Calcium- and Calmodulin-Dependent Inactivation of Calcium Channels in Inner Hair Cells of the Rat Cochlea

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Grant L, Fuchs P. Calcium- and calmodulin-dependent inactivation of calcium channels in inner hair cells of the rat cochlea. J Neurophysiol 99: 2183–2193, 2008. First published March 5, 2008; doi:10.1152/jn.01174.2007. Modulation of voltage-gated calcium channels was studied in inner hair cells (IHCs) in an ex vivo preparation of the apical turn of the rat organ of Corti. Whole cell voltage clamp in the presence of potassium channel blockers showed inward calcium currents with millisecond activation and deactivation kinetics. When temperature was raised from 22 to 37°C, the calcium currents of immature IHCs [<12 days postnatal (P12)] increased threefold in amplitude, and developed more pronounced inactivation. This was determined to be calcium-dependent inactivation (CDI) on the basis of its reliance on external calcium (substitution with barium), sensitivity to internal calcium-buffering, and voltage dependence (reflecting the calcium driving force). After the onset of hearing at P12, IHC calcium current amplitude and the extent of inactivation were greatly reduced. Although smaller than in prehearing IHCs, CDI remained significant in the mature IHC near the resting membrane potential. CDI in mature IHCs was enhanced by application of the endoplasmic calcium pump blocker, benzo-hydroquinone. Conversely, CDI in immature IHCs was reduced by calmodulin inhibitors. Thus voltage-gated calcium channels in mammalian IHCs are subject to a calmodulin-mediated process of CDI. The extent of CDI depends on the balance of calcium buffering mechanisms and may be regulated by calmodulin-specific processes. CDI provides a means for the rate of spontaneous transmitter release to be adjusted to variations in hair cell resting potential and steady state calcium influx.

INTRODUCTION

Tightly regulated synergistic processes within cochlear inner hair cells (IHCs) precisely coordinate synaptic release onto afferent dendrites. Apical stereocilia convert the mechanical energy of cochlear motion into a graded voltage change across the basolateral membrane of the IHC. Voltage-gated calcium channels respond to deviations in membrane potential, and the resulting calcium flux modulates the calcium-dependent fusion of glutamate-containing vesicles arrayed near the dense body of the ribbon synapse. Thus one might say that features of the acoustic world are encoded by the gating of the hair cell’s voltage-sensitive calcium channels. Furthermore, resting activity in these same channels is needed for ongoing, constitutive transmitter release driving spontaneous activity in the auditory nerve (Robertson and Paki 2002; Suet al. 2004). Fulfillment of these tasks must require precise regulation of calcium channel open probability, particularly when one considers the limited number of calcium channels at each ribbon synapse of mammalian IHCs (Brandt et al. 2005; Roberts et al. 1990). It is estimated that ~100 Ca,1.3, or α1D voltage-gated calcium channels (Brandt et al. 2003; Hafidi and Dulon 2004; Michna et al. 2003; Platzer et al. 2000) serve each ribbon synapse. The biophysical properties of hair cell calcium currents (I, Ca) have been well characterized (Beutner and Moser 2001; Fuchs et al. 1990; Johnson et al. 2005; Kennedy 2002; Marcotti et al. 2003; Michna et al. 2003; Nouvian 2007; Rodriguez-Contreras and Yamoah 2001; Schnee and Ricci 2003; Zidanic and Fuchs 1995). I, Ca inactivation has been shown in turtle (Schnee and Ricci 2003), frog (Rodriguez-Contreras and Yamoah 2003), and chicken (Lee et al. 2007) hair cells, shedding some light on this aspect of calcium signaling in nonmammalian vertebrates. Reports of inactivation of mammalian hair cell I, Ca range widely between publications where inactivation is not the experimental focus (Kennedy and Meech 2002; Marcotti et al. 2003; Moser and Beutner 2000; Tarabova et al. 2007). This variability likely reflects differing recording conditions and difficulties associated with blocking the much larger K+ conductances present in hair cells. Here we characterize I, Ca inactivation in mammalian IHCs and provide insights into factors that could account for variability in the extent of inactivation previously reported. We also explore potential molecular mechanisms by which inactivation could occur. This is motivated in part by the observation that, in contrast to IHC I, Ca currents through Ca,1.3 expressed in HEK cells inactivate extensively and rapidly (Yang et al. 2006). This inactivation is calcium and calmodulin-dependent. We show here that calcium-dependent inactivation (CDI) of native hair cell calcium channels also may depend on calmodulin. Finally, we show that CDI persists, albeit to a lesser extent, after the onset of hearing, and could serve to modulate the number of calcium channels available at the resting membrane potential.

