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THREE DIMENSIONAL INTRACELLULAR STRUCTURE OF THE COCHLEA USING THE A-O-D-O METHOD

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Abstract

We have observed the three-dimensional intracellular structure of the organ of Corti and the spiral ganglion of the chinchilla using the aldehyde osmium-DMSO-osmium (A-O-D-O) method, and compared our findings with previous studies in the guinea pig. In cells of the spiral ganglion and the organ of Corti, microtubules and other cytoplasmic filaments were digested by OsO_4 during processing to provide a clear view of membranous structures such as mitochondria, endoplasmic reticulum and Golgi apparatus. The appropriate digestion time with 0.1% OsO_4 solution was found to be 60-80 hrs for the organ of Corti and 70-90 hrs for the spiral ganglion. The A-O-D-O method has been especially useful to show the organization of the endoplasmic reticulum of the outer hair cells. We have made a further characterization of the apical cistern, a reticular network which appears to link the basal body of the (vestigial) kinocilium to both Hensen's body and the subsurface cisternae.

KEY WORDS: Sensory organ, inner ear, cochlea, spiral ganglion, organ of Corti, guinea pig, chinchilla, Aldehyde-Osmium-DMSO-Osmium method, intracellular organelle, endoplasmic reticulum

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Introduction

Many ultrastructural studies of the organ of Corti and the spiral ganglion have been made in various animal species using both scanning (SEM) and transmission electron microscopy (TEM). Intracellular structures had been observed mainly by TEM, (e.g. Engström and Wersäll, 1953a,1953b; Hunter-Duvar and Mount, 1978; Thomsen, 1967) while surface structures are best viewed with SEM (e.g. Lim et al., 1969; Hunter-Duvar, 1978). Some attempts had been made to observe intracellular structure of the cochlea using conventional SEM (Bredberg et al., 1970; Lim, 1971), but authors report that the cytoplasmic matrices hide or make it difficult to observe intracellular organelles and other structures. However, the recent development of high resolution SEM and new preparation techniques enabled us to reveal and describe in detail various intracellular structures, such as mitochondria, endoplasmic reticulum (ER) and the Golgi apparatus (Tanaka and Naguro, 1981; Tanaka and Mitsushima, 1984; Lim, 1986; Lim et al., 1989).

We have been using such SEM methods to demonstrate the normal stereoscopic intracellular structure of inner ear for over six years (Harada et al., 1985, 1986, 1989, 1990). In the present study we have specifically observed the chinchilla cochlea using the A-O-D-O method, and compared findings with previous observations made in the guinea pig.

Materials and Methods

Young chinchillas weighing 350-450g were used.The specimens were prepared by the A-O-D-O method (Tanaka and Naguro, 1981; Tanaka and Mitsushima, 1984) as follows. With the animal under deep anesthesia (sodium pentobarbital i.p.), the cochleas were prefixed (by cardiac perfusion) with a 0.5% glutaraldehyde and 0.5% paraformaldehyde mixture buffered at pH 7.4 with 0.1M PO_4. After rapid removal of both temporal bones, the round window and the oval window of each cochlea was perforated and the perilymphatic space gently perfused for two minutes with the same fixative. The temporal bones were then immersed in the fixative for two hours.

Following primary fixation, the specimens were rinsed with cold 0.1 M PO_4 buffer and postfixed at 4°C for 30 minutes with 1% OsO_4. During rinsing in buffer, the otic capsules were thinned until the spiral ligaments could be seen evenly. The cochleas were immersed in 25% dimethyl sulfoxide (DMSO) for 30 minutes and then in 50% DMSO for one hour; to ensure good embedding, we perfused the perilymphatic space with DMSO. They were then frozen in liquid nitrogen and cracked with a hammer and chisel. After thawing, they were well rinsed in the buffer solution, and transferred to buffered 0.1% OsO_4 at 20°C for 50-90 hours for removal of the cytoplasmic matrix. Routine conductive staining and dehydration in ethanol was carried out. After treatment with isoamyl acetate, specimens were critical-point dried (liquid CO_2) and sputter coated with platinum. The HITACHI S-570 scanning electron microscope was used for our observations.

Results

Spiral ganglion of the chinchilla.

