Ca$^{2+}$-Activated K$^+$-Current Density Is Correlated With Soma Size in Rat Vestibular-Afferent Neurons in Culture

Agenor Limón, Cristina Pérez, Rosario Vega, and Enrique Soto

Institute of Physiology, Autonomous University of Puebla, Puebla and Institute of Cellular Physiology, National Autonomous University of Mexico, Mexico City, Federal District, Mexico

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Vestibular-afferent neurons (VANs) transmit information about linear and angular accelerations during head movements from vestibular end organs to vestibular nuclei. In situ, these neurons show heterogeneous discharge patterns that may be produced by differences in their intrinsic properties. However, little is known about the ionic currents underlying their different firing patterns. Using the whole cell patch-clamp technique, we analyzed the expression of Ca$^{2+}$- and Ca$^{2+}$-activated K$^+$ currents ($I_{\text{Ca}^{2+}}$) and $I_{\text{BK}}$ in primary cultured neurons isolated from young rats (p7–p10). We found two overlapping subpopulations of VANs classified according to low-threshold Ca$^{2+}$-current (low-voltage-activated [LVA]) expression; LVA (+) neurons, formed by small cells, and LVA (+) neurons composed of medium to large cells. $I_{\text{Ca}^{2+}}$, in both cell-groups was carried through channels of high (BK), intermediate (IK), and low conductance (SK), besides a resistant channel to classical blockers (IR). BK was expressed preferentially in LVA (+) cells, whereas IK expression was preferentially in LVA (-) cells. No correlation between SK and IK expression with the soma size was found. Current-clamp experiments showed that BK participates in the adaptation of discharge and in the duration of the action potential, whereas SK and IK did not show a significant contribution to electrical discharge of cultured VANs. However, because of the low number of VANs in culture with repetitive firing it is difficult to interpret our results in terms of discharge patterns. Our results demonstrate that vestibular-afferent neurons possess different Ca$^{2+}$-activated K$^+$ channels and that their expression, heterogeneous among the cells, would contribute to explain some of the differences in the electrical-firing properties of these neurons.

Introduction

The vestibular system transforms mechanical stimuli from linear and angular accelerations during head movements into spike trains that are transmitted to the vestibular nuclei through bipolar-afferent neurons. Vestibular-afferent neurons (VANs) have a resting discharge in the absence of any stimuli that depend on the spontaneous release of a neurotransmitter from the sensory hair cells (Annoni et al. 1984; Soto and Vega 1988; Starr and Sewell 1991). Based on the coefficient of variation of their resting discharge, VANs have been classified into regular and irregular cells. However, there is no clear separation into two neuronal subgroups because the regularity of the discharge varies from the most irregular to regular cells forming a continuum among all cells (Goldberg and Fernández 1971; Honrubia et al. 1989). VAN morphology has been correlated with the regularity of the resting discharge. Calyx-ending neurons, with the largest somas and thick afferent dendrites that innervate type I hair cells mainly located in the central zones of the sensory neuroepithelia, have an irregular resting discharge. The smallest neurons, with thin dendrites that establish bouton synapses with type II hair cells in the peripheral zones of sensory epithelia, have a regular resting discharge. Dimorphic neurons, with medium soma size innervating both type I and type II hair cells, are distributed throughout the sensory epithelia and have intermediate electrical properties (Fernández et al. 1988, 1995; Kevetter and Leon 2002; Leonard and Kevetter 2002; Lysakowski et al. 1995; Si et al. 2003).

Besides this, vestibular neurons exhibit differences in their dynamic spike response to mechanical (Baird et al. 1988; Curthoys 1982; Goldberg and Fernández 1971; Lysakowski et al. 1995) and electrical stimulation (Bronté-Stewart and Lisperger 1994; Ezure et al. 1983; Goldberg et al. 1987) of the membranous labyrinth. Differences in the firing properties of VANs cannot be explained solely by their synaptic input (Smith and Goldberg 1986) nor by the type of hair cells they innervate because vestibular neurons from animals lacking type I hair cells can also be grouped into irregular and regular types with a similar regional distribution of their terminals within the neuroepithelia (Honrubia et al. 1989; Myers and Lewis 1990). Thus as suggested (Goldberg 2000; Smith and Goldberg 1986), ionic conductance of vestibular neurons may vary depending on the location of the cells within the neuroepithelia that these neurons innervate. However, there is little information about the ionic conductance expressed by vestibular neurons in mammals. Studies in embryonic and neonatal mice have shown that VANs express a tetrodotoxin (TTX)-sensitive Na$^+$ current (Chabbert et al. 1997), a hyperpolarization-activated inward current (Chabbert et al. 2001b), three voltage-dependent K$^+$ currents (Chabbert et al. 2001a), and a voltage-dependent Ca$^{2+}$ current composed of L-, N-, P/Q-, R-, and T-type channels (Chambard et al. 1999; Desmadryl et al. 1997). Only the T-type current has been shown to have a heterogeneous distribution among afferent neurons (Chambard et al. 1999; Desmadryl et al. 1997), leaving unsolved the question of a putative differential expression of ionic conductance that in turn could be correlated with the differences in the firing pattern observed in VANs.

