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MAMMALIAN COCHLEAR OUTER HAIR CELLS DENSITY EVALUATED BY MEANS OF AN OPTICAL TWEEZER

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SUMMARY : We report on the first individual measurements of guinea pig's cochlear outer hair cells densities. Cells were isolated *in vitro* and manipulated with an optical tweezer. They were levitated in an upward laser beam coaxially trapping the cells. Then they were released by switching off the laser and let fall down in upright position. Measuring their speed and using the Stokes' law, we calculated their mean density. In our experimental frame, the results suggest that the density of the cellular body (between the basal nucleus and the apical cuticular plate) remains quasi constant whatever the cells' length. This implies that density variation of the cellular body does not participate in an intrinsic tuning mechanism.

Mammalian cochlea realizes a very performing sound frequency analysis (1)(2), which is achieved by two intimately linked structures : the basilar membrane and the organ of Corti. Performances of this complex are such that neural thresholds correspond to subnanometer displacements of the basilar membrane at very precise frequencies (3). Among the different cells composing the organ of Corti, three rows of outer hair cells (OHC) play a major role in the detection and amplification of sound at hearing threshold. The OHCs possess unique electro-mechanical responses in the acoustic frequencies domain (4)(5). The mechanical properties of these cylindrical cells have been shown to be determined by a coiled cytoskeletal spring associated with multilayered membranes (lateral cisternae), beneath the plasma membrane and extending all along the cells' length between an apical cuticular plate and a basal nucleus (6). The cells' length appears therefore as a basic parameter in the longitudinal stiffness of the OHCs. It is interesting to note that there is indeed a good correlation between the OHCs' length and the distinct acoustic frequencies to which each cell is supposed to be tuned (7)(8). However, another basic mechanical parameter which has not been so far studied in detail (9) is the cellular density. Knowledge of the OHCs' densities is important for the organ of Corti/Basilar membrane micromechanics in general, and particularly if the postulated active second filter in mammals hearing is related to an intrinsic resonant process of these cells. Moreover, considering the OHCs as cylindrical cells with dense structures at both ends, a basal nucleus and an apical cuticular plate (see figure 1), could variations in cellular density between these structures (where the

0006-291X/93 $4.00

cellular spring lies) be involved in an intrinsic tuning mechanism, concurrently to length variations? The present study gives first evaluations of the OHCs' density as a function of their length.

MATERIALS AND METHODS

Cell preparation :
The cells were prepared as previously described (10). Pigmented adult guinea pigs (200-350 g) were decapitated, the temporal bones quickly removed and the bulla and bony walls of the cochlea were immediately opened. After removal of the stria vascularis and the tectorial membrane, the different turns of the organ of Corti were separated from the spiral lamina with a thin metal probe. The dissected strips of the organ of Corti were transferred to a solution of collagenase medium (type IV from Sigma; 0.5 mg/ml) and incubated for 30 minutes at room temperature. The strips were then removed from the collagenase medium and transferred into an optical glass chamber (1 mm high ; made by Thuet-Biechelin reference 430), filled with a culture medium. One hour before this chamber had been filled with rabbit serum (dilution 50% with culture medium), which contributed to reduce the cells' attachment to glass which was a major difficulty in these experiments. The dissociation procedure was completed by gentle flux and efflux of the tissue pieces by means of a Gilson 100 pipette. Both isolation procedure and experiments were performed in Hank's Balanced Salt Solution (HBSS : in mM, $CaCl_2.H_2O$ 1.25, glucose 5.55, $MgSO_4$ 0.81, KH_2PO_4 0.44, NaCl 136.9, NaH_2PO_4 0.34, KCl 5.4, HEPES (acid) 5.0, with osmolality adjusted at 300 mosm (with glucose) and pH 7.4).
We choosed the OHCs as cylindrical as possible with the nucleus in basal position, and a minimum of basal synaptic residues.

Optical trapping and method of density measurements :
The experimental set-up has been previously described (11). In our experiments we only needed a single upward laser beam, whose wavelength and beam-waist were respectively 514 nm and about 4 μm. We used the laser beam as an optical tweezer to place an isolated OHC from a prone position to an upright one (see figure 1). Then in this upright position the cylindrical cell was trapped coaxially with the laser beam, and pushed up in the sample chamber. When the cell was at about 400 μm above the bottom of the chamber, we let it fall down by switching off the

