular pH 6.0 and pH 4.0 solutions. The pH 6.0 is the concentration around pH 6.0 and pH 4.0 represents the saturating concentration. Right, Pb²⁺ right-shifted the concentration-response curve of ASIC currents in a parallel manner without altering the Hill coefficient (with Pb²⁺ 1.4, without Pb²⁺ 1.3) and maximal or minimal ASIC currents. Each point represents the average response of 5–7 neurons. All responses were normalized to the peak current induced by extracellular pH 6.0 solution (*). B, the effects of various pre-perfusion times of Pb²⁺ on ASIC inhibition. The graph indicating that the inhibitory potency of 10 μM Pb²⁺ was unchanged with the Pb²⁺ pre-perfusion time varied from 15 to 120 s (n = 8; p > 0.05, ANOVA). C, statistical data show that intracellular application of staurosporine (10 μM) and BAPTA (10 μM) did not significantly affect the inhibition of ASIC currents by 10 μM Pb²⁺ (n = 5–7, p > 0.05, unpaired Student’s t test). The Vₒ was −50 mV.

DISCUSSION

The ASIC1a Subunit Is a Novel Molecular Target of Pb²⁺—Action in the CNS—Among various molecular targets of Pb²⁺ neurotoxicity, the N-methyl-D-aspartate receptor (NMDAR) has received much attention because of its pivotal role in activity-dependent synaptic plasticity in the hippocampus, which is considered as a potential neural substrate for learning and memory, and elsewhere during both the developing and mature brain (36, 37). Consequently, Pb²⁺-induced disruption of NMDARs is considered to be one of the molecular mechanisms underlying Pb²⁺ neurotoxicity in the CNS (24, 38). Thus, Pb²⁺-induced impairment of NMDAR-dependent long term potentiation (LTP) in hippocampus is correlated with deficits in spatial memory (39–42). Although activation of NMDARs leading to influx of external Ca²⁺ has been demonstrated to be critical for induction of many forms of LTP, it is suggested that elevation of the postsynaptic Ca²⁺ level through pathways beyond NMDARs may also play important roles for the induction of hippocampal LTP (37). In particular, recent data identified ASIC1a channels as a novel pathway for [Ca²⁺]e elevation in CNS neurons (8, 16, 17). ASIC1 knock-out mice displaying a mild deficit in spatial memory and a severe deficit in a classical eyelink conditioning are accompanied by a deficit in hippocampal LTP, which indicates that ASIC1 participates in synaptic plasticity, learning, and memory (19). In this study, we show that ASIC1a-containing channel in SDH and hippocampal CA1 neurons is a novel molecular target of Pb²⁺ action. Moreover, Pb²⁺ reduces ASIC-mediated elevation of [Ca²⁺]e, and attenuates acid-induced membrane depolarization in these neurons. Considering the wide distribution of ASIC1a subunit in the central and periphery nervous system (14, 15, 18–20, 26), it is conceivable that Pb²⁺-induced inhibition of ASIC signaling could represent a novel molecular mechanism underlying Pb²⁺ neurotoxicity. On the other hand, it was recently demonstrated that a blockade of ASIC channels protects from postischemic neuronal death (17), suggesting at least some beneficial effects of ASIC1 inhibition. Therefore, although Pb²⁺ itself is neurotoxic, the structural information based on future studies of Pb²⁺-ASIC interaction would provide insights into the design of therapeutic agents against excitotoxic and acidotoxic neuronal damage. Furthermore, the subtype specificity of Pb²⁺ action suggests a role of Pb²⁺ in identifying ASIC1a-containing channels in CNS neurons.

Previous studies indicate that the accepted blood Pb²⁺ levels that cause neurocognitive deficits in children are 10 μg/dl (∼0.5 μM) or higher (43), and neurological symptoms can be seen at the blood Pb²⁺ level above 60 μg/dl (∼2.9 μM) (44). In our experiments, the concentrations of Pb²⁺ that produce direct inhibition on native ASIC channels are above 1 μM, and the IC₅₀ of Pb²⁺ inhibition of ASIC currents in SDH or hippocampal CA1 neurons is within the range of 7–9 μM (Fig. 1). These data suggest that the direct inhibition of ASICs is more likely to play a role in acute rather than chronic intoxication in vivo.

