Voltage-Gated Calcium Channel Currents in Type I and Type II Hair Cells Isolated From the Rat Crista

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Submitted 3 April 2002; accepted in final form 13 March 2003

Bao, Hong, Weng Hoe Wong, Jay M. Goldberg, and Ruth Anne Eatock. Voltage-gated calcium channel currents in type I and type II hair cells isolated from the rat cristal. J Neurophysiol 90: 155–164, 2003. First published March 20, 2003; 10.1152/jn.00244.2003. When studied in vitro, type I hair cells in amniote vestibular organs have a large, negatively activating K+ conductance. In type II hair cells, as in nonvestibular hair cells, outwardly rectifying K+ conductances are smaller and more positively activating. As a result, type I cells have more negative resting potentials and smaller input resistances than do type II cells; large inward currents fail to depolarize type I cells above −60 mV. In nonvestibular hair cells, afferent transmission is mediated by voltage-gated Ca2+-channels that activate positive to −60 mV. We investigated whether Ca2+-channels in type I cells activate more negatively so that quantal transmission can occur near the reported resting potentials. We used the perforated patch method to record Ca2+-channel currents from type I and type II hair cells isolated from the rat anterior cristal (postnatal days 4–20). The activation range of the Ca2+ currents of type I hair cells differed only slightly from that of type II cells or nonvestibular hair cells. In 5 mM external Ca2+, currents in type I and type II cells were half-maximal at −41.1 ± 0.5 (SE) mV (n = 10) and −37.2 ± 2.0 mV (n = 10), respectively. In physiological external Ca2+ (1.3 mM), currents in type I cells were half-maximal at −46 ± 1 mV (n = 8) and just 1% of maximal at −72 mV. These results lend credence to suggestions that type I cells have more positive resting potentials in vivo, possibly through K+ accumulation in the synaptic cleft or inhibition of the large K+ conductance. Ca2+ channel kinetics were also unremarkable; in both type I and type II cells, the currents activated and deactivated rapidly and inactivated only slowly and modestly even at large depolarizations. The Ca2+ current included an L-type component with relatively low sensitivity to dihydropyridine antagonists, consistent with the α subunit being Caα1.3 (α1D). Rat vestibular epithelia and ganglia were probed for L-type α-subunit expression with the reverse transcription–polymerase chain reaction. The epithelia expressed Caα1.3 and the ganglia expressed Caα1.2 (α1C).

INTRODUCTION

Transmitter release from hair cells requires Ca2+ influx through voltage-gated Ca2+ channels (reviewed in Fuchs 1996; Sewell 1996). In auditory hair cells, most of the voltage-gated Ca2+ current appears to be carried by L-type channels (Platzer et al. 2000; Schnee and Ricci 2002; Zidanic and Fuchs 1995). Such channels are selectively affected by dihydropyridines with sensitivity that varies across L-channel subtypes. Dihydropyridine (DHP) antagonists partly block both Ca2+ currents and exocytosis in mouse cochlear hair cells (Moser and Beutner 2000) and partly block the Ca2+ currents but completely block exocytosis in chick cochlear hair cells (Spassigna et al. 2001). The spontaneous and sound-evoked discharges of guinea pig cochlear afferents are depressed by DHP antagonists but not by a blocker of N-type CaV2.2 channels (Robertson and Paki 2002). The pore-forming α subunit that predominates in the chick cochlea is the Caα1.3 (α1D) subunit (Kollmar et al. 1997), which, among L-type channels, is relatively insensitive to DHP antagonists (Koschak et al. 2002; Xu and Lipscombe 2001). Mice that are null for Caα1.3 lack most of the cochlear inner hair cell Ca2+ current, have no discernable auditory brain stem response, and are deaf (Platzer et al. 2000). Remarkably, there are no obvious vestibular deficits in the Caα1.3-null mice (Platzer et al. 2000), raising the possibility that other Ca2+ channel types operate in mammalian vestibular hair cells. Here we have investigated whether hair cells from the sensory epithelium (crista) of the rat anterior semicircular canal have an L-type component and whether that component is Caα1.3.

In auditory hair cells of all vertebrates and in all hair cells of fish and amphibians, the primary afferent neuron forms bouton endings on hair cells, opposite presynaptic ribbons. In the vestibular organs of mammals, birds, and reptiles, however, there are two kinds of afferent terminals: bouton endings on type II hair cells and unusual cup-shaped endings, called calyces, on type I hair cells (Wersäll and Bagger-Sjöbäck 1974). The calyx ending differs from the much-studied calyces of Held and of the superior cervical ganglia (reviewed in Catterall 1999; von Gersdorff and Borst 2002) in being postsynaptic rather than presynaptic. It envelopes much of the basolateral membrane of the hair cell. Although this unusual morphology has long excited speculation about the nature of transmission at this synapse, the presence of numerous synaptic dense bodies and vesicles in type I hair cells of the mature rodent crista (Lysakowski and Goldberg 1997) implies that quantal transmission takes place, possibly in combination with other forms of transmission.

According to the prevailing view of quantal transmission at the hair cell synapse, some hair cell Ca2+ channels must be open near the resting potential, VR, to account for background firing levels in afferent fibers. For most hair cells, VR is −60

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mV or more positive and therefore reasonably close to the activation range of Ca\(^{2+}\) channels in hair cells of the frog saccule and chick and mouse cochleas (positive to \(-60\) or \(-50\) mV) (reviewed in Zidanic and Fuchs 1995). Type I hair cells, however, have unusually negative resting potentials (\(-70\) to \(-85\) mV) and low input resistances (10–100 M\(\Omega\)) because they express a large, negatively activating K\(^+\) conductance (\(g_{K,L}\) or \(g_{K}\)) (Bricha et al. 2002; Chen and Eatock 2000; Correia and Lang 1990; Rennie and Correia 1994; Rennie et al. 1996; Rüsch and Eatock 1996b). This is true for diverse preparations (semicircular canals and utricles from reptiles, birds, and mammals) and recording conditions (isolated hair cells vs. excised, intact epithelia; ruptured-patch vs. perforated-patch modes of whole cell recording). If these properties hold in vivo, then there should be little quantal transmitter release because even large transduction currents would not depolarize the hair cell into the activation range of the Ca\(^{2+}\) channels. Yet “calyx afferents,” which innervate type I cells, have both background and evoked discharges (Baird et al. 1988; Goldberg et al., 1990; Schessler et al. 1991).

