MODULATION OF ACID-SENSING ION CHANNELS BY Cu²⁺ IN CULTURED HYPOTHALAMIC NEURONS OF THE RAT

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Abstract—Acid-sensing ion channels (ASICs) are known to distribute throughout the nervous system and serve important roles in various physiological and pathological processes. However, the properties of ASICs in the hypothalamus, an important region of diencephalon, are little known. We herein used whole-cell patch-clamp recordings to characterize proton-induced currents in cultured hypothalamic neurons of the rat, and attributed these transient inward currents to ASICs based on their electrophysiological and pharmacological properties. We further examined the effects of Cu²⁺, the third most abundant trace element, on ASICs in hypothalamic neurons. Our results showed that this divalent cation reversibly and concentration-dependently inhibited the amplitude of ASIC currents, and slowed down the desensitization of ASIC channels. Our results also displayed that Cu²⁺ modulated ASICs independent of change in membrane potential and extracellular protons, suggesting a noncompetitive mechanism. Furthermore, micromolar concentration of Cu²⁺ attenuated the acid-induced membrane depolarization. Taken together, our data demonstrate a modulatory effect of Cu²⁺ on ASICs in native hypothalamic neurons and suggest a role of this endogenous metal ion in negatively modulating the increased neuronal membrane excitability caused by activation of ASICs. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: acid-sensing ion channels, Cu²⁺, hypothalamus, patch clamp, membrane excitability.

Acid-sensing ion channels (ASICs), members of the epithelial sodium channel/degenerin family, are proton-gated cation channels that are mainly permeable to Na⁺ and reversibly blocked by amiloride (Waldmann and Lazdunski, 1998). Four genes encoding six ASIC subunits, ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4, have been cloned and demonstrated to be broadly expressed in the CNS and in the peripheral nervous system (Krishtal, 2003; Wemmie et al., 2006). Increasing evidence has implicated ASICs in various physiological and pathological processes such as learning and memory (Wemmie et al., 2002, 2006), emotion (Wemmie et al., 2003, 2004), mechanosensation (Price et al., 2001; Krishtal, 2003; Page et al., 2004; Jones et al., 2005; Mogil et al., 2005) and pain perception (Chen et al., 2002; Ugawa et al., 2002; Sluka et al., 2003; Wu et al., 2004), ischemia (Benson and Sutherland, 2001; Johnson et al., 2001; Xiong et al., 2004, 2006; Gao et al., 2005) and epilepsy (Biangi et al., 2001).

It is known that ASICs are functionally regulated by some endogenous agents such as essential trace elements including Ca²⁺, Zn²⁺ and Mg²⁺ (Waldmann et al., 1997b; Baron et al., 2001, 2002; de Weille and Bassilana, 2001; Babini et al., 2002; Coric et al., 2003; Irmke and Mcrleskey, 2003; Chu et al., 2004; Gao et al., 2004; Paukert et al., 2004; Wang et al., 2006; Zhang et al., 2006). However, whether Cu²⁺, the third most abundant trace element, modulates ASICs remains unknown.

Previous reports show that Cu²⁺ accumulates in the CNS and can be co-released into synaptic cleft together with neurotransmitters from hypothalamic nerve terminals (Osterberg, 1980; Szerdahelyi and Kasa, 1986; Hartter and Barnea, 1988; Kardos et al., 1989). The increased concentration of Cu²⁺ due to synaptic activity may regulate the neuronal activity through its actions on neuronal membrane receptors and ion channels (Mathie et al., 2006). Notably, ASICs have been demonstrated to be involved in synaptic function (Wemmie et al., 2002, 2003, 2006). However, ASICs in the hypothalamus, a region of the brain that controls a large number of bodily functions, are little investigated. In this study, therefore, we examined the electrophysiological and pharmacological properties of proton-induced currents in cultured hypothalamic neurons of the rat, and we attributed these currents to ASICs. Furthermore, we observed a modulatory effect of Cu²⁺ on the ASICs in hypothalamic neurons and identified this endogenous divalent cation as a modulator of ASICs.

