Calcium Balance and Mechanotransduction in Rat Cochlear Hair Cells

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INTRODUCTION

The sensory hair cells of the mammalian cochlea are responsible for sound transduction, amplifying and tuning the response in the outer hair cells (OHCs), and relaying the signal to the auditory nerve afferents via the inner hair cells (IHCs) (Fettiplace and Hackney 2006; Głowatzki et al. 2008). Calcium plays a prominent role in the performance of the OHCs, which possess large amounts of cytoplasmic calcium-binding proteins (Hackney et al. 2005) to buffer Ca2+ in vivo like conditions, in which the hair bundles are bathed in extracellular fluid rather than to Na+ influx through the MT channels during stimulation controls the extent and rate of channel adaptation (Beurg et al. 2006; Ricci and Fettiplace 1998). Ca2+ may also modulate OHC electromotile behavior, both somatic contractility (Dallos et al. 1997; Frohlich et al. 2000; Murugasu and Russell 1996) and hair bundle motility (Chan and Hudspeth 2005; Kennedy et al. 2005).

The time course and localization of cytoplasmic Ca2+ excursions are dependent on binding to diffusible calcium-binding proteins (Roberts 1994) and by uptake and release by organelles such as the mitochondria, which can act in a buffering capacity. OHCs have been previously shown to contain millimolar concentrations of the proteinaceous calcium buffer parvalbumin–β (Hackney et al. 2005; Sakaguchi et al. 1998), suggesting the occurrence of large somatic Ca2+ transients as in muscle fibers, which also contain a high concentration of a parvalbumin isoform (Celio et al. 1996). Mitochondria are conspicuously concentrated in OHCs not only in a band beneath the cuticular plate, the cytoskeletal anchor for the stereocilia bundle, but also along the lateral walls adjacent to the submembranous cisternae (Furness and Hackney 2006; Weaver and Schweitzer 1994). The mitochondria are known to affect Ca2+ balance not only by generating adenosine 5′-triphosphate (ATP) that fuels the PMCA pump but also by acting as a large-capacity calcium store (Babcock and Hille 1998; Gunter et al. 2000; Nicholls 2005; Xu et al. 1997). The aim of the present work was to quantify the time course and spread of Ca2+ transients originating from the MT channels using fast imaging of fluorescent calcium indicators and to assess the resting Ca2+ influx and mechanotransduction under in vivo like conditions, in which the hair bundles are bathed in endolymph containing 20 μM Ca2+ (Bosher and Warren 1978). Imaging data were complemented with a model of Ca2+ buffering and extrusion. The results suggest that correct Ca2+ balance is crucial for optimal performance of the MT channels, which may be deleteriously affected by mitochondrial or PMCA pump dysfunction.

METHODS

Preparation and recordings

Recordings were made from OHCs in the isolated organ of Corti of Sprague–Dawley rats between 6 and 14 days postnatal (P6–P14), as previously described (Beurg et al. 2006; Kennedy et al. 2003). Animals were killed by decapitation using methods approved by the Institutional Animal Care and Use Committee of the University of Wisconsin–Madison according to current National Institutes of Health guidelines. Excised apical turns were fixed in the experimental chamber with strands of dental floss and viewed through a long working distance water-immersion objective (Olympus ×60, numerical aper-

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ture [NA] = 1.1, or Zeiss ×63, NA 0.95) and a Hamamatsu charge-coupled device (CCD) camera on a Zeiss Axioskop FS microscope. The chamber was perfused with artificial perilymph of composition (in mM): 154 NaCl, 6 KCl, 1.5 CaCl₂, 2 Na-pyruvate, 8 glucose, and 10 Na-HEPES (pH 7.4). The apical surface of the organ of Corti was separately superfused with artificial perilymph or with a saline of reduced Ca²⁺ concentration, either 0.15 or 0.02 mM, the latter being buffered with 4 mM HEDTA. In some experiments, the solution around the hair bundle was controlled by flow from a 10-μm pipette and was changed to artificial endolymph containing (in mM): 160 KCl (buffered with 4 mM HEDTA), 2 Na-pyruvate, 8 glucose, and 10 K-HEPES (pH 7.4). To block the PMCA pump, the concentration of H⁺ ions required for exchange during a pump cycle (Xu et al. 2000) was reduced by elevating the pH of the artificial perilymph to 9.0 by buffering with 10 mM TABS (Sigma–Aldrich, both agents being used to block Ca²⁺ entry into mitochondria. All experiments were performed at room temperature (20–23°C).

Hair cell recordings were made using borosilicate patch electrodes introduced at the bottom of the apical turn, a location in vivo with a characteristic frequency range of 4 to 6 kHz (Müller 1991). Patch pipettes were filled with an intracellular solution of composition (in mM): 135 CsCl, 2.5 MgCl₂, 1 EGTA, 2.5 Na₂ATP, 10 creatine phosphate, and 10 HEPES (pH 7.2) and had starting resistances of 3–6 MΩ. In some experiments, K⁺ was substituted for Cs⁺ and 1 mM BAPTA was used instead of EGTA as the calcium buffer. Also included in the pipette solution was 0.25 mM of a calcium indicator dye, Fluo-4 or the low-affinity Fluo-4FF (Invitrogen Life Sciences), which have Ca²⁺ affinities of 0.5 and 10 μM, respectively. Mitochondrial Ca²⁺ was monitored with Rhod2, which was loaded by incubating the preparation with 20 μM of the membrane-permeant Rhod2AM (+0.5% DMSO) for 30 to 45 min prior to starting the recordings. The patch electrode was connected to an Axopatch 200A amplifier and MT currents were low-pass filtered at the amplifier output at 10 kHz. During imaging experiments hair bundles were deflected by a pressure-driven fluid jet from a pipette, tip diameter 5–10 μm, placed about 10 μm from the bundle so as not to interfere with its image. The pipette was normally filled with artificial perilymph solution. Fluid pressure and pipette location were adjusted to elicit a maximal MT current. In experiments to determine current–displacement (I–X) relationships, hair bundles were deflected with a glass stylus driven by a piezoelectric step actuator (PA8/12; Piezosystems Jena, Hopedale, MA). The driving voltage to the actuator was filtered at 4 to 6 kHz, yielding a step displacement with a rise time of about 50 μs. The peak MT current in control (NA⁺, 1.5 mM Ca⁴⁺) extracellular solution ranged from about 600 to 1,000 pA (mean = 785 ± 182 pA; n = 27).

For comparing Ca²⁺ transients in normal and reduced extracellular Ca²⁺ concentrations, experiments used two pipette pipettes, one containing a control (1.5 mM Ca⁴⁺) and the other a test solution. The control pipette was first positioned near the bundle and the Ca²⁺ response to bundle deflection established; then it was moved away and the pipette with the test solution was brought up near the bundle and its response obtained; finally the control pipette was returned to its original position and a return control determined. This method was much cleaner than exposing the entire endolymphatic aspect of the preparation to reduced Ca²⁺, a procedure that was often poorly reversible and resulted in hair cell deterioration. To ensure the solution around the bundle was fully exchanged, the timing and duration of the puffer command and depolarizing voltage step were varied to give a constant outcome. To achieve sufficient sensitivity to measure calcium signals in very low extracellular Ca²⁺ concentrations, Fluo-4 was used as the fluorescent indicator.

**Imaging**

The cell apex and stereociliary bundle of a hair cell were imaged using a Photometrics Cascade 128 CCD camera (Photometrics, Tucson, AZ) at an acquisition rate of normally 500 frames/s, although this rate was reduced to 100 frames/s for measurements in low extracellular calcium. The fast camera facilitated quantification of the fast time constants of calcium accumulation and dissipation. Epifluorescence illumination was achieved with a 100 W Hg lamp through FITC or rhodamine filter sets (Chroma Technology, Brattleboro, VT). An intermediate magnification lens was inserted to accomplish a resolution at the CCD camera of 0.09 μm per pixel, about a third of the lateral resolution of the water-immersion objective. The axial resolution of the imaging system was determined from the axial intensity variation for 0.1 μm fluorescent beads, focused with a stepping motor at 0.5 μm intervals. The results were fitted with a Gaussian with SD of 1.0 μm, corresponding to a full width at half-maximum (FWHM) of 2.2 μm. This resolution is half that of the swept field confocal microscope (1 μm FWHM) used to localize stereociliary calcium transients in inner hair cells (Beurg et al. 2009). Images were captured with Metamorph (Molecular Devices, Downingtown, PA) and were analyzed with Metamorph or ImageJ software. The average intensity was calculated for small areas overlapping the stereociliary bundle or cell apex and, in most cases, the fluorescence change was corrected by subtraction of the background fluorescence measured at a depolarized potential where there is no calcium influx. To allow the pipette and cytoplasmic solution to equilibrate, images were not captured until five minutes after achieving the whole cell condition. Results are expressed as ΔF/F, the change in fluorescence normalized to the background.

