Molecular identity, ontogeny, and cAMP modulation of the hyperpolarization-activated current in vestibular ganglion neurons

Angélica Almanza,1,2 Enoch Luis,1 Francisco Mercado,1,2 Rosario Vega,1 and Enrique Soto1
1Instituto de Fisiología, Universidad Autónoma de Puebla, Puebla, Mexico; and 2Instituto Nacional de Psiquiatría “Ramón de la Fuente Muñiz,” Mexico City, Mexico

Submitted 24 April 2012; accepted in final form 20 July 2012

Almanza A, Luis E, Mercado F, Vega R, Soto E. Molecular identity, ontogeny, and cAMP modulation of the hyperpolarization-activated current in vestibular ganglion neurons. J Neurophysiol 108: 2264–2275, 2012. First published July 25, 2012; doi:10.1152/jn.00337.2012.—Properties, developmental regulation, and cAMP modulation of the hyperpolarization-activated current (Ih) were investigated by the whole cell patch-clamp technique in vestibular ganglion neurons of the rat at two postnatal stages (P7-10 and P25-28). In addition, by RT-PCR and immunohistochemistry the identity and distribution of hyperpolarization-activated and cyclic nucleotide-gated channel (HCN) isoforms that generate Ih were investigated. Ih current density was larger in P25–28 than P7–10 rats, increasing 410% for small cells (<30 pF) and 200% for larger cells (>30 pF). The half-maximum activation voltage (V1/2) of Ih was −102 mV in P7–10 rats and in P25–28 rats shifted 7 mV toward positive voltages. At both ages, intracellular cAMP increased Ih current density, decreased its activation time constant (τa), and resulted in a rightward shift of V1/2 by 9 mV. Perfusion of 8-BrcAMP increased Ih amplitude and speed up its activation kinetics. Ih was blocked by Cs+, zatebradine, and ZD7288. As expected, these drugs also reduced the voltage sag caused with hyperpolarizing pulses and prevented the postpulse action potential generation without changes in the resting potential. RT-PCR analysis showed that HCN1 and HCN2 subunits were predominantly amplified in vestibular ganglia and end organs and HCN3 and HCN4 to a lesser extent. Immunohistochemistry showed that the four HCN subunits were differentially expressed (HCN1 > HCN2 > HCN3 ≥ HCN4) in ganglion slices and in cultured neurons at both P7–10 and P25–28 stages. Developmental changes shifted V1/2 of Ih closer to the resting membrane potential, increasing its functional role. Modulation of Ih by cAMP-mediated signaling pathway constitutes a potentially relevant control mechanism for the modulation of afferent neuron discharge.

inner ear; Ih; hair cell; zatebradine; ZD7288; cAMP

The hyperpolarization-activated current (Ih) is a nonspecific cationic inward current that activates during membrane hyperpolarization with slow activation kinetics and is modulated by intracellular cAMP (Accili et al. 2002; Pape 1996). In mammalian cardiac Purkinje fibers, where the current was first referred to as the funny current (If), it participates in cardiac pacemaking, and because of its sensitivity to cAMP Ih has a significant role in the autonomic modulation of the heart rate (Accili et al. 2002; DiFrancesco and Tortora 1981). Since then it has been found that Ih is expressed in a variety of neuronal tissues, where, besides its contribution to the resting membrane potential, it also contributes to repetitive discharge. For example, Ih is essential for rhythmic oscillation of the thalamic-relay neurons and in neurons that are not spontaneously active such as hippocampal CA1 and layer V cortical-pyramidal neurons. This current also contributes to the regulation of membrane input resistance and dendritic integration of synaptic potentials (Berger et al. 2001; Magee 1998, 1999; McCormick and Pape 1990; Pape 1996).

Four full-length cDNAs encoding for Ih channels were sequenced and cloned from a variety of tissues including cardiac and neuronal tissues (Ludwig et al. 1998; Santoro et al. 1998; Seifert et al. 1999). The gene family and the corresponding products were named HCN1–4. The functional channels have a tetrameric structure and diverge with respect to other voltage-activated potassium channels in two distinct ways: 1) the current through HCN channels is carried by Na+ and K+ (permeability ratio 1:4), and 2) HCN channels are activated by membrane hyperpolarization and deactivated with membrane depolarization (Hofmann et al. 2005). All the HCN isoforms have a cyclic nucleotide binding domain in the carboxy-terminal portion. The binding of cAMP to this domain shifts the voltage dependence of the channel to positive membrane potentials and speeds up its activation kinetics (Accili et al. 2002; DiFrancesco and Tortora 1991; Wainger et al. 2001). The different isoforms show quantitative differences as a function of their activation kinetics, voltage dependence, and cAMP sensitivity. The activation kinetics of the HCN1 current is faster than that of the HCN4, with middle activation kinetics for HCN2–3. The half-activation voltage (V1/2) is more negative for HCN2 and HCN3 compared with HCN4 and HCN1. Unlike HCN2 and HCN4, whose open probability is strongly influenced by cAMP, the HCN1 isoform is weakly modulated by this nucleotide and HCN3 is inhibited rather than activated by cyclic nucleotides (Altomare et al. 2001, 2003; Robinson and Siegelbaum 2003).

In vestibular ganglion neurons from P5 mice, Ih had slow activation kinetics, was blocked by extracellular Cs+ and ZD7288, and was carried by Na+ and K+ (Chabbert et al. 2001b). However, because of its low V1/2 (~98 mV), the inhibition of Ih did not affect either the resting potential or the afterhyperpolarization (AHP) trajectory. Thus the participation of Ih in the discharge properties in the vestibular ganglion neurons is still undefined. Given their activation kinetics, it was suggested that HCN2 and HCN4 could be the subunits underlying Ih in vestibular ganglion neurons (Chabbert et al. 2001b). However, its molecular identity is still unidentified. In our work we studied 1) the possibility that modulation of Ih by cAMP shifts its activation range to more functional membrane potentials; 2) Ih expression in neurons obtained from rats in two different postnatal development stages (P7–10 and P25–28); and 3) the molecular identity of Ih.
MATERIALS AND METHODS

Animal care and procedures were in accordance with the American Physiological Society’s “Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training” and the Reglamento de la Ley General de Salud en Materia de Investigación para la Salud of the Secretaría de Salud de México. Protocols involving animal research were reviewed and approved by the Comité Institucional de Cuidado y uso de Animales de Laboratorio (CICUAL) del Consejo de Investigación y Estudios de Posgrado of the Vicerrectoría de Investigación y Estudios de Posgrado of the Universidad Autónoma de Puebla (VIEP-BUAP). All efforts were made to minimize animal suffering and to reduce the number of animals used, as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were supplied by the “Claude Bernard” animal house of the Universidad Autónoma de Puebla.