An example of the A-O-D-O prepared spiral ganglion cell region is shown in figure 1. Here we see only type I ganglion cells. Detail of the membranous structure of such a type I cell is shown in figure 2. The mitochondria appear mainly rod shaped and have a typical lamellar structure to their cristae. The endoplasmic reticulum (ER) pervades the whole cell, linking together to form a mesh-like structure. An example of an unmyelinated type II cell could not be found in the specimens of the present study; they have low numbers in the chinchilla spiral ganglion (Ruggero et al., 1982).

Cochlear hair cells in the chinchilla.

Figures 3 and 4 show internal views of the upper portions of outer hair cells (and some supporting cells) after a fracture perpendicular to the cells' axes. Clearly seen in this upper portion of the outer hair cell (OHC), just beneath the cuticular plate, are a number of mitochondria and the fine tubular structure of ER. As most clearly shown in figure 7, the mitochondria are small and spherical, mostly 300-400 nm in diameter.

The fine structure of the subsurface cisternae (SSC) is well demonstrated in figures 3 and 4. The SSC is closely associated with the hair cell plasma membrane and consists of interconnecting tubular ER. From figure 3, it appears that the SSC in the first row OHCs is thicker than that of the second row. In the first row OHCs there are 8-12 layers making up the SSC compared with 4-7 layers in cells of other rows. However, this apparent difference is possibly due to the different levels at which the hair cells have been fractured. Because SSC increases in thickness away from the apical region of the cell, those cells fractured near to the apical area may appear to have a thinner SSC layer.

In figures 5 and 6, the OHCs have been fractured about their long axes. The relationship between the many components of the ER is clearly seen. Hensen's body, the apical cistern and the SSC appear to be a continuum. Note that digestion of the cuticular plate has left a space in the apical region of the hair cell. The SSC is not present in the apical area of the hair cell, but it appears and increases in thickness some way from the cell apex.

Hensen's body (the lamellar body) is very clearly imaged in figure 6. It consists of dilated cisternae of ER and has a generally spherical form. In our three-dimensional images we can trace the continuity between Hensen's body and the SSC.

Figures 5 and 6 illustrate the structure of the apical cistern very clearly. The cistern consists of a network of tubular ER. It arises from the area where the basal body (rudimentary kinocilium) existed, and it extends to Hensen's body and the SSC.

The supranuclear region of the OHC is shown in figure 8. The central portion has relatively few organelles which allows us to observe the inner surface of the SSC, and the many mitochondria attaching to it. These mitochondria are small and spherical, similar to those in the upper portion of the OHC.

A high magnification view of this infranuclear region is shown in figure 9. In this area the SSC thins, such that near to the synaptic region the cisternae are often absent. In the cytoplasm an aggregation of mitochondria (sometimes termed a Retzius' body) could be observed; these mitochondria were variously sized and shaped, in contrast with the more uniform and generally spherical form of those in the upper portion of OHC.

Nerve endings and fibers.

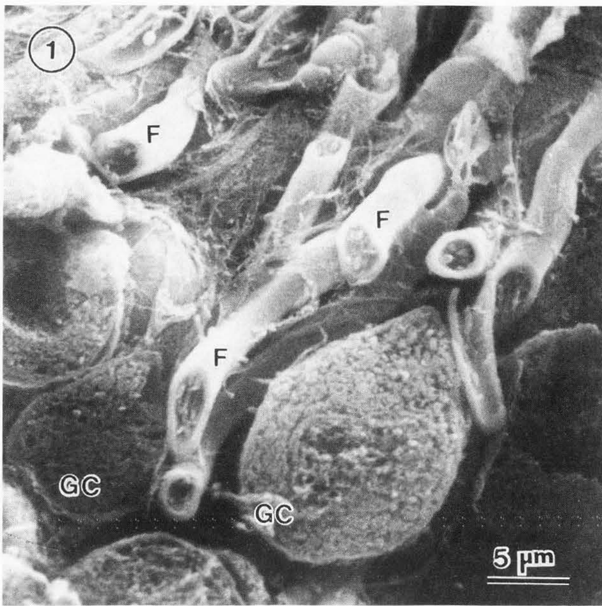
Figures 10-12 illustrate the nerve fibers in the vicinity of the OHCs and Deiter's cells. In figure 10, and the higher magnification view of figure 11, we see the basal fibers coursing between Deiter's cells and sometimes forming a dense entanglement in the synaptic area around the base of the OHCs. Figure 12 shows the more orderly arrangement of outer spiral fibers. In some cases nerve fibers appear to be very closely attached to the cell membrane of Deiter's cells.