**Immunofluorescence**

Neonatal Sprague–Dawley rats 7–13 days old (P7–P10) were decapitated using methods approved by the Institutional Animal Care and Use Committee of the University of Wisconsin. The cochleae were freshly dissected in cold HBSS. After fixation in 4% paraformaldehyde in phosphate buffer (PB) for 90 min at room temperature, the stria vascularis and tectorial membrane were removed and the cochlear coils were isolated. The apical and part of the middle turns, which included the region used for electrical recordings, was separated, permeabilized with 0.5% Triton X-100 for 30 min, washed in phosphate-buffered saline (PBS), blocked in 10% normal goat serum which included the region used for electrical recordings, was blocked in 10% normal goat serum (Invitrogen Life Sciences) for 1 h at room temperature, then incubated with rabbit anti-PMCA2 polyclonal antibody (Affinity Bioreagents, Golden, CO) diluted 1:400 in blocking solution overnight at 4°C. The control groups were processed with blocking solution instead of primary antibody. After washing in PBS, the samples were incubated with Alexa Fluor 568 goat anti-rabbit IgG antibody (1:400; Invitrogen Life Sciences) diluted in blocking solution for 2 h at room temperature, washed in PBS, incubated with Alexa Fluor 594 or 568 phalloidin (1:200; Invitrogen Life Sciences) for 45 min at room temperature, and washed in PBS. The isolated cochlear coils were mounted on coverslips with Prolong Antifade mounting medium (Invitrogen Life Sciences), viewed under a ×60 objective (Nikon ELWD Planfluor; NA 1.4) oil-immersion objective using a Bio-Rad (Hemel Hempstead, UK) MRC-1024ES laser scanning microscope system operating in confocal mode.

Theory

GEOMETRY. An OHC from the apical turn of the rat cochlea was simulated with three rows of stereocilia (4, 2, and 1.8 μm in height, all 0.25 μm in diameter) projecting from a 30 μm long cell body. Because of the repeated pattern of the OHC stereociliary columns, the three-dimensional problem was reduced to a two-dimensional one, a slice of the hair cell including a column of stereocilia and cell body being modeled. The width is the approximate distance from edge to edge along the column axis (z-axis). The thickness of the slice (y-axis) is the distance between the stereocilia columns. The depth (x-axis) is the primary direction of diffusion along the stereocilia and cell. The dimensions of the model cell body were 4.8 × 0.4 × 30 μm (width × thickness × depth). The diffusible space was divided into four sections along the z-axis: stereocilia (z ≥ 0), cuticular plate (0 > z ≥ −1), mitochondria belt (−1 > z ≥ −5), and deep cell body (z < −5). The grid size was 0.1 μm in the stereocilia and 0.2–1.0 μm in the cell body. The ratios of diffusible volume to total volume of the sections were 0.8, 0.6, 0.7, and 0.8 in the four compartments, with the smaller values in the cuticular plate and mitochondrial regions.

CALCIUM INFLUX AND REMOVAL. Ca^{2+} dynamics were described by differential equations (Lumpkin and Hudspeth 1998; Wu et al. 1999) for the following processes: 1) Ca^{2+} entry through MT channels at the tips of the stereocilia and diffusion in the cytoplasm with coefficient D_{cyt} of 0.4 μm^2·ms^{-1}; 2) buffering by cytoplasmic calcium buffers, both diffusible (1 mM EGTA + 0.25 mM Fluo-4FF, 1 mM BAPTA, or 2 mM parvalbumin-β) and fixed buffers; 3) extrusion via PMCA pumps; and 4) uptake into the subcuticular plate mitochondria (see schematic in Fig. 10A). The Ca^{2+} influx through the channel was described by

\[ \frac{dC}{dt}_{\text{influx}} = \frac{-n_{iC}(t)}{zFV_{C}} \]  

where C is the free Ca^{2+} concentration, n_{iC} is the number of MT channels per stereocilia, i_{C} is the single-channel calcium current, z is calcium ion valence, F is Faraday’s constant, and V_{C} is the diffusible volume of the compartment. Two MT channels are assumed to be present at the bottom end of the tip links (i.e., at the tops of the second and third row stereocilia), each channel carrying 1.3 pA of calcium current (in 1.5 mM external Ca^{2+}; apical OHCs have unitary MT channel currents of 8.5 pA, 15% of which is carried by Ca^{2+}; Beurg et al. 2006). Other parameter values, with their sources, are given in Table 1. Ca^{2+} extrusion by PMCA pumps was combined with leakage through the cell membrane (Sala and Hernández-Cruz 1990)

\[ \left( \frac{dC}{dt} \right)_{\text{ext+leak}} = \frac{v_{\text{max,PMCA}} A_{C}}{V_{C}} \left( \frac{C_{0} + K_{\text{PMCA}}}{C + K_{\text{PMCA}}} \right) \] 

where \( v_{\text{max,PMCA}} \) is the maximal velocity of transportation in mol·m^{-2}·s^{-1}, \( A_{C} \) is the area of membrane of the compartment, \( K_{\text{PMCA}} \) is the half-maximal activation concentration of the PMCA pump, and \( C_{0} \) is the equilibrium point established between the extrusion by PMCA pump and the leakage due to the calcium gradient through the cell membrane. An important feature of the model is that, in line with experimental observations (Apicella et al. 1997; Crouch and Schulte 1995; Dumont et al. 2001; Grati et al. 2006), the PMCA pumps were concentrated in the stereociliary membrane with a lower density in the somatic lateral membrane (here assumed to be a tenth of that in the stereociliary membrane). The properties of the fixed buffer have not been characterized experimentally but there are known to be significant concentrations of calcium-binding proteins such as calmodulin and L-plastin (fimbrin) in the stereocilia and cuticular plate (Furness and Hackney 2006). A fixed buffer dissociation constant of 10 μM was assumed, similar to that of fimbrin (Glenney Jr et al. 1981). Although the kinetic properties of the fixed buffer in hair cells are not known, the values assumed (Table 1) are close to those determined experimentally for chromaffin cells (Heinemann et al. 1994). Furthermore, these values gave good agreement between the experimental and theoretical fluorescence decay rates after a Ca^{2+} load.

MITOCHONDRIA. Transmission electron micrographs of the organ of Corti show a concentration of mitochondria beneath the cuticular plate (Weaver and Schweitzer 1994). Measurements on transmission electron micrographs provided by C. Hackney and S. Mahendrasingam (Keele University, Keele, UK) indicated that in prehearing rats, the mitochondrial layer in apical OHCs (mean lengths 32 μm) extended below the cuticular plate for 10.9 ± 0.6 μm and that the mitochondrial density (the ratio of mitochondrial volume to total cytosolic volume in a 5 μm band below the cuticular plate), determined from stereological