Two kinds of solution have been proposed. Transmission at the type I/calyx synapse may be partly mediated by other mechanisms, such as ephaptic transmission (Gulley and Bagger-Sjöbäck 1979; Hamilton 1968; Trussell 2000; Yamashita and Ohmori 1990, 1991) or depolarization by extracellular K\(^+\) accumulation (Chen 1995; Goldberg 1996). A second kind of proposal is that in vivo, modulation of \(g_{K,L}\) reduces its activation at \(V_R\) (Behrend et al. 1997; Chen and Eatock 2000), thereby depolarizing the cells and increasing their input resistances. For this study, we considered a third possibility: that the activation range of Ca\(^{2+}\) channels in type I hair cells is unusually negative and therefore overlaps the membrane potential for background levels of stimulation. We report that in type I cells of the rat crista, the Ca\(^{2+}\) channel activation range is only slightly negative to that in type II cells, not enough to compensate for the type I cell’s much more negative resting potential and low input resistance.

**METHODS**

**Cell preparation**

Hair cells were dissociated from the sensory epithelia of anterior cristas from young Long-Evans rats (postnatal days, P – 40) as previously described (Chen and Eatock 2000). All procedures for handling animals were approved by the animal care review committee at Baylor College of Medicine. All dissections were done in L-15 (GIBCO BRL; additionally buffered with 10 mM HEPES, pH 7.3, 330 mmol/kg). The ampulla was excised and placed in L-15 medium, supplemented with 1.2 mM EGTA, to lower Ca\(^{2+}\) to 100 \(\mu\)M, and the following enzymes: protease XXVII (Sigma, St. Louis MO; 500 \(\mu\)g/ml) for 10 min at room temperature and papain (crude papain, Sigma, 500 \(\mu\)g/ml), and l-cysteine (300 \(\mu\)g/ml) for 45 min at 37°C. All subsequent procedures, including recording, were at room temperature (22–25°C). The ampulla was transferred from the papain solution to L-15 containing bovine serum albumen (500 \(\mu\)g/ml; 10 min) and then to the experimental chamber. Cells were brushed off the sensory epithelium with an eyelash and allowed to settle on the glass floor of the chamber. The cells were viewed on an inverted microscope at \(\times 400\) or \(\times 600\) with differential interference contrast optics (Olympus IMT-2, Olympus, Lake Success NY). The chamber was superfused at a low rate with “standard external solution” (Table 1).

**Solutions**

Whole cell recordings were made with the perforated-patch method (Horn and Marty 1988). The internal solution contained (in mM) 75 Cs\(_2\)SO\(_4\), 35 CsCl, 5 MgSO\(_4\), 0.1 CaCl\(_2\), 5 HEPES, and 5 EGTA and 480 \(\mu\)g/ml amphotericin B; pH 7.4 and osmolality 285 mmol/kg. The external solutions used during recordings are given in Table 1. The channel blockers tetrodotoxin chloride (TEA-Cl), 4-aminopyridine (4-AP), and tetrodotoxin (TTX) were all obtained from Sigma. Seal formation was always made in the standard external solution. We ascertained that recordings were in perforated-patch mode rather than ruptured-patch mode by the gradual reduction in access resistance and by the persistence of the Ca\(^{2+}\) channel currents, which washed out rapidly in ruptured-patch mode because of the lack of ATP in the internal solution (Forscher and Oxford 1985). Final series resistance, \(R_s\), was between 5 and 25 M\(\Omega\) and was electronically compensated with the amplifier circuitry by 50–80%.

Drug- and blocker-containing solutions were applied by local perfusion. Separate lines containing the standard external solution and the test solution were fed through a peristaltic pump into needles, which were lowered into the bath after whole cell currents were recorded in the bath solution (standard external solution). The cell was then moved into the flow of solution from each needle.

Junction potentials were calculated with JPCalc software (Barry 1994). The calculated liquid junction potential in standard external solution (Table 1) was 9 mV. Local perfusion with the other solutions, in which TEA-Cl replaced NaCl as the dominant salt, added 4 mV to the total junction potential correction. No corrections were made for Donnan potentials that might arise across the perforated patch. Differences between the impermeant ion concentrations in the hair cell’s endogenous solution and the pipette solution were differences between the impermeant ion concentrations in the hair cell’s endogenous solution and the pipette solution. Such potentials are likely to be small based on other experiments on delayed rectifier currents in type II hair cells. The pipette solution was similar to that used here except that K\(^+\) replaced Cs\(^+\). Half-maximal activation voltages were \(-29 \pm 2\) (SE) mV (\(n = 8\) cells) in the ruptured-patch mode and \(-30 \pm 1\) mV (20 cells) in the perforated-patch mode, suggesting that Donnan potentials across the perforated patch were negligible (K. M. Hurley and R. A. Eatock, unpublished observations).

<table>
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<th>TABLE 1. External solutions</th>
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<td>Solution</td>
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<tr>
<td>Standard</td>
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<tr>
<td>5 Ca(^{3+})</td>
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<tr>
<td>1.3 Ca(^{2+})/3.7 Mg(^{2+})</td>
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<tr>
<td>1.3 Ca(^{2+})</td>
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<tr>
<td>0 Ca(^{2+})</td>
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<tr>
<td>5 Ba(^{2+})</td>
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All solutions had pH 7.4 and were supplemented with MEM vitamin mixture (1\(\times\)) and MEM amino acids (1\(\times\)) (GIBCO BRL).
Recording

Recordings were made with a patch-clamp amplifier (L/M EPC-7, Adams and List Associates, Great Neck, NY) and a 12-bit acquisition board (Digidata 1200, Axon Instruments, Foster City, CA), controlled by pClamp 6.1 software (Axon Instruments). The clamp rise time was on the order of \( 50 \mu s \) \((R_s \leq 10 \text{ M}\Omega) \times (C_m < 5 \text{ pF}) \). The amplifier output was low-pass filtered at 5 kHz with an 8-pole Bessel filter (Model 901, Frequency Devices, Haverhill, MA). In most protocols, test steps were 10 ms, and the filtered \( \text{Ca}^{2+} \)-channel data were sampled at 10-\( \mu \text{s} \) intervals. For longer test steps to study \( \text{Ca}^{2+} \)-channel inactivation, sampling intervals were 1.5 ms, and the data during this portion of the sweep were filtered off-line at 200 Hz with digital filtering algorithms in Clampfit (v. 8, Axon Instruments).

