Structural basis for mechanical transduction in the frog vestibular sensory apparatus: I. The otolithic membrane

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The mechanical coupling of the otoliths to the hair cell sensory stereocilia at the surface of the vestibular sensory epithelium is mediated by two layers of extracellular matrix, each one with a specific role in the mechanical transduction process. The first is a rigid layer in direct contact with the otholithic mass and is known as the otolithic membrane or gelatin membrane. This structure consists of a dense, randomly cross linked filament network that uniformly distributes the force of inertia of the non-uniform otolithic mass to all stereocilia bundles. The second layer formed by a columnar organization of filaments secures the otolithic membrane above the surface of the epithelium. The long columnar filaments are organized in parallel to the stereocilia bundles and are anchored to the apical surface of the supporting cells. The zonula adherens at the apical region of each supporting cell displays a thick polygonal bundle of actin filaments forming at the surface of the epithelium a transcellular honeycomb organization that provides mechanical ground support for the columnar filament layer. The dominant aspect of this columnar filament layer indicates that it may also have an important role in attenuating the force of inertia of the large otolithic mass during acceleration, screening stresses that would be directed to an effective bending of the stereocilia bundles.

Otolithic sensory apparatus; Otolithic membrane; Mechanical transduction; Vestibular sensory macula; Frog vestibular sensory organ

Introduction

The different organs of the acoustico-lateralis system are responsible for the detection of sound, linear and angular accelerations, water motion, and substrate vibration in vertebrates (Hudspeth, 1983). While these organs serve such a broad range of biological functions, they share a common mechano-electrical transduction mechanism that transforms an external mechanical stimulus capable of deflecting the stereociliar bundles of their hair cells into a conductance change and an electrical response of the hair cell's membrane (Howard et al., 1988). The specificity of a particular organ of the acoustico-lateralis sensory system to a particular type of mechanical stimulus de-

pends on intricate mechanical interactions between specialized accessory structures that are part of the complex architecture of each organ (Hillman, 1976; Hudspeth, 1983; Lim, 1984; Hunter-Duvar and Hinojosa, 1984).

The sacculus is one of the sensory organs of the vestibular apparatus and responds to linear accelerations and to gravity. The characteristic feature of the sacculus is the presence of the otolithic mass on top of the sensory epithelium within the endolymphatic chamber (Hillman, 1976, Lim, 1984; Hunter-Duvar and Hinojosa, 1984). This otolithic mass is made of thousands of calcareous crystals (otoliths, otoconia, or statoconia) attached to an extracellular formation called otolithic, otoconial, statoconial, or gelatine membrane (Hillman and Lewis, 1971; Hillman, 1976; Lim, 1984; Ross et al., 1987). When the animal undergoes linear acceleration, the otolithic mass is sheared in relation to the surrounding fluid and tissue because of its high density and specific gravity (Lim,

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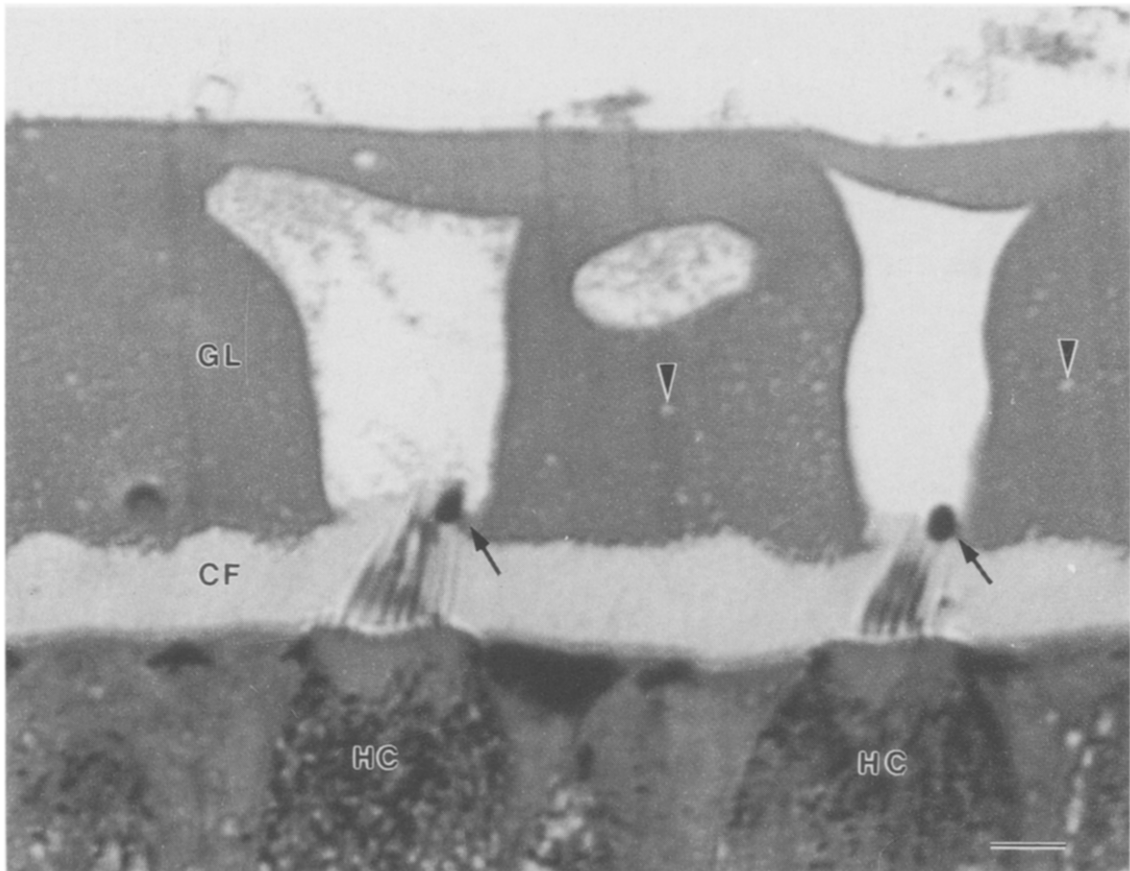
Fig. 1. Light micrograph of a 1 μm thick section of the apical region of the macular sensory epithelium of the frog. The otolithic mass was removed prior to fixation of the tissue. Each hair cell (HC) is surrounded by supporting cells. The two layers of extracellular gelatinous material are seen on the surface of the epithelium The thick darkly stained layer corresponds to the otolithic gelatin membrane (GL) and forms cavities at the site of the stereocilia bundles. The otolithic membrane is attached to the bundles through the head of the kinocilium (arrow). This layer also displays myriad small holes (arrowheads). The lightly stained layer corresponds to the columnar filament (CF) layer which fills up the gap between the otolithic membrane and the surface of the epithelium. Scale bar, 5 μm.

