Acid-Sensing Ionic Channels in the Rat Vestibular Endorgans and Ganglia

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Mercado, Francisco, Iván A. López, Dora Acuna, Rosario Vega, and Enrique Soto. Acid-sensing ionic channels in the rat vestibular endorgans and ganglia. J Neurophysiol 96: 1615–1624, 2006. First published June 21, 2006; doi:10.1152/jn.00378.2006. Acid-sensing ionic channels (ASICs) are members of the epithelial Na+ channel/degenerin (ENaC/DEG) superfamily. ASICs are widely distributed in the central and peripheral nervous system. They have been implicated in synaptic transmission, pain perception, and the mechanoreception in peripheral tissues. Our objective was to characterize proton-gated currents mediated by ASICs and to determine their immunolocalization in the rat vestibular periphery. Voltage clamp of cultured afferent neurons from P7 to P10 rats showed a proton-gated current with rapid activation and complete desensitization, which was carried almost exclusively by sodium ions. The current response to protons (H+) has a pH50 of 6.2. This current was reversibly decreased by amiloride, gadolinium, lead, acetylsalicylic acid, and enhanced by FMRFamide and zinc, and negatively modulated by raising the extracellular calcium concentration. Functional expression of the current was correlated with smaller-capacitance neurons. Acidification of the extracellular pH generated action potentials in vestibular neurons, suggesting a functional role of ASICs in their excitability. Immunoreactivity to ASIC1a and ASIC2a subunits was found in small vestibular ganglion neurons and afferent fibers that run throughout the macula utricle and cristae stroma. ASIC2b, ASIC3, and ASIC4 were expressed to a lesser degree in vestibular ganglion neurons. The ASIC1b subunit was not detected in the vestibular endorgans. No acid–pH–sensitive currents or ASIC immunoreactivity was found in hair cells. Our results indicate that proton-gated current is carried through ASICs and that anionic current activated by H+ contributes to shape the vestibular afferent neurons’ response to its synaptic input.

INTRODUCTION

Proton-gated sodium currents were originally described in nociceptive neurons (Krishtal and Pidoplichko 1981). Acid-sensing ion channels (ASICs) are ligand-gated ion channels that are directly activated by a drop in extracellular pH (pH50). Six different ASIC subunits encoded by four genes have been cloned from neuronal tissue; ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4 (Bianchi and Drischoll 2002; Waldmann et al. 1997b; Welsh et al. 2002). The functional role of ASICs in the peripheral nervous system was linked with nociception, perception of sour taste, modulation of synaptic transmission, and mechanosensory transduction (Lin et al. 2002; Price et al. 2000, 2001). The function of ASICs in central neurons is still elusive, although it is clear that there is an effective interaction between ASIC1a and the N-methyl-D-aspartate (NMDA) receptor on hippocampal CA1 neurons, where ASIC activity is necessary to maintain long-term potentiation (LTP) (Wemmie et al. 2002). Studies in rodent CNS have shown that ASICs are located predominantly at the postsynaptic endings (Alvarez de la Rosa et al. 2003; Wemmie et al. 2003). The ASIC1a subunit is highly permeable to Ca2+ (Bäsler et al. 2001; Waldmann et al. 1997b; Yermolaieva et al. 2004) and its abnormal activation may constitute an important component of the pathophysiology of ischemic stroke (Xiong et al. 2004). It has been proposed that activation of ASICs in central synapses is caused by protons that are co-released with glutamate (Bässler and Drischoll 2002; Wemmie et al. 2002). Glutamatergic vesicles with pH ≈ 5.7 use the gradient generated by an H+ ATPase to concentrate the neurotransmitter (Miesenböck et al. 1998; Ozkan and Ueda 1998). Glutamate release induced by high-frequency stimulation of Schaffer’s collaterals in hippocampal slices produces a decrease in the pH with a duration of about 10–20 ms (Krishtal et al. 1987). At the synapse between photoreceptors and bipolar neurons in the squirrel retina, acidification of the synaptic cleft caused by paired-pulse stimulation inhibits the L-type calcium current of cone photoreceptors, producing a decrease in neurotransmitter release (DeVries 2001). These reports suggest that acidification of the synaptic cleft during neurotransmitter release has a significant role in the modulation of synaptic transmission.

To date the functional expression of ASICs in the vestibular endorgans has not been evaluated. The electrical activity of vestibular afferents of the larval tiger salamander (Ambystoma tigrinum) has been shown to be sensitive to changes of pHo (Vega et al. 2003). Data suggest that the pH action was caused in part by the modulation of the excitatory amino acid receptors on the vestibular afferent neurons (Ihle and Patneau 2000; Soto and Vega 1988; Soto et al. 1994; Tang et al. 1990; Traynelis and Cull-Candy 1990). ASIC1b and ASIC4 have been cloned and identified by RT-PCR from the rat vestibular apparatus (Bässler et al. 2001; Grundler et al. 2000). Our study characterized ASIC proton-gated currents in isolated rat vestibular afferent neurons. Immunohistochemical location of the six different ASIC subunits in the vestibular sensory periphery and ganglia was also determined.

METHODS

Animal care and procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Reglamento de la Ley General de Salud en Materia Mental Retardation.

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de Investigación para la Salud of the Secretaría de Salud de México. During our work efforts were made to minimize animal suffering and to reduce the number of animals used (as outlined in the Guide to the Care and Use of Laboratory Animals, issued by the National Academy of Sciences USA).