LEAK SUBTRACTION. We usually used a voltage protocol, called “P, \(-P/4\)”, which provides on-line leak subtraction (Armstrong and Bezanilla 1974). Before each trial, the entire test waveform \( (P) \) was divided by \(-4\) and the resulting \(-P/4\) waveform was presented four times from a potential of \(-103 \text{ mV} \) (to be out of the range of activation of \( g_{\text{K,L}} \)). The method assumes that the \(-P/4\) waveform evokes only linear leak current and that the total current summed over the four \(-P/4\) steps is equal and opposite to the linear leak current evoked by \( P \). The sum of the currents evoked by the \(-P/4\) waveform was summed with the current evoked by \( P \), leaving only nonlinear current.

For our usual protocol (10-ms test steps with an inter-trial interval of 100 ms), each trial comprised, in order, four small 10-ms steps \((-P/4; \text{from} \ -103 \text{ mV})\), the test step \( (P; \text{from} \ -73 \text{ mV})\), and a final 40-ms interval at \(-73 \text{ mV} \).

Data analysis

Voltages were corrected off-line for liquid junction potentials but not for uncompensated series resistance. Maximum voltage errors were \( \pm 5 \text{ mV} \) (corresponding to total currents \( \pm 500 \text{ pA} \) and residual \( R_s \leq 10 \text{ M}\Omega \)) and most often on the order of 1 mV.

To study the voltage dependence of activation, we delivered a series of 10-ms voltage steps from the holding potential and plotted the current values at 9 ms after the step, well after activation was complete and before significant inactivation occurred. The \( I-V \) curves are nonmonotonic; at large depolarizations, when the channels are fully activated, current decreases as driving force decreases. The increasing nonmonotonic; at large depolarizations, when the channels are fully activated, current decreases as driving force decreases. The increasing

\[
I(V) = \frac{I_{\text{max}}}{1 + e^{\left(\frac{V - V_{1/2}}{S}\right)}} 
\]

where \( I_{\text{max}} \) is the maximum current, \( V \) is the voltage, \( V_{1/2} \) is the voltage at half-maximal activation, and \( S \) is the voltage change per \( e \)-fold increase of \( I(V) \).

The activation time course of \( \text{Ca}^{2+} \) channel currents was fitted with a Hodgkin-Huxley equation

\[
I(t) = I_{\text{ss}}(1 - e^{-\alpha t})
\]

in which \( I(t) \) is the current at time \( t \), \( I_{\text{ss}} \) is the steady-state current, \( \alpha \) is a time constant of activation, and \( \alpha \) is the power, usually 3.

The decay of the current (inactivation) during a 500-ms pulse was fitted by a single exponential function

\[
I(t) = I_{\text{ss}} + Ae^{-\tau t}
\]

in which \( I(t) \) is the current at time \( t \), \( I_{\text{ss}} \) is the steady-state current, \( A \) is the amplitude of the decaying component at time 0, and \( \tau \) is the time constant of decay.

Results are presented as means \( \pm \) SE.
cells in various vestibular organs (Eatock et al. 1998; Ricci et al. 1996). In a previous study of hair cells enzymatically dissociated from rat cristas (Chen and Eatock 2000), 86% (31/36) of cells that looked like type I cells had $g_{\text{K,L}}$ compared with 11% (4/36) of cells that looked like type II cells and 48% (11/23) of cells that could not be classified morphologically. The data in the present study are from 15 cells with $g_{\text{K,L}}$ and 19 cells without $g_{\text{K,L}}$. We have pooled data according to whether the cells had $g_{\text{K,L}}$, rather than by hair cell shape, because 1) $g_{\text{K,L}}$ expression is less sensitive to dissociation procedures than hair cell shape and 2) $g_{\text{K,L}}$ is the relevant attribute, as we are interested in the activation ranges of Ca\(^{2+}\) channel currents in cells with $g_{\text{K,L}}$ (see introduction).

We began each recording with our standard internal solution, which contained Cs\(^{+}\) instead of K\(^{+}\), and standard external solution, which lacked channel blockers (Table 1). To determine whether $g_{\text{K,L}}$ was present, we used the voltage protocol shown in Fig. 1A and B. $g_{\text{K,L}}$ was recognized by its substantial permeability to Cs\(^{+}\), slow activation kinetics, and, in many cells, its relatively negative activation range. Cs\(^{+}\) is poorly permeant in many K\(^{+}\) channels, but for $g_{\text{K,L}}$, the Cs\(^{+}\) permeability was calculated from reversal potential measurements to be about one-third that of K\(^{+}\) (Rüschi and Eatock 1996a). $g_{\text{K,L}}$ is also unusual in that in many cases (10/15 in this data set) it is significantly activated at the holding potential of $-69\, \text{mV}$. In such cells, there was a significant inward current at $-69\, \text{mV}$, reflecting K\(^{+}\) influx through $g_{\text{K,L}}$. As voltage was stepped to $-129\, \text{mV}$, the current transiently increased because of the increased driving force, then decayed to zero as $g_{\text{K,L}}$ deactivated (Fig. 1A). Subsequent voltage steps to between $-89$ and $-9\, \text{mV}$ re-activated $g_{\text{K,L}}$. At the most negative voltages, the inward K\(^{+}\) currents activated slowly (see trace at $-79\, \text{mV}$), consistent with the activation kinetics of $g_{\text{K,L}}$ in this voltage range (Rüschi and Eatock 1996a). Positive to $-49\, \text{mV}$, the current became outward and was presumably carried by Cs\(^{+}\).