METHODS

Dissection and preparation

Recordings were made from IHCs in the semi-intact organ of Corti preparation dissected from the cochlea of Sprague-Dawley rats. Pre-hearing (P7–P11, with the onset of hearing at P12) and older rat pups (P14–P18) were examined. Animal protocols were approved by the Johns Hopkins University Animal Care and Use Committee. Rats were anesthetized with pentobarbital 0.045 mg/g ip and decapitated. The cochlea was removed and placed in external solution containing (mM) 5.8 KCl; 145.5 NaCl; 0.9 MgCl2; 1.3 CaCl2; 5.6 glucose; and

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10 HEPES, pH 7.4, 300 mM. The apical coil was excised; stria vascularis and tectorial membrane were carefully removed; and the preparation was secured under a pin fixed to a coverslip. The coverslip was transferred to the recording chamber, where the preparation was viewed using a ×40 water immersion DIC objective (Axioskop FS microscope, Zeiss, Oberkochen, Germany); with ×4 magnification through a NC70 Newvicon camera (Dage MTI, Michigan City, IN). On transfer of the preparation to the recording chamber, the temperature for the experiment was set and regulated, and external solution was perfused at a rate of 1.5 ml/min. Recordings were made either at room temperature (22–25°C) or near-physiological temperature (34–37°C). Temperature was monitored with an in-bath thermostir and controlled (open-loop) with an inline solution heater (Warner Instruments, Hamden, CT) and a custom-built heated stage.

To gain access to neonatal IHCs for whole cell voltage-clamp recording, overlying supporting cells were removed using a large diameter pipette (−6 μm). The whole cell configuration of the patch-clamp technique was obtained using Sylogar-coated, borosilicate glass electrodes with a resistance of 4−5 MΩ.

**Solutions and recording conditions to resolve Ca^{2+} currents**

To resolve I_{Ca}, other ionic conductances were blocked. Cs+−based intracellular solutions containing tetraethylammonium chloride (TEA-Cl) blocked the majority of potassium conductances. Intracellular (pipette) solution contained (mM) 135 Cs-methanesulfonate; 13 TEA-Cl; 5 HEPES, 3.5 MgCl2; 2.5 Na3ATP; and 1 EGTA, pH 7.2, 290 mM. Where EGTA or BAPTA concentration was altered, equimolar Cs-methanesulfonate was replaced. Extracellular (bath) solution contained (mM) 3.5 KCl; 115.5 NaCl; 0.9 MgCl2; 1.3 CaCl2 and 5.6 mM NaCl. 

Solution exchange and drug application

Whole bath perfusion was used to replace external calcium with barium. The semi-intact organ of Corti preparation presents a diffusion barrier for solution exchange. Therefore to ensure complete replacement, calcium-free saline contained 5 mM BaCl2 (replacing 1.3 CaCl2 and 5.6 mM NaCl).

Benzoxyhydroquinone (BHQ; Tocris Bioscience, Ellisville, MO) was dissolved in DMSO (final concentration <0.1%) and applied by bath perfusion. Calmodulin inhibitory peptide and the paired calmodulin inhibitory control peptide (Calbiochem, San Diego, CA) were reconstituted in distilled water and introduced into IHCs via the patch pipette. E6 berbamine (Biomol, Plymouth Meeting, PA) was dissolved in DMSO (final concentration <0.1%) and applied in the patch pipette.

Data analysis and statistics

Data are presented as means ± SE, with the number of IHCs (n) presented for each experimental condition. For clarity, exemplar 60-ms I_{Ca} recordings were further low-pass filtered (3 kHz), and the sampling frequency was reduced to 10 kHz off-line. Exponential curves describing I_{Ca} activation and inactivation (for a 60-ms voltage step) were fitted with single or double exponentials, respectively. The latter equation was of the form

\[ I = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + c \]  

where \( \tau_1 \) and \( \tau_2 \) are time constants and \( A_1 \) and \( A_2 \) are proportionality constants.

To construct g-V plots, the reversal potential (E_{rev}) was measured for each cell and subtracted from the command voltage (V_{m}) for each voltage step. Initial I_{Ca} amplitude at each V_{m} was divided by this value and plotted against V:

\[ g = I_{Ca} \text{(initial)}/(V_{m} - E_{rev}) \]  

Normalized data were fit with a Boltzmann function using Origin software where

\[ I = I_{max}[1 + \exp(-V - V_{1/2}/dx)] \]  

I_{max} is the maximal current, V_{1/2} is the half activation voltage, and dx describes the slope.

To determine statistical significance of changes in I_{Ca}, inactivation under different conditions, percentage current inactivation was calculated from the difference in initial and final current amplitudes. Where appropriate, one- or two-way ANOVAs with Bonferroni posttests were used to test for statistical significance.

**RESULTS**

**Effect of temperature on I_{Ca}**

The influx of calcium through voltage-gated Ca,1.3 channels in the basolateral membrane of prehearing IHCs was recorded at room temperature and near-physiological temperature (34–37°C). In agreement with recent findings in mouse IHCs (Nouvian 2007), I_{Ca} became larger and faster at warmer temperatures. The average initial current amplitude at –24 mV was threefold larger at 37°C than at room temperature (−539 ± 37 pA at ~37°C, n = 10, compared with −163 ± 15 pA at room temperature, n = 13; Fig. 1, A, B, E, and F; 1.3 mM external calcium with 1 mM intracellular EGTA). The mean activation time constant, \( \tau_{act} \), obtained from single exponential fits was significantly (P < 0.0001) faster at ~37°C than at room temperature (Fig. 1C). For example, at −24 mV, \( \tau_{act} \) at ~37°C was 0.30 ± 0.08 ms (n = 30) compared with 0.42 ± 0.03 ms (n = 18) at room temperature. V_{1/2} activation was also significantly more negative at ~37°C compared with room temperature (P < 0.001; Table 1; Fig. 1D).