In mathematical models, interspike-interval statistics, sensitivity to galvanic currents, and the relation between discharge...
VAN regularity and galvanic sensitivity can be accounted for by interactions between the synaptic noise and the slope of the afterhyperpolarization (AHP) (Smith and Goldberg 1986), which is in part dependent on the Ca\(^{2+}\text{-}\)activated K\(^{+}\) current (\(I_{\text{KCa}}\)) characteristics (Cloues and Sather 2003; Sah 1996). At present, there is only one report of big conductance current (BK) channels in the sacular nerve of goldfish, in which it was shown that BK current is selectively expressed in a population of cells innervating the caudal portion of the saccular macula (Davis 1996). In this work, we report for the first time that in the primary afferent neurons of the vestibular system the Ca\(^{2+}\text{-}\)activated K\(^{+}\) current (\(I_{\text{KCa}}\)) is composed of four components: 1) big conductance current (BK), sensitive to iberiotoxin (IbTx); 2) small conductance current (SK), sensitive to apamin; 3) intermediate conductance current (IK), sensitive to clotrimazole (CLT) and charibdotoxin (ChTx); and 4) a resistant current (IR) that is not sensitive to any of the drugs used in this work and that is activated by extracellular Ca\(^{2+}\). In addition we found that the current density of total \(I_{\text{KCa}}\) the BK, and the IR were correlated with soma size and with the expression of a low-voltage-activated (LVA) Ca\(^{2+}\) current. Current-clamp experiments using the perforated-patch technique indicate that the BK current participates in the adaptation of discharge and in the repolarization of the action potential of cultured VANs.

Some of these results were previously presented in abstract form (Limon et al. 2003).

**METHODS**

Young Wistar rats of either gender were used for the experiments. Animal care and procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Reglamento de la Ley General de Salud en Materia de Investigación para la Salud of the Secretaría de Salud de México. All experimental procedures were done after approval by an appropriate committee within the institution. 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” issued by the National Academy of Sciences.

Cell somata of rat VANs initiate their myelinization around the eighth day after birth (p8) (Toescu 1997). To maintain accessibility to neuronal plasmalemma with patch pipettes, we decided to use rats at eighth day after birth (p8) (Toesca 1997). To maintain accessibility to the primary afferent neurons of the vestibular system the Ca\(^{2+}\text{-}\)activated K\(^{+}\) current (\(I_{\text{KCa}}\)) is composed of four components: 1) big conductance current (BK), sensitive to iberiotoxin (IbTx); 2) small conductance current (SK), sensitive to apamin; 3) intermediate conductance current (IK), sensitive to clotrimazole (CLT) and charibdotoxin (ChTx); and 4) a resistant current (IR) that is not sensitive to any of the drugs used in this work and that is activated by extracellular Ca\(^{2+}\). In addition we found that the current density of total \(I_{\text{KCa}}\) the BK, and the IR were correlated with soma size and with the expression of a low-voltage-activated (LVA) Ca\(^{2+}\) current. Current-clamp experiments using the perforated-patch technique indicate that the BK current participates in the adaptation of discharge and in the repolarization of the action potential of cultured VANs.

**Soma size measurements**

Although the electrical capacitance of a membrane depends on the membrane area of the cells, and it is an indirect measurement of soma size, we decided to analyze the correlation between soma diameter and membrane capacitance to ensure that there were no differences in cellular-membrane characteristics between small and large cells, such as differences in the membrane folding (García-Pérez et al. 2004) that could prevent the use of membrane capacitance as an indicator of soma size. For this, cell images were acquired with a CCD camera (TI-24A, NEC, Elk Grove Village, IL) mounted in a tricocular microscope (Diaphot, Nikon) and digitized with a frame-grabber board DT2867-LC (Data Translation, Marlboro, MA). The soma diameter was calculated as the average of major and minor longitudinal axes using the Global Lab Image (Data Translation) software tools.