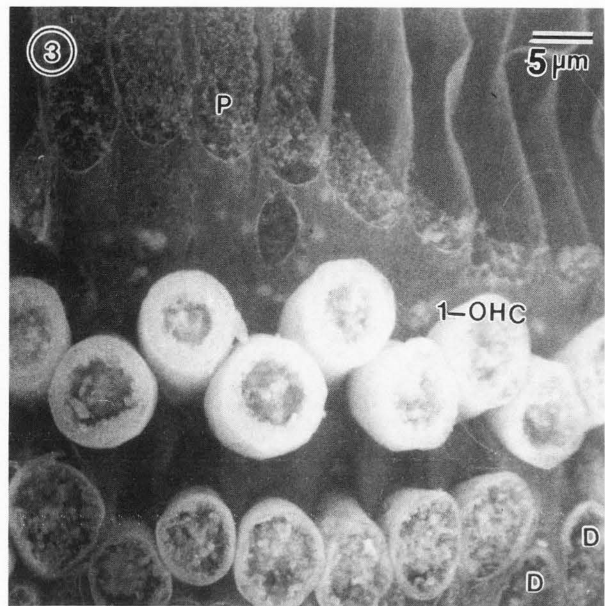
Discussion

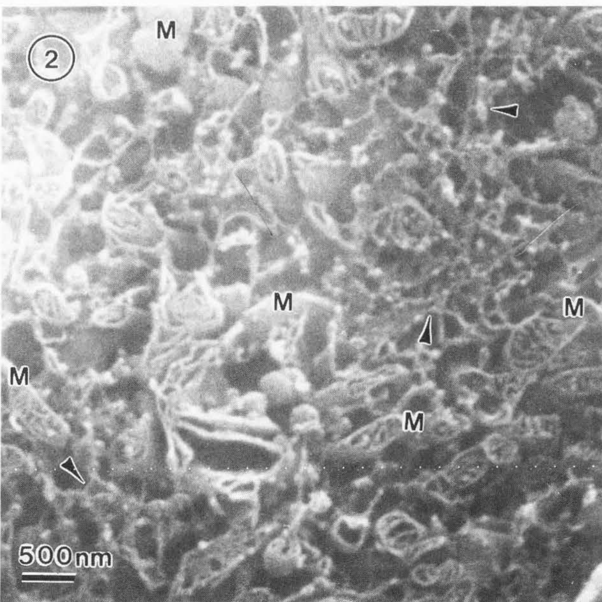
Detailed descriptions of the intracellular structure in the guinea pig cochlea have been made in our previous reports (Harada et al., 1987, 1989, 1990). In the present study we sought to compare those result with new findings in the chinchilla, specifically in respect to the spiral ganglion and cochlear hair cells.

Spiral ganglion

In both the guinea pig and the chinchilla cochlea, the majority of the spiral ganglion cells were myelinated type I cells, and rich in membranous intracellular organelles as described by Spoendlin (1972). The individual membranous organelles of the type I cell in the chinchilla are identical with those found in the guinea pig. Unmyelinated type II cells make up a much smaller population and are often reported as having fewer and less organized







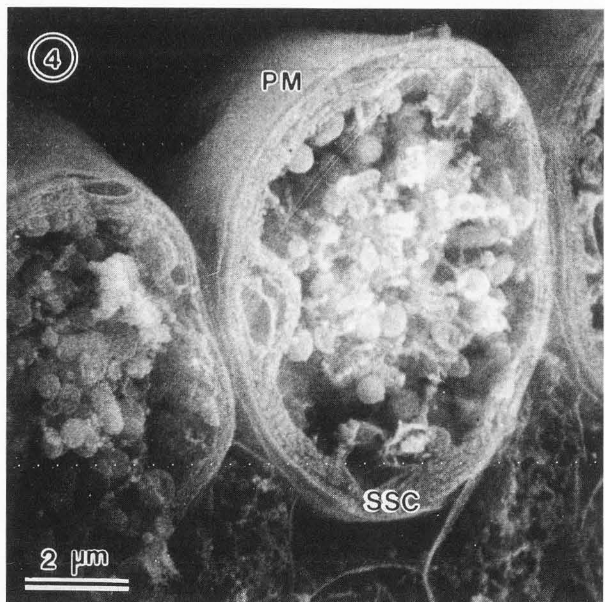
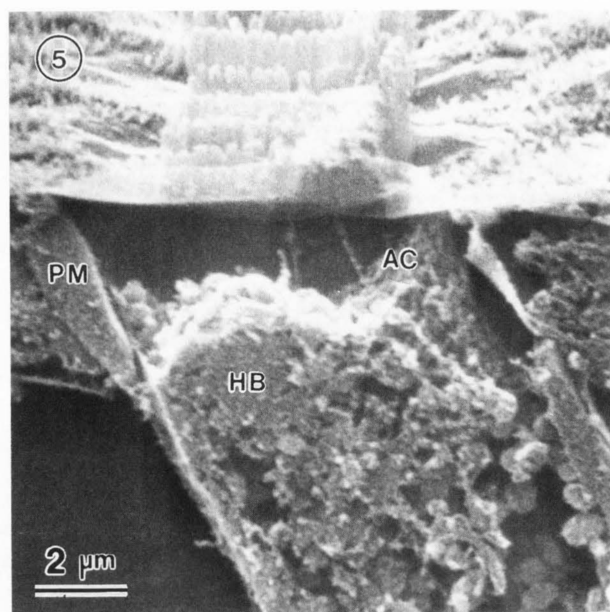


