Contribution of the Plasmalemma to Ca^{2+} Homeostasis in Hair Cells

Catherine Boyer,^{1,2} Jonathan J. Art,² Claude J. Dechesne,¹ Jacques Lehouelleur,¹ Jean Vautrin,¹ and Alain Sans¹

¹Institut National de la Santé et de la Recherche Médicale U-432, Université Montpellier II, 34095 Montpellier cedex 05, France, and ²University of Illinois, College of Medicine, Department of Anatomy and Cell Biology, Chicago, Illinois 60612

Calcium influx through transduction channels and efflux via plasmalemmal Ca^{2+} -ATPases (PMCA) are known to contribute to calcium homeostasis and modulate sensory transduction in vertebrate hair cells. To examine the relative contributions of apical and basolateral pathways, we analyzed the calcium dynamics in solitary ciliated and deciliated guinea pig type I and type II vestibular hair cells. Whole-cell patch-clamp recordings demonstrated that these cells had resting potentials near -70 mV and could be depolarized by 10 – 20 mV by superfusion with high potassium. Fura-2 measurements indicated that ciliated type II cells and deciliated cells of either type had low basal $[\text{Ca}^{2+}]_i$, near ~ 90 nM, and superfusion with high potassium led to transient calcium increases that were diminished in the presence of Ca^{2+} channel blockers. In contrast, measurements of type I ciliated cells, hair cells with large calyceal afferents,

were associated with a higher basal $[\text{Ca}^{2+}]_i$ of ~ 170 nM. High-potassium superfusion of these cells induced a paradoxical decrease in $[\text{Ca}^{2+}]_i$ that was augmented in the presence of Ca^{2+} channel blockers. Optical localization of dihydropyridine binding to the kinocilium suggests that they contain L-type calcium channels, and as a result apical calcium influx includes a contribution from voltage-dependent ion channels in addition to entry via transduction channels localized to the stereocilia. Eosin block of PMCA significantly altered both $[\text{Ca}^{2+}]_i$ baseline and transient responses only in ciliated cells suggesting that, in agreement with immunohistochemical studies, PMCA is primarily localized to the bundles.

Key words: PMCA; calcium channels; vestibular hair cells; bundles; fura-2 fluorescence; guinea pig

Auditory and vestibular hair cells are polarized epithelial cells characterized by a mechanically sensitive apical bundle formed primarily by stepped ranks of actin-filled stereocilia. Mechanical stimulation of the bundle gates transduction channels and generates a receptor potential (Hudspeth and Corey, 1977). These channels are relatively nonselective cation pores (Corey and Hudspeth, 1979; Ohmori, 1985; Crawford et al., 1991), and although potassium carries most of the current *in vivo*, they are highly selective for calcium and represent a significant calcium influx pathway (Lumpkin and Hudspeth, 1995; Ricci and Fettiplace, 1998). As with other excitable cells, receptor depolarization induces calcium influx through voltage-sensitive L-type channels (Lewis and Hudspeth, 1983; Art and Fettiplace, 1987; Art et al., 1993) that in turn evokes activation of large-conductance, calcium-activated potassium channels (Lewis and Hudspeth, 1983; Art and Fettiplace, 1987), as well as neurotransmitter release from presynaptic active zones (Roberts et al., 1990; Issa and Hudspeth, 1994; Tucker and Fettiplace, 1995).

To preserve a separation between these diverse functions requires powerful cytoplasmic (Oberholtzer et al., 1988; Roberts,

1993) and mitochondrial Ca^{2+} -buffering (Park et al., 1996; Peng and Wang, 2000) and efficient Ca^{2+} -extrusion mechanisms such as Ca^{2+} -ATPases in the plasmalemma (PMCA) (Tucker and Fettiplace, 1995; Yamoah et al., 1998) or endoplasmic reticulum (Tucker and Fettiplace, 1995) as well as Na^+ - Ca^{2+} , K^+ exchangers (Boyer et al., 1999). The polarization of hair cells into an apical domain responsible for generation of the receptor current and a basolateral domain associated with shaping the receptor potential and synaptic transmission suggests a functional dichotomy. The multiplicity of routes of calcium influx, buffering, and extrusion, however, naturally raises questions concerning interactions between calcium-dependent processes and the conditions under which the calcium level is dominated by different elements in the apical or basolateral pathways.

When solitary cells are examined *in vitro*, both apical and basal surfaces are often bathed uniformly, rather than in the asymmetric salts experienced *in vivo*. We have used this uniform environment to test the hypothesis that when the calcium is elevated on the apical surface, influx and efflux pathways in the ciliary bundle determine the resting calcium level and the transient response. Furthermore, we have examined the possibility that the apical calcium influx pathway includes contributions not only from transduction channels on the stereocilia but from calcium-selective ion channels on the kinocilium as well. Fura-2 fluorescence was used to measure $[\text{Ca}^{2+}]_i$ changes induced by high-potassium superfusion in ciliated and deciliated cells. We compared the responses of type II cells, a primitive form found throughout vertebrates, with the responses in type I cells, a more specialized cell found in the vestibular epithelia of reptiles, birds, and mammals. We identified by immunocytochemistry and microfluorimetry the presence of

Received July 20, 2000; revised Jan. 8, 2001; accepted Jan. 11, 2001.

This work was supported by National Institute on Deafness and Other Communication Disorders Grant DC 03443, Centre National d'Etudes Spatiales Grants 96-0240 and 98-793, Direction des Recherches Etudes et Techniques Grant 95-062, and the Institut National de la Santé et de la Recherche Médicale. We are grateful to Drs. A. Lysakowski, A. Pregent-Tessier, and N. Lieska for their contribution to the biochemistry experiments. We thank Dr. M. B. Goodman for her constructive comments on this manuscript and C. Travo for her excellent technical assistance.

Correspondence should be addressed to Dr. Catherine Boyer, University of Illinois, College of Medicine, Department of Anatomy and Cell Biology (mail code 512), 808 South Wood Street, Chicago, IL 60612. E-mail: cboyer@uic.edu.

Copyright © 2001 Society for Neuroscience 0270-6474/01/212640-11\$15.00/0

PMCA and calcium channels, and our physiological measures showed that the ciliary bundle plays a critical and unexpected role in the calcium fluxes and that it may intervene differently in calcium homeostasis and modulation of transduction in type I and type II hair cells.

MATERIALS AND METHODS

Cell isolation. Adult guinea pigs (150–200 gm) were decapitated under ether anesthesia. The temporal bone and bullae were quickly immersed in a modified HBSS containing (in mM): NaCl (140), KCl (6), Na_2HPO_4 (0.7), MgCl_2 (0.9), CaCl_2 (1.5), and NaHEPES (10); pH was adjusted to 7.3 with NaOH, and osmolality was adjusted to 300 mOsm/kg with sucrose. Hair cells were isolated from maculae utriculi and cristae ampullaris by gentle mechanical dissociation after 5 min of incubation in 0.5 mg/ml collagenase IV (Sigma, St. Louis, MO) in HBSS. Cells were identified on the basis of their morphology as described previously (Boyer et al., 1998). Type I cells had an attenuated and elongated region between the cuticular plate and the cell body, whereas type II cells were cylindrical in shape, lacking the constriction basal to the cuticular plate (Kevetter et al., 1994). All deciliated cells chosen for study had visible cuticular plates (see Fig. 1*B*), a diagnostic feature that distinguishes hair cells from the supporting cells that were also isolated by our procedures. Cellular viability and integrity were tested by fluorescein diacetate propidium iodide exclusion (Jones and Senft, 1985) and could be maintained *in vitro* for up to 4 hr after dissociation in a recording chamber of 1.8 ml perfused at 1 ml/min with HBSS. Data from cells with unstable resting $[\text{Ca}^{2+}]_i$ or membrane potential were excluded from the study.

Calcium measurements. Cells were loaded with the fluorescent Ca^{2+} indicator fura-2 AM (3 μM) and 0.02% (w/v) Pluronic F-127 (Sigma) in HBSS for 45 min at 37°C. Fluorescence was measured with a fast fluorescence photometer system on an Axiovert 10 microscope (Zeiss, Oberkochen, Germany) using a Plan-Neofluar 100 $\times$, 1.3 numerical aperture (NA) oil-immersion objective. Changes in $[\text{Ca}^{2+}]_i$ in the cell body (see Fig. 1*B*, white circle) were evaluated by calculating the ratio of the 530 nm fluorescence at excitation wavelengths of 340 and 380 nm. The method and its calibration were described in detail in Boyer et al. (1999). Mean values for *n* observations are reported as the mean $\pm$ SD.