In addition to these differences in activation, inactivation of I_{Ca} also was more prominent at ~37°C than at room temperature. An obvious increase in the rate and extent of inactivation of I_{Ca} at ~37°C can be seen within 60 ms (Fig. 1, A and B). Current-voltage relations for the initial and final current amplitude (Fig. 1, E and F) show the extent of inactivation. Figure 1G plots percent I_{Ca} inactivation as a function of voltage; here the increase in inactivation at 37°C is clearly shown. Indeed, inactivation was significantly enhanced (P < 0.0001) at 37°C throughout the voltage range from −44 to −4 mV. Given the greater physiological relevance, all subsequent recordings were conducted at ~37°C.

The voltage dependence of inactivation also provides a first hint of the calcium-dependence of that process. The extent of
Calcium dependence of inactivation

Ca_{1.3}, the dominant channel of cochlear hair cells, undergoes rapid and extensive calcium-dependent inactivation when expressed in HEK cells (Yang et al. 2006). L-type calcium currents in hair cells from frog, turtle, and chicken also show substantial calcium-dependent inactivation (Lee et al. 2007; Rodriguez-Contreras and Yamoah 2003; Schnee and Ricci 2003). Thus we sought to determine whether inactivation of calcium currents in rat inner hair cells also depended on calcium influx.

As a first test of calcium dependence, external calcium was replaced with barium. Barium permeates Ca_{1.3} channels more effectively than does calcium; however, barium is usually less effective at mediating calcium-dependent processes, such as CDI (Yang et al. 2006). Recordings of \( I_{\text{Ca}} \) and \( I_{\text{Ba}} \) in the same cell during a 60-ms depolarization from \(-84 \) to \(-24 \) mV are shown in Fig. 2A. Experiments were performed initially with equimolar substitution of barium for calcium in the external solution. However, it was clear from the limited change in peak current that calcium ion replacement was incomplete with only 1.3 mM barium (data not shown). Thus to ensure that calcium-dependent processes such as CDI were adequately revealed, the external barium was raised to 5 mM. In Fig. 2A, the barium current is scaled to the peak of the \( I_{\text{Ca}} \) for ease of comparison. Irrespective of intracellular buffer, in barium saline, the inward currents were substantially larger (677 + 34 dB, \( n = 10 \) calcium; \(-34-\text{mV step})\), but not doubled as would be expected even for equimolar substitution of calcium, reflecting the difficulty of calcium replacement in this tissue. Nonetheless, even with this incomplete perfusion, the extent of inactivation was significantly reduced from that in calcium (1.3 mM calcium vs. 5 mM barium; \( P < 0.0001 \)). This can be seen in records from the same IHC with calcium or barium as the external divalent (Fig. 2A). Within 60 ms, calcium currents decreased by \(-30\%\), whereas those carried by barium fell \(-10\%\), irrespective of internal buffer (\( n = 6 \)). The percent current inactivation over 60 ms was significantly lower with barium as the prevalent divalent than that with calcium over the voltage range \(-44 \) to \(+6 \) mV (Fig. 2C).

To investigate further the calcium dependence of \( I_{\text{Ca}} \) inactivation over 60 ms, the effect of intracellular calcium buffering was studied. Figure 2B shows calcium currents recorded during a step to \(-14 \) mV in two different cells with 1 or 10 mM intracellular EGTA. These traces suggest that stronger calcium buffering (10 mM EGTA) reduced the magnitude and slowed the rate of inactivation. Figure 2C shows the average extent of inactivation across a range of membrane potentials for different
cells buffered with 0.1, 1.0, or 10 mM EGTA. Buffer concentration significantly affected the extent of inactivation occurring within 60 ms ($P = 0.0007$), largely because of the reduction produced by 10 mM EGTA. There was no overall difference in percent inactivation between 0.1 and 1 mM reduction produced by 10 mM EGTA. There was no overall reduction within 60 ms (Table 1).

### TABLE 1. Properties of Boltzmann curves fit to g-V data

<table>
<thead>
<tr>
<th>Condition</th>
<th>0.1 EGTA</th>
<th>1 EGTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{1/2}$ activation</td>
<td>$-23.3 \pm 1.5$</td>
<td>$-26.5 \pm 1.4$</td>
</tr>
<tr>
<td>Slope</td>
<td>$8.9 \pm 0.7$</td>
<td>$7.6 \pm 0.8$</td>
</tr>
<tr>
<td>$n$</td>
<td>6</td>
<td>18</td>
</tr>
</tbody>
</table>

Values are means ± SE.

BAPTA (data not shown). The minimal (buffer-independent) inactivation remaining with barium as charge carrier is also shown (Fig. 2C).

