Alternative Splicing and Interaction with Di- and Polyvalent Cations Control the Dynamic Range of Acid-sensing Ion Channel 1 (ASIC1)*

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Homomeric acid-sensing ion channel 1 (ASIC1) can be activated by extracellular H⁺ in the physiological pH range and may, therefore, contribute to neurotransmission and peripheral pain perception. ASIC1a and ASIC1b are alternative splice products of the ASIC1 gene. Here we show that both splice variants show steady-state inactivation when exposed to slightly decreased pH, limiting their operational range. Compared with ASIC1a, steady-state inactivation and pH activation of ASIC1b are shifted to more acidic values by 0.25 and 0.7 pH units, respectively, extending the dynamic range of ASIC1. Shifts of inactivation and activation are intimately linked; only two amino acids in the ectodomain, which are exchanged by alternative splicing, control both properties. Moreover, we show that extracellular, divalent cations like Ca²⁺ and Mg²⁺ as well as the polyvalent cation spermine shift the steady-state inactivation of ASIC1a and ASIC1b to more acidic values. This leads to a potentiation of the channel response and is due to a stabilization of the resting state. Our results indicate that ASIC1b is an effective sensor of transient H⁺ signals during slight acidosis and that, in addition to alternative splicing, interaction with di- and polyvalent cations extends the dynamic range of ASIC H⁺ sensors.

H⁺-gated Na⁺ channels (acid-sensing ion channels; ASICs) belong to the Deg/ENaC super family of ion channels (1). Members of this super gene family form Na⁺-selective ion channels (PNa/PK, 8–100) that can be blocked by amiloride (IC₅₀, 0.2–20 μM). All family members show some common hallmarks including two hydrophobic domains, short intracellular N and C termini, and a large extracellular loop containing conserved cysteines. Channels of this gene family probably form tetramers (2, 3).

To date, six different members of the ASIC subfamily have been cloned (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4), which are encoded by four genes. ASIC1a and ASIC1b are alternative splice products of the ASIC1 gene (4, 5). Splicing exchanges approximately the first third of the protein, including the first transmembrane domain and the proximal part of the large ectodomain. In contrast, the C-terminal two-thirds are identical. We will use the term ASIC1 when we do not refer to a specific splice variant. All ASICs with the exception of ASIC4 (6) are expressed in sensory neurons of the dorsal root ganglion. Proposed functions in sensory neurons include peripheral pain perception (1, 7, 8) and mechanotransduction (9, 10). Although some evidence suggests that some of the native H⁺-gated currents in sensory neurons are mediated by heteromeric ASICs (11, 12), part of these currents are probably mediated by homomeric ASIC1 and ASIC3 (8, 11).

ASIC1a is expressed in sensory neurons and throughout the brain (13), whereas ASIC1b is specifically expressed in sensory neurons (5). Both subunits are Na⁺-selective (PNa/PK ≈ 10–15), and only ASIC1a has a low Ca²⁺ permeability (PNa/PCa ≈ 15) (4). ASIC1a and ASIC1b form rapidly activating and completely desensitizing ion channels (tᵣᵢṣ, ~100 ms; tᵦᵣᵢ)__desens__, ~1 s) (4). The expression of ASIC1 in small diameter, capsaicin-sensitive sensory neurons (5, 11, 14, 15) has led to the proposal that ASIC1 mediates excitation during tissue acidosis, which accompanies inflammation and ischemia. However, complete desensitization of ASIC1 makes it difficult to imagine how this channel can sense H⁺ signals during inflammation and ischemia when the pH persistently falls.

Here we show that both ASIC1a and ASIC1b undergo steady-state inactivation. The steady-state inactivation curve for ASIC1b is shifted by 0.25 pH units to more acidic values as compared with ASIC1a, showing that ASIC1b can operate at a more acidic resting pH. pH activation is shifted by 0.7 pH units. Differences in the sensitivity of activation and inactivation by protons are intimately linked, as both are controlled by only two amino acids in the ectodomain. These two amino acids are exchanged by alternative splicing. Moreover, we show that Ca²⁺, Mg²⁺, and spermine, when applied during the steady-state, shift the steady-state inactivation curves of both ASIC1a and ASIC1b. This leads to a potentiation of the current. Modulation by di- and polyvalent cations may be a means to adapt ASIC1 activity to changes in the extracellular concentration of these ions. Our results show that ASIC1b can replace ASIC1a as a sensor of transient proton signals during slight acidosis and that di- and polyvalent cations are modulators of ASIC activity.

