IDENTITY, EXPRESSION AND FUNCTIONAL ROLE OF THE SODIUM-ACTIVATED POTASSIUM CURRENT IN VESTIBULAR GANGLION AFFERENT NEURONS

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Abstract—Vestibular afferent neurons (VANs) transmit information from the vestibular end organs to the central nuclei. This information is encoded within the firing pattern of these cells and is heavily influenced by the K+ conductances expressed by vestibular neurons. In the present study, we describe the presence of a previously unidentified Na+-activated K+ conductance (KNa) in these cells. We observed that the blocking of Na+ channels by tetrodotoxin (TTX) or the substitution of choline for Na+ in the extracellular solution during voltage clamp pulses resulted in the reduction of a sustained outward current that was dependent on the Na+ current. Furthermore, increases in the intracellular concentration of Na+ that were made by blocking the Na+/K+ ATPase with ouabain increased the amplitude of the outward current, and reduction of the intracellular Cl− concentration reduced the TTX-sensitive outward current. The substitution of Li+ for Na+ in the extracellular solution significantly reduced the amplitude of the outward current in voltage clamp pulses and decreased the afterhyperpolarization (AHP) of the action potentials in current clamp experiments. These electrophysiological results are consistent with the presence of mRNA transcripts for the KNa subunits Slick and Slack in the vestibular ganglia and in the sensory epithelium. These results indicate that KNa channels are expressed in VANs and in their terminals. Furthermore, these data indicate that these channels may contribute to the firing pattern of vestibular neurons. © 2013 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: slack, slick, synapse, inner ear, intrinsic properties, lithium.

INTRODUCTION

Vestibular afferent neurons (VANs) transmit information regarding angular and linear accelerations from the vestibular end organs to the vestibular nuclei during head movements. The discharge properties of VANs at rest and during mechanical stimulation have been examined in several species (Goldberg and Fernández, 1971a,b; Honrubia et al., 1989; Brichta and Goldberg, 2000). In those organisms, VANs have been classified as regular and irregular using the coefficient of variation of their action potential discharge rate at rest. The regularity of the spontaneous discharge and the dynamic responses of VANs vary with the location of their afferent terminals in the sensory epithelium. The morphological characteristics of the synapses between the afferent neurons and hair cells also vary with their location in the epithelium and are likely involved in the setting of the electrical discharge pattern. Morphologically, VANs have been classified into button-shaped, calyx and dimorphic (Fernández et al., 1988). Although the regularity of the afferent neuron discharge was initially attributed to their synaptic input, cumulative evidence indicates that variations in the specific population of K+ channels that are expressed by these cells play a significant role in the determination of their discharge pattern and consequently in the coding of vestibular information (Limón et al., 2005; Iwasaki et al., 2008; Pérez et al., 2005; Kalluri et al., 2010). Several voltage-dependent K+ conductances have been described in vestibular ganglion neurons. Briefly, Chabbert et al. (2001) described three voltage-dependent K+ currents that can be identified by their kinetic properties and by their sensitivity to tetraethylammonium (TEA), to 4-aminopyridine (4-AP) and 4-dendrotoxin (4-DTX), and to 4-AP and blood depressing substance (BDS). The presence of TEA- and 4-AP-sensitive K+ currents was also observed in non-enzymatically altered ganglion neurons (Risner and Holt, 2006) and in recordings from calyx terminals (Dhawan et al., 2010). The hyperpolarization-activated current (Ih) have been described in cultured vestibular ganglion neurons and in the calyx endings of VAN (Almanza et al., 2012; Meredith et al., 2012). Cultured vestibular ganglion neurons differentially display a Ca2+-dependent K+ current (KCa), which is composed of BK, IK and SK (big-, intermediate- and small-conductance, respectively). A fourth component that is resistant to classical KCa channel blockers is also observed in vestibular ganglion neurons (Limón et al., 2005). SK-type KCa currents were...
recently reported in calyx terminals (Meredith et al., 2011). Additionally, the presence of an M-current in large soma-size ganglion neurons and calyx terminals indicates that spike-frequency adaptation in VANs is under the cholinergic control of K⁺ currents (Hurley et al., 2006; Pérez et al., 2009, 2010). Interestingly, KCNQ K⁺ channels which carry the M-type current, and α-DTX-sensitive Kv1 channels contribute to the low-voltage-activated outward current that participates in conferring irregularity to the stimuli-evoked responses of isolated vestibular ganglion neurons (Kalluri et al., 2010). Furthermore, immunohistochemical studies have demonstrated that KCNQ, Kv1.1 and Kv1.2 channels are expressed in segregated microdomains of calyx terminals (Lysakowski et al., 2011). These microdomains also express the Na⁺ channel subunit Na1.6, which is sensitive to low concentrations of tetrodotoxin (TTX), and Nav1.5, which is resistant to TTX (Lysakowski et al., 2011). Such subunits contribute, at least in part, to the Na⁺ currents that are recorded in calyx terminals (Rennie and Streeter, 2006) and may contribute to those currents that are observed in isolated vestibular neurons (Chabbert et al. 1997; Soto et al., 2002). However, the broad range of the Na⁺ current activation that is observed in electrophysiological experiments (Chabbert et al., 1997; Soto et al., 2002; Rennie and Streeter, 2006), as well as the expression of mRNA transcripts for the majority of the TTX-sensitive Na⁺ channel subunits (Wooltorton et al., 2007), indicate that the Na⁺ current in VANs is due to the activity of a combination of different Na⁺ channels, the functional roles of which may not necessarily be temporally limited to the initiation of action potentials (Wooltorton et al., 2007).

