Modulatory effects of acid-sensing ion channels on action potential generation in hippocampal neurons

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Vukicevic, Marija, and Stephan Kellenberger. Modulatory effects of acid-sensing ion channels on action potential generation in hippocampal neurons. Am J Physiol Cell Physiol 287: C682–C690, 2004. First published April 28, 2004; 10.1152/ajpcell.00127.2004.—Extracellular acidification has been shown to generate action potentials (APs) in several types of neurons. In this study, we investigated the role of acid-sensing ion channels (ASICs) in acid-induced AP generation in brain neurons. ASICs are neuronal Na⁺ channels that belong to the epithelial Na⁺ channel/degenerin family and are transiently activated by a rapid drop in extracellular pH. We compared the pharmacological and biophysical properties of acid-induced AP generation with those of ASIC currents in cultured hippocampal neurons. Our results show that acid-induced AP generation in these neurons is essentially due to ASIC activation. We demonstrate for the first time that the probability of inducing APs correlates with current entry through ASICs. We also show that ASIC activation in combination with other excitatory stimuli can either facilitate AP generation or inhibit AP bursts, depending on the conditions. ASIC-mediated generation and modulation of APs can be induced by extracellular pH changes from 7.4 to slightly <7. Such local extracellular pH values may be reached by pH fluctuations due to normal neuronal activity. Furthermore, in the plasma membrane, ASICs are localized in close proximity to voltage-gated Na⁺ and K⁺ channels, providing the conditions necessary for the transduction of local pH changes into electrical signals.

extracellular acidification in the synaptic cleft. Consistent with a role for ASICs in physiological function, ASIC1a knockout mice showed a mild defect in spatial learning and fear conditioning (31, 32).

It has been known for a long time that extracellular acidification can induce action potentials (APs) in neurons; however, the acid sensors involved in this process have not been identified (7, 8, 21, 28). The aim of this study was to determine whether ASICs can mediate acid-induced generation and modulation of APs in brain neurons. For this purpose, we compared the pharmacological and biophysical properties of acid-induced AP generation determined under current clamp in cultured hippocampal neurons with those of the ASIC-like currents in these neurons characterized under voltage clamp and with the known properties of cloned ASICs. We show that acid-induced AP generation in hippocampal neurons is essentially due to activation of ASICs. We demonstrate for the first time a direct dependence of the probability of inducing APs on the density of functional ASICs at the cell surface and the pH to which the extracellular solution is changed. We show that ASIC activation can modulate AP generation and, depending on the conditions, can facilitate AP generation or inhibit AP bursts. ASICs are localized in close proximity to voltage-gated Na⁺ and K⁺ channels in the neuronal plasma membrane. Thus the functional properties and the localization of ASICs in hippocampal neurons suggest that these channels can sense local extracellular pH changes in hippocampus and transduce them into neuronal activity.

MATERIALS AND METHODS

Isolation and culture of mouse hippocampal neurons. Murine embryonic hippocampal neurons were obtained from timed pregnant mice at 17 days’ gestation. Mice were killed by cervical dislocation according to Swiss animal care guidelines. Embryos were removed, hippocampi were dissected, and individual cells were either immediately isolated or isolated after storage of the hippocampi in Hibernate medium (Invitrogen, Carlsbad, CA) at 4°C for 1 h to 3 days (5). Dissociation of individual cells was done mechanically by trituration with a Pasteur pipette with a fire-polished tip in culture medium (Neurobasal; GIBCO BRL, Grand Island, NY) supplemented with 2% B-27 (GIBCO BRL) as well as with glutamine and glutamate at final concentrations of 500 and 25 μM, respectively. The cell suspension was centrifuged for 5 min at 1,000 rpm. The supernatant was removed, and cells were resuspended in fresh culture medium. Cells were plated on polylysine-coated coverslips, usually at a density of 10,000 cells/coverslip of 11-mm diameter, and kept in 95% air-5% CO₂ at 37°C. The ASIC current density, measured as the ratio of the pH 6-induced peak inward current (IₚH6) to the membrane capaci-
tance, changed only slightly with time in culture and was 43 ± 14 pA/pF after 2–3 days in culture (n = 10) and 34 ± 4 pA/pF after 9–14 days in culture (n = 52). For the experiments shown, the neurons were used after 9–14 days in culture.

Recombinant expression of ASICs. To obtain clues about the molecular identity of ASICs that mediate the H⁺-induced currents in hippocampal neurons, we compared ASIC currents in hippocampal neurons with currents of cloned ASICs in recombinant expression systems. For the expression of heteromeric ASICs, we performed transient transfections in COS cells. We used a COS cell line that was selected for low endogenous expression of K⁺- and H⁺-gated channels. These cells were controlled for endogenous H⁺-gated currents, which were either absent or <150 pA. Equal concentrations of cDNA in the pcDNA3.1 vector (ASIC1a, ASIC2a) were cotransfected at a 10:1 ratio with either the CD8 antigen or green fluorescent protein (for identification of transfected cells) at 3 μg/35-mm dish with the use of PerFectin (Gene Therapy Systems, San Diego, CA) according to the instructions of the manufacturer. Cells were split 1 day after transfection and were studied on days 2 and 3 after transfection. Cells were cultured in DMEM with 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C.

