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Research report

# Properties of the proton-evoked currents and their modulation by $Ca^{2+}$ and $Zn^{2+}$ in the acutely dissociated hippocampus CA1 neurons

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**Abstract**

The characterization of acid-sensing ion channel (ASIC)-like currents has been reported in hippocampal neurons in primary culture. However, it is suggested that the profile of expression of ASICs changes in culture. In this study, we investigated the properties of proton-activated current and its modulation by extracellular $Ca^{2+}$ and $Zn^{2+}$ in neurons acutely dissociated from the rat hippocampal CA1 using conventional whole-cell patch-clamp recording. A rapidly decaying inward current and membrane depolarization was induced by exogenous application of acidic solution. The current was sensitive to the extracellular proton with a response threshold of pH 7.0–6.8 and the $pH_{50}$ of 6.1, the reversal potential close to the $Na^+$ equilibrium potential. It had a characteristic of acid-sensing ion channels (ASICs) as demonstrated by its sensitivity to amiloride ($IC_{50} = 19.6 \pm 2.1$ μM). Either low $[Ca^{2+}]_o$ or high $[Zn^{2+}]_o$ increased the amplitude of the current. All these characteristics are consistent with a current mediated through a mixture of homomeric ASIC1a and heteromeric ASIC1a + 2a channels and closely replicate many of the characteristics that have been previously reported for hippocampal neurons cultured for a week or more, indicating that culture artifacts do not necessarily flaw the properties of ASICs. Interestingly, we found that high $[Zn^{2+}]_o$ (>$10^{-4}$ M) slowed the decay time constant of the ASIC-like current significantly in both acutely dissociated and cultured hippocampal neurons. In addition, the facilitating effects of low $[Ca^{2+}]_o$ and high $[Zn^{2+}]_o$ on the ASIC-like current were not additive. Since tissue acidosis, extracellular $Zn^{2+}$ elevation and/or $Ca^{2+}$ reduction occur concurrently under some physiological and/or pathological conditions, the present observations suggest that hippocampal ASICs may offer a novel pharmacological target for therapeutic invention.
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*Theme:* Cellular and molecular biology
*Topic:* Membrane composition and cell-surface macromolecules

*Keywords:* Proton-induced current; Acid-sensing ion channel; Hippocampal CA1 neuron; Patch-clamp

## 1. Introduction

Acid-sensing ion channels (ASICs) belong to degenerin/epithelial Na channel (DEG/ENaC) superfamily, which are activated by extracellular proton. To date, at least six subunits of ASICs have been cloned and expressed: ASIC1a, 1b, 2a, 2b, 3 and 4 [45]. Both homomeric and heteromeric ASICs can be formed which contribute to their functional diversity. Amongst all ASIC subunits, ASIC 1b and 3 are found only in sensory neurons [14,17,42,43] whereas all the other four subunits are also expressed in the brain [1,11,20,21,31,35,36,42]. Many studies in sensory neurons in the periphery have implicated ASICs in nociception and mechanoreception [3,14,18,21,35,43]. Recently, several studies have also characterized the functional properties of ASIC-like currents in the central nervous system (CNS). However, much of the work has been done on cultured neurons in vitro [4,10,16,30,41,46,47]. It has been suggested the profile of expression of ASICs changes in culture [25]. For example, in cultured embryonic ventral

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spinal cord neurons, the ion dependency of proton-activated currents changes over time [28]. In addition, Krishtal and Pidoplichko [26] found that the majority of acid-sensing neurons are the smallest neurons in sensory ganglion. However, such profile of expression of ASICs is true only for the cells examined immediately after acute isolation, and in culture this profile changes within a few hours in such a way that a great majority of all the neurons (i.e. not only restricted to the smallest neurons) become $H^+$-sensitive [25]. Therefore, in this study, we were stimulated to investigate the electrophysiological and pharmacological properties of proton-activated current in neurons acutely dissociated from the rat hippocampal CA1 area, which may reflect more closely to the ASIC properties under in vivo conditions.

On the other hand, both $Ca^{2+}$ and $Zn^{2+}$ have been reported to regulate the function of ASICs. For example, extracellular $Ca^{2+}$ can modulate homomeric ASIC1a- [7], homomeric ASIC2a- [16], homomeric ASIC3- [23] or heteromeric ASIC1a + 2a- [16] mediated currents in heterologous cells. In addition, extracellular $Zn^{2+}$ potentiates the ASIC-like currents in cultured hippocampal neurons [10] and augments the $H^+$-activated currents in either *Xenopus* oocytes or COS cells expressing ASIC2a or ASIC1a + 2a subunits [9]. Both $Ca^{2+}$ and $Zn^{2+}$ are the important trace elements in the brain, which can potentially change the behavior of various membrane channels and neurotransmitter receptors under physiological or pathological situations. To understand the properties of interactions of $Ca^{2+}$ and $Zn^{2+}$ with native ASICs, we further studied the modulation of the proton-activated currents by $Ca^{2+}$ and $Zn^{2+}$ in acutely dissociated CA1 neurons since the previous studies are mostly performed in heterologous cells or in cultured neurons [9,10]. Our data closely replicate many of the characteristics that have been previously reported for hippocampal neurons cultured for a week or more, indicating that culture artifacts do not necessarily flaw the properties of ASICs. Interestingly, we found that high $[Zn^{2+}]_o$ ($>10^{-4}$ M) prolonged the decay time constant of the ASIC-like current significantly in both acutely dissociated and cultured hippocampal neurons. In addition, the facilitating effects of low $[Ca^{2+}]_o$ and high $[Zn^{2+}]_o$ on ASIC-like current were not additive.