EXPERIMENTAL PROCEDURES
cDNAs and Site-directed Mutagenesis—cDNAs for ASIC1a and ASIC1b have been described previously (4). Chimeric molecules between ASIC1a and ASIC1b and point mutations were constructed by recombinant PCR using Pwo DNA polymerase (Roche Molecular Biochemicals). N-terminal sequences of ASIC1b were from ASIC1b-M3 (4). All PCR-derived fragments were entirely sequenced.

Electrophysiology—Using mMessage mMACHINE (Ambion, Austin, Texas), capped cRNA was synthesized by SP6 RNA polymerase from ASIC1a, ASIC1b, and chimeric cDNA, which had been linearized by
Sapl. We injected 10 ng of ASIC1b wild type cRNA and 0.01–0.1 ng of the other cRNAs; currents ranged from 0.5–50 µA. cRNA was injected into stage V and VI oocytes of Xenopus laevis, and oocytes were kept in OR-2 medium (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM NaHPO4, 5.0 mM HEPES, 1.0 mM MgCl2, 1.0 mM CaCl2, and 0.5 g/liter polyvinylpyrrolidone) for one to 7 days.

The bath solution for the two-electrode voltage clamp contained 140 mM NaCl and 10 mM HEPES; concentration of divalent cations was 1.8 mM CaCl2 and 1.0 mM MgCl2 if not otherwise indicated. Where appropriate, HEPES was replaced by MES buffer. Holding potential was −60 or −70 mV. Solutions with low concentrations of divalent cations contained 0.1 mM flufenamic acid or niflumic acid to block the large conductance induced in oocytes by divalent-free extracellular solutions.

For steady-state inactivation curves and Ca2+ dose-response curves, solution exchange was gravity driven. Whole cell currents were recorded with a TurboTec OTC1 amplifier (NPI Electronic Instruments, Tamm, Germany), and data were stored on hard disk. For H+ dose-response curves and recovery measurements, we used an automated, pump-driven solution exchange system together with the oocyte testing carousel controlled by the interface OTC-20 (NPI Electronic Instruments).

Complete solution exchange at whole oocytes with this system can be obtained within less than 1 s (16). Currents were recorded and filtered at 20 Hz with a TurboTec OX3 amplifier (NPI Electronic Instruments) digitized at 100 Hz using the AD/DA interface PCI 1200 (National Instruments, Austin, Texas) and stored on hard disk. Data acquisition and solution exchange were managed using the CellWorks 5.1.1 software (NPI Electronic Instruments). Amiloride, collagenase, flufenamic acid, and niflumic acid were purchased from Sigma.

Data Analysis—Data was analyzed using IgorPro software (WaveMetrics, Lake Oswego, OR). Steady-state inactivation, dose-response curves for potentiation by divalent cations, and activation by H+ were fitted using the following equation,

\[ I = I_{\text{max}} - aI(1 + C_50/[B])^n \]

where \( I_{\text{max}} \) is the maximal current, \( a \) is the residual current, \([B]\) is the concentration of H+ or of the divalent cation, \( C_50 \) is the concentration at which half-maximal response occurs, and \( n \) is the Hill coefficient. Time constants characterizing recovery from desensitization and steady-state inactivation were determined using a monoexponential fit. Statistical analysis was done with Student's t test.

All values reported represent the mean ± S.D. from \( n \) individual measurements. Currents from individual measurements were either normalized to the \( I_{\text{max}} \) obtained from a fit to the logistic function (1) or to the maximal value obtained (pH activation curves, recovery from desensitization). The mean of these normalized data from different measurements was then used to do the fit reported in the figures. Error bars on graphs indicate the standard error (except Fig. 2).