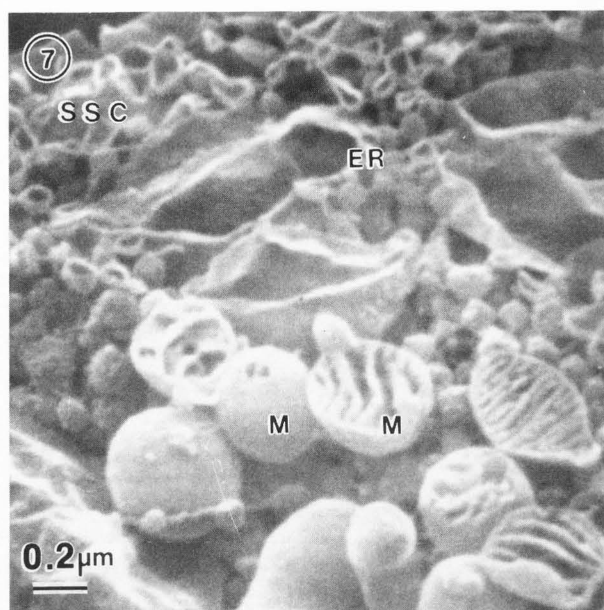
Figure 1. Ganglion cells bodies and nerve fibers of the spiral ganglion of the chinchilla. All of the cells seen in the figure are myelinated type I cells, and rich in cytoplasmic organelles. GC: ganglion cell, F: nerve fiber.

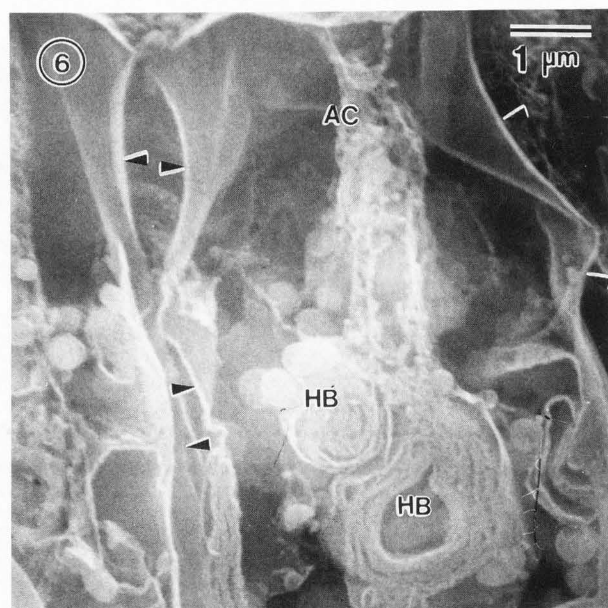
Figure 2. Intracellular view of the type I spiral gangion cell in the chinchilla. The three-dimensional form of a number of mitochondria (M). A fine tubular endoplasmic reticulum (arrowhead) runs throughout the cytoplasm.

Figure 3. View of the upper portion of outer hair cells after a horizontal fracture beneath the cuticular plate. 1-OHC: first row of outer hair cells, D: Deiter's cell, P: pillar cell.

