Acid-sensing ionic channels (ASICs) are cationic channels activated by extracellular protons. They are expressed in sensory neurons, where they are thought to be involved in pain perception associated with tissue acidosis. They are also expressed in brain. A number of brain regions, like the hippocampus, contain large amounts of chelatable vesicular Zn\(^{2+}\). This paper shows that Zn\(^{2+}\) potentiates the acid activation of homomeric and heteromeric ASIC2a-containing channels (i.e. ASIC2a, ASIC1a+2a, ASIC2a+3), but not of homomeric ASIC1a and ASIC3. The EC\(_{50}\) for Zn\(^{2+}\) potentiation is 120 and 111 \(\mu\)M for the ASIC2a and ASIC1a+2a current, respectively. Zn\(^{2+}\) shifts the pH dependence of activation of the ASIC1a+2a current from a pH\(_{1/2}\) of 5.5 to 6.0. Systematic mutagenesis of the 10 extracellular histidines of ASIC2a leads to the identification of two residues (His-162 and His-339) that are essential for the Zn\(^{2+}\) potentiating effect. Mutation of another histidine residue, His-72, abolishes the pH sensitivity of ASIC2a. This residue, which is located just after the first transmembrane domain, seems to be an essential component of the extracellular pH sensor of ASIC2a.

Acid-sensing ionic channels (ASICs)\(^{3}\) are H\(^{+}\)-gated cation channels expressed in sensory neurons and in neurons of the central nervous system. Four different genes encoding six polypeptides have been identified so far: ASIC1a (1) and ASIC1b (2), ASIC2a (3–6) and ASIC2b (7), ASIC3 (8–10), and ASIC4 (11, 12). Functional ASICs are thought to be tetrameric assemblies of ASIC subunits (13, 14). Both homomeric and heteromeric ASICs can be formed, which exhibit different kinetic, external pH sensitivities, and tissue distribution (7, 14–17). The recently identified ASIC4 subunit does not seem to form a H\(^{+}\)-activated channel on its own (11, 12).

ASIC1a is present in brain and sensory neurons, whereas its splice variant ASIC1b is found only in sensory neurons. Both ASIC1a and ASIC1b mediate fast inactivating currents upon modest but rapid acidification of the external medium. ASIC2a is substantially expressed in the brain, whereas its variant ASIC2b is present in both brain and sensory neurons. ASIC2b has no activity on its own but can form functional heteromers with other ASIC subunits and particularly ASIC3 (7). ASIC3 is specifically found in the small nociceptive sensory neurons and generates a biphasic current with a fast inactivating phase, followed by a sustained component (10). All the ASIC subunits share the same topological organization with intracellular N and C termini and two putative transmembrane domains flanking a large cysteine-rich extracellular loop (16).

In sensory neurons, ASIC currents are thought to play an important role in nociception during a tissue acidosis, for instance in muscle and cardiac ischemia (18–23) and in inflammation (24). It has been also proposed that some might participate in touch sensation (25, 26). Their function in the central nervous system is less documented. An important role of ASICs in signal transduction associated with local pH variations during normal neuronal activity has been proposed (27, 28). They might also be involved in pathological situations such as brain ischemia and epilepsy that produce significant extracellular acidification.

Some ASICs are expressed in brain regions that contain large amounts of chelatable Zn\(^{2+}\). For instance, presynaptic vesicles of glutamatergic hippocampal terminals contain Zn\(^{2+}\) in up to millimolar concentrations (29, 30). Zn\(^{2+}\) corelease with the neurotransmitter results in a transient increase of the local synaptic Zn\(^{2+}\) concentration up to 100–300 \(\mu\)M from resting levels below 500 nM (31–35), and has the potential to alter the behavior of various membrane channels and neurotransmitter receptors (35–37).

Here we show that Zn\(^{2+}\) potentiates the activation of ASIC2a-containing channels. We have used site-directed mutagenesis to investigate the structural determinants of Zn\(^{2+}\) coactivation in the extracellular loop of the ASIC2a subunit. We have identified two histidine residues, His-162 and His-339, that are essential for the Zn\(^{2+}\) potentiating effect, but also another histidine residue, His-72, which causes a large shift in pH sensitivity of ASIC2a when mutated and could be an important component of the pH sensor.