RESULTS

Splice Variants ASIC1a and ASIC1b Have Different Dynamic Ranges—Using Xenopus oocytes, we first investigated the activation of ASIC1a and ASIC1b by protons. As shown in Fig. 1A, application of an acidic solution to ASIC1 expressing oocytes elicited transient, fast activating, and completely desensitizing inward currents. Current amplitude of ASIC1a and ASIC1b increased over a pH range of 1 unit, corresponding to a 10-fold increase in agonist concentration (Fig. 1). Thus, the sensitivity of ASIC1 to its agonist is comparable with that of other ligand-gated ion channels. ASIC1b has a lower apparent affinity for H+ (EC50, pH 5.84 ± 0.09, \( n = 8 \)) than ASIC1a (EC50, pH 6.56 ± 0.06, \( n = 11 \)). Both values for pH of half-maximal activation are in good agreement with values obtained from measurements with smaller cells like COS-7 (5, 8) showing that activation curves can be confidently determined with whole oocytes. The Hill coefficient was slightly higher for ASIC1a compared with ASIC1b (3.24 ± 0.44 and 2.52 ± 0.47, respectively). A Hill coefficient bigger than 1 suggests cooperative binding of H+ to more than one subunit. As can be seen in Fig. 1B, the shift in the activation curve for ASIC1b leads to an efficient activation approximately at the pH at which the activation of ASIC1a starts to saturate.

We then analyzed the steady-state inactivation of ASIC1a and ASIC1b. In this study, the term inactivation rather than desensitization will be used whenever there is no macroscopic activation observable. Oocytes were superfused for 2 min in an extracellular solution with a pH ranging from 7.8 to 6.7, and channels were then activated by a constant test pH of 6.0. The steady-state inactivation curves were plotted in Fig. 1B together with the curves for activation. The steady-state inactivation curves of both splice variants were very steep, leading to complete inactivation in a range of 0.4 pH units corresponding to a 2.5-fold increase in agonist concentration. Correspondingly, Hill coefficients were large (10.90 ± 6.15 and 8.63 ± 4.31 for ASIC1a and ASIC1b, respectively) suggesting high cooperativity for inactivation.

A Region in the Proximal Part of the Ectodomain Controls Apparent H+ Affinity of Both Activation and Steady-State Inactivation—To determine the structural elements in the primary structure of the protein controlling apparent H+ affinity of ASIC1, we constructed a series of chimeric molecules that exchanged parts of the N terminus of ASIC1a by the corresponding part of ASIC1b (Fig. 2). These chimeras exchange the cytoplasmic N terminus, the first transmembrane domain, and various parts of the extracellular loop. All of the chimeras were functional and showed current amplitudes comparable with wild type ASIC1a.

First, we determined activation by H+ for the chimeras. The activation curves of all chimeras had the same overall shape as those of the wild type with similar Hill

**Fig. 1.** Activation and steady-state inactivation of ASIC1. **A**, top, representative current traces obtained by repetitive short (3.5 s) activation of ASIC1 with pH as indicated. pH between activation was 7.4. Bottom, representative traces of ASIC1 currents elicited by application of pH 6.0 and conditioning pH varying between 7.8 and 6.7. **B**, Dose-response relationship for pH activation (\( n = 7–11 \); ASIC1a, filled squares and ASIC1b, filled diamonds) and steady-state inactivation (\( n = 10–12 \); ASIC1a, filled circles and ASIC1b, filled triangles).
coefficients. Although the EC$_{50}$ of most chimeras did not correspond exactly to the value of either splice variant, they can be divided into the same two groups (Fig. 2). Chimeras C62, C76, and C92 showed half-maximal, steady-state inactivation at a pH similar to that of the ASIC1a wild type (values for chimeras C62–C92 ranged from 7.17 to 7.35, $n = 8$–9), whereas chimera C98 showed a significant shift of half-maximal steady-state inactivation (7.02 ± 0.02, $n = 8$–9). However, the other chimeras showed a much stronger shift in steady-state inactivation to pH values similar to or more acidic than ASIC1b (values for chimeras C106–C148 ranged from 6.74–6.93, $n = 3$–6). This is in agreement with the conclusion that the region comprising amino acids 98 to 106 of ASIC1 contains a critical element controlling the apparent affinity of the channel to H$^+$ for both activation and steady-state inactivation.