Physiological measurements. Recording pipettes were pulled from borosilicate glass capillaries (resistance, 10–15 M Ω ; tip diameter, $\sim$ 1 μm) immediately before use. Patch electrodes were filled with a K^+ -based solution containing (in mM): KCl (115), MgCl_2 (5.3), K_2EGTA (10), Na_2ATP (5), and creatine monophosphate (5). Membrane current and voltage were recorded using standard whole-cell patch-clamp methods with a List EPC-7 amplifier. Membrane current and voltage were filtered at 3 kHz, recorded with 64 $\times$ oversampling at a corner frequency of 5 kHz on Digital Data Storage-2 tape with a four-channel, 16-bit instrumentation recorder (CDAT-4; Cygnus Technology, Delaware Water Gap, PA), and analyzed off-line.

Membrane voltage was corrected for liquid junction potentials and errors caused by current flow across an uncompensated series resistance between 15 and 42 M Ω . To measure whole-cell capacitance and series resistance, each cell was voltage-clamped at -70 mV and stepped to -65 mV for 6 msec. The resulting time-dependent current was assumed to flow across the cell's capacitance and series resistance. Leak conductance was measured from the current evoked by ± 5 mV pulses from -75 mV. All data were analyzed and plotted using Igor Pro V3.14 (WaveMetrics, Lake Oswego, OR). A Levenberg-Marquardt, nonlinear least-squares minimization algorithm was used in the curve-fitting routines (Press et al., 1994).

Extracellular solutions. HBSS at 22–25°C was used in all physiological experiments. The high- K^+ solution (50 mM KCl) for cell depolarization was HBSS in which KCl was substituted on an equimolar basis for NaCl. The Ca^{2+} -free solution was prepared by addition of 0.5 mM EGTA to HBSS without added calcium. Permeation through voltage-dependent calcium channels was blocked using NiCl_2 , CdCl_2 (Sigma), and nifedipine (Sandoz, Paris, France). Eosin (Sigma) was used to inhibit PMCA. All test solutions were superfused (PV 820 Pneumatic PicoPump; WPI) onto the basolateral surface of the hair cell through a pipette (2 μm tip inner diameter) located within 20 μm of the cell. L-type calcium channels were localized *in vivo* using a fluorescent dihydropyridine (500 nm DMBODIPY-DHP; Molecular Probes, Eugene, OR) (Knaus et al., 1992). To demonstrate the plasmalemma, cells were incubated for 1 min in 1 μM FM 1–43, a styryl dye (Molecular Probes).

Immunocytochemistry. Adult guinea pigs (150–200 gm) were decapitated, and their bullae were quickly immersed in 4% paraformaldehyde in 0.1 M PBS, pH 7.2, at 15°C. Vestibular end organs were dissected, post-fixed for 2 hr in 4% paraformaldehyde at 4°C, and then rinsed in 0.1 M PBS. Utricles and cristae were then embedded in 4% agarose in PBS and cut at 50 μm on a vibratome.

The anti-PMCA monoclonal antibody clone 5F10 and the anti-neurofilament 200 monoclonal antibody clone N52 were obtained as undiluted mouse ascites fluids from Sigma. The anti-calcium channel subunit α_{1A-1D} polyclonal antibodies were obtained from Alomone Labs (Jerusalem, Israel). Single or double labeling was performed with anti-PMCA antibody (dilution 1:100), anti-calcium channel subunit antibodies (anti- α_{1A} , 1:100; anti- α_{1B-1D} , 1:200), and anti-neurofilament antibody (1:500). Sections were incubated with 10% nonimmune serum and 0.3% Triton X-100 in PBS and then with 1% nonimmune serum, 0.03% Triton X-100, and the primary antibodies alone or in combination as described above for 40 hr at 4°C.

Secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Bound primary PMCA antibody was detected by incubation for 3 hr at room temperature with indocarbocyanine-conjugated anti-mouse (1:1000). Bound primary anti-calcium channel subunit antibodies were incubated overnight at 4°C with a 1:200 dilution of biotinylated mouse anti-rabbit IgGs and detected with lissamine rhodamine-conjugated streptavidin (1:200) and, for double labeling, with additional indocarbocyanine-conjugated anti-mouse by incubation for 2 hr at room temperature. Sections were mounted in FluorSave (Calbiochem, San Diego, CA).

The specificity of all immunostaining was checked by substituting nonimmune serum for the primary antiserum or by omitting the primary antibody incubation step from the procedure. All such control sections were free of immunostaining. For *in vivo* fluorescence and immunocytochemistry studies, the staining was observed with a laser-scanning confocal microscope (LSCM-1024; Bio-Rad, Hercules, CA) on an Axiovert 100TV microscope (Zeiss) equipped with a 40 $\times$, 1.4 NA oil-immersion objective.

For electron microscopy a preembedding technique with immunogold detection was used (Jackson ImmunoResearch). Vibratome sections were incubated with the anti-PMCA antibody diluted as described above. The sections were then rinsed in 1% goat serum in Tris-buffered saline (TBS) and incubated with biotinylated goat anti-mouse IgGs (1:200) overnight at 4°C. The sections were then rinsed in TBS, incubated for 2 hr at room temperature in a 1:50 dilution of colloidal gold–streptavidin coupled to 4 nm gold particles, and then fixed in 2% glutaraldehyde in PBS for 20 min. Sections were processed for silver intensification (Amersham Pharma Biotech, Piscataway, NJ) for 10 min at 4°C. Specimens were then post-fixed by incubation in 2% osmium tetroxide in PBS for 30 min, dehydrated in alcohol, and flat-embedded in Araldite. Ultrathin sections were cut with an LKB 2088 ultratome and counterstained. They were examined using a JEOL 200CXII transmission electron microscope.

Immunoblotting. Single vestibular receptor epithelium (crista and utricle) or samples of purified red blood cells (RBC) and hemoglobin (Hb) were homogenized manually and diluted with sample buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 0.02% pyronine Y, and 10% β -mercaptoethanol). Sample volumes of 15 μl were separated on 10% SDS-PAGE at 20 V for 2 hr and then transferred to polyvinylidene difluoride membrane (Bio-Rad). After an overnight incubation in Tris-buffered saline containing 0.2% Tween 20 and 5% nonfat dry milk, PMCA was detected using the monoclonal antibody clone 5F10 (1:1000) for 4 hr at room temperature and the peroxidase-conjugated antibody (1:5000; Jackson ImmunoResearch) for 1 hr at room temperature. The protein–antibody complex was visualized using the enhanced chemiluminescence detection system (ECL-Plus; Amersham Pharma Biotech) according to the manufacturer's instructions. The detection of total protein in the different samples was made using the Silver Stain Plus Kit (Bio-Rad) according to the manufacturer's instructions. Guinea pig RBC and Hb samples were purified by differential centrifugation.

Animal care. All animals were housed and handled according to approved guidelines (French Department of Agriculture and Parks authorization no. 04890) that conform to National Institutes of Health guidelines.

Figure 1. Voltage and calcium responses in solitary guinea pig hair cells induced by high- K^+ superfusion. *A*, *B*, Ciliated (*A*) and deciliated (*B*) cells, from which recordings were obtained, that had a discernible cuticular plate at the apical pole. The white circle in *B* indicates the region of the cell body used for the integrated measurement of fura-2 fluorescence. Scale bar, 5 μm . *C*, Quiescent calcium ratios for all cells. Three populations were identified as follows: *a*, deciliated type I and both ciliated and deciliated type II cells as indicated by shading; *b*, ciliated type I cells; and *c*, cells eliminated from the study because of instabilities in their resting calcium concentration after depolarization. *D*, *E*, Voltage (*D*) and calcium (*E*) response in type I deciliated cells induced by 5 sec superfusion of 50 mM K^+ .