The extent of inactivation during these relatively short voltage steps naturally must reflect the rate of inactivation over this time course, i.e., inactivation was slowed with higher buffer concentration. Qualitatively, the initial, faster component of inactivation seemed to be particularly sensitive to calcium buffering (Fig. 2B). This observation was quantified by fitting each record with two exponentials, the faster of which ($\tau_2$) accounted for the majority of the decay in this time span (Table 2). Although this process cannot adequately describe all components of inactivation, which range from milliseconds to tens of seconds, analysis of these relatively short-duration currents allow the initial fast component to be estimated. The mean time constant for this faster component of inactivation at $-34$ mV was significantly slower in 10 mM buffer than for lower concentrations (Fig. 2D; Table 2), and its amplitude was reduced (Fig. 2E; Table 2). These differences were significant throughout the voltage range from $-44$ to $-4$ mV. ($\tau_2$: $P < 0.0001$ for both voltage and buffer; $A_2$: $P < 0.0001$ for voltage, $P = 0.01$ for buffer). Thus these observations reveal a component of calcium-dependent inactivation that occurs within 5–10 ms at physiological temperature. At the same time, it is clear that inactivation is incomplete over this time course and so the full impact of this process is not captured with these relatively short voltage steps.

### Inactivation over the course of seconds

Although the foregoing data show rapid (<10 ms) components of inactivation, as will be shown, inactivation continues to accumulate over a time course of many seconds, and these rapid components account for only a fraction of the total loss of current. Thus in the next experiment, we first evaluate the maximal extent of inactivation over the course of seconds, to be followed by examination of lesser magnitude, steady-state inactivation near the resting membrane potential. To measure the extent of inactivation, voltage ramps were used to generate instantaneous $I-V$ relations (100 ms; $-84$ to $+91$ mV) for measuring peak calcium currents before and after 2.5-s-long depolarizing voltage steps (Fig. 3A).

The 2.5-s-long conditioning steps were at, or positive to, the peak of the calcium current activation curve. Figure 3 shows ramp $I-V$s from the same cell before and after steps to $-24$, $+31$, and $+71$ mV. At $-24$ mV, the majority of channels are open, and there is a large driving force for calcium into the cell; this voltage step evokes the largest calcium influx and produced significant (51%) reduction in the succeeding peak ramp current (Fig. 3B). At $+31$ mV, all of the calcium channels are open, but less calcium influx occurs because of reduced driving...
For $\tau_b$ and $A_2$, 0.1 vs. 1 mM EGTA no significant differences; 10 mM EGTA vs. 10 mM BAPTA no significant differences; 0.1 or 1 mM EGTA vs. 10 mM EGTA or BAPTA significantly different ($P = 0.01$). For $\tau_i$ and $A_1$, there were no significant differences between any buffering conditions. IHC, inner hair cells.

### TABLE 2. Components of double exponential fits describing $I_{Ca}$ inactivation in response to 60-ms depolarizations to $-34$ mV (1.3 mM external Ca$^{2+}$; $\sim 37°C$, prehairing IHCs)

<table>
<thead>
<tr>
<th>Buffer</th>
<th>$\tau_2$ ms</th>
<th>A$_2$</th>
<th>$\tau_i$ ms</th>
<th>A$_i$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 EGTA</td>
<td>3.2</td>
<td>-111</td>
<td>28</td>
<td>-67</td>
<td>38</td>
</tr>
<tr>
<td>1 EGTA</td>
<td>3.2</td>
<td>-112</td>
<td>43</td>
<td>-82</td>
<td>6</td>
</tr>
<tr>
<td>10 EGTA</td>
<td>7.7</td>
<td>-69</td>
<td>77</td>
<td>-23</td>
<td>5</td>
</tr>
<tr>
<td>10 BAPTA</td>
<td>11.4</td>
<td>-73</td>
<td>45</td>
<td>-69</td>
<td>33</td>
</tr>
</tbody>
</table>

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Very low buffering conditions. Alternatively, there could be increased basal inactivation with submillimolar calcium buffer. However, this was not evident in the average peak current (comparison of mean peak current in 0.1 and 1 EGTA, $P = 0.2$). The effect of buffer on inactivation produced by 2.5-s-long depolarizations suggests that calcium-dependent inactivation continues to build over the course of many seconds. Indeed, visual inspection (Fig. 3A) shows that inactivation does not reach steady state even during these 2.5-s steps. Thus inactivation during still longer conditioning steps to voltages near the resting membrane potential was assessed using the ramp protocol.

The ramp protocol proved a convenient tool for assessing inactivation, because peak current, voltage-dependence, and reversal potential are all quickly obtained. At the same time, activation and inactivation of the calcium channels during the ramp raise concerns about the validity of this measure. To address these concerns, we compared currents produced by ramps of different durations, ranging from 100 to 12.5 ms. For example, in one cell, the peak ramp current dropped from 560 to 450 pA as ramp duration increased from 12.5 to 100 ms, consistent with the more rapid components of inactivation.
Steady-state calcium entry and inactivation