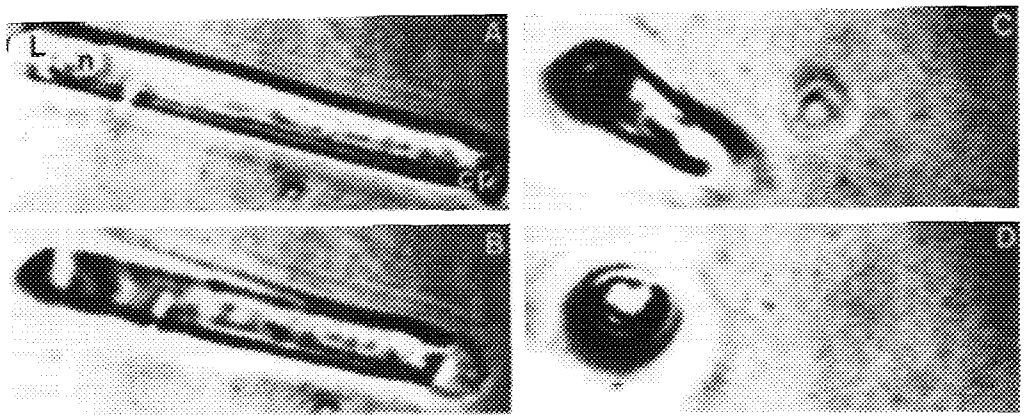

Figure 1 . Laser levitation of a 75 μm long OHC.
A) The laser beam (L) is focused at the basal pole of the OHC, and can be seen just below the nucleus (n). The apical pole is constituted by the cuticular plate (cp). Since the nucleus possesses important refractive index and volume (compared to other cellular structures), the optical tweezer was more powerful at its level. The first levitation step was to pull the cell by its nucleus in order to break the cell links to the glass. In a second step the laser beam was used to push one of the cellular poles up, and thus to trap the cell vertically.
B,C) placing the cylindrical cell in an upward position.
D) The cell is coaxially trapped in the beam and pushed up in the chamber.

laser beam. We measured the time for the cell to fall down between two vertical points of known height. This gave a sedimentation speed "v". We relate this speed to a mean cell density "d" using a simple Stokes' law : (MKSA)

$$V_c.(d-1).g.10^3 = 6.\Pi.R.\mu.K.v \quad (I)$$

In (I), the left hand side is the particle weight (V_c is the cell volume, g is equal to 9.81 $m.s^{-2}$). The right hand side is the hydrodynamic force exerted by the surrounding fluid on the falling particle. In a rough description, the particle may be viewed as cylindrical and of radius R. We write the corresponding friction coefficient as $6.\Pi.R.K.\mu$, where μ is the fluid viscosity taken equal to 10^{-3} $N.s.m^{-2}$, and K is a geometrical factor which depends on the shape and size of the particle. We used for K the values given by Happel and Brenner (12) for vertically falling prolate spheroids. Here one may question about this particular choice since the OHCs are not actually perfect spheroids neither perfect cylinders. In fact, small variations in the particle shape do not induce severe variations in K. Practically, these variations are small compared to the other sources of uncertainties in our experiments (see discussion).
Thus using (I), the value of the constant falling speed "v" gives the mean cell density "d". In fact we studied the parameter "d-1", assuming that the buffer density was equal to 1. The parameter "d-1" is proportionnal to a mean cellular organic materials concentration (13), including all the cell structures (mainly : nucleus, cuticular plate, proteic spring, cisternae, cytoplasm).
Measurements were carried out quickly in order to avoid cell damages which could occur with laser heating. Typically the laser power in the chamber was approximately 60 mW to manipulate a 80 μm long OHC, and the laser irradiation time was 1 minute.

RESULTS AND DISCUSSION

The first step in our experimental analysis of individual density measurements was to calculate the parameter "d-1" from the measured speed of fall under gravity, and from measured values (after laser manipulation) of the cell length (L_f) and radius (R_f). From these data we evaluated a cellular organic materials mass "M" for each OHC, assuming that a mean specific volume of these materials is 0.75 (13) :

$$M = (d-1).\Pi.R_f^2.L_f/(1-0.75) \quad (II)$$

Averaged masses, for trapped OHCs whose lengths range between 30-35 μm and between 70-75 μm, are respectively :

$$4.3\ 10^{-13}\ kg\ (SD = 0.6\ 10^{-13})\ and\ 5.8\ 10^{-13}\ kg\ (SD = 1.5\ 10^{-13})$$

Then in a second step, we normalized the parameter "d-1" into $(d-1)_N$. Indeed the cells' diameters generally varied between OHCs in the same experiment, and moreover the length and the diameter of a trapped cell decreased and increased respectively after laser manipulation (see comments below). Since a cell density is related to its volume, a normalization is indispensable in order to coherently compare the density of different OHCs. Thus we supposed that the mass of the organic materials composing each OHC did not vary significantly, during the biochemical and laser manipulations and whatever the corresponding cell volume changes. Such an hypothesis leads to write the following equation for $(d-1)_N$:

$$L_f.R_f^2.(d-1) = L_N.R_N^2.(d-1)_N \quad (III)$$

L_N and R_N are normalized cell length and radius respectively. Referring to previous works (7)(8), we took R_N equal to 5 μm whatever the cell. And we choosed to take arbitrarily L_N equal to the