1984). This relative inertial movement of the otoliths is coupled to the hair cell by the otolithic membrane in such a way that a fraction of the mechancial energy is used to deflect the stereocilia bundles.

Current understanding on the otolithic membrane structure is based on thin section and scanning electron microscopy studies (Hillman and Lewis, 1971; Hillman, 1976; Lim, 1984; Ross et al., 1987). It is widely accepted that it is composed of two layers of a fibrillar substance, a peripheral layer that supports the otoliths and a more loosely arranged meshwork between this layer and the macular surface (Hillman, 1971; Hillman, 1976;

Lim, 1984; Ross et al., 1987). However, the use of thin section and scanning microscopy did not produce definitive data on the detailed structure of these layers and the published schematic diagrams illustrating the otolithic membrane layers are very sketchy. According to some reports the possibility that the putative amorphous material at the surface of the sensory epithelium may correspond to floculated components of the endolymphatic fluid has remained open (Ross et al., 1987).

In the present study we have determined the detailed structure of the extracellular matrix and its relationship to the surface of the sensory epi-

thelium in the bullfrog saccular macula using the freeze-etching technique. We describe the network structure of the otolithic membrane and the layer of columnar filaments that secures the otolithic membrane above the surface of the epithelium. The characteristic structure of the otolithic membrane and the columnar filament layer are correlated to the actions that occur during the mechanical transduction process, where the initial mechanical input that acts on the very large mass of otoliths is processed into the fine action that gates the unitary molecular transducer channels in the stereocilia.

Materials and Methods

American bullfrogs *Rana catesbiana* weighing 100–200 g (Carolina Biological Supply Co.) were anesthetized with 20 μg/g of body weight of 3-aminobensoic acid ethyl ester and decapitated. The saccular macula was carefully dissected out under cooled frog's Ringer solution (Carolina Biological Supply Co.). The excised tissue was gently rinsed with fresh Ringer to remove otoliths and processed for thin section or freeze-etching electron microscopy.