In 5 of 15 cells with substantial Cs\(^{+}\) outward currents, there was relatively little current activated at $-69\, \text{mV}$. Four of the five cells were from relatively young animals (3 from P5 animals, 1 from P9, and the 5th from P18). Data from rodent utricles show that the very negative activation range is acquired during or after the first postnatal week: after P2 in semi-intact mouse utricles (Rüschi et al. 1998) and after P7 in isolated rat utricular hair cells (K. M. Hurley and R. A. Eatock, unpublished observations). We therefore think that this is more likely to be an immature, more positively activating version of $g_{\text{K,L}}$ than a completely different Cs\(^{+}\)-permeant conductance. Ca\(^{2+}\) channel currents from these 5 cells were inspected for differences from the 10 cells with negatively activating $g_{\text{K,L}}$; none were found and these cells are therefore included in the category “cells with $g_{\text{K,L}}$”.

In cells without $g_{\text{K,L}}$ (Fig. 1B), little current flowed during the depolarizing voltage steps, showing that the influx of K\(^{+}\) was largely blocked by the intracellular Cs\(^{+}\) ions. The small currents that did flow had fast kinetics, unlike $g_{\text{K,L}}$. The step from $-69$ to $-129\, \text{mV}$ evoked little inward current as few channels were open at $-69\, \text{mV}$, and no change in the steady current level, indicating that no major conductance was deactivated by the step.

To study current through Ca\(^{2+}\) channels, we applied external solutions containing 1.3 or 5 mM Ca\(^{2+}\) or 5 mM Ba\(^{2+}\), 0 K\(^{+}\), and the following K\(^{+}\) channel blockers: 130 mM TEA-Cl and 5 mM 4-AP to block external rectifiers and 5 mM CsCl to block inward rectifiers (Table 1). TTX was also present at 1 mM to block a voltage-sensitive Na\(^{+}\) current that was present in every cell studied. In these solutions, depolarizing steps from $-73\, \text{mV}$ evoked linear leak currents (subtracted out by the “P/2–P/4” routine) and small, voltage-dependent, fast-activating, sustained inward currents in cells with $g_{\text{K,L}}$ (Fig. 1C) and in cells without $g_{\text{K,L}}$ (Fig. 1D). Several observations showed that the inward current flowed through Ca\(^{2+}\) channels. First, it could be carried by Ba\(^{2+}\) (e.g., Figs. 2C and 3). Second, removal of extracellular Ca\(^{2+}\) eliminated the current (Fig. 2. A and B). Note that in our 0-Ca\(^{2+}\) external solution, Ca\(^{2+}\) was present at trace levels ($< 10\, \mu\text{M}$) because we did not add a Ca\(^{2+}\) chelating agent; at lower Ca\(^{2+}\) levels, Ca\(^{2+}\) channels become nonselective (Almers and McCleskey 1984; Art and Fettiplace 1987). Third, 25 $\mu\text{M}$ Cd\(^{2+}\) blocked peak current by 82 ± 5% ($n = 4$ cells; 5 Ba\(^{2+}\) solution; Fig. 2. C and D), consistent with reported IC\(_{50}\) values for Cd\(^{2+}\) block of L-type and N-type channels (range: 10–300 $\mu\text{M}$ across preparations) (reviewed in Brammar 1999).

It has been suggested that guinea pig vestibular hair cells have T-type Ca\(^{2+}\) currents, based on the presence of a Cd\(^{2+}\)-sensitive transient inward current (Rennie and Ashmore 1991) and a Ni\(^{2+}\)-sensitive depolarization-evoked Ca\(^{2+}\) influx (Boyer et al. 1998). Ni\(^{2+}\) sensitivity has been considered a hallmark of
component was entirely through voltage-gated \( \text{Na}^{+} \) with a rapidly inactivating component (data not shown). This prepulse was used, depolarizing steps evoked inward currents in cristal cells. When TTX was not present and a hyperpolarizing step from \( -73 \) to \( -18 \) mV in 5 Ca\(^{2+} \) solution and 0 Ca\(^{2+} \) solution; averages of 3 and 2 traces, respectively. Morphological type II, without \( \psi_{K,L} \) at P9. In this and Figs. 3 and 5, isochronal I-V relations were taken near the end of the test pulses, at 9 ms. Data were from current traces in A plus traces not shown, plus data showing recovery in a wash solution. The outward current at positive potentials in 0 Ca\(^{2+} \) is presumably carried by Cs\(^+ \) ions through Ca\(^{2+} \) channels. In support of this interpretation, the outward current is blocked by external Cd\(^{2+} \) (see D); C: inward currents recorded from a type II cell in 5 Ba\(^{2+} \) external solution (control), with 25 \( \mu \text{M CaCl}_2 \) added to the 5 Ba\(^{2+} \) solution, and back in 5 Ba\(^{2+} \) (wash). Averages of 4, 2, and 3 traces, respectively. Morphological type II, without \( \psi_{K,L} \) at P15. D: isochronal I-V relations from the traces in C and traces not shown.

T-type channels (Fox et al. 1987), although more recent studies suggest that this may not be generally true (Zamponi et al. 1996). We found no evidence for T-type Ca\(^{2+} \) currents in rat crista cells. When TTX was not present and a hyperpolarizing prepulse was used, depolarizing steps evoked inward currents with a rapidly inactivating component (data not shown). This component was entirely through voltage-gated Na\(^{+} \) channels, however, as it was eliminated in TTX or when external Na\(^{+} \) was replaced by choline\(^{+} \).