## 2. Materials and methods

### 2.1. Isolation of neurons

The experimental protocols were approved by our Institutional Care and Use of Animals Committee. Two-week-old Wistar rats were anaesthetized with pentobarbitone sodium (45–50 mg/kg, i.p.) before decapitation. Their brains were quickly excised and placed into ice-cold incubation solution. The brain was then glued to the chilled stage of a vibrotome tissue slicer [VT1000S, Leica Instruments, Wetzlar, Germany] with iced incubation solution and sliced at a thickness of 400 μm. After incubation at room temperature (22–25 °C) for 50 min (see below for the composition of the solution), the slices were transferred into well-oxygenated standard external solution (see below). Cells were prepared by a modified mechanical method [29]. In brief, a fire-polished glass pipette mounted on a vibrator and oscillating horizontally at about 5–10 Hz under the control of a pulse generator was touched lightly onto the surface of the slice. The vibration–dissociation lasted for about 3 min and then the slice was removed from the dish. Within 20 min of dissociation, isolated neurons had attached to the bottom of the culture dish and were ready for electrophysiological recordings.

### 2.2. Cultured hippocampal neurons

Hippocampal neurons from 18-day-old embryonic Sprague–Dawley rats were isolated by a standard enzyme treatment protocol. Briefly, hippocampi were dissociated in $Ca^{2+}$-free saline with sucrose (20 mM) and plated ($1.5 \times 10^6$ cell/ml) on poly-L-lysine (Collaborative Biomedical Products) coated cover glasses. The neurons were grown in DMEM (Gibco, USA) with L-glutamine plus 10% fetal bovine serum (Gibco)) and 10% F-12 nutrient mixture (Gibco). Neuronbasal medium (1.5 ml, Gibco) with 2% B27 (Gibco) was replaced every 3–4 days. Treatment with 5-fluoro-5′-deoxyuridine (20 μg/ml, Sigma, St. Louis, MO) 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% $CO_2$ humidified atmosphere. Cells were used for electrophysiological recordings 7–20 days after plating. Neurons with triangular-shaped cell bodies, a typical feature of pyramidal neurons, were selected for recording.

### 2.3. Solutions and drugs

The ionic composition of incubation solution was (mM): 124 NaCl, 24 $NaHCO_3$, 5 KCl, 1.2 $KH_2PO_4$, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 10 glucose, aerated with 95% $O_2$/5% $CO_2$ to a final pH of 7.4. The standard external solution contained (mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, buffered to various pH values with either 10 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES, pH 6.0–7.4) or 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH < 6.0). In $Na^+$-free medium, $Na^+$ was substituted with equimolar *N*-methyl-D-glucamine (NMDG), and $Ca^{2+}$-free medium contained no $Ca^{2+}$ adding 5 mM EGTA. The osmolarity of all bath solutions was adjusted to 325–330 mosM/l with sucrose (3300, Norwood, MA, USA). The patch pipette solution for whole-cell patch recording was (mM): 120 KCl, 30 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 2 Mg-ATP, 10 HEPES. The internal solution was adjusted to pH 7.2 with Tris-base. When the *I*–*V* relationships for proton-induced currents were examined, 300 nM tetrodotoxin (TTX) and 200

μM $CdCl_2$ were added to the standard external solution and $K^+$ was replaced with $Cs^+$ in the pipette solution.

Drugs used in the present experiments were purchased from Sigma. Drugs were first dissolved in ion-free water and then diluted to the final concentrations in the standard external solution just before use or dissolved directly in the standard external solution. Drugs were applied using a rapid application technique termed the 'Y-tube' method throughout the experiments [29]. In brief, a major part of the system is a Y-shaped tube. The test solution was held 20 cm above the level of the Y-tube tip, so that the solution flowed out from the Y-tube tip by gravity. Another end of the V-shaped tube was led to a drain bottle through an electric valve which is normally closed. The drain bottle was kept at a negative pressure of approximately 40 cm Hg. The orifice of the Y-tube tip was positioned 100–500 μm away from a single neuron. When solution exchange was required, the electric valve was opened for 0.5–1.5 s which was sufficient to exchange the control standard external solution with the test solution up to the V-shaped end of the Y-tube. Immediately after the electric valve was closed, the test solution started perfusing the cell by gravity. Returning to the control solution or application of another test solution was achieved by the same procedures. This system allows a complete exchange of external solution surrounding a neuron within 20 ms [48].

### 2.4. Electrophysiological recordings and data analysis

The electrophysiological recordings were performed in the conventional whole-cell patch-recording configuration under voltage-clamp conditions. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PP-830, Narishige, Tokyo, Japan). 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 (Axon 200B, Axon Instruments, Foster City, CA, USA), sampled and analyzed using a Digidata 1320A interface and a personal computer with Clampex and Clampfit software (Version 9.0.1, Axon Instruments). In most experiments, 70–90% series resistance was compensated. Unless otherwise noted, the membrane potential was held at −50 mV throughout the experiment. All the experiments were carried out at room temperature (22–25 °C).