RESULTS

Resting calcium and voltage responses of solitary cells

To evaluate the relative importance of calcium buffering and extrusion in apical and basolateral pathways we first examined the changes in voltage and calcium concentration in response to superfusion with high-potassium solutions. Solitary hair cells (Fig. 1*A,B*) were recognizable and differentiated from other cells and cell fragments isolated from the epithelium either by the

Table 1. Comparison of resting potentials

	CC I	CC II	DC I	DC II
Number of cells	10	7	14	18
Resting potential (mV)	-67.3	-65.8	-70.6	-68.7
SD (mV)	10.5	10.2	7.9	7.4
Identical population (probability)				
CCI				
CC II	0.78			
DC I	0.42	0.30		
DC II	0.73	0.53	0.48	

Four subpopulations of cells were analyzed: ciliated type I (CC I), ciliated type II (CC II), deciliated type I (DC I), and deciliated type II (DC II). The mean and SD of the zero-current level (measured in voltage clamp) or resting potential (measured in current clamp) are given. By the use of a two-tailed Student's *t* test for groups of unequal size and variance, the probability that corresponding groups are from the same underlying population is determined and tabulated at the bottom. None of the pairs was found to be significantly different at the 0.05 level.

existence of an apical ciliary bundle (Fig. 1*A*) or, in the case of deciliated cells, by the presence of a cuticular plate, a cellular organelle into which the ciliary bundle normally inserts (Fig. 1*B*). Previous studies have demonstrated that hair cells isolated from different regions of vestibular epithelia maintain their characteristic morphology after isolation (Kevetter et al., 1994; Ricci et al., 1997). Type I hair cells, enveloped by large calyceal endings *in vivo*, can be distinguished after isolation by a distinctive constriction basal to the cuticular plate (Fig. 1*A*), which is retained even if the ciliary bundle is mechanically dislodged during isolation (Fig. 1*B*). In the resting state, the whole-cell intracellular free calcium $[\text{Ca}^{2+}]_i$ as estimated by fura-2 fluorescence within the region indicated by the white circle in Figure 1*B* was relatively low in all stable cells used in our study. The cell population could be subdivided into three groups on the basis of resting $[\text{Ca}^{2+}]_i$. The first population (Fig. 1*C*, peak *a*) consisted of deciliated type I ($n = 30$) and type II ($n = 20$) as well as ciliated type II ($n = 20$) cells and had fura-2 fluorescence ratios of 0.4 ± 0.1 ($n = 70$), corresponding to basal $[\text{Ca}^{2+}]_i$ of 90 ± 10 nM. The ciliated type I cells, on the other hand, were a distinct population centered around peak *b* (Fig. 1*C*) that had nearly twice the fluorescence ratio, 0.8 ± 0.1 ($n = 16$), corresponding to basal $[\text{Ca}^{2+}]_i$ of 170 ± 20 nM. Both of these populations were distinct from a third group (Fig. 1*C*, peak *c*) composed of both ciliated and deciliated cells of either type that had fluorescence ratios of 1.8 ± 0.25 ($n = 10$), corresponding to basal $[\text{Ca}^{2+}]_i$ of 382 ± 53 nM. Members of this third group resembled the other two on the basis of morphology and the ability to exclude fluorescein diacetate propidium iodide but were unable to maintain stable $[\text{Ca}^{2+}]_i$ levels after superfusion with high potassium, and each depolarization was followed by successively higher $[\text{Ca}^{2+}]_i$. This suggested that calcium homeostasis was compromised during or subsequent to isolation, and these cells were excluded from further study.

Using whole-cell patch-clamp voltage recordings, we were unable to distinguish between cell types, or the presence or absence of a ciliary bundle based on resting potential (Table 1). Taken as a group, solitary cells had resting potentials of 68.5 ± 8.5 mV ($n = 49$), a value close to that reported for vestibular hair cells recorded *in situ* (Masetto et al., 1994; Masetto and Correia, 1997; Armstrong and Roberts, 1998). The low resting calcium levels illustrated in Figure 1*C*, peaks *a* and *b*, thus are consistent with our voltage recordings, because we would expect the L-type

Figure 2. Calcium elevation in type II and type I deciliated cells. *A, B*, Effect of extending the duration of superfusion with 50 mM K^+ for type II (*A*) and type I (*B*) hair cells is illustrated by intervals of 1, 5, and 10 sec after onset, which is indicated by the arrow. *C, D*, Pretreatment with 0.5 mM NiCl_2 and 0.5 mM CdCl_2 suppresses >90% of the response to superfusion with 50 mM K^+ (indicated by horizontal bar) as demonstrated in type II (*C*) and type I (*D*) cells.

calcium channels, the dominant voltage-activated basolateral influx pathway reported previously in vertebrate hair cells (Lewis and Hudspeth, 1983; Art and Fettiplace, 1987; Zidanic and Fuchs, 1995), to be only modestly activated at these resting potentials. Superfusion with 50 mM K^+ induced membrane depolarizations of 16 ± 7 mV ($n = 23$), as illustrated for the deciliated type I cell in Figure 1*D*. This change in potential would be expected to increase rapidly the open probability of these channels, leading to a large calcium influx and, in most cells, a rapid rise in $[\text{Ca}^{2+}]_i$ as illustrated for a deciliated type I cell in Figure 1*E*. At the end of the high-potassium challenge, as the cell repolarizes, the calcium channels would return to their resting probabilities, and ultimately, calcium extrusion processes such as the $\text{Na}^+-\text{Ca}^{2+}$, K^+ exchanger demonstrated previously in the basolateral plasmalemma (Boyer et al., 1999) would return the $[\text{Ca}^{2+}]_i$ to its quiescent level.

Calcium response to K^+ -induced depolarization in deciliated cells

The time course and degree of calcium elevation in both type I and type II deciliated cells were examined using a series of high- K^+ superfusions as indicated in Figure 2, *A* and *B*. Prolonged superfusion for 5 or 10 sec led to a plateau in the elevation of $[\text{Ca}^{2+}]_i$ during the depolarizations. In general, the calcium elevation was larger in type II than in type I cells, corresponding to fluorescence ratios of 3.8 ± 0.2 ($n = 20$) and 3.1 ± 0.2 ($n = 30$), respectively, at the peak of the 10 sec response and suggesting, in agreement with previous studies (Boyer et al., 1998), that on average the balance between calcium elevation because of influx and extrusion in the basolateral surface is shifted toward calcium elevation in type II cells. The elevation of internal calcium resulted at least in part from influx through voltage-dependent calcium channels (VDCCs), as demonstrated by the 90% block of

Figure 3. Response of type II and type I ciliated cells to superfusion with 50 mM K^+ after preincubation in saline (*A, B*) or calcium channel blockers (*C, D*). *A*, The response of ciliated type II cells to longer intervals of superfusion was similar to that seen in deciliated cells. *B*, Superfusion of type I ciliated cells with 50 mM K^+ was associated with a decrease in $[\text{Ca}^{2+}]_i$ as assayed by the fluorescence ratios. *C, D*, Preincubation in 0.5 mM NiCl_2 and 0.5 mM CdCl_2 suppressed >90% of the calcium elevation in response to superfusion with 50 mM K^+ (indicated by horizontal bar) in ciliated type II cells (*C*) and extended the reduction in $[\text{Ca}^{2+}]_i$ in ciliated type I cells (*D*).

the elevation after incubation in a saline to which 0.5 mM NiCl_2 and 0.5 mM CdCl_2 had been added (Fig. 2*C,D*). Previous studies with dihydropyridines (DHPs) (Boyer et al., 1998) demonstrated that 75% of the calcium elevation could be blocked by 500 μM nitrendipine. This small quantitative difference between the efficacy of the blocking solutions might be caused either by calcium influx pathways other than the L-type channels (Yamoah and Crow, 1994) or by the voltage dependence (Sanguinetti and Kass, 1984a) and light sensitivity (Sanguinetti and Kass, 1984b) of the DHP block.

Calcium response to K^+ -induced depolarization in ciliated cells

To examine any novel effects caused by the presence of a ciliary bundle on whole-cell calcium homeostasis, it was necessary to eliminate the well known (Hudspeth and Corey, 1977; Crawford et al., 1991; Ricci and Fettiplace, 1998) and possibly confounding influence of calcium influx through transduction channels. To disable this pathway, all experiments were performed on cells that had been transiently exposed to “zero-calcium” HBSS to which 0.5 mM EGTA had been added to reduce the free calcium to <10 nM. Such a maneuver has been shown previously to disrupt transduction (Assad et al., 1991; Crawford et al., 1991) and by these accounts leaves the channels in a closed state [but see Meyer et al. (1998)].