Isolation and culture of vestibular ganglion neurons. Neonatal Long-Evans rats from postnatal days (P)7–10 (before myelination takes place) of either sex were used for the experiments. A smaller experimental sample of young rats from P25–28 was used because at this (more mature) age the neurons of the vestibular ganglion are myelinated, thus complicating the whole cell recording (Toesca 1996). However, in some neurons this sheath had disappeared during the culture procedure, thus allowing the recording (Limón et al. 2005; Santos-Sacchi 1993). Animals were anesthetized with sevoflurane and killed by decapitation. The head was cleaned rigorously with 70% ethanol. The inferior maxilla 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 removed and treated with a combination of 1.25 mg/ml porcine trypsin and 1.25 mg/ml collagenase IA 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 4,000 rpm for 5 min. The supernatant was discarded, and this procedure was repeated three times. The isolated ganglia neurons were plated on cover slides pretreated with 100 μg/ml collagenase (Sigma-Aldrich, St. Louis, MO) in 35-mm petri dishes (Corning, Lowell, MA) with 2 ml of modified L-15 medium (supplemented with 10% fetal bovine serum, 500 IU penicillin, 25 μg/ml fungizone, 15.7 mM NaHCO₃, and 15.8 mM HEPEs; pH adjusted to 7.7 with NaOH). An initial pH of 7.7 was used to allow the medium to reach pH 7.4 after 30 min in a CO₂ incubator. The cells were maintained in a 95% air-5% CO₂ humidified incubator at 37°C for 18–24 h until recording, for which the culture dish was mounted on the stage of an inverted phase contrast microscope (TMS, Nikon).

Electrophysiological recording. Membrane ionic currents and cell-voltage responses were studied with whole cell voltage-clamp and current-clamp techniques. Experiments were done at room temperature (23–25°C). For cell recordings, the culture medium was replaced by extracellular saline solution containing (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1.2 MgCl₂, and 10 HEPEs, at pH 7.4. Three additional extracellular solutions were employed: a K⁺-rich solution in which the extracellular K⁺ concentration was increased to 50 mM and the Na⁺ concentration decreased to 90.4 mM, a Na⁺-free solution in which Na⁺ was substituted by equimolar choline chloride, and a Na⁺- K⁺-free solution where Na⁺ and K⁺ were substituted by equimolar glycine (NMGDG⁺) and choline chloride. Ionic currents from vestibular ganglion neurons were recorded with an Axopatch 200B amplifier (Molecular Devices, Union City, CA). Command pulse generation and data sampling were controlled by pCLAMP 10 software (Molecular Devices) and a 16-bit data acquisition system (Digidata 1440A, Molecular Devices). Data were sampled at 10 kHz and low-pass filtered at 2 kHz. Passive properties of the cells [membrane capacitance (Cm), membrane resistance (Rm), access resistance (Ra), and time constant (τ)] were measured online with the pCLAMP program at −70 mV. Eighty percent of the series resistance was electronically compensated. The liquid junction potential was calculated with pCLAMP 10 software (Molecular Devices). Measured liquid junction potentials were <5 mV for different solutions, for which these were not corrected. All patch-clamp recordings were made with a holding potential (Vh) of −60 mV. Normal extracellular solution was employed for current-clamp experiments, and the cell membrane potential was held at about −70 mV. The digital data were stored for off-line analysis with Clampfit 10 (Molecular Devices). Patch pipettes were pulled from borosilicate glass capillaries (TW120–3; WPI, Sarasota, FL) with a Flaming-Brown electrode puller (80/PC; Sutter Instruments, San Rafael, CA). The pipette solution contained (in mM) 125 KCl, 10 NaCl, 0.134 CaCl₂, 5 HEPES, 10 EGTA, 2 ATPMg, and 1 GTPNa, at pH 7.2. Once in the bath solution, the filled electrodes had a resistance between 1.5 and 2.5 MΩ. In the course of an experiment, seal and series resistance were continuously recorded to guarantee stable recording conditions.

An experimental series using perforated patch recording instead of a whole cell configuration was also performed. For these experiments normal extracellular solution was used, and the pipette solution was added with 300 μM amphoterin B to permeabilize the cells.

Drugs used. Drug application was made with a gravity-driven flow system (flow rate of ~0.5 ml/s) consisting of three square perfusion tubes coupled to a step motor (SF-77B; Warner Instruments, Hamden, CT) for rapid solution change. The drugs used were all from Sigma-Aldrich.

To define the inward current pharmacological profile, the effects of 2 mM Ba²⁺, 2 mM Cs⁺, 30 μM zatebradine, 80 μM ZD7288, and K⁺-rich solution were evaluated. To study the modulation of Ih by cyclic nucleotides, the cell-permeant cAMP analog 8-BrcAMP (1 mM) and cAMP (100 and 500 μM) were used. The cAMP was added to the pipette solution, and Ih was recorded 2 min after the establishment of the whole cell configuration. The current amplitude was normalized with respect to Ih and compared to the control, without cAMP. The effect of ZD7288 was studied in the presence of 200 μM cAMP added to the pipette solution. The effects of drugs were evaluated with test pulses, applied every 5 s, to −110 mV with duration of 1.5 s from a Vh of −60 mV. The amplitude of Ih was measured at least 2 min after drug perfusion.