To characterize the degree of inactivation of \( I_{Ca} \) at or near the resting membrane potential, voltage ramps (–84 to +91 mV over 12.5 ms) were applied before and after holding the membrane potential between –69 and –49 mV for 30 s (the conditioning step). Figure 4A shows a typical stimulus waveform and corresponding current (ramps are shown elongated in the stimulus waveform for clarity). To assess the effect of cytoplasmic calcium buffering, this procedure was repeated for cells buffered either with 1 or 10 mM EGTA. Figure 4, B–E, shows typical ramp I-Vs before (black) and after (gray) 30 s at –49 (B and D) or –59 mV (C and E). B and C were recorded with 1 mM internal EGTA and D and E with 10 mM internal EGTA. The percentage inactivation of \( I_{Ca} \) buffered by 1 or 10 mM EGTA for each conditioning voltage is shown in Fig. 4F (1.3 mM external calcium). The test voltage significantly affected \( I_{Ca} \) inactivation \( (P = 0.0002) \). There was also a significant difference in inactivation between \( I_{Ca} \) recorded with 1 and 10 mM internal EGTA \( (P < 0.0001) \). Thus inactivation occurred to a substantial extent at membrane potentials near the resting membrane potential. Increasing the strength of calcium entry and inactivation

Developmental changes in \( I_{Ca} \)

Figure 5 summarizes the properties of \( I_{Ca} \) recorded from IHCs of hearing rats (older than P12). \( I_{Ca} \) in response to 60-ms depolarizations from –84 mV is shown in Fig. 5A (–5°C with 1.3 mM external calcium and 1 mM internal EGTA). For direct comparison, the equivalent recording from a younger IHC (at 37°C) is superimposed (in gray). Posthearing \( I_{Ca} \) can be compared in greater detail with \( I_{Ca} \) recorded under identical conditions from IHCs of prehearing rats in Fig. 1. As described previously (Beutner and Moser 2001; Brandt et al. 2005; Johnson et al. 2005), \( I_{Ca} \) was smaller after the onset of hearing. In addition, inactivation was diminished. These examples of \( I_{Ca} \) and the respective I-V plots (Fig. 5C, posthearing; Fig. 1F, prehearing) show a marked decline in inactivation after the onset of hearing. Figure 5D quantifies the percent inactivation after 60 ms at voltages ranging from –44 to –4 mV for pre- and posthearing IHCs (prehearing data repeated from Fig. 1). Inactivation was significantly reduced \( (P < 0.0001) \) with 2°C or 10°C internal EGTA. Again this supports the contention that inactivation reflects a loss of calcium current rather than a change in other conductances that would alter the reversal potential.

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activation was also significantly more positive after the onset of hearing ($P < 0.001$; Fig. 5F; Table 1).

$I_{\text{Ca}}$ inactivation was characterized with low-level, steady-state calcium entry for posthearing IHCs as described for prehearing IHCs; voltage ramps (~84 to +91 mV over 12.5 ms) were applied before and after a 30-s conditioning voltage (~69 to ~49 mV). Figure 6A shows percentage inactivation after 30 s at different holding potentials for pre- (gray) and post- (black) hearing IHCs (1 mM EGTA; 1.3 mM calcium; ~37°C). There was significantly less inactivation in the post-hearing IHCs compared with the prehearing IHCs ($P < 0.0001$). Figure 6, B–E, shows examples of $I_{\text{Ca}}$ recorded during voltage ramps applied before (black) and after (gray) 30 s

FIG. 5. Reduced inactivation of posthearing IHC $I_{\text{Ca}}$. A: exemplar $I_{\text{Ca}}$ recorded from a posthearing IHC evoked by 60-ms depolarizations from ~84 to ~44, ~34, ~24, and ~4 mV with 1.3 mM external Ca$^{2+}$ and 1 mM internal EGTA at ~37°C. An example prehearing $I_{\text{Ca}}$ recorded under identical conditions (~24 mV) is shown for comparison (gray trace). B: black trace, posthearing $I_{\text{Ca}}$; gray trace, prehearing $I_{\text{Ca}}$; both currents were evoked by a 2.5-s depolarization to ~24 mV at ~37°C with 1.3 mM external Ca$^{2+}$ and 1 mM internal EGTA. C: I-V plot depicting initial $I_{\text{Ca}}$ amplitudes and amplitudes at 60 ms in response to depolarizations from ~84 mV in posthearing IHCs, with 1.3 mM external Ca$^{2+}$ and 1 mM internal EGTA at ~37°C. D: percent $I_{\text{Ca}}$ inactivation over 60-ms depolarizations for recordings carried out in pre- ($n = 35$) and posthearing IHCs ($n = 20$) at ~37°C, with 1.3 mM external Ca$^{2+}$ and 1 mM internal EGTA. E: $\tau_{\text{act}}$ from single exponential fit to the rising phase of $I_{\text{Ca}}$ plotted against $V$ for pre- ($n = 6$) and posthearing $I_{\text{Ca}}$ ($n = 3$), with 1.3 mM external Ca$^{2+}$ and 0.1 mM internal EGTA at ~37°C. F: normalized g-V plot with Boltzmann fits for $I_{\text{Ca}}$ recorded from pre- ($n = 35$) and posthearing IHCs ($n = 20$), with 1.3 mM external Ca$^{2+}$ and 1 mM EGTA at ~37°C. To minimize skewing of the posthearing g-V plot, because of large leak conductances at positive voltages, for normalization, the +16-mV data point was omitted.

0.0001) in posthearing IHCs compared with that in hair cells before the onset of hearing throughout the voltage range from ~44 to ~4 mV.