As illustrated in Figure 3, the presence of a ciliary bundle led to an unexpected dichotomy in the responses of type I and type II cells to depolarization and the effect of calcium channel blockers. In type II cells (Fig. 3*A*), the amplitude of the calcium

Figure 4. Effects of calcium channel blockers on the resting calcium concentration $[\text{Ca}^{2+}]_i$. *A*, In ciliated type II cells and all other cells with low resting calcium levels, superfusion with 0.5 mM NiCl_2 and 0.5 mM CdCl_2 had little effect on resting calcium. *B*, In ciliated type I cells, cells with an elevated calcium level, superfusion with 0.5 mM NiCl_2 and 0.5 mM CdCl_2 depressed the calcium level toward that found in ciliated type II cells.

elevation during depolarization was smaller in ciliated cells than what had been observed previously in deciliated ones. For example, during the 10 sec depolarizations in type II cells (Figs. 2*A*, 3*A*), the fluorescence ratio increased to 3.8 ± 0.2 ($n = 20$) for deciliated cells (Fig. 2*A*) and to 2.7 ± 0.2 ($n = 10$) for ciliated cells (Fig. 3*A*), corresponding to $[\text{Ca}^{2+}]_i$ of 850 ± 50 and 600 ± 50 nM, respectively. Moreover, the rate of calcium extrusion and the return to baseline after depolarization depended on the presence of the bundle. The time constant τ of exponential recovery to the resting level was markedly faster in ciliated cells ($\tau = 4 \pm 1$ sec; $n = 10$) than in deciliated cells ($\tau = 7 \pm 1$ sec; $n = 20$). Together these results indicate that in type II cells the net effect of adding the ciliary bundle is to enhance the rate of calcium extrusion. Block of the calcium elevation with NiCl_2 and CdCl_2 as illustrated in Figure 3*C* suggests that any additional influx because of the bundle is unremarkable, and as with the deciliated type II cells (Fig. 2*C*), much of the whole-cell calcium elevation is caused by influx through VDCCs.

The presence of a ciliary bundle on type I cells leads to responses (Fig. 3*B,D*) that were in sharp contrast to those illustrated for type II cells. As noted above, the resting calcium level in type I ciliated cells was nearly twice that observed in either type II cells or either variety of deciliated cell. Moreover, prolonged depolarizations for 5 or 10 sec consistently led to transient decreases in calcium level in ciliated type I cells (Fig. 3*B*) rather than the transient increases typically seen for ciliated type II cells (Fig. 3*A*) or either kind of deciliated cell (Fig. 2*A,B*). A simple explanation might be that the type I cells are systematically depolarized beyond the peak of the $I-V$ curve, and further depolarization led to a reduction in the driving force and an associated decrease in calcium influx. This idea is unlikely in view of the fact that the resting membrane potentials of both type I and type II cells recorded with whole-cell patch electrodes were near -70 mV, and superfusion with high K^+ led to depolarizations of ~ 10 – 20 mV. This leads to the possibility that superfusion with high K^+ has more than a single effect of changing the potassium equilibrium potential and depolarizing the cell. More intriguing still is that incubation of the cells in NiCl_2 and CdCl_2 before superfusion led to a prolongation of the reduction of the calcium level as illustrated in Figure 3*D*. These results would be consistent with the idea that elevation in external potassium increases the rate of calcium extrusion, and blockage of 90% of the calcium influx prolongs the recovery to the resting level.

The idea that the elevated resting calcium level in ciliated

type I cells is determined primarily by influx through VDCCs is supported by the experiments illustrated in Figure 4. In type II cells of either variety and deciliated type I cells, the resting fluorescence ratio was low, near 0.4, corresponding to an $[\text{Ca}^{2+}]_i$ of 90 nM (Fig. 4*A*), and addition of 0.5 mM NiCl_2 and 0.5 mM CdCl_2 has a negligible effect on the resting level. In ciliated type I cells, the elevated fluorescence ratio near 0.8, corresponding to an $[\text{Ca}^{2+}]_i$ of 180 nM, could be profoundly reduced by application of the Ni^{2+} and Cd^{2+} solution (Fig. 4*B*). Application for a period as brief as 5 sec required >4 min for complete recovery. Superfusion for 60 sec reduced the calcium to a level comparable with that observed in type II cells and deciliated type I cells. These results suggested that, at a minimum, the elevated $[\text{Ca}^{2+}]_i$ in ciliated type I cells is at least triggered by calcium influx through VDCCs in the plasmalemma.

Morphological localization of possible routes of calcium entry

In view of the effects of calcium channel blockers on the $[\text{Ca}^{2+}]_i$ in ciliated type I cells, several morphological studies were used to localize membrane proteins that might contribute to apical calcium influx. Specifically we were interested in the possibility that there was a dichotomy between the distribution of L-type calcium channels in type I and type II cells. Initially, the distribution of L-type calcium channels was investigated by immunocytochemistry in the guinea pig cristae and utricles, using a polyclonal α_{1C} subunit antibody on fixed and permeabilized epithelia. This antibody labeled the cuticular plate region and the basolateral membrane of the vestibular hair cells (Fig. 5*Aa*). Using antibodies to other α_1 subunits labeled the α_{1A} in the dark cell layer (Fig. 5*Ab*, P-, Q-type channel) and the α_{2B} at the top of the calyces (Fig. 5*Ac*, N-type channel). The use of the α_{1D} antibody resulted in higher background (data not shown), but the localization was identical to that obtained with antibody α_{1C} .

Second, because 500 nM nitrendipine was known to block at least 75% of the elevation in $[\text{Ca}^{2+}]_i$, solitary cells were labeled *in vivo* with 500 nM DMBODIPY-DHP, a fluorescently conjugated dihydropyridine (Fig. 5*Ba,c-e*). Using serial confocal sectioning through the depth of each cell, we observed that after a 1 min exposure to DMBODIPY-DHP there was a labeling of the cuticular plate region, the kinocilium (Fig. 5*Ba,c*), and the basolateral plasmalemma in all type I cells ($n = 10$). In type II cells, staining subjacent to the cuticular plate, as well as over the basolateral surface (Fig. 5*Be*), was routinely observed ($n = 6$), but staining of the kinocilium marginally above background fluorescence was observed in only one case. DHP binding was easily reversible with sufficient washing, and to verify the specificity of DMBODIPY-DHP binding (Fig. 5*Bc*), the cell was washed and pretreated with 500 nM nitrendipine before incubation with the DMBODIPY-DHP. Under these conditions, the fluorescence was markedly reduced (Fig. 5*Bd*), suggesting that the labeling was specific to DHP-binding sites. We also used FM 1–43 to visualize the plasmalemma (Fig. 5*Bb,f*) as a control for membrane integrity. FM 1–43 fluorescence appeared continuous even in the more intense locations of DMBODIPY-DHP labeling (Fig. 5*Bb,f*), suggesting that the heterogeneous distribution of the DHP we observed was not simply the result of deformations or degradation of the cell membrane. Moreover the internalization of DHP (Fig. 5*Ba,e*) and the corresponding uptake of FM 1–43 in these regions (Fig. 5*Bb,f*) are consistent with DHP uptake at sites of more active membrane turnover. In summary, these results sug-

Figure 5. Localization of voltage-dependent calcium channels in guinea pig vestibular epithelium. *A*, *a*, Double labeling of the α_{1C} subunit corresponding to L-type calcium channels (red) and of neurofilament N52 (green) that is specific for neuronal fibers. *b*, Double labeling of the α_{1A} subunit that corresponds to P-, Q-type calcium channels (red) and of neurofilament N52 (green). *c*, Single labeling of the α_{1B} subunit that corresponds to N-type calcium channels. Note that only the L-type calcium channels are present in the vestibular hair cells. *B*, *a*, L-type Ca^{2+} channels of living vestibular hair cells labeled with DMBODIPY-conjugated DHP and observed with a Bio-Rad MRC 1024 laser-scanning confocal microscope. We observed an intense labeling of the cuticular plate region and the kinocilium in all type I (*a*, *c*) but not in type II (*e*) ciliated cells. Staining with DMBODIPY-DHP in *c* was selective and reversible and

could be blocked (*d*) by preincubation with nonfluorescent DHP or removed by washing in DHP-free media. After examining ciliated cells for DHP binding (*a*, *e*), to control for the integrity of the plasma membrane, they were examined using the styryl dye FM 1–43, as shown in the corresponding images (*b*, *f*). The latter were also used to determine the relative fluorescence of apical and basolateral plasmalemma (see Fig. 6). Scale bars: *A*, *a*, *b*, *B*, 10 μm ; *A*, *c*, 1 μm .

gest that L-type channels exist in both type I and type II hair cells, but there are noticeable differences between the staining in the two types, with much greater fluorescence and presumably a much higher density of L-type channels in the kinocilia of type I cells. The failure to demonstrate an equivalent localization using the polyclonal α_{1C} subunit antibody may be caused simply by differences in the channel epitope that resulted in a failure to stain the kinocilium (Yu and Bchir, 1994).

