PHARMACOLOGICAL MANIPULATION OF ENDOLYMPH HOMEOSTASIS IN THE COCHLEA

P.J.F.M. Lohuis

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PHARMACOLOGICAL MANIPULATION OF ENDOLYMPH HOMEOSTASIS IN THE COCHLEA

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endolymfe homeostase in de cochlea

(met een samenvatting in het Nederlands)

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Voor mama Lohuis

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General introduction

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GENERAL INTRODUCTION

1.1 Mechano-electrical transduction of sound

In mammals, the transduction of sound into bio-electrical signals takes place in the cochlea (Pickles,1988). The cochlea, lying within a cavity of the temporal bone, can be described as a coiled tube, which is divided in three compartments by two membranes that run along the length of the tube: the Reissner's membrane and the basilar membrane. A cross section of the cochlear tube (Fig. 1) shows the three compartments, all being fluid-filled. The two outer compartments, which are called scala vestibuli (SV) and scala tympani (ST), contain a filtrate of cerebrospinal fluid or blood which is called perilymph. Within the membranous boundary of Reissner's membrane (RM) and the basilar membrane lies scala media (SM), which is filled with a fluid called endolymph. Perilymph and endolymph differ markedly in ionic composition (see section 1.2).

The key elements for the understanding of the neurobiology of the cochlea are located within and around SM. The auditory sensory epithelium, the spiral organ of Corti, is a well organized multicellular structure resting upon the basilar membrane. When sound waves enter the cochlea through the oval window, pressure differences occur over the basilar membrane, setting it in motion. The response of the basilar

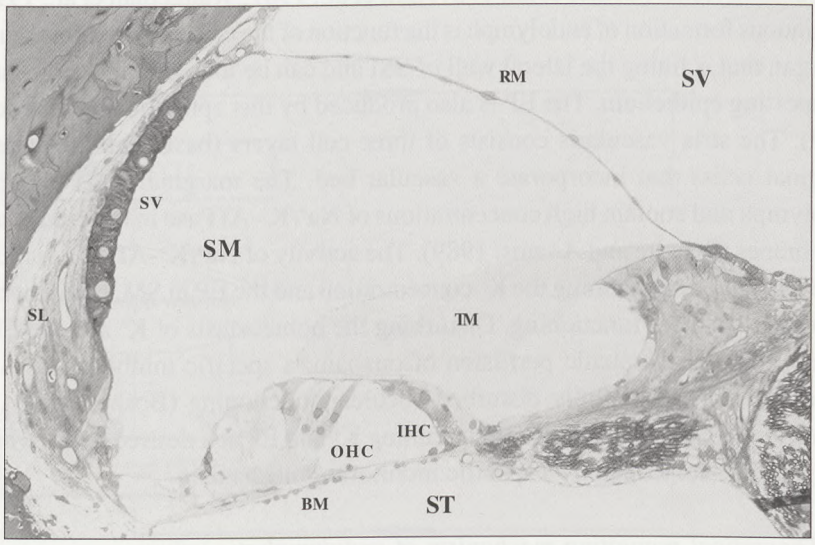

FIGURE 1. Cross section of scala media. SM-scala media, SV-scala vestibuli, ST-scala tympani, RM-Reissner's membrane, SV-stria vascularis, SL-spiral ligament, BM-basilar membrane, IHC-inner hair cell, OHC-outer hair cells, TM-tectorial membrane.

membrane to stimulation consists of a traveling wave that propagates away from the oval window towards the apex. The vibrations reach a maximum amplitude at a place along the length of the basilar membrane that varies with stimulus frequency (Von Békésy, 1947). In the organ of Corti the mechanical movements of the basilar membrane are transduced into electrical signals of the auditory nerve mainly by the inner hair cells (IHC). The outer hair cells (OHC) of the organ of Corti are thought to feed energy into the basilar membrane motion. The latter process increases the sensitivity of the organ of Corti, sharpens up the traveling wave at the place of its highest amplitude and improves the frequency resolving power of the cochlea (Murugasu and Russell, 1996).

1.2 Cochlear fluid homeostasis

SM contains endolymph and is separated from the surrounding structures by a lumenal layer of cells sealed by tight junctions (Jahnke, 1975). SM has two special features. First, the medium filling it is similar to intracellular fluid in its major ionic contents: it contains high K^+ and low Na^+ (Aniko and Wroblewski, 1986) This is in contrast to perilymph, which resembles extracellular fluid with Na^+ as its main cation and little K^+. Second, within SM, endolymph is polarized to a high positive endocochlear potential (EP) of approximately +80 mV with respect to the surrounding fluids (Offner et al., 1987; Salt et al., 1987; Wangeman et al., 1995). Continuous formation of endolymph is the function of the cells of the stria vascularis, an organ that is lining the lateral wall of SM and can be thought of as a typical ion transporting epithelium. The EP is also produced by this epithelium (Offner et al., 1987). The stria vascularis consists of three cell layers (basal, intermediate and marginal cells) that incorporate a vascular bed. The marginal cells border the endolymph and contain high concentrations of Na^+/K^+-ATPase in their basolateral membranes (Schulte and Adams, 1989). The activity of Na^+/K^+-ATPase is thought to be crucial for maintaining the K^+ concentration and the EP in SM, both important for proper cochlear functioning. Disturbing the homeostasis of K^+ and the EP, for example by perilymphatic perfusion of ouabain, a specific inhibitor of Na^+/K^+-ATPase, results in severely disturbed cochlear functioning (Bosher, 1980). The cochlea is capable of accurately maintaining K^+ and EP at a desired level, which is thought to be controlled by a specific monitoring mechanism.

1.3 Theoretical regulating mechanism of endolymphatic volume

Thus, maintenance of ion and fluid homeostasis is essential for proper mechano-electrical transduction. In order to sustain the extracellular milieu of endolymph,

the cochlea must possess a variety of monitoring mechanisms that keep track and act on the balance or imbalance of among others, K^+, EP, Cl^-, Ca^{2+}, pH and endolymph volume. Ion homeostasis and endolymph volume are correlated by osmosis, thus their homeostatic feedback systems most likely will interact. In this section we will focus on the regulation of endolymph volume, since endolymph volume forms a central theme in this thesis. Gaining knowledge about the control mechanism that regulates endolymph volume is important, because dysregulation can lead to endolymphatic hydrops (EH), a condition which is associated with several inner ear disorders, including Menière's disease (Hallpike and Cairns, 1938; Yamakawa, 1938).