**EXPERIMENTAL PROCEDURES**

**Expression of ASIC Currents in Xenopus oocytes—Xenopus laevis**

were purchased from CRBM (Montpellier, France). Pieces of the ovary were surgically removed, and individual oocytes were dissected in a saline solution (ND96) containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl\(_2\), 2 mM MgCl\(_2\), and 5 mM HEPES (pH 7.4 with NaOH). Stage V and VI oocytes were treated for 2 h with collagenase (1 mg/ml, type Ia, Sigma) in ND96 to remove follicular cells. cRNA of rat ASIC1a, WT and mutated ASIC2a and ASIC3 were synthesized with the mCAP RNA capping kit from Stratagene and injected (0.15–2.5 ng/oocyte) using a pressure microinjector. The oocytes were kept at 19 °C in the ND96 saline solution supplemented with penicillin (6 \(\mu\)g/ml) and streptomycin (5 \(\mu\)g/ml). Oocytes were studied within 1–3 days following injection. In a 0.3-ml perfusion chamber, a single oocyte was impaled in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\(^{†}\) These authors should be considered equally as first author.

\(^{‡}\) To whom correspondence should be addressed. Tel.: 33-4-93-95-77-02 or -03; Fax: 33-4-93-95-77-04; E-mail: igp@ipmc.cnrs.fr.

\(^{1}\) The abbreviations used are: ASIC, acid-sensing ion channel; MES, 4-morpholinoethanesulfonic acid; MTSET, (2-trimethylammonium)-ethylmethanethiosulfonate bromide; WT, wild type.

\(^{2}\) The abbreviations used are: ASIC, acid-sensing ion channel; MES, 4-morpholinoethanesulfonic acid; MTSET, (2-trimethylammonium)-ethylmethanethiosulfonate bromide; WT, wild type.
of ASIC currents, changes in extracellular pH were induced by rapid perfusion of the experimental chamber. Depending on the pH range, acidic solutions were buffered with HEPES (pH > 6), MES (pH between 6 and 5), or acetate (pH < 5).

Expression of ASIC Currents in COS Cells—COS cells, at a density of 20,000 cells/35-mm diameter Petri dish, were transfected with a mix of pCI-CD8 and the following plasmids: pCI-RASIC1a, pCI-RASIC2a, or pCI-rASIC3 (1:5 ratio) using the DEAE-dextran method. Cells were used for electrophysiological measurements 1–3 days after transfection. Successfully transfected cells were recognized by their ability to fix CD8 antibody-coated beads (Dynal). Ion currents were recorded using the whole cell patch-clamp technique (36). Data were sampled at 500 Hz and low-pass filtered at 3 kHz using pClamp8 software (Axon Instruments). The statistical significance of differences between sets of data was estimated by single-sided Student’s t test. The pipette solution contained (in mM): KCl 140, NaCl 5, MgCl₂ 2, EGTA 5, HEPES 10 (pH 7.35); and the bath solution contained (in mM): NaCl 150, KCl 5, MgCl₂ 2, CaCl₂ 2, HEPES 10 (pH 7.45). MES or acetate were used instead of HEPES to buffer bath solution pH ranging from 6.5 to 7.5, and from 4.5 to 3, respectively. ZnCl₂ was added to the bath solution at concentration ranging from 1 μM to 10 mM. Changes in extracellular pH were induced by shifting one out of six outlets of a microperfusion system in front of the cell. Experiments were carried out at room temperature (20–22 °C).

Site-directed Mutagenesis—Mutants were prepared by polymerase chain reaction using a modification of the method of gene splicing by overlap extension (39) or using the GeneEditor in vitro site-directed mutagenesis system from Promega. The amplified products were digested by EcoRI and XhoI and subcloned into the pBSK-Sp6-globin vector specially designed for Xenopus oocyte expression (5). cDNA were synthesized from the NotI-digested vector using the mCAP RNA capping kit from Stratagene.