Isolation of hair cells from the crista ampullaris

Wistar rats from postnatal days 14 to 17 were used for the electrophysiological experiments. The cristae ampullaris from the anterior and lateral semicircular canals were dissected from the temporal bones and immersed in extracellular saline solution [ESS (in mM): NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1.2, and HEPES 10, at pH 7.4]. Hair cells were enzymatically dissociated as previously described (Almanza et al. 2003). Briefly, the cristae were maintained in a Ca²⁺- and Mg²⁺-free ESS containing 1 mg/mL porcin-trypsin (Sigma–Aldrich, St. Louis, MO) for 10 min at 37°C, then tissue was kept in ESS supplemented with 0.1 mg/mL collagenase-1A for 7 min at 37°C, and finally washed twice in a Ca²⁺- and Mg²⁺-free ESS containing 1 mg/mL of serum bovine albumin for 10 min. The tissue was placed in the recording chamber and mechanically dissociated with fire-polished borosilicate electrodes. Hair cells were allowed to settle in the recording chamber for 10 min to promote their adhesion to the bottom of the chamber and then were continuously perfused with ESS during the course of the experiment.

Isolation and culture of vestibular afferent neurons (VANs)

Wistar rats from postnatal days 7 to 10 (before myelinization takes place; Toesca 1996) were anesthetized with halothane and killed by decapitation. The head was cleaned rigorously with 70% ethanol. The inferior maxillary was removed and the cranium immersed in L-15 medium (Gibco, Grand Island, NY). The upper part of the skull and the brain were removed and under the stereoscopic microscope (Nikon, Tokyo, Japan) the otic capsule and the vestibular ganglia were identified. The vestibular ganglia were dissected and treated with a combination of 1.25 mg/mL porcine trypsin and 1.25 mg/mL collagenase-1A dissolved in L-15 culture medium for 30 min at 37°C. The ganglia were then rinsed with fresh culture medium, triturated with a fire-polished Pasteur pipette, and centrifuged at 4000 rpm for 5 min. The supernatant was discarded and this procedure was repeated three times. The isolated ganglia neurons were plated in 35-mm Nunclon petri dishes (Nunc, Roskilde, Denmark), pretreated with 100 µg/mL poly-D-lysine (Sigma–Aldrich), with 2 mL of modified L-15 medium supplemented with 10% FBS, 500 IU penicillin, 25 µL/mL fungizone, 15.7 mM NaHCO₃, 15.8 mM HEPES, and the pH adjusted to 7.7 with NaOH. A pH of 7.7 was used to allow it to reach pH 7.4 after kept in ESS supplemented with 0.1 mg/mL collagenase-1A for 7 min, issued by the National Acad-care and Use of Laboratory Animals to reduce the number of animals used (as outlined in the Guide to the Care and Use of Laboratory Animals). During our work efforts were made to minimize animal suffering and to reduce the number of animals used (as outlined in the Guide to the Care and Use of Laboratory Animals). The isolated ganglia were then rinsed with fresh culture medium, triturated with a fire-polished Pasteur pipette, and centrifuged at 4000 rpm for 5 min. The supernatant was discarded and this procedure was repeated three times. The isolated ganglia neurons were plated in 35-mm Nunclon petri dishes (Nunc, Roskilde, Denmark), pretreated with 100 µg/mL poly-D-lysine (Sigma–Aldrich), with 2 mL of modified L-15 medium supplemented with 10% FBS, 500 IU penicillin, 25 µL/mL fungizone, 15.7 mM NaHCO₃, 15.8 mM HEPES, and the pH adjusted to 7.7 with NaOH. A pH of 7.7 was used to allow it to reach pH 7.4 after 30 min in a CO₂ incubator). Neurons from four vestibular ganglia were plated in each petri dish and were maintained in a 95% air-5% CO₂-humidified incubator at 37°C for 18–24 h until recording, during which time the culture dish was mounted on the stage of an inverted phase-contrast microscope (TMS, Nikon).

Electrophysiological recording

Membrane ionic currents and voltage changes in the cell membrane were studied using whole cell voltage-clamp and current-clamp techniques at room temperature (23–25°C). For cell recordings, the culture medium was replaced by ESS. Ionic currents from hair cells and VANs were recorded using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Command-pulse generation and data sampling were controlled by the pClamp 9.2 software (Axon Instruments) using a 12-bit data acquisition system (Digidata 1200, Axon Instruments). Data were sampled at 5 kHz and low-pass filtered at 2 kHz.

Passive properties of the cells (cell capacitance Cₛ; membrane resistance Rₛ; access resistance Rₐ; time constant τ) were measured on-line with the pClamp program at −70 mV. Series resistance was electronically compensated for by 80%. All the recordings were done at a holding potential of −60 mV. For type I hair cells holding potential was set at −90 mV to avoid the activation of low-threshold K⁺ conductance (gₖ; according to Chen and Eatock 2000). For current-clamp experiments, cell membrane potential was held at about −60 mV. The digital data were stored in a personal computer for off-line analysis.

Patch pipettes were pulled from borosilicate-glass capillaries (TW120-3; WPI, Sarasota, FL) using a Flaming-Brown electrode puller (80/PC; Sutter Instruments, San Rafael, CA). The pipette solution contained (in mM): KCl 125, NaCl 10, CaCl₂ 0.134, HEPES 5, EGTA 10, ATPMg 2, and GTPNa 1, at pH 7.2. Once in the bath solution, the filled electrodes had a resistance between 1.5 and 3.5 MΩ.