Figure 4. A magnified view of figure 3, showing the upper portion of a second row outer hair cell, fractured just beneath the cuticular plate region. Note the several layers of the subsurface cistern (SSC), attached mitochondria and tubular endoplasmic reticulum. PM: plasma membrane.







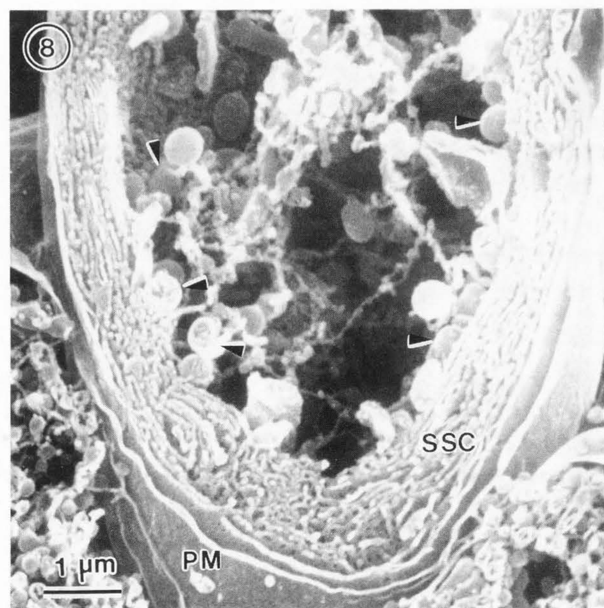
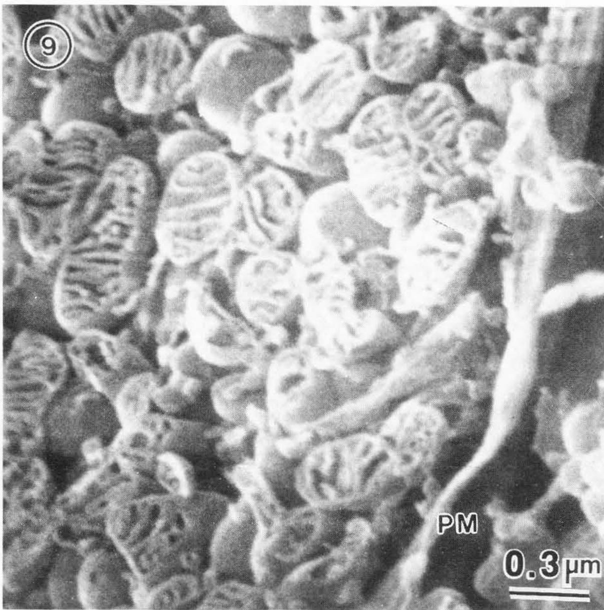


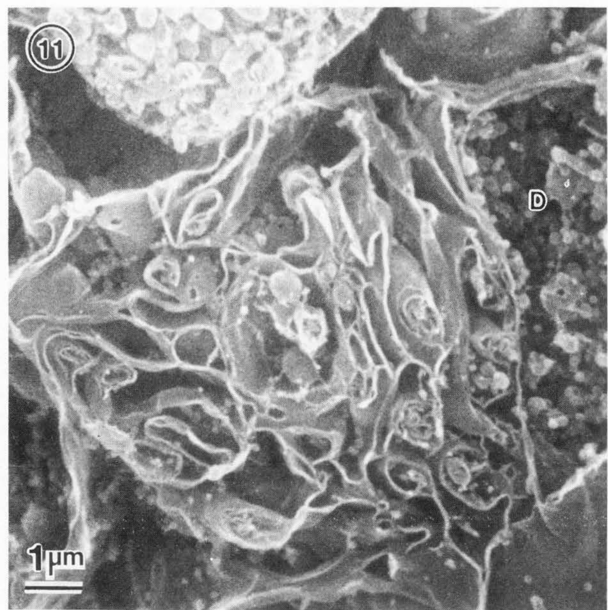
Figure 5. The upper portion of a second row outer hair cell fractured axially. Approximate structures of the apical cistern (AC) and Hensen's body (the lamellar body; HB) are seen, but the fine structure is not well revealed because of poor OsO4 digestion. However, the AC clearly arises from the area where the basal body existed, and extends to HB. PM: plasma membrane.