The Na⁺-dependent K⁺ conductance (KNa) is broadly distributed in the brain. KNa channels are tetramers formed by homomers or heteromers of two different subunits encoded by the Slack (Slc8a2; sequence like a calcium-activated K⁺ channel) and Slick (Slc8a2; sequence like an intermediate conductance K⁺ channel) genes (Dryer, 1994; Bhattacharjee et al., 2002). The KNa current (iKNa) is activated by intracellular Na⁺ and by intracellular Cl⁻, its sensitivity to these ions depending on the subunits forming the channel. In this article, we report that VANs express a KNa that is mediated by at least two different KNa channel subunits, Slack and Slick, which participate in the action potential afterhyperpolarization (AHP) and phase locking of stimuli-evoked action potentials in isolated ganglion neurons.

**EXPERIMENTAL PROCEDURES**

We used Wistar rats at postnatal days (P) 7–10 for the electrophysiological experiments and for the detection of mRNA transcripts. For the immunohistochemical experiments and to determine the expression pattern of KNa channels during postnatal development, we used P7–P10 and P21–P24 rats. All of the rats were supplied by the “Claude Bernard” animal house of the University of Puebla. The protocols that involved animal research were reviewed and approved by the Comité Institucional de Cuidado y uso de Animales de Laboratorio (CICUAL) from the Consejo de Investigación y Estudios de Posgrado de la Vicerrectoría de Investigación y Estudios de Posgrado (VIEP-BUAP). All of the animal care and experimental procedures were performed according to the Reglamento de la Ley General de Salud en Materia de Investigación para la Salud of the Secretaría de Salud de México. All 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”, which is issued by the National Academy of Sciences.

**Cell culture of vestibular ganglion neurons**

To obtain the VAN cultures, we used methods that have been previously described in detail (Soto et al., 2002; Limón et al., 2005). Briefly, the rats were euthanized by decapitation. The brain was then removed, the vestibular nerves were exposed and the vestibular ganglia were isolated. The tissue was first incubated in L-15 culture medium (Gibco, Grand Island, NY, USA) that was supplemented with 1.25 mg/mL collagenase type 1A (Sigma–Aldrich, St. Louis, MO, USA) and 1.25 mg/mL porcine trypsin (USB, Cleveland, OH, USA) for 30 min at 37 °C. This step was followed by a wash with fresh L-15 medium. Following the enzymatic treatment, the tissue was dissociated by mechanical agitation. The dissociated cells were placed in Petri dishes (Nunc, Denmark, 35 mm) in L-15 medium that was modified for CO₂ culture conditions by adding 10% fetal bovine serum (FBS) (Gibco), 10 mM NaHCO₃, 10 mM HEPES buffer and 1000 IU/mL penicillin. All of the procedures were performed in a tissue culture room under a laminar flow hood (Nuaire, Plymouth, MN, USA). The dissociated cells were incubated at 37 °C in an atmosphere of 95% air and 5% CO₂ until the electrophysiological recordings (18–24-h cultured cells).

**Electrophysiological recordings and data analysis**

We used primary cultured VANs to (i) allow for the isolated cells to recover from potential membrane alterations following the dissociation procedure and (ii) to facilitate patching of the cells after the myelin surrounding the soma disappeared during the culturing process. Whole-cell voltage and current clamp experiments were performed at room temperature (23–25 °C). The patch pipettes were pulled from borosilicate glass capillary tubing (WPI, Sarasota, FL, USA) using a Flaming-Brown micropipette puller (80/PC; Sutter Instruments, San Rafael, CA, USA). The patch pipettes were mounted on a Narishige manipulator (Narishige, Tokyo, Japan) and were electronically compensated once the whole-cell configuration was obtained. The signals were low-pass filtered at 2 kHz and digitized at 20 kHz for the voltage clamp experiments. The maximum amplitude of the iKNa was estimated by subtracting the I–V relationship obtained with the perfusion of a Na⁺-free solution, from the control I–V relationship. The normalized conductance was approximated by a Boltzmann function:

\[
g / g_{max} = 1 / (1 + e^{(V_m - V_{1/2}) / S})
\]

where \(g\) is the conductance, \(g_{max}\) is the maximum conductance, \(V_m\) is the membrane potential, \(V_{1/2}\) is the potential at which half of the maximum current is reached and \(S\) is the slope factor.

A subset of cells was recorded in current clamp conditions to examine the effects of ion substitution on their response to the current stimuli. For these experiments, the filters were open to 10 kHz, and square current pulses from –0.5 to 0.5 nA with...
0.1-nA steps and 100 ms were generated using pClamp software. The rebound discharge produced at the end of hyperpolarizing pulses and the discharge rates produced by depolarizing pulses were analyzed. The characteristics of the action potentials, elicited by 1-ms suprathreshold pulses, were analyzed off-line using Clampfit in the pClamp 10.2 (Molecular Devices) and Origin 8.0 (Microcal Software, Northampton, MA, USA). In this experiment, we analyzed the phase-plane of the response to current clamp pulses by plotting the first derivative of the voltage respect to time (dV/dt) of the first action potential versus the membrane potential (Bean, 2007). The threshold of the action potential was defined as the voltage at which dV/dt increases suddenly, and its amplitude was defined as the voltage between the maximum peak and the resting potential. The duration of the action potential was measured at 50% of the spike amplitude. The maximum depolarization and repolarization rates were measured in the phase plane as the maximum and minimum dV/dt values. The AHP was defined as the difference between the minimum voltage following the action potential and the membrane potential. Significant differences between the means were determined using Student's t-test, with P < 0.05 being considered significant. The pooled data are presented as the means ± SEM.