In the time course of an experiment, seal and series resistance were continuously recorded to guarantee stable recording conditions. The recording was not included in the analysis if the access resistance changed >10%.

Analysis of proton-gated currents and drug sensitivity

To study the current activated by H⁺, ESSs with different pH values were used. In acidic ESS (pH <6.5) 10 mM Na₂-[N-morpholino]ethanesulfonic acid (MES) was used as buffer instead of HEPES. All the drugs used were dissolved in ESS at the pH being tested. The cells were constantly perfused with ESS by using a system consisting of three square perfusion tubes coupled to a step motor (SF-77B; Warner Instruments, Hamden, CT) or with a theta tube coupled to a piezoelectric device (LSS-3200; Burleigh, Ontario, Canada) for a rapid extracellular solution exchange and drug application. The perfusion system was controlled by pClamp software and had a gravity-driven flow rate of about 1 mL/s. With these conditions the surrounding cell’s extracellular solution was changed in <100 ms with the step motor system and <15 ms with the piezoelectric system. The piezoelectric fast-perfusion changer was used in those experiments designed to analyze the rising time constant of proton-gated currents. This equipment allows a solution exchange sufficiently rapid to attain a full activation of ASICs (Büssler et al. 2001).

The period between applications of acidic ESS was 50 s to avoid desensitization of ASIC currents (see results). To construct the dose–response curves, duplicate applications (ESS at pH 5) before and after the pH to be tested were averaged and currents produced by the different pH values were normalized against current magnitudes at pH 5. For the study of drug actions, duplicate application at pH 6.1 before and during drug perfusion was compared. The washout of drug effects was followed. The drugs used were amiloride, acetylsalicylic acid (ASA), and FMRFamide (from Sigma–Aldrich). The effects of acidic ESS with added ZnCl₂, Pb(C₂H₃O₂)₂, GdCl₃, and high CaCl₂ were also investigated. Measurements of the current amplitude were made as the maximum value of the current (peak) less the holding current. Data were measured at the peak and at the sustained current component (4 s after peak current).

Ionic current recordings were analyzed with Clampfit 9.2 software (Axon Instruments) and Macrococil Origin 6.0 (Macrococil Software, Northampton, MA). Figures and statistical analysis were made using Sigma-Plot 8.0 (Systat Software, Richmond, CA). Statistical significance was evaluated with the paired or unpaired Student’s t-test in correspondence with each case. P < 0.05 was considered statistically significant. All the data are presented as means ± SE.

To evaluate the proton sensitivity of the cells and the sensitivity of the proton-gated current to amiloride, the mean of the peak current amplitude was fitted with a dose–response curve with the equation:

\[ y = A₁ + [ (A₂ - A₁) / (1 + 10^{(pH_{50} - x) / p}) ] \]

where A₁ and A₂ are the bottom and top asymptote, log xᵢ is the half-activation or inhibition between A₁ and A₂ (EC₅₀ or IC₅₀), and p is Hill’s slope. The time constant of activation and desensitization of the proton-gated current.
was approximated by adjusting a single, standard exponential function ($y = Ae^{-rt} + C$) to the rise or decay of the current. For display, the recordings were digitally filtered at 0.5 kHz.

**Immunohistochemistry**

Male rats (Wistar and Sprague–Dawley, $n = 12$ each) were anesthetized and perfused with 4% paraformaldehyde. The temporal bones were removed from the skull, immersed in the same fixative for 6 h, and decalcified by immersion in a 5% EDTA phosphate-buffered solution for 5–7 days. The auditory bullae were further microdissected and immersed in 30% sucrose for 7 days. The inner ear was properly oriented to obtain cross sections of the semicircular crista, utricle, sacule, and vestibular ganglia. Serial sections (12 microns thick) were made by using a cryostat (Microm HM 500, Zeiss, Oberkochen, Germany). Sections were mounted on glass slides (Superfrost-plus, made by using a cryostat (Microm HM 500, Zeiss, Oberkochen, Germany). Sections were mounted on glass slides (Superfrost-plus, Fisher Scientific) and stored at Germany). Sections were mounted on glass slides (Superfrost-plus, Fisher Scientific) and stored at −80°C until their use. Tissue sections were incubated at room temperature for 30 min with a blocking solution containing 5% normal goat serum, 5% normal horse serum, and 0.5% bovine serum albumin (fraction V, Sigma) in 0.1% Triton X100 in phosphate-buffered saline solution (PBS). Next, the solution was removed and tissue sections were exposed to the primary polyclonal antibody against either ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, or ASIC4 diluted 1:500 (all from Alpha Diagnostic International, San Antonio, TX).