EXPERIMENTAL PROCEDURES

The care and use of animals in this study followed the guideline and protocol approved by the Care and Use of Animals Committee of the University of Science and Technology of China. All procedures conformed to international guidelines on the ethical use of animals. Every effort was made to minimize the number of animals used and their suffering.

Primary cultures of neurons

The hypothalamic neurons were isolated by a standard enzyme treatment protocol. Briefly, time-pregnant (embryonic day 16) and postnatal day 1 Wistar rats were killed by cervical dislocation...
following anesthesia with halothane. The hypothalamic tissue was removed rapidly and incubated with 0.125% trypsin-EDTA for 10 min at 37 °C. Tissue was then triturated with fire-polished glass pipettes and plated (1–2 × 10^5 cell/ml) on poly-d-lysine (Sigma, St. Louis, MO, USA) coated cover glasses in Dulbecco’s modified eagle medium (DMEM; Gibco, Invitrogen Co., Grand Island, NY, USA) with l-glutamine plus 10% fetal bovine serum (Gibco) and 10% F-12 nutrient mixture (Gibco). After 1 day, the medium was changed to Neurobasal medium (1.5 ml, Gibco) supplemented with 2% B27 (Gibco), and replaced every 3–4 days. Treatment with 5-fluoro-β-deoxyuridine (20 μg/ml, Sigma) on the fourth 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% CO2 humidified atmosphere. Neurons were used for electrophysiological recordings 7–20 days after plating.

The preparation of cultured hippocampal and cerebral cortical neurons followed a similar protocol.

Electrophysiology

The whole-cell patch-clamp recordings were performed in voltage-clamp mode or current-clamp mode. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm (Narishige Co. Ltd., Tokyo, Japan) on a two-stage puller (PP-830, Narishige). The resistance between the recording electrode filled with pipette solution and the reference electrode was 4–6 MΩ. Membrane currents were measured using a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA, USA), filtered at 1 kHz, sampled and analyzed using a DigiData 1320A interface and a computer with the pCLAMP system (Version 9.2, Axon Instruments). The series resistance, estimated from optical cancellation of the capacity transient, was 10–30 MΩ and in most experiments, 70–90% series resistance was compensated. Under voltage-clamp condition, the neurons tested were voltage-clamped at −50 mV throughout the experiments except when the current–voltage (I–V) relationships for ASIC currents were examined. All experiments were carried out at room temperature (23–25 °C).

Solutions and chemicals

The ionic composition of the standard extracellular solution contained (mM): 120 KCl, 30 NaCl, 0.5 CaCl2, 1 MgCl2, 5 EGTA, 6.0–7.4) or 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES, buffered to various pH values with either 10 mM Hepes (pH 6.0–7.4) or 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES, pH < 6.0). In the Na+-free medium, Na+ was substituted with equimolar N-methyl-d-glucamine (NMDG). The osmolarity of all external solutions was adjusted to 325–330 mOsm with sucrose. The patch pipette solution for whole-cell patch-clamp recording contained (mM): 120 KCl, 30 NaCl, 0.5 CaCl2, 1 MgCl2, 5 EGTA, 2 MgATP and 10 Hepes; pH was adjusted to 7.2 with Tris base. The osmolarity of pipette solution was adjusted to 280–300 mOsm with sucrose. The extracellular pH was adjusted to different values by 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 nM tetrodotoxin (TTX) and 100 μM CdCl2 were added to the extracellular solutions and KCl was replaced with equimolar CsCl in the pipette solution. Cd2+ (100 μM) had no significant impact on ASIC currents. For measurement of acid-induced depolarization in current-clamp experiments, 5 μM nifedipine, 20 μM caffeine-2-amino-5-phosphohydroxamic acid (AP-5) and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were added to the bath solution to prevent activation of voltage-gated Ca2+ channels or glutamate receptors. Changes in divalent ion content of the bath solution were kept iso-osmotic. All chemicals were purchased from Sigma Chemical. CuCl2 was dissolved in distilled water at a concentration of 100 mM to make stock. The stock solutions were diluted in the extracellular solutions just before each experiment to avoid precipitation. The solutions containing Zn2+ and Pb2+ were obtained in the same way. Drugs were applied using a rapid application technique termed the ‘Y-tube’ method, which allows a complete exchange of external solution surrounding a neuron within 20 ms (Wang et al., 2006).