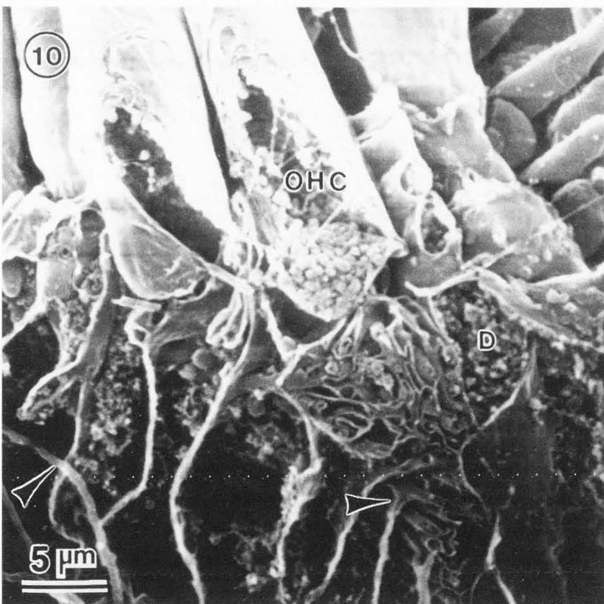
Figure 6. This micrograph demonstrates the continuity of ER network. The apical cistern (AC) consists of tubular ER; Hensen's body (HB) of lamellar ER surrounded by tubular ER network. Arrowheads indicate the plasma membranes of the hair cells.

Figure 7. High magnification of the upper portion of an outer hair cell just beneath the cuticular plate region. A number of intracellular organelles are clearly seen. In this region of the cell, mitochondria (M) are generally spherical in shape. ER: endoplasmic reticulum, SSC: subsurface cisternae.

Figure 8. The central portion of the outer hair cell. In this region, above the nucleus, the cytoplasm is not rich in organelles. Mitochondria (arrowhead) are attached the subsurface cistern (SSC). PM: plasma membrane.







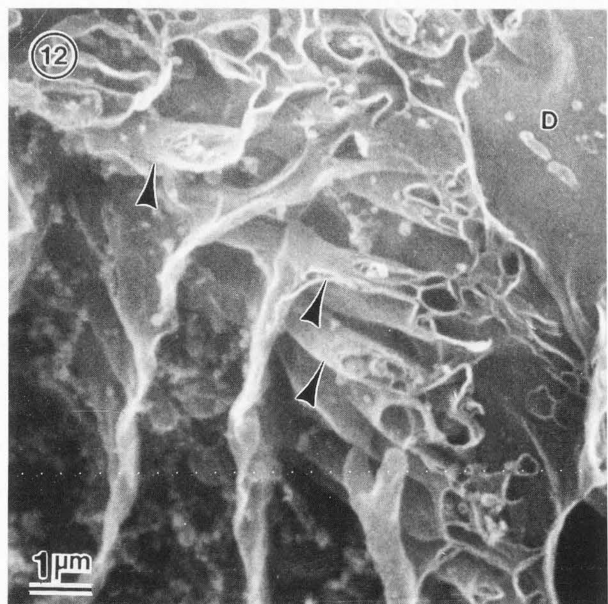


Figure 9. The basal portion of the outer hair cell. Here, in the infranuclear area, there are numerous mitochondria of differing shape and size. PM: plasma membrane.

Figure 10. View of the OHC region of the organ of Corti, fractured radially. The three dimensional structure around the base of the outer hair cell (OHC) and Deiter's cell (D) is shown. Note the basal fiber (arrowhead) running up to the base of the OHC, to join the outer spiral bundles. The outer spiral bundles entangle beneath the OHC in the region of the nerve endings.

Figure 11. Higher magnification of figure 10, showing the entanglement of the outer spiral bundle.

Figure 12. Higher magnification of figure 10, showing the outer spiral bundle (arrowhead), running beside the wall of a Deiter's cell.

intracellular organelles, and to have a more filamentous cytoplasm compared to type I cells. However, in previous studies in the guinea pig, using the digestion method (Harada at al., 1989,1990), the membranous structure of individual organelles in type II cells was found to be identical with that of type I cells. Unfortunately, in the present study, our specimens did not reveal type II cells, and thus we cannot confirm this in the chinchilla.

The detail and three dimensional structure of membranous organelles (mitochondria, ER and Golgi apparatus) of type I cells could be satisfactorily observed. On the other hand, for type II cells, the preservation of morphological appearance and integrity is poorer when OsO_4 digestion is used. This is because type II cells are so rich in microfilaments and microtubules, that their digestion leaves little support for the few remaining membranous organelles. In addition, type II cells have only a very thin myelin sheath. Such morphological characteristics often result in irregular shaped cell bodies and a loose distribution of the cytoplasmic organelles when OsO_4 digestion is used (Harada, 1989).