Ca\(^{2+} \) channel current has a dihydropyridine-sensitive component

**PHARMACOLOGY.** To test for an L-type component in the Ca\(^{2+} \) channel current, we added the DHP agonist, Bay K 8644, or the DHP antagonist, nimodipine, to the external solution. Bay K 8644 favors long channel openings (Hess et al. 1984; Nowycky et al. 1985) and therefore enhances and slows the current (Fig. 3, A and B). In four cells (2 of each type), the maximum current \( I_{\text{max}} \) in 40 \( \mu \text{M} \) Bay K 8644 was 214 ± 17% of that in control. Nimodipine reduced the current but only weakly even at high doses (Fig. 3, C and D). \( I_{\text{max}} \) was blocked 34.1 ± 8.6% by 40 \( \mu \text{M} \) nimodipine [range: 20–59%, \( n = 4 \) (2 cells of each type), P5–P12]. This relatively small block raises the possibility that the Ca\(^{2+} \) channel current includes a non-L-type component, but for reasons discussed later (Subunit composition) is not strong evidence for it.

**RT-PCR.** The \( \alpha \) subunit of Ca\(^{2+} \) channels forms the pore and in L-type channels includes the DHP binding site. L-type \( \alpha \) subunits that are expressed in the brain are CaV1.2 (\( \alpha_{1C} \)) and CaV1.3 (\( \alpha_{1D} \)); CaV1.4 (\( \alpha_{1F} \)) is expressed in retina. In chick and mouse cochlear hair cells, the CaV1.3 (\( \alpha_{1D} \)) subunit predominates (Kollmar et al. 1997; Platzer et al. 2000). We reverse transcribed mRNA from vestibular ganglia and from the epithelia of cristas and utricles of P13 rats. The epithelia were peeled from the underlying stroma after treatment with thermolysin (see METHODS) and presumably include hair cells, support cells, and nerve terminals. The cDNA was probed with primers for CaV1.2 and CaV1.3 (Fig. 4), as well as CaV1.4 (data not shown) and, as a positive control, calmodulin. All tissues were positive for calmodulin.

In the vestibular epithelia, products of the appropriate size and sequence were obtained for CaV1.3 but not for CaV1.2 or CaV1.4; the primer sets were tested on the same batch of cDNA. For both the utricle and the cristas, the sequenced product (285 nucleotides, excluding the first 20) was 100% identical to the published sequence for CaV1.3 from rat brain (Hui et al. 1991). It is possible that the PCR product includes a contribution from the supporting cells (Mori et al. 1998) or nerve terminals. Nevertheless, given the evidence for an L-type channel current, as shown by removing external Ca\(^{2+} \) (A and B) and adding the Ca\(^{2+} \) channel blocker, Cd\(^{2+} \) (C and D). A: current responses to voltage steps from \( -73 \) to \( -18 \) mV in 5 Ca\(^{2+} \) solution and 0 Ca\(^{2+} \) solution; averages of 3 and 2 traces, respectively. Morphological type II, without \( \psi_{K,L} \) at P9. B: in this and Figs. 3 and 5, isochronal I-V relations were taken near the end of the test pulses, at 9 ms. Data were from current traces in A plus traces not shown, plus data showing recovery in a wash solution. The outward current at positive potentials in 0 Ca\(^{2+} \) is presumably carried by Cs\(^+ \) ions through Ca\(^{2+} \) channels. In support of this interpretation, the outward current is blocked by external Cd\(^{2+} \) (see D), C: inward currents recorded from a type II cell in 5 Ba\(^{2+} \) external solution (control), with 25 \( \mu \text{M CaCl}_2 \) added to the 5 Ba\(^{2+} \) solution, and back in 5 Ba\(^{2+} \) (wash). Averages of 4, 2, and 3 traces, respectively. Morphological type II, without \( \psi_{K,L} \) at P15. D: isochronal I-V relations from the traces in C and traces not shown.

**FIG. 2.** The inward currents are through Ca\(^{2+} \) channels, as shown by removing external Ca\(^{2+} \) (A and B) and adding the Ca\(^{2+} \) channel blocker, Cd\(^{2+} \) (C and D). A: current responses to voltage steps from \( -73 \) to \( -18 \) mV in 5 Ca\(^{2+} \) solution and 0 Ca\(^{2+} \) solution; averages of 3 and 2 traces, respectively. Morphological type II, without \( \psi_{K,L} \) at P9. B: in this and Figs. 3 and 5, isochronal I-V relations were taken near the end of the test pulses, at 9 ms. Data were from current traces in A plus traces not shown, plus data showing recovery in a wash solution. The outward current at positive potentials in 0 Ca\(^{2+} \) is presumably carried by Cs\(^+ \) ions through Ca\(^{2+} \) channels. In support of this interpretation, the outward current is blocked by external Cd\(^{2+} \) (see D); C: inward currents recorded from a type II cell in 5 Ba\(^{2+} \) external solution (control), with 25 \( \mu \text{M CaCl}_2 \) added to the 5 Ba\(^{2+} \) solution, and back in 5 Ba\(^{2+} \) (wash). Averages of 4, 2, and 3 traces, respectively. Morphological type II, without \( \psi_{K,L} \) at P15. D: isochronal I-V relations from the traces in C and traces not shown.

**FIG. 3.** A dihydropyridine-sensitive component in the Ca\(^{2+} \) channel current. (A and B) Bay K 8644 enhanced and slowed the Ca\(^{2+} \) channel current. Cell with \( \psi_{K,L} \) type I morphology, P11. A: peak currents evoked in 5 Ba\(^{2+} \) solution (2 traces averaged) and in 5 Ba\(^{2+} \) solution plus 40 \( \mu \text{M} \) Bay K 8644 (not averaged). Steps were from the holding potential, \( -73 \) to \( -18 \) mV (5 Ba\(^{2+} \)) and to \( -33 \) mV (in Bay K 8644); a negative shift in the voltage eliciting the peak current is expected with Bay K 8644. B: isochronal I-V relations for the traces in A and others not shown. C and D: nimodipine blocked the current weakly. Cell without \( \psi_{K,L} \) type II morphology, P10. C: current evoked by a step from \( -73 \) to \( -13 \) mV in 5 Ba\(^{2+} \) solution and in 5 Ba\(^{2+} \) solution plus 40 \( \mu \text{M} \) nimodipine; each record is the average of 3 traces. Time scale applies to A and C. D: isochronal I-V relations for the data in C plus other traces.