**Electrophysiological recording**

The culture dish with attached neurons was mounted on the stage of an inverted phase-contrast microscope (TMS, Nikon). Membrane ionic currents and voltage changes in the cell membrane were studied by standard protocols of whole cell voltage-clamp and current-clamp techniques at room temperature (23–25°C) using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Some of the current-clamp experiments, as indicated in RESULTS, were done at temperatures of 35 to 37°C using the perforated-patch technique with 260 μM amphotericin B (Sigma-Aldrich). Command-pulse generation and data sampling were controlled by pClamp 7.0 software (Axon Instruments) using a 12-bit data-acquisition system (Digidata 1200, Axon Instruments). Signals were low-pass filtered at 5 or 2 kHz and digitized at 20 or 10 kHz depending on the ionic current under study. Patch pipettes were pulled from borosilicate glass capillaries (TW120-3; WPI, Sarasota, FL) using a Flaming–Browne electrode puller (80/3C; Sutter Instruments, San Rafael, CA); they typically had a resistance of 1 to 3 MΩ when filled with internal solutions. Cells were bathed with different solutions depending on the experimental protocol (Table 1). To isolate the Ca\(^{2+}\) current (\(I_{\text{Ca}}\)), Cs\(^+\), tetraethylammonium (TEA), and 4-aminopyridine (4-AP) were used to eliminate outward K\(^+\) currents (Table 1, external and internal Cs\(^+\) solutions) and TTX was used to block Na\(^+\) currents. For the analyses of...
the Ca\(^{2+}\)-activated K\(^+\) current (I\(_{\text{KCa}}\)). Na\(^+\) and 4-AP–sensitive K\(^+\) currents were blocked. The outward current elicited using internal and external choline solutions consisted of the I\(_{\text{KCa}}\) and a voltage-dependent, Ca\(^{2+}\)-independent outward current resistant to 4-AP. To evaluate the total I\(_{\text{KCa}}\) component, cells were perfused with a Ca\(^{2+}\)-free, 5 mM EGTA external choline solution (Table 1) and the I\(_{\text{KCa}}\) was obtained by subtraction. The free Ca\(^{2+}\) concentration in the internal solutions using 2 mM EGTA was about 4 nM estimated with the Maxc software (from Chris Patton, Hopkins Marine Station, Stanford University). In current-clamp experiments, internal and external solutions were the same as those used to analyze I\(_{\text{KCa}}\), except that choline-Cl was replaced by NaCl 18 (internal) and by 140 mM (external) and the CaCl\(_2\) in the external solution was set to 1.8 mM (Table 1, internal and external CC). All internal solutions also contained 2 mM ATP-Mg and 0.5 mM GTP-Na. The pH was adjusted for the external solution to 7.4 and for the internal solution to 7.2. Osmolarity was monitored by a vapor pressure osmometer (Wescor, Logan, UT) and was around 300 mOsm for internal solutions and adjusted with dextrose to 310 mOsm for external solutions.

The analysis of the pharmacological properties of I\(_{\text{KCa}}\) channels underlying the I\(_{\text{KCa}}\) was made with 100 nM IbTx, 100 nM ChTx, 100 nM CLT (Sigma-Aldrich) added to the correspond-

### TABLE 1. Solutions

<table>
<thead>
<tr>
<th>Sol</th>
<th>NaCl</th>
<th>Choline Cl</th>
<th>KCl</th>
<th>CaCl</th>
<th>CaCl(_2)</th>
<th>MgCl(_2)</th>
<th>TEA</th>
<th>4-AP</th>
<th>EGTA</th>
<th>HEPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>I(_{\text{Ca}}) External Cs(^{+})</td>
<td>5</td>
<td>3.6</td>
<td>1.2</td>
<td>130</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I(_{\text{Ca}}) Internal Cs(^{+})</td>
<td>140</td>
<td>0.134</td>
<td>10</td>
<td>2</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I(_{\text{KCa}}) External choline*</td>
<td>130</td>
<td>5.4</td>
<td>3.6</td>
<td>1.2</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I(_{\text{KCa}}) Ca(^{2+})-free choline solution*</td>
<td>130</td>
<td>5.4</td>
<td>0</td>
<td>1.2</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>I(_{\text{KCa}}) Internal choline</td>
<td>10</td>
<td>140</td>
<td>0.134</td>
<td>2</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>External CC</td>
<td>140</td>
<td>5.4</td>
<td>1.8</td>
<td>1.2</td>
<td>10</td>
<td>10</td>
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<td></td>
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</tr>
<tr>
<td>Internal CC</td>
<td>18</td>
<td>140</td>
<td>0.134</td>
<td>2</td>
<td>5</td>
<td></td>
<td></td>
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The leftmost column indicates the ionic current that each solution was used for. External and internal CC solutions were used for current-clamp experiments. *Added with TTX 200 nM.

### Action potential analysis

Some of the current-clamp experiments as indicated in RESULTS were done at temperatures of 35 to 37°C using the perforated-patch technique with 260 µM amphotericin B. In these experiments, action-potential variables were measured. For this, the voltage value of the threshold level was used as a reference point. Threshold was defined in two forms: 1) as the point where the time course of a voltage response to a suprathreshold pulse diverges from a single exponential fit to the electrical charging of the membrane [the exponential fit was of the form V(t) = exp(−t/τ) + C, where V is the voltage, τ is time, and C is the time constant of the cell membrane]; 2) as the point where the first-order derivative dV/dt of membrane potential reached a value higher than the noise 5 ms before spike onset (Leger et al. 2005), there were no significant differences using both methods. The amplitude of the action potential was defined as the peak action-potential value minus the threshold voltage. The action-potential duration was measured at 75% of the spike amplitude.

### Data analysis

Recordings were analyzed off-line using Clampfit in the pClamp 7.0 suite and Oring software (Microcal Software, Northampton, MA). Statistical differences of means were determined using a Student’s t-test, considering significant those with a P < 0.05. Curve-fitting routines were made by using a nonlinear least-squares method. Pooled data are presented as means ± SE unless otherwise stated.

