First appearance and development of motile properties in outer hair cells isolated from guinea-pig cochlea

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Cochleae from fetal guinea-pigs (37 to 64 gestation days, gd) were used to correlate the appearance of motile properties of isolated outer hair cells (OHCs) with the development of specific morphological features. Both the 'fast' electrically-driven and the 'slow' calcium-induced motilities appeared first in OHCs from basal turn of 52 gd fetuses. At 56 gd, most of basal and some apical OHCs responded positively to both types of stimulation. All tested cells were positive at 64 gd. It is noteworthy that this period closely corresponds to the onset and maturation of the gross cochlear potentials. Some structural changes in the organ of Corti may be correlated with the development of OHC motile properties: the acquisition of an adult-like cylindrical shape by the OHC, its lateral detachment from neighboring Deiters cells, and its surrounding by fluid spaces. At the ultrastructural level, the formation of a first layer of laminated cisternae regularly aligned along the OHC plasma membrane from the cuticular plate down to the nuclear level, temporally coincided with the onset of in vitro motility (52 gd). The following days, pillars and a sub-membrane lattice were clearly noticed between the outermost cisternal membrane and the plasma membrane. The results support the ideas that: motile properties observed in vitro reflect the in vivo active mechanisms, and that one single layer of laminated cisternae and its associated sub-plasma membrane material may be needed for OHC motility.

Cochlear development; Outer hair cell motilities; Outer hair cell ultrastructure

Introduction

In contrast to inner hair cells (IHCs), outer hair cells (OHCs) are now considered to be more than conventional mechanoreceptors. They are responsible for an active bio-mechanical process which feeds energy back into the cochlear partition, giving the cochlea its properties of high sensitivity and frequency selectivity. With the demonstration of electromotility in isolated guinea-pig OHCs (Brownell, 1984; Brownell et al., 1985), many models for the active bio-mechanical process have been proposed with the motile response of OHC to an exciting sound stimulus as a major factor. To date, the motile properties of OHCs, while indirectly supported by oto-acoustic emissions (Kemp, 1978), have only been documented in vitro. Two types of different mechanisms can be distinguished: respectively the so-called 'fast' and 'slow' motilities. Brownell (Brownell, 1984; Brownell et al., 1985) demonstrated that isolated OHCs contracted or elongated with electrical stimulation. This electromotility, not dependent on metabolic energy, is able to elicit forces at relatively

high frequencies (Kachar et al., 1986; Ashmore, 1987). On the other hand, the in vitro demonstration of a calcium-dependent slow OHC motility in detergent-permeabilized (Zenner et al., 1985) or intact cells (Dulon et al., 1990) may reflect an in vivo property of OHCs, namely their ability to modulate the cochlear mechanics under efferent neural control. Thus, the OHC response to a sound may consist of a unique double-transduction process: 1) the classical mechano-electro transduction through membrane depolarization after a mechanical opening of ion channels, and 2) a reverse electro-mechano transduction through the fast OHC motility depending on changes of membrane potential. Superimposed on, the central nervous system may modulate the reverse electro-mechano transduction via efferent control of slow OHC motility (see recent reviews: Santos-Sacchi, 1988; Pujol, 1989).

One way to examine the role of OHCs is to correlate their development and the development of fine tuning with the developmental onset of the motile responses. During ontogeny, OHCs have been found to develop in a completely different way than IHCs (Pujol et al., 1978; Pujol, 1986). Their shape, innervation pattern, and arrangement with surrounding support cells, all change drastically during maturation. These drastic OHC changes coincide with the acquisition by

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the cochlea of its adult-like physiological properties (Pujol et al., 1980). Roughly, the maturation of the cochlea, starting from the onset of function, could be divided in two stages: 1) a primitive stage where both types of receptors (IHCs and OHCs) look similar, and where the coding of sensitivity and frequency is crude, and 2) a stage when OHCs evolve towards a completely different type of cell, gradually giving the cochlea its fine sensitivity and tuning. It is tempting to correlate these profound OHC changes with a late development of their specific motile properties (Pujol, 1986, 1989). Indeed, the late appearance and maturation of oto-acoustic emissions (Lenoir and Puel, 1987; Norton et al., 1989) support this idea. However, to date, a direct approach of OHC motility during development is lacking.

In the present experiments we used developing guinea-pigs, from gestational age (gd) 37 days till birth, to determine the time of appearance and development of motile properties in isolated OHCs and to correlate it with ultrastructural and molecular features in the maturing OHCs. The guinea-pig was chosen for this study since most of the data on both the electrical (Brownell, 1984; Brownell et al., 1985; Zenner et al., 1987) and calcium-induced motility (Zenner et al., 1985; Dulon et al., 1990) were obtained on isolated guinea-pig OHCs. In addition, reports on structural (Thorn, 1975; Pujol and Hilding, 1973) and physiological (Romand, 1971; Romand et al., 1971; Pujol and Hilding, 1973) cochlear development in guinea-pigs allow a reliable correlation. Two types of motilities, electrically-driven and calcium-dependent respectively, were tested in one fetus from each litter. In addition, to help to discern the substrate of the motile responses, cochleae from littermates were prepared for electron microscopy and immunocytochemistry. Some preliminary observations have been presented (Pujol et al., 1990a,b).