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current of low DHP sensitivity in the hair cells and the lack of PCR products for Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.4 in the epithelium, we conclude that the hair cell Ca\textsuperscript{2+} current is at least partly carried by Ca\textsubscript{v}1.3 subunits.

In the vestibular ganglia, a PCR product was only obtained with Ca\textsubscript{v}1.2 (\(\alpha_{1C}\)) primers. This argues that Ca\textsubscript{v}1.3 expression found in the epithelia does not originate in the nerve terminals. The product obtained with Ca\textsubscript{v}1.2 primers was identical to the published sequence for Ca\textsubscript{v}1.2 from rat brain (Snutch et al. 1998; Naylor et al. 2000). PCR products corresponding to Ca\textsubscript{v}1.2 were previously obtained after reverse transcription of total RNA from the mouse cochlea (Green et al. 1996). DHP-sensitive voltage-gated Ca\textsuperscript{2+} currents have been recorded in dissociated mouse vestibular neurons (Chambard et al. 1999).

A PCR product of the correct size (442 bp) and sequence was obtained with primers for the Ca\textsubscript{v}1.4 (\(\alpha_{1P}\)) subunit in brain but not in vestibular organs or ganglia (data not shown). This subunit is predominantly expressed in retina (Bech-Hansen et al. 1998; Naylor et al. 2000).

In summary, our pharmacological and RT-PCR data indicate that mammalian vestibular hair cells express an L-type Ca\textsuperscript{2+} current carried by Ca\textsubscript{v}1.3 subunits. It is possible that there are additional contributions from other Ca\textsuperscript{2+} channel types (see Subunit composition).

Current-voltage relations

Figure 5A compares the mean isochronal I-V relations for cells with and without g\textsubscript{K,L} in 5 mM external Ca\textsuperscript{2+}. The current of the mean relations peaked at approximately −60 pA for cells with g\textsubscript{K,L} (range: −45 to −85 pA in individual cells) and approximately −80 pA for cells without g\textsubscript{K,L} (range: −45 to −125 pA). Boltzmann fits to the mean curves yielded \(V_{1/2}\) values that were on average slightly but significantly more negative in cells with g\textsubscript{K,L} (−41.1 ± 0.5 mV) than in cells without g\textsubscript{K,L} (−37.2 ± 0.2 mV) and \(S\) values that were similar (6.8 ± 0.4 vs. 6.6 ± 0.2 mV). The differences in peak currents and activation ranges have complementary effects between −70 and −40 mV, with the result that the two cell types have similar mean currents over much of the physiological range of voltages.

Figure 5B shows the I-V relation for a cell with g\textsubscript{K,L} in 1.3 mM Ca\textsuperscript{2+}. The mean current in 1.3 mM Ca\textsuperscript{2+} for eight cells with g\textsubscript{K,L} (not shown) was 1% of its peak value at −72 mV, half-maximal at −46 mV, and 90% of its peak value at −34 mV. Similar results were obtained in mouse cochlear inner hair cells in 1.3 mM Ca\textsuperscript{2+} (Platzer et al. 2000).

Time course

Activation kinetics were fitted with a Hodgkin-Huxley scheme (Eq. 2). A similar scheme has been used to fit Ca\textsuperscript{2+} channel activation in other hair cells, with the exponent, α, set to 2 (Art and Fettiplace 1987; Perin et al. 2001; Zidanic and Fuchs 1995) and 3 (Lewis and Hudspeth 1983). For our cells, α assumed values between 2 and 4 when it was allowed to vary. To compare activation time constants (\(τ\)) across cells, we set α at 3. Examples of fits are shown in Fig. 6A. Figure 6B shows the voltage dependence of mean \(τ\) values averaged from four type I and five type II cells, all in 5 Ca\textsuperscript{2+} medium. Values ranged from 400 μs at −13 mV to 730 μs at −48 mV. On average, the time constants were ~100 μs slower for the type I cells. Even the values from type II cells (100–500 μs across the voltage range) are somewhat slower than those reported from other hair cells (Art and Fettiplace 1987; Hudspeth and Lewis 1988a; Perin et al. 2001; Zidanic and Fuchs 1995).

INACTIVATION

In 5 mM external Ca\textsuperscript{2+}, no inactivation was seen during small depolarizations lasting hundreds of millisecond.
ondats (Fig. 6C, top). For large depolarizations, however, inward currents decayed with time constants of several hundred milliseconds. The example in Fig. 6C decayed during a step to -3 mV with a time constant of ~350 ms to a steady-state value ~50% of its peak value. Similar results were obtained in five other cells without $g_{K,L}$. Inward currents during long depolarizing steps also decayed in cells with $g_{K,L}$. In the latter, however, slow kinetic components in the tail currents at the offset of long steps suggest that the $Ca^{2+}$ current recorded during long steps was contaminated by currents flowing through imperfectly blocked, slowly activating channels. Such currents did not contaminate the responses to our usual 10-ms protocols.

Currents through heterologously expressed human $Ca_{v}1.3$ channels show inactivation with $Ca^{2+}$-dependent and -independent components (Bell et al. 2001). In the rat crista cells, $Ba^{2+}$ currents did not decay (Fig. 6C, bottom), consistent with $Ca^{2+}$-dependent inactivation being responsible for the decay in 5 $Ca^{2+}$.