Data analysis

Clampfit software (Axon Instruments) was used for data analysis. The continuous theoretical curves for dose–response relationships for activation of ASICs in the presence or absence of Cu2+ 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:

\[ I = I_{max} C^*/(C^* + EC_{50}^*) \]  

where \( I \) is the normalized value of the current, \( I_{max} \) the maximal response, \( C \) the drug concentration, \( EC_{50} \) the concentration that induced the half-maximal response and \( h \) the apparent Hill coefficient. The curve for the effect of Cu2+ or amiloride on ASIC currents was fitted to the following equation:

\[ I = I_{max} \frac{(C^* + IC_{50}^*)}{(C^* + IC_{50}^*) + IC_{50}^n} \]  

where \( IC_{50} \) represents the concentration that induced the half-maximal inhibition and the others are the same as the equation 1. The time constant (\( \tau \)) of desensitization of ASIC current was fitted to mono-exponential function:

\[ Y = K_y + K_x \times \exp(-t/\tau) \]  

where \( Y \) is the current amplitude at time \( t \) and \( K_y \) the residual current. The continuous theoretical curves of the I–V relationship for the ASIC currents in the absence and presence of Cu2+ were obtained from linear fit, and the I–V curve for the ASIC currents in the presence of amiloride was from polynomial fit (2nd order). The membrane conductance (Gm) was calculated from the theoretical I–V curves.

All data are shown as the mean ± S.E.M.. Statistical comparison was carried out using Student’s t-test for two groups’ comparison and analysis of variance (ANOVA) for multiple comparisons. Statistically significant differences were assessed as \( P < 0.05 \) or \( P < 0.01 \). \( P \) and \( n \) represent the values of significance and the number of neurons, respectively.

RESULTS

Characteristics of ASIC currents in cultured hypothalamic neurons of the rat

The cultured hypothalamic neurons had an average resting membrane potential of −52.8 ± 1.8 mV (\( n = 16 \)). At a holding potential (Vh) of −50 mV, a drop in extracellular pH induced transient inward currents in most of the hypothalamic neurons (115/125) we examined (Fig. 1A). These proton-induced currents could be detected approximately 4–8). The amplitude of the proton-induced current induced by pH 5.0 was remarkably reduced to 21.7 ± 4.4% of the control by replacement of extracellular Na+ with NMDG (\( n = 6 \); Fig. 1B), suggesting that Na+ is the main charge carrier of the ionic current. Moreover, amiloride, the known ASIC antagonist, revers-
ibly blocked the peak current induced by pH 5.0 in a concentration-dependent manner with an IC$_{50}$ value of 14.4 μM and a Hill coefficient of 0.9 (n=6; Fig. 1C). The Gm activated by extracellular pH 5.0 solution was significantly reduced when the membrane potential was clamped at −60 mV. Each graph was the average response of six neurons (** P<0.01, paired t-test). In this and subsequent figures, the horizontal bars indicate the drug application duration and the vertical bars show the mean±S.E.M.; the V_H is −50 mV, unless otherwise stated.

Most of the neurons (106/115) responding to extracellular low pH expressed the ASIC current with almost complete desensitization. The average τ of desensitization was 504.4±25.7 ms at pH 5.0 (n=15; Fig. 3B). However, we noted that in a minority of neurons (nine/115), the ASICs mediated biphasic currents with a fast desensitization component and a sustained current (Fig. 2A). Fitted to mono-exponential function (Equation 3), the fast desensitization component had a τ value of 351.1±46.6 ms (n=9). The ratio of the sustained current to the peak current was 12.2±2.8% (n=9). In addition, the sustained component of these ASIC currents was not significantly modified by 100 μM amiloride (P>0.05, n=3).