In some experiments sinusoidal stimulation of about 1.5× threshold amplitude (determined for each neuron by pulse injection as described above) was used. For this the output of a function synthesizer (8904A Hewlett Packard, Palo Alto, CA, USA) was connected to the external input control of the Axopatch 200B amplifier and 10-Hz sinusoids were injected into the amplifier to produce a proportional sinusoidal output stimulating current during 1 s.

Solutions and drugs used

The recording chamber was continuously perfused with a gravity-fed normal external solution or a modified external solution, as indicated (Table 1). To evaluate the total I_{Na} component of the current, the cells were perfused with a Ca^{2+}-free external choline solution. Also, the use of TTX (Alomone Labs, Jerusalem, Israel) allowed the determination of the participation of the Na\(^{+}\) current in the activation of the outward current. A Ca^{2+}-free external Li\(^{+}\) solution was used to define the Na\(^{+}\) dependence of the current activation. To determine whether the increase in the intracellular concentration of Na\(^{+}\), increased the amplitude of the outward current, we used a Ca^{2+}-free external solution added with ouabain (Sigma Chemicals Co., St. Louis, MO, USA). In the current-clamp experiments, we used a Ca^{2+}-free external solution with and without Li\(^{+}\) and the normal internal solution (Table 1).

### Table 1. Solution composition (in mM)

<table>
<thead>
<tr>
<th>Sol</th>
<th>NaCl</th>
<th>CholineCl</th>
<th>LiCl</th>
<th>Na-lasethionate</th>
<th>KCl</th>
<th>K-Gluconate</th>
<th>CaCl₂</th>
<th>MgCl₂</th>
<th>HEPES</th>
<th>EGTA</th>
<th>ATPMg</th>
<th>GTPNa</th>
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<tr>
<td>Internal</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>135</td>
<td>–</td>
<td>0.134</td>
<td>–</td>
<td>5</td>
<td>10</td>
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<td>1</td>
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<tr>
<td>Low Cl(^{-}) internal</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>10</td>
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<td>135</td>
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<tr>
<td>External (Ca(^{2+}) free)</td>
<td>140</td>
<td>–</td>
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<td>–</td>
<td>5.4</td>
<td>–</td>
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<tr>
<td>Na(^{+}) -free external (Ca(^{2+}) free)</td>
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<td>140</td>
<td>–</td>
<td>5.4</td>
<td>–</td>
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<td>4.5</td>
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<tr>
<td>External Li(^{+}) (Ca(^{2+}) free)</td>
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<td>–</td>
<td>140</td>
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<td>Low Cl(^{-}) external (Ca(^{2+}) free)</td>
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The pH was adjusted to 7.4 for external solutions and to 7.2 for the internal solutions. The osmolarities were 310 (external) and 300 mOsm (internal) and were measured using a vapor pressure osmometer (Wescor 5500, Logan, UT, USA). EGTA, ethylene glycol bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Reverse-transcription polymerase chain reaction (RT-PCR)-based detection of K\(_{Na}\) channel subunits

The mRNA levels of the K\(_{Na}\) subunits Slick and Slack (Slo2.1 and Slo2.2) were assessed using the RT-PCR technique. For each experiment, total RNA was isolated from both of the inner ears of eight rats (P7–P10) using the TRIzol Plus RNA Purification Kit (Invitrogen; Carlsbad, CA, USA). To determine whether expression of the Slack and Slick transcripts was restricted to the vestibular ganglia or to the vestibular epithelium, we performed RT-PCR experiments using tissue that was carefully microdissected from different anatomical regions under a stereomicroscope. To examine the expression of these genes in the vestibular epithelium, we used the utricule and a minimum of 2 cristae. The tissue was then homogenized in Trizol using a Pellet-Pestle in a 1.5 mL Eppendorf tube. The aqueous phase was separated from the homogenate using chloroform and was centrifuged at 12,000g for 15 min at 4°C (Beckman, Beckman Coulter Inc., CA, USA). Following incubation in 35% ethanol, the sample was transferred to the separation column, and the RNA was washed and eluted following the manufacturer’s instructions. Only RNA samples with OD 260/280 ratios of 1.6 or greater and that clearly exhibited three bands following agarose gel electrophoresis were used. The same procedure was performed using a sample of brain tissue as a positive control. Once the total RNA was obtained, cDNA synthesis (RT) and PCR were performed using the Superscript III Platinum two-step qRT-PCR kit (Invitrogen). The analyzed samples included the vestibular ganglion, the vestibular organ and the brain. As a negative control for each sample, we substituted the same volume of cDNA for the total RNA. The final volume of the PCR was 20 μL. The primers were purchased from SABiosciences (Frederick, MD; Catalog No. PPR49730A Slack and PPR55022A Slick). We used a Miniopticon thermal cycler (Bio-Rad; Hercules, CA, USA) for the gene amplification. The expected product sizes were 95 bp for Slack and 99 bp for Slick. The mRNA for the 18S ribosomal subunit, which is a housekeeping gene, was amplified and analyzed in each experiment. The primer for this gene was also acquired from SABioscience (Catalog No. PPR57734E), and the expected molecular weight of the amplification product was 184 bp. All of the samples were run in triplicate.