In this region only four amino acids are different between ASIC1a and ASIC1b (Fig. 2). We substituted these four amino acids in ASIC1a individually by those found in ASIC1b and determined the pH of half-maximal activation as well as steady state inactivation of the substituted variants. Variants ASIC1a F100L and V103L had values similar to ASIC1a wild type (pH 6.60 ± 0.13 and 6.64 ± 0.08 for activation and pH 7.19 ± 0.07 and 7.23 ± 0.06 for steady state inactivation, respectively, $n = 8$; Fig. 2). The other two variants (K105Y and N106P), however, showed a significant shift of both pH activation and steady state inactivation to values similar to or more acidic than ASIC1b (values for chimeras C62–C92 ranged from 6.74–6.93, $n = 3$–6). A chimera in which the first 106 amino acids were replaced showed an EC$_{50}$ comparable with ASIC1a (values for chimeras C62–C98 ranged from 6.32 to 6.85, $n = 6$–10). A chimera in which the first 106 amino acids were replaced, however, showed a strong shift in the EC$_{50}$ to a value even lower than for ASIC1b (5.42 ± 0.10, $n = 9$). The EC$_{50}$ for all chimeras that contained larger stretches of ASIC1b resembled the EC$_{50}$ of ASIC1b (values for chimeras C112–C148 ranged from 5.52 to 6.00, $n = 6$–9). This suggests that amino acids from 98 to 106 are critical for determining the apparent H$^+$ affinity of ASIC1 activation.

Next, we analyzed steady-state inactivation for the chimeras. Again, half-maximal, steady-state inactivation (IC$_{50}$) of most chimeras did not correspond exactly to the value of either splice variant, but they can be divided into the same two groups (Fig. 2). Chimeras C62, C76, and C92 showed half-maximal, steady-state inactivation at a pH similar to that of the ASIC1a wild type (values for chimeras C62–C92 ranged from 7.17 to 7.35, $n = 8$–9), whereas chimera C98 showed a significant shift of half-maximal steady-state inactivation (7.02 ± 0.02, $n = 8$–9). However, the other chimeras showed a much stronger shift in steady-state inactivation to pH values similar to or more acidic than ASIC1b (values for chimeras C106–C148 ranged from 6.74–6.93, $n = 3$–6). This is in agreement with the conclusion that the region comprising amino acids 98 to 106 of ASIC1 contains a critical element controlling the apparent affinity of the channel to H$^+$ for both activation and steady-state inactivation.

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Ca$_{2}^{+}$ Potentiates Currents through ASIC1a at Physiologic Concentrations—Next, we investigated the interaction of ASIC1 with divalent cations. To have constant open channel properties, we used a protocol in which we varied the Ca$_{2}^{+}$ concentration only during the conditioning period at pH 7.4 and held it constant at 1.8 mM during low pH (6.0) activation. When we increased the Ca$_{2}^{+}$ concentration during the conditioning period at pH 7.4, we observed a significant increase in ASIC1a current amplitude when pH 6.0 solution was applied (Fig. 3A). Because we used different Ca$_{2}^{+}$ concentrations during the conditioning period and low pH activation, the potentiation by Ca$_{2}^{+}$ cannot be precisely quantified. With this reservation, we observed half-maximal potentiation around the physiologic concentration of Ca$_{2}^{+}$ (EC$_{50}$, 2.10 ± 0.29 mM, $n = 8$; figure 3), suggesting that this interaction with Ca$_{2}^{+}$ is of physiological relevance. We observed a similar potentiation of currents through ASIC1a by both Ba$_{2}^{+}$ and Mg$_{2}^{+}$ (EC$_{50}$, 2.90 ± 0.52 and 1.99 ± 0.69 mM, respectively, $n = 7$–8; figure 3). The Hill-coefficient for the potentiation by divalent cations was −2 (2.45, 2.50, and 2.01 for Ca$_{2}^{+}$, Ba$_{2}^{+}$, and Mg$_{2}^{+}$, respectively), suggesting that more than one ion is necessary to potentiate the current. Potentiation was voltage independent (data not shown) suggesting a binding site outside the membrane electric...
Fig. 3. Potentiation by divalent cations of ASIC1a currents. A. Left, representative trace of ASIC1a currents elicited by application of pH 6.0 and constant amounts of extracellular Ca\(^{2+}\) (1.8 mM). Conditioning bath solution between channel activation contained 140 mM NaCl and indicated concentrations (in mM) of CaCl\(_2\). pH was 7.4. Right, dose-response relationship for potentiation by Ca\(^{2+}\), Ba\(^{2+}\) and Mg\(^{2+}\). Neutral bath solution between channel activation in experiments with Bu\(^{2+}\) and Mg\(^{2+}\) contained 140 mM NaCl and varying concentrations of BaCl\(_2\) or MgCl\(_2\). Application solution (pH 6.0) always contained 140 mM NaCl and 1.8 mM BuCl\(_2\) or 2.0 mM MgCl\(_2\). B. Left, representative traces of ASIC1b currents elicited by application of pH 6.0 and constant amounts of extracellular Ca\(^{2+}\) (1.8 mM). Conditioning bath solution between channel activation contained 140 mM NaCl and varying concentrations of CaCl\(_2\). pH was 7.4. Right, dose-response relationship.