### Table 1. Model parameters

<table>
<thead>
<tr>
<th>Component</th>
<th>Property</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT channel</td>
<td>Single-channel calcium current</td>
<td>0.15 × 8.5 pA = 1.3 pA</td>
<td>Beurg et al. (2006)</td>
</tr>
<tr>
<td>Fixed buffer</td>
<td>Concentration (plastin)</td>
<td>2 nM (stereocilia)</td>
<td>Furness and Hackney (2006)</td>
</tr>
<tr>
<td></td>
<td>Dissociation coefficient</td>
<td>10 μM</td>
<td>Glenney et al. (1981); Heinemann et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>Forward binding rate</td>
<td>10^4 M^{-1}·s^{-1}</td>
<td>Heinemann et al. (1994)</td>
</tr>
<tr>
<td>EGTA/BAPTA</td>
<td>Concentration</td>
<td>1 mM; 1 mM</td>
<td>Experimental condition</td>
</tr>
<tr>
<td>EGTA/BAPTA*</td>
<td>Dissociation coefficient</td>
<td>0.2 μM; 0.2 μM</td>
<td>Naraghi (1997)</td>
</tr>
<tr>
<td>EGTA/BAPTA*</td>
<td>Forward binding rate</td>
<td>3 × 10^4 M^{-1}·s^{-1}; 5 × 10^5 M^{-1}·s^{-1}</td>
<td></td>
</tr>
<tr>
<td>Parvalbumin-β</td>
<td>Concentration</td>
<td>4 nM binding sites</td>
<td>Hackney et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>Dissociation coefficient</td>
<td>0.5 μM</td>
<td>Henzl and Ndubuka (2007)</td>
</tr>
<tr>
<td></td>
<td>Forward binding rate</td>
<td>2 × 10^5 M^{-1}·s^{-1}</td>
<td>Assumed</td>
</tr>
<tr>
<td>Calbindin-D28k</td>
<td>Concentration</td>
<td>0.5 mM binding sites</td>
<td>Hackney et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>Dissociation coefficient</td>
<td>0.35 μM</td>
<td>Nager et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>Forward binding rate</td>
<td>4.5 × 10^7 M^{-1}·s^{-1}</td>
<td>Nager et al. (2000)</td>
</tr>
<tr>
<td>PMCA</td>
<td>Maximum transport velocity†</td>
<td>0.34 μmol·m^{-2}·s^{-1}</td>
<td>Yamoah et al. (1998)</td>
</tr>
<tr>
<td>Ca-ATPase</td>
<td>Dissociation coefficient</td>
<td>1 μmol·m^{-2}·s^{-1}</td>
<td>Measurements in text</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 μM</td>
<td>Kubitscheck et al. (1995)</td>
</tr>
<tr>
<td>Mitochondrial unipporter</td>
<td>Transportation rate</td>
<td>114 μmol·s^{-1}</td>
<td>Wingrove et al. (1984)</td>
</tr>
<tr>
<td>Mitochondrial Na–Ca exchanger</td>
<td>Transportation rate</td>
<td>1.8 μmol·s^{-1}</td>
<td>Xu et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>Dissociation coefficient</td>
<td>5 μM</td>
<td>Patterson et al. (2007)</td>
</tr>
</tbody>
</table>

*Fluo4FF in imaging had a concentration of 0.25 mM, a dissociation constant of 10 μM, and a forward rate constant identical to those in BAPTA. † 0.34 μmol·m^{-2}·s^{-1} = 2,000 pumps·μm^{-2}, each transporting 100 ions·s^{-1}; 1 μmol·m^{-2}·s^{-1} = 6,000 pumps·μm^{-2}, each transporting 100 ions·s^{-1}. OHC soma were assumed to contain one tenth the PMCA density.

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measurements, was between 0.2 and 0.4 (mean = 0.32 ± 0.08; n = 7). In the modeling, the mitochondria were assumed to be confined to a belt between 1 and 5 μm below the apical surface of the cell (−1 > z ≥ −5 μm). Due to the steep Ca²⁺ gradient along the cell depth, the effects of sparser mitochondria ≤5 μm from the cell top are negligible and would minimally affect the results. The rate of change of cytosolic free Ca²⁺ due to mitochondrial Ca²⁺ transport was composed of the flux into the mitochondria by the calcium uniporter (J_{m,uni}) and transport out of the mitochondria by the sodium-calcium exchanger (J_{m,NCX})

\[
\left( \frac{dC}{dt} \right)_{init} = -J_{m,uni} + J_{m,NCX}
\]

The rate of change of mitochondrial matrix free Ca²⁺ concentration (C_m) is

\[
\frac{dC_m}{dt} = -\kappa_m \gamma_m (J_{m,uni} + J_{m,NCX})
\]

where \( \kappa_m \) is the free to fixed Ca²⁺ in the mitochondria (\( \kappa_m = 2.5 \times 10^{-2} \); Babcock and Hille 1998) and \( \gamma_m \) is the ratio of mitochondrial volume to diffusible cytosolic volume. To assess the maximal mitochondrial contribution, a volume fraction of 0.3 was used: \( \gamma_m = 0.3/0.7 = 0.43 \). Second-order kinetics was assumed for the mitochondrial uniporter (Gunter et al. 2000; Nicholls 2005)

\[
J_{m,uni} = v_{max,uni} \frac{C^2}{K_{uni} + C^2}
\]

where \( v_{max,uni} \) is the maximum Ca²⁺ transportation rate of the uniporter in μM·ms⁻¹ and \( K_{uni} \) is the half-maximal activation point (40 μM; Xu et al. 1997). The \( v_{max} \) Value of 114 μM·ms⁻¹ was derived from the in vitro mitochondrial calcium uptake rate of 4 nmol/min per mg of mitochondrial protein at \( C = 0.5 \) μM (Wingrove et al. 1984). Ca²⁺ extrusion from the mitochondria by the sodium-calcium exchanger (NCX) was assumed to have a first-order relationship (Patterson et al. 2007)

\[
J_{m,NCX} = \frac{v_{max,NCX}}{(1 + K_{NCX}/C_m)}
\]

The maximal extrusion rate \( v_{max,NCX} \) of 1.81 μM/ms was chosen so that the mitochondrial Ca²⁺ transport is balanced at \( C = 0.5 \) μM, \( C_m = 0.05 \) μM, and \( K_{NCX} = 5 \) μM. The half-activation point of the sodium-calcium exchanger \( K_{NCX} = 5 \) μM (Patterson et al. 2007).

COMPUTATION. Finite difference equations were derived from the partial differential equations and were integrated using the Crank-Nicholson method with a time step of 0.2 μs. The code was validated by comparing the Ca²⁺ influx rate calculated analytically to the sum of the changes in free, buffered, and extruded Ca²⁺ in the simulation. The error did not exceed 1%. Initial conditions were as follows: the initial resting Ca²⁺ concentration was \( C = 0.1 \) μM, which is determined by the set point of the PMCA pump-leakage equation (Eq. 2); the initial concentrations of the buffers were obtained by static equilibrium with the 0.1 μM free calcium. Because the Ca²⁺ equilibrium point of the mitochondria (C ≡ 0.5 μM by Eq. 3; Nicholls 2005) is higher than that of the PMCA pump (C = 0.1 μM), in the initial state with the channel closed, the mitochondrial free calcium should be depleted and was set to zero.

DYE FLUORESCENCE. The fluorescence of the calcium indicator dye is intended to indicate the concentration of free Ca²⁺ in the focal plane, but it is an indirect measure because the fluorescent signal reflects Ca²⁺ binding to a dye and is integrated over the focal volume of the object defined by the radial and axial resolution of the microscopic system, 0.22 and 2.2 μm (see preceding text), respectively, which are comparable to the diameter and height of the stereocilia. Therefore it is inappropriate to equate the experimentally measured fluorescence in the stereocilia with the concentration of the calcium or calcium-bound dye in the simulation. For a better comparison of the experiment and the model, the simulated results were postprocessed to obtain the fluorescent intensity. The fluorescent intensity \( f(x, y, z) \) was calculated by integrating the concentration of Ca²⁺-bound dye, \( g(x, y, z) \), weighted by the Airy function \( w(x, y, z) \) over the focal space

\[
f(x, y, z) = \int g(x, y, z) w(x, y, z) \, dx \, dy \, dz
\]

In our case, the space is two-dimensional and the integration along the y-axis can be simplified by assuming uniform dye concentration in the y-direction

\[
f(x, z) = \int g(x, z) q(x, z) w(x) \, dx \, dz
\]

Instead of the computationally intractable Airy function, a Gaussian distribution that well matches the large central region of the Airy function was used

\[
w(x, z) = \frac{1}{\sigma_x \sqrt{2\pi}} \exp \left[ -\frac{(x - x_m)^2}{2\sigma_x^2} \right] \frac{1}{\sigma_z \sqrt{2\pi}} \exp \left[ -\frac{(z - z_m)^2}{2\sigma_z^2} \right]
\]

where \( x_m \) and \( z_m \) are the coordinates of the focal point. The SD terms \( \sigma_x \) and \( \sigma_z \) were obtained by dividing the measured radial and axial resolution by 2.355. The term \( q(x, z) \) represents the integration of the Gaussian function along the y-axis

\[
q(x, z) = \text{erf}(r/\sigma_y \sqrt{2}) \text{ stereocilia}
\]

\[
1.0 \text{ cell body}
\]

where \( r \) is the half-thickness of stereocilia at point \((x, z)\).

