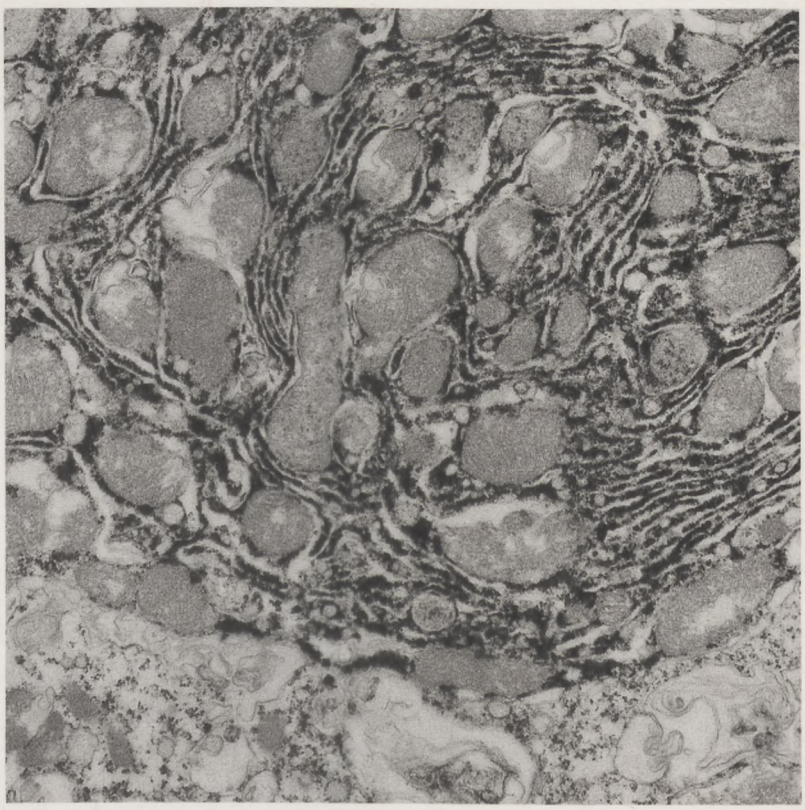
HISTOPHYSIOLOGICAL STUDIES OF THE INNER EAR

with emphasis on endolymphatic hydrops



Peter Paul G. van Benthem

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Cover illustration: Basolateral membranes of a strial marginal cell with a precipitate of lead phosphate

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HISTOFYSIOLOGISCHE STUDIES VAN HET BINNENOOR

met de nadruk op endolymfatische hydrops

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Voor Sarah
Aan mijn vader en moeder

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Contents

General Introduction

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INTRODUCTION

The inner ear is one of the most complex sensory organs in mammals. It contains several highly differentiated epithelia with specialized functions. Notwithstanding the vast number of studies investigating the inner ear, various aspects of its physiology are still not fully understood. Consequently, several inner ear disorders – such as Menière's disease – are as yet beyond our therapeutic reach. One of the more important experimental models for the investigation of inner ear pathophysiology is that of an endolymphatic hydrops, which can be experimentally induced by surgical obliteration and obstruction of the endolymphatic duct and sac, as originally described by Kimura and Schuknecht (1965). Following obliteration of the endolymphatic sac, a sequential series of functional and morphological changes take place in the inner ear, eventually resulting in a profound sensorineural hearing loss. Several of these pathological features are also found in Menière's disease: (1) a distension of Reissner's membrane; (2) the initial degeneration of the sensory cells at the apex of the cochlea; and (3) the concomitant occurrence of low-frequency threshold shifts. For this reason experimental endolymphatic hydrops has been generally considered to be a useful model for studying the pathophysiological events in Menière's disease, despite the fundamental difference in etiology. Along the above-mentioned line of research, investigations concerning functional and morphological changes in endolymphatic hydrops have been performed in our department over the past years (Van Deelen, 1987; Albers, 1988; Klis, 1988; Ruding, 1988). Investigation of the histophysiological and electrophysiological changes of the inner ear in experimental endolymphatic hydrops will not only provide more detailed information about Menière's disease, but will also add to our general knowledge of the mechanisms involved in inner ear physiology.

OBJECTIVES OF THIS STUDY

In this thesis, the ultrastructural changes in the guinea pig inner ear after surgical induction of endolymphatic hydrops are investigated using various (enzyme)-cytochemical techniques.
Chapter 1 gives a summary of our present knowledge regarding the functional morphology of the inner ear (i.e. cochlea, vestibular apparatus, endolymphatic duct and sac).
In Chapters 2 and 3, the cytochemical composition and structure of the cochlear glycocalyx and the stereociliary cross-links are investigated, both in normal and hydropic cochleas. The possible relation of these subcellular structures

to cochlear mechano-electrical transduction and electrolyte physiology of the scala media is discussed.
In Chapter 4, the glycocalyx of the epithelial cells in the endolymphatic sac is studied and related to endolymphatic sac physiology.
In Chapter 5, a comparison is made between different enzyme-cytochemical techniques for the ultrastructural demonstration of Na^+/K^+-ATPase in the inner ear. The distribution of this enzyme in the guinea pig inner ear (i.e. cochlea, vestibular apparatus, and endolymphatic sac) is described.
In Chapter 6, the effect of experimental endolymphatic hydrops on cochlear potentials and Na^+/K^+-ATPase activity is studied. Also, the effect of nimodipine – a compound reported to have Na^+/K^+-ATPase-stimulating properties – on the activity of this enzyme is investigated in both normal and hydropic cochleas. Electrocochleography and enzyme cytochemistry are used to assess the possible functional changes.
In Chapter 7, the results of this study are summarized and discussed.

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Chapter 1

Functional morphology of the guinea pig inner ear

Functional morphology of the guinea pig inner ear

THE COCHLEA

The cochlea of the rodents, and that of the guinea pig in particular, is not embedded in the temporal bone of the skull, in contrast to the cochlea of most mammalian species. Instead, it protrudes into the air-filled middle-ear space of the bulla. This facilitates easy access to the cochlea and makes it an ideal object for study of both cochlear morphology and physiology.
Surrounded by a thin bony capsule, the membranous inner parts of the cochlea spiral around a bony axis (modiolus), which contains the cochlear nerve and blood vessels. In cross-section, each one of the 3½ turns consists of three compartments: the scala media, which is filled with endolymph, and the scala vestibuli and scala tympani, filled with perilymph. The latter make contact with each other through the so-called helicotrema, located at the apex of the cochlea. The scala media (Fig. 1) has a triangular shape and is separated from the scala vestibuli by Reissner's membrane, consisting of a mesothelial and an epithelial cell layer. The scala media is separated from the scala tympani

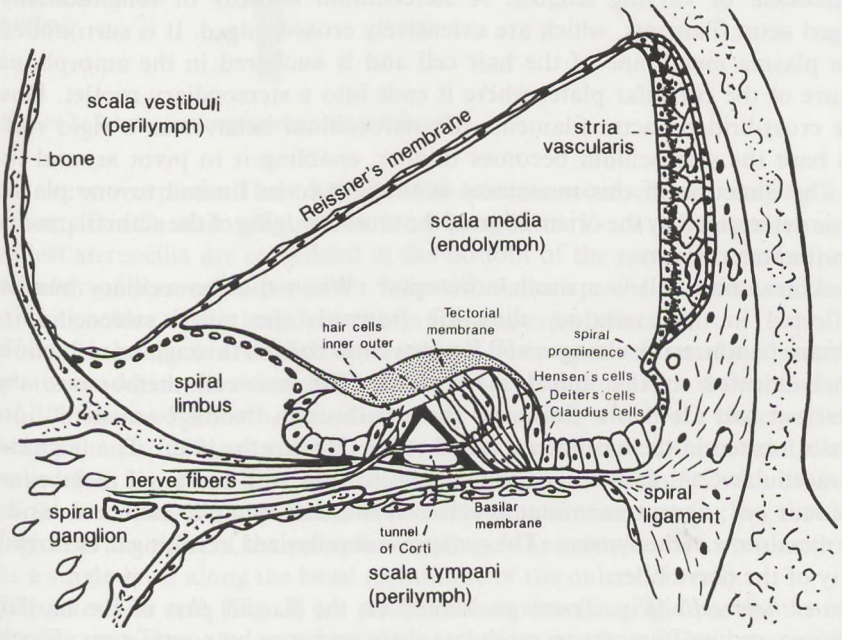

Figure 1. Schematic drawing of a transverse section of the scala media of the guinea pig cochlea.

by the basilar membrane, which supports the sensory epithelium (organ of Corti) and consists of an amorphous, extracellular matrix containing collagen fibers and proteoglycans. The lateral wall of the scala media is formed by the spiral ligament, the stria vascularis and the spiral prominence. In the stria vascularis, three cell types can be identified: (1) basal cells; (2) intermediate cells; and (3) marginal cells, the latter facing the scala media.

The organ of Corti (Fig. 1) contains two morphologically and physiologically distinct types of sensory cells (hair cells): the outer hair cells (three rows) and the inner hair cells (one row). Furthermore, various supporting cells can be distinguished in the organ of Corti: the inner phalangeal cells (supporting the inner hair cells), the outer phalangeal cells (Deiters' cells, which support the outer hair cells), the outer and inner pillar cells (forming the tunnel of Corti), and Hensen's cells.

COCHLEAR MECHANO-ELECTRICAL TRANSDUCTION: THE HAIR CELLS

Hair cells are highly differentiated epithelial cells, which form the mechanoreceptors of the sensory epithelia in the inner ear. Both afferent and efferent nerve fibers make synaptic contacts with the hair cells. The characteristic structural feature of the hair cell is the hair bundle, composed of several rows of stereocilia of varying lengths. A stereocilium consists of longitudinally arranged actin filaments, which are extensively cross-bridged. It is surrounded by the plasma membrane of the hair cell and is anchored in the amorphous structure of the cuticular plate, where it ends into a stereociliary rootlet. Due to the cross-bridged actin filaments the stereocilium behaves as a rigid rod. At its base the stereocilium becomes thinner, enabling it to pivot around its base. The direction of this movement is thought to be limited to one plane, which is determined by the orientation of the cross-bridging of the actin filaments within the stereocilium.

The cochlear hair cell is a mechanoreceptor. When the stereociliary bundle is deflected in the excitatory direction (towards the tallest stereocilium), membrane conductance changes and K^+ ions enter the cell through transduction channels located at the apical membranes. The hair-cell membranes are depolarized and Ca^{2+} ions flow into the cell through "voltage-sensitive" ion channels located in the basolateral membranes (Hudspeth, 1985). The increase in intracellular Ca^{2+} causes synaptic vesicles to fuse with the basal membrane of the hair cell; neurotransmitter is released into the synaptic cleft and binds to the membrane of the synapse. The synapse is depolarized, resulting in electrical activity of the nerve fiber.

The outer hair cells (Fig. 2) are positioned on the flaccid part of the basilar membrane and are thought to modulate their response by contraction (Flock et al., 1986; Zenner et al., 1986). Therefore, outer hair cells can be considered

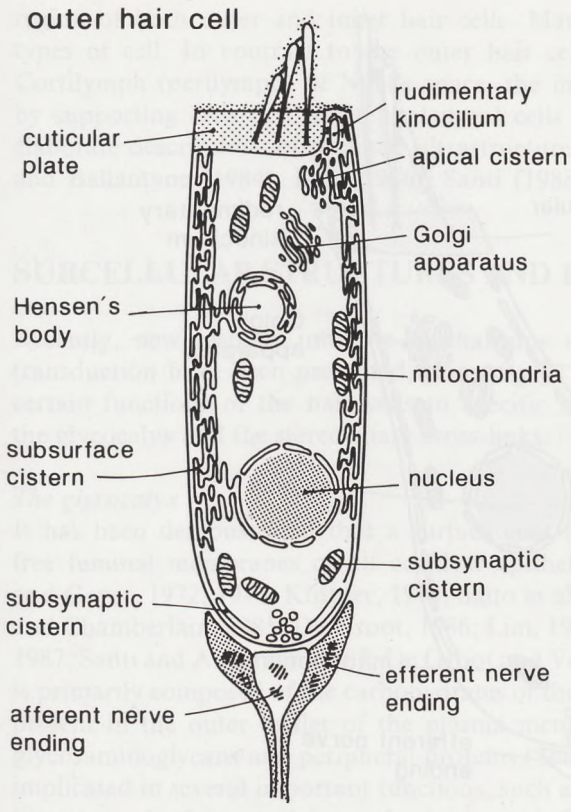

Figure 2. Schematic drawing of an outer hair cell.

as active receptors. Their hair bundles contain stereocilia of varying lengths (short, middle and tall), which are arranged in V- or W-shaped rows. The tallest stereocilia are embedded in the bottom of the tectorial membrane. The number of stereocilia per outer hair cell at the apex is less than at the base of the cochlea. Their length increases from apex to base.

The outer hair cells are cylindrical in shape. Their apex is formed by the cuticular plate into which the stereocilia are inserted. The endoplasmic reticulum consists of 3-4 layers of smooth, interconnecting cisternae, which are closely arranged along the entire inner surface of the lateral membranes; these are called the subsurface cisternae. The subsurface cisternae are in continuity with other parts of the endoplasmic reticulum, such as the apical cisternae (in the cuticle-free region at the apex of the outer hair cell) and the subsynaptic cisternae (located as a single layer along the basal membrane of the outer hair cell).

The inner hair cells (Fig. 3) are positioned on the more rigid part of the basilar membrane. They are considered to be passive receptors. Their stereocilia are also arranged in three rows, but either in a straight line or in a W-shape with

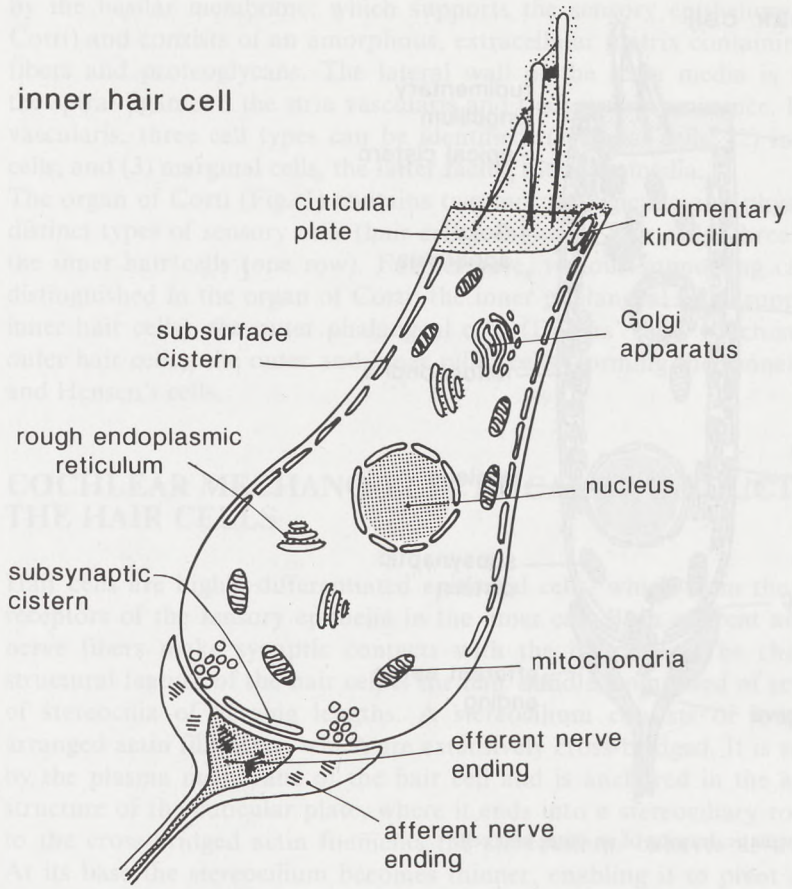

Figure 3. Schematic drawing of an inner hair cell.

a much wider angle than the stereociliary bundles of the outer hair cells. The diameter of the stereocilia is larger than that of the outer-hair-cell stereocilia. The inner-hair-cell stereocilia are not inserted into the tectorial membrane. In contrast to the outer hair cells, the number of stereocilia per inner hair cell varies only slightly along the length of the basilar membrane. However, stereocilia of apical inner hair cells are longer than their basal counterparts. Inner hair cells are flask-shaped, with the nucleus localized in the center of the cell. The apex is formed by the cuticular plate, in which the stereocilia are anchored. Subsurface cisternae of the endoplasmic reticulum, if any, are present as a single layer along the lateral membranes. Rough endoplasmic reticulum is present in the infranuclear region and is arranged in several stacks. Mitochondria are concentrated in the infracuticular region and the synaptic region of both outer and inner hair cells, most likely because these parts have a high energy requirement. Golgi saccules are situated in the infracuticular

region of both outer and inner hair cells. Many vesicles are found in both types of cell. In contrast to the outer hair cells, which are surrounded by Cortilymph (perilymph) of Nuel's space, the inner hair cells are surrounded by supporting cells (i.e. inner phalangeal cells and border cells). (For more elaborate descriptions of cochlear ultrastructure, see overviews by Friedmann and Ballantyne (1984), Lim (1986), Santi (1988), and Harada et al. (1990).)

SUBCELLULAR STRUCTURES AND HAIR CELL FUNCTION

Recently, new insights into the mechanisms underlying mechano-electrical transduction have been presented, primarily as a result of attempts to relate certain functions of the hair cells to specific subcellular structures, such as the glycocalyx and the stereociliary cross-links.

The glycocalyx

It has been demonstrated that a surface coat (glycocalyx) is present on the free luminal membranes of all cochlear epithelia (Spoendlin, 1968; Küttner and Geyer, 1972, 1974; Küttner, 1974; Saito et al., 1977; Forge, 1981; Slepecky and Chamberlain, 1985; De Groot, 1986; Lim, 1986; Prieto and Merchan, 1986, 1987; Santi and Anderson, 1987; De Groot and Veldman, 1988). This glycocalyx is primarily composed of the carbohydrates of the glycoproteins and glycolipids present in the outer leaflet of the plasma membrane (Fig. 4), and of acidic glycosaminoglycans and peripheral proteins (Schrével et al., 1981). It has been implicated in several important functions, such as intercellular recognition and filtration selectivity, and is involved in creating a specific micro-environment

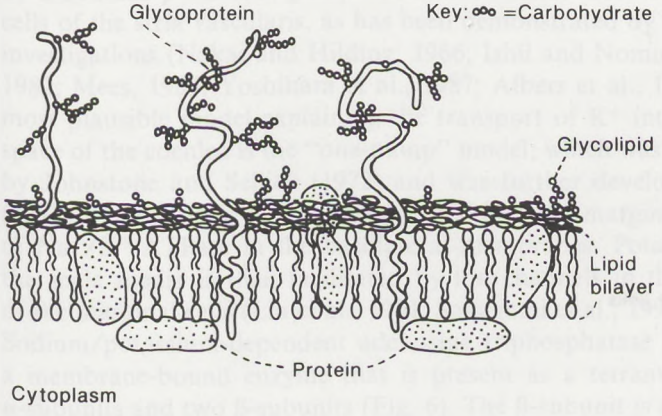

Figure 4. Artist's view of the glycocalyx present on the external surfaces of the plasma membrane. Carbohydrates of glycoproteins and glycolipids form the bulk of the glycocalyx (modified from Sharon and Lis, 1982).

near the external surface of the plasma membrane (Spicer et al., 1981; Trump et al., 1983). It has also been suggested that the glycocalyx of the cochlear hair cells plays a role in mechano-electrical transduction by creating a specific cationic micro-environment around their apical membranes and stereociliary bundles (Slepecky and Chamberlain, 1985; Hudspeth, 1985; Santi and Anderson, 1987). Furthermore, Flock et al. (1977) have suggested that the stereociliary glycocalyx – due to its net negative surface charge – prevents stereocilia from fusion, thus maintaining stereociliary integrity.

Differences in glycocalyx reactivity between the apical membranes of the sensory and supporting cells of the inner ear have frequently been reported (Lim, 1986; Prieto and Merchan, 1987; Santi and Anderson, 1987; Takumida et al., 1988a; Takumida and Bagger-Sjöbäck, 1991). These differences have also been found between the apical and basolateral membranes of outer hair cells (De Groot, 1986; Prieto and Merchan, 1986, 1987; Santi and Anderson, 1987). It has been demonstrated that the glycocalyx of different types of cell can vary in the composition of glycoconjugates, which may be related to the specific functions of these cells (Spicer et al., 1981). Therefore, determination of the composition of the glycocalyx by various cytochemical techniques could be helpful in elucidating its contribution to cell function.

Stereociliary cross-links

Since Flock et al. (1977) demonstrated the presence of lateral connections between individual stereocilia, interest in the function and subcellular morphology of these interstereociliary connections has grown (Fig. 5). Using scanning electron microscopy Pickles et al. (1984) have demonstrated the existence of three types of interstereociliary connections, or "cross-links", of the guinea pig's cochlear hair cells: (1) side-to-side links; (2) row-to-row links;

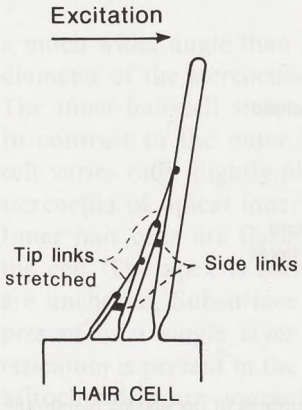

Figure 5. Schematic representation of the localization of cross-links (side and tip links) of hair cells (modified from Pickles et al., 1984).

and (3) tip-to-side links. There has been some debate as to the exact nature (and function) of these structures. Whereas some authors thought stereociliary cross-links to be only fixation artefacts, i.e. remnants of an inadequately fixed glycocalyx (Hackney and Furness, 1986, 1988), others considered them either as specializations of the glycocalyx (Csukas et al., 1987; De Groot and Veldman, 1988) or as a separate morphological and functional entity (Pickles et al., 1984; Rhys-Evans et al., 1985; Osborne et al., 1988; Takumida et al., 1989, 1990; Osborne and Comis, 1990).

The "side links" (side-to-side and row-to-row links) are generally thought to be responsible for the integral movement of the stereociliary bundle. "Tip links" (tip-to-side links) have been implicated to play a major role in mechano-electrical transduction (Pickles et al., 1984; Hudspeth, 1985). Deflection of the stereociliary bundle in the direction of the tallest stereocilia produces stretching of the tip links, that is excitation (Fig. 5), and this is thought to open the transduction channels in or near the stereociliary tips, thus facilitating the influx of K^+ ions (Moran et al., 1981; Hudspeth, 1985, 1989; Furness et al., 1991).

ENDOLYMPH PRODUCTION: THE STRIA VASCULARIS

The mechanisms underlying the mechano-electrical transduction of both the cochlear and vestibular hair cells depend on the maintenance of high K^+ and low Na^+ concentrations in the inner ear's endolymph. In the cochlea, the stria vascularis plays an important role in establishing the unique electrolyte content of the endolymph and in maintaining a positive endocochlear potential of ± 80 mV (Bosher et al., 1973; Bosher, 1980; Juhn et al., 1984; Offner et al., 1987; Sterkers et al., 1988). The high K^+ content of the endolymph is sustained by a sodium/potassium pump in the basolateral membranes of the marginal cells of the stria vascularis, as has been demonstrated by various cytochemical investigations (Nakai and Hilding, 1966; Ishii and Nomura, 1968; Kerr et al., 1982; Mees, 1983; Yoshihara et al., 1987; Albers et al., 1991). At present, the most plausible model explaining the transport of K^+ into the endolymphatic space of the cochlea is the "one-pump" model, which was originally postulated by Johnstone and Sellick (1972) and was further developed by Offner et al. (1987). Potassium is actively transported into the marginal cells by the action of Na^+/K^+-ATPase in the basolateral membranes. Potassium transport into the scala media is then facilitated by ion channels in the apical membranes of the marginal cells (Liu et al., 1991; Sakagami et al., 1991).