Our use of FM 1–43 to stain the membrane was also motivated by difficulties associated with quantifying membrane fluorescence because of regional differences in the membrane area included within a confocal volume. As shown previously, the confocal volume can be approximated by a right cylinder along the optical axis whose size is determined by the first zeros in the point spread function of the objective (Art and Goodman, 1993). Imaging in saline, a 40 $\times$, 1.4 NA objective with illumination at 488 nm and detection between 515 and 565 nm would produce a cylinder that is ~ 180 nm in diameter and 600 nm in length. As illustrated in Figure 6*A*, such an ellipsoidal confocal volume would include multiple stereocilia when imaging the ciliary bundle but would enclose relatively less total membrane when imaging the basolateral plasmalemma. Comparison of the maximum surface that might be enclosed within the volume when scanning the bundle with that enclosed along the basolateral surface suggests that although the inclusion of multiple stereocilia may be complicating, it should in theory enhance the amount of fluorescence by at most 57%. To examine this experimentally in single confocal planes, the fluorescence values across the bundle and the basolateral surface were compared in FM 1–43-stained cells. NIH Image, version 1.6, was used to quantify the profiles of the eight-bit gray scale data from three regions in the bundle and the base in each section as illustrated in Figure 6*B*. Assuming only that FM 1–43 stains both the apical and basolateral membranes uniformly, a comparison of the maximum fluorescence along each transect suggested that the peak fluorescence of the bundle was $152 \pm 9\%$ ($n = 4$) of the peaks on the basolateral surface. The agreement between theoretical and measured values supports the idea that although quantifying the relative fluorescence and density of membrane constituents such as the L-type calcium

Figure 6. Contributions to fluorescence intensity because of multiple elements within a confocal volume. *A*, The confocal volume in the LSCM, indicated by the vertical ellipse, includes multiple stereocilia when the apical bundle is scanned but generally only a single plasmalemmal element when the basolateral surface of a hair cell is scanned. Geometric estimates of the area of membrane enclosed by the confocal volume gave a maximum membrane ratio of the apical/basal surfaces within the volume. The parallel horizontal lines in the top image indicate the center of the optical section. *B*, *C*, For comparison, profiles of three direct measurements of FM 1–43 fluorescence across the apical bundle and the basolateral surface (*B*) are shown as corresponding transects (*C*). The fluorescence ratio of the peak apical versus basolateral transects gave a ratio of $152 \pm 9\%$ ($n = 4$). Scale bars, 1 μm .

channel may be complicated by regional variations in the membrane enclosed within a confocal volume, the increased fluorescence because of this factor will be on the order of 50%. This corresponds to the inherent uncertainty associated with comparing the absolute values of the fluorescence of the cilia versus that of the basolateral plasmalemma.

Effect of resting $[\text{Ca}^{2+}]_i$ on high- K^+ superfusion response

The morphological indication that there exists an additional calcium influx pathway that is heavily expressed on the kinocilium in type I cells would be consistent with the elevation in the $[\text{Ca}^{2+}]_i$ associated with these cells. To determine whether the dichotomy in the response to superfusion with high K^+ was an inherent property of the type of cell, we examined the dependence of the internal calcium concentration $[\text{Ca}^{2+}]_i$ on the external calcium concentration $[\text{Ca}^{2+}]_o$. Cells were incubated in media with 1.5, 0, or 4 mM extracellular calcium ($[\text{Ca}^{2+}]_o$) and then depolarized with a high- K^+ solution in the presence of 1.5 mM calcium. Of particular interest was whether the elevation or depression of calcium in response to superfusion with high K^+ was associated with a particular cell type or a particular resting $[\text{Ca}^{2+}]_i$.

The response of ciliated type II cells was similar to that observed previously in deciliated cells of both types (Fig. 7A), and even when $[\text{Ca}^{2+}]_o$ was effectively reduced to zero by addition of 0.5 mM EGTA, the response to the superfusion with the high K^+ in the presence of calcium was a rapid elevation in $[\text{Ca}^{2+}]_i$. When the $[\text{Ca}^{2+}]_o$ was elevated to 4 mM, the resting $[\text{Ca}^{2+}]_i$ was elevated only marginally to 100 nM (Fig. 7A), although in response to superfusion a consistent reduction in peak value and a prolonged return to baseline were observed. The data for 10 ciliated type II cells under all incubation conditions are summarized in Figure 7C (●). In all cases type II cells with stable resting calcium levels could not be elevated above fluorescence ratios of 0.5 corresponding to resting $[\text{Ca}^{2+}]_i$ of 120 ± 10 nM, and the response to superfusion with high K^+ was an increase in $[\text{Ca}^{2+}]_i$.

In contrast, the resting $[\text{Ca}^{2+}]_i$ in type I ciliated cells was highly dependent on the $[\text{Ca}^{2+}]_o$. As illustrated in Figure 7B, the response to high- K^+ superfusion after incubation in the conventional extracellular HBSS was a decrease in $[\text{Ca}^{2+}]_i$ as illustrated previously in Figures 4 and 5. After incubating the cell in the zero-added calcium HBSS to which EGTA had been added, the resting $[\text{Ca}^{2+}]_i$ had been reduced to 90 nM, a level conventionally observed in the ciliated type II cells or deciliated cells of either type. Superfusion with high K^+ in the presence of 1.5 mM Ca^{2+} resulted in the more conventional increase in $[\text{Ca}^{2+}]_i$ that had been observed for all other cell types. After incubation in 1.0, 1.5, and 4.0 mM Ca^{2+} HBSS, a consistent reduction and finally reversal in the calcium response to superfusion with high K^+ in the presence of 1.5 mM Ca^{2+} could be observed. Plotted in Figure 7C (○), the data from ciliated type I cells ($n = 10$) demonstrated that when the resting $[\text{Ca}^{2+}]_i$ was <120 nM, the response to superfusion was an elevation in calcium and that as the resting level was increased beyond this level, the response reversed and the largest reductions in calcium were associated with the largest resting $[\text{Ca}^{2+}]_i$.

Ciliary bundle calcium extrusion

Previous evidence suggests that powerful extrusion mechanisms such as the $\text{Na}^+/\text{Ca}^{2+}$, K^+ exchangers exist in the basolateral hair cell plasmalemma (Boyer et al., 1999) and that PMCA have been reported in ciliary bundles (Maurer et al., 1992; Crouch and Schulte, 1995; Apicella et al., 1997; Yamoah et al., 1998). To localize and analyze the contributions of the PMCA to whole-cell calcium homeostasis, we compared the responses to superfusion with high K^+ with those after incubation in eosin, a potent PMCA inhibitor. In Figure 8A, the eosin effect on resting calcium level is illustrated for ciliated type I cells. Eosin was increased from 0 to 20 μM , and the resting fluorescence ratio increased from

0.75 ± 0.1 ($n = 5$) to 1.30 ± 0.1 ($n = 5$), corresponding to an increase in $[\text{Ca}^{2+}]_i$ from 170 to 300 nM. Eosin is known to have some effects on VDCCs (Choi and Eisner, 1999), which can lead to a reduction in current, but the illustrated increase in the $[\text{Ca}^{2+}]_i$ is likely a result of the inhibition of the PMCA with an associated reduction in calcium efflux. The range of concentrations used is less than the concentrations known to inhibit either the sarcolemmal or smooth endoplasmic Ca^{2+} -ATPases (Gatto et al., 1995). Similarly, the eosin inhibition of the response to high- K^+ superfusion in ciliated type I cells (Fig. 8F) was dose dependent for concentrations between 0 and 20 μM as illustrated in Figure 8B.

To test whether there might be an effect on the previously demonstrated $\text{Na}^+/\text{Ca}^{2+}$, K^+ exchanger in the basolateral plasmalemma (Boyer et al., 1999), the response to high- K^+ superfusion was compared in deciliated type I and type II cells under normal conditions and after incubation in 10 μM eosin, the half-blocking concentration deduced from Figure 8B. The results illustrated in Figure 8, C and D, demonstrate that incubation in eosin had a negligible effect on the transient calcium response to superfusion, and the traces under the two conditions were superimposed.