Immunohistochemistry

The expression of Slack and Slick was assessed for cultured VANs, vestibular epithelium sections and for vestibular ganglion sections using monoclonal antibodies (IgG1) against the Slick (Anti-Slo2.1 sodium- and chloride-activated ATP-sensitive potassium channel, clone N11/33, UC Davis/NIH NeuroMab...
Facility) and Slack (Anti-Slo2.2 sodium-activated potassium channel, clone N3/26, UC Davis/NIH NeuroMab Facility). For the immunocytochemical experiments, cultured cells on glass coverslips were fixed for 30 min with 2% paraformaldehyde in a phosphate-buffered solution (PBS). The sections were then washed with a 0.1 M PBS (pH 7.4) solution that contained fetal goat serum with 0.03% Triton X-100 and 0.2% bovine serum albumin (BSA). The preparations were incubated in a humid chamber with the antibodies against Slick and Slack. The antibodies were diluted (1/1000 and 1/200, respectively) in 0.1 M PBS (pH 7.4, with 0.03% Triton X-100 and 0.2% BSA) overnight at room temperature. The preparations were subsequently washed and incubated with a fluorescein-conjugated polyclonal antibody F(ab')2 against mouse immunoglobulin (diluted 1/500; Invitrogen).

To assess the expression of Slick and Slack in the vestibular epithelium and in the vestibular ganglion of Wistar rats, the tissues were fixed for 2 h in 2% paraformaldehyde and subsequently immersed in 20% sucrose overnight. The tissues were then embedded in a cryoprotectant (Jung Tissue Freezing medium), rapidly frozen and stored at −20 °C until cryostat sectioning. The vestibular epithelium and the vestibular ganglia were cut into 20-µm sections and collected onto gelatin-coated microscope slides. The sections were then permeabilized for 4 h by immersion in a 0.1% PBS (pH 7.4) solution that contained fetal goat serum, 0.2% BSA and 0.03% Triton X-100. The sections were washed three times for 10 min each with 0.1% PBS (pH 7.4). This step was followed by incubation with the antibodies against Slick and Slack (diluted 1/1000 and 1/200, respectively) in 0.1 M PBS (pH 7.4 with 0.03% Triton X-100 and 0.2% BSA) overnight at room temperature. An Alexa 488-conjugated fluorescent secondary antibody (Invitrogen) was used to identify the primary antibody that bound to the Slack and Slick subunits.

For the observations the preparations were covered with anti-fade mounting media that contained propidium iodide to stain the cell nuclei (VectaShield, Vector Labs). The observations were performed using a confocal microscope (Pascal LSM 5, Carl Zeiss, Jena, Germany). A Zeiss LSM Image Examiner and performed using a confocal microscope (Pascal LSM 5, Carl Zeiss, Jena, Germany). The observations were performed using a 488-conjugated fluorescent secondary antibody (Invitrogen) to identify the primary antibody that bound to the Slack and Slick subunits.

The effect in the outward Na + current was not significantly different from that produced with 100 nM TTX (P = 0.391).

Given that vestibular neurons may also express TTX-resistant Na + channel subunits (Wootlorton et al., 2007), we evaluated the effects of replacing Na + with choline in the Ca 2+ -free extracellular solution (see Table 1). The perfusion of Na +-free solution abolished the inward current (Fig. 2) and reduced the peak amplitude of the outward current by 35 ± 4% (from 384 ± 25 pA/pF to 246 ± 15 pA/pF, n = 8, P < 0.001). The amplitude of the sustained component of the outward current at the end of the pulse was also reduced by 36 ± 3% (from 313 ± 29 pA/pF to 200 ± 18 pA/pF, n = 8, P < 0.001). The V 1/2 of the conductance–voltage relationship was −10 ± 2.2 mV (n = 8), which is a value that is near to the V 1/2 of the conductance–voltage curve that was blocked following TTX perfusion (−13.3 ± 4.3 mV).

VANs were identified by their birefringence under phase contrast illumination. These cells were recorded in voltage clamp conditions (V H = −60 mV). The inactivation of the Na + channels was removed using a 100-ms prepulse at −100 mV. Subsequently, 100-ms test pulses from −100 mV to +20 mV were used to generate voltage-activated currents. This protocol generated a fast-activating and fast-inactivating inward current followed by a slow-inactivating outward K + current. To remove the contribution of Ca 2+ -activated K + currents (I CaK) to the outward current, we eliminated Ca 2+ from the extracellular solution (see Table 1). In this condition a fast inward transient current which was blocked by 100 nM TTX (from −249 ± 38 pA/pF to −0 pA/pF; n = 13), was therefore identified as a Na + current (I Na). Notably the use of 100 nM TTX also reduced a sustained outward current by 18.2 ± 1.7% measured at 0 mV in this and in the following experiments (from 345 ± 50 pA/pF to 308 ± 46 pA/pF, n = 13, P < 0.001, Fig. 1A, B). The subtraction of the current measured during the TTX perfusion from that measured in the control condition revealed a TTX-sensitive inward current that was followed by a non-inactivating outward current. This latter current lasted the entire duration of the 100-ms voltage pulse (Fig. 1A). The presence of an outward current that is activated by the inflow of extracellular Na + through voltage-activated Na + channels is consistent with the presence of a I NaK. The V 1/2 of the conductance–voltage relationship for the K Na conductance was −13.3 ± 4.3 mV (range: −22.5 to −5 mV; n = 7, Fig. 1C). The use of 300 nM TTX diminished the I Na to ≥0 pA/pF (n = 5, P = 0.013) and subsequently reduced the outward K + current by 15 ± 0.9% from 328 ± 48 to 280 ± 42 pA/pF (n = 5, P = 0.002, data not shown).

