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Differential motile response of isolated inner and outer hair cells to stimulation by potassium and calcium ions

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Inner and outer hair cells were mechanically isolated from the guinea pig cochlea and subjected to stimuli known to induce shape changes in outer hair cells. Depolarization by 70 mM KCl which causes osmotic swelling of outer hair cells also swelled inner hair cells by approximately 8% of their volume. The application of the calcium ionophore ionomycin which induces cortical contractions and elongation of outer hair cells, did not affect the shape of inner hair cells. Since ionomycin increased free intracellular calcium levels in both inner and outer hair cells, the results demonstrate that inner hair cells do not possess the mechanisms necessary for a contractile response to calcium. Thus, calcium is a specific regulator of outer hair cell motility making this mechanism a likely physiological modulator of a transduction feedback process.

Outer hair cells; Inner hair cells; Motility; Potassium depolarization; Calcium

Introduction

There is strong evidence that inner and outer hair cells play different roles in the mammalian cochlea. Inner hair cells are generally considered the primary transducers while outer hair cells have been ascribed a modulatory role in the transduction process (Dallos, 1985; Neely and Kim, 1986). Differences between inner and outer hair cells range from anatomical specializations such as extensive sub-surface cisternae in outer hair cells (Saito, 1983) to differential electrophysiological responses to acoustic stimulation (Cody and Russell, 1985). Specifically, motile properties are thought to be unique characteristics that enable outer hair cells to modify basilar membrane micromechanics. Such motile responses can be elicited by a number of stimuli and via a number of different mechanisms. Fast and possibly frequency-following shape changes can be evoked by electrical stimulation of isolated outer hair cells (Brownell et al., 1985; Ashmore, 1987; Zenner et al., 1987). These fast responses appear to depend upon transmembrane potential since blocking the known ionic conductances of these cells does not interfere with the motility (Santos-Sacchi and Dilger, 1988). Slow shape changes in the millisecond-to-second time frame occur with chemical stimulation of outer hair cells, notably with depolarization by potassium and elevation of intracellular free calcium ions. K^+-depolarization induces an osmotic swelling of the cells which is accompanied by a shortening independent of calcium and structural proteins (Dulon et al., 1988). In isolated outer hair cells, where the plasma membrane has been destroyed by detergents, calcium will cause an irreversible shortening (Flock et al., 1986; Zenner, 1986). In contrast, a rise of intracellular calcium in intact isolated outer hair cells leads to reversible cortical contractions and cell elongation by a mechanism dependent on calmodulin and structural proteins (Dulon et al., 1990).

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A major question in determining the physiological correlates of such in-vitro responses appears to be whether these phenomena are unique to outer hair cells. It can be argued that a regulatory mechanism of basilar membrane micromechanics should specifically control the motility of outer hair cells. Electrical stimulation has already been shown to affect outer hair cell shape only and not inner hair cells (Brownell et al., 1985). The aim of the present study was to determine whether two different 'slow' stimuli, potassium depolarization and the elevation of intracellular calcium elicit shape changes in both outer and inner hair cells.

Methods

Hair cell isolation

Outer hair cells were isolated from the cochlea of pigmented guinea pigs as previously described (Zajic and Schacht, 1987; Dulon et al., 1989), and inner hair cells were obtained by essentially the same procedure. The whole organ of Corti was dissected in Hanks' Balanced Salt Solution (HBSS; buffered to pH 7.4 with 5 mM sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate [HEPES], and adjusted to an osmolality of 300 ± 1 mOsm with NaCl). Fragments from each turn were then transfered with a microsyringe (#705, 22-gauge; Hamilton, Reno, NV) into separate 50 μl droplets of HBSS containing 0.5 mg collagenase/ml (type IV from Sigma, St. Louis, MO). After 15 min of incubation at room temperature (20–22°C), the strips of the organ of Corti were transfered into 50 μl of collagenase-free HBSS on glass slides. The dissociation procedure was completed by gentle flux and efflux of the tissue pieces through the micropipette. After settling for 5 min, adhesion of the cells to the glass was sufficiently strong to prevent them from being dislodged by the addition of experimental solutions. All steps of the isolation procedure were carried out in a humid chamber in order to avoid evaporation and changes in the osmolality of the solutions.