In addition to changes in current amplitude and inactivation, the activation rate of $I_{\text{Ca}}$ was significantly slower in posthearing IHCs than in prehearing IHCs (comparing $\tau_{\text{act}}$ of single exponentials fit to $I_{\text{Ca}}$ activation for V steps from ~44 to ~4 mV; Fig. 5E). At ~24 mV (1.3 mM external calcium, 1 mM internal EGTA, ~37°C), $I_{\text{Ca}}$ activated with a mean $\tau_{\text{act}}$ of 0.30 ± 0.08 ms ($n = 30$) for prehearing IHCs, and 0.32 ± 0.05 ms ($n = 23$) for posthearing IHCs ($P < 0.0001$). The $V_{1/2}$ of

FIG. 6. CDI near the resting potential in posthearing IHCs. A: percent $I_{\text{Ca}}$ inactivation calculated from the difference in peak $I_{\text{Ca}}$ amplitude from voltage ramps (~84 to +91 mV; 12.5 ms) applied before and 100 ms after a 30-s step to voltages ranging from ~69 to ~49 mV. Mean ± SE percent $I_{\text{Ca}}$ inactivation for each voltage is plotted for pre- and posthearing IHCs. All recordings were at ~37°C with 1.3 mM external Ca$^{2+}$ and 1 mM internal EGTA. Numbers next to data points represent number of cells for each condition. X-axis intercepts for straight line fits to data points are ~71 mV for the prehearing plot and ~70 mV for the posthearing plot. B–E: exemplar I-V traces from posthearing IHCs evoked by ramps applied before (black) and 100 ms after (gray) 30-s voltage steps as described in A. B: ~49 mV; 23% $I_{\text{Ca}}$ inactivation. C: ~59 mV; 8% $I_{\text{Ca}}$ inactivation. D: ~49 mV; ~8% $I_{\text{Ca}}$ inactivation. E: same cell as D with 12.5 μM BHQ: ~49 mV; 40% $I_{\text{Ca}}$ inactivation.
at −49 (B and D) and −59 mV (C and E). Figure 6, B and C (1 mM internal EGTA), can be compared with the identical measure from prehearing IHCs (Fig. 4, B and C).

SERCA blockade in posthearing IHCs

The extent of CDI in posthearing IHCs was reminiscent of that in the immature IHCs with elevated calcium buffering (cf. Figs. 4F and 6A). To determine whether active calcium sequestration might underlie the reduced CDI of mature IHCs, we blocked sarco/endoplasmic reticulum calcium-ATPase (SERCA) activity with BHQ. Percent inactivation was determined following a 30-s hold at −49 mV as described in (SERCA) activity with BHQ. Percent inactivation was determined following a 30-s hold at −49 mV as described in the previous section, and this protocol was repeated in the presence of 12.5 μM BHQ. Calcium currents in response to voltage ramps applied before and after application of BHQ are shown in Fig. 6, D and E; mean ± SE percent inactivation at −49 mV for posthearing IHCs in the presence of 12.5 μM BHQ is shown in Fig. 6A (△). Blocking SERCA pumps with BHQ significantly increased the extent of inactivation (P = 0.034, paired t-test; n = 3). Even where some reduction in I_{Ca} amplitude occurred in the presence of BHQ (as shown in Fig. 6E, cf Fig. 6D), there was still an increase in I_{Ca} inactivation. Nonetheless, SERCA blockade did not completely restore the prehearing levels of CDI. Either the SERCA block was not completely effective or other calcium control mechanisms are upregulated after the onset of hearing.

Calmodulin involvement in I_{Ca} inactivation

To gain insight into possible molecular mechanisms of CDI, two calmodulin (CaM) inhibitors, CaM inhibitory peptide (CaMi) and E₆ berbamine, were introduced into prehearing IHCs via the patch pipette. CaMi is a 17 amino acid peptide based on the CaM-binding domain of myosin light chain kinase. The peptide binds to calmodulin with high affinity (Kₐ = 6 pM), displacing CaM from target peptides (Torok et al. 1998). This inhibitor can be compared with a control peptide that differs by one amino acid and does not inhibit CaM (Torok et al. 1998). Figure 7, A–C, shows representative I_{Ca} evoked by 60-ms depolarizations from −84 mV (1.3 mM external calcium; 1 mM internal EGTA; ∼37°C). Figure 7A shows I_{Ca} with 5 μM CaMi control peptide in the pipette, Fig. 7B with 5 μM CaMi in the pipette, and Fig. 7C with 5 μM E₆ berbamine in the pipette. These also can be compared with the control condition with no intracellular inhibitor present (Fig. 1B). A reduction in I_{Ca} inactivation can be seen in the presence of both CaM inhibitors. In Fig. 7, D–F, current voltage relationships are plotted for I_{Ca} peak and at 60 ms with 5 μM CaMi control peptide (D), 5 μM CaMi (E), and 5 μM E₆ berbamine (F) in the recording electrode. A reduction in the extent of inactivation with the CaM inhibitors is discernible from the current–voltage plots in Fig. 7, E and F; however, to clarify the effect, the percentage inactivation for each condition is plotted in Fig. 7G. CaMi and E₆ berbamine both markedly reduced