Cochlear hair cells

In this study we have focussed attention on the OHCs and in particular on their ER, mitochondria, and their innervation.

OHC endoplasmic reticulum. The subsurface cisternae (SSC) and lamellar bodies (Hensen's bodies) are structures which are very well revealed by the A-O-D-O method. They both consist of a complicated network of ER and somewhat dilated cisternae. The continuity between the ER of Hensen's body and the tubular ER of SSC is clearly demonstrated in the three dimensional perspective that this technique provides.

The SSC consists of tubular ER, forming layered meshwork. Gulley and Reese (1977) described, in the chinchilla, that the outermost layer consisted of a flattened cisternae, the inner layers being more tubular. In our observations in chinchilla, we find that the SSC consists mainly of linkages of fine tubular ER, and was less and only regionally lamellar. In general, we find the tubular ER in chinchilla to be finer than in the guinea pig. In the guinea pig, regional differences of SSC have been reported, specifically, the outer-most layer in the supra-nuclear region is flattened and in the infra-nuclear region it is tubular (Saito, 1983). In our present study in the chinchilla such differences were not seen, however, it is not clear how much influence the specimen fixation and preparation has on such observations.

Hensen's body (the lamellar body) is a whorl formation of lamellar ER (fig.6), and essentially similar in chinchilla and guinea pig. Differences in the absolute number of lamellae were not quantified because within animals there is much variation. For example, it has been reported that the number of lamellae the SSC and Hensen's body increase in traumatized ears, perhaps indicating a state of high or changed metabolic activity [Lim, 1986].

In the present study we have well revealed another ER system, the apical cistern. It forms cylindrically shaped network much like a spider's web or fishing net, connecting the basal body area to the ER of Hensen's body (fig.6).

In general our results support the concept, as described by others (e.g. Saito, 1983), that the ER of the OHC is essentially a single continuous system of tubules. Our observations confirm those of others that the endoplasmic reticulum of the hair cell wall is continuous with that of the apical region of the hair cell (i.e. the apical cistern). Recently attention has been given to the ability of hair cells to change their shape (Zenner et al., 1985; Brownell et al., 1985; Flock et al., 1986; Zenner, 1986). Implicated in this hair cell motility is a network of contractile proteins beneath the cell plasma membrane; this has been referred to as the cortical lattice (Holley, 1990; Holley, 1991; Holley and Ashmore, 1990). The contractile proteins involved are F. actin and possibly spectrin (Holly and Ashmore, 1990). In the hair cell transduction process modern theories propose a mechanical feedback process, wherein stimulated hair cells feed back mechanical energy to, in the case of the cochlea, the basilar membrane. This implies an activation of the motile mechanisms as a result of deflection of the stereocillia. It is therefore of some interest to speculate how ionic fluxes at the level of the stereocillia and the resultant depolarization or hyperpolarization of the cell, can rapidly affect mechanical changes to the hair cell wall. It is tempting to speculate that the apparently connected apical cistern and subsurface cisternae of the endoplasmic reticulum may have some role in distributing ionic charge, or perhaps a chemical messenger rapidly from the apical region of the cell to the cell walls.

Arima and his colleagues (1991) demonstrated the three dimensional ultrastructure of the lateral membranous system in guinea pig OHCs, and suggested that it played a role as part of the mechanical filter in active tuning movements within the cochlea (of normal hearing mammals).

OHC Mitochondria. Most mitochondria of the OHC in the guinea pig were elongate in shape and had laminated cristae. In the chinchilla we observe that they are generally more spherical and smaller. In the supranuclear region, they are closely coupled with the ER network, i.e. the apical cistern, Hensen's body and SSC; this is evidence of the high metabolic activity associated with the ER.

In the infranuclear region, mitochondria were aggregated at the bottom of the OHC, and here there was a much larger variety of size and shape to the mitochondria in comparison to those found in more apical regions of the cell. Lim and Flŏck (1985) suggested that variation in size may represent different metabolic states; "the larger ones are energetically charged and smaller are... exhausted".