Data analysis. Recordings were analyzed off-line with Clampfit 10 (Molecular Devices), SigmaPlot 8.0 (Systat Software, Richmond, CA), and Origin 6.0 (Microcal Software, Northampton, MA) software. The recordings of Ih were always done with 2 mM BaCl₂ added to the extracellular solution to block the IhBR. The current-voltage (I-V) relationship of Ih was generated by measuring current response to 1.5-s voltage steps, applied every 4 s, in 10-mV increments from −120 to −60 mV from a Vh of −60 mV. Recordings where extracellular BaCl₂ was tested were done with extracellular normal solution, and inward current was generated with voltage steps between −130 and −70 mV. The amplitude of Ih was obtained as the difference between the current at the end of the pulse (Iend) and the instantaneous current measured just after the capacitive transient (Iinst). In some cases (specified in the text), to study Ih activation kinetics the voltage pulses were hyperpolarized to −140 mV and the Ih activation τ was obtained by fitting with a double-exponential function after a delay (at 50 ms) (Altmare et al. 2001; Chen et al. 2001). When the use of a double exponential yielded a negative τ, the τ was obtained by fitting current traces with a single exponential function. These were not corrected. All data are mean ± SEM. In all cases the fitting correlation coefficient was ≥0.985.

Voltage-dependent activation was studied from the tail currents generated when voltages returned to the Vh (−60 mV). The tail current data were normalized with the equation Rnorm = (R − Rmin)/(Rmax − Rmin), where Rmin and Rmax are the minimum and maximum tail current measured. Data were normalized, plotted, and fitted by a Boltzmann distribution with the function Itail = I1/(1 +
exp(V_{test} − V_{1/2})/s, where V_{test} is the test potential, V_{1/2} is the half-maximum activation voltage, and s is the exponential slope.

Statistical analysis of the difference between various experimental conditions was done with Student’s t-test. In some cases when the normality test failed, a Mann-Whitney test was used, and this is indicated in the text. Effects were considered as significant with a P < 0.05. Results are given as means ± SE.

**RT-PCR analysis.** RNA was isolated from vestibular organs (utricle, lateral, and anterior semicircular canals were pooled), vestibular ganglion, brain cortex, and heart ventricle by a standard method with TRIzol reagent. RT-PCR experiments were done three times (n = 3); in each one, tissue from inner ear of eight rats was pooled. The tissues were homogenized with a pellet-pestle in the TRIzol reagent, and then the manufacturer’s instructions were followed to obtain the total RNA. Once the RNA was isolated it was treated with DNase I (Invitrogen, Carlsbad, CA) to eliminate the genomic DNA. RNA quantity (>10 µg/µl) and purity (A_{260}/A_{280} > 1.6) were verified in the spectrophotometer and RNA integrity by electrophoresis in a 1% agarose gel. cDNA was synthesized with the SuperScript First-Strand Synthesis Supermix kit (Invitrogen) according to the manufacturer’s instructions. PCR was done in a 25-µl reaction tube with cDNA as template. Primers to amplify HCN1, HCN2, HCN3, and HCN4 genes were purchased from SABioscience-Qiagen (Frederick, MD; catalog nos. PPM06969A-200, PPM06968A-200, PPM25737A-200, and PPM31706A-200), and 18S ribosomal primers were used as constitutive-expression controls. Controls of PCR without the controls the RT procedure was omitted. Amplification was conducted in a MiniOpticon thermal cycler (Bio-Rad, Hercules, CA). PCR products were marked with ethidium bromide in a 1% agarose gel, with photographic recording done in an UV transilluminator.

**Immunostaining.** To identify the expression of HCN channels in the vestibule, monoclonal antibodies (IgG) against HCN1, HCN2, HCN3, and HCN4 were used (all from UC Davis/NIH Neuromab Facility, clones N71/37, N70/28, N141/28, N114/10, respectively). Tissue sections and vestibular ganglion neuron cultures were obtained from Long-Evans rats (P7–10 and P25–28).

For tissue slices, the rats were killed with an overdose of sevoflurane, the vestibule and vestibular ganglia were dissected, and the samples were fixed for 2 h in parafomaldehyde (2%) and subsequently placed in sucrose (20%) overnight [both diluted in phosphate-buffered saline (PBS) 0.1 M, pH 7.4]. The vestibular epithelium and vestibular ganglia were embedded in Tissue-Tek and frozen at −20°C, and sliced sections (20 µm) were obtained in a cryostat (CryoCut 2800 Reichert-Jung), with the tissue slices then placed on gelatin-coated microscope slides. For the cultured cells, glass coverslips with the isolated vestibular ganglion neurons were fixed for 30 min with paraformaldehyde at 2% and washed with PBS.

Preparations were incubated in a blocking solution (0.2% fetal goat serum, 0.03% Triton X-100, and 0.2% bovine serum albumin in PBS) for 4 h at room temperature (−20°C). Preparations were then incubated with HCN1, HCN2, HCN3, or HCN4 antibodies (diluted 1:40 in blocking solution) overnight at 4°C. Subsequently, slides were washed in PBS three times and incubated with the F(ab')2 fragment anti-mouse IgG conjugated with Alexa 488 (diluted 1:500; Invitrogen, Grand Island, NY) for 2 h at room temperature. In all cases controls were run in which the first antibody was omitted. Medium with added propidium iodide (VectorShield, Vector Labs) was used for mounting the coverslip. Observations were done with a laser-scanning confocal microscope (LSM 5 Pascal Axioskop2MOT, Carl Zeiss, Jena, Germany). For image processing and labeling, a Zeiss LSM Image Examiner and Photoshop CS5 were used. All experiments and observations were repeated at least three times.