[FIG. 7. Effect of calmodulin inhibitors on prehearing IHC I_{Ca} inactivation. A–C: representative I_{Ca} from different IHCs evoked by 60 ms depolarizations from −84 to −44, −34, −24, and −4 mV, with 1.3 mM external Ca^{2+} and 1 mM internal EGTA at ∼37°C. A: with 5 μM calmodulin inhibitor control peptide (CaMi control) in the internal solution. B: with 5 μM calmodulin inhibitory peptide (CaMi) in the internal solution. C: with 5 μM E₆ berbamine in the internal solution. D–F: I-V curves depicting initial I_{Ca} amplitudes and those at 60 ms in response to depolarizations from −84 mV, with 1.3 mM external Ca^{2+} and 1 mM internal EGTA at ∼37°C. D: 5 μM intracellular CaMi control (n = 6). E: 5 μM intracellular CaMi (n = 8). F: 5 μM intracellular E₆ berbamine (n = 6). G: data presented in D–F expressed as percent I_{Ca} inactivated at 60 ms for control (no intracellular inhibitor, n = 10), 5 μM intracellular CaMi control (n = 6), 5 μM intracellular CaMi (n = 8), and 5 μM intracellular E₆ berbamine (n = 6).]
CDI ($P < 0.0001$, both inhibitors compared with control and CaMi control peptide). In some IHCs, there was a reduction in $I_{Ca}$ amplitude with CaMi or $E_{k}$ berbamine in the pipette or an increase in $I_{Ca}$ amplitude with CaMi control peptide in the pipette. However, even in IHCs with $I_{Ca}$ amplitude comparable to control, there was still a significant reduction in inactivation with CaM inhibitors (see examples in Fig. 7, B and C). Furthermore, posttests carried out on initial $I_{Ca}$ amplitudes comparing control, CaMi, and $E_{k}$ berbamine showed fewer significant differences than posttests comparing the extent of inactivation for the same cells. That is, the CaM inhibitors had a greater effect on inactivation than on initial $I_{Ca}$ amplitude.

**DISCUSSION**

In this paper, we show that CDI of calcium channels in rat cochlear IHCs is temperature-dependent and sensitive to calmodulin inhibitors. We show that CDI declines after the onset of hearing (P12) and propose that the reduction of CDI in mature IHCs is through molecular modifiers of the channel rather than a structural alteration of the Ca$_{1.3}$ gene product expressed in posthearing hair cells. We also show that small steady-state deflections around the IHC resting potential alter the extent of CDI and therefore the gating of calcium channels that support spontaneous transmitter release.

A central concern in studies of hair cell calcium currents is to ensure comprehensive block of the much larger potassium conductances that dominate the membrane. If incompletely blocked, activation of residual potassium conductances might be mistaken for inactivation of the much smaller voltage-gated calcium conductance. In this study, a combination of potassium channel blockers was used, including substitution of internal potassium with cesium and application of TEA, 4-AP, and apamin. The relatively slow time course of inactivation suggests that the most likely contaminating currents would be through unblocked SK channels. Because SK channels are calcium-activated, such a contaminant would behave like CDI in many respects. However, in addition to using 300 nM apamin, the conditions of these recordings make SK currents unlikely contributors to the inward current decay observed here. Taking the relative Cs/K permeability as 0.19 (Shin et al. 2005), the reversal potential for SK flux in these recordings would be $-37$ mV. Thus unblocked SK current would be minimal or inward at $-34$ to $-44$ mV, where inactivation is most prominent (Fig. 1G). In addition, the measured reversal potential of the isolated calcium current did not co-vary with the extent of inactivation, arguing that the decay of inward current did not result from an increase in outward current with a more negative equilibrium potential. Finally, only very small residual tail currents were occasionally seen after voltage commands that produced inactivation in prehearing IHCs (Figs. 1–5 and 7, initial panels). Older IHCs did more consistently present inward tail currents after depolarizing voltage commands (Fig. 5), but these cells had greatly reduced CDI.

The temperature and developmental dependence of CDI addresses the question of variability in $I_{Ca}$ inactivation in previously published work on mammalian IHCs. Under a variety of different recording conditions, the extent of $I_{Ca}$ inactivation ranged from 0% (50 ms at $-15$ mV; posthearing mouse, 35°C, 2 mM external calcium; Moser and Beutner 2000) through 8% (prehearing mouse; 50 ms at $-10$ mV; room temperature; Kennedy 2002), 30% (500 ms at $-14$ mV; P5–P7 mouse; 37°C; 1.3 mM external calcium; Marcotti et al. 2003) to 42% (440 ms at $-10$ mV; P3–P7 mouse; room temperature; 5 mM external calcium; Tarabova et al. 2007). Our findings help account for these widely ranging reports of $I_{Ca}$ inactivation and further characterize mammalian hair cell CDI. This characterization is in agreement with and builds on that described previously in immature mouse IHCs (Marcotti et al. 2003).