RESULTS

Hair bundle calcium signals

Hair bundles of OHCs were orientated vertically along the optical axis and were imaged by focusing in the middle or base of the bundle. Mechanical stimulation evoked a rapid rise in intracellular calcium that was initially confined to the bundle (Fig. 1A). A rapid onset to the calcium flux was ensured by using a protocol in which the bundle was deflected while the cell was held at a positive potential so that there was no calcium influx. The cell was then repolarized to −80 mV initiating calcium entry. With this paradigm, the average calcium in the hair bundles accumulated and dissipated rapidly for brief stimuli. Although there was often a progressive increase in background fluorescence throughout the experiment, probably reflecting calcium loading, the time course of the \( \text{Ca}^{2+} \) (\( \Delta F/F \)) was stable for the first 15 to 20 min (Fig. 1B). The bundle signal developed with two kinetic components, which could be most clearly distinguished when the duration of the bundle displacement was varied (Fig. 1C). For stimuli lasting 50 to 200 ms, the calcium decayed quickly after the stimulus, with a time constant of 25 to 70 ms (mean = 54 ± 11 ms, n = 9), but for longer stimuli, a secondary component of removal with a time constant of 100 to 300 ms appeared. These time constants are not limited by the off rate of the low-affinity dye, which will be a fraction of a millisecond. The fluorescent signals were abolished by depolarization and also by blocking the MT channels with streptomycin (Beurg et al. 2009), indicating they reflect influx via the MT channels.
Mitochondria and PMCA pumps in the clearance of hair bundle calcium

Deflection of the bundle with a train of stimuli delivered with a stiff piezoelectric probe produced fast transients superimposed on a secondary buildup of calcium (Fig. 2A) that was cleared with a slower time constant over 100 ms (mean = 115 ± 90 ms, n = 6). This secondary component was prolonged and the Ca$^{2+}$ transient was augmented if the mitochondrial uptake via the calcium uniporter was blocked by addition of 1 μM Ruthenium red (RR) to the intracellular medium (Fig. 2B). The mean time constant of the decay of the calcium transient was increased over fivefold to 607 ± 92 ms (n = 5). The increase in ΔF/ΔF₀ and slowing of the decay time constant with RR probably reflect saturation of the calcium buffer, EGTA, due to Ca$^{2+}$ loading because the removal mechanisms are too slow to contribute to fast clearance (see Modeling of calcium transients). RR, which is a polyvalent cation, has other actions including blocking the ryanodine receptor and, when present extracellularly, abolishing the MT current (Farris et al. 2004). However, there was no evidence of an effect on the MT current following intracellular perfusion of RR. Another argument against a nonspecific effect of RR is that more than a 10-fold prolongation of the Ca$^{2+}$ transients also occurred with the protonophore, FCCP (2 μM; Fig. 3B), which by eliminating the mitochondrial membrane potential also prevents Ca$^{2+}$ uptake. The effects cannot be due to loss of ATP because the nucleotide was present at 2.5 mM in the pipette solution. Furthermore, the slowing of the Ca$^{2+}$ decay occurred over a period during which the kinetics were stable in control measurements (Fig. 1B). A similar prolongation (Fig. 3A) was seen when the plasma membrane PMCA pumps were blocked by lowering the extracellular H$^+$ concentration (Duman et al. 2008; Xu et al. 2000). Comparable effects of alkalinization on the Ca$^{2+}$ transient, increasing background Ca$^{2+}$, and slowing the responses to bundle stimulation were seen in two other cells, although this maneuver had no effect on the maximum MT current in OHCs (mean current 830 ± 150 pA, pH 7.4; 846 ± 20 pA, pH 9; n = 3). For both the FCCP treatment and increasing pH, the effects of changing the extracellular perfusion medium took some time to develop. It seems likely that the slow onset of the effect (~10 min) is due in part to the time to saturate the cytoplasmic calcium buffers because, in all cases, the change in the kinetics of Ca$^{2+}$ clearance did not occur immediately after blocking the pump but only later when the background fluorescence had increased significantly.

These experiments suggest that both the PMCA pumps and mitochondrial uptake contribute to clearance of calcium from the hair bundle during mechanical stimulation. Impairment of either process results in an elevation and a protracted elimination of the bundle Ca$^{2+}$. Since the MT current is regulated by changes in stereociliary Ca$^{2+}$, hindering Ca$^{2+}$ removal would be expected to affect the MT currents. Blocking the PMCA pump had more pronounced effects on the current–displacement (I–X) relationship, both shifting it in the positive direction and substantially reducing its slope (Fig. 4A). Similar effects on the I–X relationship in OHCs have been reported by mutation or knock out of the PMCA2 isoform of the plasma membrane Ca-ATPase (Ficarella et al. 2007). The effects of FCCP (Fig. 4B) and RR (not shown) were similar to those with block of the PMCA pump, a translation of the I–X relationship,
and a decrease in its slope. The control MT currents in these types of experiment were comparable to those previously measured for apical rat OHCs (Beurg et al. 2006, 2008; Kennedy et al. 2003; Waguespack et al. 2007). In eight control measurements, the maximum MT current was $1.09 \pm 0.09$ nA, the mean adaptation time constant was $0.32 \pm 0.08$ ms, and the activation range for the $I-X$ relationships was 200 to 300 nm. For the treatments intended to elevate intracellular Ca$^{2+}$ there was a small reduction in the maximum current, a significant reduction in the extent and speed of fast adaptation, and an increase in the activation range to 500 to 1,000 nm. Maximum currents and adaptation time constants under different conditions were: $1.07 \pm 0.11$ nA, $0.98 \pm 0.12$ ms ($n = 3$; pH 9); $0.80 \pm 0.17$ nA, $0.95 \pm 0.12$ ms ($n = 3$; FCCP); $0.99 \pm 0.17$ nA, $0.98 \pm 0.13$ ms ($n = 3$; RR). Although the shift in the activation relationship resembles that during adaptation and would be expected simply by elevating stereociliary Ca$^{2+}$, the slowing of adaptation and the broadening of the $I-X$ relationship imply that other factors may be involved.

To confirm that the mitochondria in the belt beneath the cuticular plate contributed to uptake of Ca$^{2+}$ originating from the MT channels, mitochondrial Ca$^{2+}$ was monitored with Rhod2 during bundle stimulation (Fig. 5A). Hair cells had been previously loaded with the esterified form, Rhod2AM, and after 30 min incubation, it was possible to visualize a series of bright spots at the cell apex just beneath the cuticular plate that were interpreted as being the mitochondria (Duchen et al. 2001). After attaining whole cell recording, we waited 5 min to wash out any deesterified dye remaining in the cytoplasm. On bundle deflection, an increase in the mitochondrial signal was obtained in all cells studied (Fig. 5B). When we focused on the hair bundle, no fluorescence signal was measurable, indicating that the signal does not originate from Ca$^{2+}$ binding to cytoplasmic Rhod2, which should have been washed out after attaining the whole cell configuration. The intramitochondrial Ca$^{2+}$ increase began with a diffusional delay of about 20 ms and in one cell was sustained but in the others showed some decline after the stimulus. The decay time constant, probably reflecting rerelease of Ca$^{2+}$ by the mitochondrial sodium/calcium exchanger averaged over five cells, was $1.20 \pm 0.75$ s (Fig. 5B).

**PMCA exchange current**

A crucial factor in maintaining long-term Ca$^{2+}$ balance is the number of PMCA pumps. The operation of the pump is electrogenic, one Ca$^{2+}$ being extruded in exchange for one H$^+$ during each ATPase cycle (Hao et al. 1994). The resulting current, a small outward current following Ca$^{2+}$ loading, has been recorded in frog saccular hair cells (Yamoah et al. 1998). We used a similar approach to estimate the exchange current in OHCs, by comparing MT currents evoked by prolonged bundle deflections in the absence and the presence of a pump inhibitor (Fig. 6). Measurements were made in an extracellular saline in which the Na$^+$ had been replaced by the relatively impermeant Tris (see METHODS), so the MT current was carried largely by Ca$^{2+}$. At the offset of the bundle stimulus, there was a small slow outward current that could be reversibly blocked by increasing the pH around the hair bundle to 9.0 by a brief 10-s perfusion from a nearby puffer pipette (Fig. 6A). The exchange current $I_{EX}$ was derived by subtracting the currents with and without pump block (Fig. 6B). It was recorded in all seven cells studied, decayed back toward the baseline with a principal time constant of $143 \pm 23$ ms, and had an initial amplitude that grew with the Ca$^{2+}$ load and saturated at close to 18 pA (Fig. 6C), corresponding to a Ca$^{2+}$ efflux of 3.7 fmol·s$^{-1}$. The Ca$^{2+}$ load was determined by integrating the current during the bundle deflection. In some but not all cells growth of a small outward current occurred during the stimulus (see Fig. 6D).