Considering the ASICs in the CNS neurons are a mixture of complexes containing multiple ASIC subtypes (Varming, 1999; Baron et al., 2002; Askwith et al., 2004; Gao et al., 2004), we next made preliminary investigations on the ASIC subtypes responsible for the ASIC currents in cultured hypothalamic neurons using Zn$^{2+}$ and Pb$^{2+}$. Co-application of 300 μM Zn$^{2+}$ and acidic solution has been demonstrated to enhance ASIC2a-containing ASICs in the CNS (Baron et al., 2001; Chu et al., 2004; Sugiura et al., 2005; Mercado et al., 2006). We thus employed acidic solution at pH 5.0 simultaneously with 300 μM Zn$^{2+}$ to
cultured hypothalamic neurons. As shown in Fig. 2B, Zn²⁺ produced an enhancement of ASIC currents in some of the neurons tested (121.4 ± 5.6% of the control; *P < 0.01, n = 5), but had no significant effect on the ASIC currents in the majority of neurons tested (98.1 ± 3.5% of the control; *P > 0.05, n = 10).

The effects of Pb²⁺ on ASICs have recently been elucidated and employed to identify ASIC1 subtypes (Wang et al., 2006). We herein pre-perfused the hypothalamic neurons with 10 μM Pb²⁺ following by co-application of acidic solution at pH 5.0 and the same concentration of Pb²⁺ (Fig. 2B). In all neurons tested, Pb²⁺ produced a significant inhibition on the amplitude of the ASIC current (81.6 ± 5.6% of the control; *P < 0.01, n = 12).

**Effects of Cu²⁺ on ASIC currents in cultured hypothalamic neurons**

Next, we explored the effects of Cu²⁺ on ASICs, the molecular targets of a variety of endogenous divalent cations in the CNS neurons (Wemmie et al., 2006). We pre-perfused the neurons with Cu²⁺ for 15 s, and then activated the ASICs with extracellular acidic solution at pH 5.0 plus the same concentration of Cu²⁺. The amplitude of ASIC currents induced by pH 5.0 was significantly inhibited by extracellular Cu²⁺ at the V₅₀ of −50 mV (Fig. 3A). In the absence of extracellular low pH solution, Cu²⁺ alone did not evoke any detectable current. Cu²⁺ inhibited the ASIC peak current in a concentration-dependent manner with an
IC$_{50}$ value of 46.8 μM and a Hill coefficient of 1.1 ($n=4\sim8$). Prolonging the pretreatment duration from 15 to 180 s did not significantly affect the Cu$^{2+}$ inhibition of ASIC current ($P>0.05$, ANOVA; $n=5$), suggesting a rapid action of Cu$^{2+}$ on ASICs. We employed 60 μM Cu$^{2+}$ in most of subsequent experiments, for this concentration is around the IC$_{50}$.

In addition to its influence on the amplitude of ASIC current, Cu$^{2+}$ appeared to slow down the desensitization of ASIC current induced by pH 5.0. As illustrated in Fig. 3B, 60 μM Cu$^{2+}$ slowed the desensitization rate significantly (without Cu$^{2+}$, $\tau=504.4\pm25.7$ ms, and with Cu$^{2+}$, $\tau=624.7\pm43.4$ ms; $P<0.01$, $n=15$).

To understand whether the effect of Cu$^{2+}$ is specific for hypothalamic neurons, we tested neurons from two other CNS regions, in which the ASICs have been identified and characterized (Varming, 1999; Baron et al., 2002; Wemmie et al., 2002; Alvarez de la Rosa et al., 2003; Gao et al., 2004; Xiong et al., 2004). Similar effects of Cu$^{2+}$ were observed in the cultured hippocampal and cortical neurons. We pre-perfused the hippocampal and cortical neurons with Cu$^{2+}$ (60 μM) for 15 s, and then applied low pH solution plus the same concentration of Cu$^{2+}$ to these neurons. The amplitude of ASIC currents induced by pH 5.0 was reduced by extracellular Cu$^{2+}$ to 52.1±11.4% of the control in the hippocampal neurons ($n=4$, Fig. 4A), and 62.6±4.2% of the control in the cortical neurons ($n=6$, Fig. 4B), respectively. Moreover, in the absence of Cu$^{2+}$, the averaged $\tau$ value of the ASIC currents in hippocampal neurons was 500.3±64.8 ms, while in the presence of Cu$^{2+}$, the averaged $\tau$ value was significantly prolonged to 655.9±35.2 ms ($P<0.05$, $n=4$). In cortical neurons, the averaged $\tau$ values were 471.1±75.2 ms (in the absence of Cu$^{2+}$), and 673.7±179.1 ms (in the presence of Cu$^{2+}$), respectively ($P<0.05$, $n=6$). Thus, Cu$^{2+}$ appears to affect ASICs of neurons from most CNS regions.