### RESULTS

Vestibular-afferent neurons in culture were identified by their birefringent round or ovoid soma when viewed under phase-contrast optics (Fig. 1A). The relationship between membrane capacitance and neuronal size showed a linear correlation (r = 0.92), indicating that membrane capacitance can be used as an indirect measure of soma size (Fig. 1B). Previous reports indicate that in acutely dissociated VANs from embryonic mice, the largest cells express a LVA-Ca\(^{2+}\) current, whereas the high-voltage–activated (HVA) Ca\(^{2+}\) current is expressed homogeneously in all cells (Chambard et al. 1999; Desmadryl et al. 1997). Thus we analyzed the Ca\(^{2+}\) current to define whether cultured rat VANs have the same relationship between soma size and Ca\(^{2+}\) current expression.
Voltage-dependent calcium current $I_{Ca}$

The isolated Ca$^{2+}$ current ($I_{Ca}$) was studied in 35 neurons. In 26% of the cells ($n = 35$), $I_{Ca}$ was formed exclusively by HVA components, whereas in the remaining cells (74%) both LVA and HVA components were identified (Fig. 2, A and B). The $I_{Ca}$ in LVA-lacking [LVA (-)] cells activated near $-45$ mV has its maximum amplitude around $-10$ mV, inactivating partially during the 800-ms pulse ($n = 9$). The maximum peak current density in these cells was $30 \pm 6$ pA/pF. In the LVA-expressing [LVA (+)] neurons, the Ca$^{2+}$ current activated around $-60$ mV and completely inactivated with a single time constant of $31 \pm 2$ ms for pulses at $-40$ mV ($n = 26$). The HVA current in LVA (+) cells was evident around $-30$ mV and partially inactivated during the 800-ms pulse. The maximum peak current density around $-10$ mV in LVA (+) neurons was $-49 \pm 8$ pA/pF.

To analyze whether there was any correlation between HVA current density and membrane capacitance, the current density at the end of an 800-ms pulse to $-10$ mV was calculated in both neuronal subtypes. This allowed us to compare HVA current between LVA (-) and LVA (+) cells because at 800 ms the contribution of a T-type current in LVA (+) cells was negligible. The HVA current density between LVA (-) (17.5 $\pm$ 4 pA/pF; $n = 9$) and LVA (+) cells (12.5 $\pm$ 2 pA/pF; $n = 26$) was not significantly different ($P > 0.05$). In experiments made to study the $I_{KCa}$ in which the Ca$^{2+}$ current was not isolated, it was also possible to identify the LVA (-) and LVA (+) neurons by analyzing the current caused by a test pulse to $-53$ mV from a $V_H$ of $-113$ mV (Fig. 2C, insets). Membrane capacitances of cells identified using this procedure or by analysis of isolated $I_{Ca}$ were pooled according to LVA expression (Fig. 2C). Cell capacitance had a normal distribu-
tion of 18.5 ± 7 pF (mean ± SD; n = 19/68) for LVA (−) and 39 ± 15 pF (n = 49/68) for LVA (+). The statistical difference in capacitance (soma size) between these subgroups was significant (P < 0.0005). Experiments made in VANs isolated from p23 to p26 rats indicate that the separation of LVA (−) and LVA (+) cells is still present in the mature vestibular system. The mean ± SD of membrane capacitance for LVA (−) cells was 12 ± 2 pF (n = 5) and for LVA (+) it was 29 ± 1 pF (n = 10; P < 0.0005).

**Calcium-activated potassium current I\(_{\text{KCa}}\)**

The expression of I\(_{\text{KCa}}\) in VANs was analyzed by recording outward currents in external and internal choline solutions and during perfusion with Ca\(^{2+}\)-free external choline solution (Table 1). The control current was composed of I\(_{\text{Ca}}\), I\(_{\text{KCa}}\), and a voltage-dependent, 4-AP–insensitive K\(^+\) current. The perfusion with Ca\(^{2+}\)-free external choline solution reversibly reduced the outward current, leaving the Ca\(^{2+}\)-independent components. The I\(_{\text{KCa}}\) component of the outward current was obtained by subtraction of the current remaining after perfusion with the Ca\(^{2+}\)-free external solution from the control current (Fig. 3A). The I\(_{\text{KCa}}\) component of the outward currents was observed in 100% of acutely dissociated (n = 6) and cultured neurons (n = 47). The I\(_{\text{KCa}}\) activated above −40 mV reached its maximum around 10 mV, followed by a decline in the current amplitude for more depolarized values (Fig. 3B). Total I\(_{\text{KCa}}\) current density was 87 ± 7 pA/pF and it was not significantly different (P > 0.05) in cultured or acutely dissociated neurons.

The analysis of correlation between membrane capacitance and I\(_{\text{KCa}}\) shows that small LVA (−) cells express three times higher I\(_{\text{KCa}}\) current density (171 ± 32 pA/pF; n = 8) than larger LVA (+) cells (54 ± 5 pA/pF; n = 17; P < 0.01) (Fig. 3C). Two cell groups are evident based on the expression of the LVA-Ca\(^{2+}\) current. Without this association, the analysis of correlation between the membrane capacitance and I\(_{\text{KCa}}\) has a continuous pseudoeponential form.

I\(_{\text{KCa}}\) was also observed in the 100% of cells obtained from adult p23 to p26 rats (n = 15). The total I\(_{\text{KCa}}\) current density in VANs from p23 to p26 rats was 318 ± 75 (range 105 to 968 pA/pF). The difference in I\(_{\text{KCa}}\) current density between young and adult VANs was considerably significant (P < 0.0001), indicating an increase in I\(_{\text{KCa}}\) expression during the postnatal development. The I\(_{\text{KCa}}\) current density of LVA (−) cells (681 ± 99 pA/pF; n = 5) was also significantly higher than that found in LVA (+) cells (137 ± 10 pA/pF; n = 10) (P < 0.001) (Fig. 3D).