RESULTS

Effect of Zn²⁺ on the Heteromeric ASIC1a + 2a Current—The extensive colocalization of the ASIC1a and ASIC2a subunits in central neurons (3, 15) led us to first study the effect of Zn²⁺ on the heteromeric ASIC1a + 2a channel expressed in Xenopus oocytes. The properties of the current recorded after coinjection of equal quantities of both ASIC1a and ASIC2a cRNA were in good agreement with those already described for a heteromeric ASIC1a + 2a current (15). However, in the presence of 300 μM Zn²⁺, the submaximal ASIC current induced by pH 6.3 was increased ~3-fold (Fig. 1, A and C), whereas Zn²⁺ alone at pH 7.4 could not activate any current (Fig. 1A, a). The same Zn²⁺-potentiated ASIC current could be recorded when Zn²⁺ was applied either before the acidic pH jump (tested up to 3 min; Fig. 1A, d) or simultaneously (Fig. 1A, c). When the ASIC current was fully inactivated (after 5 s at pH 6.3; Fig. 1A, e), it could not be reactivated by the addition of Zn²⁺. However, application of Zn²⁺ in the course of the inactivation process was still able to increase the fraction of the ASIC current that was not inactivated (Fig. 1A, f). Based on these results, Zn²⁺ was applied simultaneously with the pH drop in all subsequent experiments.

Zn²⁺ (300 μM) induced a left shift of the ASIC1a + 2a half-maximal activation from pH 5.5 to pH 6, and an increase in the Hill coefficient from 1.05 to 1.4 (Fig. 1B, typical experiment; Fig. 2A, mean results), whereas the maximal current induced at pH 4 was not modified. Fig. 2B shows the mean factor by which the ASIC1a + 2a current amplitude was increased by 300 μM Zn²⁺ at different pH values. The potentiation by Zn²⁺ depended on the extracellular pH, with greater effects between pH 6.9 and 6.3 than between pH 6 and pH 5. The relative Zn²⁺-induced increases in current amplitude reached 4.0 ± 1.06-fold (n = 6) the control current, for pH 6.6. Zn²⁺ has no effect on the pH-dependent ASIC1a + 2a current inactivation (Fig. 2C). Zn²⁺ concentrations in between 1 μM and 10 mM were tested on ASIC1a + 2a currents activated at pH 6.3 (Fig. 2D). The Zn²⁺ concentration producing the half-maximal increase in current amplitude was 111 μM with a Hill coefficient of 0.8. A Zn²⁺ concentration of 300 μM was used in all further experiments.

Effect of Zn²⁺ on Homomeric ASIC1a and ASIC2a Currents—To determine which ASIC subunit was responsible for the Zn²⁺-potentiation of the heteromeric ASIC1a + 2a current, we have tested the effects of Zn²⁺ on homomeric ASIC1a and ASIC2a currents expressed in Xenopus oocytes. ASIC2a requires rather low extracellular pH for activation (pH₅₀ = 4.34, Fig. 3A) (6, 15). When applied simultaneously with acid, 300 μM Zn²⁺ increased the amplitude of the ASIC2a current only in the first half of the activation curve, between pH 6.3 and pH 4.5 (Fig. 3A). Even if the effect of Zn²⁺ on the ASIC2a activation curve seems moderate, the relative increases in current amplitude reached 1.21 ± 0.04-fold (n = 11) to 7.00 ± 1.89-fold (n = 4) the control current, for pH 4.5 and 6.3, respectively (Fig. 3B). Zn²⁺ coactivated the homomeric ASIC2a current induced by pH 5.7 with an EC₅₀ of 120 μM and a Hill slope factor of 1.44 (Fig. 3B, inset).

In contrast to ASIC2a, ASIC1a current is not potentiated by Zn²⁺ (Fig. 3C). Zn²⁺ instead exerted a very small inhibition on the ASIC1a current, but this effect was not significant. Fig. 3D illustrates that the effects of 300 μM Zn²⁺ on the ASIC1a, ASIC2a, and ASIC1a + 2a currents induced by pH 6 and 5 are similar after expression in COS cells (transfection) or Xenopus oocytes (cRNA injection).