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RESULTS

Removal of extracellular Na +

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The Na +-free solutions reduced a larger percentage of the outward current than the blocking of Na + channels with 100 nM TTX, suggesting that TTX-resistant currents have a significant role in the activation of the outward Na +-activated conductance or that the use of choline to replace Na + may also block outward currents (Delmas and Gola, 1995; Purohit and Grosman, 2006). To address this issue experiments applying TTX followed by Na + substitution by choline in the extracellular solution were done. The use of 100 nM TTX completely abolished the I Na ≈ 0 pA/pF (n = 5, P = 0.01) and decreased the outward K + current by 20 ± 5% (n = 5, P = 0.02). The use of Na +-free external (Na + substituted by choline) solution after TTX did not further modified the peak I Na but produced a significant reduction of the outward current of 40 ± 8% (P = 0.02). These results indicate that vestibular ganglion cultured neurons express a Na + -activated K + current that contributes to at least about 20% of the voltage-activated outward current in a Ca 2+ -free solution and that choline produced by itself a significant block of the outward currents.

Block of the Na + /K + -ATPase

Ouabain, which is a Na + /K + -ATPase blocker, was used to evaluate the effect of increasing the intracellular Na + concentration on the outward current (Fig. 3). The
perfusion with Ca\textsuperscript{2+}-free external solution and 1 mM ouabain significantly reduced the inward current (from $I_{Na} = 651 \pm 61 \ pA/pF$ to $I_{Na} = 471 \pm 51 \ pA/pF$, $P < 0.001$, $n = 9$) but did not modify the voltage dependence of the $I_{Na}$ (Fig. 3 B). These results indicate that the reduction in the inward current resulted from alterations in the Na\textsuperscript{+} equilibrium potential due to an increase in the intracellular Na\textsuperscript{+} concentration. In contrast, ouabain increased the maximum amplitude of the outward current by 23 ± 13\% (from 306 ± 44 pA/pF to 359 ± 51 pA/pF, $n = 9$, $P < 0.05$), and produced a 5 mV shift in the voltage dependence of $I_{KNa}$ to more negative voltages ($n = 9$, $P = 0.02$, Fig. 3C). The increase in the amplitude of the $I_{KNa}$ and the shift in the activation curve to more negative voltages are presumably due to the increase in the intracellular Na\textsuperscript{+} induced by ouabain, and coincide with the data found in the medial nucleus of the trapezoid body (MNTB) neurons (Yang et al., 2007).

Substitution of Li\textsuperscript{+} for extracellular Na\textsuperscript{+}

We analyzed the effect of replacing the extracellular Na\textsuperscript{+} with an equimolar concentration of Li\textsuperscript{+} on the amplitude of the outward current ($n = 10$). Li\textsuperscript{+} is known to permeate through voltage-gated Na\textsuperscript{+} channels but does not activate K\textsubscript{Na} channels (Dryer et al., 1989; Bischoff et al., 1998). Replacing Na\textsuperscript{+} with Li\textsuperscript{+} in the Ca\textsuperscript{2+}-free external solution (Table 1) reversibly decreased the amplitude of the inward current (Fig. 4A–C). The current that was carried by Li\textsuperscript{+} was 26 ± 2.4\% smaller than that carried by Na\textsuperscript{+} ($310 \pm 58 \ pA/pF$ versus $226 \pm 38 \ pA/pF$). The amplitude of the outward current was reduced by 20 ± 3.1\% at the peak and by 17.7 ± 3.1\% at the end of the pulse (from 421 ± 42 pA/pF to 333 ± 27 pA/pF and from 324 ± 44 pA/pF to 261 ± 30 pA/pF, respectively; $n = 10$, $P < 0.005$). The reduction in the outward current by Li\textsuperscript{+} substitution was similar to that obtained by blocking the $I_{Na}$ with 100 nM TTX. The substitution of Na\textsuperscript{+} by Li\textsuperscript{+} produced no significant displacement in the voltage dependence of $I_{KNa}$. The conductance of the Li\textsuperscript{+}-sensitive current had a $V_{1/2} = 18.3 \pm 2.4 \ mV$ and $S = 10.6 \pm 2.3$ (Fig. 4D). The effects of Li\textsuperscript{+} were reversed when Na\textsuperscript{+} was re-added to the extracellular medium.

Effect of low Cl\textsuperscript{−} solutions on the $I_{KNa}$ amplitude

Since the $I_{KNa}$ is also activated by intracellular Cl\textsuperscript{−} (Yang et al., 2007), experiments to analyze the effect of intracellular Cl\textsuperscript{−} on the $I_{KNa}$ were performed. For these experiments the NaCl and KCl salts in the extra and intracellular solutions were replaced by Na-isethionate and K-gluconate (Table 1), and the action of TTX in the outward current was studied. Both the intra and extracellular Cl\textsuperscript{−} were eliminated to avoid any inflow of Cl\textsuperscript{−} that might produce an uncontrolled modulation of the $I_{KNa}$. The junction potential of low Cl\textsuperscript{−} internal and external solutions (Table 1) was calculated to be 8.4 mV. The density of the outward current recorded using low Cl\textsuperscript{−} solutions was lower than that recorded using normal solutions. The use of 100 nM TTX on cells recorded with low Cl\textsuperscript{−} solutions ($n = 9$) decreased the $I_{Na}$ by