Methods

Fetuses from thirteen litters of albino guinea-pig varying in age between gestational days (gd) 37 and 64 as well as 2 newborn animals were investigated. The developmental age was assessed by comparing weight and length (crane-rump: cr) of fetuses, corrected by the number of littermates, with Draper's (Draper, 1920) tables (see Table I). Fetuses were taken by cesarian section, under ketamine/Rompun (2/3) anaesthesia of the mother. From each litter, cochleae of the first fetus were dissected for isolation of OHCs. Other fetuses were prepared for morphological and histochemical analyses.

Hair cell preparation

Hair cells were isolated from the fetal cochleae essentially as previously described in adult prepara-

TABLE I

LITTERS OF GUINEA-PIG FETUSES

Age of fetus (gd)	Number of litters	N. fetuses/ litter	Mean weight (g)	Mean cr. length (mm)
37	1	6	8	45
47	1	6	24	72
50	1	5	33	82
52	2	5/4	37	85
53	2	5/5	40	88
55	2	5/4	44	90
56	2	5/4	46	92
60	1	4	60	100
64	1	3	107	120

tions (Dulon et al., 1990). After removing the stria vascularis and the tectorial membrane, the different turns of the organ of Corti were separated from the spiral lamina with a fine forcept. This dissection was carried out under a dissecting microscope at magnification 180 to 250 in tissue-culture medium. Different strips of organ of Corti were isolated and separated in two groups referring to their location along the cochlear spiral: the base (high frequency coding region) for the lower turns, and the apex (low frequency coding region) for the apical turns. The dissected strips were transferred into a 50 μl droplet of collagenase medium (type IV from Sigma, 0.5 mg/ml) on the middle of a glass coverslide, sealed in the middle of a perforated Petri-dish (Falcon, 55 mm in diameter). After 15–20 min incubation at room temperature in a humid chamber, the dissociation procedure was completed by gentle flux and efflux of the tissue pieces through a micropipette in the droplet. A resting period of 20 min allowed the cells to settle down on the glass coverslide, then, cells were carefully rinsed with collagenase-free culture medium. Both isolation procedure and experiments were performed in Hanks balanced salt solution (HBSS, buffered to pH 7.4 with 5mM Hepes, and adjusted to an osmolarity of 300 ± 2 mosm with NaCl).

Electrical stimulation of the hair cells: 'Fast motility'

For the intracellular electrical stimulation, glass micropipettes were filled with 0.1 M-KCl, 10 mM-sodium Hepes, pH 7.2 (resistances ranging between 80 and 100 MΩ) and were fixed to a stepping micromanipulator (Leitz) for the impalement of the cells. Constant current (square pulse) between 500 pA and 1 nA was injected through a micropipette using a current generator (Microiontophoresis Current Programmer, World Precision Instruments, Sarasota, FL) at a rate of 1 Hz, reversing polarity to avoid membrane charging. Cellular response to positive hyperpolarizing current was an elongation, negative current induced a contraction of

Fig. 1. Electrically-induced motility. Isolated OHC from the basal coil of a 52 gd guinea-pig cochlea. At resting stage (left) the cell length is 21.8 μm. When a 1 nA current is injected into the cell through the micropipette, the cell contracts (right), its length is now 20.6 μm.

cell length. Cycles of movement were measured by frame-by-frame analysis of video recordings. Only cells whose motility followed the stimulus were considered responsive.

Ionomycin induced calcium-stimulation: 'Slow motility'

As previously described (Dulon et al., 90), the Ca-ionophore ionomycin increases intracellular Ca^{2+} in OHC and produces cortical contractions (Dulon et al., 1990). This stimulation was used to test the presence of the Ca^{2+}-induced slow motile property of guinea-pig fetus hair cells. Ten mM stock solutions of ionomycin (from Calbiochem, La Jolla, CA) were prepared in DMSO. The ionophores were added to the cells with a micropipette to yield a final concentration of 10 μM (final concentration of DMSO, 0.1%). Intracellular calcium was monitored by fluorescence microscopy using the Ca^{2+} probe Fluo-3 as previously described (Dulon et al., 1990). For dye loading, hair cells were incubated for 30 min at room temperature (22–25 °C) with Fluo-3/AM diluted in HBSS to a final concentration of 2μM. The cells were maintained in a 50 μl droplet of HBSS on glass cover slips (thickness, 0.13 mm) in a saturated humid chamber (to prevent evaporation and changes in osmolarity of the medium) and subsequently carefully rinsed with HBSS.

Cells loaded with Fluo-3 were observed with an inverted microscope (Leitz Fluovert) fitted with an epifluorescence system (ArcLamp HBO 100 W; a 450–490 nm band pass exciter filter, 510 nm dichroic mirror
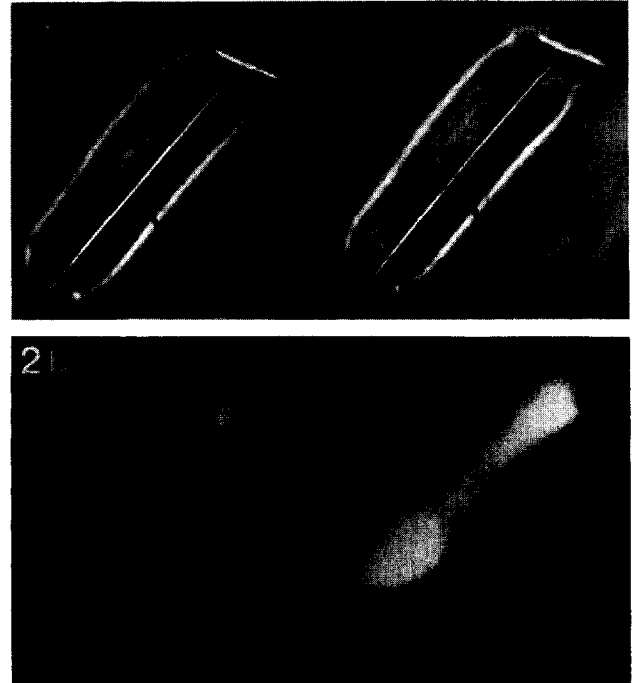