In ciliated type I and type II cells, the effects of incubation in eosin were pronounced. In type II cells, incubation in eosin elevated the resting calcium level by 25 nM, the peak amplitude during superfusion was decreased by 15%, and the rate of return to baseline after depolarization was slowed by a factor of two. In ciliated type I cells, the eosin incubation had little additional effect on the elevated resting level, but during superfusion the effect was to inhibit the reduction in calcium level. It was this reduction in calcium concentration that was plotted in the dose-response curve of Figure 8B. We conclude from these eosin experiments that the majority of the PMCA in both type I and type II hair cells is localized to the ciliary bundle and that the reduction in calcium level during superfusion with 50 mM K^+ in type I cells is consistent with the potassium sensitivity of the PMCA reported previously (Romero and Romero, 1982, 1984). In those experiments at external K^+ concentrations $[\text{K}^+]_o$ of 5 mM, the pump rate is at a minimum and increases by a factor of six when $[\text{K}^+]_o$ is increased to 40–50 mM.

To confirm the subcellular localization of the PMCA, immunocytochemistry on sections of guinea pig utricle and cristae was performed. The specificity of the monoclonal anti-PMCA antibody (clone 5F10) was checked by Western blot analysis (Fig. 9A) and revealed that guinea pig utricle (lane 7) and crista (lane 6) express a protein immunologically similar to the human erythrocyte PMCA (Heim et al., 1992) with an apparent molecular mass of 140 kDa. Using purified guinea pig red blood cells and hemoglobin samples, we showed that the pattern of PMCA protein expression in the vestibular epithelium was minimally contaminated by PMCA from blood incorporated in epithelial vessels (lane 5).

Immunocytochemistry with the anti-PMCA antibody was observed using confocal microscopy. As illustrated in Figure 9Ba, the hair cell bundles were intensely labeled, but possible staining of the basolateral membranes was below the detection threshold. PMCA localization was confirmed using immunogold-labeled antibodies at the electron microscopic level. In ultrathin sections, numerous aggregates were detected on the plasmalemma of the stereocilia (Fig. 9Bb1,b2) with a preponderance of the aggregates clustered together at the distal ends of the stereocilia (Fig. 9Bb2), whereas no aggregates were observed either at the base of the

Figure 7. Dependence of the $[\text{Ca}^{2+}]_i$ level on $[\text{Ca}^{2+}]_o$ in vestibular hair cells. *A*, Variations in the fura-2 fluorescence ratio induced by 5 sec superfusions of the 50 mM K^+ and 1.5 mM Ca^{2+} solution (horizontal bars) were recorded in ciliated type II cells incubated in saline containing 1.5, 0, or 4 mM $[\text{Ca}^{2+}]_o$ (concentration in millimolar indicated next to each trace). Two minute gaps between trials are indicated by diagonal hash marks on the x-axis. In type II ciliated cells, changes in the external calcium concentration had no significant effect on the resting fura-2 fluorescence ratio, and as with deciliated cells of either type, superfusion with 50 mM K^+ and 1.5 mM Ca^{2+} induced an increase in free calcium. *B*, Variation in fluorescence for type I ciliated cells incubated in saline of 0, 1.0, 1.5, or 4 mM $[\text{Ca}^{2+}]_o$ (indicated at the right of the figure) is shown. Note that an increase in external calcium induced an increase in the resting calcium concentration. When the resting fluorescence ratio was <0.5 (corresponding to a free calcium of 120 nM), the response to superfusion was a transient increase in calcium, and when the resting level was greater, the superfusion resulted in a transient decrease in the resting level. *C*, Amplitude of the transient response with respect to the resting level in type I (○) and type II (●) hair cells is shown. All ciliated type II cells had fluorescence ratios <0.5 , corresponding to resting calcium levels <120 nM, and increased their free calcium concentration after superfusion with the 50 mM K^+ and 1.5 mM Ca^{2+} solution. When type I ciliated cells were forced to resting calcium levels <120 nM, by incubation in low external calcium solutions, the response resembled that of type II cells, and superfusion with the 50 mM K^+ and 1.5 mM Ca^{2+} solution led to a transient increase in calcium. When incubated in external solutions resulting in resting levels >120 nM, superfusion with the 50 mM K^+ and 1.5 mM Ca^{2+} solution resulted in a transient decrease.

Figure 8. Effect of eosin, a PMCA antagonist, on $[\text{Ca}^{2+}]_i$ in hair cells. *A*, *B*, In type I cells, application of eosin led to a dose-dependent elevation in the resting calcium level (*A*), as well as a dose-dependent block of the superfusion-induced reduction in resting calcium (*B*). *C*, *D*, In the absence of ciliary bundles, superfusion with 50 mM K^+ and 10 μM eosin (horizontal bar) after a 15 min preincubation in eosin led to responses comparable with those of the controls in both type II (*C*) and type I (*D*) cells. *E*, *F*, When ciliated type II (*E*) and type I (*F*) cells were similarly preincubated in eosin before K^+ application (horizontal bar), the response was slowed and diminished in both cell types.

stereocilia or on the basolateral hair cell plasmalemma (Fig. 9*Bb3*). The immunological localization of PMCA in the bundles is consistent with our physiological results using eosin.

DISCUSSION

Cilia are a common feature in not only the inner ear but in a range of sensory as well as other eukaryote and prokaryote cells. In photoreceptors, the ciliary link between inner and outer segments serves a structural role and pathway for opsin transport (Liu et al., 1999). On the other hand, in the olfactory system, the receptor cilia are studded with cyclic nucleotide-gated channels whose open probability is modulated by binding to specific odorants (Firestein et al., 1990; Bönigk et al., 1999). In the inner ear however, a role beyond that as an attachment point to accessory structures remains unclear. Measurement of transduction currents in the mature hair cells in the vertebrate cochlea that lack a kinocilium (Kros et al., 1992) as well as measurement in vestibular

Figure 9. Immunochemical detection of PMCA in guinea pig vestibular end organs. *A*, Western blot analysis of crista (lanes 2, 6), utricle (lanes 3, 7), and red blood cells (lanes 1, 5) separated as described in Materials and Methods. A strong band at 140 kDa was visible in crista and utricle (lanes 2, 6, 7), whereas the same band was barely visible in RBC (lane 5). Samples of RBC (lanes 1, 5) and Hb (lane 4) were used as a control of blood contamination in the different vestibular epithelia. As shown by silver staining, the Hb bands are of higher density in RBC samples than in vestibular epithelium samples, suggesting that the total blood incorporated in our samples in the epithelial vessels was minimal and consequently the contamination by PMCA from RBCs in the samples was insignificant. *B*, *a*, PMCA immunostaining of the bundles of a utricle (transverse section, immunofluorescence). *b1–b3*, Detection of PMCA in bundles by immunogold electron microscopy. Low-power view of the apex and base of a cell, with areas of enlargement indicated by braces (*b1*). As shown in the enlargements, silver aggregates are numerous toward the apex of the bundles (*b2*), whereas the reaction product could not be demonstrated at the base of the bundle (*b2*), along the cuticular plate, or along the cell body (*b3*). Scale bar: *a*, 5 μm ; *b1*, 2.5 μm ; *b2*, *b3*, 1 μm .

hair cells in which the kinocilium had been dissected free from the bundle (Hudspeth and Jacobs, 1979) demonstrates that mechanical-to-electrical transduction is produced by shearing of the stereocilia. Our results suggest that at least in some hair cells a specialized kinocilium may function as an auxiliary calcium influx pathway, and it remains to be seen whether, as in paramecium (Ehrlich et al., 1984; Hinrichsen et al., 1984), the calcium influx directly modulates the mechanical properties of the cilium and has an impact on the transduction channels or perhaps even on the basolateral ionic channels.

Calcium extrusion through the bundle

Our previous work on deciliated cells has demonstrated that calcium flux through the basolateral plasmalemma is supported by L-type VDCCs and by a $\text{Na}^+/\text{Ca}^{2+}$, K^+ exchanger (Boyer et al., 1998, 1999). The resting $[\text{Ca}^{2+}]_i$ values measured in the present study agree with those reported in deciliated type I and type II cells. The present work further illustrates that elevation of $[\text{Ca}^{2+}]_i$ induced by high- K^+ superfusion is similar in both cili-

ated and deciliated type II cells. However, the K^+ -induced calcium elevation was smaller in ciliated type II than that recorded in either type of deciliated cells, suggesting that a significant pathway of whole-cell calcium extrusion is via the bundle. This idea is supported by ATPase-blocking experiments in which ciliated and deciliated cells were incubated with eosin, at a concentration specific for inhibition of PMCA (Gatto et al., 1995), and the calcium response was affected only in cells with a bundle.