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**Fig. 1.** Effects of TTX on voltage-dependent currents. (A) Whole-cell currents of a representative neuron that were obtained from P7–P10 rats using Ca\textsuperscript{2+}-free solutions to remove the K\textsubscript{Ca} currents. The stimulation protocol consisted of a 100-ms prepulse to −100 mV followed by 100-ms test pulses from −100 to +20 mV ($V_{H} = −60 \ mV$). Insets show the $I_{Na}$ in an expanded time resolution (7 times). (B) The average outward current density as a function of voltage ($n = 13$). (C) The normalized conductance–voltage relationships for the TTX-sensitive K\textsuperscript{+} current ($n = 7$). Here and in the following figures, the continuous line in the conductance plot represents the Boltzmann fit (only data from −60 to +20 mV are plotted in this and similar graphs).
99.6%, from $-252 \pm 21 \text{pA/pF}$ to $-2.1 \pm 2 \text{pA/pF}$ ($n = 9; P = 0.01$) and the outward $K^+$ current density diminished by $13 \pm 2.6\%$, from $102 \pm 8 \text{pA/pF}$ to $88 \pm 6 \text{pA/pF}$ ($n = 9, P = 0.0014$, Fig. 5A). The reduction of the outward current produced by 100 nM TTX using low Cl$^-$ solutions was below that found while using normal internal and external solutions (18%), indicating that intracellular Cl$^-$ contributes to $I_{KNa}$ activation (Fig. 5B). The use of low Cl$^-$ solutions did not significantly modify the $I_{KNa}$ activation ($V_{1/2} = -11.7 \pm 2.5 \text{mV}, n = 9$, Fig. 5C). The Cl$^-$ sensitivity of TTX-sensitive outward $K^+$ current resembles that reported previously in auditory neurons for Slack channels, producing a twofold increase in the current amplitude, when the intracellular Cl$^-$ was raised from 0.134 to 145 mM (Yang et al., 2007).

The effects of substituting Li$^+$ for Na$^+$ on the voltage response in current clamp condition

To analyze the influence of $I_{KNa}$ on the properties of action potentials, we recorded the membrane responses to current pulses and examined the effects of substituting Li$^+$ for Na$^+$ in the Ca$^{2+}$-free external solution (Table 1). The perfusion of Li$^+$ solution produced no clear effect on the resting membrane potential. To avoid interferences due to alterations in the inactivation of Na$^+$ channels, the resting membrane potential was kept close to $-60 \text{mV}$ prior to and during the perfusion of the Li$^+$ solution. In experiments in which square current pulses from $-0.5$ to $0.5 \text{mA}$ and 100-ms duration were used, depolarizing pulses produced the firing of a single action potential during the whole 100-ms pulse in 10 cells, and in seven cells, a maximum of three action potentials was produced. The use of Li$^+$ solution did not significantly modify the number of action potentials in response to depolarizing pulses nor the latency of discharge of the first action potential (control latency = 2.5 ms, with Li$^+$ = 2.7 ms, $P = 0.39$). The perfusion of Li$^+$ did not modify the threshold ($-35.4 \pm 1.6 \text{mV}$) or the amplitude ($75 \pm 2.5 \text{mV}$) of the action potential (Fig. 6A, B). Li$^+$ did increase the duration of the action potential from $1.74 \pm 0.1 \text{ms}$ to
1.87 ± 0.1 ms ($P = 0.005$) and decreased the rate of repolarization from $-62.0 ± 5.0$ mV/ms to $-46.7 ± 10$ mV/ms ($P = 0.038$). The amplitude of the AHP was reduced from $1.2 ± 0.4$ mV to $0.4 ± 0.5$ mV ($n = 18; P = 0.007$), and this effect was completely reversible. Interestingly, Li$^+$ produced a significant reduction of the hyperpolarization at the end of stimulating current pulse (from $-6.4 ± 0.8$ mV in control conditions to $-2.3 ± 0.8$ mV after Li$^+$ perfusion; $P = 0.001$, Fig. 6C). In nine out of 15 neurons Li$^+$ inhibited the rebound discharge following a hyperpolarizing current pulse injection (data not shown). Only two of the neurons examined did not fire a rebound action potential.

In experiments in which sinusoidal current injection was used, all the cells tested discharge in phase (mostly between 50–90 degrees) with the sinusoidal stimuli, and 35% of the neurons produced two or more action potentials phase locked with every stimulus cycle. This is in stark contrast with the discharge response elicited by square pulses where only 18% of the cells produced repetitive firing. The perfusion with Ca$^{2+}$-free external Li$^+$ solution produced a phase shift in the action potential discharge and in five cells decreased the number of action potentials produced in every cycle from 1.4 to 0.8 ($n = 17$, Fig. 7).

This result indicates a fast activation of $I_{KNa}$ and consequently a significant participation of this current in the setting of firing regularity, phase locking and the discharge pattern in VANs.

**RT-PCR of Slack and Slick mRNA expression**

$K_{Na}$ have been reported to consist of only Slick (Slo2.1) and Slack (Slo2.2) subunits (Gribkoff and Kaczmarek, 2009). We used RT-PCR to determine whether the mRNA transcripts for these subunits are expressed in the vestibular ganglia and the vestibular end organs. Brain and dorsal root ganglia tissue were used as positive controls (Bischoff et al., 1998; Bhattacharjee et al., 2002, 2003). We observed single bands with molecular weights of $\approx 100$ bp for both of the subunits in the samples that were isolated from the vestibular ganglia and the vestibular epithelium. The molecular weight of these bands is in close agreement with the expected size of 95 and 99 bp for Slack and Slick, respectively. These bands were obtained in the positive controls and were absent from the negative controls (Fig. 8).