Several arguments have been previously advanced that the current is not an artifact of a change in transduction (Yamoah et al. 1998). In our experiments, the puffer pipette was placed about 50 μm away from the bundle and the flow did not mechanically stimulate the bundle, as indicated by the fact that the $I-X$ relationships were superimposable with and without the blocker. $I_{EX}$ therefore seems unlikely to be attributable to a change in adaptation. Second, $I_{EX}$ cannot be an exchange current due to either the Na pump (Na$^+$ omitted from the extracellular medium) or proton extrusion (which in the hair bundle encompassing all 3 rows of stereocilia).
Calcium influx in endolymph solutions

The experiments described so far were conducted in a saline containing high (1.5 mM) Ca\(^{2+}\) bathing the hair bundle similar to perilymph, whereas rat endolymph has only 20 \(\mu\)M Ca\(^{2+}\) (Bosher and Warren 1978). Problems with maintaining homeostasis depend critically on the size of the Ca\(^{2+}\) influx under in vivo conditions and there are suggestions for hair cells of both turtle (Ricci and Fettiplace 1998) and frog (Lumpkin and Hudspeth 1998) that the Ca\(^{2+}\) influx in vivo might be substantial. To reexamine this point in mammalian cochlear hair cells, bundle Ca\(^{2+}\) transients were measured for reduced extracellular Ca\(^{2+}\) concentrations using a pair of puffer pipettes containing control (1.5 mM Ca\(^{2+}\)) and test solutions (see METHODS). Because the transduction-related Ca\(^{2+}\) signals were smaller than expected, it was necessary to use the high-affinity indicator Fluo-4. Although lowering the Ca\(^{2+}\) concentration by a factor of 53 ± 0.28, the product of these values (\(I_{K} \cdot \Delta F_{R}\)) was 82 ± 8, yielding the reduction in Ca\(^{2+}\) carried by the MT current for a 75-fold lowering of Ca\(^{2+}\) from 1.5 to 0.02 mM. When Ca\(^{2+}\) was reduced 10-fold from 1.5 to 0.15 mM, \(I_{K}\) was 1.49 ± 0.09 (\(n = 4\)), \(\Delta F_{R}\) was 7.4 ± 2.1, and (\(I_{K} \cdot \Delta F_{R}\)) was 11 ± 3. The measurements in the two Ca\(^{2+}\) concentrations indicate that the fraction of MT current carried by Ca\(^{2+}\) decreases directly in proportion to its external concentration.

In the intact cochlea, the hair bundles are exposed to endolymph, which is not only low in calcium but also has K\(^{+}\) as the major monovalent cation. To more accurately reproduce the in vivo condition, the pipette solution was changed from a Cs\(^{+}\)-based to a K\(^{+}\)-based intracellular, the maximum MT current was 800 ± 190 pA (\(n = 9\)). When Ca\(^{2+}\) was reduced from the control 1.5 mM to the artificial endolymph, the current ratio \(I_{K}/I_{R}\) was 2.1 ± 0.4 (\(n = 3\)), the fluorescence ratio \(\Delta F_{R}/F_{R}\) was 30.7 ± 3.3, and (\(I_{K} \cdot \Delta F_{R}\)) was 65 ± 5. Thus although the increase in current size was somewhat larger than that in the measurements in Na\(^{+}\), the fraction of current carried by Ca\(^{2+}\) still decreased roughly in proportion to its extracellular concentration, which

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**Fig. 3.** Effects of Ca\(^{2+}\) uptake and extrusion blockers on OHC hair bundle Ca\(^{2+}\) signals. *A:* effects of increasing the pH of the extracellular solution to 9.0 to block the plasma membrane Ca\(^{2+}\) ATPase (PMCA) pump, which requires H\(^+\) influx to exchange for Ca\(^{2+}\) efflux. This caused an increase in cytoplasmic calcium, and slows the response to a bundle stimulus. In both *A* and *B*, the stimulus was a depolarization from −80 to +80 mV followed by a bundle displacement.

**Fig. 4.** Effects of increasing the pH of the extracellular solution to 9.0 to block the plasma membrane Ca\(^{2+}\) ATPase (PMCA) pump, which requires H\(^+\) influx to exchange for Ca\(^{2+}\) efflux. This caused an increase in cytoplasmic calcium, and slows the response to a bundle stimulus. In both *A* and *B*, the stimulus was a depolarization from −80 to +80 mV followed by a bundle displacement.

The exchange current can be used to estimate the pump density, assuming all pumps are operating at their maximum rate and are located in the hair bundle. If the membrane area of the hair bundle is taken as approximately 200 \(\mu\)m\(^2\), a peak exchange current of 18 pA would require a pump density of roughly 6,000/\(\mu\)m\(^2\), assuming a pump turnover rate of 100 s\(^{-1}\), the upper limit of that measured for the erythrocyte PMCA pump (Kubitscheck et al. 1995). Such a high pump density is several times greater than that inferred for frog saccular hair cells (2,000/\(\mu\)m\(^2\); Lumpkin and Hudspeth 1998; Yamoah et al. 1998).
was 75-fold. Collected measurements from the low-Ca\textsuperscript{2+} imaging experiments are given in Fig. 9D and are well described by a linear fit. Absolute values for the fraction of current carried by Ca\textsuperscript{2+} were calculated using a control value of 0.15 for a 1.5 mM Ca\textsuperscript{2+} perilymph solution. This value was derived from experiments in which the extracellular monovalent ions Na\textsuperscript{+} and K\textsuperscript{+} had been replaced by the relatively impermeant N-methylglucamine, so the MT current was carried solely by Ca\textsuperscript{2+} (Beurg et al. 2006). The conclusion from Fig. 9D is that Ca\textsuperscript{2+} accounts for only about 0.2% of the transduction current in a saline comparable to endolymph.

One further feature of the intact cochlea is the potential difference between endolymph and perilymph, known as the endolymphatic potential, which is about 90 mV in rats (Bosher 1979). This endolymphatic potential sums with the resting potential of ~60 mV to augment the electrical driving force across the MT channels in the hair bundle. Resting potential measurements in P8 to P14 rats using EGTA as the intracellular calcium buffer gave a mean value of ~60 ± 6 mV (n = 7), implying a driving force in vivo of 150 mV. This condition was simulated by increasing the holding potential to 150 mV and measuring the MT current amplitude and calcium transient (Beurg et al. 2006). The conclusion from Fig. 9D is that Ca\textsuperscript{2+} accounts for only about 0.2% of the transduction current in a saline comparable to endolymph.

**Modeling of calcium transients**

Hair bundle and somatic Ca\textsuperscript{2+} transients were simulated using a model incorporating Ca\textsuperscript{2+} influx via the MT channels, diffusion, and binding to both mobile and fixed buffers, extrusion by PMCA pumps, and uptake into mitochondria (Fig. 10A; see METHODS). The model incorporated several new features compared with previous simulations of hair bundle Ca\textsuperscript{2+} (Lumpkin and Hudspeth 1998; Wu et al. 1999), including diffusion into the cell body, the contribution of the mitochondria, and correction for the limited resolution of the imaging system. For a single stimulus in 1.5 mM extracellular Ca\textsuperscript{2+}, Ca\textsuperscript{2+} rapidly accumulated in the stereocilia, rising after a small delay then increasing with two phases as observed experimentally (Fig. 1B). The speed of the first phase was inversely proportional to distance from the channel, whereas the second was dominated by the dimensions of the cell body, increasing as the cell width decreased. The time constant of decay of the
Ca\(^{2+}\) (Fig. 10B) was as fast as the stimulus offset until its concentration approached the equilibrium constant of the fixed buffer (10 \(\mu\)M), after which it was slowed. However, if the time course of the Ca\(^{2+}\) transient was expressed in terms of the integrated fluorescence intensity (Fig. 10C), both the onset and decay of the fluorescence signal were slower than those of the Ca\(^{2+}\) itself and were similar to the experimental results. The simulation agreed with the experiments if there was \(\approx 2\) mM of fixed buffer with dissociation coefficient \((K_{Ca})\) of 10 \(\mu\)M, in which case the decay time constant of the fluorescence was 40 ms, similar to the measurements. This decay time constant was dependent on the concentration and \(K_{Ca}\) of the fixed buffer and, when there was no fixed buffer, the time constant decreased from 40 to 10 ms.