Effect of Zn²⁺ on Heteromeric ASIC2a + 3 and Homomeric ASIC3 Currents—Since the Zn²⁺-induced potentiation of ASIC currents seemed to be linked to the presence of the ASIC2a subunit, we tested the effect of Zn²⁺ on the heteromeric ASIC2a + 3 channel expressed in Xenopus oocytes. Such an association has been characterized by Bahinski et al. (17). The ASIC3 current was not potentiated but rather slightly inhibited by 300 μM Zn²⁺ (Fig. 4A). However, when ASIC2a was coexpressed with ASIC3, Zn²⁺ potentiated both the peak and the plateau phases of the current (Fig. 4B). Fig. 4C illustrates the relative Zn²⁺-induced increase of ASIC3, ASIC2a + 3, and ASIC1a + 2a currents activated at pH 6, 5, and 4. The potentiation of the ASIC2a + 3 current by Zn²⁺ was similar to that of the ASIC1a + 2a current.
Histidines in Zn$^{2+}$ and H$^+$ Coactivation of ASICs

Involvement of Extracellular Histidines in the Coactivation of ASIC2a by Zn$^{2+}$—ASIC2a appears as the major subunit conferring Zn$^{2+}$ sensitivity to ASICs. We used site-directed mutagenesis to investigate the structural determinants of Zn$^{2+}$ coactivation in the extracellular loop of the ASIC2a subunit. Amino acids found at Zn$^{2+}$-binding sites in proteins include histidine, cysteine, and occasionally aspartate or glutamate (40). When charged water-soluble sulfhydryl reactive reagents like MTSET, a cationic methanethiosulfonate, were applied extracellularly, they had no effect on the ASIC2a current, making unlikely that a cysteine residue interact with extracellularly applied Zn$^{2+}$ (data not shown). On the other hand, diethylpyrocarbonate that reacts with several amino acid side chains, including the imidazole group side of histidine residues (41), suppressed the Zn$^{2+}$ activation of the ASIC2a current (Fig. 5B) and of the ASIC1a + 2a current (data not shown), suggesting a possible involvement of histidine residues in the Zn$^{2+}$ coactivating effect. We systematically replaced the 10 histidine residues in the extracellular loop of ASIC2a by alanine and checked the Zn$^{2+}$ sensitivity of the mutants (Table I). All mutants were still functional with no significant modifications of their pH$_{0.5}$, except the H72A mutant that had lost its ability to be activated by acidic pH down to pH 3. The expected potentiation of the ASIC2a current by zinc (up to 2 times at pH 5.5) was observed for the wild-type channel and for several mutants, but two of them, H162A and H339A, displayed no apparent potentiation, with little if any modification of their properties (Table I and Fig. 5, C and D). Each of these mutants lacked the potentiating effect of 300 μM Zn$^{2+}$, which could be as high as an 8-fold increase compared with the wild-type current amplitude at pH 6 (Fig. 5E). The absence of effect is not due to a shift in the pH dependence since the pH$_{0.5}$ of the two mutants (H162A, pH$_{0.5}$ = 4.71; H339A, pH$_{0.5}$ = 4.74) was not significantly modified compared with the wild-type channel (pH$_{0.5}$ = 4.72) (Table I).

Effect of Zn$^{2+}$ on Heteromeric ASIC Currents Involving Mutated ASIC2a Subunits—We coexpressed wild type ASIC2a or H162A and H339A mutants with ASIC1a. The pH sensitivity of the ASIC1a + 2a heteromeric currents was similar whether the ASIC2a subunit was mutated or not (Fig. 6B). Surprisingly, the ASIC1a+ASIC2a H162A current remained highly sensitive to zinc, whereas the ASIC1a+ASIC2a H339A current was practically insensitive (Fig. 6A). Even if the pH dependence found for the heteromeric ASIC1a+ASIC2a H339A current (pH$_{0.5}$ = 5.7, Fig. 6B) was intermediate between that of homomeric ASIC1a (pH$_{0.5}$ = 6.4) and ASIC2a (pH$_{0.5}$ = 4.7), this did not completely exclude a mixture of the two different homomeric currents. To confirm the heteromeric association of the ASIC1a and ASIC2a H339A subunits, we have used the G430V mutant of ASIC1a, which displays a low basal amiloride-sensitive current and is not activated by acidification (15). Co-expression of this mutant with ASIC2a H339A induced a proton-activated current with a pH$_{0.5}$ significantly different from that of the ASIC2a H339A channel alone, indicating that a heteromultimeric channel was indeed formed by association of both subunits (Fig. 6C). This confirmed that the ASIC2a H339A mutation was responsible for the lack of Zn$^{2+}$ coactivation on the heteromeric channel formed with ASIC1a.