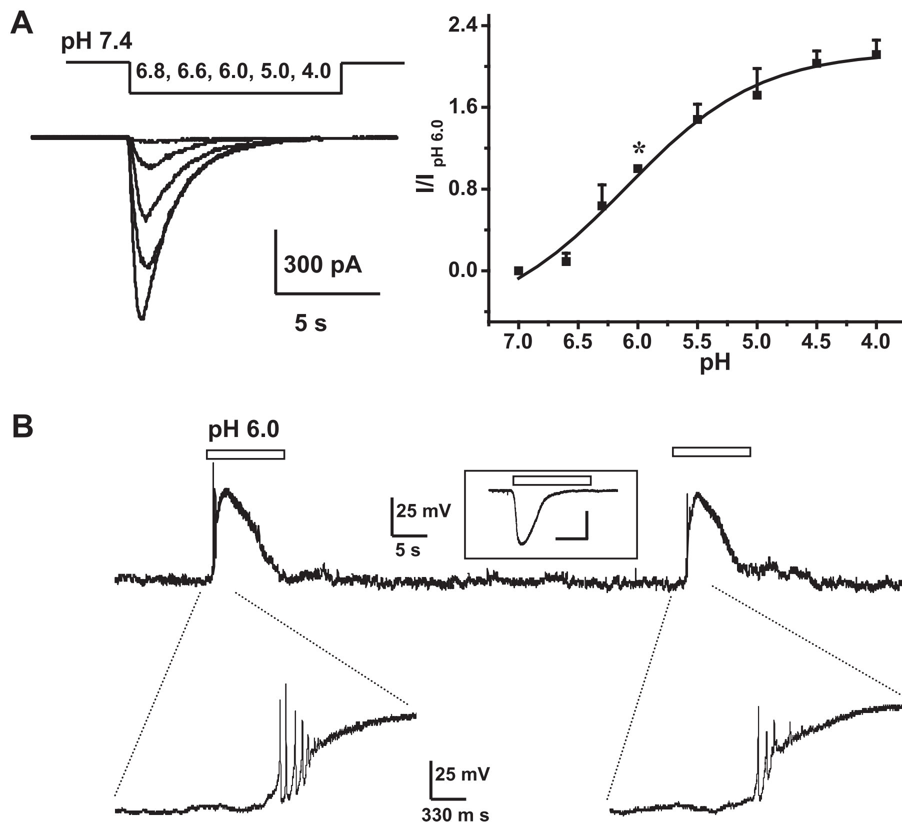


Fig. 1. The proton-evoked currents in acutely dissociated rat hippocampal CA1 neurons. (A) pH dependence of the proton-induced currents. All currents were normalized to the peak response induced by pH 6.0 (*). Each point represents the average of six to eight neurons and the vertical bars show the mean ± S.E.M. (B) Membrane depolarization was induced by tandem applied pH 6.0 in a single neuron. The initial acid-induced depolarization and the subsequently triggered action potentials are shown on a larger scale. Inset: The proton-induced rapidly decaying current activated by pH 6.0 was recorded in the same neuron in voltage-clamp mode. The scale bar is 250 pA/3 s. Similar results were obtained in other five neurons. In this and subsequent figures, the horizontal bars above each current trace indicate the application of the extracellular pH or drugs. Holding potential ($V_H$) was −50 mV.

## 3. Results

### 3.1. Proton-induced inward current and membrane depolarization in acutely dissociated hippocampus CA1 neurons

To investigate the properties of native ASICs, the responses of acutely dissociated hippocampus CA1 neurons to acidic stimuli were measured by applying various pH buffered solutions for about 15 s at a holding potential ($V_H$) of $-50$ mV. We choose $V_H = -50$ mV in the present experiments because the average resting membrane potential of our preparation was near $-50$ mV (see below). Although the amplitude of the inward currents varied from cell to cell, all of the 149 tested neurons responded to pH 6.0 and the evoked inward currents were always greater than 10 pA. In the majority of neurons (126 of 149), the acidic solution evoked a transient, rapidly decaying current (Fig. 1A). As the proton-induced rapidly decaying currents were recorded most frequently, this type of current was selected for further electrophysiological and pharmacological studies in the following experiments. The current showed a high sensitivity to pH, with an activation threshold around pH