Cell lines that stably expressed ASICs were established for homomultimeric assembly of ASIC1a and of ASIC2a. The cDNAs were subcloned into the pEAK8 expression vector (Edge Biosystems, Gaithersburg, MD). DNA (4 μg/35-mm dish) was used to transfect Chinese hamster ovary (CHO) cells, which have no endogenous transient H⁺-gated currents, with the use of Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. ASIC-expressing cells were isolated by selection in culture medium (DMEM/NutMix F-12, 3.6% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin) containing 10 μg/ml puromycin.

Electrophysiological measurements. We used an EPC-9 amplifier and Pulse and PulseFit software (HEKA Electronik, Lambrecht, Germany) for data acquisition and analysis. The sampling interval was 50–100 μs for current-clamp experiments, 1–5 ms for voltage-clamp experiments to measure ASIC currents, and 100 μs for voltage-gated currents, and filtering was set to 5 kHz in all experiments. Experiments were performed in the whole cell and the excised outside-out configuration of the patch-clamp technique (13). For rapid changes of extracellular solutions, we used either an array of nine tubes whose position in front of the cell or the excised patch could rapidly be changed (Rapid Solution Changer RSC-200; Biologic, Grenoble, France) or a micromanford that brings nine tubes into one outlet tube (Ala Scientific Instruments, Westbury, NY). The solution flow was controlled by computer-driven solenoid valves. For whole-cell measurements, the perfusion outlet was positioned close to the cell body. With excised outside-out patches, we used the RSC-200 device exclusively and placed the patch pipette containing the membrane patch in front of the tube array. Extracellular solutions contained (in mM) 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 MES, 10 HEPES, and 10 glucose, and pH was adjusted to 7.4 or to the values indicated. Pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL). When filled with the pipette solution, the pipettes used for whole cell measurements had a resistance of 2–4 MΩ and those used for excised outside-out patches had a resistance of 4–8 MΩ. The pipette solution for whole cell measurements contained (in mM) 90 K-glucuronate, 60 HEPES, 10 KCl, 1 NaCl, 1 MgCl₂, and 10 EGTA, pH 7.3. For outside-out patches, the pipette solution contained (in mM) 130 KF, 2 MgCl₂, 10 NMDG, 10 EGTA, 10 HEPES, and 5 HCl, pH 7.35. Hippocampal neurons had a resting potential of −45 ± 14 mV (n = 51). When APs were induced in current-clamp mode by short (3 ms) depolarizing current pulses from a holding potential of −75 mV in current-clamp mode, the AP threshold was −37 ± 7 mV (n = 46); the current injection needed for AP generation was 378 ± 302 pA, and the mean AP duration was 0.85 ± 0.20 ms (measured at 50% of AP amplitude, n = 46; all values shown are means ± SD). An effective holding potential of −75 mV in current-clamp experiments was chosen to reproduce the membrane potential in hippocampal neurons in vivo.

Psalmopoeus cambridgei venom was obtained from Spider Pharm (Yarnell, AZ) and was used in all experiments at a 1:20,000 dilution. The P. cambridgei venom inhibits ASIC1a currents because of the toxin Psalmoatoxin-1 contained in the venom (11). Preapplication of the venom at this dilution blocked 94 ± 1% (n = 5) of I<sub>AP</sub> in ASIC1a homomultimers expressed in CHO cells. Within the pH range 6–4, the current inhibition by the venom was not relieved by the application of more acidic stimuli (Vukicevic M and Kellenberger S, unpublished observations). Under the same experimental conditions, the P. cambridgei venom did not inhibit H⁺-induced current of all other ASICs tested (ASIC1b, ASIC2a, and ASIC3; Poiriot O and Kellenberger S, unpublished observations). The other chemicals were obtained from Sigma-Aldrich or Fluka. The pH activation curves were fit by using the Hill equation

\[ I = I_{\text{max}} \left[ 1 + \left( \frac{\text{pH}_{\text{Hill}}}{\text{pH}_0.5} \right)^n \right] \]

where \( I_{\text{max}} \) is the maximal current, \( \text{pH}_{0.5} \) is the pH at which one-half of the channels are opened, and \( n_{\text{Hill}} \) is the Hill coefficient. Data are presented as means ± SE when different mean values are compared; otherwise, as indicated, values are expressed as means ± SD; n represents the number of experiments.