In the first 100 ms, the transient Ca\(^{2+}\) profile was dominated by the mobile and fixed buffers rather than PMCA pumps and mitochondria (Fig. 10D). The PMCA pump could transport <10% of the entering calcium. The mitochondrial Ca\(^{2+}\) uptake was negligible within the first 100 ms of stimulation but, as the free Ca\(^{2+}\) concentration in the cell body increased, the mitochondrial contribution became comparable to that of the PMCA pump. The high-affinity mobile buffer (EGTA) had the greatest effect on the Ca\(^{2+}\) concentration at the stereociliary tips. At the termination of the stimulus, the buffering system returned most of the Ca\(^{2+}\) it had absorbed within a fraction of a second (Fig. 10, D and E). The Ca\(^{2+}\) was shuttled along the chain from low-affinity to high-affinity binding sites, from fixed buffer \((K_{Ca} = 10 \mu\)M) to mitochondria (equilibrium point near 0.5 \(\mu\)M), EGTA \((K_{Ca} = 0.2 \mu\)M), and finally extruded by PMCA pumps. An important conclusion from this modeling is that the kinetics of the Ca\(^{2+}\) response to a single bundle stimulus is scarcely affected by the pumping mechanisms.

The simulations in Fig. 10 were for a single stimulus starting from a condition in which the MT channels were closed. However, in reality there is a persistent calcium influx attributable to the MT channels being partially open at rest. For rat OHCs in vitro, bathed in 1.5 mM extracellular calcium, about 5% of the total MT current (\(I_{MAX} \approx 1,000\) pA) is activated at rest (Kennedy et al. 2003), equivalent to a standing Ca\(^{2+}\) current of about 8 pA. To avoid continual accumulation of intracellular Ca\(^{2+}\), the entire Ca\(^{2+}\) load must be extruded solely by the PMCA pumps. To examine the importance of the PMCA pumps, two different densities in the stereociliary membrane were simulated: 2,000 PMCA pumps\(\mu\)m\(^{-2}\), as suggested for amphibian hair cells (Yamoah et al. 1998) and 6,000 pumps\(\mu\)m\(^{-2}\), as implied from our OHC measurements (Fig. 7), in each case assuming a maximum transport rate of 100 ions/s per pump (Kubitschek et al. 1995). When there were 6,000 PMCA pumps\(\mu\)m\(^{-2}\) in the stereociliary membrane, the simulated hair cell could marginally sustain 30 pA of constant Ca\(^{2+}\) current (Fig. 11B). Although the mitochondria were still accumulating calcium, the uptake rate was decreasing (Fig. 11B). Furthermore, with this resting Ca\(^{2+}\) influx, about 99% of the mobile buffer was depleted at the tips of the stereocilia. For a constant current of 20 pA, all the Ca\(^{2+}\) was extruded by the PMCA pumps without loading the mitochondria and this therefore represents the safe rate. When the resting Ca\(^{2+}\) current was increased to 36 pA, the PMCA pumps could not extrude the Ca\(^{2+}\), which accumulated within the mitochondria (Fig. 11C). As the resting Ca\(^{2+}\) current was increased from 20 to 36 pA, the maximum Ca\(^{2+}\) concentration grew nearly 10-fold and the free cytoplasmic Ca\(^{2+}\) concentration in the mitochondrial region, 4 \(\mu\)M below the stereociliary rootlets, increased from 0.8 to 4.5 \(\mu\)M. When the PMCA density was reduced to 2,000 pumps\(\mu\)m\(^{-2}\), equivalent to the nonmammalian density, the simulated hair cell could marginally sustain a constant Ca\(^{2+}\) current of 12 pA (Fig. 11D) and could cope safely with a Ca\(^{2+}\) influx of only 8 pA (not shown). As expected, the time course of Ca\(^{2+}\) clearance was threefold slower at the lower pump rate.

Thus with 6,000 PMCA pumps\(\mu\)m\(^{-2}\), the OHCs should be able to cope with sustained Ca\(^{2+}\) influxes in both 1.5 and 0.02 mM external Ca\(^{2+}\), with resting currents three- to tenfold smaller than could be safely extruded. However, an important caveat is the contribution of the PMCA pumps in the soma. Although in the simulations, the lateral somatic membrane contained only one tenth the density of PMCA pumps, compared with that of the stereociliary membrane, the role of the somatic PMCA pumps was not negligible. If these were removed entirely, the OHC could handle a resting Ca\(^{2+}\) influx through the MT channels of <15 pA, even with the higher PMCA pump density in the stereociliary membrane.

**Effects of intracellular calcium buffer**

The model was also useful for comparing the efficacy of the endogenous mobile calcium buffer, 2 mM parvalbumin-\(\beta\) (on-
comodulin), and 0.25 mM calbindin (Hackney et al. 2005), with the extrinsic buffers used in the pipette solution. Because parvalbumin-β binds two Ca\(^{2+}\) ions and calbindin binds four Ca\(^{2+}\) ions, the effective concentration of Ca\(^{2+}\) binding sites is 5 mM. EGTA has been the principal buffer used experimentally in our studies of rat cochlear hair cells but at 1 mM, and with a slow Ca\(^{2+}\) binding rate, it was more than 10-fold less effective in buffering stereociliary Ca\(^{2+}\) than parvalbumin-β. The endogenous buffer was therefore compared with BAPTA (Fig. 12). Based on fits to the Ca\(^{2+}\) gradient along the stereocilia, the endogenous buffer was roughly equivalent to between 0.75 and 1.3 mM BAPTA (Fig. 12B), the lower concentration providing the best match to the distal part of the gradient near the stereociliary tip and the higher concentration to the proximal gradient around the rootlet. It was not possible to obtain a perfect match to the kinetics (Fig. 12A), which was faster with BAPTA due to its more rapid Ca\(^{2+}\) binding rate (Table 1). EGTA was much less effective as a buffer and ≥10 mM was needed to give a match to the endogenous buffer at the stereocilia tip (not shown). Modeling showed that the PMCA pumps contributed little to the stereociliary Ca\(^{2+}\) gradient because their inhibition had no effect in the short term until the buffers became saturated. To assess transduction under in vivo conditions, an intracellular solution containing 1 mM BAPTA was used to approximate the endogenous mobile buffer in OHC. Stereociliary Ca\(^{2+}\) gradients for different concentrations of exogenous buffers were also computed for inner hair cells. These cells in posthearing animals contain one tenth the mobile endogenous buffer of OHCs: about 0.5 mM Ca\(^{2+}\) binding sites, composed of parvalbumin and calretinin (Hackney et al. 2005). Assigning binding properties for calretinin similar to those for calbindin, stereociliary Ca\(^{2+}\) gradients at long times could be well matched to 0.5 mM EGTA (not shown). A low concentration of endogenous calcium buffer has previously been inferred for IHCs from effects on exocytosis (Moser and Beutner 2000).

The nature and concentration of the cytoplasmic calcium buffers contribute to the transient responses and to the gradients away from the source (Roberts 1993). As a consequence, they influence several aspects of the MT currents, including the fraction of current activated at rest and the kinetics of adaptation (Ricci et al. 1998). These parameters were measured in OHCs by hair bundle deflection with a glass probe attached to a piezoelectric actuator. With an intracellular solution containing K\(^+\) and 1 mM EGTA as the calcium buffer, changing the hair bundle perfusate from control (Na\(^+\), 1.5 mM Ca\(^{2+}\)) to artificial endolymph (K\(^+\), 0.02 mM Ca\(^{2+}\)) increased the maximum MT current from 989 ± 195 to 1,680 ± 190 pA and the fraction of current activated at rest from 0.04 ± 0.01 to 0.12 ± 0.02 (n = 5; Fig. 13A). Thus a mean sustained current of 200 pA flowed at rest. The artificial endolymph also slowed the fast adaptation time constant from 0.20 ± 0.05 to 0.58 ± 0.03 ms as reported previously (Ricci et al. 2005). When 1 mM BAPTA was used instead of EGTA, during perfusion with artificial endolymph a standing inward current was evident in all cells examined. Since the standing current could be blocked by 0.2 mM dihydrostreptomycin, this suggests a large resting probability of opening of the MT channels. On switching from control to artificial endolymph in OHCs recorded with 1 mM BAPTA, the peak current in five cells increased from 924 ± 128 to 1,334 ± 152 pA; the fast adaptation time constant slowed from 0.24 ± 0.08 to 0.5 ± 0.05 ms; and the fractional current turned on at rest increased from 0.07 ± 0.01 to 0.40 ± 0.08 (n = 5), the leftward shift in the I–X relationship being fully reversible (Fig. 13B). In this case, the resting current through the MT channels in endolymph amounted to 550 pA (mean in five OHCs, 523 ± 50 pA). As a consequence, the OHC depolarized from a resting potential of −49 mV (control) to −29 mV (endolymph), returning to −46 mV (wash).