The correlation between current density and membrane capacitance shows that I\(_{\text{KCa}}\) decreased as the cell membrane capacitance increased (r = 0.92), indicating a qualitatively similar relationship between p7 to p10 and p23 to p26 VANs. The range and the mean of membrane capacitance of the p23 to p26 cells were smaller because of a smaller culturing time (5 h for p23–p26 compared with 18 h in p7–p10 neurons).

The outward current that remains during perfusion with Ca\(^{2+}\)-free solution showed a significant difference (P < 0.05) between LVA (−) (58 ± 7 pA/pF; n = 8) and LVA (+) (38 ± 4 pA/pF; n = 17) cells. No efforts to characterize that current were made.

**Calcium-activated potassium current components SK, BK, IK, and IR**

To analyze I\(_{\text{KCa}}\) components in cultured vestibular-afferent neurons from p7 to p10 rats, the pharmacological agents used...
were specific blockers for BK (100 nM IbTx) (Galvez et al. 1990; Giangiacomo et al. 1992), SK (100 nM apamin) (Hugues et al. 1982), and IK (1 μM CLT) (Grissmer et al. 1993; Jensen et al. 2001; Kaczorowski and Garcia 1999). In some experiments, ChTx was also used to isolate the IK current. Because ChTx blocks both BK and IK channels (Jensen et al. 2001; Kaczorowski and Garcia 1999), BK channels were first blocked with IbTx and then cells were subsequently perfused with an external solution with ChTx + IbTx added. IK was obtained by the subtraction of the current during IbTx perfusion from the current elicited with IbTx + ChTx. We found a Ca²⁺-activated K⁺ current that was resistant to classical IₖCa blockers (100 nM IbTx, 100 nM ChTx, 100 nM apamin, and 1 μM CLT). Because no specific blockers for this current exist, we obtained the resistant current (IR) by removal of external Ca²⁺ after IbTx + apamin + ChTx/CLT application.

The cells used to study IₖCa components extend within all the range of capacitances. Values (mean ± SD) of the initial total outward current (Ca²⁺-independent K⁺ current + IₖCa) of the cells used to analyze each IₖCa component before drug application were 115.4 ± 83.8 pA/pF for SK (n = 35), 111.7 ± 86.8 pA/pF for IK (n = 15), 114.1 ± 87.7 pA/pF for BK (n = 30), and 148.7 ± 130.3 for IR (n = 15). There were no statistical differences between these data (P > 0.05), indicating that the cell sample used to make the analyses of IₖCa components was not biased.

The perfusion of 100 nM apamin reduced the outward current in 86% of the cells (n = 30/35) (Fig. 4A). Subtraction of the current during apamin perfusion from control current gave the SK current. The SK component activates for voltages positive to −30 mV and reaches its maximum amplitude between 5 and 10 mV (data not shown). The SK current had a slow activation and did not decay during the 800-ms pulse, indicating a minimal, if any, inactivation. The maximum current density of SK was classified according to LVA expression. The SK current density was not different between LVA (−) and LVA (+) cells (8.3 ± 3.8 vs. 13.6 ± 3.5 pA/pF; P > 0.05). No significant correlation between SK expression and cell membrane capacitance was found (r = 0.21), indicating that the SK current is expressed independently of VAN size (Fig. 5, A and B).

The perfusion of 100 nM IbTx reduced the outward current in 73% of the cells (n = 22/30) (Fig. 4B). Digital subtraction of the outward current before and during IbTx perfusion yielded the BK current. The BK current activated at voltages positive to −60 mV in 64% of the cells (n = 14/22). In the remaining cells BK activated around −40 mV. The maximum amplitude was observed around 20 mV (data not shown). The plot of BK current density versus cell membrane capacitance (r = 0.66) indicates that small LVA (−) cells have a smaller BK than that of larger LVA (+) cells, which have higher BK activity.

**Fig. 4.** Subtypes of Ca²⁺-activated K⁺ currents. Currents were recorded in external and internal choline solutions. A and B: effect of apamin and iberiotoxin (IbTx, 100 nM each) on outward current elicited with pulses in nominal increments of 10 mV from a V₉₀ of −61 mV. Outward current in control conditions (above), during toxin perfusion (middle), and toxin-sensitive current obtained by digital subtraction (below). C: effect of 100 nM charibdotoxin (ChTx) on apamin- and IbTx-resistant current. ChTx perfusion after big conductance current (BK) and small conductance current (SK) channel blockade with IbTx and apamin allows isolation of ionic current through intermediate conductance current (IK) channels. Current calibration bars shown in B apply for the same row in A and C. Timescale is for all recording traces. D: time course of outward current in control conditions (elicited with a pulse test to 25 mV from a V₉₀ of −61 mV) and during application of indicated toxins at a concentration of 100 nM each. E: resistant current (IR) in a LVA (−) cell, obtained by external Ca²⁺ removal and after apamin + IbTx + clotrimazole (CLT) perfusion. Ionic currents were generated with pulses in nominal increments of 10 mV from a V₉₀ of −61 mV.
membrane capacitance gave a weak correlation (\(r = 0.58\)) (Fig. 5, A and B). The perfusion of 100 nM ChTx (after BK blockade with IbTx) reduced the outward current in 86% of the cells (\(n = 6/7\); Fig. 4C). The perfusion of 1 \(\mu\)M CLT (\(n = 8\)) allowed us to isolate the outward current whose characteristics were indistinguishable from the ChTx-sensitive current. Therefore for IK analysis, data obtained from ChTx- and CLT-sensitive currents were pooled. The IK did not activate during 800-ms voltage pulses. The IK activated above -40 mV, reaching its maximum amplitude at 10 mV (data not shown). Mean IK current density was higher in LVA \((-\) than in LVA \((+)\) cells, although because of its large variability this difference was not statistically significant (26 ± 9 vs. 11 ± 3.8 pA/pF; \(P > 0.05\)). The analysis of correlation between IK current density and membrane capacitance gave a weak correlation \((r = 0.58)\) (Fig. 5, A and B).