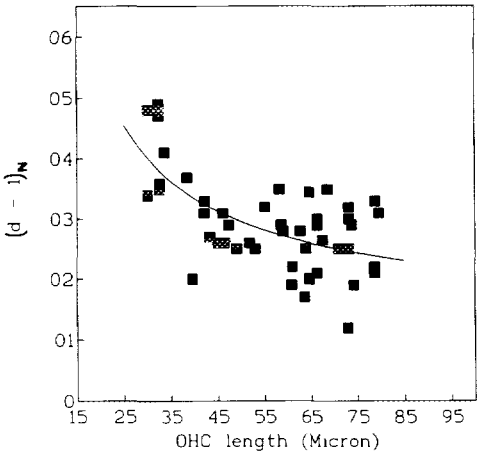

Figure 2 . *Normalized OHCs' density minus 1 versus length.*
A parameter "d-1" was calculated for each cell from hydrodynamical measurements, using the Stokes' law. Then "d-1" was normalized into $(d\text{-}1)_N$, in order to coherently compare the OHCs' density.

cell length before laser manipulation. The individual measurements of 47 OHCs of different lengths between 30 μm and 80 μm, isolated from 8 guinea pig cochleas, gave values of the parameter $(d\text{-}1)_N$ ranging between 0.049 and 0.012 (figure 2). The cellular densities are similar to those obtained in Percoll density gradients (9).

The results plotted in figure 2 show that the shortest OHCs have higher values of $(d\text{-}1)_N$ compared to the longest ones. This behaviour can be explained by the fact that the nucleus and cuticular plate masses contribute more and more in the mean cellular density as the cell length decreases. Now if we simply assume that the sum "S" of nucleus and cuticular plate masses does not vary significantly with the cell length, we can separate the total organic cell mass in two parts : the sum "S" and the mass of the organic materials composing the cell body, mainly between the nucleus and the cuticular plate. Thus we can write the following equation :

$$V_c.(d\text{-}1)_N.10^3 = S + (V_c - V_n).(d_b\text{-}1).10^3 \qquad (IV)$$

V_n is the nucleus volume which is supposed to be constant, and d_b a mean organic materials density of the cell body between the nucleus and the cuticular plate. Equation (IV) can be rewritten in the following form which can be fitted to the experimental data :

$$(d\text{-}1)_N = A/L_N + (d_b\text{-}1) \qquad (V)$$

A is a constant including in particular the nucleus and cuticular plate mean masses. The fit is qualitatively correlated to the following realistic argument : if we consider the mean density of an infinitely long OHC, it will be close to the mean density of the cell body between the nucleus and the cuticular plate. Such a fit gives the following estimation :

$$d_b = 1 + 0.014 \quad (SD = 0.003)$$

In addition it has to be noted that our results interpretation suggests that the density of the OHCs' cellular bodies (between the nucleus and the cuticular plate) remains quasiconstant whatever the cells' length. Therefore, the participation of OHCs' cellular body density variations to a frequency coding, at the level of the cytoskelettal spring, is ruled out in our experimental frame.

Now let us discuss the uncertainties in these results. One may try to calculate errors on the basis of equation (I). However, the main difficulty comes from the fact that many cells (in fact the longest ones) are sligthly curved and do not fall vertically along a distance much larger than their lengths. In practice the resulting error cannot be calculated reliably. In a more realistic way, one could estimate the error on the density from the statistical dispersion obtained from many experiments carried out with each cell. Unfortunately it is not possible to repeat the same experiment more than two or three times without seriously damaging the cell. Practically, the only way is to take the experimental error as the dispersion of data corresponding to different cells, in other words the thickness of the data cloud such as in figure 2.
Finally, we want to precise the effects of the laser manipulation on the cells' volume, such as the length decrease and the diameter increase (observed in 90% of the cells). As the cellular bodies are entirely trapped in a focused laser beam of large power density, we believe that these effects are certainly the result of the cellular response to the laser heating (14), rather than a cellular response to the opto-mechanical stress exerted by the radiation pressure (15). Indeed if the laser manipulation of a cell is prolonged for several minutes, an irreversible alteration of the cell structure occurs : it begins by an important shortening and swelling, with a simultaneous decrease of the cell refringence, followed by a quick cell reextension.

In conclusion, we have weighed individual mammalian cochlear outer hair cells by placing these cylindrical cells, with an optical tweezer, in such a position that hydrodynamical measurements using the Stokes' law were possible. In the frame of our experiments, the results suggest that there is no variation of the cellular bodies density which could participate to a sound frequency coding at the cellular level, concurrently to what is suspected for the cell length (7)(8). The mean density of the OHCs' cellular bodies between the basal nucleus and the apical cuticular plate, whatever the cells' length but with diameters taken equal to 10 μm, has been estimated to :
$d_b = 1.014$ (SD = 0.003).

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