The idea that PMCA is primarily localized in the bundle is further supported by specific immunostaining of the bundles by the PMCA antibody, in both types of cells. These results are consistent with previous findings in mammalian cochlea (Crouch and Schulte, 1995; Apicella et al., 1997) and the amphibian vestibular system (Yamoah et al., 1998).

Elevated resting $[\text{Ca}^{2+}]_i$ in ciliated type I cells

A novel result of this study was the observation that ciliated type I cells differed from all other cell types in their resting $[\text{Ca}^{2+}]_i$. The fact that the resting $[\text{Ca}^{2+}]_i$ in ciliated type I cells was twice that found in deciliated cells suggests that the elevation in the former is a consequence of the bundle. In principle the additional calcium load might have resulted from continuous activation of transduction channels in the stereocilia leading either to calcium influx through these channels or to a depolarization activating the basolateral VDCCs. Such an explanation seems unlikely, because the elevated resting level remained in ciliated type I cells after brief exposure to calcium-free medium, a manipulation that would be expected to inactivate irreversibly the transduction channels and hyperpolarize the cells (Assad et al., 1991; Crawford et al., 1991). It is more likely that other bundle constituents may be responsible for the elevated resting $[\text{Ca}^{2+}]_i$.

In ciliated cells, the resting $[\text{Ca}^{2+}]_i$ was found to increase in the presence of eosin, and if the cell possessed an elevated resting $[\text{Ca}^{2+}]_i$, a decrease could be produced by calcium channel blockers. In deciliated cells, however, the relatively low resting $[\text{Ca}^{2+}]_i$ remained unaffected by incubation with any of these agents. These observations suggest that ciliated type I cells equilibrate to a high resting $[\text{Ca}^{2+}]_i$ because of a balance between the Ca^{2+} extrusion process and a continuous Ca^{2+} influx through the L-type channels that can be demonstrated on the kinocilium by DMBODIPY-DHP staining.

Origin of the two types of K^+ -induced calcium responses

An unexpected result in our study is the differential responses elicited by superfusion with high- K^+ solutions. In cells with a significant potassium conductance, such superfusion would be expected to depolarize the cell and lead to a calcium influx through VDCCs. Indeed this is the result for both types of deciliated cells. For the ciliated cells, however, the results dichotomize with respect to the resting $[\text{Ca}^{2+}]_i$. For both type I and type II cells, if the resting $[\text{Ca}^{2+}]_i$ was <120 nM, then the response to high- K^+ superfusion would lead to transient calcium elevation. However, if the resting level was >120 nM, as it is for the ciliated type I cells, high- K^+ superfusion resulted in a decrease in $[\text{Ca}^{2+}]_i$ from the resting level. Such results may be explained by considering that elevating K^+ not only affects membrane potential, and by extension the VDCCs (Boyer et al., 1998), but may also affect the exchanger on the basolateral surface (Boyer et al., 1999) and the PMCA on the stereocilia. The frank reduction in $[\text{Ca}^{2+}]_i$ observed during high- K^+ superfusion of ciliated type I cells requires a net increased rate of extrusion. One

such possibility is that the hair cell PMCA is similar to that in human red cells (Romero and Romero, 1984) and has a minimal K^{+} -sensitive pump rate at 5 mM $[\text{K}^{+}]_o$ that increases by a factor of six when $[\text{K}^{+}]_o$ is elevated to 50 mM. To produce the observed change would require only that the increased extrusion rate via the PMCA be greater than the combined increased load through VDCCs, and the decreased extrusion rate by the exchanger.

Although it is apparent how the addition of VDCCs in the bundle might raise the resting $[\text{Ca}^{2+}]_i$ and dominate the combined rates of calcium extrusion of the exchanger and PMCA at rest, the question remains as to why an increased number of such channels in ciliated type I cells would be associated with a reduction rather than a strong elevation in $[\text{Ca}^{2+}]_i$ during K^{+} superfusion. The answer may lie in the expected calcium-dependent inactivation of L-type channels (Yue et al., 1990; Yamaoka and Seyama, 1996). Cells with a relatively elevated resting $[\text{Ca}^{2+}]_i$ will have fewer VDCCs available for activation and consequently a smaller increase in calcium influx during K^{+} superfusion. Alternatively, the VDCCs in the kinocilium may resemble the VDCCs in the apical membrane of mammalian epithelial cells (for review, see Yu and Bchir, 1994) and possess an unusual pattern of voltage activation in a window near the resting potential (Tan and Lau, 1993; Zhang and O'Neil, 1996). In principle the results could be explained by a K^{+} -dependent increased pump rate of the PMCA significantly larger than the combined effect of K^{+} on promoting calcium influx through a relatively inactivated population of L-type channels and decreasing the calcium efflux via the exchanger. This, in turn, leads to the suggestion that the primary difference between type I and type II cells with respect to calcium homeostasis may be only a quantitative difference between the numbers of L-type channels and the resting $[\text{Ca}^{2+}]_i$ that is produced by their presence. All other features with respect to the exchanger and the PMCA might be identical, and the difference between the typical response of type I and type II cells derives from differences in the resting $[\text{Ca}^{2+}]_i$ and the degree to which the VDCCs are inactivated. For cells with low basal calcium, the VDCCs are free to activate, and the cytosolic calcium can be elevated; in cells with high basal calcium, a majority of these channels are inhibited, and the K^{+} -dependent increased activity of the PMCA is revealed.

Calcium extrusion and homeostasis in the intact epithelium

We demonstrated immunocytochemical heterogeneity in the sensory epithelium calcium influx pathways that may be caused by an ensemble of either L- or N-type VDCCs localized in hair cells and in calyceal endings, respectively. To understand the impact of the hair cell basolateral exchanger and the bundle PMCA in context, it is important to remember that these cells exist within complex epithelia noted for enveloping synapses and an asymmetric ionic environment bathing the apical and basal poles of the cells. The role of the PMCA should be considered in context of the unusual ionic composition, relatively high in K^{+} and low in both Na^{+} and Ca^{2+} , bathing the bundles. In such an environment we would expect the apical PMCA to function at a maximum rate as very powerful loci of calcium extrusion. Thus our experiments indicate that the apical PMCA and L-type channels of the kinocilium make significant contributions to the whole-cell calcium homeostasis, and under some conditions these elements may set the $[\text{Ca}^{2+}]_i$. A more precise analysis of the differences in resting $[\text{Ca}^{2+}]_i$ between different types of cells awaits an analysis

in a context in which the ionic asymmetries of the intact epithelium can be maintained.