The source of antigens used to produce ASIC antibodies according to the manufacturer was ASIC1a: 20 amino acid peptide sequence (AAPS) near the extracellular N-terminus of rat ASIC1a; ASIC1b: 18 AAPS near the extracellular N-terminus of rat ASIC1b; ASIC2a: 20 AAPS near the extracellular N-terminus of rat ASIC2a; ASIC2b: 18 AAPS, near the extracellular N-terminus of rat ASIC2b (MDEG2); ASIC3: 21 AAPS, C-terminal, cytoplasmic of rat ASIC3 (DRASIC); and ASIC4: 19 AAPS after the first transmembrane domain (extraacellular) human-rat ASIC4. To further confirm the immunohistochemical results, ASIC antibodies from two additional commercial sources (Chemicon International and Alomone Laboratories) were also tested. For Alomone Labs: polyclonal antibodies against rabbit: anti-ASIC2a antibody directed to a region situated at the N-terminus of the human ASIC2a protein. ASIC2a, immunogen: DLKESPSEGSLQPSSIQC corresponding to residues 2–18 of human ASIC2a protein, ASIC3: directed against a region located in the intracellular N-terminus domain. Epitope: KPRSGLEEAAQS(C) corresponding to residues 2–16 of rat ASIC3, ASIC4 antibody directed to the intracellular N-terminus domain of the rat ASIC4 protein. Epitope: corresponding to residues 7–26 of rat ASIC4. Immuneigen peptide: CKKKFAEEDAKPKEKEAGDE. For Chemicon polyclonal antibodies against guinea pig: ASIC1b. Epitope: N-terminus, immunogen: synthetic peptide from rat ASIC beta residues 3–18. ASIC3. Epitope: 285–304 extracellular domain of rat ASIC3, immunogen: synthetic peptide from the extracellular domain of rat ASIC3 (C)TASLDPDDDFDPEPSPDPGLSP.

Tissue sections were incubated in a humid chamber at 4°C for 48 h. After the incubation, the tissue sections were washed three times in PBS, 10 min each time. At the end of the incubation, tissue sections were postincubated with secondary antibodies against rabbit labeled with Alexa Fluor 488 (1:1,000) (Molecular Probes, Eugene, OR) for 1 h at room temperature in the dark. The tissue sections were washed with PBS then mounted with Vectashield solution (Vector Labs, Burlingame, CA).

Negative controls were made by incubating preabsorbed primary antibody with the corresponding blocking peptide (Alpha Diagnostic International) or by omitting the ASIC antibodies during the immunohistochemical procedure. Antibody absorption was made by mixing the primary antibody with the blocking peptide 1 μg/l μg. The mixture was placed at 37°C for 1 h, after which immunostaining was done as described above. Only a faint background was detected. The rat dorsal-root ganglia (DRG) were used as positive control for all subunits, with cerebellar cortex used as positive control for ASIC2a. Figure 5 summarizes positive and negative controls.

Immunostained tissue sections were viewed and imaged in an Eclipse E800 microscope (Nikon, Tokyo, Japan) equipped with RTSilder spot digital camera (Diagnostics Instruments, Sterling Heights, MI) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD). All images were prepared using the Adobe Photoshop software program.

Immunoreactive cells were measured to determine their soma capacitance. Surface areas of the soma cells were assumed to be spherical and the major and minor diameters were determined and averaged to obtain a radius. The area was calculated using the equation for a sphere, $4\pi r^2$. The area was converted to capacitance using the specific capacitance value for biological membranes of 1 μF/cm².

**RESULTS**

**Hair cell response to pHo shifts**

Rat hair cells isolated from the crista ampullaris maintained their characteristic soma shape (flask shaped for type I hair cells and predominantly cylindrically shaped for type II) with their electrophysiological properties similar to those previously reported (Almanza et al. 2003; Chen and Eatock 2000). Perfusion of hair cells with solution with pHo from 7.4 to 5.5 (for periods ≤30 s) did not affect the holding current in either type I ($n = 7$) or type II ($n = 7$) vestibular hair cells (data not shown). These results are in good agreement with immunohistochemical data indicating no expression of ASICs in hair cells (see **Immunohistochemical location of ASIC subunits**).

**VANs response to pHo shift**

Vestibular afferent neurons held in primary culture express a proton-gated current with fast activation kinetics and almost complete desensitization in a 5-s pulse of acidic extracellular solution (Fig. 1A). Replacement of extracellular Na⁺ for choline decreased the current to 2 ± 0.9% of the peak control current produced by pH 6.1 perfusion ($n = 9$). This indicates that the pH-sensitive current was carried mainly by Na⁺ (Fig. 1A). At pH 6.1 the current has a peak density of $-56 ± 4$ pA/pF ($n = 86$). Its dependency on the extracellular protons was estimated with a dose–response curve (Fig. 1B) with a pH₀.₅ of 6.2 and a Hill number of 1.8.

The rise of the extracellular Ca²⁺ ion concentration from 1.8 to 3.6 mM produced a significant rightward shift in the dose–response pH curve, with a pH₀.₅ of 6.2 (Fig. 1B). This effect is consistent with the model of ASIC gating proposed previously where Ca²⁺ is displaced from the channel pore by protons (Immke and McCleskey 2003).

The activation process of the current caused by pH 6.1 was measured by adjusting a single-power exponential function to the rising phase (10–90%) with a time constant ($\tau_{on}$) of 33 ± 4.7 ms ($n = 17$). For the activation measurement we included only the cells (recordings) that were perfused with the theta-tube piezoelectric device. The time course of the desensitization was adjusted with a single-power exponential function and the time constant ($\tau_{desens}$) obtained at pH 6.1 was 129 ± 4 ms ($n = 86$) (Fig. 1A). The percentage of nondesensitizing current was 6 ± 0.7% ($n = 86$). The recovery of desensitization was measured with repetitive applications of pH 6.1. Data were
fitted with a sum of two single-power exponential functions with a fast time constant ($\tau_f$) of 0.9 ms and a slow time constant ($\tau_s$) of 4 s (Fig. 1C). The desensitization process was partially inhibited when pH 4 was used to produce the proton-gated current. In this case the current became biphasic with a fast activating current followed by a slower one (Fig. 2). The percentage of the desensitization-resistant current (measured 4 s after the peak) at pH 4 was 42% (n = 3), different from the 6% at pH 6.1 (P < 0.001, Student’s t-test). Under our experimental conditions, proton-gated current was restricted to cells with a smaller capacitance forming two well-differentiated groups of cells, the first with a capacitance of 18.9 ± 0.7 pF (n = 146) and a second with 37 ± 1.5 pF (n = 49). This difference was statistically significant with a P < 0.001 (Student’s t-test; Fig. 1D).