Field. Under identical conditions, ASIC1b currents were only slightly potentiated by Ca\(^{2+}\) (Fig. 3B).

Extracellular Divalent Cations Shift the Steady-state Inactivation Curve of ASIC1a and ASIC1b—We then addressed whether extracellular Ca\(^{2+}\) changes the affinity of the channel to H\(^+\). Low extracellular concentrations of divalent cations (0.1 mM Ca\(^{2+}\), 0 mM Mg\(^{2+}\)) during the conditioning period did not significantly shift the pH dose-response curve for ASIC1a and ASIC1b (EC\(_{50}\) = 6.53 ± 0.11, n = 11, and 5.86 ± 0.08, n = 7, respectively; Fig. 4A). For ASIC1a, at any given pH there was an increase in current amplitude with high concentrations of divalent cations compared with low concentrations (Fig. 4A, inset). Thus, Ca\(^{2+}\) potentiation is not due to a shift in the pH activation curve. We then decreased Ca\(^{2+}\) concentration during low pH activation (0.1 mM Ca\(^{2+}\)) and held constant (1.8 mM Ca\(^{2+}\), 10 mM Mg\(^{2+}\)) in the conditioning period at pH 7.4. Under these conditions, we observed a shift in the pH activation curve to a lower agonist concentration for ASIC1a (EC\(_{50}\) = 6.83 ± 0.02, n = 8, p < 0.01; Fig. 4B) and to a lesser extent for ASIC1b (EC\(_{50}\) = 5.95 ± 0.12, n = 8, p = 0.05; Fig. 4B). The shift of the activation curve during activation but not steady-state suggests fast on and off rates of Ca\(^{2+}\) at this modulating site. The observed shift in ASIC3 (17) is a complex interaction of ASICs with divalent cations.

Next, steady-state inactivation curves were determined with low concentrations of divalent cations (0.1 mM Ca\(^{2+}\)) between activation steps. For both splice variants, there was a significant shift of the steady-state inactivation curves by about 0.2 pH units to higher pH values (IC\(_{50}\), 7.48 ± 0.07 for ASIC1a, n = 12, and 7.22 ± 0.04 for ASIC1b, n = 10, p < 0.01; Fig. 4C) compared with normal concentrations of divalent cations. This shift of the steady-state inactivation curves will lead to a significantly lower number of available ASIC1a channels at pH 7.4 and low concentrations of extracellular divalent cations. This accounts for the current potentiation by Ca\(^{2+}\) at this pH.

Because the steady-state inactivation curve for ASIC1b is shifted to more acidic values compared with ASIC1a, this suggests that ASIC1b may be potentiated by Ca\(^{2+}\) at a more acidic conditioning pH. In fact, at a conditioning pH of 7.1 we observed a strong potentiation for ASIC1b also (Fig. 4D). At this pH, current amplitude rose with increasing Ca\(^{2+}\) concentration. Half-maximal potentiation was reached at a Ca\(^{2+}\) concentration similar to ASIC1a (2.00 ± 0.68 mM, n = 9), suggesting: (i) a conserved binding site for Ca\(^{2+}\); and (ii) a physiologic relevance for Ca\(^{2+}\) potentiation of ASIC1b. Because Ca\(^{2+}\) potentiation of ASIC1b can be seen only at a slightly acidic resting pH, ASIC1b is a more effective sensor of transient H\(^+\) signals during slight acidosis.