**Modulation of ASIC currents by Cu$^{2+}$ was independent of membrane voltage and extracellular pH**

To explore the mechanisms underlying the Cu$^{2+}$ effects on ASICs in hypothalamic neurons, we determined whether the Cu$^{2+}$ effects on ASICs are influenced by...
alternation in membrane potentials. At various \(V_H\) values ranging from \(-60\) mV to \(+20\) mV, ASIC currents were recorded in the absence or presence of \(60 \mu M\) \(Cu^{2+}\) (Fig. 5A). When the membrane potential was clamped at \(-60\) mV, \(60 \mu M\) \(Cu^{2+}\) significantly reduced the \(G_m\) activated by extracellular \(pH\) 5.0 solution (without \(Cu^{2+}\), \(G_m=4.3\pm1.1\) nS, and with \(Cu^{2+}\), \(G_m=3.0\pm0.8\) nS; \(P<0.05, n=7\); Fig. 5B). Similarly, when the membrane potential was clamped at \(+20\) mV, the \(G_m\) was significantly reduced by \(Cu^{2+}\) from 2.1\pm0.7 nS to 1.3\pm0.4 nS (\(P<0.05, n=7\)). Moreover, we found that the inhibitory effect of \(60 \mu M\) \(Cu^{2+}\) on ASICs was not significantly affected by change in membrane potential (\(P>0.05\), ANOVA; \(n=5\); Fig. 5C).

We also studied whether the \(Cu^{2+}\) effects on ASICs is modified by extracellular protons. The activation curves of ASIC currents were generated in the presence and absence of \(60 \mu M\) \(Cu^{2+}\), respectively (Fig. 6). \(Cu^{2+}\) reduced the \(I_{\text{max}}\) of the \(pH\) activation curve approximately by 47.3\% (\(n=8\)), indicating a non-competitive mechanism. The apparent \(EC_{50}\) and Hill coefficient were 5.8 and 1.0 in the absence of \(Cu^{2+}\), and 5.7 and 1.2 in the presence of \(Cu^{2+}\) (\(n=4–8\)).

Fig. 4. Representative traces showing that the amplitude of the ASIC-like currents induced by extracellular \(pH\) 5.0 was significantly inhibited by \(60 \mu M\) \(Cu^{2+}\) in cultured hippocampal (A) or cerebral cortical (B) neurons of the rat. Similar results were obtained from other three or five neurons for hippocampal and cortical neurons respectively (see text for details). Note that the extent of inhibition by \(Cu^{2+}\) was similar among neurons prepared from different regions (see Fig. 4A for comparison).

Fig. 5. The effects of \(Cu^{2+}\) on ASIC-like currents were independent of changes in membrane voltage. (A) Representative traces of ASIC currents elicited by extracellular \(pH\) 5.0 solution in the absence or presence of \(60 \mu M\) \(Cu^{2+}\) in a single hypothalamic neuron voltage-clamped at various \(V_H\) values (from \(-60\) to \(+20\) mV). (B) The column graph showing that the \(G_m\) activated by \(pH\) 5.0 was significantly reduced by \(60 \mu M\) \(Cu^{2+}\), when the membrane potential was clamped at \(-60\) mV and \(+20\) mV, respectively. Each graph was the average response of seven neurons (*\(P<0.05\), paired \(t\)-test). (C) The scatter graph showing the relative inhibition of the peak currents recorded at various \(V_H\) values in the presence of \(60 \mu M\) \(Cu^{2+}\). \(Cu^{2+}\) inhibition of ASIC currents did not depend on \(V_H\) change (\(n=5\); \(P>0.05\), ANOVA).
Comparison of Cu\(^{2+}\) effects on ASICs in different drug application modes