On the average, one guinea pig cochlea yielded approximately 250 viable outer and 20 to 30 inner hair cells. Contrary to outer hair cells, the yield of isolated inner hair cells was higher from the basal part of the cochlea than the apex.

Measurement of intracellular calcium

Loading of the calcium-sensitive dye and fluorescence measurements were performed as previously described (Dulon et al., 1989, 1990). Hair cells were incubated for 30 min at room temperature (22–25°C) with 2 μM fluo-3/AM (Molecular Probes Inc., Eugene, OR) in HBSS and observed with an inverted microscope (Leitz Fluovert fitted with an epifluorescence system [ArcLamp HBO 100W; 450–490 nm band-pass exciter filter, 510 nm dichroic mirror and 520 nm barrier filter for excitation and emission, respectively] and a Leitz 160/- NPL FLUOTAR 50/1.00 oil immersion objective). Cell fluorescence was monitored with a Silicon Intensified Target video camera (SIT 66, Dage-MTI Inc., Michigan City, IN) and analyzed with a Quantex QX-7–210 image processing system (Quantex Corp., Sunnyvale, CA). The concentrations of intracellular calcium were calculated (Dulon et al., 1990) according to the equation (Koa et al., 1989) $[Ca2+]_i = K_d(F - F_{min})/(F_{max} - F)$. K_d is the dissociation constant for the fluo-3/calcium complex; F is the observed fluorescence in arbitrary units of intact fluo-3 loaded cells; F_{max} (fluorescence maximum) was calculated from the equation $F_{max} = (F_{Mn} - F_{bkg})/0.2 + F_{bkg}$. F_{Mn} is the fluorescent signal obtained after treatment of the cells with ionomycin (10 μM; 60 s) and $MnCl_2$ (2 mM; 3 to 5 min); F_{bkg} is the fluorescent signal obtained after lysis of the cells with 100 μg saponin/ml; 0.2 is the ratio of the signal of the Mn^{2+}-saturated fluo-3 to that of the Ca^{2+}-saturated dye. F_{min} (fluorescence minimum) was calculated from the equation $F_{min} = (F_{max} - F_{bkg})/40 + F_{bkg}$ where 1/40 is the ratio of the fluorescent signal of metal-free fluo-3 to the Ca^{2+} complex.

Application of stimuli

Ten mM stock solutions of ionomycin (from Calbiochem, La Jolla, CA) were prepared in DMSO. The ionophore was added to the cells to yield a final concentration of 10 μM (final concentration of DMSO, 0.1%).

For $[K^+]$-depolarization (70 mM KCl final concentration), 50 μl of a 140 mM KCl-HBSS solution (Na^+ replaced by KCl and osmolality adjusted to 300 ± 1 mosm) was gently added with

a pipette to 50 μl of normal HBSS containing the cells.

Measurement of hair cell dimensions

Cell length was simultaneously monitored on bright field images and analyzed with the Quantex-QX-7-210. The system provided measurements with a resolution of ±0.2 μm as tested with calibrated fluorescent microspheres (Polysciences, Warmington, PA). The cell lengths reported are the distance between the cuticular plate and the synaptic ending of the cell. The cell volume was calculated from the two-dimensional video images of the cells by treating these as projections of defined three-dimensional bodies. Outer hair cells were divided into five segments whose diameter and length were individually determined, and volume was calculated assuming a cylinder. Inner hair cells were divided near the neck region into two segments, and volume was calculated assuming a truncated cone for the neck and a sphere for the lower portion of the cell.