To determine the identity of proton-gated currents and to study the possible expression of some other proton-sensitive currents such as two-pore K⁺ channels (TPKC: TREK, TASK, TWIK) or the transient receptor potential vanilloid-1 channel (TRPV1), the pharmacology of ASIC currents was studied using different blockers and coactivators.

FIG. 1. Proton-gated currents in vestibular afferent neurons (VANs). A, left: a typical record of a proton-gated current in a VAN (holding potential = −60 mV); middle: in a Na⁺-free extracellular solution (replaced by choline); right: after wash with normal extracellular solution. Current has fast activation kinetics and an almost complete desensitization during acidic pH 6.1 use (bars above records). Gray-dotted line represents the single-power exponential fit adjusted to the falling phase of the current (desensitization; $\tau_s = 154$ ms). B: pH dependency of the proton-gated current. Currents recorded using an extracellular solution with 1.8 mM Ca²⁺ (filled circles) and with 3.6 mM Ca²⁺ (open circles) concentration. Curves were adjusted with a dose–response function with pH0.5 values of 6.2 and 6.05, both with slope of 1.8 ($r^2 = 0.98$). Each point is the mean of ≥4 cells. C: rate of the recovery of the desensitization, with the line fitting to the data representing the sum of 2 single-power exponentials (n = 7; $\tau_f = 0.9$ s, $\tau_s = 4$ s). Inset: representative current recording and the pH stimulation protocol that was used. D: frequency histogram of the capacitance related to the expression of proton-gated currents. Neurons with a mean capacitance of 18.9 ± 0.7 pF (n = 146; black bars) expressed proton-gated currents. In contrast, those neurons with a mean capacitance of 37 ± 1.5 pF (n = 49; gray bars) did not. Capacitance calculated from measurements of soma size of the immunoreactive cells to acid-sensing ionic channels (ASICs) are also shown. For these cells mean estimated capacitance was 9 ± 3 pF (n = 166; crosshatched bars). Lines represents the Gaussian fit to the data for the three histograms. Normality test revealed that the 3 populations were normal (P > 0.05, Kolmogorov–Smirnov test). Inset: typical responses to pH 6.1 of each group; the capacitance values are from the three specific neurons where the recordings were obtained.

FIG. 2. Proton-gated current from a VAN at pH 4. Current showed a biphasic response to pH 4. One component with fast activation and partial desensitization is followed by a second component with slower activation that increased in amplitude during the use of the pH 4 solution.
The proton-gated current was blocked reversibly by the diuretic amiloride (a typical and unspecific blocker of the ENaC/DEG family; Kellenberger and Shild 2002). The use of amiloride reduced the peak current with an IC₅₀ of 4.5 μM and a Hill number of 2 without affecting the sustained component. At 100 μM, amiloride blocked 80 ± 3% of the peak current and did not affect the sustained current (Fig. 3, A and B; n = 3–8 in each point). Also, the proton-gated current was partially blocked by coapplication of 100 μM GdCl₃, a well-known mechanoreceptor and heteromeric ASIC blocker (Babinski et al. 2000). This trivalent cation blocked the peak current 76 ± 2% and produced a nonsignificant increase in the sustained component of 40 ± 25% (P = 0.17, paired Student’s t-test; n = 6; Fig. 3C). Other proton-sensitive currents such as TRPV1 and TPKC are not blocked by Gd³⁺ or amiloride (Tousova et al. 2005; Ugawa et al. 2002) at the concentrations used in our study.

Acetylsalicylic acid (ASA) has been shown to be an ASIC blocker with greater affinity for the ASIC3 subunit (Voilley et al. 2001). The use of 100 μM ASA in our preparation significantly increased the peak and the sustained component of the proton-gated current in a reversible fashion without affecting the holding current. G: perfusion of 300 μM ZnCl₂ increased the peak current in a significant form. Dotted lines in the recordings are the zero current. Currents were produced by 5 s of extracellular perfusion at pH 6.1. Calibration values in C apply to all the recordings. H: summary of drug effects of ASIC blockers and enhancers on protonated currents in VANs. Bars represent the mean ± SE of the magnitude of the peak current (black bars) and the sustained component (gray bars) after the application of the tested drug. Data are presented as the percentage change vs. the control value. Asterisks indicate statistically significant change with respect to the control value (∗P < 0.05, paired Student’s t-test).
significantly reduced the peak proton-gated current $23 \pm 4\%$ ($n = 7$; $P = 0.03$, paired Student’s $t$-test), and also significantly decreased the sustained component of the current $23 \pm 6\%$ (Fig. 3D) ($P = 0.017$, paired Student’s $t$-test).