Shift in Steady-state Inactivation Is due to a Stabilization of the Resting State—The shift in the steady-state inactivation curves by divalent cations can be explained either by a difference in the rate of recovery from desensitization and/or by a difference in the rate of steady-state inactivation. First, we determined time constants of recovery. We used a conditioning pH of 7.4. At this pH value we did not observe significant steady-state inactivation for ASIC1b with both low and normal concentrations of divalent cations (see Fig. 3); for ASIC1a we observed substantial inactivation at pH 7.4, but recovery at pH 7.7 was too fast (complete in less than 3 s) to be reliably determined. Time constants of recovery were similar for ASIC1a and ASIC1b under physiological concentrations of divalent cations (5.23 ± 2.37 s, n = 27, and 7.66 ± 1.87 s, n = 24, respectively; Fig. 5A). With low concentrations of divalent cations (0.3 mM Ca\(^{2+}\)), we observed a significantly slower recovery from desensitization for both ASIC1a and ASIC1b (15.39 ± 14.14 s, n = 23, and 17.03 ± 3.96 s, n = 19, respectively, p < 0.01).

The time constant of the onset of steady-state inactivation was determined by a varying period at pH 7.05 (ASIC1a) or 6.5 (ASIC1b) and normal concentrations of divalent cations or a varying period at pH 7.15 or 6.6 and low concentrations of divalent cations. These pH values were chosen to have maximal inactivation without significant activation (see Fig. 1B). The number of available channels was then assessed by a short (3.5 s) test pulse of pH 6.0, after which channels were allowed to recover from inactivation for 100 s at pH 7.4 (Fig. 5B). With low concentrations of divalent cations (0.3 mM Ca\(^{2+}\)), we observed a significantly faster steady-state inactivation for ASIC1a and a slightly faster inactivation for ASIC1b (ASIC1a, 14.08 ± 3.99 s, n = 13, compared with 78.92 ± 54.48 s, n = 16, respectively, p < 0.01; ASIC1b, 38.95 ± 8.63 s, n = 23, compared with 47.48 ± 18.88 s, n = 18, respectively, p = 0.06). Thus, Ca\(^{2+}\) seems to favor the resting state of the channel, thereby increasing the rate of recovery as well as slowing the rate of inactivation during steady-state. Both mechanisms work in concert to increase the number of available channels during steady-state, which explains the shift in the steady-state inactivation curves.

The Polyvalent Cation Spermine Also Potentiates ASIC1 Currents—The extracellular concentration of the polyvalent cation spermine can significantly vary, and it has been shown that spermine modulates different ion channels. Potentiation of ASIC1 currents by divalent cations led us to ask whether spermine also interacts with ASIC1. We determined steady-state inactivation in the presence of 0.25 mM spermine and 0.1 mM Ca\(^{2+}\) during the conditioning period. As can be seen in Fig. 6, 0.25 mM spermine led to a significant shift of the steady-state inactivation curves of both ASIC1a and ASIC1b. This shift was
A pH dose-response curves obtained with low divalent cation concentration (0.1 mM CaCl₂) during the conditioning period (ASIC1a, open squares and ASIC1b, open diamonds). n = 7–11, unless otherwise indicated. Inset, plot of absolute current amplitudes of measurements with ASIC1a. Dose-response curves obtained with normal concentrations of divalent cations (1.8 mM CaCl₂/1.0 mM MgCl₂) from figure 1 are shown for comparison (filled symbols). B, pH dose-response curves showing that currents through both splice variants can be strongly potentiated by spermine and that interaction with spermine significantly extends the dynamic range of ASIC1.

**DISCUSSION**

Our study has two key findings. 1) ASIC1b is an effective sensor of transient H⁺ signals under slight acidosis, extending the dynamic range of ASIC1. 2) We show that extracellular divalent and polyvalent cations interact with ASIC1, leading to a potentiation of the current.

**Physiological Relevance of Splice Products ASIC1a and ASIC1b**—Our results indicate that agonist sensitivity is the major difference between ASIC1a and 1b. We show that ASIC1b can still function at a resting pH down to 6.9, which is of special importance because ASIC1b is specifically expressed in sensory neurons (5). This property may, thus, enable ASIC1b to sense acidic transients even under conditions of slight acidosis, for example during inflammation. The finding that Ca²⁺ potentiation of ASIC1b is efficient only at a resting pH below 7.15 additionally shows that ASIC1b has its full operational capacity only at slightly acidic resting pH. ASIC1a is expressed in the dorsal root ganglion mainly in small diameter neurons (5, 13, 14), whereas ASIC1b is expressed in both small and large diameter neurons (5). Whether both splice variants are co-expressed in some small-diameter neurons is at present unknown. Irrespective of differential expression, our results suggest that alternative splicing at the ASIC1 gene increases the operational range of H⁺ receptors in sensory neurons.