**RESULTS**

**Hydropolarization-activated inward current identification.** Under control conditions with normal extracellular solution, hyperpolarizing voltage steps generated in all vestibular ganglion neurons (n = 104) an inward current with two components: a quasi-instantaneous linear component with fast activation (I_{inst}) and a slow-activating component, which could correspond to I_{h} (Fig. 1A). To corroborate the identity of the instantaneous inward-rectifier component, 2 mM extracellular BaCl2 was used (Fig. 1B). I_{inst} amplitude (measured just after the capacitive current) was sensitive to BaCl2 (n = 10), decreasing from −520 ± 70 pA under control conditions to −230 ± 30 pA at −130 mV (P < 0.001), whereas the slow activated component (I_{end} − I_{inst}) did not change with the use of BaCl2 (control −400 ± 40 pA and −400 ± 30 pA with BaCl2; n = 10; P = 0.76) (Fig. 1C). The use of 2 mM CsCl (n = 11), 30 µM zatebradine (n = 9), and 80 µM ZD7288 (n = 13, data not shown) decreased the slow component 93 ± 2%, 94 ± 5%, and 79 ± 2% respectively, without significant effect on I_{inst} (Fig. 2, A and B).

Perfusion of the Na+-free solution decreased the slow component amplitude 53 ± 7% (n = 7), whereas perfusion of the Na+-K+-free solution decreased the amplitude 83 ± 8% (n = 4) (data not shown). These results indicate that the slow-activating component flows through nonspecific cationic channels. Additionally, and because K+ ions have been shown to have an activation-like effect on some of the inward currents (DiFrancesco et al. 1986), an extracellular K+-rich solution...
was tested on the current caused by hyperpolarizing voltage steps \((n = 5)\). At \(-120\) mV, the use of high extracellular \(K^+\) (50 mM) solution increased the amplitude of the slow-activating component \(721 \pm 140\%\) (Fig. 2, C and D), while only increasing \(I_{\text{inst}}\) by \(60 \pm 29\%\) \((P = 0.1)\). More detailed studies of \(I_{\text{inst}}\) identity were not made.

In summary, the \(I_{\text{inst}}\) sensitivity to extracellular \(BaCl_2\) suggests that this component flows at least in part through an inward-rectifier \(K^+\) channel. In contrast, the lack of sensitivity to \(BaCl_2\) and the sensitivity to a relatively low concentration of extracellular \(Cs^+, zatebradine, ZD7288, external K^+\) concentration, and a nonspecific cationic permeability are characteristics of \(I_h\), which indicates that the slow-activating current flows through HCN channels.

**Cyclic nucleotide modulation of \(I_h\).** In all the following recordings, 2 mM BaCl$_2$ was used to isolate \(I_h\) from the inward instantaneous rectifying current. Typically, \(I_h\) activated with hyperpolarizing steps to potentials below \(-60\) mV \((n = 44)\). To evaluate whether or not \(I_h\) was modulated by cAMP, initially 8-BrcAMP was used. Extracellular perfusion of 1 mM 8-BrcAMP significantly increased \(I_h\) amplitude at voltages between \(-140\) mV and \(-110\) mV \((25 \pm 10\%\) at \(-140\) mV; \(n = 7, P < 0.05)\) (Fig. 3A). At \(-140\) mV current density was 13 \pm 2.5 pA/pF under control conditions and 16 \pm 2.5 pA/pF with 8-BrcAMP \((P = 0.002)\). 8-BrcAMP also increased the \(I_h\) activation rate. Current activation at \(-140\) mV was fitted with a single exponential with \(\tau = 302 \pm 38\) ms under control conditions and with \(\tau = 191 \pm 17\) ms with 8-BrcAMP \((n = 7, P = 0.016;\) Fig. 3, B and C). Similarly, at \(-130\) mV the activation rate changed from \(\tau = 292 \pm 21\) ms under control conditions to \(\tau = 251 \pm 18\) ms with 8-BrcAMP \((n = 7, P = 0.03)\). Activation \(V_{1/2}\) was not significantly modified by perfusion of 8-BrcAMP \((V_{1/2} = -99 \pm 0.6\) mV and \(s = 9 \pm 0.6\) mV in control condition, \(V_{1/2} = -100 \pm 0.7\) mV and \(s = 9 \pm 0.7\) mV; \(n = 7, P = 0.094\)). An increase in the amplitude of \(I_h\) with extracellular 8-BrcAMP without significant change in its \(V_{1/2}\) has also been reported in primary auditory neurons \((Chen 1997)\).

In experiments in which 100 \(\mu\)M cAMP was added to the internal solution of the recording pipette, the current density measured at \(-140\) mV increased from \(12 \pm 2\) pA/pF \((n = 17)\) in the control group to \(18 \pm 2\) pA/pF \((n = 17, P = 0.02;\) Fig. 4A). It should be noted that current densities of control groups for experiments with perfusion of 8-BrcAMP and those with intracellular cAMP were not statistically different \((P = 0.69)\). Similarly, the increase in \(I_h\) density observed with 8-BrcAMP was analogous to the increase in its density with intracellular...
The activation kinetics were fitted with a double exponential only cAMP (P = 0.44). Intracellular cAMP shifted the activation \( V_{1/2} \) from \(-102 \pm 1.4 \) mV to \(-93 \pm 2 \) mV (\( P < 0.001; \) Fig. 4B), with no significant change of the slope in the I-V curves \( (s = 8 \pm 0.8 \) mV and \( 9 \pm 0.9 \) mV). Under control conditions, in 11 of 17 cells the \( I_{h} \) activation kinetics was fitted with a simple exponential whereas in 6 of 17 cells the activation kinetic was better fitted with a double exponential (Table 1). Figure 4C shows family traces of \( I_{h} \) activation fitted with a single- or double-exponential function in control conditions and with 100 \( \mu \)M cAMP (no difference in the activation rate was found between control groups for 8-BrcAMP and intracellular cAMP; \( P = 0.26 \)). The activation kinetics of \( I_{h} \) was voltage dependent; the activation \( \tau \) for two voltages in different conditions are shown in Table 1. The activation rate measured at \(-140 \) mV and fitted with a simple exponential was significantly different between the controls and cells treated with cAMP (\( P = 0.03 \)), whereas between the groups in which the activation kinetics were fitted with a double exponential only the \( \tau_{\text{slow}} \) was significantly different (\( P = 0.017 \) for \( \tau_{\text{slow}} \) and \( P = 0.052 \) for \( \tau_{\text{fast}} \); Mann-Whitney test used in both cases) (Table 1). Analysis of the voltage dependence separately for cells fitted with a single or a double exponential shows that activation kinetics in the control condition and with intracellular cAMP did not differ. At higher cAMP concentrations, i.e., 500 \( \mu \)M, \( V_{1/2} \) shifted to \(-87 \pm 1.2 \) mV (\( n = 13; \) \( P < 0.001; \) Fig. 4B) without significant change in the slope (\( s = 8 \pm 1.2 \) mV; \( P = 0.09 \)). A good linear correlation between \( C_{m} \) of vestibular ganglion neurons in culture and their neuronal size is well established, indicating that \( C_{m} \) can be used as an indirect measure of soma size (Limón et al. 2005). \( C_{m} \) was not significantly different in the group of neurons in which fitting of \( I_{h} \) activation was single compared with those in which it was double exponential (Fig. 4D; \( P = 0.4 \) and 0.7 for control conditions and with intracellular cAMP, respectively), indicating that \( I_{h} \) activation characteristics are not segregated between neurons with different soma sizes.