For thin sections, specimens were fixed using either of the two following protocols: (a) immersion in 1% glutaraldehyde in 0.05 M phosphate buffer solution at pH 6.8 for 30 min, washed in 0.1 M phosphate buffer for 10 min; postfixed in 1% OsO4 in 0.1 M phosphate buffer at pH 6.3 for 45 min at 0°C, washed in distilled water and en bloc stained with 0.5% uranyl acetate for 3 h; (b) immersion in 3% glutaraldehyde, 2% paraformaldehyde, 2% tannic acid, 0.5% calcium chloride in 0.1 M sodium cacodylate buffer solution at pH 7.2 for 1 h, rinsed in 0.1 M sodium cacodylate buffer and postfixed in 1% OsO_4, 1.5% potassium ferrocyanide for 2 h on ice. Specimens were rinsed in 0.1 M sodium cacodylate buffer, pH7.2, dehydrated in a series of graded methanol, embedded in Polybed 12 and polymerized for 24 h at 60°C. Thick sections (1 μm) were cut and stained with a mixture of 1% azure II, 2% methylene blue, and 2% borax in distilled water. Ultrathin sections were stained with lead citrate and examined in a JEOL 100CX electron microscope.
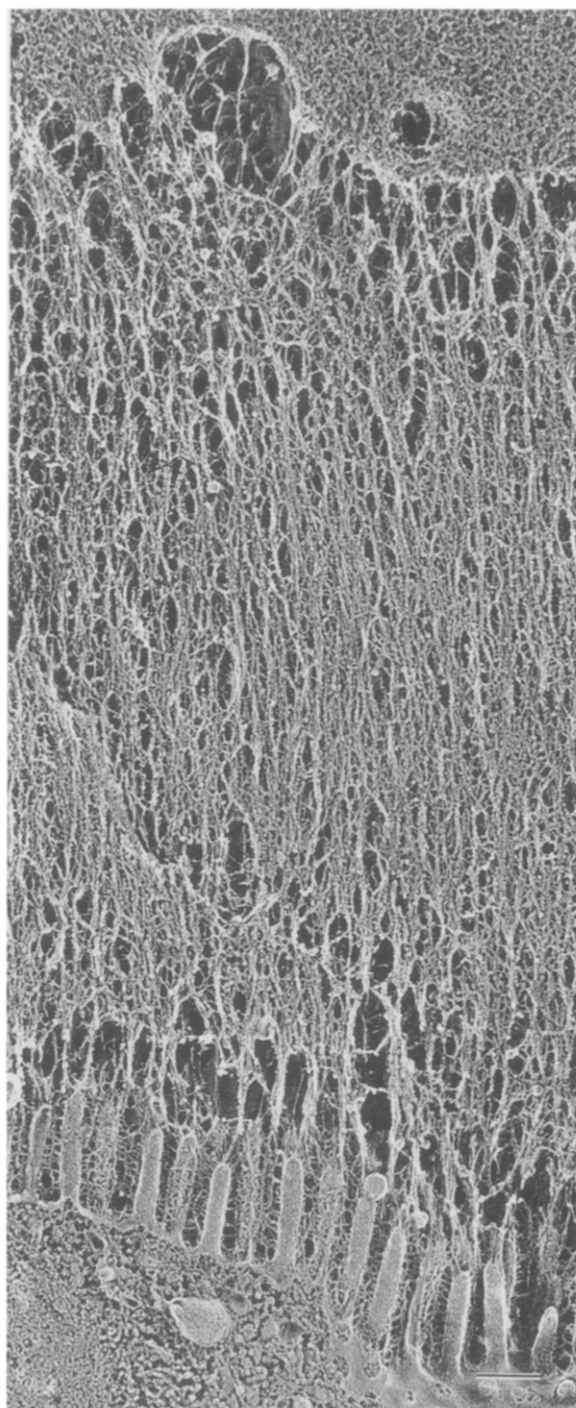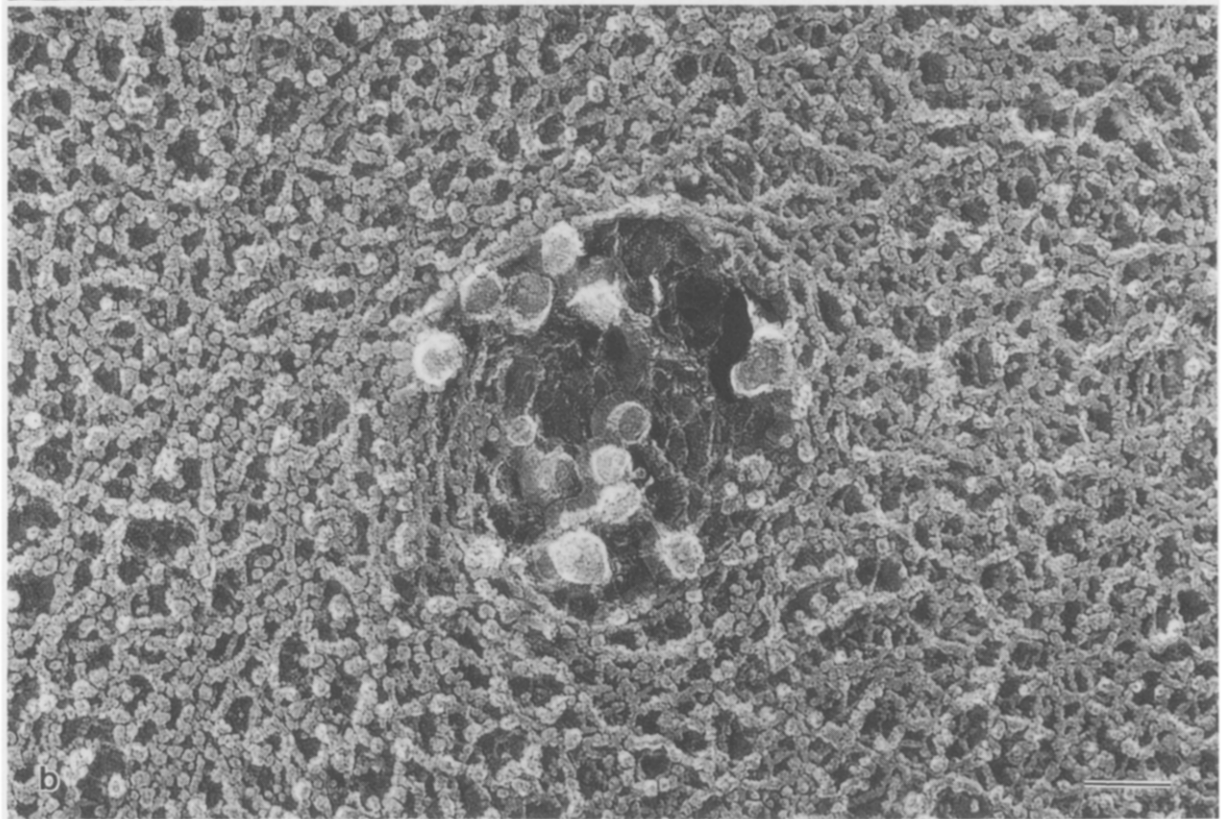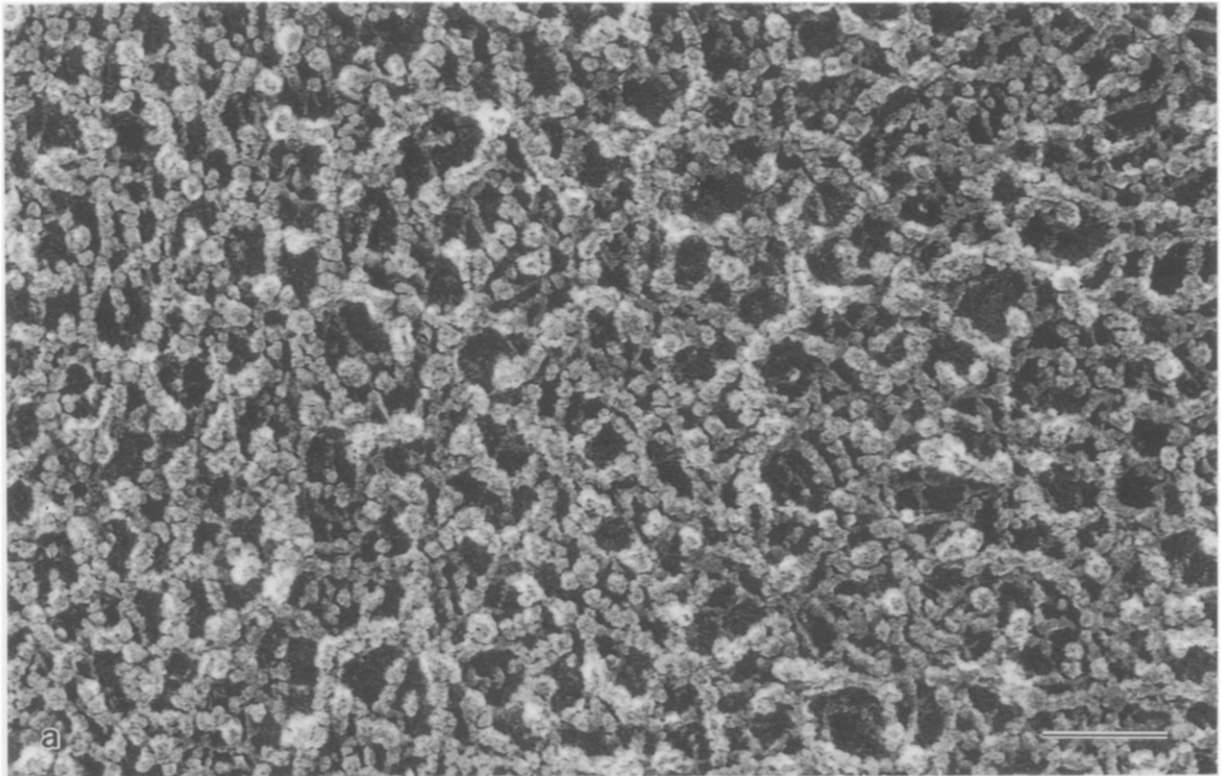

Fig. 2. Freeze-etching replica showing the columnar filament layer connecting the dense cross-linked matrix of the otolithic membrane to the microvilli on the surface of the supporting cell of the sensory epithelium. Scale bar, 0.5 μm.

For freeze-etching, specimens were fixed by immersion in 3% glutaraldehyde, 2% paraformaldehyde, 2% tannic acid, 0.5% calcium chloride in 0.1 M sodium cacodylate buffer solution, pH 7.2, for 1 h, rinsed extensively in distilled water and fast-frozen by contact against the surface of a liquid helium cooled copper block (Heuser and Kirchner, 1980). The fast-frozen samples were fractured at $-100°C$ and allowed to etch until a thin layer of ice was removed and then rotary replicated in a Balzers 301 apparatus. Electron micrographs were taken with either a 200CX or a 100CX Jeol electron microscope.