Alvarez de la Rosa et al. (15) recently reported an EC₅₀ value for activation by H⁺ of ASIC1a expressed in Xenopus oocytes that is substantially higher (pH 5.3) than the value we obtained. ASIC1 activates within few msec (4) and, therefore, fast solution exchange is essential for concerted activation of ASICs. This is especially relevant for large cells like Xenopus oocytes. One possible explanation for lower agonist affinity reported in their study is, thus, slow solution exchange in their experimental set-up.

**Physiological Relevance of Ca²⁺ and Spermine Modulation**—Our results point to an important role for extracellular Ca²⁺ in modulation of neuronal receptor channels. The Ca²⁺-sensing receptor (CaR), a G-protein-coupled cell-surface receptor for divalent cations, is activated by Ca²⁺ over a concentration range of 0.5 mM to 10 mM (18, 19). ASIC1 has a Ca²⁺ sensitivity comparable with the CaR, which is specialized in the sensing of extracellular Ca²⁺. The CaR has a role in body homeostasis of Ca²⁺ but is also significantly expressed in different brain regions, suggesting that fluctuations of extracellular Ca²⁺ concentrations do occur in the brain. In a recent elegant study, Hofer et al. have shown that mobilization of Ca²⁺ in one cell will lead to extrusion of Ca²⁺ and concurrent activation of neighboring cells expressing the CaR (20). This suggests that there is a form of intercellular communication using extracellular Ca²⁺. In addition to these local changes in Ca²⁺ concentration, experiments with Ca²⁺ selective microelectrodes have revealed more global changes in Ca²⁺ concentration in the brain during neuronal activity (21–23). The extracellular Ca²⁺-tained 140 mM NaCl and 1.8 mM CaCl₂/1.0 mM MgCl₂, pH 6.0. Steady-state inactivation curves obtained with normal concentrations of divalent cations (1.8 mM CaCl₂/1.0 mM MgCl₂) from figure 1 are shown for comparison (filled symbols). D, Left, representative trace of ASIC1b currents elicited by application of pH 6.0 and constant amounts of extracellular Ca²⁺ (1.8 mM). Conditioning bath solution between channel activation contained 140 mM NaCl and indicated concentrations (in mM) of CaCl₂ pH was 7.1. Right, dose-response relationship for potentiation by Ca²⁺.
recover completely from desensitization (pH 7.4 for 100 s). Next, oocytes were activated shortly (pH 6.0 for 3.5 s, black bars) and was then allowed to undergo time in conditioning pH.

Current amplitude at the end of a steady-state inactivation protocol was performed with low and normal concentrations of divalent cations during the conditioning period at pH 7.4. We observed in some measurements significant channel rundown. We observed in some measurements significant channel rundown. Right, current peak amplitudes were normalized to the largest current amplitude during a recovery protocol and are plotted against the corresponding time of recovery. Left, ASIC1 was activated shortly (pH 6.0 for 3.5 s, black bars) and was then allowed to recover completely from desensitization (pH 7.4 for 100 s). Next, oocytes were exposed to a conditioning pH for varying periods of time (hatched bars). Right, current peak amplitudes were normalized to the largest current amplitude at the end of a steady-state inactivation protocol (time in conditioning pH = 0 s) and are plotted against the corresponding time in conditioning pH.

Concentration can decrease from a resting value around 1.2 mM to values as low as 0.08 mM in extreme conditions (22). Although Ca²⁺ concentrations usually decreased by only about one fourth of the resting concentration, local changes may well be higher.

In the skin it has been shown that there is a standing gradient of extracellular Ca²⁺ (24) and it has been proposed that tissue injury may cause a spread of high Ca²⁺ from the outer epidermis to the inner epidermis (25), which would then increase the number of available ASIC1. Chelation of divalent cations by metabolites like lactate may also contribute to variations in the extracellular concentration of divalent cations (17).