In trying to unravel the etiology and pathophysiology behind EH, we will use a model according to Klis et al. (1999). Fig. 2 depicts a reference frame which helps in discussing the minimally required components needed in controlling cochlear fluid homeostasis, e.g. endolymph volume. The reference frame is set up as a feedback system containing four components: a monitor, a comparator, three effectors and a signaling pathway between the monitor, the comparator and the effectors.

We will discuss the different components separately:

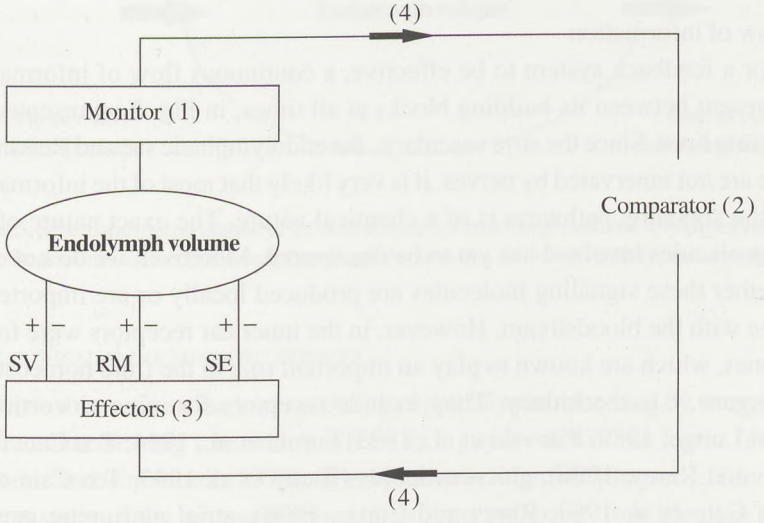

FIGURE 2. Putative feedback system responsible for endolymph homeostasis, e.g. endolymph volume. SV=stria vascularis; RM=Reissner's membrane; SE=endolymphatic sac.

1. The monitor

The monitor keeps track of the volume of SM, probably by measuring endolymphatic pressure instead of the actual volume. The outer hair cells could well be a candidate for this monitor function. They are mechanically sensitive and have been shown to play a role in monitoring the position of the basilar membrane (Zenner, 1993). However, the spiral prominence and Reissner's membrane are also potential candidates.

2. The comparator

The comparator in the given schedule would serve as a device which compares the actual endolymph volume or pressure with the desired one. In case of an existing difference it gives orders for an appropriate action to one of the effectors. We know nothing of this putative comparator.

3. The effectors

More is known about the effectors. Potential candidates for the effectors concerned with the homeostasis of endolymph volume are the stria vascularis and the endolymphatic sac, considered to be primarily connected to endolymph production and endolymph absorption, respectively. Another important effector could be Reissner's membrane, regulating endolymph homeostasis by changing its permeability to water and ions.

4. The flow of information

In order for a feedback system to be effective, a continuous flow of information must be present between its building blocks at all times, in Fig. 2 represented by the connecting lines. Since the stria vascularis, the endolymphatic sac and Reissner's membrane are not innervated by nerves, it is very likely that most of the information traveling the signaling pathways is of a chemical nature. The exact nature of the signaling molecules involved has yet to be discovered. Moreover, we do not even know whether these signaling molecules are produced locally or are imported in the cochlea with the bloodstream. However, in the inner ear receptors were found for hormones, which are known to play an important role in the fluid homeostasis of other organs, e.g. the kidney. They include receptors for mineralocorticoids (Rarey and Luttge, 1989; Pitovski et al., 1993; Furuta et al., 1994; Ten Cate et al, 1994; Yao and Rarey, 1996), glucocorticoids (Rarey et al. 1993, Ten Cate et al. 1992; Ten Cate et al. 1993; Rarey and Curtis, 1996), atrial natriuretic peptide (Lamprecht and Meyer zum Gottesberge, 1988; Suzuki et al., 1998), and vasopressin (Kitano et al., 1997).

1.4 Some aspects of endolymphatic hydrops

Some important aspects of endolymphatic hydrops are discussed shortly.

1.4.1 ETIOLOGY OF ENDOLYMPHATIC HYDROPS

EH is per definition the result of a dysbalance between endolymph production and endolymph absorption (Fig. 3). The production of endolymph takes place in the cochlea by the stria vascularis, while the absorption of endolymph is thought to take place in the endolymphatic sac. Several experimental animal models have been developed to study the etiology of EH as well as the associated physiological alterations (Klis et al., 1999). Most of these models are based on decreasing the absorption of endolymph by obstruction of the endolymphatic sac, one of the effectors proposed in Fig. 2. However, it is not at all clear that EH is caused by disturbed absorption of endolymph. Instead, EH could well be based on an increased production of endolymph. This idea is central to the studies described in this thesis. By affecting strial activity, we are trying to get more insight into the way strial activity might be responsible for pathological changes in cochlear physiology.

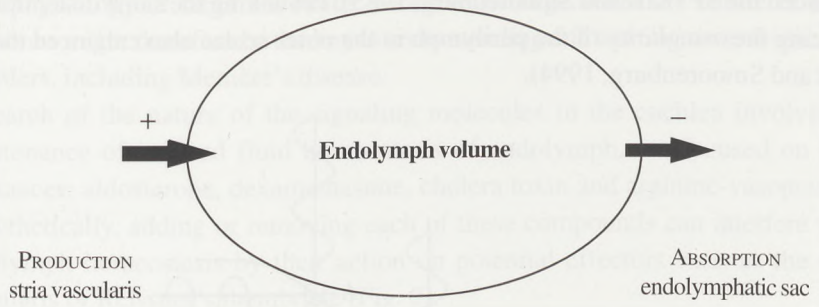

FIGURE 3. Schematic representation of the maintenance of endolymph volume. Endolymphatic hydrops can be the result of an increased endolymph production or a decreased endolymph absorption.