The specific actin filament fluorescent dye rodhamine-phalloidin was applied in a 0.1 M phosphate buffer solution to specimens fixed in 1% glutaraldehyde and permeabilized with a 0.2% solution of the detergent Brij 58 (Sigma)

Results

The basic layered organization of the sensory epithelial surface and the associated extracellular components of the frog saccular macula are shown in Fig. 1. In order to allow for either the fast-freezing or thin sectioning procedures the otoliths of the vestibular organ had to be removed from our preparations. However, in the intact macula the otoliths are held together by a gluey substance on top of the otolithic or gelatinous membrane (Lim, 1984). Information on the detailed structure of the otolith aggregate is available from scanning electron microscopy studies (Lim, 1984).

The sensory macula is a tall columnar epithelium which consists of hair cells separated and completely surrounded by supporting cells (see also Fig. 7b). The supporting cells form a continuum with the adjacent epithelium (Fig. 7a) that covers the whole internal surface of the sacculus. Two layers of extracellular material cover the surface of the sensory epithelium. The top layer that appears darkly stained in the 1μm sections corresponds to the otolithic membrane also called gelatin, statoconial, or otoconial membrane (Lim,

1984). The lightly stained layer which we call here columnar filament layer fills up the space between the otolithic membrane and the surface of the epithelium (Fig. 1) . Stereocilia bundles span the thickness of the columnar filament layer and attach to the otolithic membrane through the head of their kinocilium (Fig. 1; see also Eatock et al., 1987).

Otolithic membrane

The otholithic membrane consists of a 25–30μm thick layer of dense matrix forming large cavities at regions overlaying the apex of the hair cells. In addition to the large cavities this layer displays smaller round cavities (arrows, Fig. 1). Freeze-etching permits the visualization of the fine texture of the otolithic membrane (Figs. 2 and 3). It consists of a homogeneous matrix of a densly packed and randomly cross-linked filament network (Fig. 3). The mesh size of the network is 40–60 nm. The filaments display a beaded appeareance and are 19 ± 2.4 nm ($N = 28$) thick. The random texture of the filament network is occasionally interrupted by round cavities (Fig. 3b). Vesicular structures (50–100 nm diameter) are frequently seen within these cavities (Fig. 3b).

Columnar filament layer

The columnar filament layer is 5–8 μm thick and connects the surface of the epithelium to the otolithic membrane. It consists of a columnar organization of parallel 20 ± 5 nm ($N = 28$) thick filaments cross-bridged by 12 ± 1.4 nm ($N = 28$) thick filaments (Fig. 4). The columnar filaments have a beaded appearance similar to the filaments of the otolithic membrane layer. The substructure of the thin filaments cross-linking the columnar filaments cannot be resolved in the replicas.

The long columnar filaments terminate at the surface of the apical membrane of the supporting cells either at the base or at the tip of the microvilli structures (Figs. 2 and 6) . Filaments that terminate at the base of the microvilli form thin cross-links with the microvilli membrane. Such

Fig. 3. Detailed views of the cross-linked filament lattice that forms the gel matrix of the otolithic membrane. This randomly cross-linked dense matrix is occasionally interrupted by 0.2–2 μm diameter holes that often contain globular particles (b). Scale bar: (a) 0.1 μm; (b) 0.2 μm.
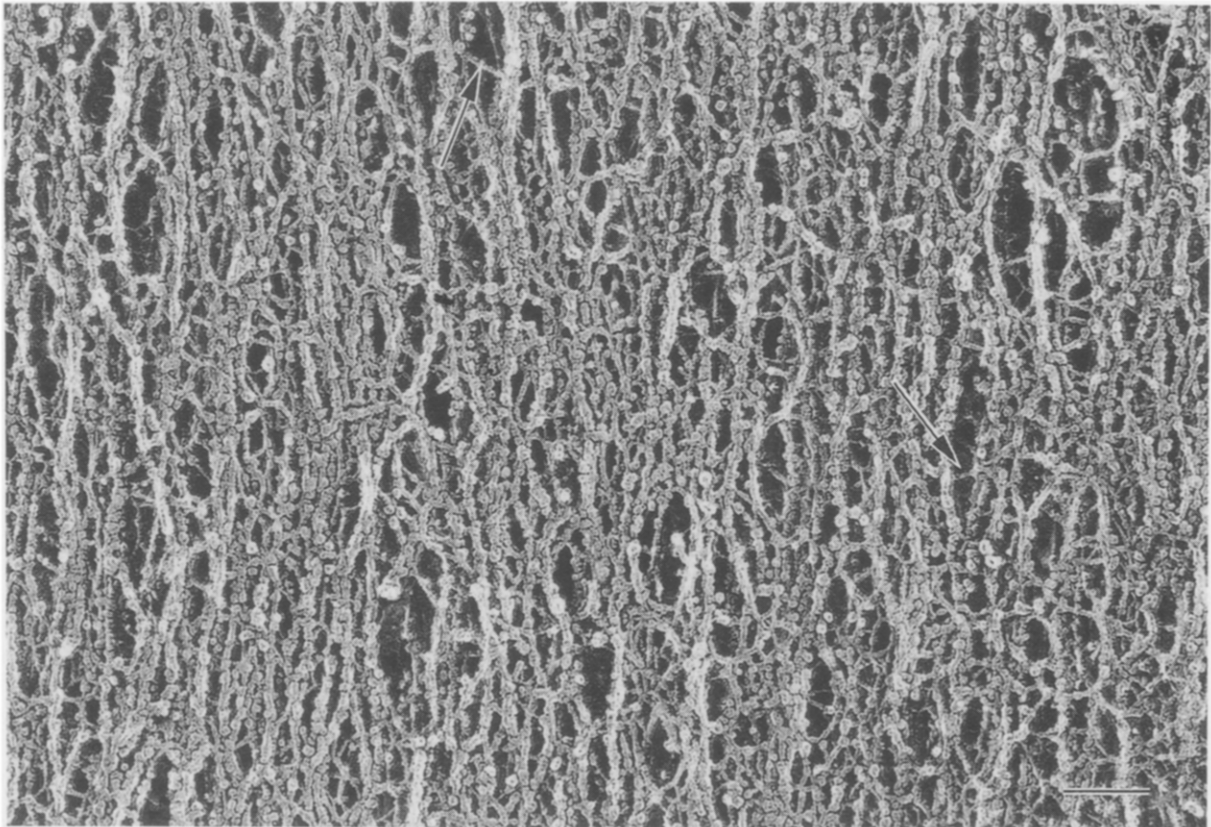
Fig. 4. Detailed view of the columnar filament layer. Columnar filaments 20 nm thick with a beaded appearance are cross bridged at different angles by 12 nm thin filaments (arrows). Scale bar, 0.2 μm.

filamentous material is found on supporting cells that are part of the sensory macula and covered by the otolithic membrane. In transverse sections they show a very regular pattern formed by an arrangement of filaments, microvilli and cross bridges. These filaments are never found associated to the stereocilia or occasional microvilli on the surface of the hair cells. The cross-linking pattern of columnar filaments, and microvilli is not interrupted at the boundary of contiguous supporting cells (Fig 5a). Epithelial cells that are contiguous with the supporting cells outside the macular region are devoid of the extracellular filaments and are not as well organized as the microvilli of the sensory region.