Sodium/potassium-dependent adenosine triphosphatase (Na^+/K^+-ATPase) is a membrane-bound enzyme that is present as a tetramer consisting of two α-subunits and two ß-subunits (Fig. 6). The ß-subunit is a 50-kD glycoprotein with unknown function. The α-subunit is a 120-kD nonglycosylated polypeptide with a catalytic site for ATP hydrolysis and three high-affinity sites for binding

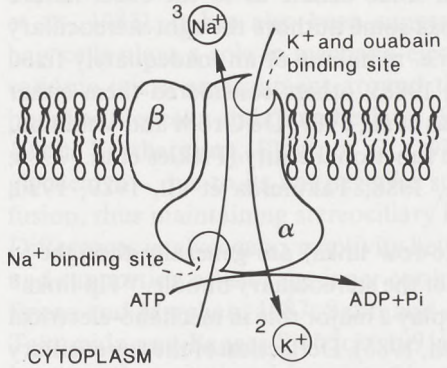

Figure 6. Schematic diagram depicting the enzyme Na^+/K^+-ATPase, which acts as a Na^+/K^+-pump which actively pumps Na^+ out and K^+ into the cell (modified from Alberts et al., 1989)

Na^+ ions at the cytoplasmic surface. Two high-affinity binding sites for K^+ ions as well as a binding site for the cardiac glycoside ouabain are present at the outer surface of the membrane. During transport three Na^+ ions are moved out of and two K^+ ions are moved into the cell per hydrolysed ATP molecule. ATP hydrolysis proceeds in two steps:

(1) Na^+-dependent phosphorylation of the enzyme, coupled to outward transport of Na^+

(2) K^+-dependent dephosphorylation of the enzyme, coupled with the inward transport of K^+. This last step is inhibited by ouabain.

(1) Enzyme + ATP $\xrightarrow{Na^+}$ Enzyme-P + ADP phosphorylation

(2) Enzyme-P $\xrightarrow{K^+}$ Enzyme + P_i dephosphorylation

The phosphatase site can also catalyze K^+-dependent hydrolysis of some simple monophosphate substrates, such as p-nitrophenyl phosphate. This characteristic has frequently been used to localize the enzyme cytochemically at the light- as well as the electron-microscopic level (Ernst, 1972; Firth, 1974; Mayahara and Ogawa, 1980; Mayahara et al., 1980; Hulstaert et al., 1983).
Kuijpers (1969) and Kuijpers and Bonting (1969) used biochemical methods to demonstrate relatively high concentrations of the enzyme in homogenates of the stria vascularis and the spiral ligament of the cochlea. They found that the enzyme activity decreases from the base to the apex of the cochlea. Using various cytochemical detection methods, efforts were made to more specifically localize the membrane-bound enzyme. It was shown to be present in the basolateral membranes of the marginal cells of the stria vascularis and in the membranes of the fibrocytes of the spiral ligament (Ishii and Nomura, 1968;

Nakai and Hilding, 1966; Kerr et al., 1982; Mees, 1983; Yoshihara et al., 1987; Iwano et al., 1990; Albers et al., 1991; Spicer and Schulte, 1991).

THE VESTIBULAR APPARATUS

Unlike the cochlea, the vestibular apparatus is situated in the medial wall of the bulla and completely surrounded by bone. It consists of the sacculus, utriculus, and the three semicircular canals. The sacculus and utriclus are juxtaposed, with the utriculus in the upper position and in direct contact with the semicircular canals. The lumen of the sacculus communicates with the scala media of the cochlea through the ductus reuniens. The three semicircular canals are positioned on top of the utriculus at approximately right angles to one another. In transverse section, the endolymph-containing lumen of the semicircular canals is delineated by an epithelium, whereas the perilymphatic space is filled with a subepithelial, loose connective tissue. The endolymphatic space of the semicircular canals communicates directly with the utriculus. Each semicircular canal forms an ampulla, which contains the crista ampullaris projecting into the lumen.

The vestibular sensory cells are located in the macula sacculi, the macula utriculi (Fig. 7), and the cristae ampullares of the semicircular canals (Fig. 8). These epithelia demonstrate a remarkable uniformity in their general morphological structure. They contain two types of hair cells (Types I and II), presumably of different functional significance. The Type-I hair cell is flask-shaped and enveloped by an afferent nerve ending (calyx). Efferent nerve fibers have synaptic

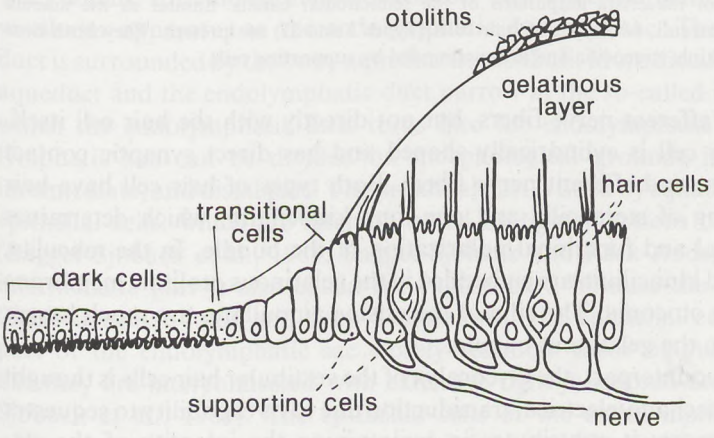

Figure 7. Morphological structure of the macula utriculi. Flask-shaped (Type I) and cylindrically shaped (Type II) hair cells are surrounded by supporting cells. The longest cilium is the kinocilium, the others are stereocilia. The sensory epithelium of the macula sacculi has a similar morphology but lacks the dark cells.

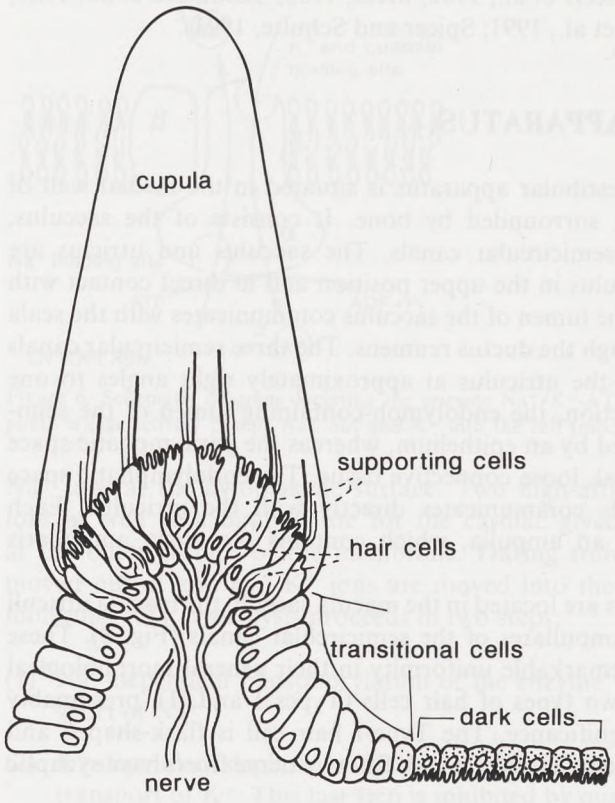

Figure 8. Structure of the crista ampullaris of the semicircular canals. Similar to the macula utriculi and macula sacculi, two types of hair cells (Types I and II) are present. They both bear a kinocilium and multiple stereocilia and are surrounded by supporting cells.

contact with the afferent nerve fibers, but not directly with the hair cell itself. The Type-II hair cell is cylindrically shaped and has direct synaptic contact with both afferent and efferent nerve fibers. Both types of hair cell have hair bundles consisting of stereocilia and one long kinocilium, which determines the morphological and functional polarization of the bundle. In the maculae, the stereocilia and kinocilium are embedded in the gelatinous otolithic membrane which carries the otoconia. The cilia of the cristae ampullares are much longer and protrude into the gelatinous cupula.

Like its cochlear counterpart, the glycocalyx of the vestibular hair cells is thought to play a role in mechano-electrical transduction due to its capability to sequester cations. Furthermore, it contributes in maintaining the integrity of the stereociliary bundle (Takumida et al., 1989; Khan et al., 1991; Takumida and Bagger-Sjöbäck, 1991). Stereociliary cross-links have also been reported to be present between the stereocilia of vestibular hair cells (Bagger-Sjöbäck and

Wersäll, 1973; Hillman, 1973; Jeffries et al., 1986; Takumida et al., 1988a; Oda et al., 1992). The side links connect the flanks of the stereocilia and might be involved in the simultaneous movement of all individual stereocilia within the bundle. Tip links, as described for the stereocilia of the cochlear hair cells, have also been demonstrated in the vestibular apparatus (Jeffries et al., 1986; Takumida et al., 1989; Takumida et al., 1990); they have been suggested to be of importance to the mechano-electrical transduction process of these hair cells. The sensory epithelia of the vestibular apparatus are surrounded by a zone of transitional epithelium. Beyond this zone the so-called "dark cells" can be found in the utriculus and the ampullae, but these cells are not present in the sacculus (Kimura, 1969). Dark cells resemble the marginal cells of the stria vascularis of the cochlea. Their basolateral membranes have numerous infoldings and demonstrate a high activity of the enzyme Na^+/K^+-ATPase (Nakai and Hilding, 1968; Mees et al., 1983; Burnham and Stirling, 1984; Yoshihara et al, 1987; Albers et al., 1991). This high activity corresponds with the production of K^+-rich endolymph by the vestibular dark cells (Sterkers, 1985; Sterkers et al., 1988; Ferrary et al., 1992). In contrast to the cochlea, the positive endolymphatic potential is less explicit (Juhn, 1984; Drescher and Kerr, 1985).

THE ENDOLYMPHATIC DUCT AND SAC

The endolymphatic sac lies partly within a bony niche on the medial surface of the temporal bone (intraosseous part) and partly outside this niche, covered by the dura mater of the posterior cranial fossa (extraosseous part; Fig. 9). The ductus utriculo-sacculari connects the endolymphatic spaces of the vestibular apparatus to the endolymphatic duct and sac. The endolymphatic duct is surrounded by the bony aqueductus vestibuli. More distally, the vestibular aqueduct and the endolymphatic duct narrow at the so-called isthmus, beyond which the endolymphatic duct turns into the endolymphatic sac. The endolymphatic sac can be divided on morphological grounds into a proximal, intermediate, and distal part. The proximal part is lined by squamous or cuboidal epithelial cells, which are interconnected by tight junctions of the leaky type (Bagger-Sjöbäck et al., 1981; Bagger-Sjöbäck and Rask-Andersen, 1986). The intermediate part is also called the rugose part, because the epithelial lining forms irregular folds and deep depressions. The epithelial cells of the distal part of the endolymphatic sac closely resemble those of the proximal part, but they are interconnected with extensive tight-junctional networks (Bagger-Sjöbäck et al., 1981). The epithelial cells of the endolymphatic sac rest on a basement membrane, which separates it from the subepithelial space consisting of loosely arranged collagen fibers and blood and lymph vessels. The lumen of the endolymphatic sac is filled with colloid and contains free-floating cells such as macrophages and leucocytes.

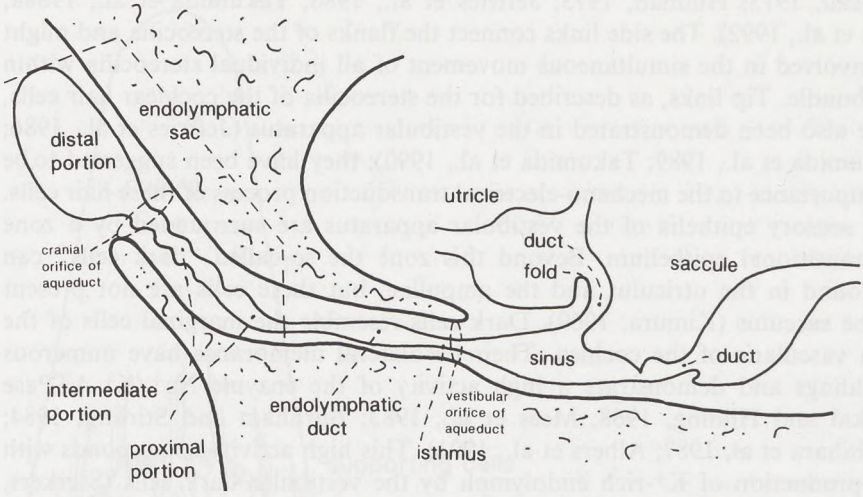

Figure 9. The endolymphatic duct runs through the aqueductus vestibuli within the medial portion of the temporal bone and connects the vestibular apparatus to the endolymphatic sac.

Two types of epithelial cells can be distinguished in both the intermediate and distal part of the endolymphatic sac, the so-called "dark" and "light" cells. On the basis of their ultrastructural appearance, it has been suggested that they have different functions (Lundquist, 1976; Friberg et al., 1985; Fukazawa, 1991). The light cells are assumed to be involved in transepithelial transport of fluids, while the dark cells are thought to have a phagocytic function. Differences in thickness or reactivity of the glycocalyx between these two types of cell could not be demonstrated (Takumida et al., 1988b). The basolateral membranes of both cell types do not exhibit much Na^+/K^+-ATPase activity (Albers et al., 1991). This is in concordance with the low concentration of K^+ in the lumen of the endolymphatic sac (Sterkers et al., 1988).
A multitude of functions has been allotted to the endolymphatic sac: (1) resorption of endolymph (Lundquist, 1965; Kimura and Schuknecht, 1965; Adlington, 1967, 1984; Manni and Kuijpers, 1987); (2) degradation and absorption of otoconia (Imoto et al., 1983; Erwall et al., 1988); (3) regulation of endolymph pressure (Friberg et al., 1986; Rask-Andersen et al., 1987; Wackym et al., 1987); (4) a secretory function (Adlington, 1984; Friberg et al., 1986; Barbara et al., 1988; Jansson et al., 1991); and (5) to be a site of inner ear immunological responses (Rask-Andersen and Stahle, 1979; Arnold et al., 1984; Altermatt et al., 1990).

EXPERIMENTAL ENDOLYMPHATIC HYDROPS

Since Hallpike and Cairns (1938) and Yamakawa (1938) suggested that

endolymphatic hydrops of the cochlea is the pathological substrate of Menière's disease, various efforts have been made to develop an experimental model of endolymphatic hydrops (Lindsay, 1947; Lindsay et al., 1952; Kimura and Schuknecht, 1965; Beal, 1968; Konishi and Shea, 1975; Manni et al., 1986). Kimura and Schuknecht (1965) were the first to succeed in obtaining an endolymphatic hydrops in the guinea pig cochlea with a 100% success rate. The endolymphatic sac was surgically obliterated through an intradural posterior-fossa approach, and the endolymphatic duct was subsequently obstructed. This animal model proved to be valid and reliable in the guinea pig, and it has been studied extensively. The observation that, following obliteration of the endolymphatic duct and sac, Reissner's membrane gets distended – as a consequence of disturbed or even absent absorption of endolymph – supported the hypothesis of Guild (1927) that a longitudinal flow of endolymph exists within the cochlea (Kimura et al., 1980).

Morphological changes in endolymphatic hydrops

After induction of endolymphatic hydrops, a series of pathological changes develops, which start at the apex and progress, in time, towards the base of the cochlea (Kimura and Schuknecht, 1965; Kimura, 1967; Albers et al., 1987a; Ruding et al., 1991). The most prominent change of all is the distension of Reissner's membrane (Fig. 10), with the loss of intercellular contacts between individual mesothelial cells. Later, connections can be seen between Reissner's membrane and the bony wall of the scala vestibuli, whereas ruptures and folds have been observed occasionally (Shinozaki and Kimura, 1980; Lawrence, 1983; Ruding et al., 1987; Albers et al., 1987b). Initially, slight strial edema can be seen, but at a later stage more prominent changes appear, such as the loss of intermediate cells and of the basolateral invaginations of the marginal cells (Albers et al., 1987b).

In the organ of Corti, degenerative changes are observed, especially in the outer hair cells. One of the earliest changes is a disturbance of the stereociliary bundle, which is eventually followed by a complete loss of the stereocilia. Horner et al. (1988) and Rydmaker and Horner (1991) reported atrophy of the short- and middle-sized stereocilia of the outer hair cells as one of the early degenerative changes in endolymphatic hydrops. In the same preparations, they also observed loss of cross-links. These findings have been corroborated by Ruding et al. (1991). Finally, the hair cells degenerate and are replaced by Deiters' cells forming so-called "phalangeal scars" (Kimura and Schuknecht, 1965; Kimura, 1967; Shinozaki and Kimura, 1980; Albers et al., 1987a). Albers et al. (1987c) reported a disorganization of the glycocalyx of the epithelial cells lining the scala media in an early stage of endolymphatic hydrops.

Functional changes in endolymphatic hydrops

In endolymphatic hydrops a discrepancy is found between the loss of hair cells in the cochlea and the recorded threshold shifts of the compound action

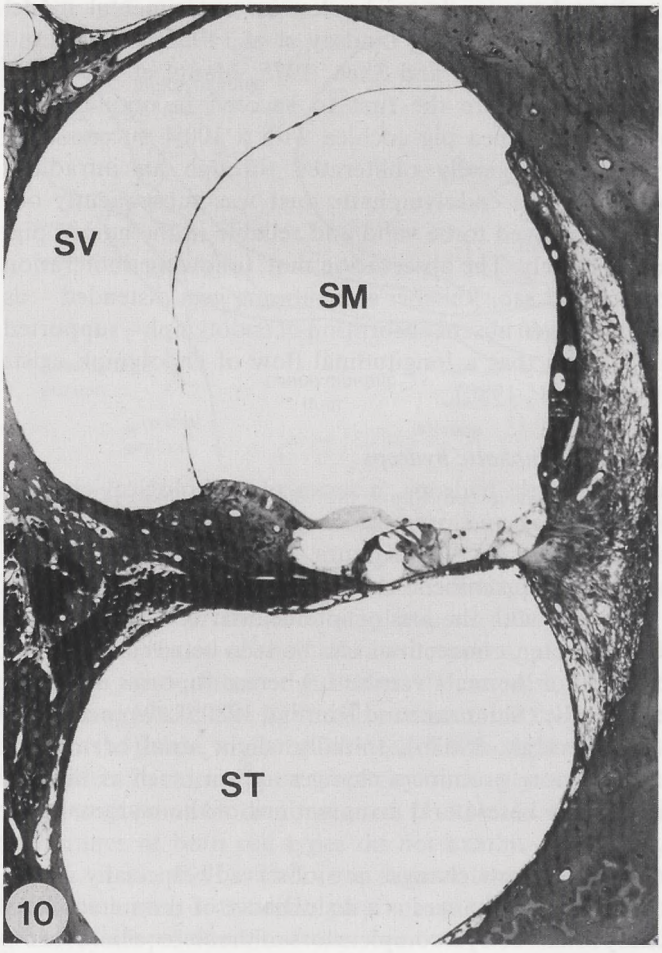

Figure 10. Light micrograph of a cross-section through a guinea pig cochlear turn showing an endolymphatic hydrops with Reissner's membrane distended. SM = scala media; SV = scala vestibuli; ST = scala tympani (x 80).

potential during electrocochleography. The functional changes are more elaborate than the loss of hair cells can account for (Rydmaker and Horner, 1991). More subtle, subcellular defects of hair cells – such as a disorganization of the hair cell's glycocalyx or loss of stereociliary cross-links – might be responsible for these early functional changes. Functional changes that have been reported after obliteration of the endolymphatic duct and sac include: (1) fluctuating and, at a later stage, permanent threshold shifts of the compound action potential (Aran et al., 1984; Harrison et al., 1984; Horner and Cazals, 1987; Van Deelen et al., 1987); (2) an increased negative summating potential

(Kumagami and Miyazaki, 1983; Aran et al., 1984; Van Deelen et al., 1987); and (3) decreased cochlear microphonics (Konishi and Kelsey, 1976; Konishi et al., 1981; Morizono et al., 1985; Klis and Smoorenburg, 1988). Furthermore, the endocochlear potential is reported to be decreased following obliteration of the endolymphatic duct and sac (Konishi and Kelsey, 1976; Cohen and Morizono, 1984; Sziklai et al., 1989).

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Chapter 2

Glycocalyx heterogeneity in normal and hydropic cochleas of the guinea pig

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The free, luminal surfaces of various types of cell exhibit a coat (glycocalyx) consisting largely of glycoproteins, glycolipids, and glycosaminoglycans. These glycoconjugates serve several important functions, one of which is to create a selective filtration boundary for both fluids and ions (Spicer et al., 1981). In this respect the cochlear hair-cell glycocalyx is thought to play an important role in the mechano-electrical transduction process by creating a cation-rich micro-environment along the endolymphatic surfaces of the hair cells (Slepecky and Chamberlain, 1985; Prieto and Merchan, 1987). Furthermore, the stereociliary glycocalyx is likely to prevent fusion of stereocilia through repulsion by its net negative surface charge. Consequently, stereociliary integrity is maintained (Flock et al., 1977).

The variability in cell surface glycoconjugates, which can be revealed by cytochemical markers, is considered to reflect differences in cell function (Spicer et al., 1981). Whether or not differences in composition exist between the endo- and perilymphatic glycocalyx of the cochlear duct, as well as between the sensory and supporting cells of the organ of Corti, is still subject to discussion (Lim, 1986; De Groot, 1986; Santi and Anderson, 1987; Albers et al., 1987; De Groot and Veldman, 1988).

A growing number of ultrastructural and cytochemical studies indicate that the glycocalyx lining the endolymphatic compartment of the inner ear changes biochemically after administration of ototoxic drugs (Forge, 1981; Furness and Hackney, 1986; De Groot and Veldman, 1988; Takumida et al., 1989a) and following acoustic trauma (Takumida et al., 1989b). Up till now, only limited data are available on the dynamics of cell surface glycoconjugates in experimental endolymphatic hydrops (Albers et al., 1987). Since this condition appears to involve a fluid/ion imbalance in the endolymphatic compartment (Konishi and Shea, 1975), it is pertinent to determine whether cell surface glycoconjugates are involved in the pathophysiology of this disorder.

Therefore, we investigated the cochlear glycocalyx in normal cochleas as well as in cochleas in which the endolymphatic duct and sac had been surgically obliterated. The electron-dense markers cationized ferritin and colloidal thorium were used to visualize the glycocalyx ultrastructurally. These cationic markers have particular affinities for the anionic sites of the cell membranes. In addition, normal cochleas were treated with neuraminidase and hyaluronidase in order to identify the reactive groups responsible for colloidal thorium and cationized ferritin reactivity.