RESULTS

Acidification-induced AP generation is mediated by ASICs. It is known that extracellular acidification can induce APs in neurons (7, 8, 21, 28). However, the activity of a number of electrogenic transporters depends on extracellular pH, and the contribution of ASICs to acid-induced AP generation has not been specified to date. To determine the role of ASICs in acid-induced AP generation, we investigated the pharmacological and biophysical aspects of acid-induced AP generation in murine hippocampal pyramidal neurons. A previous study showed that the dominant ASIC type in these neurons is the homomultimeric ASIC1a and that, in addition, heteromultimeric channels exist that are composed of ASIC1a together with ASIC2a and/or ASIC2b (2). In neurons of intermediate size, we measured an average whole-cell capacitance of 32 ± 96 pF (mean ± SD; n = 93). Neurons were studied under current clamp, and current injection was adapted to obtain a membrane potential of −75 mV in the absence of stimuli. Extracellular acidification to pH 6 induced a transient depolarization of the membrane potential and generated APs, as illustrated in Fig. 1A (solid trace). After a short delay due to the perfusion system, the membrane potential was depolarized and APs were generated ~200 ms after the onset of depolarization. The membrane potential then returned to the baseline, while the extracellular pH was still 6. In the same neuron under voltage clamp, extracellular acidification to pH 6 induced a transient inward current (Fig. 1B), with kinetics typical of ASIC currents (30). In neurons, no transient H⁺-gated Na⁺ currents other than ASIC currents are known. Further indications that the transient acid-induced currents in hippocampal neurons are indeed ASIC currents are the observations that they display the same pharmacological properties as, and pH dependence similar to that of, cloned ASICs (see Figs. 1C and 7). In addition to transient currents, extracellular acidification may induce sustained currents due to effects on ion channels other than ASICs that are potentially present in hippocampal neurons. Activation of the capsaicin receptor TRPV1 or inhibition of the background K⁺ channels TASK-1 or TASK-3...
ASIC-MEDIATED ACTION POTENTIALS IN NEURONS

C684

A

0 mV

pH 6

+ amiloride

20 mV

2 s

B

pH 6

+ amiloride

100 μA

2 s

C

amiloride (μM)

relative I\text{Na}

0.1

1

10

1000

depolarization ΔV\text{m}/m

0

50

100

D

amiloride

**

Fig. 1. Acidification-induced action potential (AP) generation is mediated by acid-sensing ion channels (ASICs). APs were induced in whole cell current clamp. The current injection was adjusted to obtain a membrane potential of −75 mV at pH 7.4. A: representative experiment showing the generation of an AP train by a pH change to 6.0 (solid trace) and the absence of APs when the experiment was performed in the presence of 100 μM amiloride (shaded trace). B: current trace obtained under voltage clamp to −60 mV from the same cell in the absence (solid trace) and presence (shaded trace) of 100 μM amiloride. The durations of the depolarization measured in current clamp had kinetics roughly similar to those of the pH 6-induced inward current under voltage clamp. An exponential fit to the data points (n = 5) and yields a concentration for half-maximal block of 5.1 ± 0.3 μM. D: pH 6-induced depolarization in current clamp in the absence or presence of 100 μM amiloride, shown as the change in membrane potential (ΔV\text{m}). **P < 0.01, Student’s paired t-test; n = 12.

could induce a sustained inward current (6, 25). However, as illustrated in Fig. 1B, the main current response to acidification was transient. The transient current was only in some cells followed by a sustained current the amplitude of which in all experiments was <10% of the transient peak current (data not shown). Comparison of the traces in Fig. 1, A and B, shows that the pH 6-induced depolarization in current clamp had kinetics roughly similar to those of the pH 6-induced inward current under voltage clamp. An exponential fit to the data points of the pH 6-induced depolarization or the inward current in such experiments yielded similar time constants (τ\text{m}) of 145 ± 32 ms for depolarization under current clamp and 176 ± 27 ms (n = 6) for the inward current measured in voltage clamp. The durations of the depolarization measured in current clamp and of the inward current measured under voltage clamp, at 20% of the peak amplitude, also were similar: 4.0 ± 0.3 s (depolarization) and 3.4 ± 0.3 s (inward current) (n = 6). This close correlation of the time course suggests that the pH 6-induced depolarization was due to ASIC activation.

To further test the role of ASICs for acid-induced AP generation, we inhibited ASICs with the use of 100 μM amiloride, a known blocker of ENaC/degenerin channels. At this concentration, amiloride inhibits 93 ± 3% of the acid-induced current in hippocampal neurons, as illustrated in Fig. 1, B and C. The shaded trace in Fig. 1A shows the response of the same neuron under current clamp to acidification to pH 6 in the presence of 100 μM amiloride. This illustrates that amiloride strongly inhibited the depolarization of the membrane potential and prevented AP generation. In the presence of 100 μM amiloride, the pH 6-induced depolarization (ΔV\text{m}, Fig. 1D) was 15 ± 3 mV, while it was 48 ± 3 mV (measured between APs) in the absence of amiloride. In 10 of 12 experiments, H\text{+}-induced AP generation was prevented by coapplication of 100 μM amiloride. The failure of amiloride to prevent AP generation in two experiments is likely due to the high expression of functional ASICs in these two neurons, allowing AP generation caused by activation of the fraction of ASICs not blocked by amiloride, although implication of other H\text{+}-sensitive electrogenic transporters in these two experiments cannot be completely excluded.