To further examine the mechanisms of the Cu\(^{2+}\) effects on ASICs, we employed three different modes of Cu\(^{2+}\) application. In co-application protocol (protocol a), extracellular Cu\(^{2+}\) and pH 5.0 solution were co-applied to the neurons; in sequential application protocol (protocol b), ASICs were activated by pH 5.0 solution alone following 15 s pre-perfusion of neurons with Cu\(^{2+}\); in pre-treatment protocol (protocol c), ASICs were pre-treated with Cu\(^{2+}\) for 15 s and then activated by pH 5.0 solution plus the same concentration of Cu\(^{2+}\). All responses were normalized to the \(I_{5.0}\) (\(\dagger\)). Each point represents the average response of four to eight neurons.

Cu\(^{2+}\) attenuated the acid-induced membrane depolarization

It has been demonstrated that Cu\(^{2+}\) influences neuronal excitability (Mathie et al., 2006), and acid-induced ASIC-mediated membrane depolarization has been demonstrated in native CNS neurons (Baron et al., 2002; Chu et al., 2004; Vukicevic and Kellenberger, 2004; Wu et al., 2004; Xiong et al., 2004). Therefore, we tested the effects of this endogenous metal ion on acid-induced membrane depolarization in hypothalamic neurons. As shown in Fig. 8, acidic solution at pH 6.5, a slight drop in extracellular pH, induced an increased membrane depolarization (change in membrane potential of 28.4±2.8 mV), which was reversibly blocked by 100 μM amiloride (change in membrane potential of 16.0±2.6 mV, \(n=5\)) in current-clamp mode with no current injection. This acid-induced change in membrane depolarization was reversibly reduced by 60 μM Cu\(^{2+}\) to 13.4±3.4 mV in the same neurons. Furthermore, more acidic solution at pH 6.0 induced a larger...
depolarization (change in membrane potential of 35.6 ± 4.4 mV, n=6). This acid-induced depolarization was similarly attenuated by 100 μM amiloride (change in membrane potential of 23.2 ± 4.9 mV) and 60 μM Cu²⁺ (change in membrane potential of 22.50 ± 3.92 mV).

DISCUSSION

The ASICs in hypothalamic neurons

It is generally accepted that the mammalian hypothalamus is an important part of diencephalon that controls and regulates endocrine, autonomic nervous system, circadian rhythm and emotion. A variety of neuronal membrane receptors and ion channels have been implicated in maintaining and regulating hypothalamic functions (Chen et al., 2002; Albus et al., 2005; Argiolas and Melis, 2005; Jezova, 2005; Caterina, 2006; Giordano et al., 2006; Osei-Hyia- man et al., 2006). Although ASICs are involved in synaptic function and serve roles in many functions in various regions of the CNS, to date, little is known about these channels in the hypothalamus (Wemmie et al., 2006). We recorded proton-induced cation currents in cultured hypothalamic neurons of the rat. These transient inward currents with an activation threshold of around pH 6.8, were mainly carried by Na⁺, and reversibly blocked by amiloride, the known ASIC antagonist. Based on these typical electrophysiological and pharmacological properties for ASICs observed in sensory neurons (Sutherland et al., 2001; Benson et al., 2002), we attribute these currents in hypothalamic neurons to ASICs.

It is unlikely that the acid sensitivity reported in this study is related to culture and age factors, because before ASICs were cloned and identified, Ueno et al. (Ueno et al., 1992; Akaike and Ueno, 1994) had already reported a proton-induced sodium current in freshly dissociated ventromedial hypothalamic neurons. The properties of the proton-induced sodium currents described in their report are similar to those of the ASIC currents described in the present study. Thus, we consider the currents in both freshly dissociated and cultured hypothalamic neurons as ASIC currents.