MATERIALS AND METHODS

Twenty-six female albino guinea pigs (Dunkin Hartley, body weight 250-300 g) were used. The cochleas of six animals (N=6) were used for enzymatic digestion experiments. In 20 animals the endolymphatic sac and duct of the left ear were surgically obliterated by the extradural posterior fossa approach (Konishi and Shea, 1975). The right ear served as a control. Three (N=10) or six months (N=10) after surgery, the animals were sacrificed. Tissue fixation was performed by means of intravascular perfusion with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The bullae were removed and immersed in the same fixative for an additional 2 h at 4°C.

Enzymatic digestion of normal cochleas

Of the 12 cochleas of the non-operated animals (N=6), a group of six cochleas were post-fixed with 1% aqueous OsO_4 containing 1% $K_4Ru(CN)_6$ for 2 h at 4°C. The other group of six cochleas were post-fixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C. Three cochleas of each group were microdissected, followed by incubation of the cochlear turns in 40 IU/ml neuraminidase (Type V, Sigma, St. Louis, USA) in 0.05 M sodium acetate buffer (pH 4.5) for 4 h at 37°C to remove sialic acid residues. The remaining six cochleas (i.e., three post-fixed with OsO_4 and three post-fixed with $OsO_4/K_4Ru(CN)_6$) were microdissected and the cochlear turns were subsequently incubated in 150 IU/ml hyaluronidase (Type IX, Sigma, St. Louis, USA) in 0.05 sodium acetate buffer (pH 5.0) for 4 h at 37°C to remove hyaluronic acid.

The cochlear turns of the specimens post-fixed with OsO_4 were incubated overnight in 1% colloidal thorium (Thoria-sol®, Polysciences, Warrington, USA) in 3% acetic acid (pH 2-2.5) at room temperature. The cochlear turns post-fixed with $OsO_4/K_4Ru(CN)_6$ were incubated in 5 mg/ml cationized ferritin in phosphate-buffered saline (pH 7.4) for 1 h at room temperature.

Glycocalyx cytochemistry of hydropic vs. control cochleas

From each operated group (three (N=10) and six months (N=10) after operation), both the hydropic and the control cochleas of five animals were post-fixed with 1% aqueous OsO_4 containing 1% $K_4Ru(CN)_6$ for 2 h at 4°C. The cochlear turns were then microdissected and incubated in 5 mg/ml cationized ferritin in phosphate-buffered saline (pH 7.4) for 1 h at room temperature.

Cochleas of the other five operated animals from each group were post-fixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C. After microdissection, the cochlear turns were incubated overnight in 1% colloidal thorium (Thoria-sol®, Polysciences, Warrington, USA) in 3% acetic acid (pH 2-2.5) at room temperature.

Tissue processing and sectioning
All cochlear turns were dehydrated in a graded ethanol series and embedded in Spurr's low-viscosity resin. Ultrathin sections were contrast-stained with 7% uranyl acetate in 70% methanol and Reynolds' lead citrate; they were then examined by transmission electron microscopy (Philips EM 201c; 60 kV).

RESULTS

Normal cochleas
Cationized ferritin showed a delicate pattern of reactivity. All surfaces in the cochlear duct reacted equally strongly with this marker (Figs. 1A, B). Regional differences in reactivity between the various epithelial surfaces could not be observed.
Colloidal thorium reactivity of the cochlear glycocalyx was exhibited by a granular, flocculent precipitate with high electron density. Thorium reactivity of the basolateral membranes of the outer hair cells was less pronounced than reactivity of the apical membranes (Fig. 2A). Occasionally, a more prominent reactivity of the apical membranes of the outer hair cells was seen as compared to the membranes of the adjacent Deiters' cells (Fig. 2A). The endo- and perilymphatic surfaces of Reissner's membrane reacted equally strong with colloidal thorium (Fig. 2B). Thorium reactivity of the endolymphatic surface of the stria vascularis appeared to be less prominent than that of the surfaces of the organ of Corti and Reissner's membrane (Fig. 2C).
Digestion with either neuraminidase or hyaluronidase did not affect cationized ferritin reactivity. However, after enzymatic digestion with neuraminidase, thorium reactivity of the endolymphatic glycocalyx of the cochlear duct was greatly reduced (Figs. 3A, B). Thorium reactivity of the perilymphatic glycocalyx of the epithelia was only slightly affected (Fig. 3A), except for the basolateral membranes of the outer hair cells and Deiters' cells of the organ of Corti, which showed greatly reduced thorium reactivity (Fig. 3B). Digestion with hyaluronidase did not affect thorium reactivity. The results are summarized in Table 1.

Hydropic cochleas
Examination of the cochleas by transillumination during microdissection showed a moderate to severe distension of Reissner's membrane in all turns of the operated cochleas, indicating an endolymphatic hydrops. This phenomenon was never observed in the non-operated control ears.
In the apical turns of the three-month hydropic cochleas, several ultrastructural changes characteristic of an endolymphatic hydrops could be observed. These included defects in the mesothelial cell layer of Reissner's membrane, edema of the stria vascularis, and disarrangement and loss of the stereocilia of the outer hair cells. The glycocalyx of all cochlear epithelia displayed reactivity

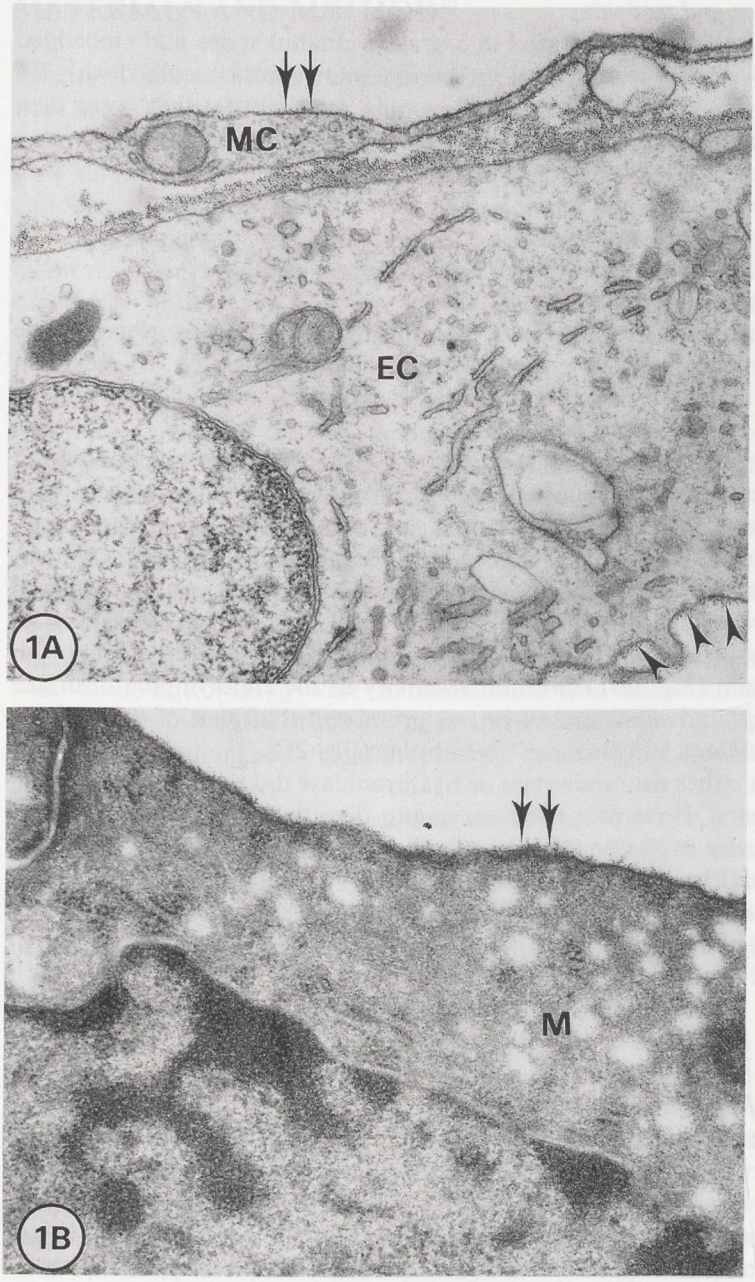

Fig. 1. (1A) Cationized ferritin reactivity of the endolymphatic (arrowheads) and perilymphatic surfaces (arrows) of Reissner's membrane; MC = mesothelial cell, EC = epithelial cell (x 16,000). (1B) Cationized ferritin reactivity of the endolymphatic surface (arrows) of the stria vascularis; M = marginal cell (x 24,000).

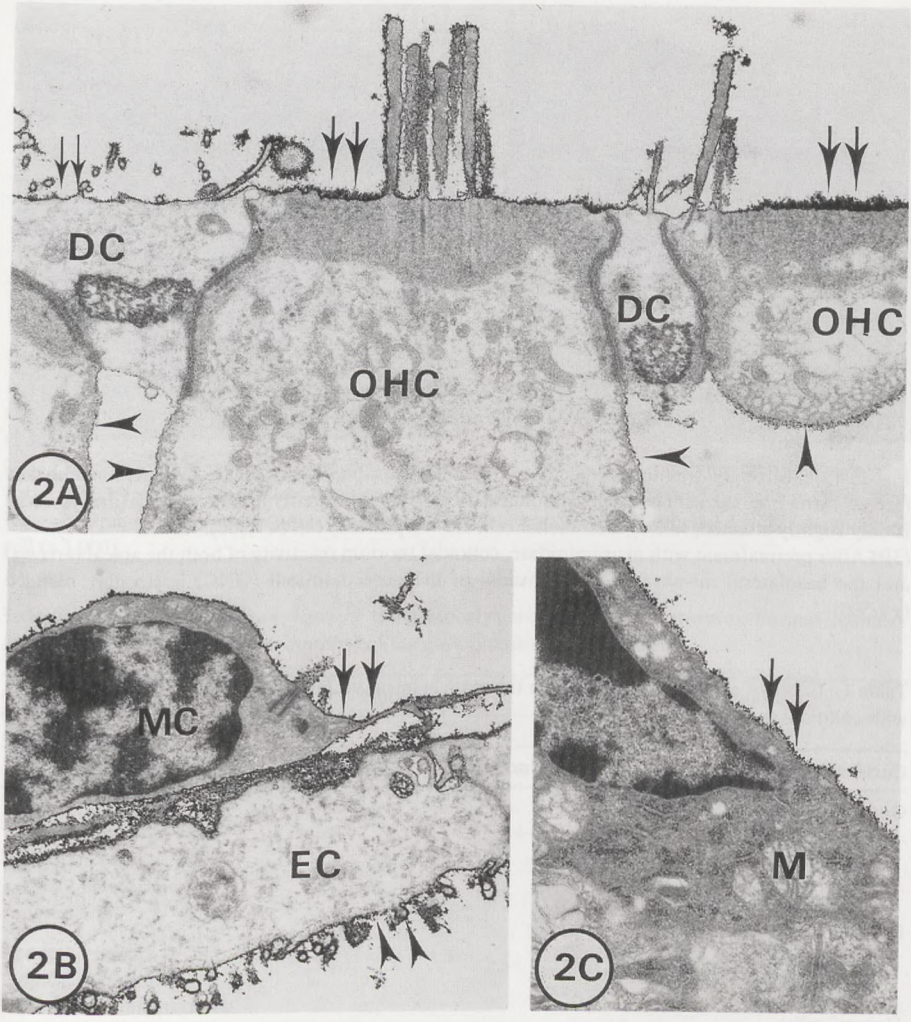

Fig. 2. (2A) More pronounced colloidal thorium reactivity of the apical membranes (arrows) of the outer hair cells (OHC) as compared to the basolateral membranes (arrowheads) and the apical membranes (small arows) of the Deiters' cells (DC) (x 15,000).
(2B) Equally strong colloidal thorium reactivity of the endolymphatic (arrowheads) and perilymphatic surfaces (arrows) of Reissner's membrane; MC = mesothelial cell, EC = epithelial cell (x 10,000).
(2C) Less prominent colloidal thorium reactivity of the apical membranes (arrows) of the marginal cells (M) (x 15,000).

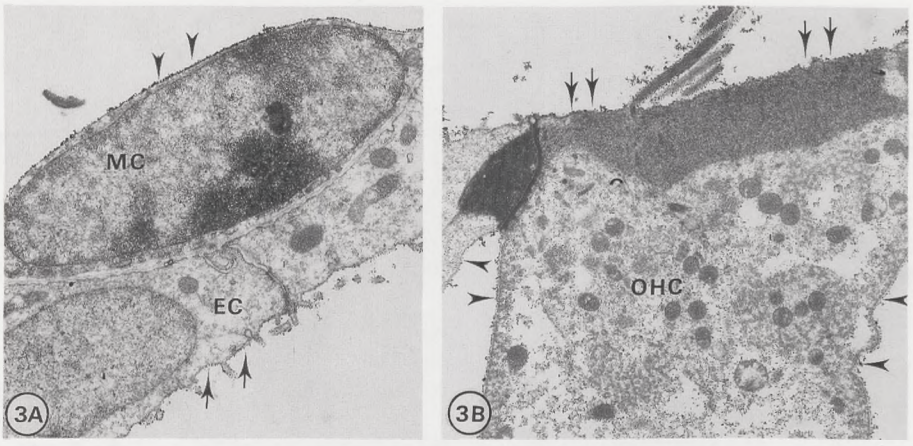

Fig. 3. (3A) Strongly diminished reactivity of the endolymphatic surface of Reissner's membrane (arrows) after pretreatment with neuraminidase. Reactivity of the perilymphatic surface (arrowheads) is only slightly affected; MC = mesothelial cell, EC = epithelial cell (x 7,500).
(3B) After pretreatment with neuraminidase, colloidal thorium reactivity of both the apical (arrows) and the basolateral membranes (arrowheads) of the outer hair cell (OHC) is strongly reduced (x 7,500).

Table 1. Effect of enzymatic digestion on the cytochemical visualization of the cochlear glycocalyx with colloidal thorium.

Cochlear tissues	Normal	Neuraminidase	Hyaluronidase
Outer hair cells:			
apical membranes	++	-	++
stereocilia	+	-	+
basolateral membranes	±	-	±
Inner hair cells:			
apical membranes	+	-	+
stereocilia	+	-	+
Deiters' cells:			
apical membranes	+	-	+
basolateral membranes	±	-	±
Basilar membrane:			
tympanal cells	+	±	+
Stria vascularis:			
marginal cells	±	-	±
Reissner's membrane:			
endolymphatic surface	+	-	+
perilymphatic surfaces	+	±	+

(++ = strong reactivity, + = positive reactivity, ± = weak reactivity, - = strongly reduced reactivity)

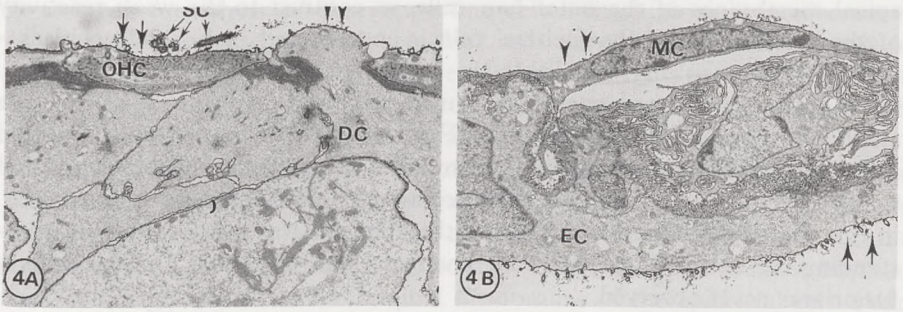

Fig. 4. (4A) Colloidal thorium reactivity of the organ of Corti in a three-month hydropic cochlea. No differences in reactivity between the apical surfaces of Deiters' cells (DC) (arrowheads) and the outer hair cells (OHC) (arrows) can be seen. The stereociliary bundle (SC) is disarranged (x 3,000).
(4B) Colloidal thorium reactivity of the endolymphatic (arrows) and perilymphatic (arrowheads) surface of Reissner's membrane remains unaffected in a six-month hydropic cochlea. Note that Reissner's membrane demonstrates pathological alterations; MC = mesothelial cell, EC = epithelial cell (x 3,000).

Table 2. Cytochemical visualization of the glycocalyx with colloidal thorium in normal cochleas and after obliteration (3 and 6 months) of the endolymphatic duct and sac.

Cochlear tissues	Normal	3 months	6 months
Outer hair cells:			
apical membranes	++	+	+
stereocilia	+	+	+
basolateral membranes	±	±	±
Inner hair cells:			
apical membranes	+	+	+
stereocilia	+	+	+
Deiters' cells:			
apical membranes	+	+	+
basolateral membranes	±	±	±
Basilar membrane:			
tympanal cells	+	+	+
Stria vascularis:			
marginal cells	±	±	±
Reissner's membrane:			
endolymphatic surface	+	+	+
perilymphatic surfaces	+	+	+

(++ = strong reactivity, + = positive reactivity, ± = weak reactivity)

for both markers. Nonetheless, the difference between the strongly reactive apical membranes of the outer hair cells, in contrast to the weak reactivity of the membranes of the Deiters' cells - as observed in normal cochleas - was no longer seen (Fig. 4A, Table 2).
In the six-month hydropic cochleas, the ultrastructural changes were more severe and had progressed to the lower turns. Frequently, the stria vascularis appeared atrophied, and folds in Reissner's membrane could be seen. In the organ of Corti the outer hair cells had been replaced by Deiters' cells. Nevertheless, in this group glycocalyx reactivity of the epithelia was still present, notwithstanding the extent of degenerative changes (Fig. 4B, Table 2). Stereociliary fusion was never observed.

DISCUSSION

In the present study we investigated the glycocalyx of the various epithelia in normal cochleas and during experimentally induced endolymphatic hydrops using the electron-dense markers cationized ferritin and colloidal thorium. Both markers displayed excellent visualization of the glycocalyx of the cochlear epithelia. Thorium reactivity of the endolymphatic glycocalyx was greatly reduced after neuraminidase treatment, but appeared to be unaffected following hyaluronidase treatment. Therefore, it is likely that this reactivity is due to the presence of sialic acid residues. In contrast, cationized ferritin reactivity was not altered by pretreatment with either enzyme. This is in agreement with the observation that these cationic protein molecules bind non-selectively to all anionic sites of the glycocalyx, at the pH used in our experiments (Schrével et al., 1981).
In the normal cochlea, colloidal thorium reactivity of the endolymphatic surface of the stria vascularis is less abundant than that of the endolymphatic surfaces of the organ of Corti and Reissner's membrane. This seems to indicate that the glycocalyx of the stria vascularis has a relatively lower content of sialic acid residues. Similar results have been reported by Nishiyama et al. (1991) using lectin-gold probes. Sialic acid is considered to influence transport of cations across cell membranes (Glick and Githens, 1965; Langer et al., 1976; Roth and Taatjes, 1985). We thus speculate that the relatively low content of sialic acid may be functionally related to the intensive transport of cations (i.e. potassium) across the endolymphatic (i.e. apical) membranes of the strial marginal cells.
The apical membranes of the outer hair cells display a more pronounced reactivity with colloidal thorium than their basolateral membranes, or the membranes of the Deiters' cells. This implies a higher content of sialic acid of the endolymphatic glycocalyx of the outer hair cells. That would concur with the recent findings of Takumida and Bagger-Sjöbäck (1991). They reported stronger binding of wheat germ agglutinin (specific for polymeric N-acetyl-

glucosamine and sialic acid) to the apical membranes of vestibular hair cells as compared to those of the supporting cells. The high sialic acid content of the endolymphatic glycocalyx of hair cells may play a significant role in creating a specific (cationic) micro-environment in order to facilitate the mechano-electrical transduction process (Slepecky and Chamberlain, 1985; Prieto and Merchan, 1987; Takumida and Bagger-Sjöbäck, 1991).

As is demonstrated in the present study, the endolymphatic surfaces of the scala media appear to be rich in sialic acid, unlike the glycocalyx of the perilymphatic compartments of the cochlea. A recent investigation of the endolymphatic sac epithelium has also demonstrated a high content of sialic acid of the glycocalyx of the luminal membranes of the epithelial cells (Van Benthem et al., 1992). In view of its suggested role in transmembranous cation transport (Glick and Githens, 1965; Langer et al., 1976; Roth and Taatjes, 1985), the high sialic acid content of the glycocalyx lining the endolymphatic duct may be of functional importance to the longitudinal concentration gradient of sodium and potassium ions in the endolymphatic compartment of the inner ear (Rask-Andersen et al., 1981).

Within three months, the differences in thorium reactivity between the apical membranes of the outer hair cells and the membranes of the Deiters' cells - as observed in normal cochleas - could no longer be demonstrated. This effect on the glycocalyx, as well as the evident loss of stereociliary cross-links (Horner et al., 1988), probably contributes to the initial functional changes in the outer hair cells at an early stage of endolymphatic hydrops.

Glycocalyx reactivity of the other cochlear epithelia was not reduced in three- or six-month hydropic cochleas, using either marker. This observation does not entirely confirm the findings of Albers et al. (1987), who reported diminished glycocalyx contrast-staining of the endolymphatic surfaces of both Reissner's membrane and the stria vascularis - and, to a lesser extent, of the endolymphatic surfaces of the organ of Corti - in experimental endolymphatic hydrops. They only used routine post-fixation with $OsO_4/K_4Ru(CN)_6$ for the visualization of the cochlear glycocalyx. This might explain the discrepant findings reported in the present study, especially since different moieties are involved in $OsO_4/K_4Ru(CN)_6$ post-fixation and subsequent glycocalyx contrast-enhancement (De Groot, 1986; De Groot and Veldman, 1988). We did not observe any reduction of glycocalyx reactivity of the endolymphatic surfaces of either Reissner's membrane or the stria vascularis in experimental endolymphatic hydrops. Yet, this does not exclude the possibility that non-reactive moieties of the glycocalyx are actually affected.

In our preparations, stereociliary fusion was never observed, whereas it is reported during aminoglycoside intoxication (Takumida et al., 1989a), after acoustic trauma (Spoendlin, 1968; Takumida et al., 1989b), and in hereditary inner ear disease (Ernstson, 1972). In ototoxicity and hereditary inner ear disease, stereociliary fusion is thought to be due to a failure of hair cells to produce negatively charged glycoconjugates to keep the stereocilia separated. During

acoustic trauma the mechanical impact is considered to be large enough to neutralize the repulsive forces between stereocilia and therefore to induce fusion (Flock et al., 1977). Since we did not observe stereociliary fusion in our specimens, this could mean that glycoconjugates responsible for interstereociliary repulsion are still present during hair-cell degeneration in experimental endolymphatic hydrops. This is supported by the observation that the reactivity of the stereociliary glycocalyx for both colloidal thorium and cationized ferritin is not reduced in hydropic cochleas.

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Chapter 3

Structure and composition of stereociliary cross-links in normal and hydropic cochleas of the guinea pig

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Structure and composition of stereociliary cross-links in normal and hydropic cochleas of the guinea pig

The stereocilia of cochlear hair cells exhibit a glycocalyx which is in continuity with the apical membranes of the hair cells. This glycocalyx is largely composed of the sugar residues from membrane glycoproteins and glycolipids, and of glycosaminoglycans (Spicer et al., 1981). Two important functions have been attributed to the stereociliary glycocalyx: (1) it is thought to provide a cation-rich microenvironment around the stereociliary membrane to facilitate transduction (Slepecky and Chamberlain, 1985); and (2) it may prevent stereociliary fusion through repulsion by means of its net negative surface charge, whereby it maintains stereociliary integrity (Flock et al., 1977).