Probability of inducing APs by acidification correlates with amplitude of ASIC inward current. We tested whether there is a correlation between the density of functional ASICs at the plasma membrane and the probability of inducing APs. As a measure of the density of functional ASICs at the plasma membrane, we determined the I\text{pH6} density, which we defined as the ratio of the I\text{pH6} under voltage clamp to cell capacitance. To establish this correlation, we took advantage of the fact that the I\text{pH6} density showed great cell-to-cell variation (34 ± 31 pA/pF, mean ± SD; n = 52). To calculate the probability of AP induction, we determined in each experiment whether acidification to pH 6 under current clamp induced APs (one or more). Experiments were then grouped according to their I\text{pH6} density, and for each group, the probability of AP induction was calculated as the frequency of experiments with successful pH 6-induced AP generation. In Fig. 2A, we plot the probability of AP induction against the I\text{pH6} density. This graph shows that the probability of AP induction increased with higher I\text{pH6} density and that it was maximal at I\text{pH6} densities >40 pA/pF. For comparison, in Fig. 2A, we also plotted the pH 6-induced depolarization ΔV\text{m}, which illustrates that the capacity of the extracellular acidification to induce APs strictly correlates with its capacity to induce depolarization.

We then tested, in the pH range 7–6, whether the probability of successful AP generation depends on the pH to which the extracellular solution is acidified. We were especially interested in the pH values close to 7 that are sufficient for ASIC activation (threshold for ASIC activation pH ~6.9; see Fig. 7A) and that may be reached during neuronal activity. To reduce variation due to ASIC current density, we considered for this analysis only neurons with a pH 6 density >25 pA/pF. Acidification from pH 7.4 to 7.0 never induced APs under our experimental conditions (n = 7) (Fig. 2B). However, pH changes to 6.8 induced APs in 50 ± 22% of the neurons tested, and the probability of AP induction further increased with more acidic pH and was maximal at pH 6.4 and 6 (Fig. 2B). This shows that submaximal activation of ASICs in many hippocampal neurons is sufficient for AP induction. The comparison with the dependence of the depolarization ΔV\text{m} on pH (Fig. 2B) shows that the increased probability of inducing APs at more acidic pH in the range of 6–7 is due to the increased depolarization.

pH dependence of AP train duration. In most experiments, ASIC activation induced bursts (“trains”) of APs. In the pH range 6.0–6.8, AP trains were of longer duration at less acidic...
Acidiﬁcation to pH 6 or 6.4 induced strong depolarizations ($V_m$ of 54 ± 2 and 47 ± 2 mV, respectively) (Table 1) that lasted several seconds. AP trains, however, were short despite continued depolarization. The absence of APs during this plateau phase likely reﬂects the accumulation of voltage-gated Na$^+$ channels in the inactivated state, in which they remain trapped until the membrane is repolarized. A similar phenomenon was recently observed in hippocampal neurons in which AP trains were induced by activation of heterologously expressed TRPV1 channels (34). Thus, for pH changes from 7.4 to the 7–6 range, the duration of induced AP trains is inversely correlated with the extent of acidiﬁcation, while the probability of AP induction correlates with the extent of acidiﬁcation (Fig. 2B).

Modulation of AP generation by ASIC activation. So far, our analysis has shown that activation of ASICs in a resting neuron can induce APs. The analysis shows also that the duration of AP bursts decreases with more acidic pH, thus suggesting a potential inhibitory effect of ASICs. To test for an inhibitory action of ASICs, we induced long-lasting bursts of APs by injecting a constant depolarizing current for several seconds. We then repeated the same current injection and, just after the beginning of the AP burst, simultaneously activated the ASICs by extracellular acidiﬁcation. This ASIC activation depolarized the membrane further and terminated the AP burst. In some experiments, APs reappeared when the transient acid-induced

### Table 1. Parameters of trains of action potentials induced by acidiﬁcation

<table>
<thead>
<tr>
<th>pH</th>
<th>Duration of AP trains, ms</th>
<th>Depolarization $\Delta V_m$, mV</th>
<th>AP threshold, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.8</td>
<td>1,015 ± 257</td>
<td>27 ± 3</td>
<td>-43 ± 2</td>
</tr>
<tr>
<td>6.6</td>
<td>643 ± 148</td>
<td>43 ± 2</td>
<td>-42 ± 1</td>
</tr>
<tr>
<td>6.4</td>
<td>319 ± 108</td>
<td>47 ± 2</td>
<td>-42 ± 1</td>
</tr>
<tr>
<td>6.0</td>
<td>131 ± 24</td>
<td>54 ± 2</td>
<td>-42 ± 1</td>
</tr>
</tbody>
</table>