1.4.2 CLINICAL ENDOLYMPHATIC HYDROPS

Menière's disease is an inner-ear pathology clinically characterized by vertigo, tinnitus and fluctuant hearing loss which affects to a greater extent the lower frequencies (Schmidt and Antvelink, 1979). The systematic identification of EH in the post-mortem study of the temporal bones of patients suffering from Menière's disease, has led to the assumption that the increased endolymphatic volume is responsible

for the inner ear dysfunction (Hallpike and Cairns, 1938; Yamakawa, 1938). Loss of auditory sensitivity was mimicked in numerous studies in which EH was induced (Horner and Cazals, 1986; Klis et al., 1998).

1.4.3 ENDOLYMPHATIC HYDROPS AND THE SUMMATING POTENTIAL

The summating potential (SP) is a positive or negative direct current response, which can be picked up by an electrode placed either on or in the cochlea during stimulation. Its polarity and magnitude depend on the position of the recording electrode and on the frequency and the sound pressure of the stimulus (Dallos et al., 1972; Van Deelen and Smoorenburg, 1986).
Gibson et al. (1979) showed that the SP amplitude recorded from the promontory of patients suffering from Menière's disease was larger than the SP amplitude recorded from normal subjects. In addition, many animal studies support the concept of hydrops enhancing the SP. In the guinea pig, electrophysiological evaluation of the effect of endolymphatic sac obliteration showed enhancement of the SP up to two months after obliteration (Van Deelen et al., 1987). Low-frequency biasing, simulating distention of the basilar membrane towards ST as expected in hydrops, enhanced the SP (Klis and Smoorenburg, 1985). Provoking the same distention by reducing the osmolarity of the perilymph in the outer scalae also enhanced the SP (Klis and Smoorenburg, 1994).

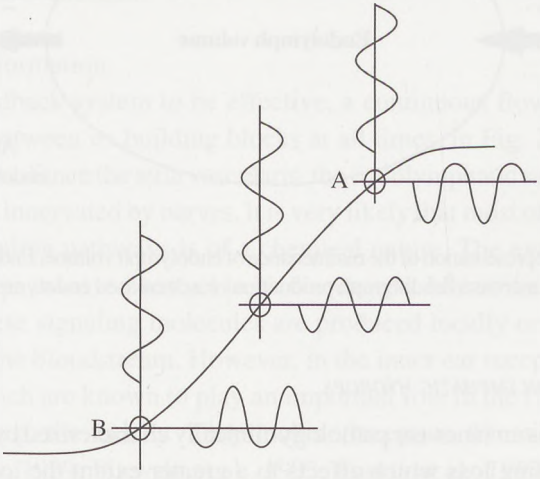

Figure 4. Boltzman curve. A symmetrical saturating transduction function (f(x) = -f(-x)). The resting or operating point is positioned below (A), at, or above (B) the point of symmetry. For each operating point the response (horizontal) to a sinusoidal stimulus (vertical) is shown. In point A the response contains a positive DC potential, in point B the response contains a negative DC potential (printed by courtesy of M.G. van Emst).

Based on these experiments it was hypothesized that the increase of endolymphatic volume results in a distention of the basilar membrane towards ST, shifting the operating point of the cochlear transducer out of its normal resting position. A change in the SP amplitude is then to be expected because of the nonlinearity of the cochlear transducer (Fig. 4).
It is believed that the enhancement of the SP mainly occurs in the acute phase of EH, when RM still has sufficient stiffness to produce a counterforce of a magnitude that can displace the basilar membrane. Therefore, a distinction should be made between an initial stage and an advanced stage of Menière's disease.

1.5 Signaling molecules in the cochlea

It is highly probable that the regulation of endolymphatic volume involves hormonal factors (see section 1.3, Fig. 2). In the cochlea, membrane incorporation of Na^+/K^+-ATPase, ion channels and water channels via synthesis or transcription of genetic information could, to some extent, be under hormonal control. Moreover, hormonal regulation of endolymph volume might form the link between the discovery that several hormone receptors are present in the cochlea and the physiology and pathophysiology of inner ear fluids. More knowledge about the nature of these hormones might therefore be the key to understanding and treating several inner ear disorders, including Menière's disease.
In search of the nature of the signaling molecules in the cochlea involved in maintenance of ion and fluid homeostasis of endolymph, we focused on four substances: aldosterone, dexamethasone, cholera toxin and arginine-vasopressin. Hypothetically, adding or removing each of these compounds can interfere with endolymph homeostasis by their action on potential effectors such as the stria vascularis or Reissner's membrane (Fig. 2).

1.5.1 ALDOSTERONE

In various tissues, e.g. the kidney, the Na^+/K^+-ATPase activity is indirectly regulated by inducing the synthesis of new active pump-units at the nuclear level by adrenocortical hormones, notably aldosterone (Ewart and Klip, 1995). Based on the following investigations, it has been hypothesized that the latter also occurs in the stria vascularis. First, the presence of protein receptors for mineralocorticoids was demonstrated in inner ear tissues (Rarey and Luttge, 1989; Pitovski et al., 1993; Furuta et al., 1994; Ten Cate et al, 1994; Yao and Rarey, 1996). Second, injection of aldosterone increased the binding of [^{3}H]ouabain in the cochlear lateral wall, indicative of an increase of the number of Na^+/K^+-ATPase sites (Pitovski et

al., 1993). Third, removal of endogenous levels of adrenal hormones by bilateral adrenalectomy caused a significant decrease of ATPase activity in the cochlear lateral wall as was shown by fluorometric assay (Rarey et al., 1989).
Since the stria vascularis is involved in the production of endolymph, these studies suggest that perturbations in the activity of strial Na^+/K^+-ATPase can influence endolymph homeostasis and therefore might be related to Menière's disease.

1.5.2 DEXAMETHASONE

Dexamethasone is often used clinically to treat inner ear disorders, including Menière's disease and sudden deafness. Transtympanic injection of dexamethasone (a synthetic adrenal steroid with glucocorticoid action and effects) was reported by Itoh and Sakata (1991) and by Shea and Ge (1996) to give relief of dizzy-spells and to improve hearing in patients suffering from Menière's disease. The recent demonstration of the presence of glucocorticoid receptors in human inner ear tissues (Rarey and Curtis, 1996) provides a basis to consider dexamethasone to act directly on specific inner ear cells, rather than assuming a systemic anti inflammatory or immunosupressive effect. In the cochlea, glucocorticoids may therefore play part in cellular metabolism through regulation of protein synthesis of metabolic enzymes or even influence electrolyte balance.