DISCUSSION

Transmission at the type I-calyx synapse

In vitro measurements of resting potential from type I hair cells are generally negative to ~70 mV because of the large $K^+$ conductance at negative potentials; in the rat crista, ruptured-patch recordings with standard solutions yielded a mean resting potential of ~81.3 ± 0.2 mV ($n = 144$) (Chen and Eatock 2000). At ~70 mV and in physiological $Ca^{2+}$, $Ca^{2+}$ current is ~1% maximal (Fig. 5B). One would predict on this basis that there would be little background release of quanta. Furthermore, the observation that $Ca^{2+}$ current is not appreciable negative to ~60 mV (Fig. 5B) together with other information from in vitro studies argues that there would be little stimulated quantal release, as follows. For a cell with a large $g_{K,L}$, the input resistance is frequently as low as 20 MΩ. The largest transduction currents that have been recorded from rodent vestibular hair cells are ~400 pA (M. A. Vollrath and R. A. Eatock, unpublished results). Such a current would depolarize such a cell by just 8 mV, barely into the voltage range at which significant $Ca^{2+}$ current flows. In vivo, however, rodent calyx afferents do have both background and evoked discharges (Baird et al. 1988; Goldberg et al. 1990). The background rates and gains (evoked rates per unit stimulus) can be lower than for other afferents but are nevertheless substantial. Although other transmission mechanisms may operate at this synapse, the presence of the presynaptic machinery makes it likely that chemical transmission operates at least some of the time. In that case, type I cells must have, at least some of the time, more positive resting potentials, larger input resistances, or both. The responsible mechanisms may include down-modulation of $g_{K,L}$, which would have both effects (Behrend et al. 1997; Chen and Eatock 2000), and accumulation of $K^+$ in the synaptic cleft, which would depolarize the hair cells (Chen 1995; Goldberg 1996).

Comparison with $Ca^{2+}$ channel currents in other hair cells

BIOPHYSICAL PROPERTIES. We were interested in the possibility that the $Ca^{2+}$ channels of type I cells have distinctive properties that explain how they control transmitter release in these low-resistance, negatively resting hair cells. But the differences that we saw were modest: type I $Ca^{2+}$ channel currents were slightly slower and smaller and activated at slightly more negative potentials relative to type II $Ca^{2+}$ channel currents. More striking are the similarities between the currents of both hair cell types and those described for hair cells from other inner ear organs. All are fast to activate and deactivate, show little inactivation for small depolarizations, and activate at fairly similar voltages.

The aspect in which we were most interested, the voltage dependence of type I $Ca^{2+}$ currents, is a few millivolts more negative than that of type II $Ca^{2+}$ currents (Fig. 5A). The source of this modest difference and the small difference in activation kinetics is not clear. If there are multiple $Ca^{2+}$ channel types—reflecting differences in $\alpha$ or $\beta$ subunits or posttranslational modifications—the differences in biophysical
properties between the two cell types might reflect a difference in the proportions of channel types. The slightly more negative activation range in cells with \( \delta_{\text{K, L}} \) is in the right direction to function with the more negative resting potentials encountered in these cells. On the other hand, because the average peak current was smaller in cells with \( \delta_{\text{K, L}} \), the average current levels in the voltage range of the greatest physiological significance, between \(-70\) and \(-40\) mV, were indistinguishable (Fig. 5A).

The voltage ranges of activation for both cell types span the range reported in other hair cells in comparable concentrations of charge carrier. In 2.8 mM Ca\(^{2+}\), Ca\(^{2+}\) currents in isolated turtle cochlear cells were half-maximal at approximately \(-40\) mV (Art et al. 1993). In 4–5 mM external Ca\(^{2+}\), Ca\(^{2+}\) currents in hair cells from the frog amphibian papilla (an auditory organ) and frog crista were half-maximal at \(-40\) to \(-45\) mV (Martini et al. 2000; Perin et al. 2001; Smotherman and Narins 1999). In 1.8 mM Ca\(^{2+}\), Ca\(^{2+}\) current is half-maximal at \(-38\) mV in enzymatically dissociated cells from the frog sacculus (Armstrong and Roberts 1998). In the latter study, the papain dissociation shifted the activation range by \(+7\) mV. Our dissociation procedure includes a lengthy exposure to papain and a \(-5\)- to \(-10\)-mV shift would go a long way to aligning the Ca\(^{2+}\) channel activation range with type I membrane potentials at background levels of stimulation: the current might be 1% of maximal at \(-80\) mV (rather than \(-72\) mV, Fig. 5B) and 10% of maximal at \(-65\) mV. Any papain effect would act on both cell types, however, so that there still would not be a major difference in their Ca\(^{2+}\) channel activation ranges.

The activation kinetics of the Ca\(^{2+}\) currents in rat crista hair cells were slightly slower than reported in other hair cells. Assuming a thermal Q\(_{10}\) of 2.3, correction for mammalian body temperature brings the time constants into line with time constants measured at room temperature in poikilothersms: turtle cochlea (Art and Fettiplace 1987), frog sacculus (Hudspeth and Lewis 1988a), and frog crista (Perin et al. 2001), but they are still slower than those in chick cochlea (Zidanic and Fuchs 1995) after correction for chick body temperature. The kinetics of rat crista channels may have been slowed by the papain dissociation (Armstrong and Roberts 1998). Alternatively, cochlear Ca\(^{2+}\) channels may really have faster kinetics to reduce low-pass filtering of the afferent signal at acoustic frequencies. A single exponential fit of activation in the rat crista cells at \(-28\) mV yields time constants at room temperature of \(-1\) ms, for a half-power low-pass frequency of 160 Hz—in the acoustic frequency range and well above the frequencies of head movements. We therefore know of no functional significance for the small difference between the activation time constants of type I and type II hair cells. Inactivation was modest in overall extent and quite slow; even for large depolarizations, the time constant at room temperature was several hundred milliseconds, corresponding to a high-pass corner frequency of \(-0.4\) Hz, and there was a substantial steady-state component.

Maximal Ca\(^{2+}\) current amplitudes and estimated conductances in hair cells vary over an order of magnitude, from \(-50\) pA to 1 nA. In the frog sacculus, voltage-gated Ca\(^{2+}\) channels are localized at presynaptic active zones (Issa and Hudspeth 1994; Roberts et al. 1990; Rodriguez-Contreras and Yamoah 2001). In chick cochlear hair cells, Martinez-Dunst et al. (1997) found that Ca\(^{2+}\) channel number varies with “presynaptic release area” [the area of hair cell membrane adjacent to each presynaptic dense body] \times\) (the number of dense bodies)] and showed that data from the turtle cochlea and frog saccule lie along the same continuum. This relationship accounts for the increase in Ca\(^{2+}\) current amplitude with best frequency in the chick cochlea and possibly in the auditory organs of other nonmammals (Art et al. 1993; Smotherman and Narins 1999).