In 80% of the cells (\(n = 12/15\)), the application of IKCa blockers (100 nM IbTx, 100 nM ChTx, 100 nM apamin, and 1 \(\mu\)M CLT) did not remove a Ca\(^{2+}\)-dependent outward current (Fig. 4D). This resistant current (IR) was evident by removal of external Ca\(^{2+}\) after IbTx + apamin + ChTx/CLT application (Fig. 4E). The IR current density in LVA \((-\) cells was significantly higher than that in LVA \((+)\) cells \((86 ± 3 vs. 6.2 ± 1.8\) pA/pF; \(P < 0.01\)). The plot of IR current density versus membrane capacitance shows a significant correlation \((r = 0.82)\) and confirms the existence of two separated cell groups (Fig. 5, A and B). The first was composed of LVA \((-\) cells with higher IR current density dispersed in a wide range from 36 to 165 pA/pF. The second was composed of LVA \((+)\) cells that did not express a significant IR component, with a range of IR current density from 0 to 14 pA/pF. Note that the IR current density is always underestimated because it is contaminated with \(I_{Ca}\). This is because removal of Ca\(^{2+}\) from the external solutions used to obtain IR simultaneously abolishes the Ca\(^{2+}\) current. The range of \(I_{Ca}\) current density at 800 ms (time at which IR was measured) was between -4 and -43 pA/pF, so the IR could be underestimated within this range. Because HVA-Ca\(^{2+}\) current density was not different between LVA \((-\) and LVA \((+)\) subgroups, the difference observed in IR current density between LVA \((-\) and LVA \((+)\) cells is produced exclusively by differences in IR expression.

Effect of IKCa blockers on the voltage response to current pulses

To analyze the influence of IKCa components on the discharge of the cultured vestibular neurons, the effect of IKCa channel blockers on the electrical response of VANs was studied. For this, the perforated patch-clamp technique was used to avoid dialysis of internal constituents of VANs. Recordings were done at temperatures between 35 and 37°C to approximate to more physiological conditions. Voltage responses were recorded using external and internal CC solutions (Table 1), and with holding membrane potential of -60 mV (close to the zero current potential of VANs). In these conditions, 60% of VANs fired one or two action potentials (APs) during 200-ms and 2-s pulses of suprathreshold current injection (\(n = 21\)). The remaining 40% of the cells showed a slowly adapting response, firing no more than eight action potentials with long-duration pulses (2 s). The threshold of the first AP in control conditions was -36 ± 1 mV (\(n = 21\)). AP amplitude from the threshold to the maximum peak was 53 ± 3 mV. The AP duration (measured at 75%) was 4 ± 0.4 ms (note that external CC solution contains 10 mM 4-AP).

The perfusion of 100 nM apamin or 1 \(\mu\)M CLT did not significantly modify any of these variables (Fig. 6, A and B).
However, in 38% of the cells, either apamin or CLT perfusion produced a slight (5–10 mV) oscillatory behavior in the voltage response after the first action potential. This oscillatory response continuously decreased and vanished within 150 ms.

The effect of IbTx was variable among cells as expected from correlation analysis between $I_{KCa}$ current density and membrane capacitance. The perfusion of IbTx in small cells did not significantly modify AP waveform, whereas in large cells the AP duration increase was ≤77% ($r = 0.78, n = 7$) (Fig. 6C). In those cells with repetitive discharge, the perfusion of 100 nM IbTx removed the adaptation of the response during 200-ms and 2-s current pulses (Fig. 6D).

These results indicate that the BK current contributes to repolarization of the action potential and to discharge adaptation, and this contribution is correlated with the membrane capacitance of VANs.

Voltage-clamp experiments indicate that the IR is the principal $I_{KCa}$ component expressed by small LVA (−) cells and its contribution is correlated with the membrane capacitance. Because of the lack of a specific channel blocker for this current, there is no possibility of making a pharmacological analysis of its contribution to the VANs’ discharge.