1.5.3 CHOLERA TOXIN

Although cholera toxin is not a endogenous compound in mammals, it is known to cause prolonged elevation in cyclic AMP (cAMP) levels within intestinal epithelial cells by activation of adenylate cyclase (AC), which is an enzyme involved in regulatory processes. This results in a large efflux of Na^+ and water into the gut, which is responsible for the severe diarrhea that is characteristic of cholera. Cholera toxin binds to GM1, a ganglioside that is also found in most epithelial cells lining the endolymphatic surface of scala media, including those of the stria vascularis (Santi et al., 1994). In addition, several biochemical and histochemical studies have localized AC in the cochlea, notably in the stria vascularis and RM (Zajik et al., 1983; Schacht, 1982). The endolymphatic injection of cholera toxin was shown to gradually decrease the concentration of a simultaneously injected isotope (inuline), indicative of a developing EH (Feldman and Brusilow, 1976). Since cholera toxin is not a endogenous substance it is hypothesized that the latter effect may be crosstalk on receptors that normally interact with more specific inner ear ligands.

1.5.4 ARGININE-VASOPRESSIN

Arginine-vasopressin (AVP) is the natural vasopressin in the guinea pig and is known to have both a vasoconstrictive effect (via V_1 receptor, activation of phospholipase C) and a antidiuretic effect (via V_2 receptor, activation of AC).
In the kidney, vasopressin binds to basolateral membrane V_2 receptors and stimulates AC. Activation of protein kinase A by cAMP is then believed to insert intracellular vesicles containing functional water channels (aquaporines) into the apical plasma membrane (Knepper, 1997). Subsequently, water is reabsorbed from the collecting duct by osmosis.
Zenner and Zenner (1979) reported that vasopressin stimulated AC in the guinea pig inner ear in an *in vitro* experiment. Furthermore, recently V_2 AVP receptor mRNA was identified in the rat inner ear, using the reverse transcription-polymerase chain reaction method (Kitano et al., 1997). Perilymphatic perfusion of vasopressin (Fig 5) has been shown to dramatically reduce the EP (Mori et al., 1989). This could be the effect of an increased permeability of water of the cells that border SM. However, this effect could also arise from a vasoconstrictive effect in the vessels of the lateral wall, resulting in anoxia which is well known to reduce EP (Tilanus et al., 1992). Interestingly, the plasma levels of vasopressin appeared to be significantly elevated in patients suffering from Menière's disease (Takeda et al., 1995). In addition, these levels were found to be significantly higher in patients whose symptoms were active as compared with those in remission.

THIS THESIS

In this thesis we tried to influence cochlear ion and fluid homeostasis by interference with signaling molecules that are assumed to be capable of affecting the organs responsible for the maintenance of endolymph homeostasis. Histological and electrophysiological techniques were used in *in vivo* experiments.

Chapter 2 describes a method to assess the relative volume of marginal cells in standardized regions of the rat stria vascularis. Changes in marginal cell volume may be indicative of a disturbed homeostasis of endolymph. The marginal cells, bordering SM, contain high concentrations of Na^+/K^+-ATPase. Na^+/K^+-ATPase serves an important role in the maintenance of the production of endolymph and its ionic composition.

In *chapter 3* endogenous levels of adrenal hormones in rats were removed by adrenalectomy in order to decrease the Na^+/K^+-ATPase activity in the stria vascularis (Rarey et al., 1989). The method described in chapter 2 was used to study if the latter had any effect on the relative volume of the strial cells.
In *chapter 4* we tried to gain more insight into the nature of the different adrenal hormones that caused the volume effect found in chapter 3. Therefore we investigated whether or not this volume effect could be counteracted by subcutaneous implantation of mini-osmotic pumps that deliver constant rates of either aldosterone or dexamethasone.

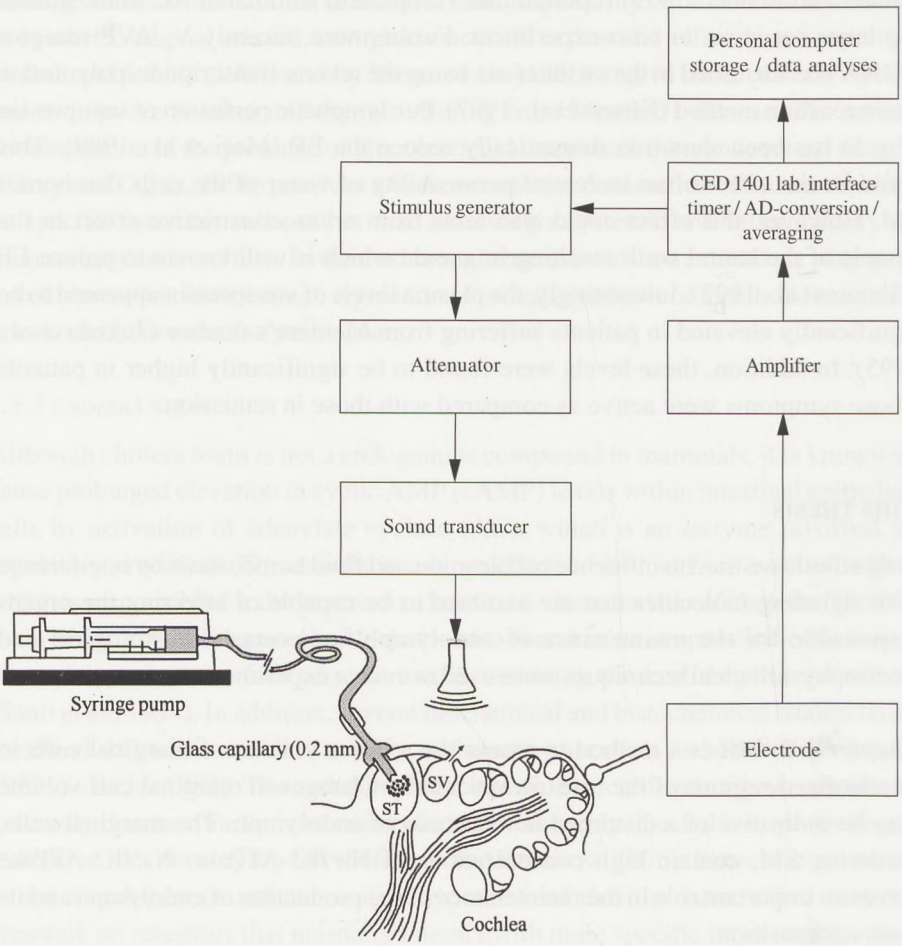

Figure 5. Schematic drawing of experimental set-up of *in vivo* perilymphatic perfusion of guinea pig cochleas.