The blocking effect of lead acetate on ASIC1a and ASIC1b subunits at micromolar concentrations was recently described (Wang et al. 2006). To further define the participation of the ASIC1 subunits in the current caused by pH 6.1, $10 \mu M$ Pb(C$_2$H$_3$O$_2$)$_2$ was coapplied with the acidic solution. Lead acetate produced a significant reversible decrease of $11 \pm 4\%$ of the peak proton-gated current ($n = 5$; $P = 0.04$, paired Student’s $t$-test). Furthermore, lead acetate increased the sustained current a nonsignificant $29 \pm 18\%$ (Fig. 3E) ($P = 0.2$, paired Student’s $t$-test).

To determine the nature of proton-gated currents expressed in VANs, we tested the effect of FMRFamide and Zn$^{2+}$ (two enhancers of the ASIC currents). The snail neuropeptide FMRFamide and related mammalian peptides (e.g., neuropeptide FF or SF) have been reported to inhibit ASIC desensitization acting mainly on ASIC1 and ASIC3 subunits (Asksmith et al. 2000; Deval et al. 2003; Lingueglia et al. 2006; Xie et al. 2003). The use of $100 \mu M$ FMRFamide for 35 s did not cause any current nor affect the holding current, although FMRFamide significantly increased the peak current induced by pH 6.1 with a significant increase of $52 \pm 3\%$ ($P = 0.002$, paired Student’s $t$-test; $n = 9$). The sustained component of the current also significantly increased from $8 \pm 2\%$ under control conditions to $36 \pm 10\%$ ($P = 0.023$, paired Student’s $t$-test) (Fig. 3F).

The divalent ion Zn$^{2+}$, a specific ASIC2a coactivator at high $\mu M$ concentrations (Baron et al. 2001; Chu et al. 2004), was used to determine whether this subunit forms part of the ASIC channels on VANs. Perfusion of VANs with $300 \mu M$ ZnCl$_2$ significantly increased the peak current induced by pH 6.1 with the current rising $20 \pm 6\%$ ($P = 0.04$, paired Student’s $t$-test; $n = 9$). A greater effect was noticed on the sustained current with a significant increase of $52 \pm 32\%$ ($P = 0.03$, paired Student’s $t$-test) (Fig. 3G).

To test whether TRPV1 channel activation was also contributing to proton-gated current, $1 \mu M$ (n = 11) and $10 \mu M$ (n = 5) capsaicin (a specific TRPV1 activator; Balaban et al. 2003; Tominaga et al. 1998) was also studied under the same conditions as those used to characterize the pH dose–response relationship. Capsaicin produced no significant effect on the holding current nor on the current caused by low pH perfusion of VAN (data not shown).

In current-clamp conditions the cultured-afferent vestibular neurons typically discharge one or two action potentials when square current injection is used (Limón et al. 2005). Perfusion of ASIC-expressing neurons with acidic solutions (pH 6.1) caused the firing of action potentials followed by a slow depolarization. Use of $100 \mu M$ amiloride ($n = 8$) with the acidic solution prevented the generation of action potentials and reduced the slow depolarization (Fig. 4). In neurons that did not express ASIC currents, only a minor depolarization was measured when they were exposed to acidic solutions (data not shown). This depolarization was probably caused by the blocking of voltage-gated and leak-K$^+$ channels by protons as previously reported in DRG neurons, where leak-K$^+$ channels were especially sensitive to blocking by acidic pH (Baumann et al. 2004).

**FIG. 4.** Action potentials caused by acidic solutions in VANs in current clamp. A: perfusion of an acid solution (pH = 6.1) caused the action-potential discharge that is followed by a slow depolarization lasting for about 420 ms with a decay time constant of 375 ms. B: coapplication of amiloride prevented the action-potential generation and reduced the slow depolarization. C: removal of amiloride. Inset: time amplification of the action potentials shown in A. Dotted line represents 0 mV.

**Immunohistochemical location of ASIC subunits**

To determine the expression and location of the different ASIC subunits in vestibular endorgans, immunohistochemical experiments were done using specific antibodies against ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4 subunits on vestibular endorgans and ganglia sections. The control expression assays were done in DRG and cerebellar slices (Fig. 5).

Neurons in the vestibular ganglia were immunoreactive to all ASIC subunits (Fig. 6, A and C–F) except to ASIC1b (Fig. 6B). No immunoreactivity to any of the ASIC subunits was found at the type I or type II hair cells or supporting cells either from the crista ampullaris or the utricular macula (Fig. 6, A1–F1 and A2–F2). In addition, antibodies against ASIC1a and ASIC2a subunits immunostained peripheral processes running throughout the crista ampullaris and the macula utriculus (Fig. 6, A1, C1 and A2, C2).

The immunoreactive neuronal cell-body diameter was measured and its soma capacitance was estimated. Measurement of stained somas in the ganglia slices showed the mean diameter was 16.7 ± 0.3 $\mu$ m with a range from 10 to 25 $\mu$ m ($n = 166$). The immunoreactive cells for the different ASIC subunits have the same estimated soma size distribution (not shown). Based on this, the pooled immunoreactive cells had an estimated capacitance value of 9 ± 3 pF, lower than that found experimentally (18.9 ± 0.7 pF, $n = 146$) in neurons expressing proton-gated currents (Fig. 1D). The origin of this discrepancy is probably caused by the estimation method used to convert area to capacitance.