Values are means ± SE. The pH was changed from 7.4 to the pH indicated. Duration of action potential (AP) trains and depolarization (determined between action potentials as maximal change in membrane potential, $\Delta V_m$) were significantly different between different pH conditions ($P < 0.05$, ANOVA; $n = 4–13$). The fact that the AP threshold did not depend on pH indicates that in this pH range, there were no direct pH effects on voltage-gated Na$^+$ channels.

pH, as shown in Fig. 3 and Table 1. Acidiﬁcation to pH 6 or 6.4 induced strong depolarizations ($\Delta V_m$ of 54 ± 2 and 47 ± 2 mV, respectively) (Table 1) that lasted several seconds. AP trains, however, were short despite continued depolarization. The absence of APs during this plateau phase likely reﬂects the accumulation of voltage-gated Na$^+$ channels in the inactivated state, in which they remain trapped until the membrane is repolarized. A similar phenomenon was recently observed in hippocampal neurons in which AP trains were induced by activation of heterologously expressed TRPV1 channels (34). Thus, for pH changes from 7.4 to the 7–6 range, the duration of induced AP trains is inversely correlated with the extent of acidiﬁcation, while the probability of AP induction correlates with the extent of acidiﬁcation (Fig. 2B).

Fig. 2. Probability of acid-induced AP induction depends on ASIC expression and pH. A: graph relating the ASIC current density [peak current induced by acidiﬁcation to pH 6 ($I_{pH6}$) measured under voltage clamp, normalized to the capacitance of the cell] to the induced depolarization (expressed as $\Delta V_m$, plotted as $\Delta V_m$ and to the probability of AP induction by acidiﬁcation to pH 6 (plotted as )), calculated as frequency of occurrence of neurons that responded with APs to extracellular acidiﬁcation to pH 6 in current clamp; see text). The $I_{pH6}$ density for each neuron was determined in parallel in voltage clamp to ~60 mV, and neurons were classiﬁed for this analysis according to their $I_{pH6}$ density. B: pH dependence of acidiﬁcation-induced depolarization and probability of AP induction (measured as described for A). Neurons with $I_{pH6}$ density >25 pA/pF were included in the analysis in B; $n = 5–9$ per condition.

Fig. 3. Dependence of AP train duration on pH. AP trains were induced by different pH changes. Traces were obtained in whole cell current clamp, and the pH was changed from 7.4 to the value indicated under each trace. The current injection was adjusted to obtain a membrane potential of ~75 mV at pH 7.4.
depolarization was over. Figure 4 shows traces from a representative experiment. The extracellular acidification increased the depolarization and reduced the number of APs in the burst, as shown in Table 2. The kinetics of the acid-induced depolarization resemble closely those of the ASIC-mediated depolarization shown in Figs. 1 and 3, indicating that this inhibition is due to ASIC activation. Because short bursts rather than single APs are the predominant information carriers in the central nervous system, the premature termination of the bursts is expected to have an inhibitory effect on neuronal signaling.

A modulatory excitatory role of ASICs may also be possible. In neurons with weak excitatory input, ASIC activation may add to other incoming subthreshold stimuli to facilitate AP induction. We found that in a substantial fraction of neurons that had relatively low $I_{\text{pH6}}$ density, acidification to pH values > 6 was not sufficient to induce APs. To test whether facilitation can occur in such situations, we combined two activating stimuli in hippocampal neurons that displayed a comparably low $I_{\text{pH6}}$ density: 1) a short (3 ms) depolarizing current injection through the patch pipette and 2) activation of ASICs by extracellular acidification. First, a current-clamp protocol with current injections of increasing amplitude was performed to determine the current injection required for AP generation. In a second protocol, a series of acidic pH solutions were tested for their capacity to induce APs. The combined stimulation was then achieved by switching the extracellular solution to a pH that was not sufficient in this particular neuron to generate APs. A depolarizing current was injected simultaneously with the peak of the acid-induced depolarization, and the current injection needed for AP generation was determined under this condition. Figure 5A illustrates an experiment in which the combination of subthreshold current injection and subthreshold acidification induce an AP. The results of such experiments are summarized in Fig. 5B. They show that the current injection needed for AP generation is decreased if ASICs are activated at the time of current injection, indicating that ASIC activation can have a modulatory excitatory role in AP generation.