Results

Hair cell isolation

The dimensions of isolated inner hair cells varied somewhat along the cochlear spiral. The length ranged from 25 to 40 μm with apical hair cells being longer, and their diameter, measured across the nucleus, from 15 to 20 μm. These variations were small compared to the length of outer hair cells which varies three- to four-fold from approximately 20 μm in the base to over 70 μm in the apex of the guinea pig cochlea.

Inner hair cells can be distinguished from basal outer hair cells of the same length by several features (Fig. 1). Inner hair cells are flask- or pear-shaped while outer hair cells are cylindrical. Outer hair cells will also often have numerous nerve terminals attached to their base while this is not seen in inner hair cells. The diameter of isolated inner hair cells is almost twice as large as the corresponding diameter of outer hair cells (8 to 10 μm) whether they are basal or apical. The apex of

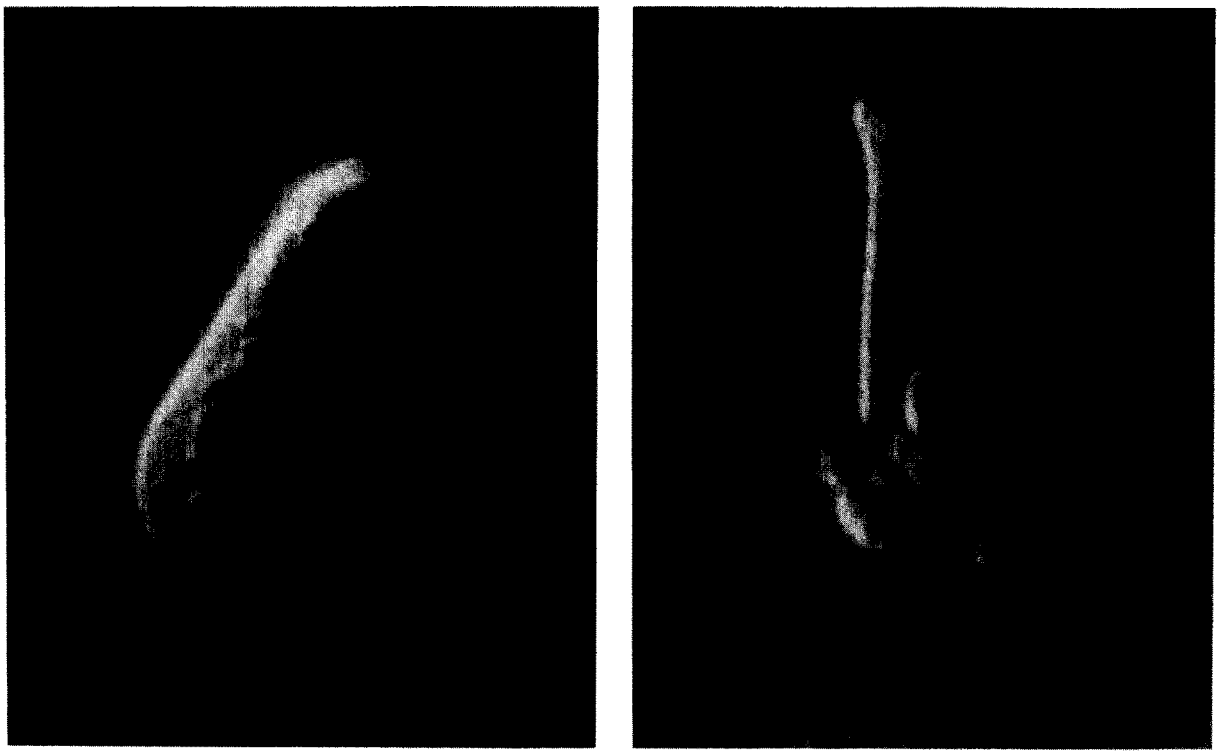

Fig. 1. Isolated inner and outer hair cells from the base of the cochlea. Left: inner hair cell, right: basal outer hair cell. Cells were isolated and maintained in culture as described in 'Methods'. Calibration bar: 5 μm.