In *chapter 5* we investigated the issue of whether the absence of circulating adrenal hormones has an effect on endolymphatic volume and cochlear function, as measured electrophysiologically. This is to be expected, because the strial Na^+/K^+-ATPase is thought to be involved in endolymph production and the marginal cells of the stria vascularis are immediately adjacent to endolymph.

In *chapter 6* and *chapter 7* a perfusion technique (Fig. 5) is used to introduce signaling molecules into the cochlea that could be capable of influencing endolymph production. The perilymph in the outer scalae is replaced by artificial perilymph supplemented with cholera toxin (chapter 6) or vasopressin (chapter 7). In these investigations the SP and the EP were measured as a function of time.

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CHAPTER II

Quantitative assessment of the rat stria vascularis

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Abstract

Stria vascularis tissues from standardized regions in the basal, middle and apical turns of the rat cochlear duct were assessed quantitatively. Strial width, number of marginal cells across the strial width, radial area, as well as the volume density of the different components of the stria vascularis were determined for each standardized region. Strial width, number of marginal cells across the strial width and the radial area were greatest in the basal region and least in the apical region of the cochlea. The volume density of intermediate cells and capillary space was statistically unchanged in the three examined regions of the stria vascularis. However, the volume density of marginal cells and that of basal cells were different between regions. The volume density of marginal cells was highest in the basal turn while the volume density of basal cells was greatest in the apical turn. An objective assessment of the response of the stria vascularis to environmental conditions can be made by kant of its cellular architecture, providing a means to compare the effects of various agents between animal models used to study human inner ear dysfunction.

INTRODUCTION

Transduction of sound is dependent upon maintenance of ionic gradients between the fluid compartments of the cochlea (Dallos, 1973; Konishi and Kelsey, 1973, 1978). Within the cochlear duct, the ionic composition of endolymph (high K^+, low Na^+) is thought to be conserved by the stria vascularis (Davis et al., 1958; Smith et al., 1954; Offner et al., 1987; Salt et al.,1987). This hypothesis is based in part upon immunocytochemical localization of dense concentrations of ion-transporting enzymes such as Na^+/K^+-ATPase (Kerr et al., 1982; Schulte and Adams, 1989; Iwano et al., 1989), carbonic anhydrase (Lim and Karabinas, 1982; Lim and Karabinas and Trune, 1983; Watanabe and Ogawa, 1984) and adenylate cyclase (Schacht, 1982) along the contraluminal membrane of the marginal cells of the stria. In addition, strial marginal cells, which are in direct contact with cochlear endolymph, appear morphologically similar to renal principal cells (Quick et al., 1973). Such cells have been demonstrated to be involved with active ion transport and have been shown to contain Na^+/K^+-ATPase sites (Jorgensen, 1986).
Numerous investigations have been performed to reveal mechanisms associated with the physiology of the structurally complex stria vascularis. These include studies which correlate electrophysiological changes within the cochlear duct with structural changes of the different strial cells after administration of enzyme inhibitors, such as ethacrynic acid (Bosher, 1973; Brumett et al., 1977), bumetanide (Santi et al., 1983) and ouabain (Bosher, 1980). Perilymphatic perfusion of ouabain, a specific inhibitor of Na^+/K^+-ATPase, was shown to cause inhibition of Na^+/K^+-ATPase activity, an increased ratio of Na^+ to K^+ in endolymph, a reduction of the cochlear potential, an inhibition of cochlear microphonics (Konishi and Mendelsohn, 1970; Sellick and Johnstone, 1974; Konishi et al., 1978; Melichar and Syka, 1986), marginal cell swelling and intermediate cell shrinkage (Bosher, 1980). Although changes in cellular architecture of the stria vascularis under certain experimental conditions have been reported in several other experimental animal models, such changes have not been routinely quantitated. Quantitative analysis of the stria vascularis tissue has been previously performed in the chinchilla and guinea pig model under normal and experimental conditions (Santi et al., 1983ab, 1985; Forge et al., 1987; Carlise and Forge, 1989). The estimated relative volume of strial cells and capillaries, strial width and the number of marginal cells across the strial width were determined. The objective of this study was to quantitate similar parameters for standardized areas of the normal stria vascularis of the rat by the application of stereological methods. This technique serves as a basis whereby the effect of different agents on the metabolically active stria vascularis tissue may be determined quantitatively.

MATERIALS AND METHODS

Tissue processing

Five randomly selected, healthy male Long-Evans rats (Charles River, Wilmington, Ma., 175-200 g) were anesthetized with intramuscular injections of ketamine hydrochloride (100 mg per kg body weight) and xylazine (10 mg per kg body weight). After opening the thorax of each animal and clamping the descending vessels, the right auricle was incised and 12 ml phosphate buffered saline (PBS: 20 mM NaH_2PO_4. H_2O and 42 mM Na_2HPO_4), pH 7.4, followed with 36 ml fixative (2.5% glutaraldehyde and 2.0% paraformaldehyde in PBS, pH 7.4) was perfused through the left ventricle. Temporal bones were quickly extracted following decapitation, and after the oval and round windows were opened, each cochlea was perilymphatically perfused with fresh fixative and stored overnight at 4°C. Each temporal bone was washed 3X in PBS, post-fixed with 1% osmium tetroxide for 2 hours, washed 3X in PBS and placed in 5% EDTA (pH 7.4) for 5 days at 4°C. Following decalcification, a midmodiolar section of the cochlea between the oval and round windows was made with a sharp razor blade. Each cochlear sample was processed for embedding in epon/araldite (Polysciences, Warrington, Pa.).