Fig. 2. Calcium-induced motility. Isolated OHCs from the apical coils of 55 gd (a) and 56 gd (b) guinea-pig cochleae. The initial stage (left) is taken after Fluo-3 dye loading. The elongated stage (right), after 30 sec application of ionomycin (10 μM). (a): The cell elongation (46.4 to 48.8 μm); (b): the strong Ca^{2+} increase accompanying the cell elongation is observed with epifluorescence microscopy.

and a 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 via a Silicon Intensified Target video camera (SIT 66, Dage-MTI Inc., Michigan City, IN) and recorded for analysis on a video cassette recorder (Mitsubishi SuperVHS, model HS-423UR). The recorded images were analyzed with a Quantex QX-7-210 image processing system (Quantex Corp., Sunnyvale, CA). Cell length was measured from digitalized bright field microscopy images before and after the addition of the ionophore. An increase in intracellular calcium had to be observed for a cell to be included in the length measurements.

Transmission (TEM) and scanning (SEM) electron microscopy

Fetuses were perfused (10 min, intracardiac) with buffered glutaraldehyde (3%) and paraformaldehyde

TABLE II

MOTILE RESPONSES OF OHCs ISOLATED FROM GUINEA-PIG FETUSES

Age (gd) of fetuses	Electrically-induced responses (positive/total cells)		Ca^{2+}-induced responses (positive/total cells)	
	base	apex	base	apex
52–53	6/9	0/5	5/7	1/4
55–56	4/5	2/5	11/11	3/9
60–64	all tested cells were positive		all tested cells were positive	
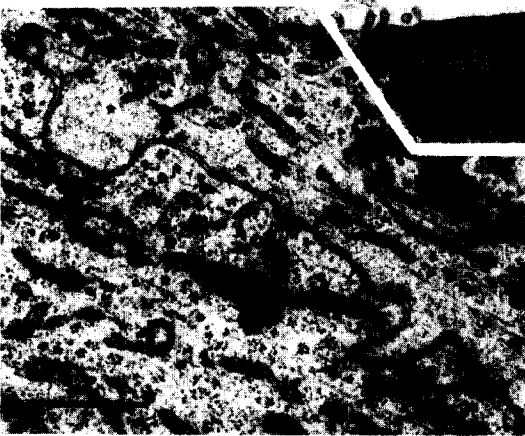
(2%). The cochleae were dissected out and kept in the same fixative for 1 h, postfixed with osmium (2% in phosphate buffer), and routinely processed. For TEM, ultra-thin sections were observed using a Jeol 2000 or a Jeol 1200EX electron microscope. At each developmental stage, sections taken randomly from at least 5 basal and 5 apical OHCs were examined. For SEM, specimens were critical point dried, coated with gold and analyzed using an Amray 1000B electron microscope.

Fluorescence histochemistry

Fetuses were perfused and fixed with 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The organ of Corti was immunolabeled with cytokeratin-specific antibodies (monoclonal anti-CK pan, Boehringer Mannheim Biochemicals) and double-labeled with phalloidin (Molecular Probes) to localize actin. The procedure for labeling was essentially as described by Raphael and Altschuler (1991). Segments (surface preparations) containing half-turns of the organ of Corti were mounted in Gelvatol 20/20 (Monsanto, St. Louis) and photographed using a Leitz Orthoplan microscope.

Results

Motilities of isolated OHCs (Table II)

Prior to 50 gd, OHCs whatever their location on the cochlear partition could barely be isolated under the standard procedures, even with prolonged incubation in collagenase medium. Dissociation became more successful with advancing gestational age. Cells from the basal turn of embryonic cochleae were easier to harvest than those from apical turns until gd 56. At that time,

an equal but still small number of well-preserved basal and apical OHCs could be obtained. In the adult guinea-pig, typically 200–300 cells can be isolated and the yield of apical OHCs far exceeds that of basal OHCs. Beginning at 50 gd, cells responded to applied ionomycin with an increase in intracellular calcium but a motile response was not observed.

At 52–53 gd, the electrically-driven fast motility was tested on 14 healthy well dissociated OHCs. Among the basal cells, 6 out of 9 followed the stimulation for several cycles by a contraction-elongation sequence (Fig. 1); they were considered as positive cells. Conversely, none of the 5 apical OHCs tested exhibited a stimulation-following response. However, some of them showed a single and strong contraction, before dying, when the applied current was increased to 1 nA. The Ca^{2+}-induced slow motility was tested on 11 isolated OHCs. Five out of seven OHCs from the basal coil elongated within seconds when applying ionomycin, simultaneously they showed a strong fluorescence increase indicating an increase in their cytoplasmic free calcium. The other 2 cells did not show any response. Only 1 out of 4 apical OHCs tested had a positive motile response with ionomycin. Noteworthy, at this developmental stage, all OHCs either basal or apical are small (20 to 30 μm in length): the elongation amplitude was 4 to 5% of the initial length, as found in adult preparations (Dulon et al., 1990).