inner hair cells tapers to a neck region which is smaller than that of outer hair cells. This region is regularly tilted to one side probably corresponding to the angular anchoring of inner hair cells in the cuticular plate in vivo. The neck widens at the cuticular plate which looks concave in contrast to the usually flat-appearing cuticular region of an outer hair cell. The length of the stereocilia is another distinguishing characteristic. The tallest stereocilia of inner hair cells are longer than those of basal outer hair cells. Our measurements show inner hair cell stereocilia to average 6.5 μm (6.45 + 0.71 μm; $N = 7$) while those of basal outer hair cells do not exceed 5 μm (4.03 $\pm$ 1.15 μm; $N = 9$). Only at more apical levels of the cochlea do outer hair cell stereocilia approach the same length and exceed those of inner hair cells (Lim, 1980). Another distinguishing feature is the size and position of the nucleus. The nucleus of outer hair cells (8.01 $\pm$ 0.35; $N = 12$) is smaller than that of inner hair cells (9.71 $\pm$ 0.56; $N = 6$); it is located at the extreme base of the cell in outer but higher in inner hair cells. By these criteria, a previously published 'inner' hair cell (Yamashita et al., 1990) most likely represents a short outer hair cell.

Both outer and inner hair cells maintain their distinctive shape after isolation indicative of a rigid lateral cell wall. Swelling does occur in some cells after isolation, probably as a result of exposure to mechanical or chemical stress. It is usually confined to the synaptic regions of either cell species. In isolated inner hair cells, the swollen region shows a clear cytoplasm, with intracellular organelles remaining more apically. If swelling occurs at the base of isolated outer hair cells they may resemble inner hair cells in shape. The base of the outer hair cells will, however, mostly maintain a structured cytoplasm.

While outer hair cells could be maintained in culture for several hours, inner hair cells appeared less stable and the useful lifespan of a preparation was approximately 60 min. After this time, inner hair cells began to deteriorate showing increased Brownian motion in the cytoplasm and excessive swelling.

Effects of K^+-depolarization

Viable isolated inner hair cells, as previously described for outer hair cells, were capable of hydrolyzing the membrane permeant form of the calcium sensitive probe fluo-3/AM to its fluorescent derivative compound fluo-3 and of accumulating it in their cytoplasm. In the absence of stimulation, the concentration of intracellular free calcium of inner hair cells was 182 $\pm$ 88 nM (Table I). This calcium concentration is of the same magnitude as found in isolated outer hair cells (Dulon et al., 1989, 1990). The external application of 70 mM KCl increased intracellular calcium in 20 of 20 inner hair cells (Table I) similar to the increase reported in outer hair cells (Dulon et al., 1989).

TABLE I

EFFECTS OF K^+-DEPOLARIZATION ON ISOLATED INNER HAIR CELLS

	Before K^+	After K^+	% Change [1]
Calcium (nM)	182 ±88	346 ±189	+91 ±52 [2]
Length (μm)	37.5± 9.3	37.7± 9.4	+0.4± 1.8 [3]
Diameter (μm)	19.1± 3.5	20.0± 4.0	+4.4± 3.7 [4]
Volume (pL)	3.7± 0.5	4.0± 0.5	+7.7± 2.6 [2]

Cells were isolated and treated as described in 'Methods' and exposed to the stimulus for 1 min. Results for calcium and for cell dimensions are from separate experiments. Diameter was measured at the widest area across the nucleus. Numbers are means ± SD; $N = 20$ (calcium) and 7 (dimensions). [1] Percent change calculated from individual cells before and after treatment; [2] $P < 0.01$ by paired t-test on individual cells before and after treatment; [3] not significant; [4] $P = 0.03$ by paired t-test on individual cells before and after treatment.