Tissue sampling

A second cut parallel to the midmodiolar cochlear plane was made with a fine jewelers saw after epon polymerization, resulting in a slice (approximately 1 mm thick) containing standardized samples of five different regions of the cochlear duct (Fig. 1). Each of these five sample regions was divided with a sharp razor blade under a stereomicroscope and reembedded in epon. Morphometric analysis was performed on three of the five stria vascularis regions: (1) Region B, from the basal turn of the stria; (2) Region M, from the middle strial turn; (3) Region A, from the apical region of the stria. Ultra-thin (70 nm) cross sections of each of the three tissue regions were cut transversely through the scala media with a Diatome MT401 diamond knife on a Reichert-Jung ultra-microtome. Thin sections were collected on Formvar/carbon coated copper slot grids (2 x 1 mm; Electron Microscopy Sciences, Ft. Washington, Pa.), stained with uranyl acetate (5 min) and lead citrate (10 min), and examined in a JEOL IOOS electron microscope.

A series of photographs was taken along the stria vascularis from each strial region (B, M and A) at a magnification of 2000 x . These micrographs were enlarged 2.67 x and were used to form a cross-sectional photomontage of the stria vascularis, uninterrupted by gridbars. Each montage represented a single tissue sample from

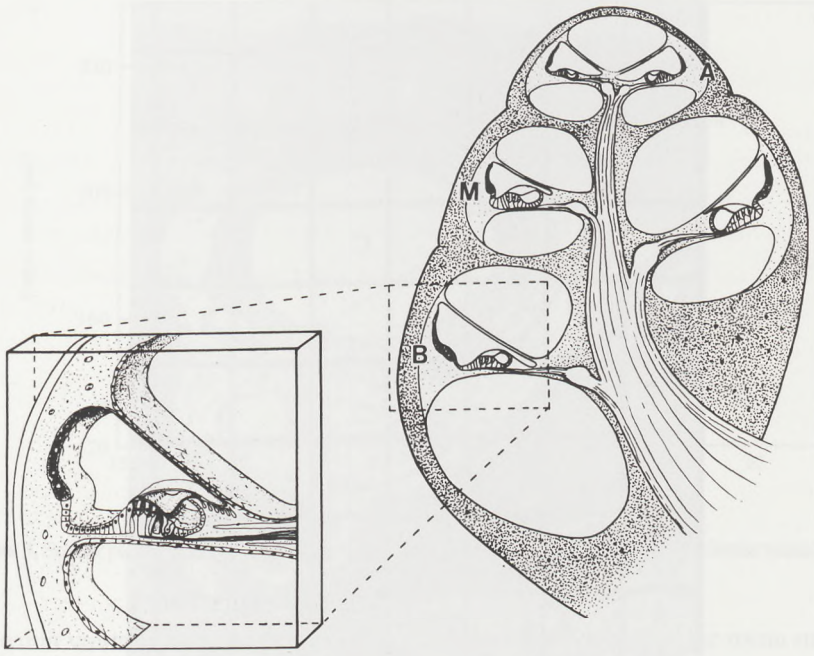

FIGURE 1. Diagram of a midmodiolar section of the rat cochlea. Morphological analysis was performed on three regions (B,M and A). This diagram illustrates how an excised block of Epon containing a cross section of a standardized region of the cochlear duct, e.g. in the basal turn (Region B), was obtained. Region M, middle turn of cochlea; Region A, apical turn of cochlea.

the corresponding region. Magnification was calibrated prior to each printing session by photographing a carbon replica grid containing 2160 lines/mm.

Analysis of the stria vascularis

Boundaries of the stria vascularis were outlined as described by Santi et al. (1983), and each strial cell type was identified. Strial width, the number of marginal cells across the strial width, the volume density of the different strial components, and the radial area of the stria vascularis were determined for each of the stria regions examined as follows:

STRIAL WIDTH. Strial width was determined by measuring the distance between the spiral prominence and Reissner's membrane along the endolymphatic surface of the marginal cells. Measurements were performed with an image analysis system

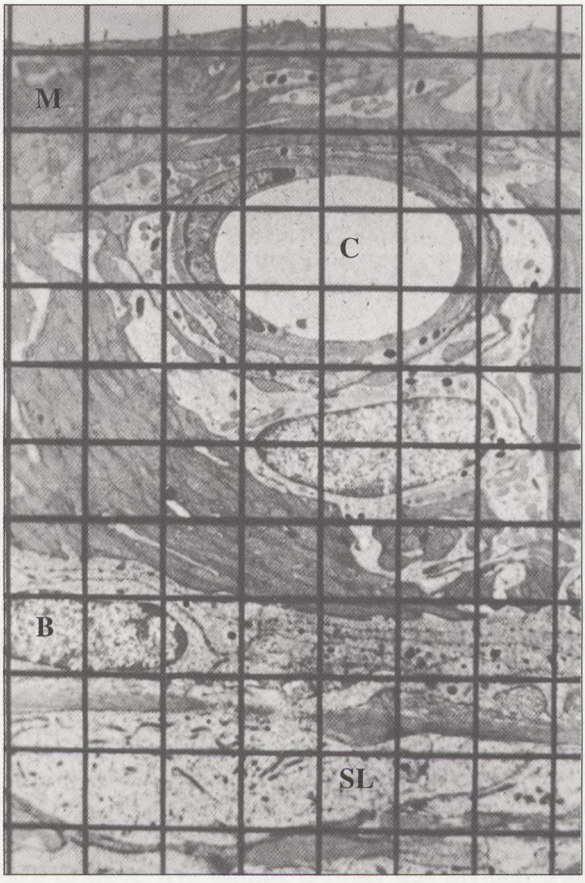

FIGURE 2. Transmission electron micrograph of an area of the stria vascularis overlaid with the point-count test grid. Marginal cell,(M); intermediate cell,(I); basal cell,(B); capillary,(C); spiral ligament,(SL). The arrow indicates a test point. Magnification, x5340.

(Southern Micro Instruments, Atlanta, Ga.) interfaced to an IBM PC/AT microprocessor with a digitizing tablet.

NUMBER OF MARGINAL CELLS OVER THE STRIAL WIDTH. The number of marginal cells along the endolymphatic surface was determined by identifying apical tight junctions between adjacent marginal cells.