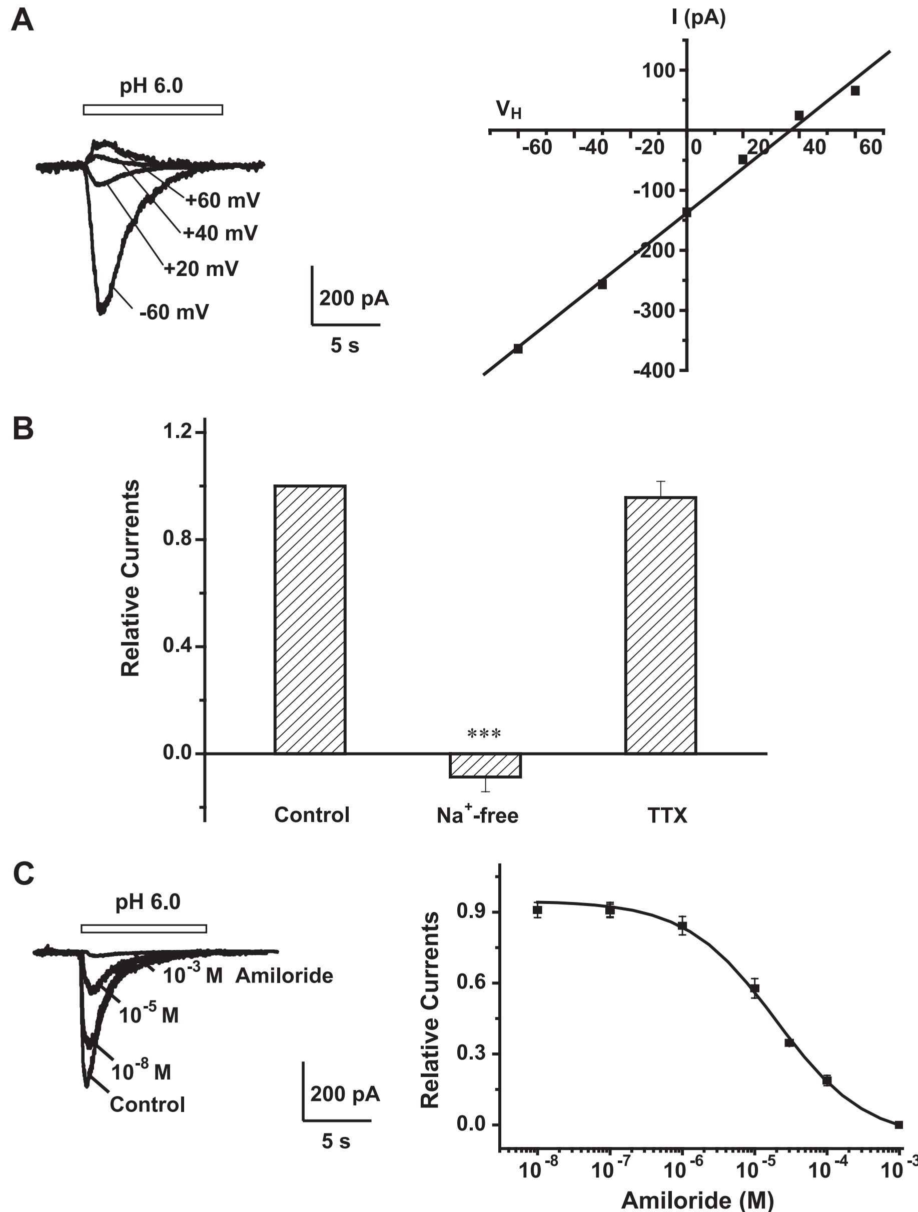


Fig. 2. The electrophysiological and pharmacological properties of the proton-induced current. (A) $I$–$V$ relationship for proton-induced current. Proton-induced currents recorded at various $V_H$s and the current reversed at about +40 mV. All the current traces and the linear fitted curve were obtained from the same cell. (B) Statistical data from the triggered current by pH 6.0 in the presence of 300 nM TTX and in $Na^+$-free saline experiments. All currents were normalized to the control response of pH 6.0. Each column represents the average of six to eight neurons and the vertical bars show the mean ± S.E.M. ***$P < 0.001$. (C) Amiloride inhibited the proton-induced current in a concentration dependent manner. All traces were obtained from the same neuron. The statistical data were fitted to the Hill equation and $IC_{50} = 19.6 \pm 2.1$ μM. Each point represents the average of six neurons and the vertical bars show the mean ± S.E.M.

7.0–6.8 (Fig. 1A). Between pH 7.4 and 4, the activation of the peak current could be fitted by a sigmoid curve, with a half-maximal activation at pH 6.1 and a Hill slope factor ($n_H$) of 1.42 (Fig. 1A).

As was reported for the cultured hippocampal neurons [10], proton also induced membrane depolarization in acutely dissociated CA1 neurons. Membrane potential variations had been examined under current-clamp conditions and the resting potentials of the neurons were $-54.7 \pm 3.2$ mV ($n = 16$). A typical change of membrane depolarization by pH shifting from 7.4 to 6.0 was recorded as shown in Fig. 1B. Activation of rapidly decaying current of about 500 pA (as shown in inset) transiently depolarized the neurons to approximately 0 mV, with a train of attenuated action potentials triggered at the initial transient depolarization (as enlargement shown in Fig. 1B).