At 55–56 gd, the proportion of positive responses increased in both types of experiments. The electrically-driven motility was observed on 4 out of 5 basal, and on 2 out of 5 apical OHCs. The Ca^{2+} induced elongation was observed on all of the 11 basal OHCs tested, and on 3 out of 9 apical OHCs (Fig. 2). On positive cells, the elongation amplitude was again in the order of 4 to 5% of the length.

Figs. 3–6. 37 gd guinea pigs.

Fig. 3. Transverse section at the base of the organ of Corti. OHCs (O) are short (20 μm) with a big nucleus. A large number of mitochondria are seen in the supra-nuclear region. A small cuticular plate is just forming; it supports both microvilli and short stereocilia. Deiters cells (D) are small, cuboidal with a thin phalangeal process which separates OHCs from the different rows. Scale bar = 5 μm.

Fig. 4. Enlargement from the apical pole of the row 1 OHC. On this serial section, the beginning of formation of the cuticular plate is better seen. Note the kinocilium (arrow) on the right. Scale bar = 1 μm.

Fig. 5. More apically, lateral membranes from two adjacent OHCs (rows 1 and 2) are often in direct contact, especially at the nuclear level. Scale bar = 0.5 μm.

Fig. 6. Cytoplasmic indentations between an OHC (O) and its neighbouring Deiters cell (D). Scale bar = 1 μm.

Figs. 7–8. 53 gd guinea pigs.

Fig. 7. The cuticular plate of an OHC is now thicker and almost devoid of microvilli (compare with Fig. 4). Scale bar = 1 μm.

Fig. 8. Below the cuticular plate of an OHC (O), the smooth endoplasmic reticulum is abundant and sometimes it begins to get aligned (arrowheads) along the lateral membrane. D: Deiters cell. Scale bar = 0.5 μm.

At 64 gd, just prior to birth, all tested cells in both basal and apical coils responded positively, either with electrical or ionomycin stimulations.

Morphological correlates

With regard to the above physiological results, three periods of development were particularly interesting to study: prior to 50 gd, when no motile responses were observed; at 52–53 gd, when motile responses appeared in some of the basal OHCs; at 56 gd, when almost all basal and some apical OHCs responded. In all cases, the fine morphology of developing OHCs, particularly the structures which are concerned with the motility, was compared to what is found in mature OHCs.

Prior to 50 gd. At 37 gd, whatever their position on the cochlear spiral, OHCs were clearly immature. Short (about 20 μm), round-shaped, with a basal nucleus, they were closely packed within the supporting elements, leaving no intercellular fluid space (Fig. 3). The supra-nuclear region of the OHC was rich in mitochondria and a thin cuticular plate was beginning to form, supporting small growing stereocilia and a kinocilium (Fig. 4). The OHC lateral membrane showed no specialization of any sort. The Deiters cells were small, cuboidal and had a thin phalangeal process separating the different rows of OHCs and reaching the luminal surface (Fig. 3). In the most apical and immature regions of the cochlea, this Deiters cell phalangeal process did not completely separate the different rows of OHCs, leaving two adjacent hair cells closely apposed by their lateral membrane, especially in the nuclear region (Fig. 5). Frequently, cytoplasmic indentations were seen tightly attaching hair cell and supporting cell (Fig. 6); desmosomes were also present in the OHC-supporting cell junctions, close to the luminal surface. These features probably accounted for most of the difficulty to isolate OHCs at this developmental stage.

Very few morphological changes were noticed on most of OHCs from 47 gd fetuses. However, in the basal turn, OHCs exhibited two criteria of maturation: 1), the cuticular plate was thicker and almost devoid of small microvilli (Fig. 7) and 2), in the upper region of the lateral plasma membrane, a first sign of formation of a laminated cistern was noticed (Fig. 8).

52–53 gd. As indicated by the gross morphology, the organ of Corti was now entering the last stage of its maturation: i.e., opening of the tunnel and formation of the Nuel's spaces between OHCs. This process was more advanced at the base than at the apex of the cochlea. A basal OHC was now 20 to 35 μm in length (Fig. 9). Its cuticular plate had developed and the roots of stereocilia were visible (Fig. 10); however the kinocilium was still present on the nearby apical surface. The most striking feature concerned the increase in density of mitochondria and of smooth endoplasmic reticulum in the supra-nuclear region, below the cuticular plate (Figs. 9,11). Round-shaped clusters of endoplasmic reticulum were observed closely associated with portions of the reticulum near the lateral plasma membrane (Figs. 9,11,12). Lining the lateral wall of the OHC, the laminated cisternae complex has been formed, with a first layer now almost completed. At this stage, the distance between the plasma membrane and the first layer of cisternae was irregular and greater than in adult specimens; neither pillars nor sub-plasma membrane could be clearly discerned in between. The formation of the laminated cistern proceeded from the luminal surface to the synaptic pole of the OHC. In a given cell, at this stage, the process is much more advanced apically (Fig. 12) than at nuclear level (Fig. 13) where the first layer is not continuous.