**Current-clamp recordings.** To analyze the functional role of \( I_{h} \), current-clamp experiments were done. Current was injected...
to hold the cells around −70 mV, a potential near the resting membrane potential (−62 ± 4 mV, n = 45), and voltage responses were produced with current pulses between 1.7 and 3 nA and a duration of 1 ms. Action potential (AP) characteristics and the zatebradine effect were analyzed at the minimum current that caused an AP. The waveform of the AP and quantitative characteristics were determined in accordance with Bean (2007). Under control conditions (n = 10) the threshold of the AP was −30 ± 1.5 mV, the overshoot was 44 ± 3 mV, the spike height was 118 ± 4 mV, the AP width was 1 ± 0.1 ms (measured at half-maximum spike amplitude), and the AHP was 8.7 ± 1.8 mV. Perfusion of 30 μM zatebradine (n = 10) did not significantly modify the cell membrane potential or the AP threshold (P = 0.06 and P = 0.7). However, it decreased the overshoot to 30 ± 4 mV (P = 0.004) and the spike height to 103 ± 5 mV (P = 0.005), increased the AP width to 1.2 ± 0.1 ms (P = 0.006), and decreased the AHP to 4.4 ± 1.4 mV (P = 0.04) (Fig. 5A). The reduction of AP amplitude produced by zatebradine might be due to nonspecific action of the drug, which has been reported to also affect the Na+ current (Soto et al. 2008). In another experimental series cell responses were caused with 200-ms pulses between −0.5 and 0.9 nA (n = 12). In this condition, typically after the onset of hyperpolarization current, a depolarizing sag, thought to be due to Ih activation, slowly develops (the sag was measured as the ratio between the voltage change at the end and at the beginning of the pulse). At the end of the hyperpolarizing current step, in 83% of the cells a postpulse rebound AP was produced upon repolarization. The use of 30 μM zatebradine significantly decreased the sag from 0.7 ± 0.04 to 0.9 ± 0.04 (P < 0.001). In 90% of the cells the postpulse AP was prevented (Fig. 5B), and in 10% of the cells zatebradine increased the postpulse AP latency (from 20.8 ms to 33.6 ms). Perfusion of 2 mM Cs+ (n = 5) and ZD7288 (n = 12) had a similar effect on the voltage response produced with hyperpolarizing current injection and on the postpulse AP. Thus, although the action of zatebradine may be in part due to its effect on currents participating in AP generation other than Ih, the action of Cs+ and ZD7288 did not affect the height of the AP and still prevented rebound discharge, indicating that activation of Ih plays a significant role in the increase in excitability and AP generation that follows a hyperpolarization.

Perforated patch recordings. An experimental series using perforated patch clamp in P7–10 rats was developed with the aim of defining the functional expression of Ih when the intracellular milieu is not dialyzed. Hyperpolarizing voltage steps generated inward current similar to that found in whole cell recordings, showing two components: a quasi-instantaneous linear component with fast activation (Ihinst) and a slow-activating component corresponding to Ih (Fig. 6A). At −140 mV, the mean activation rate (128 ± 21 ms; n = 4) was faster than that found under control conditions in whole cell recordings (P < 0.01), and also faster than that found when 100 μM cAMP was added to the internal solution (P < 0.05). Ih conductance measured at the tail showed $V_{1/2} = −93 ± 4$

Table 1. Ih activation kinetics at two voltages in the different conditions studied

<table>
<thead>
<tr>
<th>Group</th>
<th>P7-10</th>
<th>P25-28</th>
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<tr>
<td></td>
<td>τinst</td>
<td>τslow</td>
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<tr>
<td>Control</td>
<td></td>
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<tr>
<td>n = 10</td>
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<tr>
<td>−140 mV</td>
<td>11 ± 2 ms</td>
<td>6 ± 0.8 ms</td>
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<tr>
<td>−110 mV</td>
<td>1.5 ± 0.4 ms</td>
<td>4.8 ± 1.2 ms</td>
</tr>
<tr>
<td>n = 10</td>
<td></td>
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</tr>
<tr>
<td>−140 mV</td>
<td>12 ± 2 ms</td>
<td>5 ± 0.8 ms</td>
</tr>
<tr>
<td>−110 mV</td>
<td>911 ± 178 ms</td>
<td>1.7 ± 0.7 ms</td>
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Values are mean ± SE activation time constants (τ); n for each case is indicated. P value corresponds to statistical comparison between activation kinetics at 2 voltages in the same group. Because n = 2 for τslow and τfast at P25–28 with cAMP, no P value is reported. *Statistical difference between control and cAMP groups.