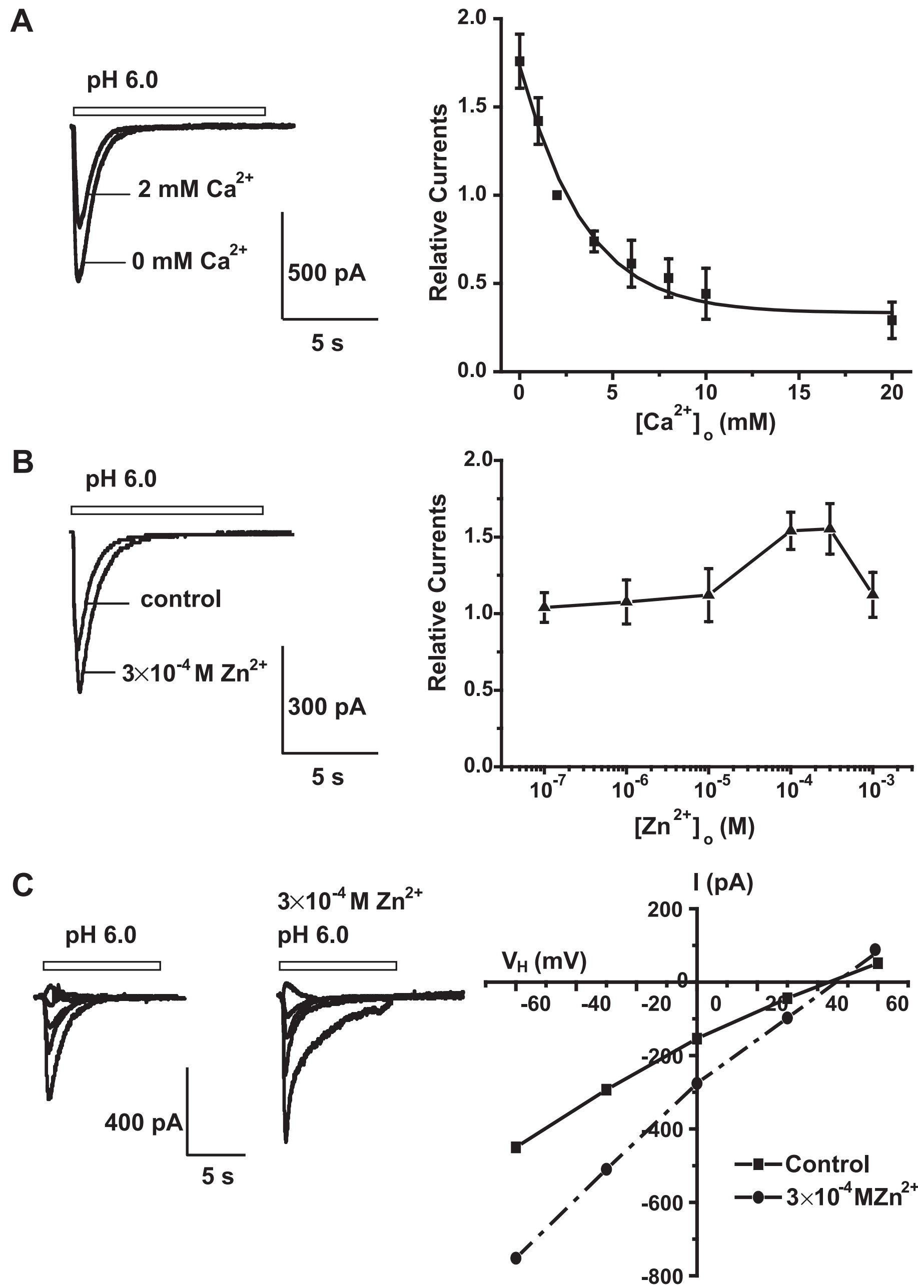


Fig. 3. Modulation of the ASIC-like current by extracellular $Ca^{2+}$ and $Zn^{2+}$. (A) Inhibition of the proton-induced current by high extracellular $Ca^{2+}$ ($[Ca^{2+}]_o$). However, high extracellular $Zn^{2+}$ ($[Zn^{2+}]_o$) co-applied with acidic pH significantly increased the amplitude of the proton-induced current by pH 6.0 (B). In both (A) and (B), each point represents the average of six to eight neurons and the vertical bars show the mean ± S.E.M. (C) Voltage-independent enhancement of the proton-evoked current by $Zn^{2+}$. Proton-induced current at different holding potentials from the same patch in the presence or absence of $3 \times 10^{-4}$ M $Zn^{2+}$ was illustrated. Similar results were obtained in other four neurons.

### 3.2. Pharmacological properties of the proton-induced current

When the currents triggered by pH 6.0 were recorded at various $V_H$s, they reversed between potentials of +30 and +50 mV (Fig. 2A). The reversal potential of the currents, estimated from the $I-V$ relationships, was +46.9 ± 2.5 mV ($n=12$), which is close to the theoretical $Na^+$ equilibrium potential (+41.4 mV) calculated with the Nernst equation for the given extra- and intracellular $Na^+$ concentrations (150 and 30 mM, respectively) (a representative current was shown in Fig. 2A). When the extracellular $Na^+$ was substituted by NMDG, the amplitude of the current was markedly depressed. However, the current persisted when the neuron was perfused with the 300 nM TTX medium (Fig. 2B). These results indicate that $Na^+$ primarily carries the proton-induced currents in the present preparation.

Amiloride, the inhibitor for acid-sensing ion channels (ASICs) [41,44], reduced the current concentration-dependently (Fig. 2C). The half-maximal inhibition ($IC_{50}$) for amiloride inhibition was 19.6 ± 2.1 μM.

### 3.3. Potentiation of the ASIC-like current by low $[Ca^{2+}]_o$ and high $[Zn^{2+}]_o$

The ASIC-like currents of the present preparation were sensitive to extracellular $Ca^{2+}$ ($[Ca^{2+}]_o$). As shown in Fig. 3A, the effect of the $[Ca^{2+}]_o$ from 0 to 20 mM were tested on the currents activated by pH 6.0. The proton-induced current was blocked by high $[Ca^{2+}]_o$ and was almost eliminated in 20 mM $[Ca^{2+}]_o$. The dose–effect curve in Fig. 3A shows that $[Ca^{2+}]_o$ in excess of 2 mM inhibited the amplitude of the proton-induced currents in a concentration-dependent manner. The $IC_{50}$ occurred between 2 and 5 mM $[Ca^{2+}]_o$.

The effect of extracellular $Zn^{2+}$ ($[Zn^{2+}]_o$), a well-known endogenous divalent cation, on the ASIC-like current was next examined. Contrary to the effect of the $[Ca^{2+}]_o$, $[Zn^{2+}]_o$ exerted a significant enhancing effect on the amplitude of the currents (Fig. 3B). In the presence of $10^{-5}$ M to $3 \times 10^{-4}$ M $Zn^{2+}$, the amplitude of the currents induced by pH 6.0 was increased in a dose-dependent manner, and even increased by 55.3 ± 1.6% in the presence of $3 \times 10^{-4}$ M $Zn^{2+}$. $Zn^{2+}$ did not change the reversal potential of the proton-induced currents (46.79 ± 2.3 vs. 44.86 ± 2.1 mV of the control