OHC nerve endings and fibers. Both afferent and efferent nerve endings on the OHCs, have been well described from TEM observations. (e.g. Engstrom, 1958). In the present study in the chinchilla, and in the guinea pig, several nerve

endings could be observed at the bottom of each OHC. Abundant mitochondria were observed within some endings, perhaps indicating them to be efferent. However, we cannot be definite because the synaptic structures which characterize afferent and efferent nerve endings could not be clearly observed. Further study will be necessary to characterize the synaptic region.

Conventional SEM preparation techniques tend to show only Corti's tunnel (Hunter-Duvar, 1978; Harada, 1990). The three dimensional views offered by the A-O-D-O method allows the observation of fibers between the OHCs and Deiter's cells. In the present study in chinchilla, we have been able to visualize the outer spiral bundles and the basal fibers running up to the bases of the OHCs; these axons are usually hidden among Deiter's cells or the OHCs. We believe this technique will be useful for further study of the cochlea, particularly of pathological specimens.

Methodological considerations

Freeze cracking method. The main problem in this type of preparation is the method for cracking the cochlea. The organ of Corti is suspended in the peri/endolymphatic space, and the cochlea is completely covered by bone. Our method for successful cracking was as follows: To obtain good embedding in DMSO, we perfused it gently through the perilymphatic space with a pipette. For easier cracking the chinchilla cochlea was dissected so as to thin the bony otic capsule until the spiral ligament could be clearly seen. This dissection was carried out during post-fixation rinsing.

OsO_4 digestion. A number of hours of 0.1% OsO_4 immersion is required to remove the matrices of cytoplasm. The spiral ganglion requires a longer digestion time with OsO_4 than the organ of Corti because it has a more compact arrangement of elements. In our experience, optimum digestion time for the spiral ganglion is 70-90 hours; that for the organ of Corti is 60-80 hours.

Acknowledgements

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Discussion with Reviewers

B.A. Bohne: Does the A-O-D-O technique permit the examination of nerve fibers in the habenulae perforata and inner spiral bundle? What about nerve endings on the inner hair cell?
Authors: So far we have not been able to observe the habenular region or the nerve endings on inner hair cells because it is difficult to obtain a freeze fracture near to the edge of the osseous spiral lamina.

B.A. Bohne: On average, how far can nerve fibers within the outer spiral bundles be traced?
Authors: The outer spiral bundle cannot be traced far because the fractures in the exact plane of the bundle rarely extend for more than a few hair cell widths.

B.A. Bohne: What fraction of the organ of Corti from a particular cochlea is available for examination with this technique? If only a small fraction of the organ of Corti can be examined, this may limit the applicability of the A-O-D-O technique to pathological specimens. Several ototraumatic agents (noise, ionizing radiation, aging) produce spotty or focal damage within the organ of Corti rather than a generalized lesion.
Authors: Only a small proportion of the cochlea is revealed with this method. The whole cochlea is usually cracked into two large fragments along the cochlear axis. In some cases, lines of fracture are through Rosenthal's canal, or the spiral ligament/outer hair cell region. It is not a good method for assessing the condition of the whole cochlea.

N. Slepecky: Is the apical cistern (as viewed in Fig.6) a small structure composed of only one cistern, or is it composed of a layer of cisterns that underlies the entire cuticular plate?
Authors: It is not a layered structure but rather a network of tubular ER

N. Slepecky: You state that the three-dimensional view offered by the A-O-D-O method allows better observation of the nerve fibers between the OHCs and Deiter's cells than are offered by conventional SEM preparations. One would expect that in the absence of myelin sheaths, fibers would totally collapse when their axoplasmic matrix material (microfilaments and microtubules) has been digested with osmium tetroxide. How do your results with A-O-D-O compare with those seen in conventional SEM preparations by Bredberg (Bredberg, G. 1981: SEM studies of Corti's organ with special reference to its innervation. Biomedical Res 2: 403-413)?
Authors: A high concentration of OsO_4 (e.g. 1%) and/or a long maceration time can, of course, digest almost any membrane. However, in our study we used low concentration (0.1% OsO_4) and carefully controlled the maceration time such that nerve fiber membranes were preserved.

Y. Nakai: The shape and number of Hensen's bodies may be always changing. In this respect, is there any difference in each turn, or each row, or because of various conditions of the inner ear?
Authors: This is an interesting question. In the present study we did not quantity the characteristics of Hensen's bodies. This was largely because of the wide variation in size and number even within hair cells of the same animal. A very large sample size would be necessary for such a study.