**Immunoreactivity of the Slack and Slick subunits**

Given that the mRNA for Slick and Slack was detected in the vestibular ganglion and the vestibular sensory epithelium, we sought to determine the expression of these subunits in cultured vestibular ganglion neurons.
In agreement with the electrophysiological experiments, cultured neurons expressed the Slick and Slack subunits (Fig. 9). The somatic diameter of P7–P10 rat neurons that were immunoreactive for Slick was $21 \pm 1 \mu m$ ($n = 26$). For Slack-immunoreactive neurons, this value was $19 \pm 1 \mu m$ ($n = 30$). No
appreciable difference in the Slack and Slick expression in terms of neuronal size was found. The expression of Slack and Slick was also observed in P21–P24 rat neurons. The somatic diameter of Slick- and Slack-positive neurons was also similar between them. The expression of these subunits was investigated in the vestibular ganglion and in the vestibular sensory epithelium of P7–P10 and P21–P24 rats. Slick and Slack immunoreactivity was detected in the neuronal somas of the vestibular ganglia at both examined ages. We also observed clear immunoreactivity in the fibers that innervate the vestibular epithelium and in the afferent terminals that contact the hair cells in the crista ampullaris and the macula utricle (Fig. 9). The control expression assays were performed using sections from the dorsal root ganglion, in which $K_{Na}$ expression has been previously demonstrated (Bischoff et al., 1998). No immunoreactivity was observed in any of the negative controls.

**DISCUSSION**

Our results showed that a $K_{Na}$ conductance is expressed in the primary afferent vestibular neurons. The mRNA for the two subunits constituting the $K_{Na}$ conductance (Slack and Slick) was identified in the vestibular ganglia. The pattern of expression of these subunits showed that $K_{Na}$ subunits were found to be expressed by the vestibular ganglion neurons. It was also found that Slack and Slick antibodies produced a staining pattern that is consistent with the expression of $K_{Na}$ channels in the somas and in dendrites of the vestibular primary afferents. Electrophysiological recordings showed that vestibular ganglion neurons expressed an outward current whose characteristics are consistent with the properties of $I_{KNa}$ as discussed below. This current accounts for about 20% of the outward current in Ca$^{2+}$-free solutions. Its contribution in other regions such as the calyx- or button endings cannot be determined from our experiments.

$K_{Na}$ has been reported to be expressed in cardiomyocytes (Kameyama et al., 1984; Luk and Carmeliet, 1990) and in several types of neurons throughout the central and peripheral nervous system (Dryer et al., 1989; Schwindt et al., 1989; Haimann et al., 1990; Egan et al., 1992; Dale, 1993; Bischoff et al., 1998; Liu and Leung, 2004; Hess et al., 2007). However, its presence in the vestibular system had not been explored. Here, we describe for first time the expression of a sustained outward current that was blocked by the removal of extracellular Na$^{+}$ and by the blocking of Na$^{+}$ channels with TTX. This current was potentiated by the increase in the intracellular Na$^{+}$ following the block of Na$^{+}$/K$^{+}$-ATPase. This outward current is consistent with the functional expression of $K_{Na}$ channels in VANs. Interestingly, the contribution of $I_{KNa}$ to the outward current that was obtained using Na$^{+}$-free solutions was larger than that obtained using 100 nM TTX. To the extent of our knowledge choline, albeit affecting some K$^{+}$ currents, does not affect $K_{Na}$ channels and for that reason is one of the most widely
used methods to isolate and study $I_{KNa}$ currents. This result suggests that in our experimental conditions, where recordings were made from the cell body of isolated neurons, most of $KNa$ channels are coupled to TTX-sensitive $Na^+$ current; however it cannot be excluded that a fraction of the $I_{KNa}$ may be generated by TTX-resistant $Na^+$ channels (most probably Nav 1.5 subunits) that have been reported in microdomains of VANs (Lysakowski et al., 2011).

The presence of mRNA for Slick and Slack in the vestibular ganglia is consistent with the electrophysiological recordings of $I_{KNa}$; moreover, the detection of...
the mRNA for these subunits in the sensory epithelium may have several explanations. Given that (i) there is a clear immunoreactivity for Slick and Slack in the afferent terminals that contact the hair cells in the vestibular epithelium and (ii) it is known that several mRNAs localize to dendrites (Martin and Zukin, 2006), we speculate that the Slick and Slack mRNA in the vestibular epithelia is present in the nerve terminals of the vestibular afferents. Another possibility is that hair cells or certain epithelial cells exhibit an \( I_{\text{KNa}} \). The expression of Slick and Slack subunits in the calyx terminals opens interesting possibilities. For example, the presence of the Na\(^+\) channel subunits Nav1.5 and Nav1.6 in the microdomains of calyx terminals was recently reported (Lysakowski et al., 2011). Nav1.6 is known for its capacity to generate persistent Na\(^+\) currents in axonal initial segments and for its role in the regulation of neuronal spike timing (Chatelier et al., 2010; Osorio et al., 2010). Therefore, whether \( I_{\text{KNa}} \) open probability is high at resting potential in VANs and participates in the low-voltage-activated outward current that is observed in calyx terminals should be further investigated. Moreover, the immunoreactivity of Slick and Slack subunits in the hair cells and in certain calyx and button endings suggest an important role for the \( I_{\text{KNa}} \) in the firing of the vestibular afferents. The Na\(^+\) influx through ionotropic channels has been considered as a charge carrier without effect on synaptic integration; however it was shown that \( I_{\text{KNa}} \) may play a significant modulatory role in glutamatergic synapses, such as hair cell afferent synapse, in which Na\(^+\) entering through AMPA receptors may activate \( I_{\text{KNa}} \) thus limiting the depolarization induced by AMPA receptor activation and contribute to shaping of synaptic potentials (Nanou and