The Ca²⁺ and Mg²⁺ concentration at which half-maximal potentiation of ASIC1 occurred (EC₅₀, about 2 mM) matches well with the resting concentration of these ions. Therefore, small variations in the physiologic extracellular Ca²⁺ and/or Mg²⁺ concentration will have significant effects on channel activity. The inhibition of ASIC1 by Ca²⁺ during H⁺-activation and the potentiation in the steady-state endows this channel with the property to translate the time when Ca²⁺ concentration changes into two opposing effects. Changes in Ca²⁺ concentration both in the conditioning period and during low-pH activation have only a small net effect on channel activity (results not shown), adding to the possible responses of ASICs to changes in extracellular Ca²⁺ concentration.

Natural polyamines, spermine, spermidine and putrescine, are synthesized and released upon brain trauma (26, 27). The concentration of spermine in secretory granules and nerve terminals from ox neurohypophyses has been calculated to be 0.26 mM (32) and it has been proposed (32) that tissue acidosis and may contribute to increased excitotoxicity during seizures and ischemia when the extracellular pH falls. A more detailed analysis will show whether spermine acts through the same or a different mechanism than divalent cations and what the apparent affinity is of ASIC1 for polyvalent cations.

**Ca²⁺ Modulation of Other Ion Channels**—ASICs share the property of Ca²⁺ potentiation with P2X receptors (25), nicotinic acetylcholine receptors (29), and metabotropic glutamate receptors (30). They also share the property of Mg²⁺ potentiation with the NMDA receptor (31). This underlines the relevance of potentiation. Potentiation of P2X₃ receptors by Ca²⁺ is especially interesting because P2X₃ is an ion channel that is specifically expressed in small-diameter sensory neurons. P2X₃ currents are potentiated by speeding recovery from desensitization, and potentiation has the special property of endowing P2X₃, which shows a very slow (> 5 min) recovery, with a memory for Ca²⁺ transients (25). Because recovery of ASIC1 is much faster and, additionally, the memory will vanish because of steady-state inactivation, the memory for Ca²⁺ transients during steady-state will be short (several seconds) for ASIC1.

**Mechanism of Ca²⁺ Potentiation**—The most likely explana-
tion of the potentiation by Ca$^{2+}$ and other di- and polyvalent cations is a direct competitive binding with H$^+$ to a common binding site. Alternatively, there may be a distinct binding site for di- and polyvalent cations, which would change the affinity at the H$^+$ binding site.

The H$^+$ sensor or ligand binding domain of ASICs is unknown. It has been suggested that His-72 may be involved in activation of ASIC2a (32). However, this residue is not the ligand binding site of ASIC1 or ASIC3 (32). Our results suggest that residues 105 and 106 of ASIC1a are critical in modulating the pK$_a$ of the H$^+$ binding site. We propose that they are contained within a pocket creating an altered electrostatic environment thereby changing pK$_a$ of the H$^+$ binding site. This does not necessarily imply that they are close to the H$^+$ binding site in the primary structure of the channel. The similar EC$_{50}$ of Ca$^{2+}$ potentiation of ASIC1a and ASIC1b suggests a conserved binding site for Ca$^{2+}$. Negatively charged amino acids are the best candidates to contribute to this Ca$^{2+}$ binding site.

Site-directed mutagenesis will show whether any of the numerous aspartates/glutamates in the extracellular loop of ASICs are involved in Ca$^{2+}$ binding and if these residues constitute also the H$^+$ sensor.

The fact that steady-state inactivation occurs at a pH at which there is no macroscopic activation has been taken as evidence that the inactivated state can be reached without prior opening of the channel (7, 15). However, models where the channel has to open before inactivation occurs cannot be ruled out. Our results (not shown) indicate that time constant of recovery from steady-state inactivation is not significantly different from time constant of recovery from desensitization suggesting that there is only one type of inactivated state and we favor a simple model where closed, open and inactivated states are linearly connected.

In summary, our results show that ASIC1b is still active at more acidic resting pH than ASIC1a, suggesting that it is a sensor of transient H$^+$ signals during slight acidosis. Moreover, we show that di- and polyvalent cations modulate gating of ASIC1, which may link ASIC activity to the extracellular concentrations of these ions.

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