Structure of the apical surface of the supporting cells

Just beneath the apical surface, associated with the apical junctional band of each supporting cell, there is a bundle of actin filaments as part of the adherens zone (Fig. 7). Actin filaments are identified by their characteristic 6–7 nm thick electron microscopy appearance (Fig. 7c) and by labelling with the actin specific marker rhodamine-phalloidin (Fig. 7a and b). This very dense bundle of actin filaments is 1–2 μm thick and forms a polygonal ring around the apex of each cell. The actin bundle is enmeshed in the dense network of intermediate filaments also present in this region. Many of these intermediate filaments are associated with the numerous desmosome junctions between adjacent cells (Fig. 7c). The combination of the intercellular junctions and juxtaposed actin polygonal structures in the same plane form a transcellular structure with a honeycomb appearance (Fig. 7). This transcellular honeycomb plate formation is well visualized in the whole mount preparation of the macula (Fig. 7a,b).

Discussion

The mechanical qualities of accessory structures in the organs of the acoustico-lateralis system provide the basis for their specialized mechanical tranduction functions. The analysis of their fine structure and microarchitecture provides important complementary information to the often
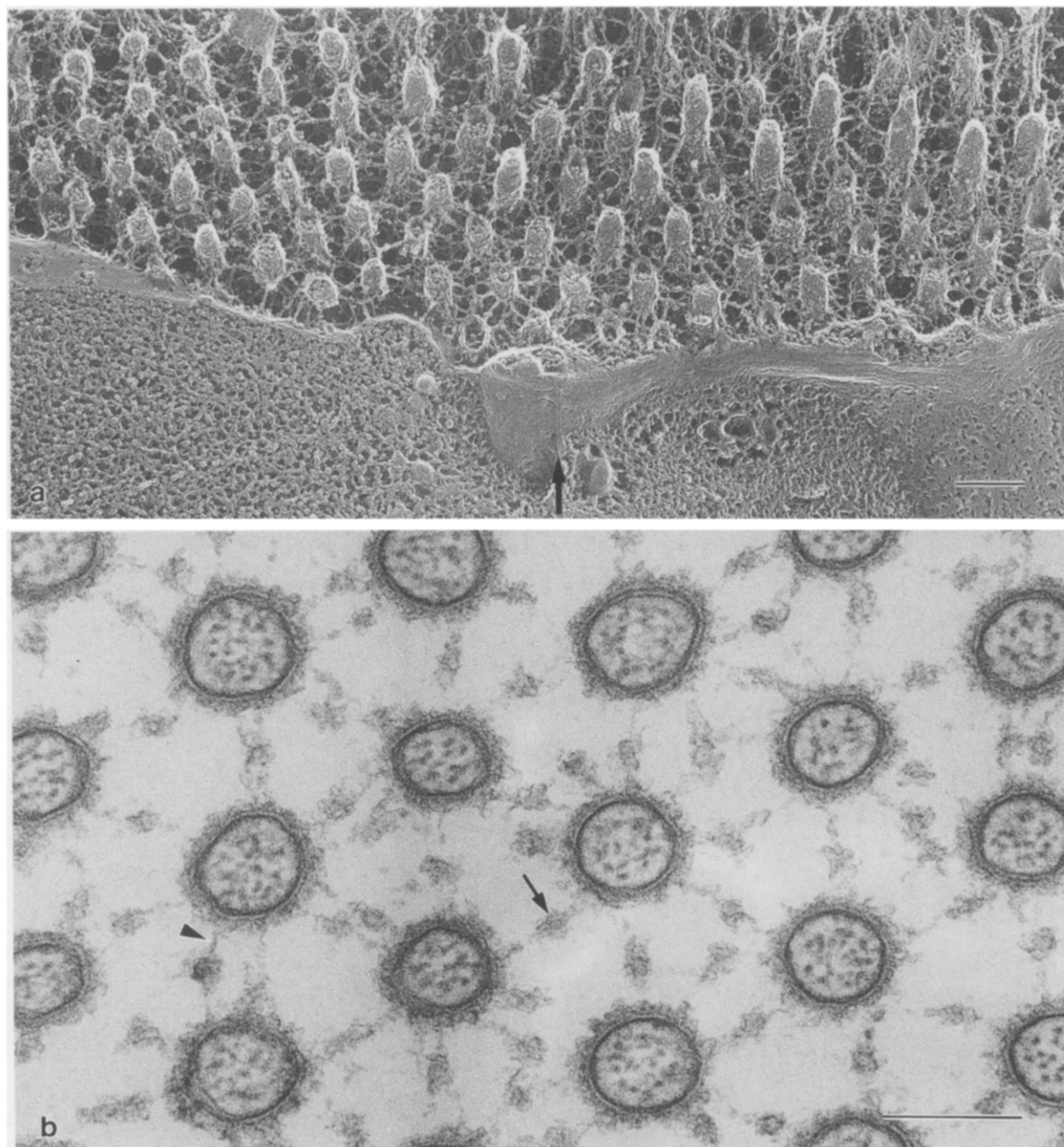