### Table 2. Inhibition of AP burst activity by simultaneous ASIC activation

<table>
<thead>
<tr>
<th>pH</th>
<th>Maximal depolarization $\Delta V_{\text{mem}}$, mV</th>
<th>No. of APs/5 s</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>34 ± 2</td>
<td>88 ± 16</td>
</tr>
<tr>
<td>6.8</td>
<td>47 ± 2</td>
<td>40 ± 21</td>
</tr>
<tr>
<td>6.4</td>
<td>57 ± 2</td>
<td>38 ± 9</td>
</tr>
<tr>
<td>6.0</td>
<td>64 ± 3</td>
<td>18 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE. AP bursting activity was induced by injection of a depolarizing current for 10 s. Shortly after the start of this depolarization, the extracellular solution was changed for 7 s from pH 7.4 to the pH values indicated. The maximal depolarization, determined between APs, and the number of APs during the first 5 s of the current injection were significantly different between pH conditions ($P < 0.05$, ANOVA; $n = 8 – 13$). In this series of experiments, the current injection required to induce AP bursts was 42 ± 10 pA and acid-sensing ion channel (ASIC) current ($I_{\text{pH6}}$) was 1.104 ± 267 pA ($n = 13$).
resistant current contributed 42 ± 10% (n = 8) of the total $I_{\text{AP}}$. These results confirm two previous analyses of ASIC currents in rat hippocampal neurons (1, 2).

The pH dependence of the venom-resistant ASIC current is shown in Fig. 7B. This current had a pH½ of 5.9 ± 0.1 and a Hill coefficient of 1.9 ± 0.3 (n = 7). For comparison, in parallel to the experiments in hippocampal neurons, we determined the pH dependence of peak currents of cloned ASICs that were expressed in COS or CHO cells (Fig. 7B). The comparison indicates that the pH dependence of the venom-resistant H⁺-induced current is similar but not equal to that of ASIC1a heteromers. Therefore, in agreement with the study by Baron et al. (2), the venom-resistant component is most likely mediated by one type or several types of heteromeric ASICs composed of ASIC1a together with ASIC2a and/or ASIC2b. This finding is also consistent with the absence of ASIC currents in hippocampal neurons of ASIC1a knockout mice (32). The functional importance of the ASIC1a homomeric current (>95% of $I_{\text{AP}}$ in 60% of neurons, 42% of $I_{\text{AP}}$ in the remaining 40% of neurons) is illustrated by our observation that the presence of the P. cambridgei venom prevented AP generation in the pH range 5.5–6.8 (n = 8).

**DISCUSSION**

By comparing the pharmacological and biophysical properties of acid-induced AP generation with those of ASIC currents in hippocampal neurons, we show in this study that acid-induced AP generation is essentially due to ASIC activation. We demonstrate for the first time that the probability of inducing APs by extracellular acidification correlates with current entry through ASICs. It increases with increasing density of functional ASICs at the plasma membrane and with the acidity of the test solution in the pH range of 7–6, which

1.4 ± 0.2, indicating cooperative gating (mean of 3 preparations and 3–8 measurements each). The P. cambridgei venom, which selectively blocks currents mediated by ASIC1a homomultimers (11) (see MATERIALS AND METHODS), inhibited ≥95% of the $I_{\text{AP}}$ in 60% of the tested neurons ($n_{\text{total}} = 20$), indicating that in these neurons, the ASIC-like current was essentially due to activation of ASIC1a homomultimers. In the remaining 40% of neurons, the H⁺-induced current was only partially blocked by the P. cambridgei venom, suggesting the presence of one or several additional ASIC types. In these neurons, the venom-

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**Fig. 5.** Subthreshold ASIC activation can facilitate AP generation by other stimuli. The minimal current injection required for AP generation was determined first at pH 7.4 and then when the current injection was applied simultaneously to the peak of the ASIC-mediated depolarization due to extracellular acidification. The extracellular pH was changed to a pH that by itself did not induce APs in the neuron tested. A: trace showing that the threshold for AP generation can be reached by the combination (bottom) of a subthreshold current injection (top left) and a subthreshold extracellular acidification (top right). Note the different time scales. The current injection was adjusted to obtain a membrane potential of −75 mV in the absence of stimulation. B: summary of the results of the experiments, plotting the minimal current injection required for AP generation (shown as the difference of the current injected for AP generation to the current injected to maintain membrane potential of −75 mV) under different conditions, either at pH 7.4 or when the current injection coincided with ASIC activation by pH change to 6.4 or 6.8. Acidification decreased the minimal current injection required for AP generation. **P < 0.01, Student’s paired t-test; n = 4. For each experiment, it was verified that this pH change alone did not induce APs.

**Fig. 6.** Simultaneous presence of ASICs and voltage-gated Na⁺ and K⁺ channels in a single excised outside-out patch. A: current induced by extracellular acidification to pH 5.8: current carried by voltage-gated Na⁺ and K⁺ channels in extracellular Na⁺ and intracellular K⁺ solution. The Na⁺ current is visible as the transient downward deflection. B: K⁺ currents in extracellular and intracellular K⁺ solution. Holding potential was −80 mV for ASIC currents and −120 mV for voltage-gated currents. The voltage-gated currents were elicited by step depolarizations to test potentials of −60 to +70 mV in 10-mV increments. Transients and leak were subtracted with the use of the P/4 procedure.
be reached locally by the pH fluctuations due to normal neuronal activity (7, 8), suggesting a role of ASICs in neuronal signaling.