**DISCUSSION**

**Calcium-activated current expression**

Rat VANs in primary culture expressed a $Ca^{2+}$ current formed by LVA and HVA components. Whereas the HVA-$Ca^{2+}$ current was present in 100% of VANs, the expression of LVA-$Ca^{2+}$ was heterogeneous among cells and preferentially polarized to medium and large neurons. Correlation between T-type $Ca^{2+}$-current expression and diameter has been reported in vestibular-ganglion neurons of mice (Chambard et al. 1999; Desmadryl et al. 1997) and in the dorsal root and nodosus-ganglion neurons of rat (Fedulova et al. 1985; Lambert et al. 1997). The percentage of cells expressing the LVA-$Ca^{2+}$ current in this study (74%) was greater than that reported in newborn mice (20%). The differences are because in mice only the largest cells express the LVA-$Ca^{2+}$ current, whereas in the rat, medium and large cells express this current. This may be an interspecies difference or caused by distinct culturing times of isolated neurons (2–8 h as compared with 18–24 h in the present study). Another possible source for the differences could arise from the distinct age of the animals used (p4–p8 compared with p7–p10 in the present study). In mice, the separation of VANs into two groups on the basis of T-type current starts around E17, and at p4 it is possible to observe two completely separated groups (Chambard et al. 1999). As shown in this work, in rats this separation is still present in the nearly mature (p23 to p26) neurons. The expression of the LVA-$Ca^{2+}$ current in medium to large cells suggests that the T-type current could contribute to discharge differences between small and large cells in situ. The T-type current is preferentially expressed in dendrites of central neurons and participates in the threshold for action-potential generation, in synaptic integration, and in the configuration of spike discharge (Gauck et al. 2001; Perez-Reyes 2003; Pouille et al. 2000). Therefore as proposed for mice (Desmadryl et al. 1997), the presence of an LVA-$Ca^{2+}$ current in large cells may decrease the threshold for spike generation, which could explain the increased sensitivity of thick axons that innervate central zones of the neuroepithelium compared with thinner axons that innervate peripheral zones.

For the HVA-$Ca^{2+}$ currents, although we did not perform a pharmacological dissection, its characteristics were similar to those reported in mice (Desmadryl et al. 1997), where it has been found that the HVA-$Ca^{2+}$ current is composed of L-, P/Q-, N-, and R-type currents (Chambard et al. 1999; Desmadryl et al. 1997). The whole HVA-$Ca^{2+}$ current was found in 100% of the rat VANs, and no correlation between its current density and soma size (membrane capacitance) was observed. However, we cannot discard the possibility that some of the ionic channels that make up the HVA-$Ca^{2+}$ current have a polarized distribution or are coupled differently with...
some of the $K_{Ca}$ channels reported in this work. Therefore further studies focused on the characterization of channels underlying the HVA-Ca$^{2+}$ current and its functional coupling with $K_{Ca}$ channels are needed to determine its particular role in the electrical firing of VANs.

Calcium-activated potassium current expression

The removal of Ca$^{2+}$ from the external solution decreased the outward current, indicating the presence of an outward current that is activated by the influx of extracellular Ca$^{2+}$. The $I_{KCa}$ current was found in 100% of the studied cells. Current-density analysis showed that small LVA (-) neurons had up to four times higher current density than that of larger LVA (+) cells. Our results indicate that in both cell groups four different currents compose the $I_{KCa}$—SK, IK, BK, and IR—although in different proportions. Of these, only BK and IR have a clear correlation with soma size (membrane capacitance) and with the expression of the LVA-Ca$^{2+}$ current.

The BK current was preferentially expressed in LVA (+) cells, whereas the IR was strongly expressed in LVA (-) cells. In 60% of the cells, BK was activated at voltages below the AP threshold, indicating its physiological coupling with LVA-Ca$^{2+}$ channels, similar to data reported in central-vestibular neurons (Smith et al. 2002). If the correlation between soma diameter and BK expression and the functional coupling with T-type currents are present in VANs in situ, it might have important functional consequences. The T-type current decreases the AP threshold, but the major contribution of BK may tend to repolarize the cells, thus producing failures in the AP generation and irregularity of discharge. Actually, the participation of BK in the adaptation of the response to current pulses, revealed by IbTx effects, indicates that BK inhibition converts the firing pattern from phasic to tonic. Furthermore, the BK participates in the repolarization of the action potential and contributes to the spike duration, supporting the hypothesis that the expression of BK significantly contributes to determination of the discharge properties of VANs in situ.

Our current-clamp experiments with the perforated-patch technique, which should maintain endogenous intracellular buffers, reveal that SK and IK have an insignificant participation in the discharge of VANs caused by constant-current pulses, despite the fact that electrical firing was recorded using external solutions with 4-AP, which enhances the excitability of sensory neurons (Sculptoreau et al. 2004; Soto et al. 2002; Stansfeld et al. 1986). However, the possibility that SK and IK currents participate in the more dynamic and complex conditions in situ cannot be discarded.

SK as well as IK activity depends largely on the concentration of intracellular Ca$^{2+}$. However, the $I$-$V$ relationships of SK and IK did not follow the expected decrease for voltages > 0 mV (at which the inward Ca$^{2+}$ current decreases). This can be attributable to contamination with voltage-dependent channels (such as BK currents), although this is unlikely because ChTx and CLT inhibit similar currents and to date there are no reports about nonspecific effects of CLT and apamin on voltage-dependent channels at the concentrations used in this work. Another possibility is that Ca$^{2+}$ may accumulate by an incomplete clearance by mechanisms of extrusion or sequestration of Ca$^{2+}$ between voltage pulses. In dorsal root ganglion neurons (Thayer and Miller 1990) and in chromaffin cells of the rat (Herrington et al. 1996) large Ca$^{2+}$ loads (1–2 µM) produce a rapid initial Ca$^{2+}$ uptake by mitochondria, but intracellular [Ca$^{2+}$] decay to resting levels can last > 2 min. The modulation of $I_{KCa}$ currents by Ca$^{2+}$ released from intracellular stores will also be taking place in our system and that particular issue should be addressed in future studies.