It is generally thought that in the CNS, ASIC currents represent a mixture of homo- and heteromeric tetramers containing multiple ASIC subtypes (Krishtal, 2003; Wemmie et al., 2006). The diverse electrophysiological and pharmacological properties of ASIC currents we found in hypothalamic neurons strengthen this notion. The dose–response relationship for activation of ASICs by extracellular low pH showed that the apparent EC₅₀ and the Hill coefficient were 5.8 and 1.0, respectively. And the majority of the ASIC currents we examined desensitized almost completely with the τ value of 504.4 ± 25.7 ms at pH 5.0. These results suggest the presence of homomeric ASIC1α or heteromeric ASIC1a-containing channels in hypothalamic neurons (Sutherland et al., 2001; Hesselager et al., 2004). This conclusion is further supported by the Pb²⁺ inhibition of the proton-induced currents in hypothalamic neurons because ASIC1α-specific modulation of ASICs by Pb²⁺ has been reported recently (Wang et al., 2006). Our results also showed that 300 μM Zn²⁺ could significantly enhance the ASIC currents in some hypothalamic neurons, indicating the presence of ASIC2α subunit (Fig. 2B) (Baron et al., 2001; Chu et al., 2004; Sugiura et al., 2005; Mercado et al., 2006). The observed EC₅₀ and the Hill coefficient of the proton-induced currents in hypothalamic neurons are also consistent with the electrophysiological characteristics of heteromeric ASIC1α–ASIC2α channels (Hesselager et al., 2004). Taken together, we suggest that homomeric ASIC1α channels and/or heteromeric ASIC1α–ASIC2α channels are the most likely configuration of ASICs in rat hypothalamus.

In addition, we observed biphasic ASIC currents characterized by a fast desensitization component with the τ value of 351.1 ± 46.6 ms, and a sustained current component insensitive to amiloride. These properties match those of ASIC3 subtype described in heterologous expression system (Waldmann et al., 1997a; Benson et al., 2002; Hesselager et al., 2004). These findings suggest the presence of ASIC3 in rat hypothalamus. The inability of 300 μM Zn²⁺ to enhance proton-induced current in some hypothalamic neurons is consistent with the reported Zn²⁺ effect on ASIC3 channels (Baron et al., 2001). Furthermore, a previous study described ASIC3-like currents in other regions of the rat brain (Bolskakov et al., 2002). However, the presence of ASIC3 subtype in the murine CNS is doubted (Waldmann et al., 1997a; Babinski et al., 2000;
Hruska-Hageman et al., 2004; Wemmie et al., 2006). Further investigations on ASIC3-like currents in the murine CNS and efforts for detecting the molecular identity of ASIC3 subtype in the hypothalamus will eventually address this doubt.

Another finding in this study is that membrane depolarization in hypothalamic neurons could be induced by a mild and moderate drop in extracellular pH that is within the range locally reached by pH fluctuations during normal synaptic transmission or due to tissue acidosis occurring in inflammation, tumor, ischemic stroke and seizure (Krishtal, 2003; Wemmie et al., 2006). Therefore, it is conceivable that in addition to their established roles in other CNS regions, hypothalamic ASICs may regulate hormone release from hypothalamic neurons via ASIC-triggered membrane depolarization.

Although proton-induced response seems ubiquitous and exists in nearly all hypothalamic neurons, our data indicated that various subtypes of ASIC channels (ASIC1a, ASIC2a and possibly ASIC3) are present in this particular region, which plays an important role in the homeostatic regulation of blood acid–base balance, body temperature and energy balance. Considering that different ASIC subtypes participate in distinct physiological and pathological processes (Krishtal, 2003; Wemmie et al., 2006), there is a possibility that subtype-specific expression of ASICs in the different hypothalamic subnuclei mediates distinct functions. Interestingly, several ASIC subtypes are indeed modulated by temperature change and extracellular osmotic pressure (Askwith et al., 2001; Allen and Atwell, 2002; Sugiuira et al., 2003). Apparently, further experiments are required to display the subregional distribution of ASIC subunits in the hypothalamus.