**AP induction and modulation by extracellular acidification.** ASICs are not the only neuronal ion channels whose function depends on extracellular pH. Extracellular acidification, for example, has been shown to inhibit N-methyl-D-aspartate channels, voltage-gated Na$^+$ channels, and the two-pore domain TASK channels; to activate the capsaicin receptor TRPV1 and the chloride channel CLC-2; and to be a coactivator for the inhibitory GABA$_A$ receptors (8, 14, 15, 17, 25). Thus extracellular acidification might depolarize the membrane by inhibiting the background K$^+$ channels TASK-1 or TASK-3 or by activating TRPV1. ASIC currents are transient, in contrast to the TASK and TRPV1 currents, which do not inactivate. Extracellular acidification induces predominantly a transient inward current in hippocampal neurons (>90% of total pH 6-induced current). These transient, acid-induced currents display pH dependence similar to that of cloned ASICs and are blocked by the ENaC/degenerin inhibitor amiloride, which clearly identifies them as ASIC-mediated currents. Two recent studies that either used a pharmacological approach (2) or took advantage of ASIC1 and ASIC2 knockout mice (1) indicated that ASIC1a homomultimers and heteromers composed of ASIC1a together with ASIC2a and/or ASIC2b mediate ASIC currents in hippocampal neurons. We confirmed these observations and found a higher prevalence of ASIC1a homomultimers than of the heteromers. The presence of functional ASICs in cultured hippocampal neurons is consisent with observations of ASIC1a, ASIC2a, and ASIC2b mRNA in rodent hippocampus and of ASIC-like currents in acutely isolated hippocampal neurons (4, 12, 22, 29).

It previously was shown that extracellular acidification induces APs in hippocampal neurons, and recently it was shown that the induced depolarization correlates with acidification and ASIC current density, suggesting a role for ASICs in acid-induced AP generation (2). The aim of this study was to provide direct evidence for the implication of ASICs in acid-induced AP generation, to show how acidification induces APs by activating ASICs despite a potential inhibition of voltage-gated Na$^+$ channels (14), and to investigate potential modulatory roles of ASICs in AP generation. The following observations in our analysis indicate that acid-induced AP generation in hippocampal neurons is essentially mediated by ASICs.

1) The kinetics of acid-induced depolarization are similar to the kinetics of ASIC currents measured under voltage clamp. 2) Acid-induced membrane depolarization and AP generation are inhibited by amiloride. 3) The probability of successful acid-induced AP generation depends on Na$^+$ entry through ASICs, because it is higher in neurons with an increased density of functional ASICs at the membrane and increases with the extent of acidification in the pH range in which ASIC activity is steeply pH dependent. The observations that the probability of inducing APs by acidification increased with more acidic pH in the range 7.0–6.0 and that the voltage-threshold for AP generation did not depend on pH indicate that in this pH range, the pH-dependent inhibition of voltage-gated Na$^+$ channels did not affect AP generation. Because homomultimeric ASIC1a is activated at higher pH than the other ASIC types in these neurons ($pH_{0.5}$ = 6.4 (ASIC1a) compared with 5.9 (venom-resistant, acid-induced currents in hippocampal neurons in presence of venom, $n = 7$), 7.4 ± 0.1 nA (ASIC1a, $n = 9$), 9.1 ± 2.1 nA (ASIC1a2a, $n = 6$), and 11.0 ± 2.6 nA (ASIC2a, $n = 5$).

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**Fig. 7. Properties of H$^+$-gated currents in murine hippocampal neurons.** Measurements were obtained in whole cell voltage clamp to −60 mV. A: pH dependence of ASIC activation in hippocampal neurons from a representative example obtained from one preparation of hippocampal neurons ($n = 8$ measurements). Line represents a fit of the data to the Hill equation (see MATERIALS AND METHODS) and yields a pH for half-maximal current (pH$_{0.5}$) of 6.1 ± 0.1 and a Hill coefficient of 1.8 ± 0.5. The absolute peak current amplitude at pH 5.0 was 1.1 ± 0.3 nA. B: comparison of the pH dependence of activation of *Psalmodoeus cambridgei* venom-resistant ASIC current in hippocampal neurons with that of cloned ASICs expressed in CHO or COS cells. Activation curves shown are of the venom-resistant current in hippocampal neurons in the presence of 1:20,000 dilution venom (○) and of the following recombinant ASICs: ASIC1a (○), ASIC2a (○), and ASIC1a2a (○). The lines represent fits to the Hill equation (see MATERIALS AND METHODS) and yield the following respective values for pH$_{0.5}$ and Hill coefficients: 5.9 ± 0.1 and 1.9 ± 0.3 (hippocampal neurons in presence of venom), 6.4 ± 0.0 and 3.4 ± 0.7 (ASIC1a), 5.6 ± 0.1 and 3.3 ± 1.4 (ASIC1a2a), and 4.7 ± 0.1 and 1.3 ± 0.5 (ASIC2a) ($n = 3$–10). The peak currents were normalized for comparison. The absolute maximal current amplitudes were 1.1 ± 0.7 nA (hippocampal neurons in presence of venom, $n = 7$), 7.4 ± 0.1 nA (ASIC1a, $n = 9$), 9.1 ± 2.1 nA (ASIC1a2a, $n = 6$), and 11.0 ± 2.6 nA (ASIC2a, $n = 5$).
pal neurons), it probably mediates AP generation induced by pH changes to pH ≥6.5. The important role of ASIC1a homomultimers is further supported by our observation that the presence of the P. cambridgei venom prevented acid-induced AP generation.