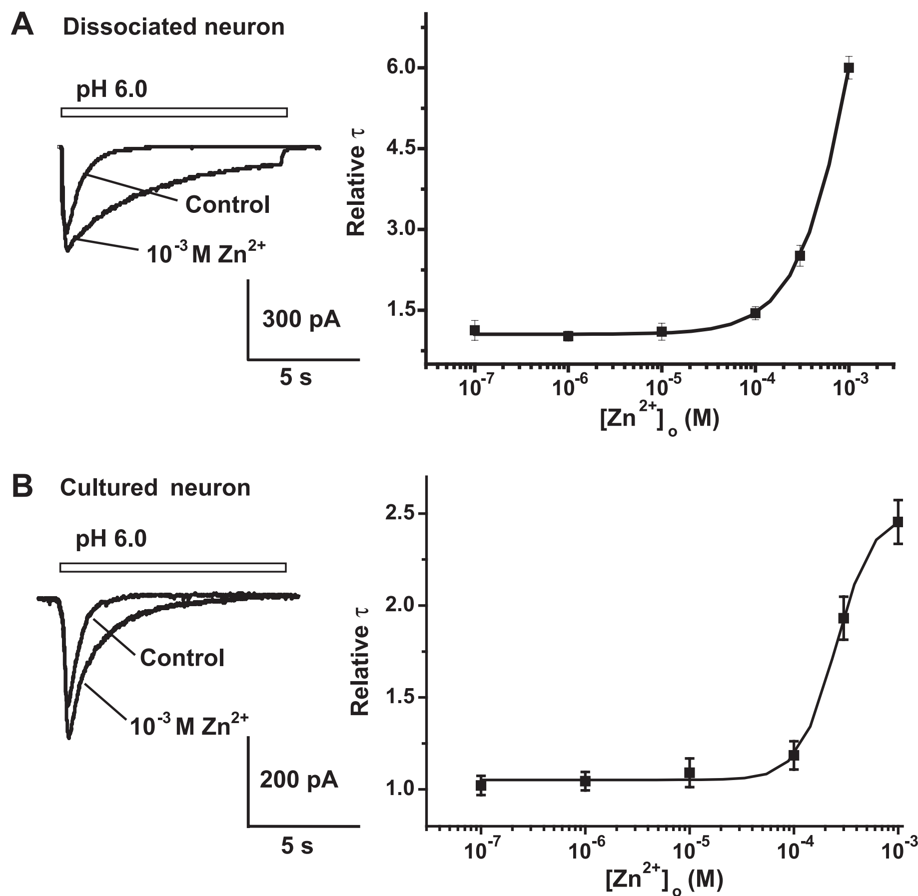


Fig. 4. $Zn^{2+}$ slowed the inactivation of the ASIC-like currents. $Zn^{2+}$ ($3 \times 10^{-4}$ M) markedly increased the decay time constant (τ) of the ASIC-like currents in both dissociated (A) and cultured hippocampal neurons (B). Left: A typical trace. Right: Graph plotting of the relative τ of the proton-induced current at various concentrations of $Zn^{2+}$. Each point represents the average of six neurons and the vertical bars show the mean ± S.E.M.

currents, $n = 7$, Fig. 3C). Moreover, the enhancement did not depend on the holding potentials in a range from − 60 to + 60 mV. Interestingly, less potentiation (by 12.3% ± 0.14, $n = 8$) was observed in the presence of higher concentration of $[Zn^{2+}]_o$ ($10^{-3}$ M $Zn^{2+}$, Fig. 3B). Instead, we found high concentration (>$10^{-4}$ M) of $Zn^{2+}$ significantly prolonged the decay time constant ($\tau$) of the currents ($\tau = 2.74 \pm 0.51$ s in the presence of $10^{-3}$ M $Zn^{2+}$ vs. $\tau = 0.61 \pm 0.18$ s of the control responses, $n = 6$) (Fig. 4A). To test whether this prolongation is specific to acutely dissociated neurons, we examined $10^{-3}$ M $Zn^{2+}$ on cultured hippocampal neurons. As shown in Fig. 4B, a similar prolongation of the decay time by $10^{-3}$ M $Zn^{2+}$ was observed in cultured hippocampal neurons, though the extent of augment was less than that of the acutely dissociated neurons (cultured neurons vs. acutely dissociated neurons: times of 2.45 ± 0.12 vs. times of 6.00 ± 0.21, $n = 7$).

### 3.4. *Interactions of $Ca^{2+}$ and $Zn^{2+}$ on the ASIC-like currents*

Since both extracellular $Ca^{2+}$ and $Zn^{2+}$ affected the proton-evoked currents, we then asked whether their modulation sites are identical or separate. If they are separate, the effect of $Ca^{2+}$-free and high $Zn^{2+}$ would be additive. Our results favored this hypothesis, showing that the potentiation of the ASIC-like current by co-applying $Ca^{2+}$-free plus high $Zn^{2+}$ external solutions was comparable to that of the theoretical summation of their individual modulation (Fig. 5). This result suggests that $Ca^{2+}$ and $Zn^{2+}$ modulation site on ASICs might be independent and therefore both divalent ions produced little overlapped effect on the ASIC-like current.

## 4. Discussion

The principal finding of the present study is that the electrophysiological and pharmacological characteristics of the proton-activated ASIC-like currents of the acutely dissociated hippocampal CA1 neurons closely replicate many of the characteristics that have been described for hippocampal neurons cultured for a week or more [10]. These results are in agreement with the idea that a mixture of homomeric ASIC1a and heteromeric ASIC1a + 2a channels may mediate the proton-gated currents in hippocampus

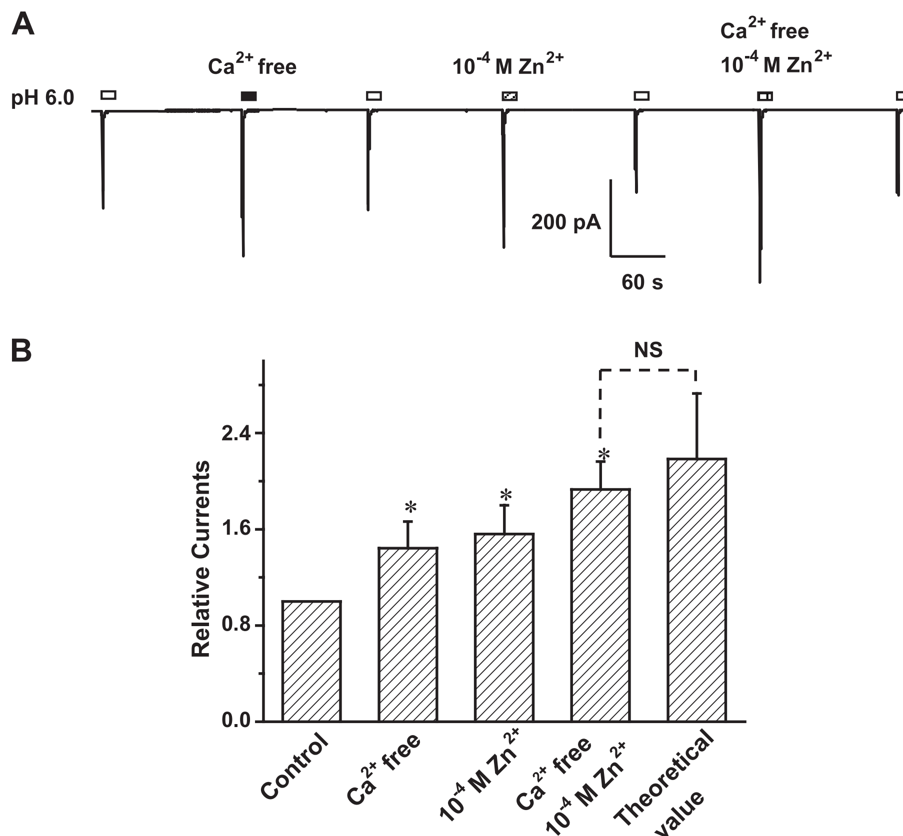


Fig. 5. Effect of co-application $Ca^{2+}$ and $Zn^{2+}$ on the ASIC-like currents. (A) Typical recordings of the currents evoked by pH 6.0 under $Ca^{2+}$-free or $10^{-4}$ M $Zn^{2+}$-alone administration, or co-application. (B) Pooled data from experiments shown in (A). Theoretical value is the linear summation of the proton-evoked currents under conditions of external 0 $Ca^{2+}$ and in the presence of $10^{-4}$ M $Zn^{2+}$, respectively. All currents were normalized to the peak response evoked by normal pH 6.0 external solution. Each column is the average of 8–10 neurons. *$P < 0.05$. NS means no statistic significance.