We coexpressed wild type or H162A and H339A mutants of ASIC2a with ASIC3. The pH sensitivity of the heteromeric ASIC2a + 3 current was similar whether the ASIC2a subunit was mutated or not (Fig. 6E). When coexpressed with ASIC3, the H162A and the H339A mutated forms of ASIC2a decreased the effect of Zn$^{2+}$ on both transient and sustained current. The decrease in zinc effect was more pronounced for the H339A mutation than for the H162A mutation (Fig. 6D), as observed previously with the ASIC1a + 2a current.

Involvement of Extracellular Histidines in the Activation of ASIC2a by Protons—The ASIC2a H72A mutant is not activated by increasing the external H$^+$ concentration (Table I). This can reflect a loss of the capability of the channel to sense extracellular pH. However, this could also be due to a disrup-
tion of the necessary association with other subunits to form a homomeric channel. Analysis of the pH sensor of ASICs is complicated by the absence of other modes of activation, such as capsaicin and/or temperature for the VR1 channel (42). To analyze in more detail the effect of the H72A mutation on the ASIC2α properties, we have used a previously described gain-of-function mutant of ASIC2α that displays a medium to high amiloride-sensitive basal current and retains the property to be activated by external protons (5, 6). This mutant corresponds to a single gain-of-function mutation (H72A) introduced in this mutant, and the properties of the double mutant have been analyzed. Interestingly, the double mutant was functional (Fig. 7A), demonstrating that the H72A mutation is not a loss-of-function mutation and that subunit association was not disrupted. Like the single gain-of-function mutant (6), the double mutant showed a large constitutive current, which remained activated by low pH with no apparent inactivation (Fig. 7A), but the pH dependence was largely modified, shifted toward more acidic pH by almost 2 orders of magnitude (pH0.5 = 4.9; Fig. 7B). This drastic modification of the pH sensitivity of ASIC2α by the H72A mutation could explain the lack of activation of the single H72A mutant.

Fig. 3. Effects of Zn2+ on homomeric ASIC1α and ASIC2α currents. A, effect of Zn2+ on the pH-dependent activation of the ASIC2α current expressed in Xenopus oocytes. Current amplitude was expressed as a fraction of the current induced by pH 5 (I/IpH 5), and plotted as mean ± S.E. (n = 3–22). The control curve (○) showed a half-maximal activation at pH3.58 = 4.34 and a Hill coefficient of 1.3. Zn2+ (300 μM (●) increased the amplitude of ASIC2α currents activated by pH values under the pH3.58 value. Inset, current traces showing the effect of Zn2+ on ASIC2α current activated at pH 5. Holding potential, −70 mV. B, relative effect of Zn2+ on ASIC2α current expressed in Xenopus oocytes for each pH value. The ratio between the current amplitude in presence and in absence of Zn2+ (300 μM (Iz+Zn2+/Icontrol) was plotted as mean ± S.E. (n = 3–22). *, significantly different from the pH 4 ratio (p < 0.005). Inset, dose-response curve of Zn2+ potentiation of ASIC2α current. Currents were activated by pH 5.7 in absence and presence of Zn2+ concentrations ranging from 1 μM to 10 mM. The current amplitude increase was normalized to its maximal value (Iz+Zn2+/Icontrol) and plotted as mean ± S.E. as a function of Zn2+ concentration (n = 8–12). The calculated Hill coefficient is 1.44. C, effect of Zn2+ on the pH-dependent activation of the ASIC1α current expressed in Xenopus oocytes. Current amplitude was expressed as a fraction of the current induced by pH 5 (I/IpH 5), and plotted as mean ± S.E. (n = 3–9). The control activation curve (○) showed a half-maximal activation at pH3.58 = 6.37 and a Hill slope factor of 1.64. Zn2+ at 300 μM (●) did not increase ASIC1α current. Inset, current traces showing ASIC1α currents activated at pH 5 in absence and presence of Zn2+. Holding potential, −70 mV. D, relative effect of Zn2+ on ASIC1α+2α, ASIC1α, and ASIC2α currents activated at pH 6 (white bars) and pH 5 (black bars), expressed in COS cells (left) or Xenopus oocytes (right). The ratio between the current amplitude in presence and in absence of Zn2+ (300 μM (Iz+Zn2+/Icontrol) was calculated for each pH value and plotted as mean ± S.E. (n = 4–22).