TABLE II

EFFECTS OF CALCIUM IONOPHORE ON INNER HAIR CELLS

	Before (μm)	After (μm)	Change [1] (%)
Calcium	[2]	[2]	+84 ±129 [3]
Length (μm)	31.5±4.7	31.3±4.8	−0.6± 0.8 [4]
Diameter (μm)	17.8±1.8	17.9±1.9	+0.7± 1.2 [4]

Cells were isolated and treated as described in 'Methods' and exposed to the stimulus for 1 min. Results for calcium and for cell dimensions are from separate experiments. Diameter was measured at the widest area near the base of the cell. Numbers are means ± SD; $N = 19$ (calcium), and 12 (dimensions). [1] Percent change calculated from individual cells before and after treatment; [2] Calcium determined in arbitrary units; [3] $P = 0.015$ by paired t-test on individual cells before and after treatment; [4] Dimensions of individual cells did not change significantly.

Potassium-depolarization also caused inner hair cells to swell consistently with 7 out of 7 cells responding (Table II). As determined from individual cells before and after treatment, cell volume increased by 7.7% due to an increase in cell diameter by 4.4% ± 3.7. Cell length essentially remained unaffected (±0.4% ± 1.8). This is analogous to the well-documented behavior of isolated outer hair cells to swell in response to depolarization with KCl by 5 to 10% (Zenner 1986; Dulon et al., 1988).

Effects of ionomycin

The external application of the calcium ionophore ionomycin to inner hair cells raised their intracellular free calcium concentrations (Fig. 2) within seconds. The increase in calcium fluorescence averaged 91% and was observed in 16 of 19 cells (Table II). Their shape, however, remained unaffected by the addition of ionophore: there were no significant changes in length or diameter and, consequently, in volume. Under identical conditions, outer hair cells consistently elongated in agreement with our previous results (Dulon et al., 1990). Seven out of 7 outer hair cells responded with a 50% ± 33 increase in calcium fluoresence ($P < 0.01$ by paired t-test) and another 9 out of 10 outer hair cells responded with a 5.2% ± 3.6 increase in their length (from 69.6 ± 8.7 to 73.0 ± 7.9 μm; $P < 0.01$ by paired t-test).