Other structures likely to be of importance in maintaining stereociliary integrity are the stereociliary cross-links (Pickles et al., 1984; Hackney and Furness, 1986, 1988; Osborne et al., 1988; Takumida et al., 1988; Osborne and Comis, 1990). Pickles et al. (1984) have proposed the existence of three types of cross-links: side-to-side, row-to-row, and tip-to-side links. The first two types (side links) are considered to be responsible for the integral movement of the stereociliary bundle. The tip-to-side links are of a more delicate structure, and have been thought to play an essential role in the mechano-electrical transduction process (Hudspeth, 1985; Pickles et al., 1984). Pickles et al. (1987a, 1987b) have shown that tip links are vulnerable to acoustic overstimulation as well as to chronic kanamycin application. Ultrastructural changes of the stereociliary cross-links and glycocalyx during experimentally induced endolymphatic hydrops have been occasionally documented (Albers et al., 1987; Horner et al., 1988; Ruding et al., 1991).

In this study the stereociliary cross-links and glycocalyx were investigated in normal and hydropic cochleas of the guinea pig. The electron-dense markers cationized ferritin and colloidal thorium were used for ultrastructural visualization of the stereociliary cross-links. In order to characterize the reactive groups involved, enzymatic digestions were performed with neuraminidase and hyaluronidase.

MATERIALS AND METHODS

Twenty-three female albino guinea pigs (Dunkin Hartley, body weight 250-300 g) were used in this study. In 20 animals the endolymphatic sac and duct of the left ear were surgically obliterated using the extradural posterior fossa

approach. The right ear served as control. Three animals were not operated on and were used for enzymatic digestion experiments.
The operated animals were sacrificed three (N=10) or six months (N=10) after surgery. Initial tissue fixation was performed by means of intravascular perfusion with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The bullae were removed and immersed in the same fixative for an additional 2 h at 4°C.

Cationized ferritin
From the three- and six-month group, the cochleas of five animals were post-fixed in 1% aqueous OsO_4 containing 1% $K_4Ru(CN)_6$. They were then microdissected and incubated in 5 mg/ml cationized ferritin in phosphate-buffered saline (pH 7.4) for 1 h at room temperature.

Colloidal thorium
The cochleas from the remaining five operated animals from both groups were post-fixed in 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C. After microdissection, the cochlear turns were incubated overnight in 1% colloidal thorium (Thoria-sol®, Polysciences, Warrington, USA) in 3% acetic acid (pH 2.5) at room temperature.

Enzymatic digestions
The cochleas (N=6) of the three non-operated animals were post-fixed in 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at 4°C. Microdissection of three cochleas was followed by incubation of the cochlear turns in 40 IU/ml neuraminidase (Type V, Sigma, St. Louis, USA) in 0.05 M sodium acetate buffer (ph 4.5) at 37°C for 4 h to remove sialic acid residues. The remaining three cochleas were incubated in 150 IU/ml hyaluronidase (Type IX, Sigma, St. Louis, USA) in 0.05 M sodium acetate buffer (pH 5.0) at 37°C for 4 h to remove hyaluronic acid. All specimens were incubated overnight in the colloidal thorium suspension as described above.

Tissue processing and sectioning
Following the incubations, the cochlear turns were dehydrated in a graded ethanol series and embedded in Spurr's low-viscosity resin. Ultrathin sections were contrast-stained with 7% uranyl acetate in 70% methanol and lead citrate according to Reynolds. They were then examined by transmission electron microscopy (Philips EM 201c; 60 kV).

RESULTS

Normal cochleas
Cationized ferritin incubation resulted in a delicate pattern of reactivity, which

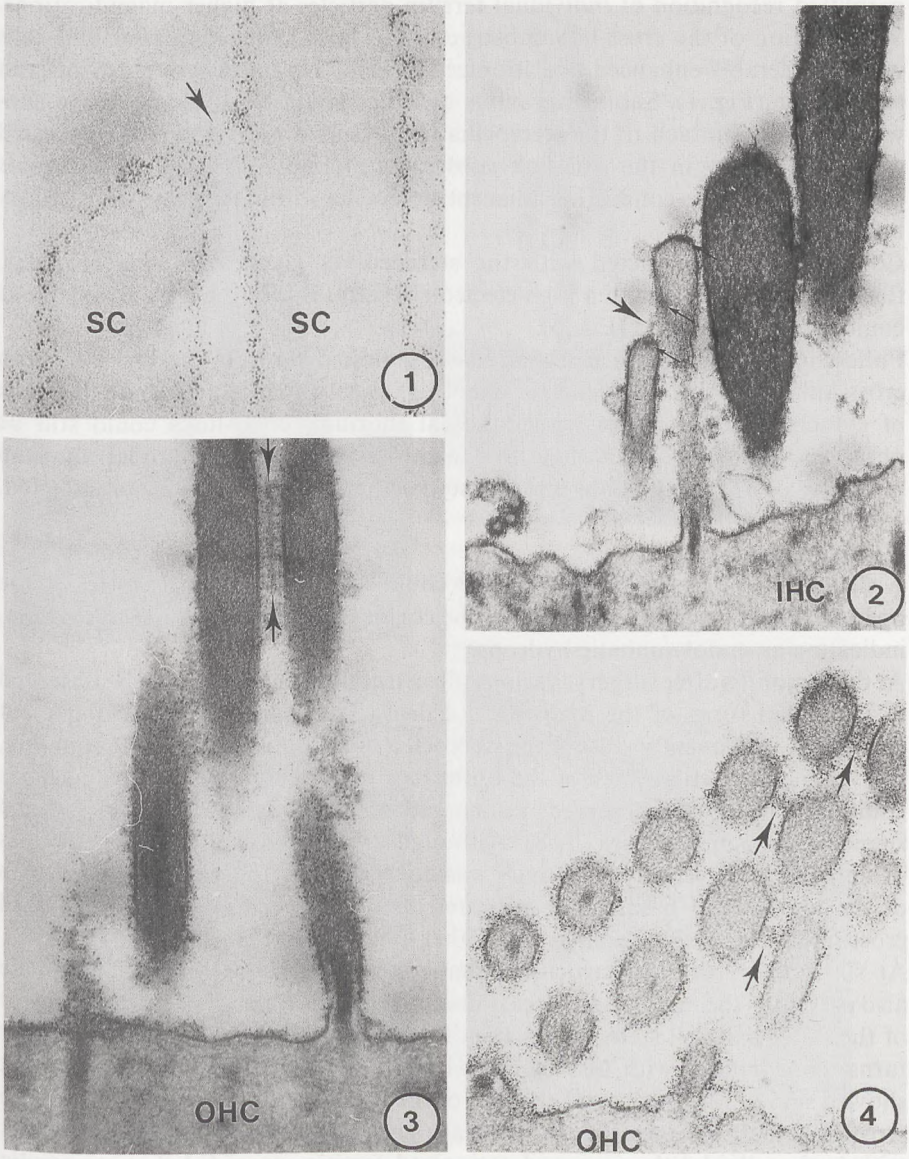

Fig. 1 Cationized ferritin reactivity of tip link (arrow) between two stereocilia (SC) (x 60,000).
Fig. 2 Cationized ferritin reactivity of inner hair cell (IHC) stereociliary bundle. Tip link (large arrow) with submembranous densities (small arrows) (x 26,000).
Fig. 3 Cationized ferritin reactivity of outer hair cell (OHC) stereocilia and side link. Note the central density (arrows) (x 40,000).
Fig. 4 Cationized ferritin labeling of an obliquely sectioned OHC stereociliary bundle, with a regular pattern of side links (arrows) in the upper region of the bundle (x 20,000).

permitted recognition of individual ferritin particles at higher magnifications. Visualization of the cross-link substructure of both outer and inner hair cells was considerably enhanced by cationized ferritin. Tip links were more difficult to visualize (Fig. 1). Submembranous densities could be observed at the sites where tip links attach to the stereocilia (Fig. 2). Cationized ferritin visualized a central density in the side-link substructure (Fig. 3) as well as a regular pattern of side links connecting adjacent stereocilia within the same stereociliary bundle (Fig. 4).

Colloidal thorium reacted with the stereociliary glycocalyx as a granular, flocculent precipitate with a high electron density. Both tip links and side links could also be visualized (Fig. 5).

Following digestion with neuraminidase, colloidal thorium reactivity of both cross-links and the stereociliary glycocalyx was abolished. Despite the lack of cytochemical reactivity with colloidal thorium, cross-links could still be observed (Fig. 6). After digestion with hyaluronidase, colloidal thorium reactivity of both cross-links and the stereociliary glycocalyx was not affected.

Hydropic cochleas

A distension of Reissner's membrane could be observed in all turns of the operated ears by transillumination of the cochlear turns during microdissection, indicating an endolymphatic hydrops.

At three months after surgery, various ultrastructural changes could be observed in the apical turns of the hydropic cochleas. These included disarrangement and - more infrequently - loss of the stereocilia of the outer hair cells. Frequently, the stereociliary cross-links of the outer hair cells at the apex of the cochleas could no longer be observed, concomitant with a disarrangement of the stereociliary bundles (Figs. 7, 8). Although cross-links had disappeared, loss of stereociliary glycocalyx reactivity was not seen (Fig. 7). Stereociliary bundles of the middle and basal turns appeared to be unaffected and demonstrated cross-links which were reactive with either cytochemical marker.

At six months, the ultrastructural changes were more extensive. They were also present in the middle and, occasionally, in the basal turns. A disarrangement of the outer hair cell stereociliary bundles was then also observed in the lower turns, concomitant with loss of cross-links. The inner hair cells at the apex showed similar ultrastructural changes of the stereociliary cross-links. Although cross-links had disappeared, stereociliary glycocalyx reactivity was still intact with both colloidal thorium and cationized ferritin, provided that stereocilia were still present.

DISCUSSION

In this study the stereociliary cross-links were investigated in normal and hydropic cochleas of the guinea pig using cationized ferritin and colloidal

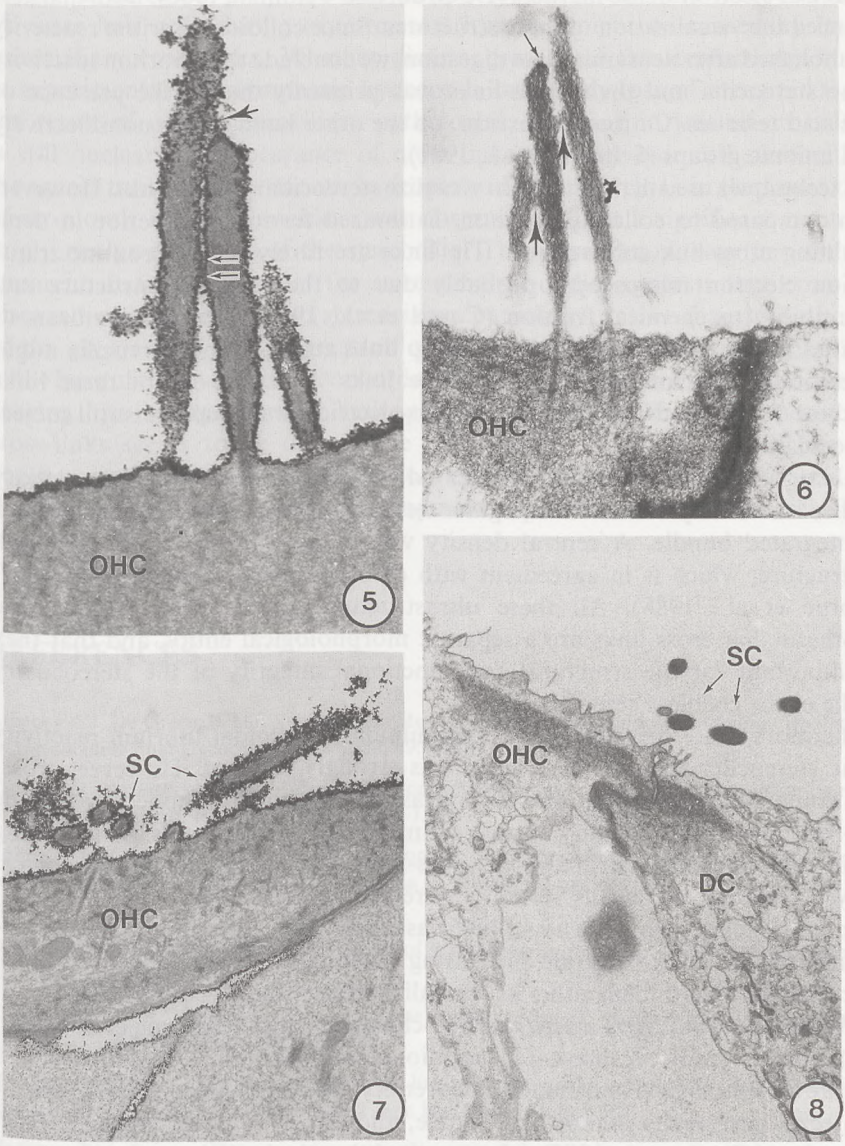

Fig. 5 Colloidal thorium reactivity of an OHC sterociliary bundle. Note the tip link (large arrow) and the side link (small arrows) (x 26,000).

Fig. 6 Colloidal thorium reactivity of this OHC stereociliary bundle is abolished after digestion with neuraminidase. Side links (large arrows) and part of a tip link (small arrow) can still be observed (x 20,000).

Fig. 7 Colloidal thorium reactivity of disarranged OHC stereocilia (SC) in experimental hydrops. Glycocalyx reactivity is still intact, while cross-links have apparently disappeared (x 20,000).

Fig. 8 Cationized ferritin reactivity of obliquely sectioned, disarranged OHC stereocilia (SC) in experimental hydrops. No cross-links can be seen. DC = Deiters' cell (x 6,000).

thorium as electron-dense markers (De Groot and Veldman, 1988). Both markers enhanced the visualization of these structures. Since colloidal thorium reactivity was abolished after neuraminidase digestion, we conclude that thorium reactivity of the stereocilia and their cross-links was primarily due to the presence of sialic acid residues. Cationized ferritin, on the other hand, binds nonselectively to all anionic groups (Schrével et al., 1981).

Both techniques used in this study do visualize stereociliary cross-links. However, when compared to colloidal thorium, cationized ferritin is superior in demonstrating cross-link substructure. Tip links are rarely seen in routine transmission electron microscopy, probably due to their delicate structure and vulnerability to chemical fixation (Comis et al., 1985). The submembranous densities we observed at the sites where tip links attach to the stereocilia might represent the site for anchorage of these links. Whether or not these links and these densities are involved in mechano-electrical transduction is still subject to speculation.

We also found that the side links are located in the upper parts of the stereociliary bundle, where they constitute a regular network, grouping the stereocilia into an integrated bundle. A central density was often observed in the side-link substructure, which is in agreement with earlier studies (Csukas et al., 1987; Osborne et al., 1988). All these ultrastructural observations support the hypothesis that cross-links are a separate morphological entity, and that they are important for the structural and functional integrity of the stereociliary bundle of the cochlear sensory cells.

Furthermore, after digestion with neuraminidase, colloidal thorium reactivity of the stereocilia and the cross-links was strongly reduced. However, cross-links could still be observed due to contrast enhancement obtained by routine post-fixation with OsO_4 and contrast-staining with heavy metals. This finding implies that the basic structure of cross-links, as a separate morphological entity, differs cytochemically from the stereociliary glycocalyx.

Although Hackney and Furness (1986) assumed that cross-links are condensation artefacts due to routine processing techniques (i.e. chemical fixation), other authors regard them either as specializations of the glycocalyx (De Groot and Veldman, 1988) or as a separate cytochemical entity (Osborne et al., 1988, 1990). In this study we presented additional evidence that cross-links are a separate morphological and cytochemical entity. The cross-links might represent an integral part of the stereociliary lattice, and this is in close agreement with the results of Flock et al. (1977), who reported that the lateral coupling between the individual stereocilia did not alter after removing the stereociliary membranes (including the glycocalyx) with the detergent Triton X-100. Moreover, the recent experiments of Osborne and Comis (1990) suggest that tip links consist of a protein core coated with a carbohydrate layer. This carbohydrate layer is probably identical to the stereociliary glycocalyx, since we found that colloidal thorium reactivity of both cross-links and the stereociliary glyocalyx is strongly reduced after digestion with neuraminidase.

Horner et al. (1988) proposed that atrophy of the middle and short stereocilia of outer hair cells during experimental hydrops might be caused by loss of stereociliary cross-links. Whether disarrangement of the stereociliary bundle in experimental endolymphatic hydrops is caused by loss of cross-links or is the result of other stereociliary pathologies, resulting in loss of cross-links, is still unclear. Disappearance of cross-links of outer hair cell stereocilia in the apical turns of three-month hydropic cochleas as well as in the lower turns of six-month hydropic cochleas was observed without a significant reduction in glycocalyx reactivity of the stereocilia. This observation, together with the apparent disarrangement of the stereociliary bundle, suggests that cross-link disorganization is related to stereociliary pathology, rather than to pathology of the glycocalyx. The progression of cross-link degeneration from the apex to the base of the cochlea is in concordance with the development of sensorineural hearing loss in experimental hydrops (Horner, 1991). The disappearance of cross-links seems to be one of the early pathological features of hair cell degeneration after surgical induction of endolymphatic hydrops and may therefore be responsible for the development of permanent sensorineural hearing loss.

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Chapter 4

Cell membrane polarity of the epithelial cells in the endolymphatic sac of the guinea pig

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Cell membrane polarity of the epithelial cells in the endolymphatic sac of the guinea pig

The endolymphatic sac (ES) is generally thought to have several functions, such as degradation of waste products and absorption of endolymph (Lundquist, 1965; Kimura and Schuknecht, 1965), and endolymphatic pressure regulation (Bagger-Sjöbäck et al., 1986). It also acts as a site for immunological reactivity (Rask-Andersen and Stahle, 1979; Arnold et al., 1984). Although the mechanisms by which fluid homeostasis and ion composition of the endolymphatic fluid are regulated are not yet fully understood, the glycocalyx and basement membrane of the ES epithelial cells have been implicated to play an important role in the transport of water and solutes across this epithelium (Takumida et al., 1988). Identification of surface glycoconjugates and their distribution along the ES epithelial lining may increase our knowledge of ES function, especially in relation to endolymphatic fluid and ion balance.

Lundquist (1976) distinguished two types of cells in the intermediate part of the guinea pig ES, the "light cell" and the "dark cell". On the basis of their ultrastructural features, he postulated that the light cells are actively involved in fluid transport, whereas the dark cells have a phagocytic function. It could be speculated that functional differences between these cell types may also be accompanied by a different pattern of glycocalyx reactivity.

It is commonly accepted that the apical membranes of epithelial cells differ significantly from the basolateral membranes in their biochemical composition, which may be associated with different functions of the respective domains (Simons and Fuller, 1985). If this holds also for the epithelial cells in the ES, it could be reflected in different patterns of glycocalyx reactivity.

To verify these hypotheses the glycocalyx of the epithelial membranes in the ES was studied using the electron-dense markers colloidal thorium and cationized ferritin. Also digestions with neuraminidase and hyaluronidase were performed prior to colloidal thorium incubation, in order to identify the reactive groups responsible for colloidal thorium labeling.

MATERIALS AND METHODS

Female, albino Dunkin-Hartley guinea pigs (N = 11), with a body weight of 250-300 g, were used for all experiments. The animals were fixed by means of intravascular perfusion with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The temporal bones were removed and immersed in the same fixative for two hours at 4°C followed by dissection of the ES.

Colloidal thorium

Eight specimens were post-fixed in 1% OsO_4 in 0.1M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C. After microdissection, the ES were incubated overnight in 1% colloidal thorium dioxide (Thoria-sol^R, Polysciences, Warrington, USA) in 3% acetic acid (pH 2-2.5) at room temperature. This was followed by dehydration in a graded ethanol series, embedding in Spurr's low-viscosity resin, and ultrathin sectioning.

Enzymatic digestions

After microdissection and post-fixation as described above, three specimens were incubated in 40 IU/ml neuraminidase (Type V, Sigma, St. Louis, USA) in 0.05 M sodium acetate buffer (pH 4.5) at 37°C for 3 h to remove sialic acid. Three specimens were incubated in 150 IU/ml hyaluronidase (Type IX, Sigma, St. Louis, USA) in 0.05 M sodium acetate buffer (pH 5.0) at 37°C for 3 h to remove hyaluronic acid. Subsequently, all six specimens were incubated overnight in the colloidal thorium suspension and processed as described above.

Cationized ferritin

Eight specimens were post-fixed in 1% aqueous OsO_4 containing 1% $K_4Ru(CN)_6$ for 2 h at 4°C. Microdissection was followed by incubation for one hour in 5 mg/ml cationized ferritin in phosphate buffered saline (pH 7.4) at room temperature. The specimens were dehydrated in a graded ethanol series, embedded in Spurr's low-viscosity resin, and ultrathin sectioned.

Ultrathin sections were contrast-stained with 7% uranyl acetate in 70% methanol and lead citrate according to Reynolds, and examined in a Philips EM 201c operating at 60 kV.

RESULTS

Colloidal thorium

Reactivity for colloidal thorium was noted by the presence of an electron-dense, granular precipitate. The apical membranes of both light and dark cells reacted equally strong with colloidal thorium (Fig. 1). When electron-dense intraluminal colloid was present, and in close contact with the apical membranes, no labeling was noticed.

Reactivity of the basolateral membranes was absent when the basement membrane appeared to be intact (Fig. 2). Whenever the basement membrane was disrupted, a moderate reactivity of the basolateral membranes was found (Fig. 1). The basement membrane itself also exhibited strong reactivity (Fig. 2).

Enzymatic digestions

Digestion with neuraminidase resulted in a strongly reduced reactivity for colloidal thorium, of both the apical and basolateral membranes of the ES

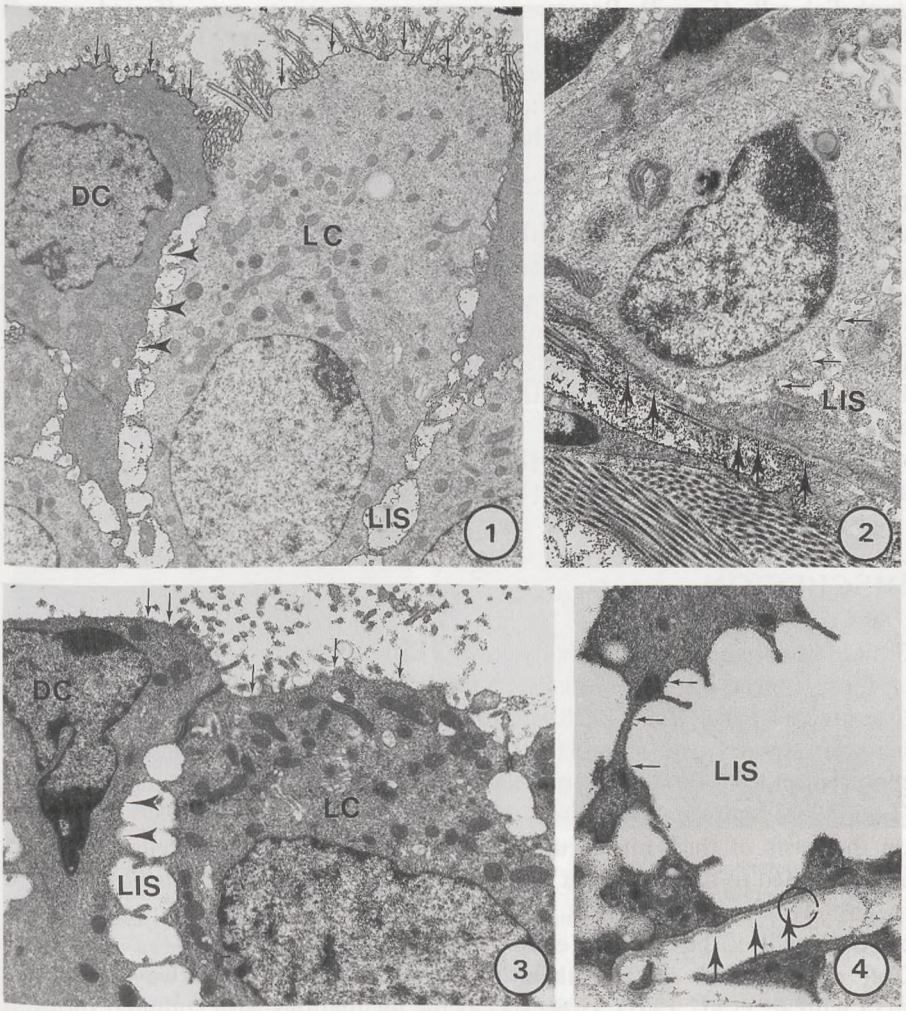

Fig. 1 Colloidal thorium reactivity of the apical (arrows) and basolateral (arrowheads) membranes of the light cell (LC) and dark cell (DC) of the ES epithelial lining. LIS = lateral intercellular space (x 8,000).