Fig. 4. Effects of Zn2+ on homomeric ASIC3 and heteromeric ASIC2α+3 currents expressed in Xenopus oocytes. Current traces showing the effect of Zn2+ (300 μM) on ASIC3 (A) and ASIC2α+3 (B) current activated by pH 5 and recorded at −70 mV. C, relative effect of Zn2+ on ASIC3, ASIC2α+3, and ASIC1α+2α currents activated at pH 6 (white bars), pH 5 (gray bars), and pH 4 (black bars). The ratio between the current amplitude in presence and in absence of Zn2+ (300 μM (Iz+Zn2+/Icontrol) was calculated for each pH value and plotted as mean ± S.E. (n = 5–10). *, p < 0.05; **, p < 0.005, significantly different from pH 4 ratio.
Alanine substitution of ASIC2a extracellular histidines and effect on zinc coactivation activity

Schematic representation of the ASIC2a subunit showing the mutated histidines in the extracellular loop and the two membrane-associated domains (M1 and M2) is shown on the left. The pH for half-maximal activation (pH50) and the effect of co-application of Zn2+ with acidic pH (pH 5.5) calculated as the ratio between the current amplitude in presence and in absence of Zn2+ 300 μM (I/Imax) were determined for each mutant at a holding potential of −70 mV. Means ± S.E. are shown (n = 3–10). **, p < 0.005, significantly different from wild type ASIC2a (unpaired t test). NA, nonactivated by acidic pH.

Table I

| Mutant | pH50  | I Zn2+/I Ctrl (pH 5.5) |
|--------|-------|----------------------|
| WT     | 4.72 ± 0.12 | 1.98 ± 0.04 |
| H72A   | 4.72 ± 0.12 | 1.63 ± 0.14 |
| H109A  | 4.96 ± 0.40 | 1.43 ± 0.07 |
| H127A  | 4.51 ± 0.29 | 1.76 ± 0.27 |
| H158A  | 4.52 ± 0.34 | 1.48 ± 0.13 |
| H162A  | 4.71 ± 0.18 | 1.07 ± 0.05 ** |
| H180A  | 4.48 ± 0.11 | 2.00 ± 0.19 |
| H249A  | 4.43 ± 0.24 | 1.74 ± 0.15 |
| H326A  | 4.90 ± 0.31 | 1.44 ± 0.10 |
| H339A  | 4.74 ± 0.19 | 1.05 ± 0.05 ** |

because shifting the pH50 of the wild-type ASIC2a channel (pH50 = 4.7) to more acidic values by several pH units would lead to a channel virtually insensitive to proton activation.

DISCUSSION

Coactivation of ASIC Currents by Zn2+—Zn2+ is known to exert a variety of inhibitory effects on ion channels, but stimulatory effects are rare. For instance, potentiation by Zn2+ has been described for the activation of purinergic P2X3, P2X5, and P2X7 channels by ATP (44–46).

We show that both homomeric ASIC2a channels and heteromeric ASIC2a-containing channels are potentiated when Zn2+...
Histidines in Zn$^{2+}$ and H$^+$ Coactivation of ASICs