CA1 neurons [10]. Identification of the channel subtypes in neurons in vivo is essential to understand their functional implications and to develop pharmacological strategies able to interfere selectively with specific neuronal functions. In addition, we found that high $[Zn^{2+}]_o$ ($>10^{-4}$ M) prolonged the decay time constant of the ASIC-like current significantly in both acutely dissociated and cultured hippocampal neurons. Moreover, the facilitating effects of low $[Ca^{2+}]_o$ and high $[Zn^{2+}]_o$ on the ASIC-like current were not additive.

### 4.1. Proton-activated ASIC-like currents in acutely dissociated CA1 neurons

In the present study, the proton-induced current in the acutely dissociated hippocampal CA1 neurons showed a high sensitivity to acidic solution, with an activation threshold of pH 7.0–6.8, the $pH_{50}$ of pH 6.1 and a Hill slope factor ($n_H$) of 1.42. Moreover, the currents were sensitive to amiloride ($IC_{50} = 19.6 \pm 2.1$ μM), a commonly used inhibitor for the ASICs [41,44]. These properties were between those of homemeric ASIC1a and heteromeric ASIC1a + 2a currents ($pH_{50} = 6.4$ and $n_H = 1.65$; $pH_{50} = 5.5$ and $n_H = 1.10$, respectively) [9,19], and were similar to the result by Baron et al. obtained from cultured hippocampal neurons [10] (Table 1), suggesting that, at least for hippocampal CA1 neurons, culture artifacts do not flaw the previous studies [10]. These results are in agreement with the idea that a mixture of different ASIC subunit composition may mediate the proton-gated currents in hippocampus CA1 neurons. Following observations support this speculation: (1) It has been reported that ASIC1a channel expressed in *Xenopus* oocytes could be inhibited by high $[Ca^{2+}]_o$ [7], and the heteromeric ASIC1a + ASIC2a channel is sensitive to high $[Ca^{2+}]_o$ too [16]. In the present preparation, $[Ca^{2+}]_o$ had an inhibitory effect on the proton-induced current. This observation thus suggests the existence of ASIC1a underlying the proton-induced currents in the present preparation. (2) Baron et al. [9] has indicated that extracellular $Zn^{2+}$ can enhance the current mediated by homomeric and heteromeric ASIC2a-containing channels expressed in *Xenopus* oocytes. In our study, $Zn^{2+}$ enhanced the proton-gated currents, indicating that ASIC2a was also involved. (3) ASIC1a, ASIC2a and ACIC2b isoforms are detected in hippocampus [4,31,44]. However, whether ASIC2b subunit participates the composition of ASICs in hippocampal neurons awaits further studies.

Table 1
The comparison of proton-evoked currents between cultured and acutely dissociated hippocampus neurons

| Functional properties | Cultured hippocampus neurons (Ref. [10]) | Acutely dissociated hippocampus CA1 neurons (present preparation) |
|---|---|---|
| Transient component | Yes | Yes |
| Sustained component | Yes | No |
| $pH_{threshold}$ | ~ 6.9 | ~ 6.8 |
| $pH_{50}$ | 6.2 | 6.1 |
| Ion selectivity | highly selective for $Na^+$ | highly selective for $Na^+$ |
| Sensitivity to amiloride | transient component is sensitive to amiloride, while sustained one not | amiloride (1 mM) almost inhibited the current completely |
| Inhibition by 10 mM $[Ca^{2+}]_o$ | NT | ~ 60% |
| Effect of $[Zn^{2+}]_o$ on the amplitude of the current | yes | yes |
| Effect of $[Zn^{2+}]_o$ on the inactivation of the current | yes[a] | yes |

"NT" stands for "not tested."
[a] Data from the present study.

### 4.2. Modulation of ASIC-like current by $Ca^{2+}$ and $Zn^{2+}$

Both $Ca^{2+}$ and $Zn^{2+}$ are the prevalent trace elements in the brain and are involved in synaptic plasticity and modulation of ion channels or receptors under some physiological or pathological situations. A number of studies have demonstrated that extracellular $Ca^{2+}$ inhibits homomeric ASIC1a, ASIC2a-, ASIC3- or heteromeric ASIC1a + ASIC2a-mediated currents in transfected cells [16,22,23]. For example, $[Ca^{2+}]_o$ has a fairly high-affinity binding site on ASIC3, exerting an inhibitory effect on the channel [23]. However, the effect of $Ca^{2+}$ on the ASIC1a-mediated currents is more complex [7,16]. The data from de Weille and Bassilana [16] suggest that $[Ca^{2+}]_o$ (ranging from 0 to 10 mM) potentiates the ASIC1a-mediated currents while higher concentration of $Ca^{2+}$ produces inhibitory effects. Contradictorily, Babini et al. [7] indicate that ASIC1 is inhibited during channel activation but the channel is augmented in the resting state by $[Ca^{2+}]_o$. In the present study, we also observed an inhibition of $[Ca^{2+}]_o$ on proton-gated ASIC-like currents. The potentiation of ASICs by low$[Ca^{2+}]_o$ may have functional significance in vivo, since external $Ca^{2+}$ falls during neuronal activity [12,34,37] and can decrease from a resting value around 1.2 to 0.08 mM in extreme conditions [34].