Fig. 2 Colloidal thorium reactivity of the basement membrane (large arrows) of the ES epithelial lining. Basolateral membranes (small arrows) along the lateral intercellular space are not reactive (x 8,000).

Fig. 3 Strong reduction of colloidal thorium reactivity of the apical (small arrows) and basolateral (arrowheads) membranes of light and dark cells after neuraminidase digestion (x 8,000).

Fig. 4 Strongly reduced colloidal thorium reactivity of the basement membrane (large arrows) and the basolateral membranes (small arrows) of the ES epithelial lining after digestion with neuraminidase (x 9,000).

epithelial cells (Fig. 3). Basement membrane reactivity was affected as well (Fig. 4).
Digestion with hyaluronidase resulted in a virtually unchanged reactivity of the apical and basolateral membranes of the epithelial cells (Fig. 5). The reactivity of the basement membrane was hardly affected (Fig. 6).

Cationized ferritin
Incubation with cationized ferritin resulted in a more delicate staining of the glycocalyx as compared to colloidal thorium. At higher magnifications individual ferritin particles could easily be identified. The apical membranes of light and dark cells reacted equally strong, provided that no electron-dense intraluminal colloid was present on these membranes (Fig. 7).
Basolateral membranes demonstrated moderate reactivity, but only when the basement membrane appeared to be disrupted. With an intact basement membrane reactivity was absent (Fig. 8). The basement membrane itself reacted strongly with cationized ferritin (Fig. 8).

DISCUSSION

Studies investigating the glycocalyx of the ES epithelium are limited, in contrast to those concerning the epithelia of the cochlea or the vestibular end organs (Takumida et al., 1988; Barbara, 1989). Differences in glycocalyx reactivity between sensory and supporting cells of the cochlea have been frequently reported and are thought to be related to differences in membrane properties (Lim, 1986; Santi and Andersen, 1987; Prieto and Merchan, 1987). Although on the basis of their ultrastructural features, dark and light cells of the ES are presumed to have different functions (Lundquist, 1976), this is not reflected in any significant differences in glycocalyx reactivity between the light and the dark cells of the ES using either technique.
Cationized ferritin reacts with anionic sites in a nonspecific way at neutral pH (Schrével et al., 1981). However, as the incubations with colloidal thorium were performed at pH 2-2.5, the only ionized groups that could possibly have reacted with colloidal thorium are the carboxyl groups of sialic acid and hyaluronic acid, and the sulfate groups of sulfated glycoconjugates such as chondroitin sulfate and keratan sulfate (Schrével et al., 1981; De Groot and Veldman, 1988). Colloidal thorium reactivity in the ES was strongly reduced following neuraminidase digestion, whereas glycocalyx reactivity appeared to be unaffected after digestion with hyaluronidase. This finding indicates that glycocalyx reactivity for colloidal thorium in ES epithelial cells is mainly due to the presence of sialic acid. Sialic acid has been implicated in the sequestration and transport of electrolytes, especially cations, across cell membranes (Glick and Githens, 1965; Langer et al., 1976; Roth and Taatjes, 1985). Since no differences in colloidal thorium reactivity were found between light and dark

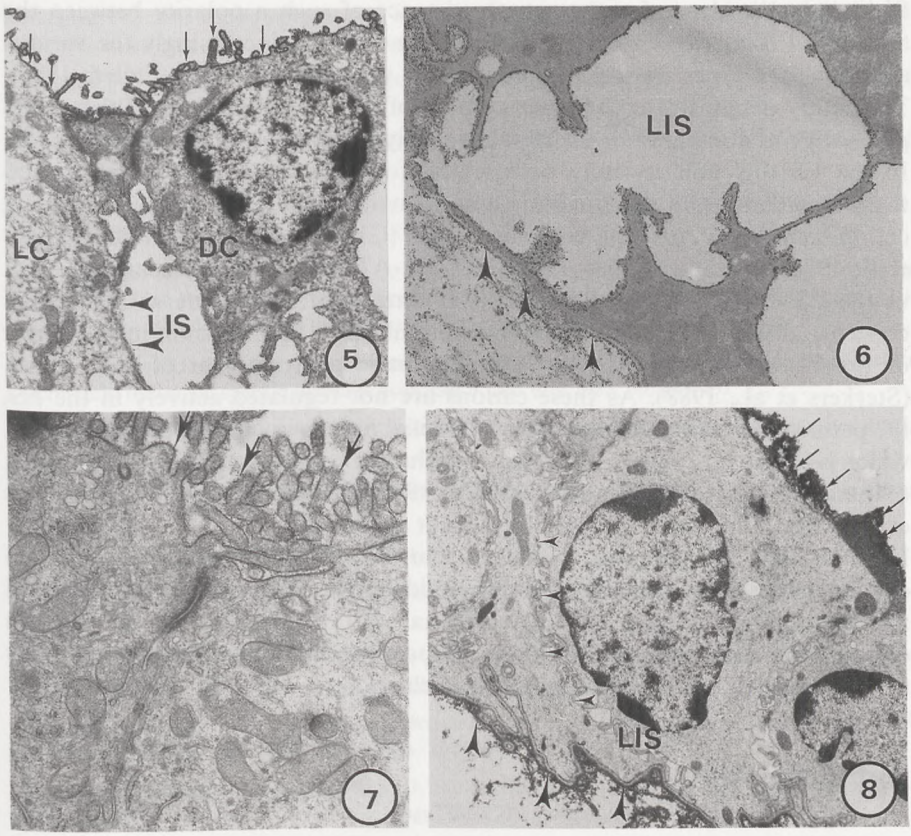

Fig. 5 Virtually unaffected reactivity of the apical (small arrows) and basolateral (arrowheads) membranes of the light and dark cells of the ES following digestion with hyaluronidase (x 8,000).
Fig. 6 Virtually unaffected reactivity of the basement membrane (arrowheads) of the ES epithelial lining following hyaluronidase digestion (x 9,000).
Fig. 7 Cationized ferritin reactivity of the apical membranes of the ES epitelium (arrows) (x 14,000).
Fig. 8 Cationized ferritin reactivity of the apical membranes of the ES epithelium (arrows). The basement membrane (large arrowheads) also reacts strongly, whereas the basolateral membranes lining the LIS do not demonstrate any reactivity (small arrowheads) (x 4,000).

cells, these cells probably behave in a similar way as far as transmembranous cation transport is concerned.

The basolateral membranes of the epithelial cells did not react with either colloidal thorium or cationized ferritin when the basement membrane appeared intact, suggesting that both markers are not able to pass through the intact basement membranes or the junctional complexes between the epithelial cells. In case of disruption of the basement membrane, reactivity for both colloidal thorium and cationized ferritin could be noted along the basolateral membranes, although the reactivity was not as strong as that on the apical membranes.

Biochemical evidence for the actual existence of such a polarity between the apical and basolateral membranes has been reported increasingly for various epithelial cell types during the last several years (Simons and Fuller, 1985). This study demonstrates a higher content of sialic acid residues of the apical glycocalyx as compared to the basolateral glycocalyx of the ES epithelial cells. In the ES this polarity may be essential for both electrolyte filtration and the maintenance of a specific ionic micro-environment, facilitating or delaying cation transport across the cell membranes (Glick and Githens, 1965; Langer et al., 1976; Roth and Taatjes, 1985). Na^+/K^+-ATPase is not significantly present in the ES (Albers et al., 1991). This finding is in agreement with the high concentration of sodium and the low concentration of potassium in the lumen of the ES as compared to the endolymphatic compartment of the cochlea (Sterkers et al., 1988). As these cations are not regulated actively in the ES, the permeation characteristics of the epithelial lining – including the glycocalyx, which is rich in sialic acid – are likely to influence passive transcellular transport of these electrolytes (Rask-Andersen et al., 1981; Roth and Taatjes, 1985; Sterkers et al., 1988; Albers et al., 1991; Sugyama et al., 1991).

In conclusion, this study demonstrates that the glycocalyx of the ES epithelial cells is rich in sialic acid, and presents evidence for the existence of an apical and basolateral domain of the epithelial cell membranes in the ES. In concordance with earlier publications, we postulate that these features are likely to be of importance to the equilibrium of electrolytes in the ES lumen.

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Chapter 5

Cytochemical localization of ouabain-sensitive, potassium-dependent p-nitrophenylphosphatase in the guinea pig inner ear.

Evaluation of the lead-based method vs. the cerium-based method

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Cytochemical localization of ouabain-sensitive, potassium-dependent p-nitrophenylphosphatase in the guinea pig inner ear

Evaluation of the lead-based method vs. the cerium-based method

The perilymphatic compartment of the inner ear resembles plasma ultrafiltrate, whereas the endolymphatic compartment is characterized by a high potassium and low sodium concentration as well as by a positive transepithelial potential. The specific chemical composition of endolymph and the generation of the transepithelial potential are considered to be regulated by a membrane-bound, ouabain-sensitive, sodium-potassium-activated adenosine triphosphatase (Na^+/K^+-ATPase). This hypothesis is confirmed by the observation that inhibition of Na^+/K^+-ATPase by perilymphatic perfusion with ouabain results in a decline of the endolymphatic potential and the endolymphatic electrolyte (sodium and potassium) gradients (Kuijpers and Bonting, 1970; Bernard et al., 1986).

Biochemical investigations of the intracochlear distribution of Na^+/K^+-ATPase have demonstrated the highest activity present in the stria vascularis with a decrease in enzyme activity from the base to the apex of the cochlea (Kuijpers, 1969; Kuijpers and Bonting, 1969). In biochemical studies of the vestibular labyrinth a high activity of Na^+/K^+-ATPase has been found in the region of the dark cells in the utricle and the cristae ampullares of the semicircular canals (Thalmann, 1971).

The first studies on the cytochemical localization of Na^+/K^+-ATPase in the inner ear were performed by Nakai and Hilding (1966, 1968) according to the Wachstein-Meisel method. More specific information concerning the regional distribution of Na^+/K^+-ATPase in the inner ear was obtained in investigations using the cytochemical technique of Ernst (Ernst, 1972; Kerr et al., 1982; Mees, 1983). In recent studies the cytochemical localization of the ouabain-sensitive, K^+-dependent phosphatase activity of the Na^+/K^+-ATPase complex in the cochlear and vestibular labyrinth, and in the endolymphatic sac, has been demonstrated by the one-step lead citrate technique according to Mayahara using p-nitrophenylphosphate as a substrate (Mayahara and Ogawa, 1980; Mayahara et al., 1980; Yoshihara et al., 1987; Mizukoshi et al., 1988).

In phosphatase cytochemistry the cerium-based method is regarded to be superior to the lead-based method for the demonstration of plasma membrane phosphatase activities (Veenhuis et al., 1980; Hulstaert et al., 1983; Kalicharan et al., 1985). In this study the cytochemical properties of the lead-based method and the cerium-based method have been investigated for their usefulness in

the demonstration of ouabain-sensitive, potassium-dependent p-nitrophenyl-phosphatase activity in the guinea pig inner ear.

MATERIAL AND METHODS

Healthy, female albino guinea pigs (strain Dunkin Hartley, weight: 350-450 g) were used for the experiment. Primary fixation was performed by means of intravascular perfusion with respectively 4% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) or 2% formaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), followed by immersion of the temporal bones in the same fixative for 60 min at 4°C. After removal of the bony capsule the lateral cochlear wall, the utricle, the ampullae of the semicircular canals, and the endolymphatic sac were microdissected in 0.1 M sodium cacodylate buffer (pH 7.4). The specimens were rinsed overnight at 4° C in 0.1 M sodium cacodylate buffer (pH 7.4) containing 10% DMSO and 8% sucrose.

Lead-based method

The incubation medium according to the lead-based method (Mayahara and Ogawa, 1980; Mayahara et al., 1980) consisted of 0.01 M p-nitrophenylphosphate (Mg-salt, Sigma, St. Louis, USA), 25% v/v DMSO, 4mM leadcitrate (in 50 mM KOH stock solution), 2.5mM levamisole (Sigma, St. Louis, USA) and 0.25 M glycine-KOH buffer (pH 8.8). Overnight pre-incubation in incubation medium without the substrate p-nitrophenylphosphate at 37°C was followed by incubation in the complete incubation medium at 37°C for respectively 10, 20 and 30 min.

Cerium-based method

The incubation medium according to the cerium-based method (Veenhuis et al., 1980; Hulstaert et al., 1983; Kalicharan et al., 1985) consisted of 0.01 M p-nitrophenylphosphate (Mg-salt, Sigma, St. Louis, USA), 25% v/v DMSO, 1 mM cerium chloride, 2.5 mM levamisole (Sigma, St. Louis, USA) and 70 mM Tris-maleate buffer (pH 8.8). Overnight pre-incubation in the incubation medium without the substrate p-nitrophenylphosphate at 37°C was followed by incubation in the complete incubation medium at 37°C for respectively 15, 30 and 60 min.

In the control experiments the specimens were incubated in the incubation medium without the substrate p-nitrophenylphosphate, or in the complete incubation medium containing 10 mM ouabain.

Tissue processing and sectioning

After the cytochemical incubation procedures the specimens were rinsed overnight at 4°C in 0.1 M sodium cacodylate buffer (pH 7.4), containing 8% sucrose. Post-fixation was performed in 1% OsO_4 and 1% $K_4Ru(CN)_6$ (ICN Pharmaceuticals, New York, USA) in 0.1 M sodium cacodylate buffer (pH

7.4) at 4°C for 30 min. The specimens were dehydrated in a graded ethanol, 2,2-dimethoxypropane, propylene oxide series and embedded in Spurr's low-viscosity resin. Ultrathin sections were cut using an LKB Ultrotome V and mounted on Pioloform-coated, single-slot copper grids. The ultrathin sections were contrast-stained with methanolic uranyl acetate and Reynolds' lead citrate, and examined by transmission electron microscopy (Philips EM 201c; 60 kV).

RESULTS

The cytochemical localization of potassium-dependent p-nitrophenylphosphatase according to the lead-based method resulted in an electron-dense precipitate, consistently present in all experiments. Incubation for 10 min showed an optimal deposition of reaction product, whereas incubation for 20 and 30 min resulted in a significant increase of non-specific lead precipitates masking the ultrastructural details.

The reaction product obtained with the cerium-based method appeared as a delicate, granular precipitate and was not consistent throughout the present study. Prolongation of the incubation time showed an increase of non-specific precipitates without significantly improving the intensity of the potassium-dependent p-NPPase activity.

Primary fixation with 2% formaldehyde and 0.5% glutaraldehyde resulted in a more optimal preservation of the inner ear ultrastructure as compared to primary fixation with 4% formaldehyde. However, a significant decrease in p-NPPase activity was observed after primary fixation with 2% formaldehyde and 0.5% glutaraldehyde.

In the control experiments no reaction was observed following incubation in the incubation medium without the substrate, p-nitrophenylphosphate. After incubation in the complete medium containing 10 mM ouabain the cytochemical reaction was strongly reduced.

Cochlear labyrinth

Strong activity of p-NPPase using both the lead-based and the cerium-based methods was observed on the basolateral plasma membranes of the marginal cells of the stria vascularis. (Figs. 1A and B). The reaction product was absent at the luminal cell surface facing the endolymphatic compartment. The cytoplasmic processes of the intermediate cells did not show any p-NPPase activity. No reaction product was found on the basolateral infoldings of the epithelial cells in the spiral prominence. The stromal cells of the spiral ligament, adjacent to the epithelial lining of the spiral prominence, demonstrated a weak p-NPPase activity only when using the lead-based method.

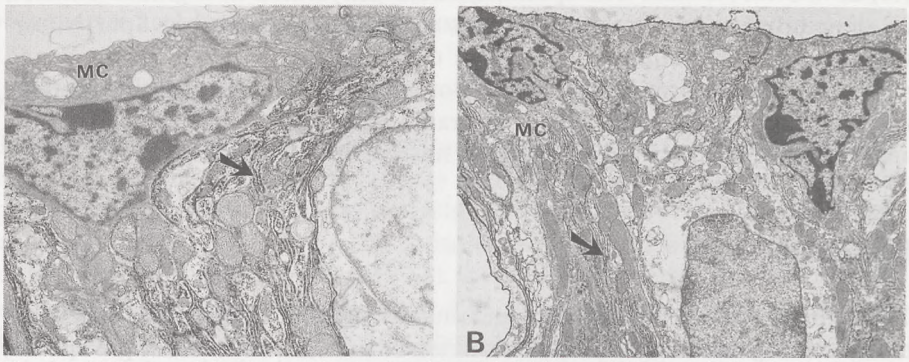

Fig. 1 p-NPPase activity (arrow) in the basolateral infoldings of the marginal cells (MC) in the stria vascularis using (A) the lead-based method and (B) the cerium-based method (x 7,500).

Vestibular labyrinth

The basolateral interdigitations of the dark cells in both the macula utriculi and cristae ampullares of the semicircular canals demonstrated intense p-NPPase activity as demonstrated by the lead-based method. However, only a weak p-NPPase activity was observed using the cerium-based method (Figs. 2A and B). The p-NPPase activity in the dark cells of the macula utriculi and the cristae ampullares of the semicircular canals did not show significant differences in intensity. No reaction products were found on the transitional epithelium surrounding the sensory epithelia of both the urticle and the cristae ampullares.

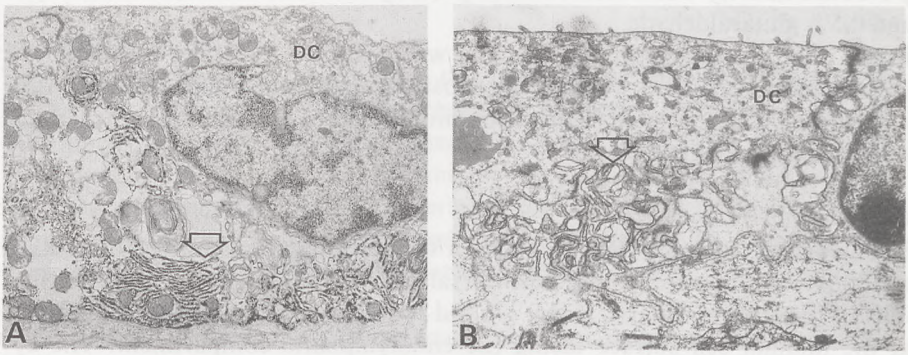

Fig. 2 p-NPPase activity (arrow) in the dark cells (DC) of a crista ampullaris of the semicircular canal using (A) the lead based method and (B) the cerium-based method (x 7,500).

Endolymphatic sac

In the intermediate part (pars rugosa) of the endolymphatic sac the light and dark cells could be clearly identified. Using the lead-based method no reaction products indicating p-NPPase activity were observed in the two different cell types, although non-specific precipitates were frequently found in the specimens

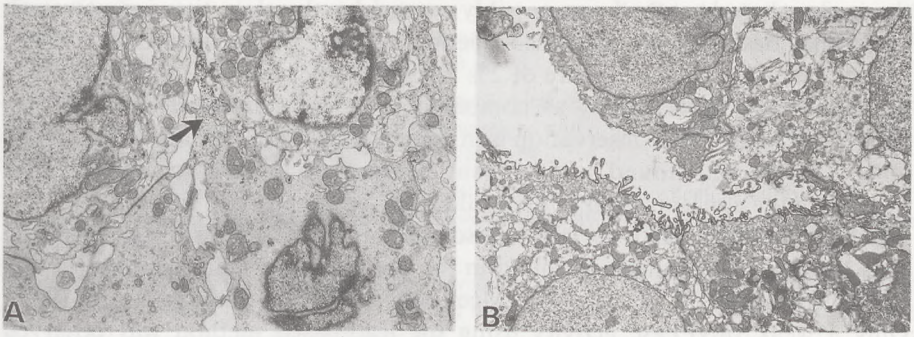

Fig. 3 No p-NPPase activity was observed in the epithelial cells of the intermediate part of the endolymphatic sac using the lead-based method (A) or the cerium-based method (B). Non-specific precipitates (arrow) were found throughout the specimen after the lead-based method (x 5,000).

(Fig. 3A). The cerium-based method revealed neither specific nor non-specific reaction products in the epithelial lining of the endolymphatic sac (Fig. 3B).

DISCUSSION

In the present study the cytochemical localization of p-NPPase activity in the inner ear was investigated using either lead citrate or cerium chloride as capture agent and p-nitrophenylphosphate as substrate.

Wachstein and Meisel (1957) introduced the lead-salt precipitation method for the demonstration of Na^+/K^+-ATPase activity. The standard concentration of lead nitrate in the Wachstein-Meisel incubation medium, however, resulted in a strong inhibition of ouabain-sensitive Na^+/K^+-ATPase with less inhibitory effect on non-specific ATPases (Marchesi and Palade, 1967; Jacobsen and Jørgensen, 1969). In the one-step method of Mayahara (Mayahara and Ogawa, 1980; Mayahara et al., 1980) for the cytochemical localization of ouabain-sensitive, potassium-dependent p-nitrophenylphosphatase (p-NPPase) activity the incubation medium contained p-NPPase as substrate, lead citrate as capture agent and dimethylsulfoxide (DMSO) as activator. The p-NPPase activation by DMSO caused a significant reduction of the lead inhibition resulting in a sensitive demonstration of p-NPPase activity. In the present study, the high sensitivity of the lead-based method according to Mayahara could be confirmed. However, the interpretation of ultrastructural details is sometimes made difficult by the presence of non-specific lead precipates.