Fig. 5. Effect of zatebradine on electric activity of vestibular ganglion neurons. A: 30 μM zatebradine significantly modified the action potential waveform. B: voltage response produced with a 200-ms 400-pA hyperpolarizing pulse in the control condition and with 30 μM zatebradine. The voltage change following the onset of hyperpolarizing pulse shows a depolarizing sag and rebound action potential; both are blocked by zatebradine. In both graphs, dotted lines indicate the zero voltage; current was injected to hold the cell at about −70 mV.
During postnatal development the amplitude and current density of \( I_h \) increased significantly in all neurons studied (Fig. 7, A and B). However, the increment of \( I_h \) density was greater in smaller neurons, which probably means that there is a selective expression among the different neuron sizes present in the vestibular ganglion (Kevetter and Leonard 2002). On the basis of previous observations (Limón et al. 2005; Mercado et al. 2006), neurons were divided into small (\( C_m < 30 \text{ pF} \)) and large (\( C_m > 30 \text{ pF} \))-soma diameter neurons. In young rats (P25–30) \( I_h \) density was 37 ± 6 pA/pF in neurons with \( C_m < 30 \text{ pF} \) (mean capacitance of 20 ± 1 pF; \( n = 11 \)) and 14 ± 1 pA/pF in neurons with \( C_m > 30 \text{ pF} \) (mean capacitance of 46 ± 3 pF; \( n = 11 \)) (Fig. 7B). \( I_h \) density in small neurons from P25–30 was 410% higher than in small neurons from P7–10 (9 ± 1 pA/pF; mean capacitance of 19 ± 1 pF; \( n = 24 \); \( P < 0.001 \)) and 200% higher than in large neurons from P7–10 (7 ± 1 pA/pF; mean capacitance of 36 ± 2 pF; \( n = 10 \); \( P = 0.001 \)).

The \( V_{1/2} \) of \( I_h \) in P25–28 rats was −95 ± 1 mV (\( n = 22 \)), a value shifted to positive voltages compared with the \( V_{1/2} \) in neonatal rats (−101 ± 0.8 mV; \( n = 24 \); \( P < 0.001 \); Fig. 7C). With 200 \( \mu \text{M} \) cAMP (in the recording pipette) the \( V_{1/2} \) of P25–28 rats was −86 ± 1 mV significantly shifted to more positive potentials (\( n = 9 \); \( P < 0.001 \); Fig. 7C). No significant changes were observed in the \( s \) factor (\( P = 0.6 \)). The activation kinetics of \( I_h \) in P25–28 rats was significantly faster than in neonatal P7–10 rats. In 59% of cells from P25–28 rats the activation kinetics was well fitted with a simple exponential, and in the remaining 41% of cells it was better fitted with a double-exponential function (Table 1). With 200 \( \mu \text{M} \) intracellular cAMP in seven cells the \( I_h \) activation kinetics was fitted with a simple exponential function and in two cells was better fitted with a double exponential (Table 1). Both simple and double exponential fits were faster in P25–28 rats than in P7–10 rats (\( P < 0.05 \) for each comparison).

Similar to neurons obtained from P7–10 rats, \( C_m \) of P25–28 neurons was independent of whether \( I_h \) activation was fitted with a single or double exponential (\( P = 0.2 \) and 0.5 for control conditions and with intracellular cAMP respectively) (Fig. 4D).

**RT-PCR identification of HCN in vestibular ganglion and epithelia.** To determine the molecular identity of the HCNs underlying the \( I_h \), RT-PCR of vestibular ganglia and vestibular epithelia (anterior and lateral semicircular canals and utricle) was done. Specific primers against the four HCN genes were used, and cDNAs from the brain and the heart ventricle were used as positive controls. HCN1, HCN2, HCN4, and, to a lesser extent, HCN3 were amplified, whereas the expressions of HCN3 and HCN4 were not significant. Similar results in both tissues were found in rats from P25–28 (data not shown); although the results are not quantitative, transcripts for HCN3 were more evident in vestibular ganglia. The positive control from the brain tissue amplified the four HCN genes as expected (Thollon et al. 2007), and the heart ventricle only expressed HCN2 and HCN4, as previously reported (Moosmang et al. 2001; Shi et al. 1999).

**HCN immunoreactivity in vestibular ganglia and epithelia.** To determine the tissue expression of the HCNs, the immunoreactivity for each of the HCN subunits was studied in P7–10 and P25–28 rats, both in vestibular tissue (semicircular canal cristae and ganglia) and in cultured vestibular ganglion neurons.

**Fig. 6.** Perforated patch recording of \( I_h \) in P7–10 rats. A: inward current shows a large slow-activating component (pulses from −140 to −60 mV in every 20 mV). B: current-voltage relationship for the slow-activating component. C: voltage response produced with 2-s hyperpolarizing pulses from −140 to −50 pA (every 50 pA) show a depolarizing sag and rebound action potential. Depolarizing pulse to 250 pA produced 2 action potentials. Dotted line indicates zero voltage.

Mv and \( s = 11 \pm 1 \text{ mV} \) (Fig. 6B), which is 9 mV rightward shifted compared with control whole cell \( V_{1/2} \) but not significantly different from that found with 100 \( \mu \text{M} \) cAMP (\( P > 0.05 \)). \( I_h \) open probability obtained from perforated patch recordings was 6.4% (at −70 mV), significantly larger than that measured from whole cell recordings with cAMP (1%).

Under current clamp, injection of hyperpolarizing pulses produced a voltage sag whose trajectory followed that of the \( I_h \) activation under voltage clamp; upon repolarization of the membrane potential to −60 mV, a rebound AP was produced in all recordings (\( n = 4 \); Fig. 6C). Depolarizing pulses produced from one to three (at most) APs in the cells studied.

**Recordings in neurons from young rats (P25–28).** Because of the negative activation voltage reported for \( I_h \) in vestibular ganglion neurons from neonatal mice (Chabbert et al. 2001b), the possibility that in more mature stages the activation range of \( I_h \) could shift to more positive voltages has been considered.
(n = 3 for each experimental condition). Immunostaining for the four HCN subunits was found in vestibular ganglia and in cultured vestibular ganglion neurons at both ages studied. The expression of the four subunits was not the same: immunoreactivity was clearly higher in all the cases studied for HCN1, and qualitatively the staining intensity was graded as HCN1 > HCN2 > HCN3 ≥ HCN4 (Figs. 9 and 10). In all cases controls were clearly negative (Fig. 9, J–L).