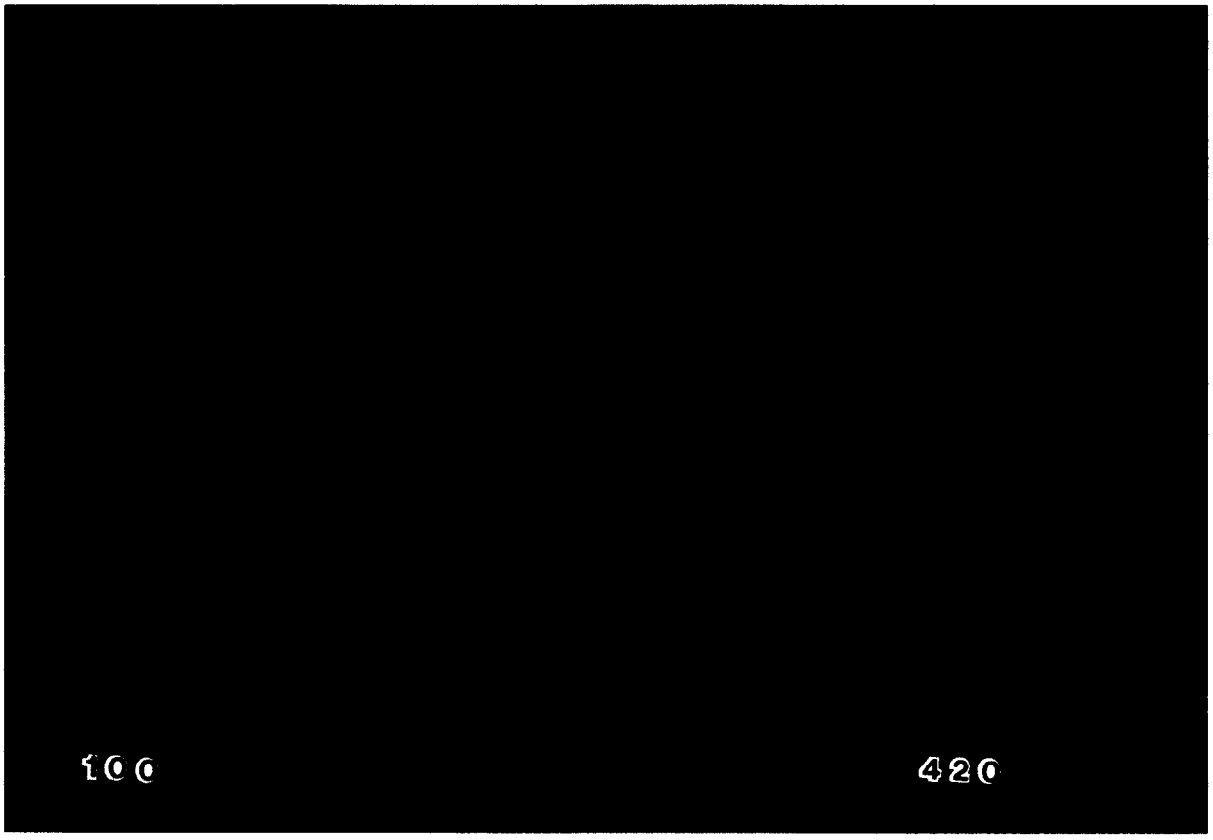

Fig. 2. Imaging of intracellular free calcium in an isolated inner hair cell. Left: cell before stimulus; right: 60 sec after stimulus. The cell was isolated and treated with ionomycin as described in 'Methods'. Levels of intracellular free calcium ion are displayed in pseudocolors. Calibration ramp: concentration of free calcium ion [nM].

Discussion

Isolated inner and outer hair cells demonstrate a differential response to chemical stimuli that induce slow shape changes. Potassium-induced depolarization equally affects the shape of both cell types while the response to elevated intracellular free calcium is unique to outer hair cells. These are compatible results since potassium and calcium operate via different mechanisms.

We speculate that the depolarization-induced shape change in inner hair cells is the result of a passive osmotic swelling independent of contractile proteins as has been demonstrated for outer hair cells (Zenner et al., 1985; Dulon et al., 1988, 1989). This osmotic phenomenon seems to be based on chloride influx since it could be abolished in outer hair cells when potassium gluconate was substituted for potassium chloride (Ulfendahl, 1988). However, while both cell shortening and increase in diameter is associated with this swelling in outer hair cells, inner hair cells mainly change their diameter. This contrasting behavior may stem from differences in the rigidity of the lateral wall of the two cell types and the elongated tubular shape of outer hair cells. Furthermore, we have proposed that the depolarization-induced rise of intracellular calcium in outer hair cells is mediated by voltage-gated calcium channels (Dulon et al., 1989). Although more pharmacological information is needed, it is likely that calcium entry in inner hair cells similarly occurs via voltage-gated channels.

Ionomycin provides a ionophoric action for calcium at the plasma membrane. Inner hair cells did not respond with a shape change to this stimulus. This is in contrast to outer hair cells in which ionomycin triggers calcium entry followed by a calmodulin-mediated circumferential contraction leading to cell elongation (Dulon et al., 1990). The first step of the ionomycin-induced sequence, namely calcium entry, is clearly evident in inner hair cells. Therefore, the absence of a shape change suggests that inner hair cells do not possess a calcium-dependent contractile apparatus.

In conclusion, this analysis of inner and outer hair cell motility strongly reinforces intracellular calcium as a specific regulator of outer hair cell motility. Thus, this mechanism is a most likely physiological modulator of a transduction feedback mechanism.

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