Recent studies have produced excellent results in the cytochemical demonstration of phosphatases using cerium chloride as capture agent (Veenhuis et al., 1980; Hulstaert et al., 1983; Kalicharan et al., 1985). In our experiments the fine granularity of the reaction product and the absence of non-specific deposits resulted in a considerably improved ultrastructural localization of p-NPPase activity as compared to the lead-based method. The less consistent results using

the cerium-based method are possibly caused by the slow tissue penetration of cerium, despite a pre-incubation period of 24 h (Kalicharan et al., 1985). Primary fixation with a mixture of 2% formaldehyde and 0.5% glutaraldehyde decreased p-NPPase activity as compared to primary fixation with 4% formaldehyde alone. This observation is in agreement with the results of Mayahara et al. (1980), who found a significant decrease in p-NPPase activity after increasing the concentration of glutaraldehyde in the primary fixative.

The high p-NPPase activity in the basolateral interdigitations of the marginal cells in the stria vascularis as found in the present study is in accordance with earlier reports (Kerr et al., 1982; Mees et al., 1983; Yoshihara et al., 1987). Only a weak p-NPPase activity using the lead-based method was observed in the subepithelial stromal cells of the spiral ligament. The epithelial cells of the spiral prominence did not show any enzyme activity, wich makes a possible involvement of these cells in the homeostasis of endolymph doubtful. The highest p-NPPase activity in the vestibular labyrinth was found in the basolateral infoldings of the dark cells in the macula utriculi and the cristae ampullares of the semicircular canals. The abcence of p-NPPase activity on the endolymphatic cell surface of the dark cells is in agreement with the observations of Yoshihara et al. (1987).

Besides non-specific lead precipitates no reaction product indicating p-NPPase activity was observed in the epithelial cells of the intermediate part of the endolymphatic sac using either the lead-based or cerium-based method. As mentioned earlier, non-specific lead deposits are a frequently encountered feature during the cytochemical localization of p-NPPase using lead as a capture agent (Veenhuis et al., 1980; Hulstaert et al., 1983; Kalicharan et al., 1985). These findings are in contrast with the results of Mizukoshi et al. (1988) who, using the one-step lead citrate method, reported a weak p-NPPase activity localized at the basolateral sides of the epithelial cells of the endolymphatic sac.

In conclusion, the high enzymatic activity of ouabain-sensitive, potassium-dependent p-NPPase in the stria vascularis and in the dark cells of the vestibular labyrinth indicates an active role of these structures in the regulation of endolymphatic cation transport and the generation of the endocochlear potential. Furthermore, the absence of significant p-NPPase activity as well as the low concentration of potassium and the high concentration of sodium in the endolymphatic sac suggest a passive mechanism for cation transport in this region of the inner ear.

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Chapter 6

The effect of nimodipine on cochlear potentials and Na^+/K^+-ATPase activity in normal and hydropic cochleas of the guinea pig

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The effect of nimodipine on cochlear potentials and Na^+/K^+-ATPase activity in normal and hydropic cochleas of the guinea pig

Experimental endolymphatic hydrops (EEH) can be evoked by the induction of an imbalance between endolymph production - considered to be connected with the activity of the stria vascularis - and endolymph absorption, which is considered to be connected with the activity of the endolymphatic duct and sac. In guinea pigs, EEH can be created by destruction of the endolymphatic sac and obstruction of the endolymphatic duct (Kimura and Schuknecht, 1965). As endolymph is then no longer absorbed and will accumulate in the scala media, Reissner's membrane becomes distended and EEH develops. As a result, degenerative changes in all cochlear epithelia will gradually occur (Kimura and Schuknecht, 1965, 1976; Konishi and Kelsey, 1976; Ruding et al., 1987; Albers et al., 1987, 1988; Horner et al., 1988). Simultaneously, various electrophysiological changes can be found: (1) fluctuating and permanent threshold shifts of the compound action potential (CAP) (Aran et al., 1984; Harrison et al., 1984; Horner and Cazals, 1987; Van Deelen et al., 1987); (2) an increase of the summating potential (SP) (Kumagami and Miyazaki, 1983; Aran et al., 1984; Van Deelen et al. 1987); and (3) a decrease in cochlear microphonics (CM) (Konishi and Kelsey, 1976; Konishi et al., 1981; Morizono et al., 1985; Klis and Smoorenburg, 1988). Following obliteration of the endolymphatic duct and sac, morphological and physiological alterations progress with time (Horner, 1990). However, CAP threshold shifts have been reported to fluctuate in the early stages of EEH, i.e., within three months after obliteration, indicating reversibility of EEH pathophysiology, while they tend to stabilize after this period (Aran et al., 1984; Harrison et al., 1984; Horner and Cazals, 1987). The mechanisms responsible for this reversibility remain to be elucidated. Insight in these mechanisms might provide clues for treatment of endolymphatic hydrops in patients suffering from Menière's disease. The maintenance of the endocochlear potential (EP) is crucial to normal cochlear function. The EP is thought to be produced by the strial marginal cells (Offner et al., 1987). One of the key findings in EEH is degeneration of the stria vascularis (Konishi and Kelsey, 1976; Albers et al., 1987) concomitant with a decrease in EP (Konishi and Kelsey, 1976; Cohen and Morizono, 1984; Sziklai et al., 1989). Most investigators ascribe the decreased EP to reduced activity of the enzyme Na^+/K^+-activated adenosine triphosphatase (Na^+/K^+-ATPase), which is abundantly present in the stria vascularis (Kuypers and Bonting, 1969),

especially in the basolateral infoldings of the marginal cells (Yoshihara et al., 1987; Albers et al., 1991). One might therefore hypothesize that stimulation of Na^+/K^+-ATPase in the EEH condition could restore the EP and thus lead to improved cochlear function. On the other hand, it is also conceivable that by stimulating Na^+/K^+-ATPase, excessive endolymph might be produced and evoke or augment EEH, as was suggested by Feldman and Brusilow (1976). The present study was designed to assess the effects of a systemically applied Na^+/K^+-ATPase stimulator, nimodipine, on Na^+/K^+-ATPase activity and cochlear potentials of both normal and hydropic cochleas. Nimodipine is a compound first described as an L-type Ca^{2+}-channel antagonist. It was later found to also have Na^+/K^+-ATPase-stimulating properties at concentrations as low as 1.5 nM (Pan and Janis, 1983; Godfraind et al., 1986). It should be noted that locally applied nimodipine at higher concentrations (>1 μM) has strong effects on cochlear physiology (Bobbin et al., 1990), including depression of the CAP, depression of the SP, and depression of the EP. These effects were assumed to be a consequence of the Ca^{2+}-channel-blocking properties of the compound (Bobbin et al., 1991). The possible Na^+/K^+-ATPase-stimulating properties of nimodipine were not considered in their studies.
This investigation addresses the following questions:
(1) What is the effect of surgically induced EEH on the activity of the enzyme Na^+/K^+-ATPase in the cochlea, as determined by the enzyme-cytochemical technique according to Mayahara et al. (1980), and on concomitant cochlear electrophysiological changes as measured by recording responses to tone bursts?
(2) What is the effect of systemically applied nimodipine on the volume of the scala media, on the activity of strial Na^+/K^+-ATPase, and on the cochlear potentials in the normal cochlea?
(3) What is the effect of systemically applied nimodipine on the activity of the enzyme Na^+/K^+-ATPase, and on the cochlear potentials in endolymphatic hydrops?

MATERIALS AND METHODS

Twenty-five female albino guinea pigs (Dunkin Hartley, body weight 250-300 g) were used. In 13 animals the endolymphatic sac and duct of the left ear were surgically obliterated using the extradural posterior fossa approach (Konishi and Shea, 1975). The animals were anesthetized with preoperative intramuscular injections of 0.18 ml Thalamonal (a mixture of 2.5 mg/ml droperidol and 0.05 mg/ml fentanyl) per 100 g bodyweight, followed by ventilation with a gas mixture containing 33% O_2, 66% N_2O and 1% Halothane. During surgery rectal temperature was kept at 38°C.
Eleven weeks after surgery, seven of the 13 operated animals were treated with nimodipine (Nimotop®, Bayer AG, Wuppertal, Germany) dissolved in sterile sesame oil by twice-daily intraperitoneal injections (15mg/kg/day) for

three days. Five of the 12 non-operated animals were treated the same way. The remaining six operated animals and four of the non-operated animals received a sham injection with sesame oil only. Because of light sensitivity, handling of nimodipine was carried out under light from a sodium vapor lamp as recommended by the supplier.
Finally, we ended up with four groups of animals (Table 1): (1) untreated controls (N=4) (code: C); (2) nimodipine-treated non-operated control animals (N=5) (code: Cn); (3) untreated animals with EEH (N=6) (code: EEH); and (4) nimodipine-treated animals with EEH (N=7) (code: EEHn).
Three of the non-operated animals were treated with nimodipine, as described above, to determine nimodipine serum concentrations as a function of time following the last injection.

Table 1 Classification of the different groups investigated in this study.

	Hydrops	**Control**
Nimodipine	EEHn (N = 7)	Cn (N = 5)
Control	EEH (N = 6)	C (N = 4)

Electrocochleography
Immediately following the last injection with nimodipine, the animals were anesthetized as described above. The cochlea was exposed through a ventrolateral approach. The CAP, CM and SP of the cochlea were recorded with a silverball electrode at the apex (Fig. 1) (Van Deelen and Smoorenburg, 1986). The reference electrode was placed in the muscles of the neck. Stimulus generation and data acquisition were controlled by computer. Tone bursts were calculated and stored in a revolving memory consisting of 1024 points with 12-bit resolution. Tone bursts of 2, 4 and 8 kHz were used. Rise and fall envelopes of the tone bursts were cosine-shaped (1 ms each) and were separated by a plateau of 6 ms. The stimuli were led to a Beyer DT48 dynamic transducer, which was connected to a hollow ear bar that was fitted into the exposed outer ear canal. The inter-burst interval was 99 ms. Consecutive tone bursts were presented with alternating polarity. The responses were amplified (5K or 10K), bandpass-filtered, (-12 dB/octave, -3 dB at 1 Hz and 10 kHz), AD-converted, averaged, and stored on disc for off-line analysis. The responses to the tone bursts of opposite polarity were stored separately to allow accurate measurement of the CAP, the CM and the SP. The SP was based on the DC level measured approximately 6 ms after the start of the 8-ms tone burst (Fig. 1). The CAP (N_1) was measured relative to the SP. CM was measured as the peak-to-peak value halfway through the sinusoidal response.

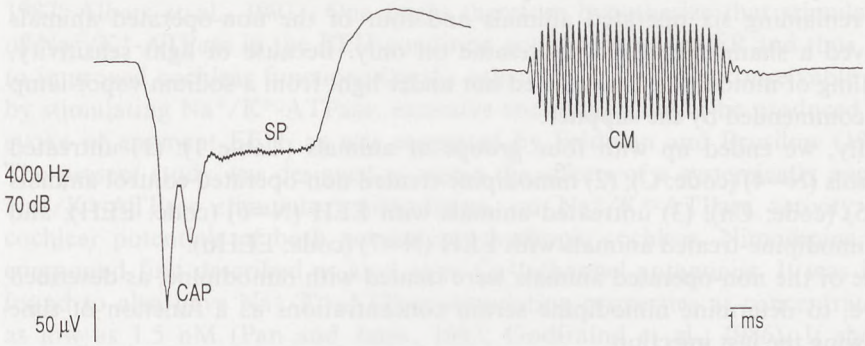

Fig. 1 Method of measurement of CAP, SP and CM. The given response was evoked by a 4-kHz, 70-dB (SPL) tone burst of 8 ms duration. By adding the responses to tone bursts of opposite polarity, the left-hand result is obtained. Subtraction yields the response on the right-hand side. The SP was measured as the DC level at approximately 6 ms after the start of the response. The CAP was measured as the distance between the SP and the largest negative peak (N_1). CM was measured as the peak-to-peak value in the middle of the subtraction response on the right-hand side.

Cytochemistry and light microscopy

After electrocochleography, the animals were sacrificed. Primary tissue fixation was performed by means of intravascular perfusion with 4% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) (buffer). Both left and right cochleas of the operated animals and one cochlea of each of the non-operated animals (the other one was used for routine light-microscopical preparation) were immersed in the same fixative for 60 min at 4°C. After removal of the bony capsule of the lateral cochlear wall, the cochlear turns were microdissected in buffer. Distension of Reissner's membrane, indicating endolymphatic hydrops, was seen in all operated cochleas and in none of the non-treated, non-operated cochleas. The specimens were rinsed overnight at 4°C in buffer, containing 10% DMSO and 8% sucrose.

To determine the activity of K^+-dependent, ouabain-sensitive p-nitrophenylphosphatase (p-NPPase, a functional part of the enzyme Na^+/K^+-ATPase), the method described by Mayahara et al. (1980) was used. Cochlear turns were incubated at 37°C for 15 min in a medium consisting of 0.01 M p-nitrophenylphosphate (Mg-salt, Sigma, St. Louis, USA), 25% v/v DMSO, 4 mM lead citrate (in 50mM KOH stock solution), 2.5 mM levamisole (Sigma, St. Louis, USA), and 0.25 M glycine-KOH buffer (pH 8.8). Control experiments were performed by incubating specimens in the same medium without the substrate p-nitrophenylphosphate, or in the complete incubation medium containing 10 mM ouabain.

The incubation was followed by overnight rinsing in buffer, containing 8 % sucrose. Post-fixation was performed with 1% OsO_4 and 1% $K_4Ru(CN)_6$ in buffer at 4°C for 30 min. The specimens were dehydrated in a graded ethanol

series and embedded in Spurr's low-viscosity resin. Ultrathin sections were contrast-stained with methanolic uranyl acetate and lead citrate according to Reynolds and examined by transmission electron microscopy (Philips EM 201C; 60 kV).

One cochlea per non-operated animal was not used for the cytochemical experiments. These cochleas were fixed in tri-aldehyde fixative (3% glutaraldehyde, 2% formaldehyde, 1% acroleine, 2.5% DMSO in 0.08 M sodium cacodylate buffer, pH 7.4) for 3 h at room temperature. They were then rinsed in buffer and decalcified in 10% EDTA.2Na (pH 7.4) for 4-5 days at room temperature. Subsequently, the specimens were rinsed in buffer and post-fixed with 1% OsO_4 and 1% $K_4Ru(CN)_6$ in buffer for 2 h at 4°C, after which they were dehydrated in a graded ethanol series and embedded *in toto* in Spurr's low-viscosity resin. Next, the cochleas were divided along their midmodiolar plane and re-embedded. Semithin sections (1 μm) were stained with methylene blue and azure B in borax for light-microscopical evaluation.

Nimodipine serum concentrations

In order to determine nimodipine concentrations in serum as a function of time after injection, three animals received the same nimodipine treatment as described above. Immediately following the last injection, the animals were anesthetized as during electrocochleography; the carotid artery was then exposed and canulated. As the electrocochleographic measurements started approximately 30 min after the last intraperitoneal injection and took about 60 min, we were interested in blood samples taken 30, 60 and 90 min following the last injection with nimodipine. Blood samples were centrifuged for 10 min at 12,000 rpm. The serum was removed and stored at -80°C. Determination of nimodipine concentrations was performed at the laboratories of Bayer AG (Institute of Pharmacokinetics, Wuppertal, Germany) by gas chromatography (Ramsch et al., 1985).

RESULTS

Electrocochleography

Fig. 2 depicts the input-output curves for the CAP at the three frequencies used. Mean and standard error of the mean (s.e.m.) are shown for each of the four groups of animals at each level of stimulation. Repeated Measures ANOVA was used, whereby, for each animal, the measurements at different levels were considered repeated measurements. Statistical significance ($p < 0.05$) was addressed using SPSS-PC$^+$ software. Only those levels at which responses above visual threshold were obtained in all four groups were included in the analysis. It had to be accepted that this approach neglects effects at levels yielding above-threshold responses in the control group (C), but not in all treated groups (Cn, EEH, EEHn). The main effects tested were experimental

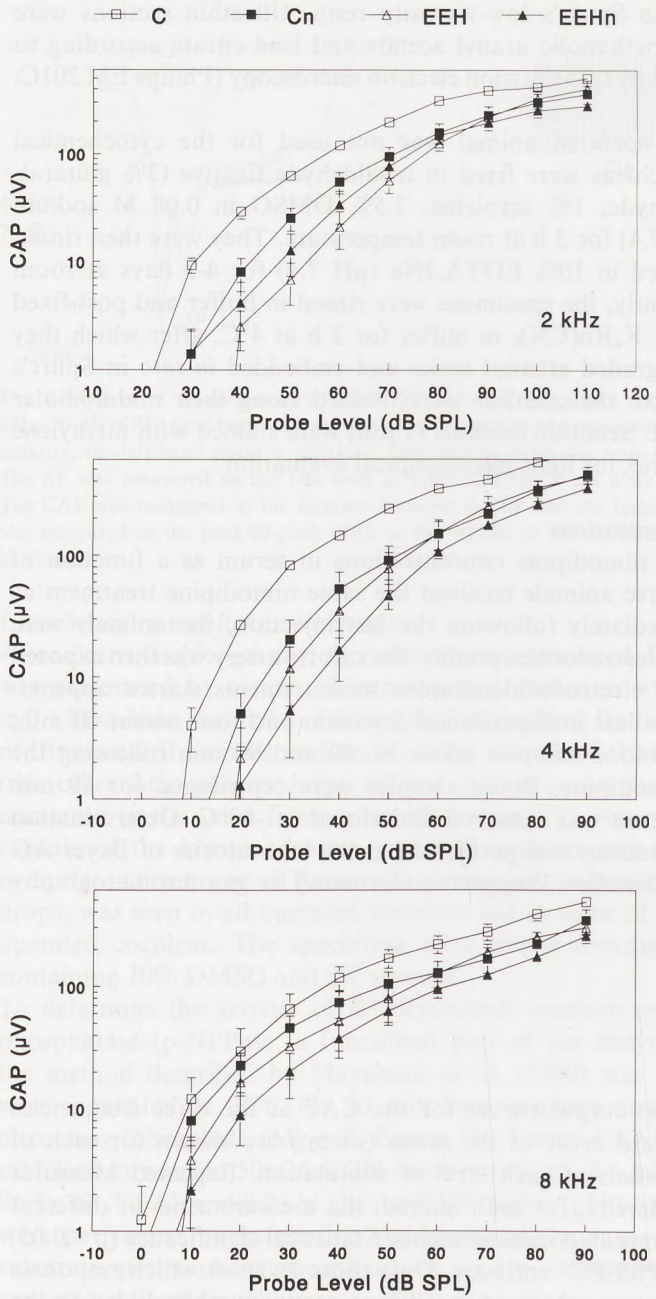

Fig. 2 CAP input-output curves for 2-, 4- and 8-kHz probe tone bursts. Mean and s.e.m. are shown at each level of stimulation for each of the 4 groups of animals. Squares represent normal cochleas, triangles represent cochleas that were operated to evoke EEH. Closed symbols represent the cochleas of animals treated with nimodipine.

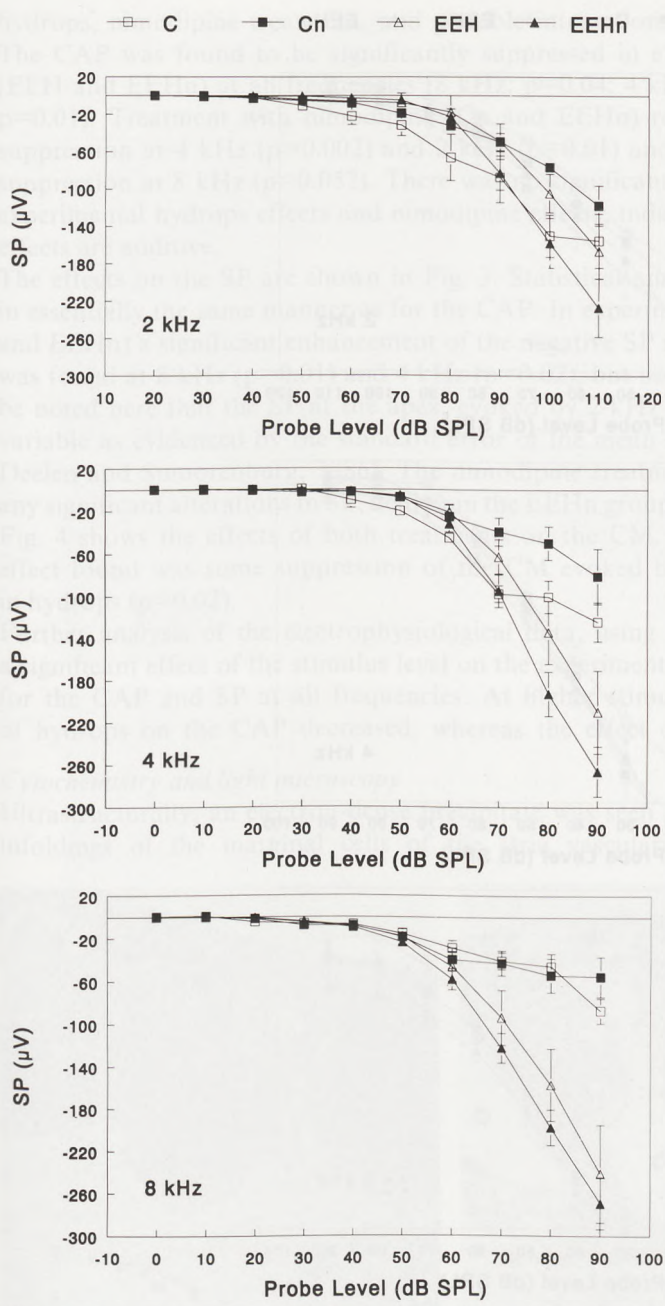

Fig. 3 SP input-output curves for 2-, 4- and 8-kHz probe tone bursts. Mean and s.e.m. are shown at each level of stimulation for each of the 4 groups of animals. Squares represent normal cochleas, triangles represent cochleas that were operated to evoke EEH. Closed symbols represent the cochleas of animals treated with nimodipine.