The identity of the $K_{Ca}$ channels underlying the IR current will require further studies. However, the electrophysiological properties of the IR current are similar to drug-resistant currents from oocytes expressing BK channels coupled with the β4 subunit (Meera et al. 2000) and astrocytes expressing the β4 subunit (Gebremedhin et al. 2003). The β4 subunit has been reported to confer resistance to IbTx to the BK channels (Gebremedhin et al. 2003; Meera et al. 2000). Unfortunately, the lack of selective blockers of the IR current deter an adequate analysis of its contribution to the firing discharge of VANs. The IR has current densities ≤ 160 pA/pF, which are considerably greater than those of other currents reported in this study. Thus the differences found in outward current among cells seems to be caused by the expression of IR.

In mathematical models of VAN, it has been proposed that the AHP slope accounts for differences in sensibility and regularity of discharge (Smith and Goldberg 1986). According to this, the irregular discharge of large neurons innervating type I hair cells may be caused by a smaller slope of postspike voltage trajectory compared with small regular neurons innervating type II hair cells. Although the AP duration did not have a significant linear correlation with total outward current, there is a tendency of small cells to have shorter AHPs, as observed in VANs in situ innervating the peripheral zones of chick vestibular-neuroepithelium (Yamashita and Ohmori 1990).

It is worth noting that the electrophysiological properties of VANs in situ are changing during the first weeks after the birth until reaching a stable phenotype around p23 (Curthoys 1979). Therefore because most of our results were obtained from rats between p7 and p10, our conclusions are mainly circumscribed by this time window in the development of the vestibular system. However, at this age irregular neurons almost show a mature phenotype except for the presence of firing, bursting, and silent neurons at rest (Curthoys 1979). The percentage of regular neurons between p7 and p10 is in the range of 10 to 15%, which is nearly half of those in the adult animal (32%). Regular neurons at p7 to p10 have an electrical discharge at rest of about 20 spikes/s, which is around one third of the rate in the adult rat (Curthoys 1979, 1982). The inability of our cultured VANs to fire tonically during sustained pulses in current clamp suggests that they are in an intermediate stage of maturity or the spatial relationships of ionic channels is not the same as that in the vestibular system in situ. That total $I_{KCa}$ increases in adult p23 to p26 rats reinforces the idea of the immaturity of VANs at p7 to p10 and is in agreement with studies of vestibular-nucleus neurons, which show that electrical properties of vestibular-nucleus neurons change during the embryonic ages (Peusner and Giaume 1997) and beyond birth (Dutia and Johnston 1998). Despite the increase in $I_{KCa}$ current density, the correlation between total $I_{KCa}$ and membrane capacitance in p23 to p26 adult VANs indicates that the differences in the expression of $I_{KCa}$ are maintained in the mature vestibular system. However, the individual contribution of each $I_{KCa}$ subtype to total $I_{KCa}$ in p23 to p26 neurons was not defined. It is also possible that some of the kinetic and elec-
trophysiological properties of $I_{K_{Ca}}$ change during the development as previously reported in central neurons (Kang et al. 1996).

The $I_{K_{Ca}}$ has been shown to significantly contribute to the shaping of the action potential waveform and to the firing-discharge pattern of neurons (Dutia and Johnston 1998; Faber and Sah 2002; Johnston et al. 1994; Sah and Faber 2002; Smith et al. 2002). In lamprey motoneurons, blockade of the SK current increased the variation coefficient of the spike discharge (El Manira et al. 1994). The heightened contribution of the $I_{K_{Ca}}$ in small LVA (−) cells could determine a faster AHP slope, thus significantly influencing discharge regularity of VANs (Smith and Goldberg 1986).

The functional role of $K_{Ca}$ channels is not restricted to the modulation of the electrical firing but also may participate at the central synapse controlling neurotransmitter release (Hu et al. 2001; Robitaille et al. 1993). The particular role of $K_{Ca}$ channels at central terminals should be analyzed in experimental models keeping intact the synapses between primary afferents and second-order neurons. It is worth noting that our results are limited to analysis of the $K_{Ca}$ currents expressed in specific developmental stages and that there is no evidence indicating whether the complement of these currents is also expressed in afferent dendrites or central terminals of the mature VAN. Our results indicate that cultured VANs express an $I_{K_{Ca}}$ carried by several channel subtypes. As shown in other systems $I_{K_{Ca}}$ may participate in determining the threshold, latency, repolarization rate, and afterhyperpolarization of the action potential. Its differential expression among VANs may have important functional implications in the coding of vestibular sensory information. Future studies to analyze the expression and distribution of $I_{K_{Ca}}$ subtypes within the primary sensory neuron in the mature vestibular system, including recordings in the vestibular nerve in situ, would contribute to our understanding of the role of the intrinsic properties of VANs in the coding of the vestibular information.

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