REFERENCES

- Apicella S, Chen S, Bing R, Penniston JT, Llinas R, Hillman DE (1997) Plasmalemmal ATPase calcium pump localizes to inner and outer hair bundles. *Neuroscience* 79:1145–1151.
- Armstrong CE, Roberts WM (1998) Electrical properties of frog saccular hair cells: distortion by enzymatic dissociation. *J Neurosci* 18:2962–2973.
- Art JJ, Fettiplace R (1987) Variation of membrane properties in hair cells isolated from the turtle cochlea. *J Physiol (Lond)* 385:207–242.
- Art JJ, Goodman MB (1993) Rapid scanning confocal microscopy. *Methods Cell Biol* 38:47–77.
- Art JJ, Fettiplace R, Wu YC (1993) The effects of low calcium on the voltage-dependent conductances involved in tuning of turtle hair cells. *J Physiol (Lond)* 470:109–126.
- Assad JA, Shepherd GMG, Corey DP (1991) Tip-link integrity and mechanical transduction in vertebrate hair cells. *Neuron* 7:985–994.
- Bönigk W, Bradley J, Muller F, Sesti F, Boekhoff I, Ronnett GV, Kaupp UB, Frings S (1999) The native rat olfactory cyclic nucleotide-gated channel is composed of three distinct subunits. *J Neurosci* 19:5332–5347.
- Boyer C, Lehouelleur J, Sans A (1998) Potassium depolarization of mammalian vestibular sensory cells increases $[\text{Ca}^{2+}]_i$ through voltage-sensitive calcium channels. *Eur J Neurosci* 10:971–975.
- Boyer C, Sans A, Vautrin J, Chabbert C, Lehouelleur J (1999) K^{+} -dependence of Na^{+} - Ca^{2+} exchange in type I vestibular sensory cells of guinea-pig. *Eur J Neurosci* 11:1955–1959.
- Choi HS, Eisner DA (1999) The effects of inhibition of the sarcolemmal Ca^{2+} -ATPase on systolic calcium fluxes and intracellular calcium concentration in rat ventricular myocytes. *Pflügers Arch* 437:966–971.
- Corey DP, Hudspeth AJ (1979) Ionic basis of the receptor potential in a vertebrate hair cell. *Nature* 281:675–677.
- Crawford AC, Evans MG, Fettiplace R (1991) The actions of calcium on the mechano-electrical transducer current of turtle hair cells. *J Physiol (Lond)* 434:369–398.
- Crouch JJ, Schulte BA (1995) Expression of plasma membrane Ca^{2+} -ATPase in the adult and developing gerbil cochlea. *Hear Res* 92:112–119.
- Ehrlich BE, Finkelstein A, Forte M, Kung C (1984) Voltage-dependent calcium channels from *Paramecium* cilia incorporated into planar lipid bilayers. *Science* 225:427–428.
- Firestein S, Shepherd GM, Werblin FS (1990) Time course of the membrane current underlying sensory transduction in salamander olfactory receptor neurons. *J Physiol (Lond)* 430:135–158.
- Gatto C, Hale CC, Xu W, Milanick MA (1995) Eosin, a potent inhibitor of the plasma membrane Ca^{2+} pump, does not inhibit the cardiac Na^{+} - Ca^{2+} exchanger. *Biochemistry* 34:965–972.
- Heim R, Iwata T, Zvaritch E, Adamo HP, Rutishauser B, Strehler EE, Guerini D, Carafoli E (1992) Expression, purification, and properties of the plasma membrane Ca^{2+} pump and of its N-terminally truncated 105-kDa fragment. *J Biol Chem* 267:24476–24484.
- Hinrichsen RD, Saimi Y, Hennessey T, Kung C (1984) Mutants in *Paramecium tetraurelia* defective in their axonemal response to calcium. *Cell Motil* 4:283–295.
- Hudspeth AJ, Corey DP (1977) Sensitivity, polarity, and conductance change in the response of vertebrate hair cells to controlled mechanical stimuli. *Proc Natl Acad Sci USA* 74:2407–2411.
- Hudspeth AJ, Jacobs R (1979) Stereocilia mediate transduction in vertebrate hair cells (auditory system/cilium/vestibular system). *Proc Natl Acad Sci USA* 76:1506–1509.
- Issa NP, Hudspeth AJ (1994) Clustering of Ca^{2+} channels and Ca^{2+} -activated K^{+} channels at fluorescently labeled presynaptic active zones of hair cells. *Proc Natl Acad Sci USA* 91:7578–7582.
- Jones KH, Senft JA (1985) An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *J Histochem Cytochem* 33:77–79.
- Kevetter GA, Correia MJ, Martinez PR (1994) Morphometric studies of type I and type II hair cells in the gerbil's posterior semicircular canal crista. *J Vestib Res* 4:429–436.
- Knaus HG, Moshhammer T, Friedrich K, Kang HC, Haugland RP, Glossman H (1992) In vivo labeling of L-type Ca^{2+} channels by fluorescent dihydropyridines: evidence for a functional, extracellular heparin-binding site. *Proc Natl Acad Sci USA* 89:3586–3590.
- Kros CJ, Rusch A, Richardson GP (1992) Mechano-electrical transducer currents in hair cells of the cultured neonatal mouse cochlea. *Proc R Soc Lond B Biol Sci* 249:185–193.
- Lewis RS, Hudspeth AJ (1983) Voltage- and ion-dependent conductances in solitary vertebrate hair cells. *Nature* 304:538–541.
- Liu X, Udovichenko IP, Brown SD, Steel KP, Williams DS (1999) Myosin VIIa participates in opsin transport through the photoreceptor cilium. *J Neurosci* 19:6267–6274.

- Lumpkin EA, Hudspeth AJ (1995) Detection of Ca^{2+} entry through mechanosensitive channels localizes the site of mechanoelectrical transduction in hair cells. *Proc Natl Acad Sci USA* 92:10297–10301.
- Masetto S, Correia MJ (1997) Electrophysiological properties of vestibular sensory and supporting cells in the labyrinth slice before and during regeneration. *J Neurophysiol* 78:1913–1927.
- Masetto S, Russo G, Prigioni I (1994) Differential expression of potassium currents by hair cells in thin slices of frog crista ampullaris. *J Neurophysiol* 72:443–455.
- Maurer J, Mann W, Baggelmann M (1992) Histochemical localization of calcium ATPase in the cochlea of the guinea pig. *Eur Arch Otorhinolaryngol* 249:176–180.
- Meyer J, Furness DN, Zenner HP, Hackney CM, Gummer AW (1998) Evidence for opening of hair-cell transducer channels after tip-link loss. *J Neurosci* 18:6748–6756.
- Oberholtzer JC, Buettger C, Summers MC, Matschinsky FM (1988) The 28-kDa calbindin-D is a major calcium-binding protein in the basilar papilla of the chick. *Proc Natl Acad Sci USA* 85:3387–3390.
- Ohmori H (1985) Mechano-electrical transduction currents in isolated vestibular hair cells of the chick. *J Physiol (Lond)* 359:189–217.
- Park YB, Herrington J, Babcock DF, Hille B (1996) Ca^{2+} clearance mechanisms in isolated rat adrenal chromaffin cells. *J Physiol (Lond)* 492:329–346.
- Peng Y-Y, Wang K-S (2000) A four-compartment model for Ca^{2+} dynamics: an interpretation of Ca^{2+} decay after repetitive firing of intact nerve terminals. *J Comput Neurosci* 8:275–298.
- Press WH, Teukolsky SA, Vetterline WT, Flannery BP (1994) Numerical recipes in C. Cambridge, UK: Cambridge UP.
- Ricci AJ, Fettiplace R (1998) Calcium permeation of the turtle hair cell mechanotransducer channel and its relation to the composition of endolymph. *J Physiol (Lond)* 506:159–173.
- Ricci AJ, Rennie KJ, Cochran SL, Kevetter GA, Correia MJ (1997) Vestibular type I and type II hair cells. 1: Morphometric identification in the pigeon and gerbil. *J Vestib Res* 7:393–406.
- Roberts WM (1993) Spatial calcium buffering in saccular hair cells. *Nature* 363:74–76.
- Roberts WM, Jacobs RA, Hudspeth AJ (1990) Colocalization of ion channels involved in frequency selectivity and synaptic transmission at presynaptic active zones of hair cells. *J Neurosci* 10:3664–3684.
- Romero PJ, Romero E (1982) The affinity of the Ca^{2+} pump of human erythrocytes for external Na^{+} or K^{+} . *Biochim Biophys Acta* 691:359–361.
- Romero PJ, Romero E (1984) The modulation of the calcium pump of human red cells by Na^{+} and K^{+} . *Biochim Biophys Acta* 778:245–252.
- Sanguinetti MC, Kass RS (1984a) Voltage-dependent block of calcium channel current in the calf cardiac Purkinje fiber by dihydropyridine calcium channel antagonists. *Circ Res* 55:336–348.
- Sanguinetti MC, Kass RS (1984b) Photoalteration of calcium channel blockade in the cardiac Purkinje fiber. *Biophys J* 45:873–880.
- Tan S, Lau K (1993) Patch-clamp evidence for calcium channels in apical membranes of rabbit kidney connecting tubules. *J Clin Invest* 92:2731–2736.
- Tucker T, Fettiplace R (1995) Confocal imaging of calcium microdomains and calcium extrusion in turtle hair cells. *Neuron* 15:1323–1335.
- Yamaoka K, Seyama I (1996) Regulation of Ca channel by intracellular Ca^{2+} and Mg^{2+} in frog ventricular cells. *Pflügers Arch* 431:305–317.
- Yamoah EN, Crow T (1994) Two components of calcium currents in the soma of photoreceptors of *Hermisenda*. *J Neurophysiol* 72:1327–1336.
- Yamoah EN, Lumpkin EA, Dumont RA, Smith PJ, Hudspeth AJ, Gillespie PG (1998) Plasma membrane Ca^{2+} -ATPase extrudes Ca^{2+} from hair cell stereocilia. *J Neurosci* 18:610–624.
- Yu ASL, Bchir MB (1994) Calcium channels. *Curr Opin Nephrol Hypertens* 3:497–503.
- Yue DT, Backx PH, Imredy JP (1990) Calcium-sensitive inactivation in the gating of single calcium channels. *Science* 250:1735–1738.
- Zhang MIN, O'Neil RG (1996) An L-type calcium channel in renal epithelial cells. *J Membr Biol* 154:259–266.
- Zidanic M, Fuchs PA (1995) Kinetic analysis of barium currents in chick cochlear hair cells. *Biophys J* 68:1323–1336.