In a vestibular organ, the frog crista, Ca\(^{2+}\) channel current amplitude and presynaptic release area may also co-vary. Perin et al. (2001) found that Ba\(^{2+}\) current amplitude varies considerably with region of origin, and noted that the larger Ba\(^{2+}\) currents occur in cells with larger synaptic bodies (Lysakowski 1996).

The presynaptic release areas, numbers of such areas per hair cell, and current amplitudes of rodent crista hair cells appear to be comparable to those of chick tall hair cells. Inspection of Figs. 15 and 16 in Lysakowski and Goldberg (1997) suggests that presynaptic release areas are similar to those in chick cells (Martinez-Dunst et al. 1997). There are \(-17\)–\(-18\) dense bodies in mature rodent crista hair cells (Lysakowski and Goldberg 1997) and \(-15\) in chick tall hair cells (Martinez-Dunst et al. 1997). Peak Ca\(^{2+}\) channel currents are also consistent with each other given that they were recorded in different concentrations of Ba\(^{2+}\). For rat crista type II hair cells, currents doubled when we increased the Ca\(^{2+}\) or Ba\(^{2+}\) concentration fourfold, from 1.3 to 5 mM (data not shown). Thus the \(-80\)-pA currents that we got in 5 mM Ba\(^{2+}\) are comparable to the 100- to 300-pA currents recorded at fourfold higher Ba\(^{2+}\) concentration in chick hair cells (Martinez-Dunst et al. 1997; Zidanic and Fuchs 1995). Presynaptic release areas can be larger in type II hair cells than in type I hair cells (Lysakowski and Goldberg 1997), which might contribute to the difference in peak current size between type I and type II cells (Fig. 5).

Another possible factor in the relative sizes of Ca\(^{2+}\) currents in hair cells from different inner ear organs is whether the currents are used to drive the activation of Ca\(^{2+}\)-gated K\(^+\) channels in addition to synaptic transmission. Those hair cells with the largest Ca\(^{2+}\) currents also have sizeable Ca\(^{2+}\)-gated K\(^+\) currents, and both channel types participate in electrical tuning (Art and Fettiplace 1987; Hudspeth and Lewis 1988a;b; Lewis and Hudspeth 1983). In rodent vestibular hair cells, in contrast, K(Ca) conductances are not the dominant outward rectifiers and electrical tuning is broad (Rennie and Correia 1994; Rennie et al. 1996; Rüscher and Eaton 1996a,b).

SUBUNIT COMPOSITION. Our pharmacological and RT-PCR data suggest that rat crista hair cells express L-type channels made up of Ca\(_{\text{L, 1.3}}\) (\(\alpha_{\text{1, 1.3}}\)) \(\alpha\)-subunits as do other hair cells (Green et al. 1996; Kollmar et al. 1997; Platzer et al. 2000).

Are there other components to the whole cell Ca\(^{2+}\) current? The evidence so far is equivocal. By itself, modest block by high doses of nimodipine does not constitute strong evidence for non-L-type components. DHP block is strongly enhanced by depolarization (Bean 1984; Koschak et al. 2001); thus the evidence so far is equivocal. By itself, modest block by high doses of nimodipine does not constitute strong evidence for non-L-type components. DHP block is strongly enhanced by depolarization (Bean 1984; Koschak et al. 2001); thus the efficacy of the blocker in our experiments was reduced by holding at relatively negative potentials and using short depolarizations. Heterologously expressed Ca\(_{\text{L, 1.3}}\) subunits form channels that are less sensitive to DHP block at any voltage than are those channels formed by other L-type subunits (Koschak et al. 2001; Xu and Lipscombe 2001). The DHP-sensitive component of the mouse inner hair cell current is
even less sensitive to DHP block than are heterologous CaV1.3 channels (Koschak et al. 2001). For a voltage protocol similar to ours, the Ca^{2+} channel currents of mouse inner hair cells are blocked ~40% by 10 μM nimodipine (Platzer et al. 2001), similar to the effect that we observed at 40 μM nimodipine. The DHP sensitivities of heterologously expressed CaV1.2 and CaV1.3 channels and hair cell Ca^{2+} channels follow the same progression as the amounts by which the channels inactivate (CaV1.2 > CaV1.3 > hair cell Ca^{2+} channels), consistent with the proposal that DHP blockers preferentially bind inactivated channels (Bean 1984).

Nevertheless, a number of observations make it likely that there is a non-L-type component. First, there is evidence from CaV1.3 null mutants. The inner hair cells in the CaV1.3-null mice have a small residual current (<10% of wild-type) (Platzer et al. 2000). This small current may not sustain synaptic transmission; the animals are deaf. They lack obvious vestibular deficits, however, suggesting that non-CaV1.3 components may be more important in vestibular hair cells (Platzer et al. 2000). Second, an R-type component has been expressed in frog crista cells, based on its resistance to L- and N-type blockers (Martini et al. 2000). Third, there is evidence for N-type subunits in hair cells. An antibody to N-type subunits (CaV2.2, α1B) labeled the basolateral membranes of type I and type II hair cells in the chinchilla crista (Lopez et al. 1999). In frog saccular hair cells, single-channel recordings reveal both L-type channels and a second type with some N-type properties (Rodriguez-Contreras and Yamoah 2001; Su et al. 1995), and antibody to N-type channels labels discrete spots on the basolateral membrane (Rodriguez-Contreras and Yamoah 2001). Further experiments with different pharmacological agents and/or single-channel recordings are required to determine whether there are multiple voltage-gated Ca^{2+} channels in mammalian vestibular hair cells.

We thank Drs. Julian Woolorton and Melissa Vollrath for comments on the manuscript and D. Himes for technical help.

This work was supported by National Institute on Deafness and Other Communication Disorders Grant DC-02058.

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