Possible Mechanisms Underlying the Inhibition of ASICs by Pb²⁺—We employed patch clamp recording to study the effects of Pb²⁺ on ASICs, and we demonstrated that Pb²⁺ action site(s) are located on the exterior surface of the ASIC1 subunit based on the following findings. First, the Pb²⁺-induced inhibition of ASIC current, which did not necessitate any pretreatment (Fig. 5B), was immediate and reversible (Fig. 1). In addition, we did not observe any significant differences in Pb²⁺-induced inhibition of ASIC currents between short term and prolonged Pb²⁺ pretreatment (Fig. 6B). Second, Pb²⁺ has been demon-
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FIGURE 6. Distinctions between Pb2+ and Ca2+ in modulating ASIC currents in acutely dissociated SDH neurons. A and B, representative traces illustrating 10 mM Ca2+ (A) or 10 μM Pb2+ (B) modulated ASIC currents in three different modes of drug application (a–c). C, statistical results showing the relative values of ASIC currents in the presence of 10 mM Ca2+ or 10 μM Pb2+ in the three modes indicated in A and B. All responses were normalized to the peak current induced by extracellular pH 6.0 solution (dotted line). Each graph was the average response of eight neurons. ** and *** mean significant difference (**, p < 0.01; ***, p < 0.001; ANOVA). The V0.5 was −50 mV.

strated to stimulate intracellular protein kinases (23–25, 29, 30) that may be associated with ASIC activity (31–33). However, in our experiments, the intracellularly loaded nonselective protein kinase inhibitor staurosporine or the Ca2+ chelator BAPTA did not affect Pb2+ inhibition of the ASIC currents, excluding the involvement of intracellular signaling messengers in the modulation of ASIC currents (Fig. 5C). Finally, the Pb2+ -induced inhibition was independent on membrane potentials, suggesting that Pb2+ action site(s) are not within the channel pore where it would experience the membrane electric field (Fig. 4C).

The finding that Pb2+ affects ASICs via the extracellular domain of this channel suggests the presence of structural determinants of Pb2+ -binding site(s) in the extracellular loop. Because the negatively charged amino acids, glutamate and aspartate (45, 46) as well as cysteine (47), have been shown to be associated with Pb2+ coordination in some other proteins, and our data from the heterologous expression experiments indicate that only ASIC1 and -3 subunits are sensitive to Pb2+ inhibition, we speculate that glutamate, aspartate, and cysteine, which are conserved in the extracellular portions of ASIC1 and -3, may be the site(s) of Pb2+ inhibition. Therefore, Asp-303, Glu-359, and Glu-364 of ASIC1a and Asp-290, Glu-344, and Glu-349 of ASIC1b, which are conserved only in ASIC1a and -1b but not present in ASIC2a and -2b, and -3 subunits might represent the high affinity Pb2+ inhibition site(s). In addition, similar residues conserved in ASIC1 and -3 or present only in ASIC3 may constitute the low affinity site(s) for Pb2+ inhibition. Apparently, further investigations are required for the establishment of the action site(s) of Pb2+ on ASICs.

Another finding in our experiments is that Pb2+ -rightward-shifted the concentration-response curve for ASIC currents in a parallel manner without significantly altering the maximal value and Hill coefficient (Fig. 5A), which suggests that Pb2+ reduced pH sensitivity of ASICs through a competitive mechanism. Comparatively, Ca2+ has also been shown to modulate ASIC currents in a competitive manner (5, 10, 13). Therefore, we hypothesized that the two divalent cations may interact with ASIC1a in a similar fashion. However, in our experiments, enhancement or inhibition of ASIC currents by Ca2+ depended on the open or closed state of ASICs, whereas Pb2+ always exerted inhibitory effects on ASIC currents. When Ca2+ was applied at the resting state of ASICs, a significant enhancement of ASIC currents was observed. How-
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We demonstrate that ASICs of CNS neurons are a novel target of Pb²⁺ action. However, the definite relationship between the effect of Pb²⁺ on ASICs and Pb²⁺-induced neurotoxicity should be established through further investigations. We observed a direct Pb²⁺-induced inhibition of ASIC currents, which may play an important role in acute Pb²⁺ intoxication. As for the chronic Pb²⁺ exposure, the effects of Pb²⁺ on neuronal ASIC expression at the transcriptional or translational levels should be examined. Moreover, to explore the role of ASICs in Pb²⁺ intoxication-related pathological processes, more knowledge in the involvement of ASICs themselves in synaptic physiology and cellular functions is required.

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