VOLUME DENSITY OF STRIAL COMPONENTS. Volume density (V_v) was defined as the volume of the components (strial cells, capillaries, and intercellular space) contained

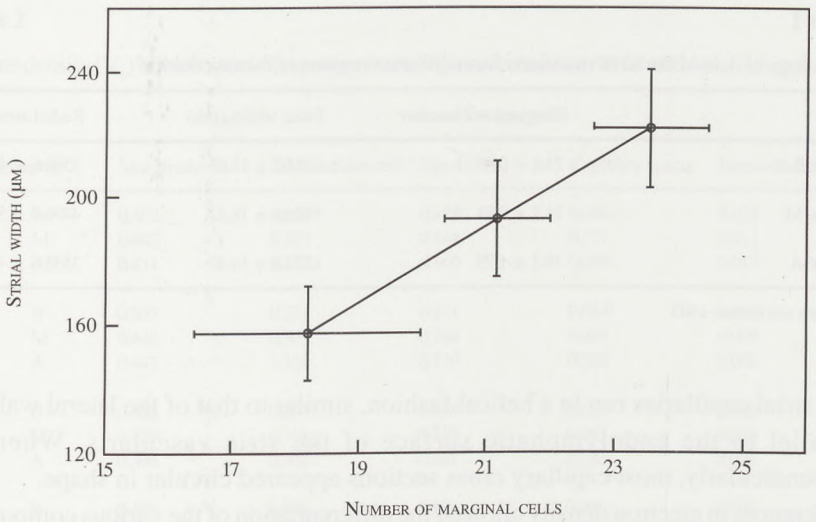

FIGURE 3. Scatter plot of strial width as a function of number of marginal cells. Note the linear relationship.

in the unit volume of the structure (stria vascularis). Assuming that the mean surface proportion of a given strial component (obtained from a 2-D image) is equal to the mean volume proportion of that component, it was possible to estimate the relative volume fractions of the strial components (Steer, 1981). Surface area was determined using a stereological point-counting method (Weibel, 1979, 1980; Santi et al., 1983). A grid with a test-point density of 1 cm was placed randomly over the strial photomontage (Fig. 2). The estimated relative volume of each strial component was determined by dividing the amount of points falling on that component by the amount of points falling on the total stria vascularis.

RADIAL AREA OF THE STRIA VASCULARIS. The radial area was defined as the total surface area of individual strial cross sections, and was estimated by multiplying the total number of point-counts falling on the stria vascularis by d^2, where $d = 1.92$ μm is the distance between adjacent gridlines divided by the magnification. The carbon replica grid was required for an accurate determination of d.

Morphometry

Because perpendicular sectioning is of great importance when morphological parameters such as strial width and radial area, are compared between different animals, thick sections were used to determine proper alignment. The orientation of the sensory cells and the strial capillaries were used to determine such alignment.

TABLE 1

Stereological data of the stria vascularis from different regions of the rat cochlea

	Marginal cell number	Strial width (μm)	Radial area (μm²)
Region B	23.6 ± 0.89	221.02 ± 18.67	5586.6 ± 450.9
Region M	21.2 ± 0.84	192.61 ± 18.22	4496.4 ± 517.6
Region A	18.2 ± 1.79	157.18 ± 14.49	3533.6 ± 483.0

* Values are means ±SD

The strial capillaries run in a helical fashion, similar to that of the lateral wall and parallel to the endolymphatic surface of the stria vascularis. When cut perpendicularly, most capillary cross sections appeared circular in shape.
Differences in electron density allowed the differentiation of the various components of the rat stria vascularis. Electron dense marginal cells were easily distinguished from the relatively lucent intermediate cells. It was more difficult to distinguish between basal cells and intermediate cells, as well as to distinguish between basal cells and the type II fibroblasts of the adjacent spiral ligament, due to similar cytoplasmic electron densities. Endothelial cells, pericytes and basal lamina were counted to be within the structure of the strial capillary. Pigment-containing cells were present in the stria since pigmented rats were used, and were included as intermediate cells.

Statistical methods

Differences in volume density between the given regions examined were tested with a multivariate analysis of variance (MANOVA) (O'Brien and Kaiser, 1985). MANOVA techniques allow for a general correlation structure when testing factor effects for significance and were required because the measurements within an experimental animal were correlated. The probability level for accepting the null hypothesis was set at 0.05.

RESULTS

Morphological data of the stria vascularis from the three regions of the rat cochlea are shown in Table I. The number of marginal cells across the strial width was 23% greater in the basal portion of the cochlea as compared to the apical portion, and was demonstrated to increase in linear fashion with the increase of the strial width (Fig. 3). The mean strial width was 29% greater in the cochlear base (Region B)

TABLE 2

Volume density (V_v) of each strial component in the regions examined (B, M and A)

Animal	region	Strial components				
		Marginal cell	Intermediate cell	Basal cell	Capillary space	Intercellular space
No. 1	B	0.462	0.294	0.128	0.088	0.029
	M	0.402	0.323	0.144	0.121	0.011
	A	0.431	0.303	0.160	0.089	0.017
No. 2	B	0.507	0.273	0.131	0.084	0.004
	M	0.442	0.307	0.154	0.088	0.009
	A	0.447	0.326	0.139	0.088	0.000
No. 3	B	0.448	0.297	0.154	0.101	0.000
	M	0.429	0.315	0.170	0.085	0.000
	A	0.396	0.301	0.181	0.122	0.000
No. 4	B	0.455	0.306	0.140	0.096	0.002
	M	0.391	0.325	0.190	0.094	0.000
	A	0.403	0.377	0.152	0.108	0.000
No. 5	B	0.485	0.342	0.122	0.052	0.000
	M	0.474	0.290	0.159	0.071	0.006
	A	0.360	0.333	0.185	0.122	0.000

than in the cochlear apex (Region A). The radial area of the stria was 37% greater in the basal turn of the cochlea as compared to the apex.

An estimation of the mean endolymphatic distance across an individual marginal cell (i.e., apical distance between a cells tight junctions in perpendicular section) was determined by dividing the total strial width by the number of marginal cells over the strial width. The mean endolymphatic distance across a given marginal cell was estimated to be 9.04 μm (± 0.76), and it did not significantly differ between the given regions examined.