SEM analysis revealed a surface maturation of the guinea-pig organ of Corti which mimics what has been described for the rat pup (Lenoir et al., 1987). Therefore, results are not shown but for stage 52 gd, when the OHC motility begins. At this stage, specimens revealed the base to apex gradient of maturation in the surface of the organ of Corti (Figs. 14a,b). Apical OHCs were irregularly arranged, especially those of second and third rows (Fig. 14a). In contrast, OHCs at

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Figs. 9–13. 53 gd guinea pigs.

Fig. 9. Basal OHC at the onset of in vitro motilities. As compared with Fig. 3 (37 gd), the OHC is taller (27 μm) and more cylindrical in shape. The cuticular plate and stereocilia are about mature (see also Fig. 10). In the supra-nuclear region, the cytoplasm contains an exhuberant endoplasmic reticulum, sometimes forming round-shaped clusters (see also Fig. 11). A first layer of laminated cisternae is formed (best seen in Fig. 12). Scale bar = 1 μm.

Fig. 10. OHC stereocilia: note the staircase arrangement and the roots deeply implanted into the cuticular plate. Scale bar = 1 μm.

Fig. 11. Clusters of endoplasmic reticulum in the supra-nuclear cytoplasm of an OHC. Note the relationships between this reticulum (arrows) and the first layer of laminated cisternae (arrowheads). Scale bar = 1 μm.

Fig. 12. Regular alignment of the first cistern (arrowheads) along the plasma membrane in the apical region of an OHC. Scale bar = 1 μm.

Fig. 13. Irregular and incomplete cistern (arrows) in the nuclear region of an OHC. Scale bar = 0.5 μm.
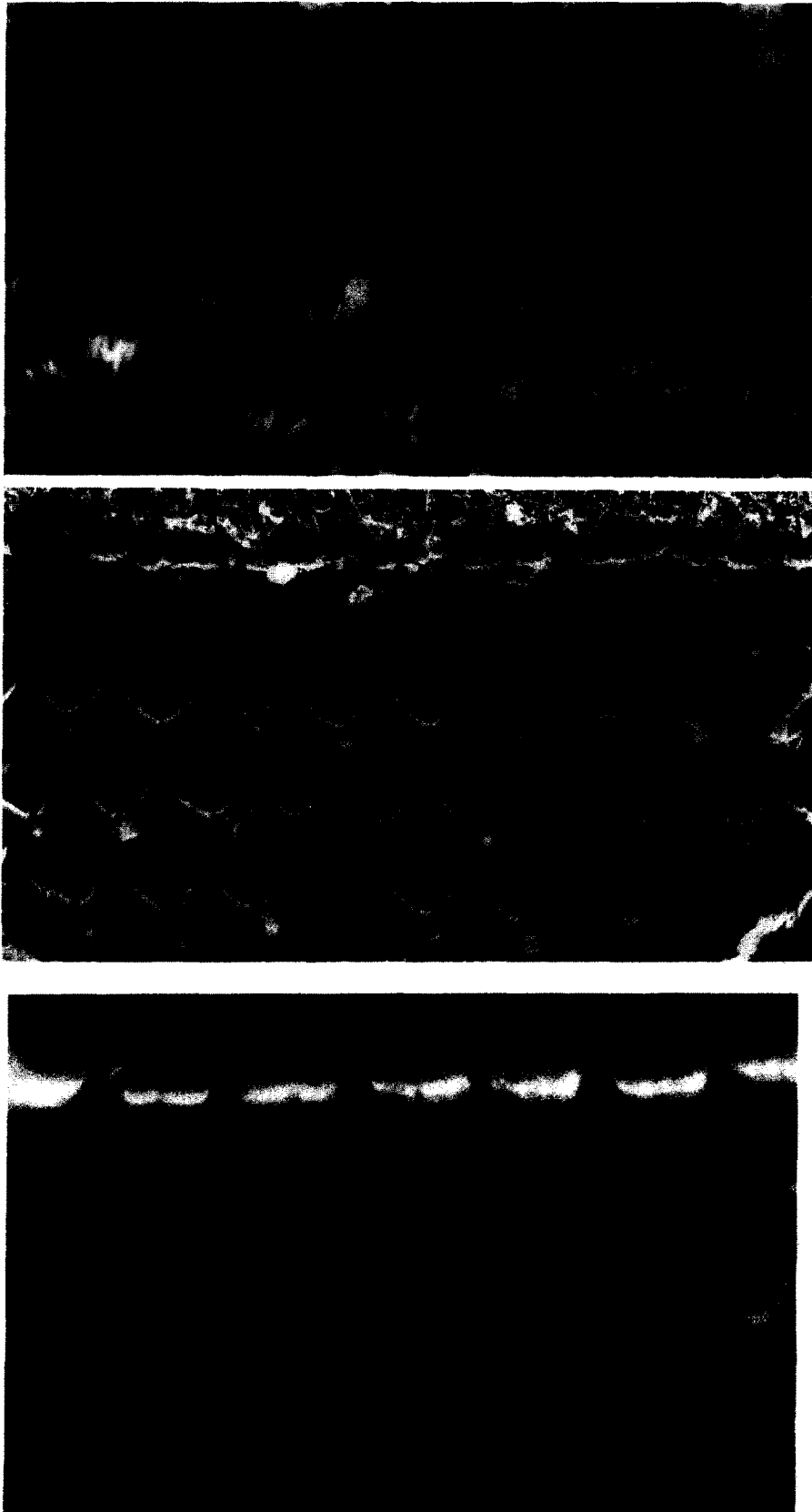
Fig. 14. Apical (A) and basal (B) scanning electron microscopic views from the surface of a 52 gd organ of Corti (at time of onset of motility in base). Note the base-to-apex gradient of maturation: mainly characterized by the more regular pattern of hair cell arrangement in B.