In the vestibular ganglion at P7–10, staining with HCN1, HCN3, and HCN4 antibodies was homogeneous, with no particular selectivity in relation with the cell soma size (Fig. 9, A–D), although HCN3 and HCN4 intensity were very low compared with HCN1. The HCN2 antibody produced a less homogeneous staining, with large cells scattered through tissue sections. At P25–28 HCN1 staining was homogeneous and the most intense within vestibular ganglion (Fig. 10A). Selective neuronal body staining for HCN2 and HCN4 was clearly noticeable in P25–28 rats (Fig. 10, B and D), and staining for HCN3 was just above noise level (Fig. 10C).

In the cultured neurons the four HCN subunits were found at both ages studied, although differences in the staining intensity and staining patterns were noted (Fig. 9, E–H). HCN2 was the most difficult to identify, and stained cells were scarce. At P25–28 HCN4 was clearly identifiable at the neuronal membrane (Fig. 10E).

In the neuroepithelia, HCN1 staining of hair cells was intense and homogeneous in both P7–10 and P25–28 rats (Fig. 9I). This coincides with previous reports showing that HCN1 is the main subunit sustaining $I_h$ in mouse hair cells (Horwitz et al. 2011). HCN2 and HCN3 produced a nonhomogeneous staining of vestibular neuroepithelia that did not allow definition of the cellular site of expression. At both ages studied, HCN4 produced a defined staining suggesting its expression at afferent calyceal endings. The HCN4 antibody also produced a punctate staining within the neuroepithelia suggesting its expression in bouton endings of either efferent or afferent terminals (Fig. 10, F and G). This result raises the interesting possibility that HCN4 could be preferentially expressed at the synaptic regions of vestibular afferent neurons.
DISCUSSION

We found the expression of mRNA for all HCN subunits in rat vestibular ganglion neurons harvested from neonatal (P7–10) and young (P25–28) animals. The neurons from neonatal rats predominantly express the mRNA for HCN1, HCN2, and HCN4 and, to a lesser extent, for HCN3, whereas in young rats the neuron transcripts for HCN3 were more evident, suggesting an increase in HCN3 expression with inner ear maturation. Immunolabeling with specific primary antibody showed that in the ganglia and the cultured neurons the four HCN subunits were expressed with a rank order HCN1 > HCN2 > HCN3 > HCN4. Notably, immunoreactivity for HCN4 was identifiable in the calyceal terminals specifically in domain 1 (Lysakowski et al. 2011). In the vestibular neuroepithelia the HCN1 and HCN2 mRNAs were the most abundant in both neonatal and young rats. Our results are in concordance with data obtained in the rainbow trout saccular hair cell layer, where the expression of HCN1:HCN2:HCN4 is 7:2:1 (Cho et al. 2003), and in mouse utricle, where expression of HCN1–4 mRNA was shown and hair cells were stained by HCN1, 2, and 4 antibodies (Horwitz et al. 2011). Vestibular hair cells obtained from Hcn1 knockout mice lacked $I_h$ entirely, while in Hcn2 knockout mice $I_h$ was similar to wild type, suggesting that HCN1 is both necessary and sufficient to form the channels that carry $I_h$ (Horwitz et al. 2011). Our results also match up with results in guinea pig spiral ganglion neurons, where all HCN subunits are localized (Bakondi et al. 2009), although no immunolabeling for HCN3 was found in either the spiral ganglion or the afferent dendrites in the rat cochlea (Yi et al. 2010).

Recordings of the inward current showed two components: 1) an instantaneous component (that we referred to as $I_{inst}$) decreased by extracellular BaCl₂, suggesting that this component flows at least in part through an inward-rectifier K⁺ channel, and 2) a slow-activating component whose pharmacology and non-specific cationic permeability are characteristic of $I_h$. The $V_{1/2}$ for $I_h$ in whole cell recording (around $-101$ mV) is coincident with the $V_{1/2}$ reported in mouse vestibular primary neurons (Chabbert et al. 2001b), in guinea pig and murine spiral ganglion neurons (Chen 1997; Mo and Davis 1997), and in afferent dendrites in the rat cochlea (Yi et al. 2010), where $V_{1/2}$ values were in the range of $-96$ to $-110$ mV. It is worth noting that $V_{1/2}$ under perforated patch was significantly lower (around $-93$ mV). In heterologous expression systems the voltage dependence of the HCN channels is limited by the isoform that activates at the most negative membrane voltages (Ulens and Tytgat 2001). This can explain why, although the immunoreactivity signal was stronger for HCN1, the $V_{1/2}$ determined for $I_h$ is closer to that of the HCN2 and HCN4 subunits. This may also account for the fact that even
though voltage dependence was analyzed separately for cells with double-exponential or single-exponential activation kinetics, significant differences were not found. In our experiments, two cellular groups could be differentiated. In the first set, $I_h$ activation was fitted with a simple exponential falling within the activation range for the HCN2, HCN3, and HCN4 channels (Moosmang et al. 2001; Santoro et al. 2000; Seifert et al. 1999). In the second set, $I_h$ activation was better fitted with a double exponential, for which the $\tau_{\text{slow}}$ is in the activation range for HCN2 and HCN4 and the $\tau_{\text{fast}}$ is closer to the HCN1 and HCN2 activation range (Altomare et al. 2003; Santoro et al. 2000). The presence of multiple HCN isoforms in vestibular ganglion neurons and the variability of $I_h$ activation kinetics suggests that current results from both homomeric and heteromeric channels.

We found that raising intracellular cAMP increased the amplitude of the $I_h$, shifted its $V_{1/2}$ toward positive membrane voltages, and speeded up its activation kinetics. However, $I_h$ open probability at $-70 \text{ mV}$ remained around 1% with or without cAMP. Removal of intracellular factors that might be lost during recording in the whole cell configuration could produce a negative shift of $I_h$ (Bal and Oertel 2000; Biel et al. 2009; Seifert et al. 1999; Southan et al. 2000). The use of perforated patch recordings showed that this is the case, and maintaining a more homogeneous and long-lasting intracellular milieu produced a significant shift of $V_{1/2}$ toward positive values and the open probability at $-70 \text{ mV}$ increased to $-6.5\%$. The $V_{1/2}$ shift produced by cAMP in the vestibular ganglion neurons is similar to that found in auditory neurons, where displacements between 7 and 16 mV have been reported (Banks et al. 1993; Mo and Davis 1997; Rodrigues and Oertel 2006; Yi et al. 2010). The increase of cAMP significantly decreased the $\tau$ in cells whose $I_h$ activation was fitted with a simple exponential and the slow $\tau$ in cells whose $I_h$ activation was fitted with a double exponential. The fast $\tau$ was not significantly modified by cAMP, consistent with the expression of HCN1.