Zinc modulation of ASICs has been reported by Baron et al. [9,10]. They showed that $Zn^{2+}$ enhanced ASIC2a-containing channel [10] as well as proton-induced current in cultured hippocampal neurons [10]. In heterologous cells, Baron et al. [9,10] demonstrated that $Zn^{2+}$ enhanced ASIC1a + 2a currents at concentrations ranging from 1 μM to 10 mM. In the acutely dissociated CA1 neurons, we also observed that $Zn^{2+}$ facilitated the ASIC-like currents dose-dependently but in a narrower $Zn^{2+}$ concentration window (from 10 μM to 0.3 mM). This discrepancy may result from the different expression of ASIC subunits and/or differences in heteromultimeric assembly [28]. More interestingly, we found that higher concentrations of $Zn^{2+}$ ($>10^{-4}$ M) not only enhanced the amplitude of the ASIC-like currents, but also prolonged the inactivation kinetics of the currents in both acutely dissociated and cultured hippo-

campal neurons. This finding is important considering that the ASICs desensitize very rapidly. The interaction with $Zn^{2+}$ could thus provide a gain-control mechanism for ASICs to sense the prolonged acidosis during persistent neuronal excitation and/or neural injury when a fall in external pH coincides with a remarkable elevation of extracellular $Zn^{2+}$. Additional experiments are required to understand how the interaction of ASICs with $Zn^{2+}$ alters the synaptic function and/or contributes to acidosis-induced injury.

ASICs are modulated not only by $Ca^{2+}$ and $Zn^{2+}$, but also by other cations. For example, $Mg^{2+}$, $Ba^{2+}$ and spermine all potentiate the homomeric ASIC1a- or ASIC1b-mediated currents in *Xenopus* oocytes [7], while $Gd^{3+}$ has an inhibitory effect on heteromeric ASIC2a + 3 channels [8]. The mechanism and the function of such modulation in vivo remain uncertain. According to a recent study by Babini et al., interaction with di- and polyvalent cations could extend the dynamic range of ASIC $H^{+}$ sensors.

### 4.3. Functional significance of ASICs in hippocampus

In the central nerve system (CNS), ASICs are implicated in synaptic plasticity and neural injury. For example, recent studies have indicated that ASIC1 enrich in synaptosome, primarily locating in hippocampal postsynaptic neurons [46]. Moreover, ASIC1 contributes to the deficit in learning and memory and fear conditioning in ASIC1 knockout mice [46,47]. Synaptic vesicles are acidic (pH–5.7) [32], and release of neurotransmitter likely ejects protons into the synaptic cleft [15,27,40]. Accumulating evidence indicates that the release of vesicular protons modulates synaptic transmission [15,27,40,41,44,46]. In the present study, we showed that extracellular acidosis could induce an ASIC-like current in acutely dissociated hippocampus CA1 neurons. The characteristics of the ASIC-like currents resemble those of homomeric ASIC1a and heteromeric channels containing 2a subunits. In addition, the ASIC-like current could further induce a membrane depolarization and trains of action potentials in these neurons, which may release NMDA receptor $Mg^{2+}$ blockage and consequently increase cytosolic $Ca^{2+}$ concentration and modulate the synaptic function. Although our results confirm ASICs as a key component of hippocampal channels that open in response to extracellular acidification, the question remains if in vivo extracellular pH changes are large enough to activate ASIC channels. In addition, it is still unclear whether $H^{+}$ is the only natural ligand for ASICs in the CNS. In this regard, the present data further indicates that low $[Ca^{2+}]_o$ and high $[Zn^{2+}]_o$ augmented hippocampal ASIC-like currents. Furthermore, we found that high $[Zn^{2+}]_o$ prolonged the decay time constant of the currents significantly. Alternatively, it is possible that under physiological conditions other endogenous ligands or modulators of ASICs such as $Zn^{2+}$, $Ca^{2+}$, or spermine, FMRFamide-related peptides, which are regulated by synaptic activities [5,7,9,10,23,44], may facilitate the ASIC activation or stabilize/prime the channel in a 'ready-to-activate' state [7]. Interestingly, ASIC1a channel itself could provide a direct $Ca^{2+}$ entry into neurons [39,44,49]. A role of ASIC1 that is strongly expressed in the CNS in synaptic function has been suggested by examining ASIC1 knockout mice [46,47]. Apparently, further experiments are needed to clarify the actual mechanisms by which each ASIC subunits of the hippocampus serve their functions.

Brain injury such as global ischemia and epilepsy can induce the entire surface of brain to become more acidic than necessary to activate the majority of ASICs [33,38]. Moreover, the ischemia-related signals, for example, arachidonic acid up-regulates the ASIC currents in the cerebellar Purkinje cells [2]. In addition, the increased production of lactate during ischemia also facilitates the activities of ASICs by chelating extracellular $Ca^{2+}$ [22]. More recently, it has been suggested that $Ca^{2+}$ influx through homomeric ASIC1a contributes to cell death in vitro in heterologous cells and in hippocampal neurons [49]. In the present study, we showed that the ASIC-like currents were not just controlled by the extracellular pH, but were potentiated by low $[Ca^{2+}]_o$ and high $[Zn^{2+}]_o$ in the hippocampal CA1 neurons. Moreover, we found that the effects of low $[Ca^{2+}]_o$ and high $[Zn^{2+}]_o$ were not additive. Extracellular $Ca^{2+}$ abruptly falls to $\sim$ 0.1 mM at the time of the anoxic depolarization, 60–90 s after the start of ischemia [13]. On the other hand, zinc is released together with glutamate into the extracellular space upon brain injury, where it can reach a concentration of several hundred micromolar [6,24]. Since tissue acidosis, extracellular $Zn^{2+}$ elevation and/or $Ca^{2+}$ reduction occur concurrently under some physiological and/or pathological conditions, our data suggest that hippocampal ASICs may offer a novel pharmacological target for therapeutic invention.

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