Fig. 5. Freeze etching (a) thin section (b) of the apical surface of the supporting cells. The microvilli and the associated extracellular filaments form a regular hexagonal pattern that is not interrrupted at the boundaries between cells (arrow in a). This organization of microvilli, columnar filaments (arrow in b) and crossbridges (arrowhead in b) form the base of the columnar filament layer. Scale bar = 0.2 μm.
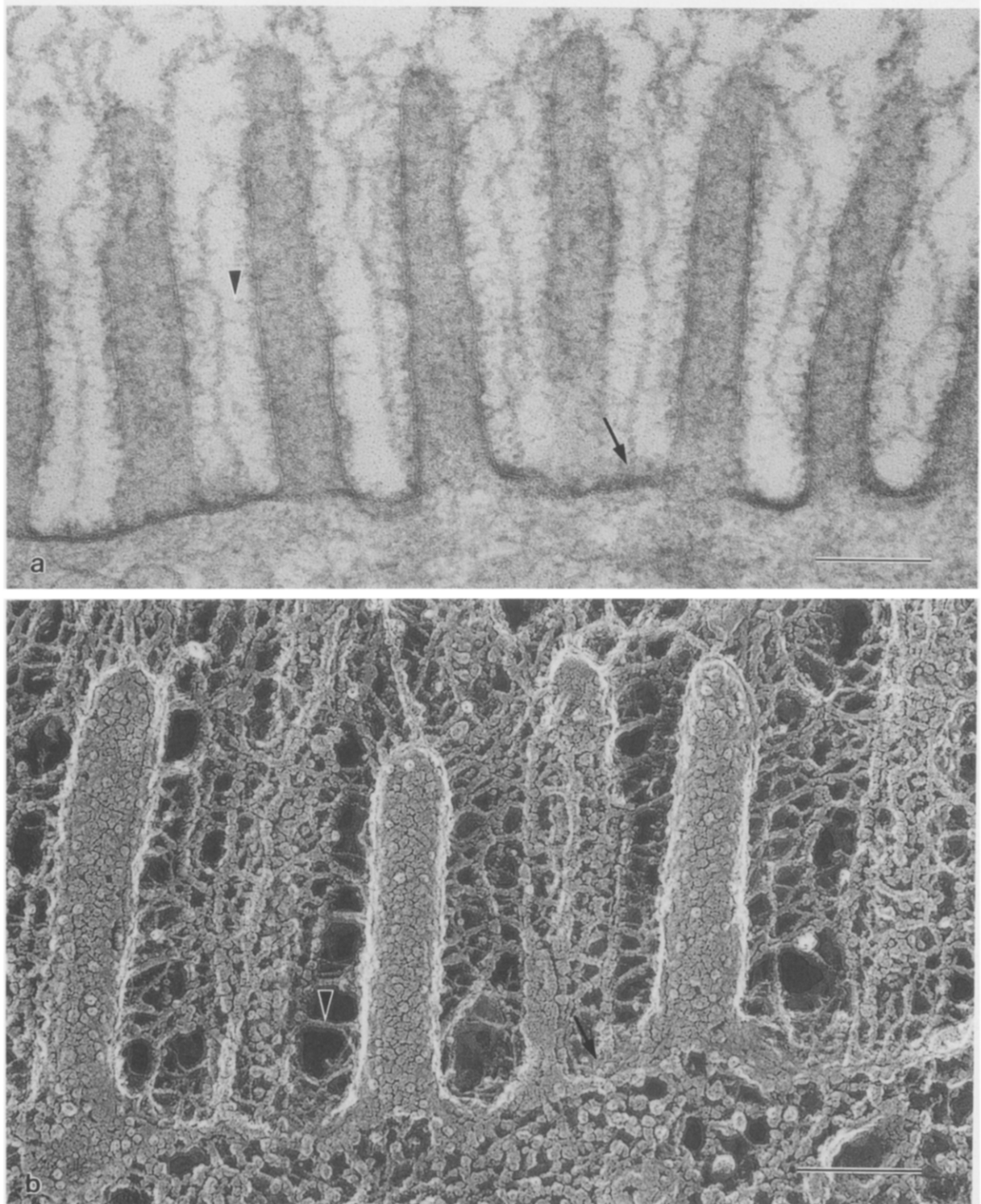
Fig. 6. Thin section and freeze-etching views of microvilli of supporting cells. The columnar filaments are typically anchored to the apical membrane between microvilli structures (arrow). These filaments are cross-linked to the lateral surface of the microvilli by 12 nm filaments (arrowheads). Scale bar: 0.2 μm.
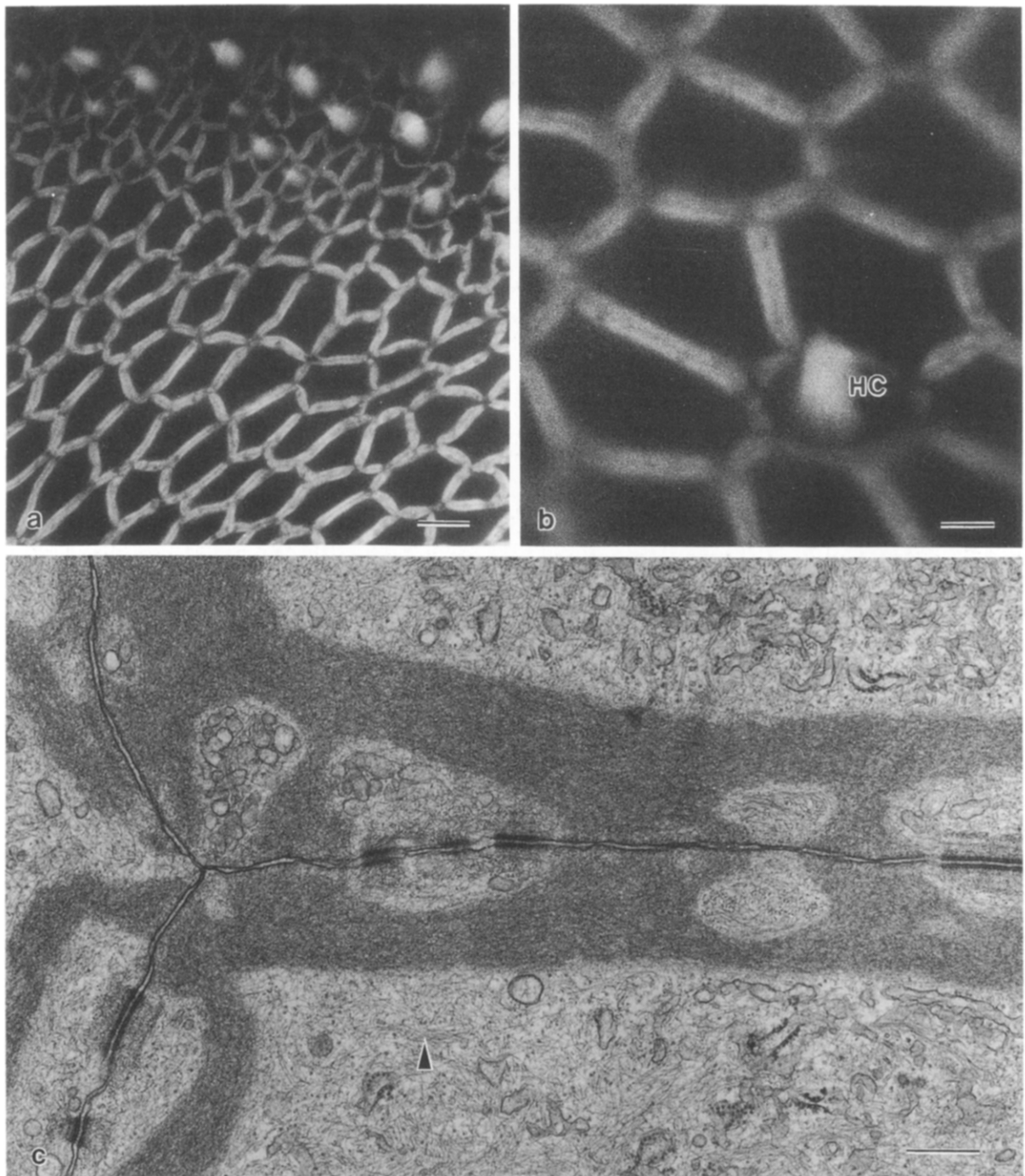
Fig. 7. Polygonal bundles of actin at the apical region of supporting cells visualized by fluorescence light microscopy (a and b) and by thin section electron microscopy (c). The actin bundles are part of the adherens junction complex which also includes desmosomes. A high density of intermediate filaments (arrowheads) also populate the apical region of these cells. Scale bar: (a) 10 μm; (b) 5 μm (c) 0.5 μm.