Fig. 15. Basal region of the cochlea from a 52 gd guinea-pig (onset of in vitro motilities). The distribution of actin showed with fluorescein phalloidin restricted in the reticular lamina, and stereocilia bundle is almost mature.

the base of the cochlea were regularly arranged and their surface appeared mature apart from the presence of a kinocilium (Fig. 14b). In both cases, pillar cells and Deiters cells were covered with numerous small microvilli (Figs. 14a,b). The distribution of actin in the apical domain of the OHC was similar to the mature stage, with intense labeling in the cuticular plate and stereocilia (Fig.15). Conversely, from 50 gd on, cytokeratin immunoreactivity was no longer detected in hair cells, but only in supporting cells.

56 gd. Within the next few days, the maturational processes reported above have quickly progressed. At 56 gd, the tunnel of Corti was completely open and Nuel's spaces had an adult-like appearance. Basal OHCs had reached their definitive length: (40 μm, in the upper basal turn, Fig. 16). Their cuticular plate had now a mature appearance and stereocilia also bore adult characteristics: a staircase arrangement, cross-links, tip-links and roots deeply implanted in the electron-dense material of the cuticular plate (Fig. 17). Within the supra-nuclear region of the cytoplasm, the intense production of reticular membranes was still obvious (Fig. 16). On the opposite pole of the OHC, another indication for maturity was the appearance of dense intercellular material at the basal junction between the OHC and the Deiters cell. On the Deiters side of that junction numerous microtubules were noticed (Fig. 18). The laminated cisternae complex was composed of a first layer very regularly apposed to the plasma membrane. This first layer extended now from the cuticular plate down to the synaptic pole of the OHC. Second or third layers were irregularly scattered in several locations (Figs. 19, 20). Between the plasma membrane and the first layer of cisternae, the sub-plasma membrane material such as pillars were distinguishable (Fig. 20). OHCs from apical turns had also matured, approaching an adult length (60 to 70 μm). Their cuticular plate, stereocilia, and lateral membrane were very similar to basal OHCs at this stage.

60–64 gd. The organ of Corti had largely completed its development, as judged by gross morphology and ultrastructure. OHCs and supporting Deiters cells showed an adult-like ultrastructural aspect.

Discussion

OHC motile responses: Fetal versus adult data

The difficulty in isolating OHCs from the youngest fetuses used in this study is due to the overall immaturity of the organ of Corti, as determined by TEM, SEM and molecular analysis of the cytoskeleton. The lack of extracellular fluid spaces, and the extensive junctions OHCs have with the surrounding cells at this stage, are particularly significant in that respect. Once OHCs

were isolated from fetal cochleae (around 50 gd), they could be loaded with the fluorescent calcium indicator Fluo-3 by incubation with its membrane permeant ester, Fluo-3/AM. This indicated the presence of active intracellular esterases and the integrity of the plasma membrane of the fetal isolated hair cells, as previously described for mature OHC (Dulon et al., 1989, 1990).

Prior to 52 gd, all hair cells (basal and apical) did not show any sign of cortical contraction (elongation), when increasing their $[Ca^{2+}]i$ under ionomycin stimulation. This indicated that at this stage of development the OHCs do not possess a functional contractile apparatus. In some aspect, they behaved more like IHCs, which also fail to respond to calcium stimulation (Dulon et al., 1991). The appearance of the Ca^{2+}-induced motility under ionomycin stimulation occurred first in basal, then a few days later in apical OHCs. An obvious potential criticism arises, as most of basal OHCs from 52–53 gd fetal cochleae are positive, while no clear response was noticed at earlier stages when isolation was difficult. Prior to 52 gd, some OHCs might have responded positively if we could have isolated more of them. On the contrary, the date of appearance of the Ca^{2+} driven motility in apical OHCs is less ambiguous, as only a third of OHCs are positive at 55–56 gd, a time when isolating hair cells is no longer a problem. Once present, the amplitude and time course of these cortical contractions (elongations) were rather similar to those observed previously in mature OHCs (Dulon et al., 1990).

Responses to electrical stimulation followed the same pattern of appearance of the Ca^{2+}-contractions. They appeared at 52–53 gd for basal OHCs and at 55–56 gd for apical OHCs. Although the OHC ability to follow current stimulation at high frequencies was not tested, these electrically-induced motile responses might reflect the first appearance, during development, of the cochlear amplifier (Ashmore, 1987; Zenner et al., 1987).

Development of motile responses as part of the overall cochlear maturation

Our results indicate that a critical stage of the OHC development occurs in the guinea-pig cochlea between 50 and 60 days of pregnancy. This is the period when isolated OHCs begin to show and develop two types of motile responses, an electrically-driven and a Ca^{2+}-induced response. In the meantime, the morphology of OHCs changes drastically as these cells acquire ultrastructural and immunocytochemical adult-like characteristics.