Possible mechanisms of the Cu^{2+} effects on ASICs

Using whole-cell patch-clamp recordings, we found endogenous divalent cation Cu^{2+} reversibly inhibited the amplitude of ASIC current, and slowed down the desensitization of ASICs in the cultured hypothalamic neurons. A similar Cu^{2+} effect was also observed in the hippocampal and cerebral cortical neurons. These results indicate that Cu^{2+} is an endogenous modulator of ASICs in mammalian brain.

Our results showed that the Cu^{2+} effects on ASICs were independent of changes in membrane potential, ruling out the possibility that Cu^{2+} acts on ASIC channel solely via its positive ionic charge and suggesting that the acting site(s) is outside the channel pore. On the other hand, the inability to overcome the Cu^{2+} inhibition at very high concentrations of proton is consistent with a non-competitive mechanism, implying an allosteric modulation. The finding that Cu^{2+} modified the desensitization of ASIC channel further supports an allosteric modulation. Additionally, our results showed that the inhibitory effect in pre-treatment protocol was statistically identical to that of the linear summation from co-application protocol and sequential application protocol, suggesting that the inhibitory effects of Cu^{2+} in the resting state and open state of the channel are additive. Based on this observation, we propose that two separate action sites located at the external side of ASIC channels might account for the effects of Cu^{2+} on these channels. One action site is through the blocking site. When extracellular low pH opens the ASIC channel in the co-application mode, the simultaneously applied Cu^{2+} bound to this site, resulting in channel block effect (Fig. 7Aa). Another action site is a modulating one. Binding of Cu^{2+} to this site in the resting state of the ASIC channel might result in a conformational change, which could stabilize the channel, consequently making the channel less sensitive to extracellular protons (Fig. 7Ab). This stabilization of ASICs reduces the number of open and desensitized channels elicited by extracellular low pH, leading to the inhibition of the amplitude of ASIC currents and the prolongation of channel desensitization (Fig. 3B). Since the blocking site and modulating site are separate, we thus observed a cumulative inhibition (Fig. 7Ac) caused by Cu^{2+} binding to both sites.

Functional relevance of the Cu^{2+} effects on ASICs

The Cu^{2+} concentrations that inhibit the ASIC currents (IC_{50}=46.8 \mu M in hypothalamic neurons) are compatible with the physiological concentration of synaptically released Cu^{2+} (>100 \mu M) (Szerdahelyi and Kasa, 1986; Kardos et al., 1989). Thus our results suggest that ASIC channel in CNS neurons could be a physiological target for Cu^{2+}. Moreover, during pathological insults, the concentration of Cu^{2+} increases in specific regions of the brain (Linder and Hazegh-Azam, 1996; Uru-Adams and Keen, 2005). Alternatively, although most Cu^{2+} is protein-bound, following synaptic activity or in neurodegenerative disease, the loosely bound Cu^{2+} is estimated to reach micromolar concentration (Gutteridge, 1984; Kardos et al., 1989; White et al., 2004) that is effective at ASICs. Therefore, we consider that the modulation of ASICs by Cu^{2+} occurs in conditions of increased synaptic activity or in some brain diseases such as Wilson’s disease (Linder and Hazegh-Azam, 1996).

Finally, our data demonstrated that inhibition by Cu^{2+} of hypothalamic ASIC current could participate in the modulation of neuronal excitability by reducing the membrane depolarization induced by mild pH fluctuations (Fig. 8). This suggests a role of Cu^{2+} in negatively modulating the increased neuronal membrane excitability caused by activation of ASICs. A presumable advantage of this negative feedback is to maintain normal neuronal excitation at a moderate level in physiological conditions. This negative feedback may also occur in pathological conditions such as Wilson’s disease, seizure and ischemic stroke, providing a possible role for preventing overexcitation caused by local or global tissue acidosis. Nevertheless, whether Cu^{2+} antagonism of acid-induced membrane excitability has a therapeutic value requires further investigations.

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