more difficult direct mechanical measurement procedures and other physiological studies. In the present study we have used the freeze etching technique to investigate the fine structure and microarchitecture of the extracellular gelatinous material present at the surface of the otolithic vestibular sensory epithelium. This gelatinous material is bathed by the endolymphatic fluid and fills up the space between the otoliths and the apical surface of the sensory cells. It consists of two structurally distinct layers, the otolithic membrane and the columnar filament layer. Their fine structure and relationship to the surface of the sensory epithelium as revealed by the freeze etching technique provides a framework within which to consider the process of mechanical transduction.

The relatively more rigid nature of the otolithic membrane layer when compared to the other structures in the sensory macula can be inferred by the fact that it remains intact when mechanically removed by peeling it from the surface of the epithelium with a pair of forceps (Howard and Hudspeth, 1987). The otolithic membrane transmits the force of inertia of the otoliths to the stereocilia bundle through lateral cross-links between the head portion of the kinocilium and the otolithic membrane layer (Eatock et al., 1987). The highly cross-linked isotropic texture of this layer indicates that this membrane would function as a rigid plate and equally distribute the force of inertia of the large number of otoliths to all the stereocilia bundles.

The second layer is not connected to the stereocilia but connects the otolithic membrane layer to the surface of the epithelium, more precisely to the surface of the supporting cells. The characteristic anisotropic structure of the columnar filament layer would provide an optimal support for the otolithic membrane securing it in the vertical axis position but still allowing for lateral movements through pivoting of the columnar filaments. This way the columnar filament layer would screen the stress transmitted to stereocilia so that the stereocilia are subject only to the stress that cause angular deflection of the bundle.

The columnar filament layer is anchored to the surface of the supporting cells. In fact, the combination of columnar filaments, microvilli and
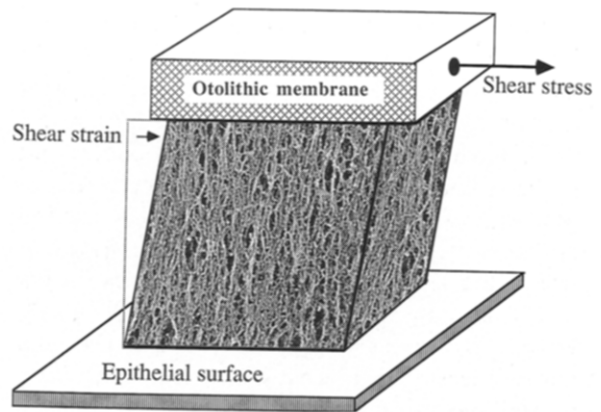

Fig. 8. Schematic diagram illustrating the relationships between the otolithic (gelatin) membrane, the columnar filament layer and the epithelial surface. Shear stress applied to the rigid gelatin membrane will result in shear strain on the columnar filament layer. The epithelial surface functions as ground support.

cross-bridges between the filaments and the microvilli form a crystalline-like hexagonal organization. The actin core of the microvilli is typically ancored to the terminal web (Hull and Staehelin, 1979). The terminal web of each supporting cell is particularly well developed and includes a prominent adherens zone that consists of thick and dense polygonal bundles of actin filaments. The combination of juxtaposed polygonal bundles of neighboring cells forms an isodiametric transcellular network with a honeycomb appearence. This honeycomb actin network in combination with the terminal web and the cross-linking between microvilli and the columnar filaments within a narrow plane at the surface of the epithelium forms a second rigid plate that provides mechanical ground support for the columnar filament layer.

Shear stress due to relative acceleration of the otolithic mass results in shear strain of the columnar filament layer (Fig. 8) and the stereocilia bundles which are intercalated between two rigid plates, the otolithic membrane and the rigid complex of cytoskeletal components at the surface of the epithelium. It is important to determine to what extent the structurally prevalent columnar filament layer absorbes shear stress energy from the accelerated otoliths, thus modulating the transfer of energy to the stereocilia.

Stereocilia are rigid rods of actin filament bundles (Tilney et al. 1980). In vivo, the bundle is held together by a web of connections and linkages (Ernston and Smith, 1986; Furness and Hackney, 1985; Neugebauer and Thurm, 1985; Osborne at al. 1984). The site of the actual molecular transducer is presumed to be at the so called tip-link apparatus which involves a gentle thread that bridges the tip of a stereocilia to the side of a taller neighbor (Pickels et al., 1984; Howard et al., 1988) . Spontaneous displacement of stereocilia bundles measured in in vitro preparations, where the otolithic membrane was removed, show that hair bundle damping is sensitive even to changes of viscosity of the surrounding fluid (Denk and Webb, 1987). While the degree of deflection of the bundle under physiological conditions in vivo is not known, the configuration of the bundle indicates that even for small angular deflection the tips of the stereocilia would undergo significant relative shear (Howard and Hudspeth, 1987). The cross-linking material at the narrow spaces between the stereocilia (Ernston and Smith, 1986; Furness and Hackney, 1985; Osborne at al. 1984) is in a favorable condition to resist the shear stress on the bundle. The columnar filament layer may exert additonal damping effect when the stereocilia bundle is subjected to a large shear force. The columnar filament layer may not only support the large otholithic mass in the vertical direction, but it may also absorb some mechanical energy in the direction of the shear force reducing the shear strain in the bundle and the load on the molecular transducing apparatus. Shear stress energy could be absorbed by bending the columnar filaments or sliding the cross-links between the columnar filaments.