The $I_h$ density in vestibular ganglion neurons shows a developmental increase. The $V_{1/2}$ was more depolarized in young than in neonatal rats, thus increasing the channel open probability at $-70 \text{ mV}$ to 3%. Also, the current activation kinetics was developmentally regulated, being faster in young rats. These changes determine an increase of $I_h$ participation in the setting of the membrane potential and in shaping neuronal responses. The changes observed in the $V_{1/2}$ and activation kinetics of $I_h$ can be caused by a variation in the expression ratio of HCN subunits or by changes in the expression of the auxiliary subunit TRIP8b (Santoro et al. 2009).

The increase in $I_h$ amplitude when the extracellular K$^+$ concentration is increased may have a special relevance in the synapse between the type I hair cells and the primary neurons, because K$^+$ may accumulate in the intersynaptic space (Goldberg 1996; Lim et al. 2011; Soto et al. 2002b). The accumulation of K$^+$ in the synaptic cleft in calyx afferents will also affect the postsynaptic element (Eatock and Lysakowski 2006; Goldberg 1996). Thus the amplitude of $I_h$ in situ may be higher than that observed under experimental conditions that use a low K$^+$ concentration similar to the perilymphatic fluid.

The voltage responses produced by hyperpolarizing pulses showed a sag back toward the resting potential, enough to cause postpulse APs in 83% of the cells. Zatebradine and ZD7288 decreased the sag amplitude and blocked the postpulse spike discharge. Both drugs decreased the AHP and increased AP duration and decreased its amplitude, with no significant changes in the resting membrane potential. In our recording conditions the AHP reached values close to $-80 \text{ mV}$ (similar to mouse vestibular ganglion neurons; Chabbert et al. 2001b), a voltage at which $I_h$ opening probability increased to 14%...
with cAMP. After a hyperpolarization, the activation of \( I_h \) will lead to an increase in neuron excitability and AP discharge.

Electrophysiological properties of central and peripheral terminals of vestibular afferent neurons most probably differ from the cell body. Even though there are differences in the specific ionic channels between afferent process and somas, data suggest that the use of cultured cells is adequate for study of vestibular afferent neuron electrical properties. Furthermore, short-term primary culture of vestibular neurons gives rise to the outgrowth of neurites, and to the expression of various membrane proteins including ionic channels and neurotransmitter receptors that are usually located at the nerve terminals (Soto et al. 2002a). In addition, part of the set of voltage-activated ionic channels that underlie the firing pattern generation at the dendrites seem to be expressed at the cell body. In both peripheral dendrites and cell body regions voltage-activated Na\(^+\) channels (Chabbert et al. 1997; Rennie and Streeter 2006), KCNQ channels (Hurley et al. 2006; Pérez et al. 2009), calcium-activated K\(^+\) channels (Limón et al. 2005; Meredith et al. 2011), and voltage-activated K\(^+\) currents (Chabbert et al. 2001a; Dhawan et al. 2010; Iwasaki et al. 2008; Kalluri et al. 2009), calcium-activated K\(^+\) (Streeter 2006), KCNQ channels (Hurley et al. 2006; Pérez et al. 2009), and soma size, while although all older neurons expressed \( I_h \), in this population there was a correlation between \( I_h \) density and small cell size. Thus most probably the great majority of vestibular ganglion neurons express the \( I_h \), including calyx, dimorphic, and bouton afferents. In fact, the immunostaining of the ganglion neurons shows no selectivity for soma size, and staining of both bouton- and calyxlke terminals can be identified within the vestibular neuroepithelia. To date, most studies on vestibular afferent neurons are from neonatal rats because of the difficulty of recording in mature neurons because of myelination of the soma after P10 (Toesca 1996). Results obtained in P25–28 neurons are physiologically significant, indicating that functional changes take place after P7–10, extending the range over which maturation processes are thought to occur. In hair cells from mouse utricle electrophysiological maturity is reached after P6 (Rüsch et al. 1998), while in afferent neurons some features of mature discharge patterns (like the regularity of the spontaneous discharge) begin after P3 (Desmadryl et al. 1986) and the sensitivity of the vestibular nerve also is increased after birth (Desmadryl 1991). Thus developmental changes after P7–10 in current density, voltage sensitivity, and kinetics could enhance differences in spiking of afferent neurons between neonatal and young rats. Furthermore, modulation of \( I_h \) by activation of cAMP-mediated signaling pathways and by other factors in the intracellular milieu could constitute a control mechanism by which neurotransmitters or intracellular process may modulate vestibular afferent neuron discharge.

**ACKNOWLEDGMENTS**

The authors thank cDr. Emmanuel Seseña Mendez for performing the perforated patch experiments, cDr. Blanca Cervantes Sanchez for her participation in the immunohistochimical experiments, and Dr. Ellis Glazier for editing the English-language text.

**GRANTS**

This work was supported by Grants PIFI-2010 and VIEP-BUAP 2011 to R. Vega and National Council of Science and Technology of México (CONACyT) Grants 10110/127/08 to E. Soto and 10013/119257 to A. Almanza.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: A.A., E.L., F.M., R.V., and E.S. performed experiments; A.A., E.L., F.M., and E.S. analyzed data; A.A. and E.S. interpreted results of experiments; A.A., E.L., F.M., R.V., and E.S. prepared figures; A.A. and E.S. drafted manuscript; A.A., R.V., and E.S. edited and revised manuscript; A.A., E.L., F.M., R.V., and E.S. approved final version of manuscript; R.V. and E.S. conception and design of research.

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