The H72A mutation modifies the pH dependence of homomeric ASIC2a current. A, the G430T mutation is associated with a basal amiloride-sensitive current (5), but the mutated channel is still activated by acidic pH with modified pH sensitivity compared with wild-type channel (6). Introduction of the H72A mutation in the G430T ASIC2a mutant does not eliminate the basal activity and the activation by acidic pH. The H72A mutation is therefore not loss-of-function, but it drastically alters the pH dependence of ASIC2a. The bar above the current recordings represents the pH pulse, value being indicated for each trace. The zero current base line is indicated by a dotted line. B, effect of the ASIC2a H72A mutation on the pH dependence of the ASIC2a G430T gain-of-function current. The pH$_{0.5}$ of the G430T mutant is 6.6 (▲). The double mutant H72A + G430T has a pH$_{0.5}$ of 4.9 (●), although the H72A simple mutant is not activated by pH down to pH 3.0. Current amplitude was expressed as a fraction of the current induced by pH 3.0 (I/I$_{\text{max}}$). Each point represents the mean ± S.E. of 5–8 oocytes. Holding potential for A and B = −70 mV.

Physiological Significance of ASIC Current Coactivation by Zn$^{2+}$—The Zn$^{2+}$ concentrations that potentiate the ASIC2a-containing channels only appeared between pH 6.9 and pH 5.5, independent of the pH dependence of the current, suggesting the involvement of titrable residues that would less efficiently chelate Zn$^{2+}$ as pH decreases.

In summary, the present study has revealed interesting structural features of acid-sensing ion channels, with special binding site located in the extracellular domain of the channel. The side chain of histidine has been involved in the zinc-binding sites of numerous metalloproteins (40). Alanine substitution of each of the 10 histidine residues present in the extracellular loop of ASIC2a demonstrates that His-162 and His-339 are sensitive to Zn$^{2+}$. The data presented here using co-expression of mutated ASIC2a subunits with ASIC1a or ASIC3 definitely demonstrate that Zn$^{2+}$ sensitivity in heteromeric channels is carried by the ASIC2a subunit. A comparison between sequences of ASIC1a, ASIC2a, and ASIC3 shows that, although His-162 is highly conserved in all these subunits, His-339 is specific of ASIC2a (Fig. 8). Because ASIC2a is the main subunit responsible for the Zn$^{2+}$ sensitivity of ASICs, it is then tempting to assign to His-339 a predominant role in this property. However, the selective replacement of His-162 by alanine can also completely abolish the Zn$^{2+}$ sensitivity of the homomeric ASIC2a channel, whereas it has moderate or no effect on heteromeric channels. This could reflect some difference between the Zn$^{2+}$ binding sites of homomeric and heteromeric channels comprising the ASIC2a subunit.

His-72 Is Involved in the pH Sensor of ASIC2a—Titrable histidine residues are major determinants of pH modulation in many ion channels (48, 49), whereas glutamate residues have also been shown to be important for acid sensing as for the capsaicin receptor VR1 (42). The His-72 residue adjacent to the first transmembrane domain of ASIC2a (Table I) drastically changes its pH sensitivity since the ASIC2a H72A channel has become insensitive to acidic pH. However, it is not the unique determinant of the pH sensitivity because the ASIC2a G430T+H72A double mutant remains activable by low pH (Fig. 7). Other positions have been shown previously to be involved in the pH dependence of ASIC2a like the Gly-430 residue situated just before the second transmembrane segment (6) and the region preceding the first transmembrane domain (50). The His-72 residue of ASIC2a is conserved in ASIC1a and ASIC3 (Fig. 8). These positions have been mutated in both ASIC1a and ASIC3, but in that case no modification of the pH dependence of the mutant channels was observed, with pH$_{0.5}$ = 6.5 and 6.1 for the ASIC1a H73A and ASIC3 H73A peaks, respectively, compared with pH$_{0.5}$ of 6.4 for ASIC1a and 6.3 for ASIC3 (data not shown). These results support for such a situation with two-P domain K$^+$ channels (48, 51); His-98 has been shown to be important for the pH dependence of TASK-3 near physiological pH, but TWIK-1, which also contains a histidine residue at the equivalent position, does not display a pH dependence in the same pH range.

In summary, the present study has revealed interesting structural features of acid-sensing ion channels, with special...
emphasize on ASIC2a, which is coactivated both by external Zn$^{2+}$ and extracellular acidification. Several histidines have been identified as candidate electron donors to the Zn$^{2+}$ coordination site, and another histidine residue is a candidate for the pH sensor. However, there are clearly other residues directly or indirectly involved in pH sensing that will also need to be identified.

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