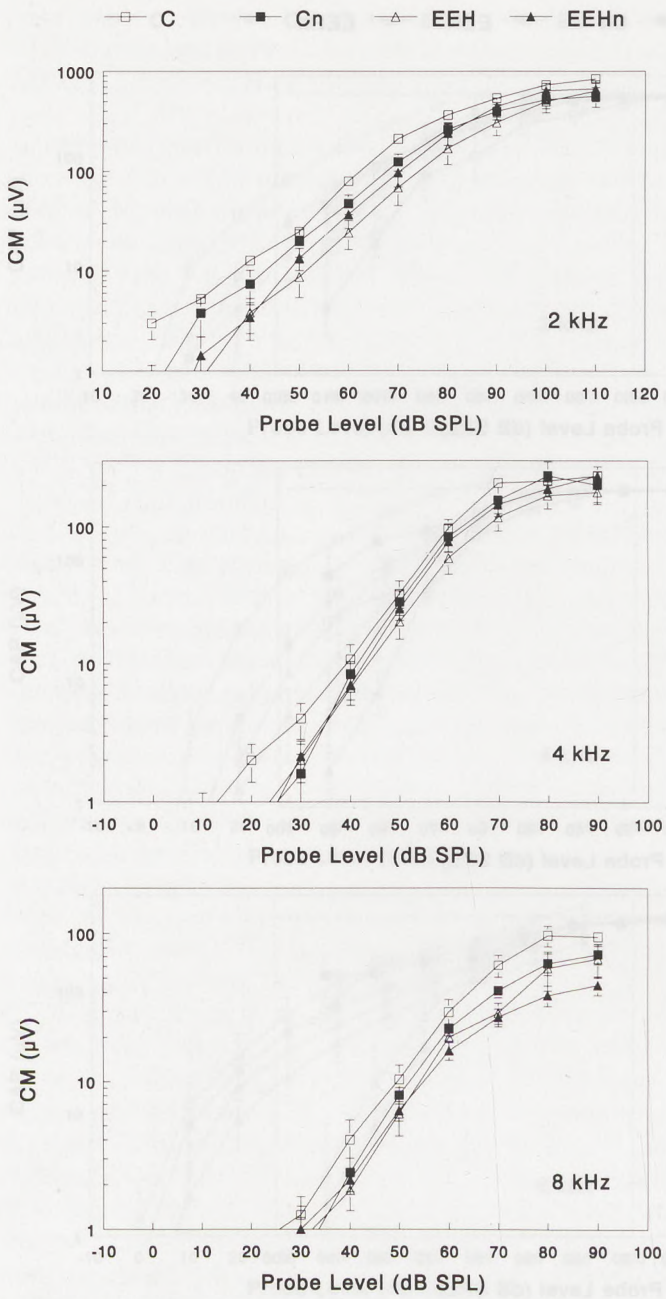

Fig. 4 CM input-output curves for 2-, 4- and 8-kHz probe tone bursts. Mean and s.e.m. are shown at each level of stimulation for each of the 4 groups of animals. Squares represent normal cochleas, triangles represent cochleas that were operated to evoke EEH. Closed symbols represent the cochleas of animals treated with nimodipine.

hydrops, nimodipine treatment, and possible interactions between these two. The CAP was found to be significantly suppressed in experimental hydrops (EEH and EEHn) at all frequencies (8 kHz: p=0.04; 4 kHz: p=0.002; 2 kHz: p=0.01). Treatment with nimodipine (Cn and EEHn) resulted in significant suppression at 4 kHz (p=0.002) and 2 kHz (p=0.01) and (almost) significant suppression at 8 kHz (p=0.052). There was no significant interaction between experimental hydrops effects and nimodipine effects, indicating that these two effects are additive.

The effects on the SP are shown in Fig. 3. Statistical analysis was performed in essentially the same manner as for the CAP. In experimental hydrops (EEH and EEHn) a significant enhancement of the negative SP measured at the apex was found at 8 kHz (p=0.01) and 4 kHz (p=0.02), but not at 2 kHz. It should be noted here that the SP at the apex, evoked by 2-kHz tone bursts is highly variable as evidenced by the standard error of the mean shown in Fig.3 (Van Deelen and Smoorenburg, 1986). The nimodipine treatment did not result in any significant alterations in SP, neither in the EEHn group nor in the Cn group. Fig. 4 shows the effects of both treatments on the CM. The only significant effect found was some suppression of the CM evoked by 8-kHz tone bursts in hydrops (p=0.02).

Further analysis of the electrophysiological data, using Wilks' test, revealed a significant effect of the stimulus level on the experimental hydrops treatment for the CAP and SP at all frequencies. At higher stimulus levels, the effect of hydrops on the CAP decreased, whereas the effect on the SP increased.

Cytochemistry and light microscopy

Ultrastructurally, an electron-dense precipitate was seen along the basolateral infoldings of the marginal cells of the stria vascularis (Fig. 5A) and in

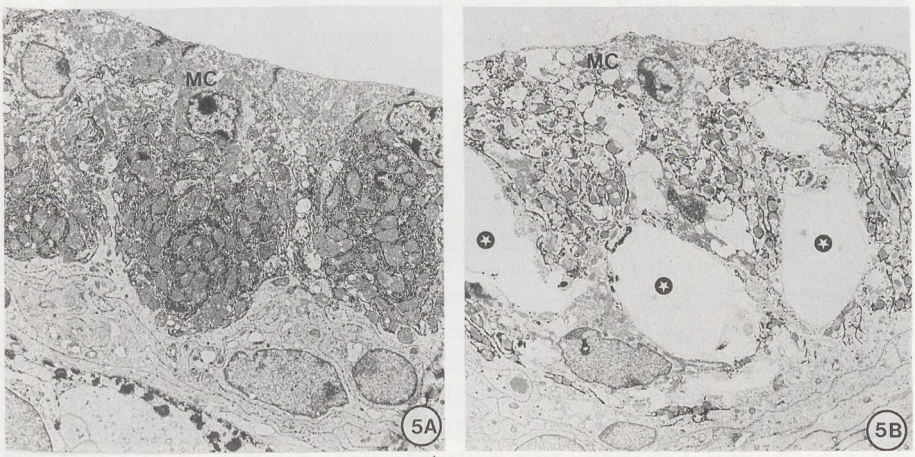

Fig. 5 Lead phosphate precipitates at the basolateral membranes of the strial marginal cells, where Na^+/K^+-ATPase activity is located. (A) In the normal cochlea. (B) In experimental endolymphatic hydrops. MC = marginal cell, asterisk = oedema (x 3,000).

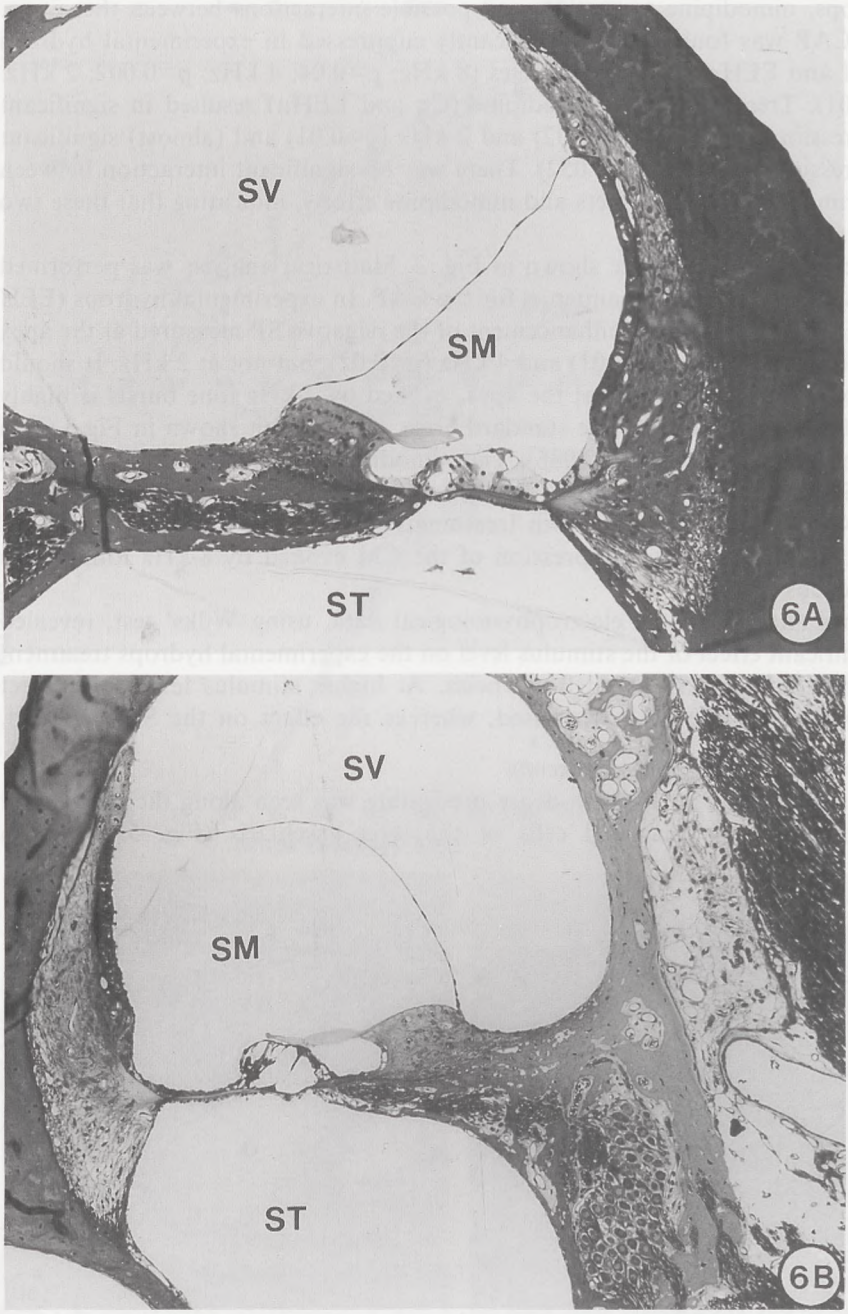

Fig. 6 Light micrograph of the scala media. (A) In the normal situation. (B) Mild distension of Reissner's membrane following nimodipine treatment. SV = scala vestibuli, SM = scala media, ST = scala tympani (x 80).

the stromal cells of the spiral ligament, indicating p-NPPase activity. Differences in the intensity of the precipitates between cochleas from the C and Cn group were not observed. In experimental hydrops, edema of the stria vascularis was noted (Fig. 5B). This degenerative feature appeared to reduce the total amount of precipitates of the stria vascularis, suggesting a decrease in p-NPPase activity. Differences in reaction product of the stria vascularis and the spiral ligament between cochleas of the EEH and EEHn group were not found.
Light microscopy demonstrated a mild hydrops of the endolymphatic compartment of the lower cochlear turns in two out of five cochleas of the Cn group (Fig. 6B). Hydrops was never seen in the cochleas of the C group (Fig. 6A).

Serum concentrations of nimodipine
The serum concentrations of nimodipine 30, 60 and 90 minutes after the last intraperitoneal nimodipine injection are presented below, together with their standard error of the mean.

30 min: 152.8 ± 15,9 nM
60 min: 169.4 ± 21,2 nM
90 min: 163.2 ± 28,6 nM

DISCUSSION

Effects of EEH
The results presented in this paper confirm that 11 weeks after obliteration of the endolymphatic duct and sac, evident morphological and electrophysiological alterations have occurred in the cochlea. A distension of Reissner's membrane was found in all operated cochleas, but in none of the non-treated, non-operated cochleas. Electrophysiologically, a depression of the CAP at 2, 4 and 8 kHz, an enlargement of the negative SP at 4 and 8 kHz, and a tendency of CM depression, significant at 8 kHz, were measured. Similar effects were reported previously in several other studies (Konishi and Kelsey, 1976; Konishi et al., 1981; Kumagami and Miyazaki, 1983; Aran et al., 1984; Harrison et al., 1984; Morizono et al., 1985; Horner and Cazals, 1987; Van Deelen et al., 1987; Klis and Smoorenburg, 1988). The effect of EEH on the CAP was found to be dependent on the level of stimulation. The effect of EEH is more pronounced at the lower levels of stimulation; this phenomenon can be interpreted to resemble recruitment. The reverse is found for the SP. Here, the EEH effect is more prominent at higher levels of stimulation.
The total Na^+/K^+-ATPase activity in the stria vascularis of hydropic cochleas was found to be reduced. This reduction was connected with degenerative changes in the stria vascularis, such as edema, at those sites where Na^+/K^+-ATPase activity is normally observed. Bosher (1980) reported a significant

reduction of the EP after blocking strial Na^+/K^+-ATPase with ouabain. Therefore, it seems logical to assume that the reduction of the EP found in EEH (Konishi and Kelsey, 1976; Cohen and Morizono, 1984; Sziklai et al., 1989) is the result of the reduced Na^+/K^+-ATPase activity in the stria vascularis of the hydropic cochleas, as we have demonstrated cytochemically in this study. Within the scope of the present study, quantification of lead precipitation was not performed. For proper quantification of Na^+/K^+-ATPase activity, biochemical assays seem more adequate.

Effects of nimodipine

Previous studies have emphasized the Ca^{2+}-channel-blocking effects of nimodipine in the normal cochlea (Jastreboff and Brennan, 1988; Bobbin et al., 1990, 1991). However, nimodipine is reported to have a distinct Na^+/K^+-ATPase-stimulating effect as well (Pan and Janis, 1984). The concentration necessary for 50% stimulation of Na^+/K^+-ATPase activity is around 10 nM (Pan and Janis, 1984; their Fig. 3). In view of the nimodipine serum concentrations produced with the present protocol (around 160 nM) and considering the observation by Jastreboff and Brennan (1988) of a 10-fold reduction of the nimodipine concentrations in perilymph as compared to serum, it must be assumed that the effects of nimodipine in the present study mainly stem from stimulation of Na^+/K^+-ATPase in the stria vascularis. This is further supported by our finding of an almost 50% reduction of the CAP evoked at 8 kHz, at the same levels of stimulation at which Bobbin et al. (1990; their Fig. 4) demonstrated only a 10% reduction of the CAP at 10 kHz, following perilymphatic perfusion with 100 nM nimodipine. The presence of a mild endolymphatic hydrops in normal cochleas after nimodipine treatment is in line with the assumption that the effects found mainly stem from stimulation of strial Na^+/K^+-ATPase. This mild endolymphatic hydrops in the presence of an intact endolymphatic sac appears to be due to (a history of) increased endolymph production, probably resulting from a nimodipine-evoked increased activity of strial Na^+/K^+-ATPase. However, the possibility of some Ca^{2+}-channel-blocking effect of nimodipine in the present study cannot be excluded on the basis of our data.

A pharmacologically induced increase of endolymph production resulting in hydrops has been suggested for cholera toxin (Feldman and Brusilow, 1976). Increased activity of the stria, as measured by an increased EP, has been found with forskolin (Doi et al., 1990). Both cholera toxin and forskolin probably exert their action through the cAMP intracellular messenger system, which stimulates the activity of Na^+/K^+-ATPase; nimodipine is thought to do so through a yet unknown mechanism.

The effect of nimodipine treatment on the CAP for the lower frequencies could be a direct effect of the nimodipine-induced hydrops. In EEH, the initial effects also occur at the lower frequencies (Horner and Cazals, 1987). Significant effects of nimodipine treatment on the SP were not observed, although these are

common in EEH. This could be due to the fact that the animals were treated with nimodipine for only three days, resulting in only very mild endolymphatic hydrops. Absence of effects on the SP does, however, lend support to the assumption of mainly Na^+/K^+-ATPase-stimulating activity of nimodipine in the present study, because at higher concentrations strong depressing effects on the SP have been reported. These are interpreted to be connected with the Ca^{2+}-channel-blocking effects of the compound (Bobbin et al., 1990).
Obvious effects on lead phosphate precipitation in the stria vascularis of the nimodipine-treated animals were not observed, neither in the normal nor in the EEH cochleas. One explanation could be that no effects on Na^+/K^+-ATPase activity have occurred at all, but this does not seem very likely, considering the development of mild hydrops and in view of the electrophysiological changes described above, both suggesting excessive production of endolymph. On the other hand, it is conceivable that after a certain period of endolymph over-production, some internal feedback mechanism might start down-regulating Na^+/K^+-ATPase activity, thereby counteracting the stimulatory effect. Alternatively, the cytochemical method we have used might just not be sensitive enough to detect small differences in Na^+/K^+-ATPase activity.

Interactions between EEH and nimodipine effects
Statistically, no significant interaction was found between the EEH and nimodipine effects on cochlear function. The effects seem to add, and nimodipine certainly does not improve function in guinea pig cochleas affected by EEH. There have been some optimistic reports about nimodipine treatment of various inner ear pathologies associated with tinnitus (Theopold, 1985; Jastreboff and Brennan, 1988; Coleman et al., 1991). However, this animal study indicates that caution is warranted with regard to its use in the treatment of tinnitus in Menière's disease. The results of this study point to an excessive production of endolymph following treatment with nimodipine. This could augment hydrops in patients with Menière's disease.
The finding of mild endolymphatic hydrops in some of the nimodipine-treated normal cochleas suggests the possibility of a pharmacologically induced hydrops which might serve as a new model to study its pathophysiology. This model differs from the classical hydrops model in one important respect. In the classical "obstruction of absorption" model a decrease in the EP is found; in this "excessive production" model, the EP should be increased, at least temporarily. This might prove to be a better model for studying endolymphatic hydrops in Menière's disease. There are further advantages of a pharmacological model of endolymphatic hydrops. Such a model would allow the study of changes in the endolymphatic duct and sac during hydrops. Moreover, it might offer the possibility to study reversibility with respect to both morphological and functional changes in the hydropic inner ear.

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Chapter 7

Summary and Discussion

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THE GLYCOCALYX

In general, the membranes of various types of cell exhibit a cell surface coat (glycocalyx) that is mainly composed of glycoproteins, glycolipids, and glycosaminoglycans. The glycocalyx present on the luminal surfaces of the epithelia of the inner ear is assumed to contribute to several aspects of inner ear physiology. In this thesis (Chapters 2 and 4) the glycocalyx of the epithelia lining the endolymphatic compartment of both the cochlea and the endolymphatic sac has been studied. The cationic markers colloidal thorium and cationized ferritin were used; these have been demonstrated to react abundantly with the glycocalyx (cf. De Groot and Veldman, 1988). Experiments in which enzymatic digestions were performed prior to incubation with colloidal thorium revealed that mainly sialic acid residues are responsible for the observed reactivity in both cochlea and endolymphatic sac.

The apical membranes of the hair cells demonstrate a high content of sialic acid, which might be functionally related to the mechano-electrical transduction process. This is in agreement with the assumption that the glycocalyx is responsible for creating a distinct micro-environment around the stereociliary bundles and the apical membranes of the hair cells, by reason of its net negative surface charge (Hudspeth, 1985; Slepecky and Chamberlain, 1985; Santi and Anderson, 1987).

A similar situation has been observed in the proximal tubules and the thin limb of Henle's loop in the rat kidney (Roth and Taatjes, 1985). There the high sialic acid content of the epithelial membranes is thought to be related to the sequestration of cations by the glycocalyx, prior to facilitated uptake via ion-selective channels. Interestingly, enzymatic removal of sialic acid has been shown to result in impairment of K^+ transport across membranes (Glick and Githens, 1965). Because of these findings, the fact that the endolymphatic glycocalyx of both the cochlea and the endolymphatic sac is rich in sialic acid would suggest that it is somehow involved in K^+ transport in the inner ear. In our experiments we observed only a weak reactivity for colloidal thorium of the apical membranes of the strial marginal cells, as compared to the endolymphatic surfaces of the organ of Corti and Reissner's membrane. This seems to indicate that the apical membranes of the marginal cells are relatively low in sialic acid, which is in agreement with the previous results of Saito et al. (1982) and Santi and Anderson (1986). Assuming that sialic acid plays a significant role in the uptake of K^+, it seems plausible that sialic acid is low in the apical membranes of the marginal cells since K^+ is transported

out of the marginal cells into the scala media.
Potassium in the endolymphatic compartment of the inner ear can easily leak away through the tight junctions of the epithelia in the endolymphatic duct and sac (Bagger-Sjöbäck et al., 1981; Rask-Andersen et al., 1981; Bagger-Sjöbäck and Rask-Andersen, 1986; Wackym et al., 1986). In addition, K^+ is constantly drained from the endolymph, for instance, during transduction. The glycocalyx of hair cells is rich in sialic acid, with which it can sequester cations like K^+. Hair cells need K^+ for depolarization of their membranes. This is followed by expulsion of K^+ from the hair cells into the Cortilymph. These deficits are compensated by the energy-dependent production of K^+-rich endolymph by the strial marginal cells and vestibular dark cells, thus maintaining the high K^+ concentration (140-150 mM) in the endolymph of the cochlea and the vestibular apparatus. In the endolymphatic sac, the K^+ concentration of endolymph is only 15 mM (Sterkers et al., 1988; Ikeda and Morizono, 1991). This correlates well with our finding that Na^+/K^+-ATPase activity is low in the endolymphatic sac (Chapter 5).

The glycocalyx in endolymphatic hydrops
Except for slightly decreased reactivity of the apical membranes of the outer hair cells, changes in glycocalyx reactivity for colloidal thorium or cationized ferritin could not be demonstrated in the cochlear epithelia within the first six months following endolymphatic sac obliteration (Chapter 2). This seems to indicate that neither membrane nor glycocalyx changes occur at this stage of endolymphatic hydrops. However, it can not be excluded that moieties other than those detected by colloidal thorium and cationized ferritin might be affected during endolymphatic hydrops. In support of this, Albers et al. (1987), using $OsO_4/K_4Ru(CN)_6$ post-fixation to visualize the glycocalyx, reported glycocalyx disorganization of the non-sensory epithelia of the cochlea just two months following endolymphatic sac obliteration. Since we cannot exactly identify the reactive groups (carbohydrates, glycolipids, glycoproteins, lipids) responsible for the enhancing effect of $OsO_4/K_4Ru(CN)_6$, it remains unclear which particular glycocalyx alterations occur in experimental hydrops.

STEREOCILIARY CROSS-LINKS

Recently, there has been some discussion about the exact subcellular morphology, biochemical composition, and function of interstereociliary connections (stereociliary cross-links) of hair cells in the inner ear. In Chapter 3, the subcellular morphology and composition of the stereociliary cross-linkage system of cochlear hair cells have been cytochemically studied. In close agreement with Pickles et al. (1984) we have distinguished two types of cross-links: (1) side links connecting the stereocilia, either of adjoining rows (row-to-row links) or within the same row (side-to-side links); and (2) tip links (tip-

to-side links) connecting the tips of shorter stereocilia to the flank of an adjacent, longer stereocilium.

The side links are positioned perpendicular to the stereociliary surface. They consist of a system of multiple strands, which react with the cationic markers colloidal thorium and cationized ferritin. The network of multiple strands is assumed to be continuous with the stereociliary lattice (Flock et al., 1977; Furness and Hackney, 1985; Takumida et al., 1989).

The tip links were found to connect the tips of the shorter stereocilia with the flanks of the longer stereocilia of an adjoining row within the same stereociliary bundle. A thin, single strand was demonstrated to be surrounded by anionic material, reacting with the cationic markers colloidal thorium and cationized ferritin. At both ends of the tip link, a submembranous density was frequently seen (cf. Furness and Hackney, 1985; Osborne et al., 1988). These densities may represent the sites of the subcellular anchoring system of the tip links.

The exact composition of the cross-links has not yet been elucidated. The strands seem to contain an elastin or an elastin-like protein, since cross-links disappear after treatment with elastase, leaving a collapsed stereociliary bundle; this is not the case following treatment with chondroitinase, hyaluronidase, keratanase or collagenase (Osborne and Comis, 1990; Pickles et al., 1990). When incubation with colloidal thorium is preceded by digestion with neuraminidase, the stereociliary cross-links lose their reactivity, indicating that sialic acid is present in or around the stereociliary cross-links (Chapter 3). Preliminary results obtained with lectin cytochemistry demonstrate that cross-links contain not only sialic acid but also other carbohydrates (De Groot, personal communication). On the basis of these findings and in analogy with the model proposed for cell adhesion through connections of the cell's cytoskeleton, *a new concept of the composition of cross-links* can be presented. We hypothesize that cross-links consist of a proteinaceous core, which connects to an integrin-like protein in the stereociliary membrane and through that to the actin skeleton of the stereocilium (cf. Plinkert et al., 1992). In addition, cross-links contain and/or are surrounded by glycoconjugates.

Because of their subcellular morphology and characteristic biochemical composition, we think that side and tip links are a separate morphological and functional entity, and do not represent mere remnants of an inadequately preserved stereociliary glycocalyx.