**DISCUSSION**

By using an electrophysiological and immunohistochemical approach in VANs, we have identified a proton-gated current whose biophysical and pharmacological characteristics parallel
those reported for ASIC channels. Immunoreactivity to ASIC1a, ASIC2a, ASIC2b, ASIC3, and ASIC4 subunits was found in vestibular ganglion neurons and ASIC1a and ASIC2a in the nerve fibers, with only the ASIC1b subunit not detected in the vestibular ganglion neurons. In contrast, no acid-pH-sensitive currents or ASIC immunoreactivity was found in hair cells.

We found that raising the extracellular Ca$^{2+}$ concentration produced a rightward shift in the dose–response curve to protons. Based on the competitive activity of Ca$^{2+}$ and H$^+$, it has been proposed that ASIC gating implies a displacement by protons of Ca$^{2+}$ from the channel (Immke and McCleskey 2003). In VANs, raising the extracellular Ca$^{2+}$ concentration from 1.8 to 3.6 mM displaced the pH$^{0.5}$ from 6.2 to 6.06. Proton-gated currents typically had rapid activation and fast desensitization. The percentage of ASIC current resistant to desensitization was small (≈6%) compared with the peak current that is caused at pH near the half-maximum activation. The constant rate of desensitization was similar to that produced by ASIC1a, ASIC2a, and ASIC3 heteromers, as reported in DRG neurons of the mice and rat, particularly those found in the DRG neurons of types 3 and 7 classified on the basis of a "current signature" method (Petruska et al. 2000) and in heterologous expression systems (Benson et al. 2002; Hesselager et al. 2004).

The proton-gated currents in VANs were biphasic when the neurons were stimulated at pH 4. This type of response was also observed in heterologous expression systems in which ASIC3 is expressed (Hesselager et al. 2004; Waldmann et al. 1997a), indicating that most probably the ASIC3 subunit is involved in the channel formation in VANs. Coexpression of TRPV1 may also lead to this type of biphasic current activation by pH (Tominaga et al. 1998). However, activators of TRPV1 such as capsaicin produced no effect in our experiments (Szallasi and Blumberg 1999) and Gd$^{3+}$ blocked the pH response (Tousova et al. 2005). No TRPV1 immunoreactivity was found in VANs (not shown), indicating that ASIC3 subunit expression may be responsible for the biphasic current response. The pH$^{0.5}$ of the ASIC current in VANs was 6.2, similar to that reported for channels formed by various combinations of ASICs involving ASIC1 subunits. Combinations such as homomeric arrangements of ASIC2a (pH 4.5–4.9) or ASIC3 (6.4–6.6) have a pH dependency that is relatively far from that recorded in VANs.
High concentrations of Zn$^{2+}$ enhanced the proton-gated current in VANs, suggesting the participation of ASIC2a in the formation of functional channels (Baron et al. 2001; Chu et al. 2004). Coincidently, immunoreactivity for the ASIC2a subunit was detected. In spiral ganglion neurons of the mouse, the ASIC2a subunit was also shown to contribute to suprathreshold functions of the cochlea (Peng et al. 2004).

ASA blocked ASIC currents from VANs in a reversible fashion as it did in proton-gated currents from DRG neurons and in ASIC3 channels expressed in heterologous systems (Voilley et al. 2001). This result suggests the interesting possibility that part of the symptoms produced by ASA intoxication (such as tinnitus and vertigo) could be caused by the effect of ASA on ASIC currents. Although it was described that salicylate (the active metabolite of ASA) may act as a blocker of the $N$-methyl-$d$-aspartate (NMDA) function in the cochlea (Guittton et al. 2003), recent experimental evidence suggests that NMDA receptors might not participate in the afferent synaptic transmission in this organ (Glowatzki and Fuchs 2002).

Amiloride, which is a prototypical inhibitor of the ASIC-ENaC/DEG-channel superfamily, inhibited the proton-gated currents in VANs. Amiloride has been used as a tool to differentiate between ASIC-mediated currents and other proton-gated currents such as those caused by TRPV1 or TPKC that are not significantly inhibited by amiloride (Ugawa et al. 2002).

Most significant was the action of FMRFamide, which reduced the desensitization rate of ASIC currents similarly to the effect described for ASIC1 and ASIC3 subunits expressed in heterologous systems or native ASIC currents in rodent DRG neurons (Askwith et al. 2000; Xie et al. 2003). This result reinforces the idea that proton-gated currents in VANs are primarily conducted through ASIC channels and that ASIC1 and ASIC3 subunits form part of these channels. In preliminary experiments done in the isolated vestibule of the rat, recording the multiunit electrical discharge of the semicircular canal afferent neurons, the microperfusion of 1 mM FMRFamide produced a significant increase (about 150%) in the resting discharge of the afferent neurons (A Ortega, R Vega, F Mercado, and E Soto, unpublished results). This indicates that ASIC channels are already active contributing to the resting discharge of the afferent neurons and that proton-gated currents significantly contribute to shape the discharge properties of the vestibular afferent neurons. Taken together, our electrophysiological and pharmacological results suggest that ASIC currents in VANs may be mainly produced by ASIC1a, ASIC2a, and ASIC3 subunit heteromultimers (Benson et al. 2002; Hesselager et al. 2004; Waldmann et al. 1997a,b).

Neuropeptide FF (NPFF) receptors have also been reported to mediate FMRFamide responses, although we cannot exclude that these receptors may also be expressed in afferent neurons. It has been described that the influence of these peptides can be mediated by activation of opioid receptors (Askwith et al. 2000). However, the use of FMRFamide altered the ASIC function in cultured VANs, indicating that FMRFamide affects the ASIC current independently of other possible actions.