Despite the common use of the guinea-pig for experimental studies of the cochlear physiology, few data are available concerning the cochlear development in this species. However, a general schedule of the matu-

ration can be proposed. The onset of cochlear function occurs around 52–53 gd, as indicated by recording gross cochlear potentials (Romand, 1971; Romand et al., 1971; Pujol and Hilding, 1982). The main events of structural maturation and synaptogenesis have also been reported to occur between 45 gd and birth, (Thorn, 1975; Pujol and Hilding, 1982). At birth, a mature cochlea is found. It is of interest to correlate
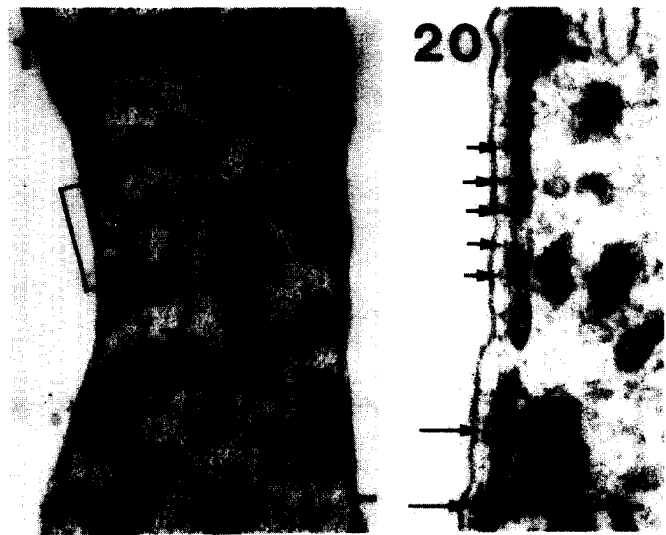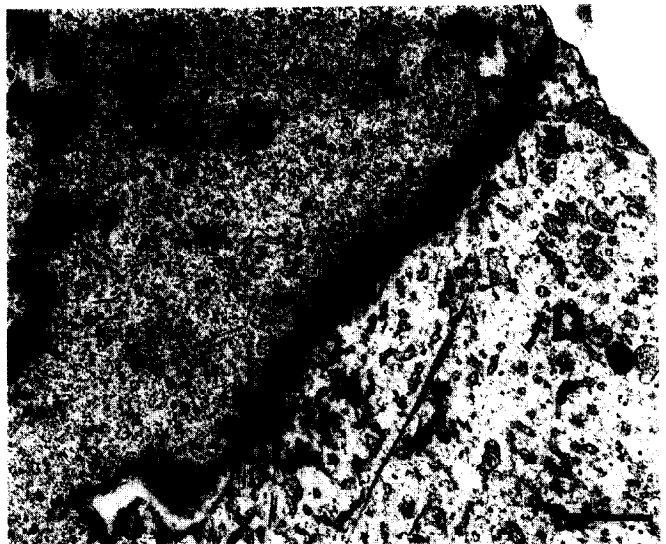
the maturation of OHCs motilities with that of tuning curves or oto-acoustic emissions, features which are thought to be associated with the active bio-mechanism. In the absence of data from guinea-pig, correlations can be made using the rat for which such data have been published (Carlier et al., 1979; Puel and Uziel, 1987; Lenoir and Puel, 1987; Norton et al., 1991). Thus, taking into account the available developmental criteria, is it possible to consider that a guinea-pig at 52 gd (at the time of onset of cochlear function) corresponds to a 10-day-old rat pup, and a guinea-pig at birth corresponds to a 20- to 25-day-old rat pup (see also the comparative Table in Pujol and Hilding, 1973). At this later stage in the rat, both the compound action potential tuning curves (Puel and Uziel, 1987) and the distortion product emissions (Lenoir and Puel, 1987) are almost mature, and this is also probably true in the newborn guinea-pig. This correlates quite well with our finding of adult-like motile properties of OHCs isolated from guinea-pigs at 60 gd and over.

It is noteworthy that the appearance and development of OHC motilities in vitro follows a base-to-apex gradient similar to maturation in vivo. This, together with the good timing coincidence discussed above, strongly supports the idea that motile properties of OHCs observed in vitro reflect the in vivo active mechanisms.

Structural corrrelates

Physiological and anatomical studies were performed on separated littermates and, obviously, not on the same cells. Then, even if particular attention was made to work on littermates having the same gross developmental aspects (same weight and size), and to select OHCs from similar levels of the cochlea, one cannot exclude differences in the maturation of fetuses and/or differences in the maturation of OHCs belonging to slightly different levels of the cochlea.

During the early stages (37–47 gd) the immaturity of the organ of Corti as a whole is obvious,: hair cells and supporting cells being densely packaged, with no fluid spaces in between. Moreover, the difficulty of separat-

ing the OHCs probably increases because of the particularly tight OHC-Deiters cell junction at this stage. Especially, close to the luminal surface both the OHCs and the Deiters cells are tightly linked by desmosomes and cytoplasmic indentations. These features regress and totally disappear by 50 gd. In the meantime, spaces of Nuel are forming: they are clearly differentiated at 52 dp, a stage when OHC isolation becomes much easier. Prior to the onset of motility, OHCs acquire an adult-like distribution of microfilaments and intermediate filaments. The finding that cytokeratin-specific immunoreactivity disappeared from OHCs at this stage is consistent with earlier results on down regulation during development (Raphael et al., 1987) and may suggest that the presence of these rigid and stable proteins is incompatible with motility, and related functions of OHCs. Actin is present in conspicuous amount in stereocilia, cuticular plate and adherens junctions of mature OHCs; it may also be present in small quantities in association to the lateral wall of OHCs (Thorne et al., 1987; Slepecky et al., 1988). Actin starts to accumulate in the cuticular plate and stereocilia more than a week prior to the onset of motility, but further studies are needed to conclusively determine its distribution along the lateral wall of developing OHCs, and its relation to the development of the calcium-induced motility.