Cross-links in endolymphatic hydrops

In experimental endolymphatic hydrops of the guinea pig cochlea, a progressive loss of cross-links has been suggested by Albers et al. (1987) using transmission electron microscopy. This was confirmed with scanning electron microscopy by Horner et al. (1988), Rydmaker and Horner (1991), and Ruding et al. (1991). Furthermore, Oda et al. (1992) demonstrated loss of stereociliary cross-links of vestibular hair cells three months after surgical induction of endolymphatic

hydrops. Moreover, we observed loss of stereociliary cross-links - both tip and side links - of outer hair-cell stereociliary bundles within six months after obliteration of the endolymphatic sac (Chapter 3). In addition, disarrangement and collapse of the stereociliary bundle was noted; the changes progressed from the apex to the base of the cochlea, which is in agreement with the results of other investigators (Horner et al., 1988; Rydmaker and Horner, 1991; Ruding et al., 1991). These findings support the proposed function of cross-links in maintaining structural integrity within the stereociliary bundle. Furthermore, and concomitant with the atrophy of both short- and middle-sized stereocilia, the loss of stereociliary cross-links of the outer hair cells coincides with permanent threshold shifts of the compound action potential at corresponding frequencies (Rydmaker and Horner, 1991). This, in part, corroborates the hypothesis that tip links are key structures in mechano-electrical transduction.

Na^+/K^+-ATPase

Chapter 5 presents a comparative study that has been conducted to investigate different enzyme-cytochemical methods for the ultrastructural localization of the enzyme Na^+/K^+-ATPase in the normal inner ear of the guinea pig. These methods make use of the enzyme's potassium-dependent, ouabain-sensitive phosphatase activity, and visualization is based on hydrolysis of the substrate p-nitrophenylphosphate resulting in the release of free, inorganic phosphate. This phosphate is immediately captured by either lead or cerium ions, resulting in electron-dense, insoluble precipitates of lead phosphate and cerium phosphate, respectively. The precipitates, hence, represent the subcellular sites where the enzyme demonstrates its activity.

Although the use of cerium as capture agent generally results in distinct precipitates, in our study this method proved to be inconsistent, probably due to poor penetration of cerium ions into the tissues (Kalicharan et al., 1985). By means of the lead-based method, heavy precipitates were observed with high consistency, in particular at the basolateral membranes of the marginal cells of the stria vascularis. A weaker, but still significant, reaction was seen on the invaginated membranes of the fibrocytes of the spiral ligament, and especially in those cells situated in the stroma of the spiral prominence. Precipitates were also seen at the basolateral infoldings of the vestibular dark cells. Virtually no enzyme activity could be demonstrated in the epithelial lining of the endolymphatic sac, contrary to earlier reports by Mizukoshi et al. (1988). This subcellular distribution of Na^+/K^+-ATPase activity in inner ear tissues correlates well with the high K^+ concentrations present in endolymph (Juhn, 1984; Drescher and Kerr, 1985; Sterkers et al., 1988). In the cochlea, the K^+ concentration in endolymph is about 150 mM, in the utricule and saccule, about 140 mM; and in the endolymphatic sac - where virtually no Na^+/K^+-ATPase activity could be demonstrated - it is only about 15 mM (Juhn, 1984;

Sterkers et al., 1988). Various studies point to a direct dependence of the endocochlear potential on strial Na^+/K^+-ATPase activity (Bosher et al., 1973; Bosher, 1980; Juhn, 1984; Offner et al., 1987). The observation that the concentrations of K^+ in the cochlear endolymph and the magnitude of the endocochlear potential both decrease from the basal to the apical turns (Sterkers, 1985; Sterkers et al., 1988) correlates remarkably well with the concomitant decrease in Na^+/K^+-ATPase activity from the basis to the apex of the cochlea (Kuijpers and Bonting, 1969). The low endolymphatic potential of the utricle does not correspond with the presence of Na^+/K^+-ATPase activity in this structure (Juhn, 1984; Dresher and Kerr, 1985). At present no explanation for this phenomenon is at hand.

Na^+/K^+-ATPase in endolymphatic hydrops

Na^+/K^+-ATPase activity in experimental endolymphatic hydrops was investigated in Chapter 6. Edema of the stria vascularis of the hydropic cochleas was noted with a concomitant reduction in Na^+/K^+-ATPase activity. As mentioned earlier, a direct relationship between Na^+/K^+-ATPase activity and the endocochlear potential is assumed for the cochlea. The decrease in endocochlear potential in endolymphatic hydrops (Konishi and Kelsey, 1976; Cohen and Morizono, 1984; Sziklai et al. 1989) can therefore be explained by the decrement of strial Na^+/K^+-ATPase activity, as we have demonstrated in Chapter 6. Hozawa et al. (1988), however, did not observe any decrease in Na^+/K^+-ATPase activity up to six months following obliteration of the endolymphatic sac. It must be stated that variability in degenerative changes of the stria vascularis as observed in endolymphatic hydrops makes evaluation of Na^+/K^+-ATPase activity difficult.

Steel et al. (1987) suggested that the absence of intermediate cells in the stria vascularis of a certain strain of mutant mice is responsible for the low endocochlear potential in these animals. In analogy to this concept, the absence of intermediate cells in the affected stria vascularis of hydropic cochleas could be an alternative explanation for the decreased endolymphatic potential in endolymphatic hydrops.

Nimodipine and cochlear function

Apart from the decrease of the endocochlear potential in experimental endolymphatic hydrops as dicussed above, a depression of the compound action potential and an enlargement of the negative summating potential are found (Konishi and Kelsey, 1976; Konishi et al., 1981; Kumagami and Myazaki, 1983; Aran et al., 1984; Harrison et al., 1984; Morizono et al., 1985; Horner and Cazals, 1987; Van Deelen et al., 1987; Klis and Smoorenburg, 1988). In Chapter 6, an attempt was made to restore the endocochlear potential (and thus cochlear function) during endolymphatic hydrops by stimulating strial Na^+/K^+-ATPase activity. To this end, both normal guinea pigs and guinea pigs with hydropic ears were systemically treated with nimodipine, an L-type Ca^{2+}-channel-blocking

agent with Na^+/K^+-ATPase-stimulating properties. This treatment was found to result in nimodipine serum levels above the EC_{50} for stimulation of Na^+/K^+-ATPase (Pan and Janis, 1984) but well below the IC_{50} for depression of the compound action potential due to Ca^{2+}-channel blockage (Bobbin et al., 1990). Therefore, we assumed that the effects of nimodipine on cochlear function mainly stem from Na^+/K^+-ATPase stimulation.

Light-microscopic evaluation of normal cochleas treated with nimodipine showed a mild distension of Reissner's membrane in the lower turns of some of the specimens. Furthermore, in normal animals treated with nimodipine, threshold shifts of the compound action potential occur at the lower frequencies, as in animals with hydropic cochleas. Both findings suggest that nimodipine treatment might result in an overproduction of endolymph. The cytochemically-determined strial Na^+/K^+-ATPase activity, however, appeared to be unaltered following nimodipine treatment in both normal and hydropic cochleas. Since excessive production of endolymph can only be explained by an increased activity of Na^+/K^+-ATPase, we have concluded that the cytochemical method used might not be sensitive enough to detect the apparent changes in enzyme activity caused by nimodipine treatment. On the other hand, after nimodipine-evoked stimulation of Na^+/K^+-ATPase activity, some internal feedback mechanism might start down-regulating Na^+/K^+-ATPase activity, eventually counteracting the stimulatory effect.

Nimodipine treatment does not restore cochlear function in hydropic ears. There have been several optimistic reports about nimodipine treatment of tinnitus due to inner ear pathologies (Theopold 1985; Jastreboff and Brennan, 1987; Coleman et al., 1991). Yet the results obtained in Chapter 6 indicate that caution is warranted in nimodipine treatment of patients suffering from Menière's disease. Such treatment may enhance the production of endolymph, thus augmenting endolymphatic hydrops.

Pharmacologically induced endolymphatic hydrops

During the last decades, the most frequently used model to study and to explain endolymphatic hydrops has been the so-called "obstruction of absorption" model. This model is based on the fact that endolymphatic hydrops can be induced by surgical obliteration and obstruction of the endolymphatic duct and sac. Due to blockage of the longitudinal flow of endolymph, accumulation of endolymph results and Reissner's membrane becomes distended.

In Chapter 6, a more dynamic model for the investigation of endolymphatic hydrops has been proposed. Pharmacological stimulation of strial Na^+/K^+-ATPase activity could result in "excessive production" of endolymph (cf. Feldman and Brusilow, 1973), which the endolymphatic sac might not be able to absorb immediately; subsequent endolymph accumulation eventually results in a distension of Reissner's membrane.

Pathophysiology of Menière's disease

Since the development of the "obstruction of absorption" model by Kimura and Schuknecht (1965), many investigators have used the obstruction-of-absorption concept to explain the pathophysiological events in Menière's disease. As evidenced by the histopathological changes observed in the endolymphatic sac of patients suffering from Menière's disease (Hallpike and Cairns, 1938; Schuknecht, 1977; Arenberg et al., 1970; Lim and Glasscock, 1981; Ge and Shen, 1989), dysfunction of the human endolymphatic sac may lead to slowly progressive degenerative changes in the inner ear. Similar changes can also be observed in experimental endolymphatic hydrops.

However, many questions remain unanswered. For instance, no conclusive explanation has been found for the combined attacks of vertigo, tinnitus, and sensorineural hearing loss. These attacks are thought to be due to rupturing of Reissner's membrane, resulting in transient contamination of perilymph (and Corti-lymph) with neurotoxic, K^+-rich endolymph (Schuknecht, 1982; Schmidt, 1983). However, the clinical observation that exacerbations of Menière's disease often coincide with periods of stress (Groen and Schmidt, 1984) cannot be explained by the "rupture and repair" theory.

Rarey et al. (1988, 1989, 1991) and Ten Cate et al. (1990) demonstrated that adrenal hormones influence Na^+/K^+-ATPase activity in the rat inner ear. As adrenal activity is increased during stress, this might incite Na^+/K^+-ATPase activity, resulting in an overproduction of endolymph.

When the classical "obstruction of absorption" model and the proposed "excessive production" model are combined, a more complete description for the pathophysiological events in Menière's disease may be presented.

FUTURE STUDIES

As far as glycoconjugate cytochemistry is concerned, future studies with lectins are needed to further elucidate the composition of the endolymphatic glycocalyx of the inner ear. Lectin cytochemistry will also be useful to determine in more detail the composition of stereociliary cross-links. Future studies of cross-links should also focus on their system of anchoring to the stereocilia, and on their suggested continuity with the stereociliary lattice.

Studies concerning Na^+/K^+-ATPase activity in the inner ear should focus on the possibility to influence the activity of this enzyme in order to study the proposed model of "excessive production" for the investigation of endolymphatic hydrops. This model allows us to study the endolymphatic duct and sac during experimental endolymphatic hydrops. Furthermore, it could shed more light on the issue whether endolymphatic sac pathology itself is a direct cause for the development of endolymphatic hydrops or is just a consequence of endolymphatic hydrops. Also, the reversibility of the observed degenerative changes in hydropic inner ears could be studied by simply withdrawing the

causative (pharmacological) agent. This could be of special interest, since it may add to a better understanding of the functional recovery of the inner ear following attacks of Menière's disease.

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Samenvatting

Samenvatting

In *Hoofdstuk 1* werd een uiteenzetting gegeven van de huidige inzichten over de mechano-electrische transductie van de haarcellen en over de fysiologie van het endolymfatische compartiment van het binnenoor, met name in relatie tot de subcellulaire morfologie van het binnenoor. Daarnaast werd de pathofysiologie van experimentele endolymfatische hydrops in het binnenoor besproken, een proefdiermodel dat overeenkomsten vertoont met de ziekte van Menière wat betreft de pathologische en functionele veranderingen van het binnenoor.

In *Hoofdstuk 2* werd de luminale glycocalyx – een onderdeel van de plasmamembraan gevormd door met name de glycolipiden en glycoproteinen die in de "outer leaflet" aanwezig zijn – van de cochleaire epithelia bestudeerd, zowel in normale als hydrops cochlea's. De elektronen-dichte, cytochemische markers colloïdaal thorium en gekationiseerd ferritine werden gebruikt voor de ultrastructurele aantoning van de glycocalyx.
De apicale glycocalyx van de cochleaire haarcellen bleek sterk te reageren met colloïdaal thorium, terwijl de glycocalyx van de stria vascularis in minder sterke mate reageerde. Behandeling met het enzym neuraminidase voorafgaand aan incubatie met colloïdaal thorium resulteerde in een verminderde reactiviteit van de glycocalyx die het endolymfatisch compartiment bekleedt. Dit duidt op aanwezigheid van siaalzuur-bevattende eiwitten en/of lipiden in de luminale membranen van de epithelia die het endolymfatische compartiment van de cochlea bekleden. Er is gesuggereerd dat de apicale glycocalyx van de cochleaire haarcellen een rol speelt in het proces van mechano-electrische transductie. De glycocalyx aanwezig op de apicale membranen van de stria vascularis zou betrokken zijn bij de instandhouding van de samenstelling van de endolymfe. In hydrops cochlea's werd de sterke reactiviteit van de apicale glycocalyx van de haarcellen voor colloïdaal thorium niet meer gezien. Biochemische disorganisatie van de glycocalyx van de haarcellen zou een veranderde glycocalyxfunctie tot gevolg kunnen hebben. Dit zou een rol kunnen spelen bij de functionele veranderingen die optreden in de hydrops cochlea.

Andere subcellulaire structuren die mogelijk belangrijk zijn voor het transductie proces zijn de zogenaamde stereociliaire "cross-links". Zowel "side links" als "tip links" worden onderscheiden. Side links verbinden de flanken van de stereocilia en komen voor in het bovenste deel van de stereocilaire bundel. Een centrale verdichting van de side links werd geobserveerd. Waarschijnlijk hebben de side links tot doel een geïntegreerde beweging van de stereociliaire bundel te bewerkstelligen. Een tip link verbindt de tip van een korter stereocilium met de flank van een langer, naburig stereocilium. Op de plaats van insertie

van de tip link wordt een submembraneuze verdichting gezien in het stereocilium. Mogelijk dat dit de plaats is waar de tip links zijn verankerd aan het cytoskelet van het stereocilium.

In *Hoofdstuk 3* werden deze cross-links bestudeerd met behulp van de cytochemische markers colloïdaal thorium en gekationiseerd ferritine. Digestie met neuraminidase voorafgaande aan incubatie met colloïdaal thorium resulteerde in een sterk gereduceerde reactiviteit van de cross-links. Echter, cross-links konden nog steeds worden waargenomen als gevolg van de routinematig toegepaste post-fixatie en nacontrastering met zware metalen. Op basis van bovengenoemde structurele en cytochemische argumenten nemen wij aan dat cross-links een aparte entiteit zijn.
In experimentele endolymfatische hydrops is het verlies van cross-links een van de eerste pathologische veranderingen die optreden in de cochleaire haarcel. Het verlies van cross-links heeft tot gevolg dat de stereociliaire bundel uiteen valt. Waarschijnlijk is het verlies van cross-links in combinatie met het uiteenvallen van de stereociliaire bundel een van de factoren die bijdragen aan het ontstaan van een permanent perceptief gehoorsverlies in endolymfatische hydrops.

In *Hoofdstuk 4* werd de glycocalyx van de epitheelcellen van de saccus endolymphaticus bestudeerd. Colloïdaal thorium en gekationiseerd ferritine werden gebruikt om de glycocalyx aan te tonen. Er werden geen verschillen in glycocalyx reactiviteit tussen de zogenaamde "light" en "dark cells" waargenomen. Na incubatie met colloïdaal thorium werd wel een sterkere reactiviteit van de apicale membranen ten opzichte van de basolaterale membranen gezien. Digestie met neuraminidase resulteerde in sterk gereduceerde reactiviteit van alle celmembranen, iets wat duidt op de aanwezigheid van siaalzuurgroepen. Het feit dat de apicale membranen sterk reageerden met colloïdaal thorium zou kunnen wijzen op een hoog gehalte aan siaalzuur in deze membranen. Aangezien siaalzuur van belang lijkt te zijn voor het lokaal accumuleren van elektrolyten en het transport van elektrolyten over de plasmamembraan, denken wij dat de specifieke samenstelling van de glycocalyx van de apicale celmembraan van invloed is op de samenstelling van endolymfe in de saccus endolymphaticus.

In *Hoofdstuk 5* werden diverse methodieken bestudeerd voor de aantoning van het enzym Na^+/K^+-ATPase, dat sterk betrokken is bij de instandhouding van het hoge gehalte aan kalium in de endolymfe van de cochlea alsmede bij het handhaven van de hoge transepitheliale potentiaal in de scala media. De enzymcytochemische aantoning van Na^+/K^+-ATPase activiteit is gebaseerd op het vermogen van het enzym om de fosfaatgroep van het, door ons gebruikte, substraat para-nitrofenylfosfaat af te splitsen. De fosfaatgroep wordt tijdens de incubatie direct weggevangen door zwaar metaal-ionen, zoals lood-ionen of cerium-ionen, waarbij een precipitaat gevormd wordt dat voldoende elek-

tronendicht is om in het elektronen microscoop te kunnen worden geobserveeerd. De methode waarbij lood-ionen worden gebruikt blijkt een meer consistent resultaat te geven dan de methode waarbij cerium-ionen worden gebruikt. Elektronen-dichte precipitatie, wijzend op Na^+/K^+-ATPase activiteit, werd gezien langs de basolaterale membranen van de marginale cellen van de stria vasularis, maar ook op de membranen van de stromale cellen in het spirale ligament. De basolaterale membranen van de "dark cells" van de cristae ampullares en de macula utriculi in het vestibulair apparaat vertoonden ook een significante activiteit. Nagenoeg geen activiteit werd gezien in de saccus endolymphaticus.

Volgens diverse studies is de sterk positieve transepitheliale potentiaal in de scala media verlaagd in chirurgisch geïnduceerde hydrops. Dit zou in verband kunnen staan met een verminderde activiteit van het enzym Na^+/K^+-ATPase in de stria vascularis.

In *Hoofdstuk 6* werd het effect van nimodipine – een calcium-antagonist waarvan ook Na^+/K^+-ATPase stimulerende eigenschappen zijn beschreven – op het enzym Na^+/K^+-ATPase in normale en hydrops cochlea's bestudeerd. Daarnaast werd het effect van nimodipine op de cochleaire functie met behulp van elektrofysiologische methoden bestudeerd. In chirurgisch geïnduceerde hydrops werden verhoogde actiepotentiaal drempels gemeten na laagfrequente stimulatie. De sommatiepotentiaal was sterk verhoogd met name bij 4 en 8 kHz. Door degeneratieve afwijkingen van de stria vascularis leek een verminderde precipitatie van loodfosfaat – en dus een verminderde activiteit van het enzym Na^+/K^+-ATPase – aanwezig te zijn. Ook systemische toediening van nimodipine resulteerde in een verhoging van de actiepotentiaal drempels na laagfrequente stimulatie. Het effect van hydrops en nimodipine op de actiepotentialen was additief en niet interactief. Toediening van nimodipine alleen leek geen invloed te hebben op de sommatiepotentialen of op de Na^+/K^+-ATPase activiteit in de stria vascularis. Wel werd in sommige controle cochlea's een milde hydrops waargenomen na nimodipine toediening. Dit laatste zou toch weer pleiten voor een verhoogde activiteit van Na^+/K^+-ATPase in de cochlea. Ook veranderingen in de actiepotentialen na nimodipine toediening lijken op de actiepotentiaal veranderingen die werden gemeten in chirurgisch geïnduceerde hydrops. Deze bevindingen lijken aan te tonen dat farmacologische stimulatie van het enzym Na^+/K^+-ATPase kan leiden tot een endolymfatische hydrops in de cochlea.

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Curriculum Vitae

Petrus Paulus Germain van Benthem werd op 28 oktober 1961 geboren te Arnhem. In 1980 behaalde hij het diploma Atheneum-B aan het Hoogveld College te Rosmalen. In datzelfde jaar begon hij met de studie geneeskunde aan de Erasmus Universiteit Rotterdam. Van maart tot juli 1983 deed hij onderzoek naar receptor-binding aan het Child Study Center van de Yale University in New Haven, Verenigde Staten (Hoofd: Prof. A.J. Solnit). In november en december 1984 deed hij onderzoek naar de beoordeling van röntgenfoto's bij ongevallen aan de afdeling radiologie van de University of Cambridge, Cambridge, Groot-Brittannië (Hoofd: Prof. T. Sherwood). In september 1987 voltooide hij de studie geneeskunde. Vanaf oktober 1987 was hij in opleiding tot keel- neus- en oorarts op de afdeling keel- neus- en oorheelkunde van het Academisch Ziekenhuis Utrecht (Opleiders: Prof. Dr E.H. Huizing en Prof. Dr G.J. Hordijk). De opleiding werd 1 oktober 1992 voltooid.

Tijdens de opleiding heeft hij op het laboratorium voor histofysiologie en experimentele pathologie (Hoofd: Dr J.E. Veldman) van de afdeling keel- neus- en oorheelkunde (Hoofd: Prof. Dr E.H. Huizing) aan het onderzoek gewerkt dat heeft geresulteerd in dit proefschrift.

Op dit moment is hij als keel- neus- en oorarts verbonden aan bovengenoemde afdeling. Vanaf januari 1993 zal hij werkzaam zijn in het Lukas Ziekenhuis te Apeldoorn.

De auteur is getrouwd met Sarah Myriam Irene Triest.

STELLINGEN

STELLINGEN

1 Farmacologische stimulatie van het enzym Na^+/K^+-ATPase van de stria vascularis kan leiden tot een endolymfatische hydrops van het binnenoor.

2 Stereociliaire *cross-links* zijn een aparte morfologische en functionele entiteit.

3 De glycocalyx die het endolymfatisch compartiment van het gehele binnenoor bekleedt is van invloed op de samenstelling van de endolymfe.

4 De activiteit van het enzym Na^+/K^+-ATPase in de stria vascularis is bij chirurgisch geïnduceerde hydrops verlaagd.

5 De ontwikkeling van de functionele endoscopische chirurgie van het ostiomeatale complex van de neusbijholten, heeft het indicatiegebied voor het primair creëren van een rhinantrostoma van de onderste neusgang sterk verkleind, doch niet opgeheven.

6 Op dit moment is er geen plaats voor orale hyposensibilisatie-therapie bij de behandeling van IgE gemedieerde allergische rhinitis.

7 Consensus en protocol in de geneeskunde zijn in het algemeen kwaliteit verhogend; echter kritiekloos toegepast kunnen zij een bedreiging vormen voor de individuele patient.

8 Ziektekostenverzekeraars werpen zich op als bewakers van de kwaliteit van de gezondheidszorg; zij zullen waarschijnlijk andere criteria en parameters hanteren dan de medisch beroepsbeoefenaars zelf.

9 Opmerkingen die in de dagbladpers worden gemaakt ten aanzien van het universitaire onderwijs – zoals "studie-rendement", "business-denken" en "op maatschappelijk nut toetsen van een universitaire opleiding" – leiden tot een gevoel van heimwee naar de ivoren toren die de universiteit eens was.

10 De toepassing van het begrip "management" in het ziekenhuis leidt tot het ontstaan van een vergader- en memo-cultuur die contraproductief is voor genees- en verpleegkundig handelen.

11 Net als de golfsport, is promoveren een gevecht met jezelf.

12 Evenmin als mannen altijd gelijk willen hebben, zijn vrouwen geen zandbanken der wetenschap.