Immunohistochemical experiments were made to elucidate the subunit composition and location of the ASICs within the vestibule. A prominent immunoreactivity of ASIC1a and ASIC2a, and also the 2b, 3, and 4 ASIC subunits, was found in VANs (ASIC2b and ASIC4 at lower levels). Expression of various ASIC subunits (ASIC1a, 2a, 2b, 3, and 4) suggests a heteromeric arrangement of ASIC channels on the membrane of the VANs. Immunoreactivity for the ASIC1b subunit was not detected. This last result is in contrast with a report in which ASIC1b was cloned from a cDNA library specific for the vestibular system of the rat (Bässler et al. 2001). The lack of coincidence in these results cannot be explained by the use of different animal models because the ASIC expression in the vestibular endorgans, ganglia, cochlea, and the spiral ganglia has been found to be similar in rats, mice, and humans using the same experimental procedures as described here (I Lopez, unpublished observation). The lack of immunoreactivity to ASIC1b indicates that most probably a conditional expression of this subunit could be occurring. To validate the specificity of the immunohistochemical staining, we used antibodies from three commercial sources. A similar pattern of ASIC immunoreactivity in the vestibular ganglia was found. In situ hybridization and RT-PCR studies would give a definitive answer regarding the specific population of subunits in the vestibular ganglia.

ASICs seem to be expressed in a non-uniform population of vestibular ganglion neurons with an apparent selectivity among the different neuron sizes present in the vestibular ganglia (Leonard and Kevetter 2002). The proton-gated current was recorded only from neurons of lower capacitance, suggesting that smaller-soma-diameter neurons express functional ASIC channels on the cellular membrane. Measurement of immunoreactive cell somas and calculation of their capacitance indicate that the immunoreactive cells fall close to capacitance values of the group of cells expressing a proton-gated current, showing that only small-size cells express ASIC immunoreactivity and proton-gated currents. The difference between the capacitance calculated from morphological measurement of immunoreactive cells and that measured electrophysiologically is most probably explained by the fact that for morphological capacitance calculation we assumed that cell body is a perfect sphere. Although cultured afferent neurons may have not a smooth surface, in fact lamelopodia outgrowth attaching the cell to culture surface could account for the difference in the capacitance measured experimentally from that calculated from size measurements (Limon et al. 2005; Soto et al. 2002). Studies comparing the surface area in phase-contrast microscopy with that found with electron microscopy showed that the light microscopy measurement underestimates the cell surface by $\geq 25\%$ (García-Pérez et al. 2004).

The expression of ASIC currents in the vestibular afferent neurons suggests the possibility that ASICs may constitute an important part for the normal function of the glutamatergic-afferent synaptic transmission between hair cells and afferent neurons. Synaptic activity may lead to synaptic-junction pH variations, as reported in the CNS and retinal glutamatergic synapses (Devries 2001; Kristtal et al. 1987; Miesenböck et al. 1998; Palmer et al. 2003). The inner-ear hair cells form ribbon synapses with afferent terminals (Fuchs et al. 2003; Sterling and Matthews 2005). These kinds of synapses are specialized for a high rate of vesicle release (Sterling and Matthews 2005), constituting an endogenous source of H$^+$ as shown in retinal-cone bipolar cells (Devries 2001; Hosoi et al. 2005; Palmer et al. 2003). Thus neurotransmitter release from hair cells could increase the excitability of the VANs by
activation of ASICs, providing an additional source of postsynaptic excitation. Data indicating a nonquantal sustained depolarization were previously reported in bouton- and calyx-bearing vestibular afferent neurons (Holt et al. 2006; Yamashita and Ohmori 1990). This nonquantal-like depolarization of afferent neurons has been attributed to glutamate spillover at the afferent junction (Holt et al. 2006). However, other mechanisms such as K⁺ accumulation or ASIC activation cannot be discarded. Moreover, our results show that extracellular acidification caused the generation of action potentials in vestibular neurons, suggesting a functional role of ASICs in their excitability. This observation is similar to reports indicating that ASICs expressing neurons (i.e., DRG and CA1 neurons) produced action potentials when exposed to an acidic pH (Deval et al. 2003; Peng et al. 2004; Vukicevic and Kellenberger 2004; Wu et al. 2004).

Because of the action of protons on other voltage-gated currents such as the Ca²⁺ current, proton accumulation in the synaptic cleft will, at the same time, introduce a negative current such as the Ca²⁺-activated K⁺ current (Limón et al. 2005). In this small size group of VANs, the current density of the Ca²⁺ current (I KCa) has been shown to be up to fourfold higher than that in larger cells. The heightened contribution of the I KCa in small LVA (−) cells could determine a faster afterhyperpolarization slope, thus significantly influencing discharge regularity of VANs (Smith and Goldberg 1986). Differential expression among vestibular afferents of ASIC channels may have important functional implications in the coding of vestibular sensory information. ASIC-mediated input could contribute considerably to heighten the synaptic input to these neurons and significantly contribute to shape their discharge pattern.

Finally, ASIC channels may also significantly participate in the physiopathology of some vestibular disorders, including vestibular symptoms of lead poisoning, and would also participate in the action mechanism of amiloride in the inner ear. Furthermore, pan-ASIC knockout mice have been found to have important alterations in the maintenance of balance (Price et al. 2005), suggesting a significant role for ASICs in the function of the vestibular system.

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