The onset of motility in isolated guinea-pig OHCs (52 to 56 gd) coincides, at gross morphological level, with an increase in the length of the cell, which also attains its size (length and diameter) and its very regular cylindrical shape. However, the most prominent ultrastructural OHC changes are at the level of the subsurface cisternae complex. Namely, a first layer of laminated cisternae is differentiated, although it does not bear yet its mature appearance. Incidently, our results point out the strong relationship existing between the clusters of endoplasmic reticulum, strikingly abundant at this stage in the supra-nuclear region of the cytoplasm, and the cisternae (see also, Thorn, 1975). Such an abundance of smooth endoplasmic reticulum has already been found in OHCs under

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Figs. 16–20. 56 gd guinea pigs.

Fig. 16. Upper basal OHC showing an almost mature morphology, except for its lateral contact with phalangeal processes of Deiters cell (D), as spaces of Nuel (stars) are not completely formed. Scale bar = 2 µm.

Fig. 17. Cuticular plate from a basal OHC. Mature appearance of the stereocilia with cross (arrowheads) and tips (thin arrows) links. Scale bar = 1 µm.

Fig. 18. Basal junction between an OHC (O) and a Deiters cell (D). Note the dense material at the junction and microtubules (arrows) on the Deiters cell. Scale bar = 1 µm.

Fig. 19–20. Basal OHC: supranuclear region. The laminated cisternae complex is now well formed, with sometimes a second and third layer (arrowheads). Between the first layer and the plasma membrane, pillars (short arrows) and other filamentous material (tall arrows) are clearly seen. Scale bar = 0.2 µm.

different pathologic situations, such as acoustic trauma where the clusters have been called Hensen bodies (Spoendlin, 1970; Dodson et al., 1982) or lamellar bodies (Saito, 1983). A possible explanation for finding these formations in pathological cases would be a restoration process of an injured cisternae complex.

In adults, the laminated, or subsurface cisternae complex (Engström, 1967; Saito, 1983) may have a role in both 'fast' and 'slow' motile responses. The fast motility either conceived as an electro-osmotic (Kachar et al., 1986), an electrostrictive (Ashmore, 1987), or a displacement generator mechanism (Dallos et al., 1991) needs the linear arrangement of cisternae along the plasma membrane for the generation of longitudinal forces. Furthermore, recent observations suggest that the electrically-driven motility is indeed related to ultrastructural changes in cisternae (Evans, 1990). More precisely, the contractile apparatus is supposed to be located in the sub-plasma cortical system interposed between the plasma membrane and the first layer of cisternae (Holley and Ashmore, 1989; 1990). It has also been proposed that in slow motility the laminated cistenae could play a role in storing and releasing Ca^{2+}, as the T-tubules in muscles (Flock et al., 1986) acting on a cortical contractile apparatus associated with the cell membrane along the OHC lateral wall (Slepecky, 1989; Ulfendahl and Slepecky, 1988).

The cisternal complex, at onset of motility, corresponds to a regular alignment of one layer of cistern along the lateral plasma membrane, although this formation is not yet completely down to the base. This tends to indicate that if cisternae are involved in motility, it is not the number but only the first layer that is critical. A similar conclusion can be drawn by comparing, at adult stage, the differences in number of cisternae among OHCs from different species or different turns in a same species: there is no logical correlation between this number and the respective physiology of OHCs; this has been already suggested (Spoendlin, 1966) and discussed (Ekström von Lubitz, 1981). Actually, both the horseshoe bat (Vater et al., 1991) and the mole-rat (Raphael et al., 1991) OHCs have a single layer of cisternae: yet, the former with their specialization to ultra-high frequencies should have more powerful active mechanisms than the latter which is only concerned with low frequencies. If the cisternal complex is to account for the physiological differences presumed to be based upon OHC motilities, the critical feature is not the number of cisternae but more likely the organization of the first layer and attached lattice (Holley and Ashmore, 1990; Forge, 1991; Dieler et al., 1991). Moreover, as already proposed (Evans, 1990; Holley and Ashmore, 1990; Dallos et al., 1991), only the apparatus above the nucleus may be important for motility.

The onset of motility also coincided with the forma-

tion of the arms (Raphael and Wroblewski, 1986) or pillars (Flock et al., 1986; Bannister et al., 1988; Lim et al., 1989; Arnold and Anniko, 1990), stuctures which link the plasma membrane to the outermost layer of cisternae. More precisely the pillars seem to be attached to a fibrillar meshwork on the outer surface of the first cisternal membrane (Forge, 1991). This sub-plasmalemmal filamentous material (among others: Gulley and Reese, 1977; Saito, 1983; Lim, 1986, Ulfendahl and Slepecky, 1988) is thought to be composed of the cortical lattice (Holley and Ashmore, 90) and is probably involved in the two types of motility (Slepecky, 1989; Holley and Ashmore, 1990; Dulon et al., 1990). The formation and development of this lattice and pillars, probably account for setting the motile responses of fetal OHCs at an adult-like appearance. However, the present data limited to classical TEM do not bring enough information on this point and further analyses using freeze-fracture or freeze-etching (Forge, 1991) are needed.

Acknowledgements

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