The calcium dependence and kinetics of $I_{Ca}$ inactivation in prehearing rat IHCs are similar to those described for turtle hair cells where three inactivation time constants of 6, $\sim 75$, and $\sim 1,000$ ms (Schnee and Ricci 2003) are reminiscent of the fast and slow phases of inactivation reported here. In contrast, CDI of $I_{Ca}$ in chicken cochlear hair cells was described by a single time constant of 1.9 s (Lee et al. 2007), except when cytoplasmic calcium was elevated by SERCA blockade. However, that work was carried out at 20–22°C, cooler than chicken body temperature ($\sim 40^\circ$C) (Crumling and Saunders 2005) and therefore inactivation may still be faster and more extensive under physiological conditions. What is clear is that CDI is more pronounced in hair cells of turtle and chicken than in mature rat IHCs, suggesting that the developmental modulation of CDI may be a specialization of mammalian hair cells.

What might determine the time course of CDI? The multiple time constants could simply reflect the dynamics of calcium accumulation and dissipation in hair cell cytoplasm. The fastest time constants would reflect near-channel accumulation of calcium. This is consistent with the observation that short-term CDI is more prominent at negative than at positive membrane potentials. Such asymmetry is often explained as the differential importance of near-channel versus longer range accumulation of calcium. Slower components of inactivation would result from gradual accumulation of cytoplasmic calcium, consistent with the effect of SERCA blockers to increase CDI that accrues during a 30-s depolarization. It is conceivable that multiple calcium binding sites subserve these different dynamics of CDI, with low affinity sites for rapid, and higher affinity sites for slower, components of CDI. Given the proposed role of CaM in CDI, such sites could correspond to the N- and C-terminal IQ domains of CaM (Peterson et al. 1999).

The developmental modulation of CDI may reflect the different demands on calcium signaling in mature and immature IHCs. Before the onset of hearing, mammalian IHCs generate spontaneous, regenerative calcium action potentials (Kros et al. 1998; Marcotti et al. 2003). IHC spiking activity ceases with the drop in calcium current density and the development of a rapidly activating calcium activated K$^+$ conductance $I_{K, f}$ around the onset of hearing (Kros et al. 1998; Marcotti et al. 2003). It is possible that the ability of prehearing IHC $I_{Ca}$ to inactivate to a greater extent in a more rapid fashion than mature $I_{Ca}$ is important for shaping calcium action potential waveforms in the immature IHC. The faster component of $I_{Ca}$ inactivation shown in this paper in prehearing IHCs had a time constant of $\sim 4$ ms, which is fast enough to affect the waveform of calcium spikes, with half-amplitude durations exceeding 10 ms (Marcotti et al. 2003).

This study shows that, in both immature and mature (post-onset of hearing) IHCs, CDI of $I_{Ca}$ occurs around the resting...
potential, to an extent that depends on the cytoplasmic calcium buffer. Measurements from mouse inner hair cells suggest that intrinsic calcium buffering corresponds to ~1 mM BAPTA (Marcotti et al. 2004). Under equilibrium binding conditions, CDI should reach levels consistent with those seen with 1 mM EGTA buffering, i.e., 10–20% in the voltage range between −60 and −50 mV in mature IHCs. Although seemingly modest, nonetheless such negative feedback could have significant effects. Spontaneous activity will drop back toward the original resting level. IHC acoustic sensitivity will be lowered, so that equivalent sound-evoked depolarizations will produce a smaller increase in calcium-dependent transmitter release.

$I_{\text{Ca}}$ through Ca$_{\text{a},1.3}$ heterologously expressed in HEK cells shows a much stronger, more rapid inactivation at room temperature than that seen in IHCs at room temperature (Yang et al. 2006). The mechanism regulating CDI of Ca$_{\text{a},1.3}$ in IHCs is intriguing, particularly concerning the developmental aspect. Although CDI in immature IHCs is weaker than that of heterologously expressed Ca$_{\text{a},1.3}$, CDI in mature IHCs is even further reduced. It has been shown that CDI of Ca$_{\text{a},1.3}$ expressed in HEK cells is CaM-dependent and that it can be reduced by co-expression with CaM-like calcium binding proteins (CaBPs). Furthermore, CaBP4 is present in the mammalian organ of Corti where its expression alters during development, becoming restricted to IHCs in the mature cochlea (Yang et al. 2006). CaBP4 has been proposed as a putative molecule controlling CDI in cochlear IHCs (Yang et al. 2006). More recently, CaBP1 also has been shown in IHCs and proposed as a modifier of hair cell CDI (Cui et al. 2007). The results presented in this paper reveal further properties of IHC CDI that go some way toward delineating the mechanism of control of IHC $I_{\text{Ca}}$. CDI is at least in part CaM-dependent, as has been shown for Ca$_{\text{a},1.3}$ expressed in HEK cells. This is important as CaM-like CaBPs act on CaM-dependent CDI (Haeseleer et al. 2000). Furthermore, the fact that inactivation can be enhanced by blocking SERCA pumps in posthearing IHCs, whose $I_{\text{Ca}}$ inactivates to a lesser extent than that of prehearing IHCs, is revealing. This shows that the reduction in CDI in posthearing IHCs is not an irreversible change. Under “normal” circumstances, CDI is constrained; however, this can be overwhelmed by elevating cytoplasmic calcium, thereby favoring calcium–CaM binding. This fits with increased expression of a molecule with the capability to inhibit Ca$_{\text{a},1.3}$ CDI, as proposed for CaBP4 and CaBP1 in the mammalian cochlea (Cui et al. 2007; Yang et al. 2006).

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REFERENCES