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**Fig. 9.** Slack and Slick immunoreactivity in cultured vestibular afferent neurons and in sections from the vestibular ganglia and the vestibular neuroepithelia. The green signal indicates Slack and Slick immunofluorescence. The red signal indicates propidium iodide staining of cell nuclei. The cells were isolated from P7–P10 and from P21–P24 rats (indicated). In the left column, the negative controls (primary antibody omitted) did not exhibit immunolabeling for Slack or Slick in any of the conditions that are shown. The most thinner and ordered nuclei presumably correspond to glial cells; they are particularly conspicuous in the cultured P25–28 photograph done at lower amplification. The sections of vestibular ganglia from P7–10 exhibited staining for Slack and Slick. Slack and Slick expression is clearly observed in the soma of cultured neurons from P7–P10 and P21–P24 rats. The sections of the vestibular epithelia from P21–P24 rats exhibited very distinct immunoreactivity for Slack and Slick. For Slack staining, a clear calyx surrounding a hair cell is shown (asterisk). For Slick staining, the trajectory of an afferent neuron and calyx ending are visible as a series of fluorescent puncta, depicting the neuron terminal trajectory (arrows). Scale bars = 20 μm in all the images.
El Manira, 2007). Also, preliminary information from our laboratory suggests that $I_{\text{KNa}}$ can be activated by inflow of Na$^+$ through acid sensing ionic channels.

No differences were found in the Slack and Slick expression among all the neurons investigated irrespective of their size, nor a significant developmental change in the distribution of Slack and Slick.

In the current clamp experiments, the AHP of Li$^+$-driven action potentials, which does not activate $I_{\text{KNa}}$ channels, was significantly smaller than the AHP of Na$^+$-driven action potentials. These observations indicate that a single action potential was sufficient to activate the $I_{\text{KNa}}$ and that the fast activation of $I_{\text{KNa}}$ was most likely due to the clustering of Na$^+$ and $I_{\text{KNa}}$ channels (Koh et al., 1994). Apart from the reduction of the AHP, the use of Li$^+$ also modified the postpotential trajectory, dampening the oscillations of membrane potential and reducing further spiking activity, effect that was particularly evident while studying the repetitive discharge produced by sinusoidal stimuli in which Li$^+$ reduced the spiking activity of vestibular neurons. In our experiments evidence suggest that $I_{\text{KNa}}$ activation contributes to membrane potential oscillations and to further action potential discharge. These actions have a significant impact in the phase locking of the action potential discharge, particularly affecting the number of spikes per cycle. Importantly, Li$^+$ significantly reduced the postpulse hyperpolarization that has been usually attributed to the activation of SK channels that are sensitive to apamin (Cangiano et al., 2002). In our case we have no Ca$^{2+}$ in the extracellular solution thus uncovering the participation of $I_{\text{KNa}}$ in the postpulse hyperpolarization.

Our electrophysiological results are in agreement with those obtained in neocortical intrinsically bursting neurons, in which the substitution of extracellular Li$^+$ for Na$^+$ did not significantly affect the shape of the action potentials but inhibited the slow AHP that controls their intrinsic bursting, even in single-action potentials (Franceschetti et al., 2003). In the lamprey spinal neurons that control locomotion, one component of the AHP that follows a single spike appears to result from a $I_{\text{KNa}}$ conductance (Cangiano et al., 2002).

In summary, we demonstrate the presence of Slick and Slack subunits in the vestibular ganglia and the vestibular epithelium. The expression of these subunits is consistent with a Na$^+$-dependent sustained outward K$^+$ current in vestibular ganglion neurons. Given that (i) this current participates in the AHP that follows single-action potentials and (ii) $I_{\text{KNa}}$ are expressed in the regions of most probable Na$^+$ accumulation (e.g., the afferent terminals), this current may participate in firing regularity and spike timing precision in the transmission of vestibular information.

Finally it is worth noting that binding of fragile X mental retardation Protein (FMRP), an RNA-binding protein, to the Slack subunit has been shown to potentiate the $I_{\text{KNa}}$ current, and that the FMRP is damaged in fragile X syndrome a disorder due to trinucleotide repeat expansions (Zhang et al., 2012). It has been found that a common sign in patients with fragile X syndrome is the presence of nistagmus and in many patients balance problems are significant, thus indicating the involvement of the vestibular system (Buscà Safor-Tria, 2001). Although, the potential impact of $I_{\text{KNa}}$ in neuronal activity is still in the process of definition, vestibular alterations in fragile X syndrome may well be related to $I_{\text{KNa}}$ current alterations and subsequent degradation of the sensory coding in the vestibular system. However, the overall functional significance of $I_{\text{KNa}}$ is surely not marginal and it may be a leading K$^+$ current in determining the properties of some neuronal systems, opening a significant new window for the understanding of neuronal activity, pathological processes and constitute a new potentially relevant pharmacological target.

Acknowledgments—This work was supported by grants from PIFI 2011, CONACyT grant 167052 to E.S. and VIEP-BUAP grants 2011 to E.S. and to R.V. B.C. was supported by CONACyT fellowship 22839. The authors thank Dr. Francisco Mercado for performing the PCR experiments. The English text was edited by Nature Publishing Group Language Editing service.

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