We show that ASIC activation induces APs but that the duration of the induced AP bursts decreases with stronger depolarization. Consistent with these observations, ASIC activation facilitates AP generation when combined with subthreshold excitatory stimuli, and if it occurs during bursting activity of a neuron, the ASIC activation can terminate the burst. The mechanism of this inhibitory effect of ASIC activity on neuronal signaling is analogous to that of depolarizing blocking agents at the neuromuscular junction (e.g., suxamethonium) that cause a maintained depolarization. Thus ASICs are modulators that, depending on the conditions present, are excitatory or inhibitory. The type of electrical response of a neuron to acidification thus depends on the extent of the pH change, on the expression level of ASICs present in the neuron, and on the momentary signaling activity of the neuron. ASIC expression is likely to change in response to the (patho)physiological situation. For example, upregulation of ASIC2a expression in brain after global ischemia and downregulation of ASIC1a and ASIC2b after status epilepticus were recently demonstrated (3, 16).

Localization of pH changes and ASICs. Brain ischemia, hypoxia, and epilepsy are accompanied by acidosis (7, 23). On the basis of the current understanding of ASIC function, during long-lasting acidification such as that induced by brain ischemia or hypoxia, ASICs are expected to be briefly activated and then to inactivate and remain inactive. During global brain ischemia, extracellular pH decreases by ~1 pH unit (reviewed in Ref. 23). Under these conditions, ASIC1a homomultimers and ASIC1a-containing heteromers are inactivated. During seizure activity, rapid extracellular acidification by 0.2–0.5 pH unit has been observed (19, 27, 33). In these studies, pH was measured using pH-sensitive microelectrodes. Because of limitations in spatiotemporal resolution, the actual pH changes may have been underestimated with the use of this approach. Thus ASICs may be activated during seizure activity and are expected to be inhibitory under these conditions. Fluctuations of extracellular pH also occur during normal brain function. Several studies with brain slices have indicated that neuronal activity causes rapid changes in extracellular pH (7, 8, 21). Much interest has been focused on the pH changes in the synaptic cleft during synaptic activity. Direct information regarding the exact pH changes in extracellular microdomains such as the synaptic cleft is currently not available (7). However, the pH in hippocampal synaptic vesicles has been determined to be pH 5.7 (24), and extracellular acidification due to the release of presynaptic vesicular contents has been detected indirectly (10). In addition to synaptic pH changes, there is evidence for glial acid secretion in response to neural activity. This acid secretion is expected to induce pH changes with a time course on the order of seconds (7); thus these pH changes would also be fast enough to activate ASICs. Currently, it is not clear whether the extent of acidification by glial acid secretion would be sufficient to activate ASICs. The pH changes reported during seizure activity were relatively small but might have been underestimated because of the limited spatiotemporal resolution of pH microelectrodes that were used in these studies (8).

For a better appreciation of the role of ASICs with regard to their potential involvement in synaptic functions, it is important to know their subcellular localization with respect to synapses. The subcellular localization in brain neurons has thus far been addressed only for the ASIC1a subunit. Studies conducted at two different laboratories produced somewhat divergent results. Wemmie and colleagues (31, 32) showed evidence for preferential localization of ASIC1a at synapses and for involvement in synaptic functions. de la Rosa et al. (9), however, reported that the ASIC1a protein is equally distributed in plasma membrane of soma, axons, and dendrites of hippocampal and other brain neurons.

While functional roles of synaptic ASICs can be imagined and were proposed in recent studies (31, 32), the potential roles of extrasynaptic ASICs are less clear. Extrasynaptic ASICs might be activated by glial cell-dependent acid secretion or during seizure activity, or they may play as yet undefined roles in signaling during ischemia. To elucidate the role of the venom-resistant ASIC current in hippocampus, it is important to determine in addition the subcellular and synaptic vs. extrasynaptic localization of ASIC2a and ASIC2b.

In conclusion, we have shown in this study that acid-induced AP generation in hippocampal neurons is due to the activation of ASICs. Our study suggests that ASICs in hippocampus are likely to change the electrical properties of neurons in response to even small pH changes. The effect on hippocampal neuron excitability due to ASIC activation depends on the extent of the pH change, on the expression level of ASICs present in the neuron, and on the activity of the neuron at the moment of ASIC activation.

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