To illustrate the mechanical interactions between the different structures in the otolith sensory epithelium, we can use a mechanical model of springs similar to the one presented by Howard and Hudspeth (1987). To their model we add a spring corresponding to the mechanical analog of the columnar pivoting layer. We also attribute two separate springs in the stereocilia bundles, one for the structural cross-links and one for the elastic element that actually gates the transducer channel. In this model the spring equivalent to the columnar filament layer acts in parallel to the stereo-
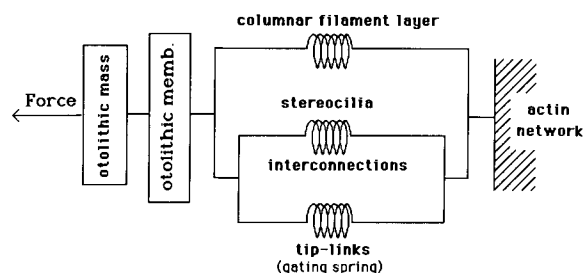

Fig. 9. Mechanical model of the saccular sensory epithelium. This model is based on the Howard and Hudspeth (1987) model to which we have added a spring corresponding to the columnar filament layer. We have also changed the spring, which in their model corresponds to the stereocilia pivot, to include the stereocilia interconnections that are now widely reported in the literature. The gelatin layer or otolithic membrane would act as a rigid plate to equally distribute the force derived from the thousands of otoliths to all the stereocilia bundles.

cilia bundle springs and dissipates energy modulating the action of the large inertia of the accelerated otolithic mass on the gating channel.

The biochemical composition of the otolithic membrane and the columnar filament layer are not known. Because of the difficulty in obtaining sufficient amounts of material, biochemical studies are difficult to perform. A new purification procedure that exploits the adhesion of specific components of the sensory epithelium to nitrocellulose was recently used for a preliminary study of the protein composition of the different structures in the frog sensory macula. Gel electrophoresis of otolithic membrane purified by the nitrocellulose method shows broad polypeptide bands at 33, 40, 59 and 190 kDa (Shepherd et al., 1989). Immunocytochemical studies are needed to establish the relationship of these polypeptide bands with the filament structure of the otolithic membrane and the columnar filament layer.

Another point to consider is the relationship between the endolymphatic fluid and the structure of the gel layers. It is known that swelling pressure of gels is affected by the distribution of ions between the liquid inside and outside the gel structure (Hermans, 1949), particularly in the case of protein gels which contain ionizable groups. It is possible that physiological and pathological changes in the structure of the gelatin layers may occur as a result of modulation of the composition

of the endolymphatic fluid. In addition, a possible complementary function of the gel structure of both otolithic membrane and columnar filament layers is to allow for diffusion of metabolites while reducing the convection currents in the endolymph that would add to the 'noise' of the receptor.

Acknowledgment

References

Denk, W. and W.W. Weeb (1987) Fluid viscosity dominates hair-bundle damping in transducer cells of the frog sacculus. Biophys. J. 51, 519a.

Eatock, R.A, D.P. Corey and J. Hudspeth. (1987) Adaptation of mechanoelectrical transduction in hair cells of the bullfrog's sacculus. J. Neurosci. 7, 2821–2836.

Ernstson, S. and C.A. Smith (1986) Stereo-kinociliar bonds in mammalian vestibular organ. Acta Otolaryngol. 101, 395–402.

Furness, D.N. and C.M. Hackney. (1985) Cross-links between the stereocilia in the guinea pig cochlea. Hear. Res. 18, 177–188.

Hermans, P.H. (1949) Chapter XII — Gels. In: H.R. Kruyt (Ed.), Colloid Science, Vol. II, Elsevier, Amsterdam.

Heuser, J.E. and M. W. Kirshner (1980) Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. J. Cell Biol. 86, 212–234.

Hillman, D.E. (1976) Vestibular and lateral line system: Morphology of peripheral and central vestibular system. In: R. Llinas and W. Precht (Eds.), Frog Neurobiology, Springer-Verlag, Berlin, pp. 452–479.

Hillman, D.E. and E.R. Lewis. (1971). Morphological basis for a mechanical linkage in otolithic receptor transduction in the frog. Science 174, 416–418.

Howard, J. and A.J. Hudspeth. (1987) Mechanical relaxation of the hair bundle mediates adaptation in mechanoelectrical transduction by the bullfrog's saccular hair cell. Proc. Natl. Acad. Sci. USA 84, 3064–3068.

Howard, J., W.M. Roberts and A.J. Hudspeth. (1988) Mechanoelectrical transduction by hair cells. Ann. Rev. Biophys. Chem. 17,99–124.

Hudspeth, A.J. (1983) Mechanoelectrical transduction by hair cells in the acousticolateralis sensory system. Ann. Rev. Neurosci. 6, 187–215.

Hunter-Duvar, I. M. and Hinojosa, R. (1984). Vestibule: Sensory Epithelia. In: I. Friedman and J Ballantyne (Eds.), Ultrastructural Atlas of the Inner Ear. Butterworths, London. 211–244

Hull, B. E and L. A. Staehelin. (1979) The terminal web: a reevaluation of its structure and function. J. Cell Biol. 81,67–82.

Lim, D.J. (1984) The development and structure of the otoconia. In: I. Friedmann, and J. Ballantyne (Eds.), Ultrastructural Atlas of the Inner Ear. Butterworths, London, 245–269.

Neugebauer, D.Ch and U. Thurm. (1985) Interconnections between the stereovilli of the fish inner ear. Cell Tissue Res. 240, 449–455.

Osborne. M.P., Comis, S.D. and J.O. Pickles. (1984) Morphology and cross-linkage of stereocilia in the guinea-pig labyrinth examined without the use of osmium as a fixative. Cell Tissue Res. 237, 43–48.

Pickles, J.O., Comis, S.D. and M.P. Osborne. (1984) Cross-links between stereocilia in the guinea pig organ of Corti, and their possible relation to sensory transduction. Hear. Res. 15, 103–112.

Ross, M. D., Komorowski, T.E. Donovan, K.M. and K.G. Pote. (1987) The suprastructure of the saccular macula. Acta Otolaryngol. 103, 56–63.

Shepherd, G. M., Barres, B. A and D. P. Corey. (1989) 'Bundle blot' purification and initial protein characterization of hair cell stereocilia. Pro. Natl. Acad. Sci. USA 86, 4973–4977.

Tilney, L.G., Derosier, D.J. and M.J. Mulroy. The organization of actin filaments in the stereocilia of cochlear hair cells. J. Cell Biol. 86,244–259, 1980.