![Figure 6](http://jn.physiology.org/)

**FIG. 6.** Quantification of the PMCA pump rate from the exchange current I\(_{\text{EX}}\). A: MT currents to 0.6 μm hair bundle displacements before, during, and after increasing the pH of the saline around the bundle from 7.4 to 9.0 to block the PMCA pump. Three responses are superimposed: the pH 7.4 (control and wash) as black lines and the pH 9.0 (pump block) as gray. B: I\(_{\text{EX}}\) obtained by subtracting the pH 9.0 from the pH 7.4 in A. The decline in I\(_{\text{EX}}\) after termination of the stimulus is fitted with 2 time constants of 128 ms and 2.2 s (dashed line). C: the I\(_{\text{EX}}\) in 7 cells plotted against the Ca\(^{2+}\) load, obtained by integrating the MT current during the stimulus. For the 2 smallest Ca\(^{2+}\) loads, the hair bundle stimulus was 400 ms in duration, whereas that for the rest it was 800 ms. D: MT currents to 0.6 μm hair bundle displacements before (control) and several minutes after replacing the bath saline, with one containing 50 μM carboxyeosin diacetate succinimidyl ester to block the PMCA. Inset shows I\(_{\text{EX}}\) obtained by subtracting the test from the control in D. The decline in I\(_{\text{EX}}\) is fitted with 2 time constants of 125 ms and 3.3 s (dashed line). All experiments were performed on P7 to P10 rats in salines in which the Na\(^+\) had been replaced by Tris so the MT current is carried mainly by Ca\(^{2+}\).
DISCUSSION

Calcium homeostasis in vivo

We have used fast millisecond imaging to monitor calcium changes in the hair bundle and cell apex of OHCs during MT channel activation. For brief stimuli the time course of the hair bundle transient was dictated largely by diffusion and binding to cytoplasmic buffers and not by extrusion mechanisms, which operate on a slow timescale of seconds. However, significant perturbation of the Ca$^{2+}$/H$^{+}$ dynamics occurred during prolonged inhibition of the PMCA pumps or the mitochondrial uptake system as the calcium buffers became saturated. Quantification of the PMCA pump rate indicated that it was at least threefold larger than that in nonmammalian hair cells, which should allow the OHCs to cope with large Ca$^{2+}$/H$^{+}$ loads incurred during periods of high stimulation. Initial results were obtained in isolated preparations of the organ of Corti that differ in several respects from the intact cochlea. Ca$^{2+}$/H$^{+}$ influx through the MT channels will be altered because of a low endolymph Ca$^{2+}$ concentration, an endolymphatic potential, and an elevated body temperature. This led us to examine the Ca$^{2+}$/H$^{+}$ load under conditions more closely resembling those in vivo in which the solution enveloping the hair bundles had a reduced Ca$^{2+}$ concentration (20 mM), resulting in a smaller Ca$^{2+}$/H$^{+}$ influx. Our measurements indicated that the fraction of current carried by Ca$^{2+}$ decreased in proportion to the extracellular concentration of the divalent cation and, in 20 mM external calcium, only about 0.2% of the current was due to Ca$^{2+}$. This result differs from previous conclusions that in nonmammalian vertebrates, such as frogs or turtles (Jørgensen and Kroese 1995; Ricci and Fettiplace 1998), a much larger fraction of current (~10%) is carried by calcium in endolymph. The reasons for the discrepancy are unclear, although the results carry implications for the resting Ca$^{2+}$ load in vivo and for the role of Ca$^{2+}$ in fast adaptation.

The much reduced Ca$^{2+}$ influx in endolymph is puzzling in light of the proposed role of Ca$^{2+}$ in adaptation, which is only weakly sensitive to extracellular Ca$^{2+}$. Lowering Ca$^{2+}$ from 1.5 to 0.02 mM slows the fast adaptation time constant by no more than a factor of three to four (Ricci et al. 2005; present study). One explanation for this result is that the adaptation time constant in high Ca$^{2+}$ is overestimated because of a limitation in the speed of stimulus delivery. Alternatively, the time constant, when it gets briefer, may become independent of intracellular Ca$^{2+}$ because other factors, such as conformational transitions in the MT channel underlying adaptation, become rate limiting.

Hair bundle calcium load in vivo

Our results allow a determination of the Ca$^{2+}$ load under in vivo like conditions. With $K^+$ as the monovalent cation, as in endolymph, and with the addition of an endolymphatic potential, we estimate that the maximum Ca$^{2+}$ load in apical OHCs is 7 pA. Using an intracellular calcium buffer (1 mM BAPTA) roughly equivalent to the endogenous mobile buffer, about 40% of the MT current is activated at rest, so the sustained calcium load is 3 pA under ionic conditions resembling those in vivo. These currents can be compared with the ability of the OHC to extrude the accumulated Ca$^{2+}$ via the PMCA pumps in the stereocilia. Simulations with brief calcium loads showed that the pump can safely clear a sustained influx of 20 pA (Fig. 11A), several times larger than either the resting or maximum MT currents, thus providing a reasonable safety margin. This safety margin arises because of a larger than expected PMCA pump density inferred from measurements of the electrogenic
pump current that are greater than those recorded in bullfrog saccular hair cells (Yamoah et al. 1998). The electrogenic exchange current does not directly yield the pump density but requires assumptions about the turnover rate and the membrane area. We assumed a turnover rate of 100 ions/s, equivalent to the maximum measured in biochemical experiments on erythrocytes (Kubitscheck et al. 1995), but the turnover rate in the PMCA2 isoform, which is regarded as a fast neuronal pump (Brini et al. 2003), may be larger than the PMCA1 and -4 isoforms in erythrocytes. The PMCA pumps in OHCs were assumed to be largely confined to the hair bundle because they were inhibited by brief perfusion with a high pH saline that enveloped the hair bundles, but was unlikely to gain access to the OHC basolateral membrane. However, some PMCA pumps must occur in the soma to extrude Ca\(^{2+}\)/H\(^{+}\) that has entered as the voltage-dependent Ca\(^{2+}\)/H\(^{+}\) current, although this current is small in rats older than P8 (Beurg et al. 2008).

The present conclusions apply only to the apical low-frequency OHCs. High-frequency OHCs may be more vulnerable to becoming Ca\(^{2+}\) loaded because of larger MT currents (Ricci et al. 2005) and shorter stereocilia (Roth and Bruns 1992). Because of tonotopic gradients in MT channel size and numbers of stereocilia (Beurg et al. 2006; Ricci et al. 2003, 2005), there could be up to a fivefold greater MT current flowing into hair bundles of high-frequency OHCs compared with low-frequency ones. As a consequence, the Ca\(^{2+}\) load during transduction will be substantially larger than that in low-frequency OHCs. The measured pump density may then be only marginal to extrude the larger load and it will be important to establish whether there is a concomitant increase in the PMCA pump rate in high-frequency cells to deal with this load. One factor not accounted for in the present experiments is the raised body temperature (37°C) in vivo, which will increase both Ca\(^{2+}\) influx and efflux. However, the effect of temperature on the Ca-ATPase rate (Q\(^10\)) by analogy with other ion channel conductances; Hille 2001), which therefore widens the safety margin between the maximum rates of influx and removal.

**Inner and outer hair cells**

These effects of the calcium buffer on the resting open probability recall similar measurements in turtle auditory hair cells. In those cells, recording with a 0.5 mM BAPTA electrode equivalent to the endogenous calcium buffer and exposing the hair bundles to low-calcium endolymph...
yielded a fraction of MT current activated at rest as 28% (Ricci et al. 1998). In turtle, as in rat OHCs, the high resting open probability of the MT channels caused a sustained inward current that depolarized the resting potential (Farris et al. 2006). The differing calcium buffer concentrations in OHCs (≈1 mM BAPTA) and IHCs (≈0.5 mM EGTA) suggests that larger fractions of MT channels will be activated at rest in OHCs than those in IHCs. This implies that, other things being equal, the receptor potentials in vivo will be more symmetrical and the resting potentials more depo-

**FIG. 9.** Hair bundle Ca$^{2+}$ signals in artificial endolymph (160 mM K$^+$, 0.02 mM Ca$^{2+}$). A: measurements in control (Na$^+$, 1.5 mM Ca$^{2+}$) solution. B: responses in artificial endolymph (160 mM K$^+$, 0.02 mM Ca$^{2+}$). C: responses in artificial endolymph (160 mM K$^+$, 0.02 mM Ca$^{2+}$), with the addition of a hyperpolarization to −150 mV to simulate the endolymphatic potential. The current response without the bundle stimulus is superimposed on that with the bundle deflection. Note the MT current amplitude is about 4 nA. The stimulus was a depolarization from −80 to +80 mV followed by a bundle displacement elicited by a fluid jet from a puffer pipette containing either Na$^+$, 1.5 mM Ca$^{2+}$ or K$^+$, 0.02 mM Ca$^{2+}$ solution. D: collected measurements of the fraction of MT current carried by Ca$^{2+}$ ($I_{Ca}/I_{Tot}$) evaluated as described in the text and plotted against the extracellular Ca$^{2+}$ concentration. Open symbols, extracellular Na$^+$, intracellular Cs$^+$; filled symbols with extracellular and intracellular K$^+$. Numbers of experiments are given beside each point. The line is a straight line fit to the points.

**FIG. 10.** Model of Ca$^{2+}$ homeostasis in an OHC. A: components of the model: Ca$^{2+}$ influx through MT channels, binding to fixed and mobile buffers, absorption by mitochondria, and extrusion via PMCA pump (filled circles). Note the pumps are concentrated in the stereocilia and virtually absent from the cell body. See METHODS for more details. B: simulated change in Ca$^{2+}$ at the base of the bundle. C: change in fluorescence at the base of the bundle. The time course is slowed relative to B due to the Fluo-4FF dye and the averaging over the focal volume; offset time constant = 40 ms. Compare with Fig. 1C. D: contribution of the different factors, buffers, PMCA pumps, and mitochondria to absorbing Ca$^{2+}$ entering. E: contribution of the different buffers, the high-affinity EGTA ($K_{Ca} = 0.2$ μM) absorbing most compared with the lower-affinity fixed buffer and dye ($K_{Ca} = 10$ μM).
larized in OHCs than those in IHCs. This conclusion differs from what has been reported with sharp electrode recordings in vivo (Cody and Russell 1987; Dallos 1985), in which the resting potentials of OHCs were very negative, about \(-70\) mV. We suggest that such negative resting potentials are unphysiological and reflect compromise of the MT apparatus, indicated by small receptor potentials \(-10\) mV peak to peak (Cody and Russell 1987; Dallos 1985), and therefore reduction of the large inward standing current via MT channels open at rest.

Role of organelles

Although \(\text{Ca}^{2+}\) signals from the mitochondrial belt below the cuticular plate were detectable only during transduction 1.5 mM external calcium, such results demonstrate a potential contribution of these organelles in acting as a fixed buffer to block \(\text{Ca}^{2+}\) diffusion into the cell body. A role for mitochondria in \(\text{Ca}^{2+}\) homeostasis has been demonstrated experimentally in other cell types (Babcock and Hille 1998; Brini 2003; Duchen 2000). Furthermore, by clearing submembranous calcium, mitochondria can influence the inactivation of voltage-dependent \(\text{Ca}^{2+}\) channels in both chromaffin cells and heart muscle (Hernández-Guijo et al. 2001; Sánchez et al. 2001). Here, we demonstrated that block of the \(\text{Ca}^{2+}\) removal systems, both mitochondria and PMCA pumps, shifted the working range of the MT channels in OHCs by causing \(\text{Ca}^{2+}\) accumulation in the stereocilia. This is consistent with the proposed role of stereociliary \(\text{Ca}^{2+}\) in regulating adaptation.

It might be argued that the main function of mitochondria in hair cells is to supply energy for the PMCA pump, but this role is

FIG. 11. Theoretical assessment of sustainable \(\text{Ca}^{2+}\) current through the MT channels. A: resting \(\text{Ca}^{2+}\) influx of 20 pA. Top: \(\text{Ca}^{2+}\) concentration along the 3 stereociliary rows as a function of distance \(z\), from the base of the bundle \((z = 0)\). Bottom: time course of contribution of the PMCA pump (thin line), buffers (dashed line), and mitochondria (thick line). B: for a resting calcium influx of 30 pA, almost all the \(\text{Ca}^{2+}\) was extruded, but there was a small accumulation in the mitochondria. This case is marginal. C: for a resting \(\text{Ca}^{2+}\) influx of 36 pA, only about 75% was extruded, the remainder accumulating in the mitochondria, which over prolonged timescale will become saturated. Simulations in A–C performed with a PMCA density of 6,000/\(\mu\text{m}^2\) (PMCA = 1). D: when the PMCA density was reduced to 2,000/\(\mu\text{m}^2\) (PMCA = 0.33), a sustained \(\text{Ca}^{2+}\) influx of 12 pA was now marginal and resulted in \(\text{Ca}^{2+}\) accumulation in the mitochondria. Individual stereociliary rows: R1, R2, and R3. All simulations with endogenous calcium buffer, 2 mM parvalbumin-\(\beta\), and 0.25 mM calbindin-28K.

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FIG. 12. Theoretical comparison of native OHC calcium buffer with BAPTA. A: time courses of \(\text{Ca}^{2+}\) transient with different concentrations of BAPTA and with the native buffer as the mobile buffer at a position \(z = 1.5 \mu\text{m}\) from rootlet. B: \(\text{Ca}^{2+}\) gradients along the middle row stereocilium in the 3 buffers 100 ms after stimulus onset. The \(\text{Ca}^{2+}\) source, the MT channels, located at 2 \(\mu\text{m}\). The mobile \(\text{Ca}^{2+}\) buffer in OHCs of posthearing rats is assumed to be 2 mM parvalbumin-\(\beta\) and 0.25 mM calbindin (Hackney et al. 2005). Based on the matches to the \(\text{Ca}^{2+}\) kinetics and the gradients along stereocilia, the native buffer is equivalent to 0.75 to 1.3 mM BAPTA.
intimately linked to the hair cell Ca\textsuperscript{2+} homeostasis. In other neurons, the majority of ATP produced is known to be expended in fueling ATPase ion pumps (Nicholls 2008). However, with even a modest decline in mitochondrial oxidative metabolism (as may occur in aging; Beal 2005) so that the ATP supply is diminished, the PMCA pumps may no longer cope with the calcium influx through MT channels open at rest; the extra calcium load will accumulate in the mitochondria and may further impair their ability to supply energy. Thus there is likely to be positive feedback between mitochondrial insult and loss of PMCA activity. Moreover, the problems will be exacerbated in high-frequency OHCs in which the Ca\textsuperscript{2+} load is larger and may account for their preferential vulnerability.

Several cytoplasmic factors were not considered in the present experiments, including the endoplasmic reticulum and the mitochondria and submembranous cisternae that line the OHC lateral wall (Lim 1986). Extracellular ATP is known to increase cytoplasmic Ca\textsuperscript{2+} in the OHC by influx via P2X receptors and by release from intracellular compartments such as Hensen’s body (Ashmore and Ohmori 1990; Mammano et al. 1999). The physiological significance of this ATP pathway is unclear and its link to OHC mechanotransduction is unknown. It is conceivably a patho-
cance of this ATP pathway is unclear and its link to OHC

buffering calcium entering through the MT channels and hindering its access to the soma. The role of the lateral cisternae in Ca\textsuperscript{2+} homeostasis is also controversial, although they may be involved in Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release secondary to acetylcholine discharge from the efferent terminals at the OHC base (Dallos et al. 1997; Lioudyno et al. 2004; Murugasu and Russell 1996). The Ca\textsuperscript{2+} released may decrease axial compliance of OHCs, thereby modulating their motor activity (Dallos et al. 1997; Frolokov et al. 2003). However, a larger Ca\textsuperscript{2+} load emanating from the hair bundle would activate proteases and irreversibly digest the somatic cytoskeleton. It will be important in future experiments to explore the factors dictating Ca\textsuperscript{2+} balance in the cell soma and whether there is any interaction with Ca\textsuperscript{2+} signals in the hair bundle.

A C K N O W L E D G M E N T S

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D I S C L O S U R E S

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