The volume density of each strial component from the individual strial cross sections examined are presented in Table II. The volume density data from the basal region are displayed in a histogram (Fig. 4) to show the low variability between animals. A minute value for the volume density of the intercellular space was recorded in some of the tissues, yet the majority of the stria vascularis tissues examined contained no intercellular space (Table II).

Mean volume density of the strial components in regions B, M and A are shown in Table III. There was a significant increase (15%) in the estimated volume density of the marginal cells and a significant decrease (11%) in the volume density of the

TABLE 3

Comparison of stereological data of the three strial regions examined (B, M and A) by Manova

Region	Marginal cell	Intermediate cell	Basal cell	Capillary space
B (n=5)	0.471 ± 0.024	0.302 ± 0.025	0.135 ± 0.012	0.084 ± 0.019
M (n=5)	0.428 ± 0.033	0.312 ± 0.014	0.163 ± 0.018	0.092 ± 0.018
A (n=5)	0.407 ± 0.034	0.320 ± 0.017	0.163 ± 0.019	0.106 ± 0.017
F value:	34.35	1.13	9.90	1.31
P value:	0.009	0.430	0.048	0.390

* Values are mean volume densities (V_v) ±SD

basal cells when comparing the apical region (Region A) to the basal region (Region B) of the cochlear duct. There was no significant difference in volume density among the different examined regions for both intermediate cells and capillary space.

DISCUSSION

The findings of this study demonstrate that concomitant with an increase in radial area of the stria vascularis, the strial width and the number of marginal cells across the strial width increased towards the base in the rat stria vascularis. This increase in strial width and radial area is presumed to be due to the increase in the number of marginal cells. Identical results were reported for chinchilla (Santi et al., 1983), guinea pig (Forge et al., 1987) and mouse (Carlisle and Forge, 1989).

These data for the rat stria vascularis were consistent in part with that for the chinchilla model. The volume density of the intermediate cells and that of the capillary space of the rat stria vascularis did not change between the regions examined, but there was a statistically significant increase (15%) in the volume density of the marginal cells of the basal region of the cochlea as opposed to that of the apical region. The volume density of all the different strial components was reported to be unchanged over the entire length of the stria vascularis for the chinchilla model (Santi et al., 1983a). This difference between the rat and the chinchilla model might be due to species variation.

The increase of the radial area (37%), the volume density of the marginal cells and the number of marginal cells (23%) across the strial width may be correlated with

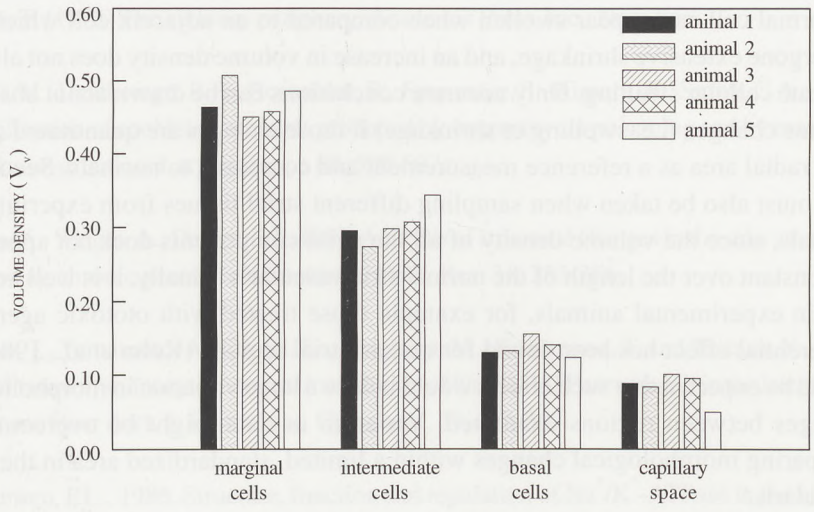

FIGURE 4. Histogram of the volume density (V_v) of each strial component in Region B. Note the low variability between animals for each component.

distinctive, regional, biochemical and/or electrophysiological characteristics of the stria vascularis. For example, Kuijpers observed a greater Na^+/K^+-ATPase activity in the basal part of the stria vascularis than in the apex (Kuijpers and Bonting, 1969).

It can be concluded from the results of this investigation that the increase of volume density of the marginal cells towards the base is not only due to an increase of marginal cell number, but also may be due to an increase in absolute volume of each individual cell. Such an increase in individual cell volume could be caused by a greater cell height and/or a greater number of cell processes. In the latter case, such an increase in volume may also indicate a greater basolateral surface area of the marginal cells, and with an equal amount of binding sites per unit length of membrane, an increase in Na^+/K^+-ATPase in the cochlear base. If it were possible to quantitate the basolateral membrane length with a Merz grid as has been accomplished for the dark cells in vestibular organs (unpublished data), a direct correlation of Na^+/K^+-ATPase with basolateral membrane length could be made. However, quantitation of basolateral membrane length appears impractical to apply to the stria vascularis at this time due to the great density of the processes of the marginal cells.

Examination of morphological changes in the stria vascularis requires three considerations. First, it is important to be able to assess strial cell volume objectively.

A normal cell can appear swollen when compared to an adjacent cell which has undergone extensive shrinkage, and an increase in volume density does not always indicate cellular swelling. Only accurate conclusions can be drawn about absolute volume changes (i.e. swelling or shrinkage) if those changes are quantitated along with radial area as a reference measurement and compared to normals. Secondly, care must also be taken when sampling different strial tissues from experimental animals, since the volume density of all the strial components does not appear to be constant over the length of the normal stria vascularis. Finally, it is well known that in experimental animals, for example those treated with ototoxic agents, a preferential effect has been found for certain strial regions (Kohn et al., 1988). It would be expected that such effects would induce a large variation in morphological changes between regions examined. Variation in data might be overcome by comparing morphological changes within a limited, standardized area in the stria vascularis.

In summary, morphological data from standardized regions of the rat stria vascularis have been compiled using stereological methods. Such methods form an objective, quantitative basis by which to investigate the regulation of the physiological mechanisms within the stria vascularis and their influence on the microenvironment of the cochlear duct.

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CHAPTER III

Modulation of the rat stria vascularis in the absence of circulating adrenocorticosteroids

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Acta Otolaryngol. (Stockh.) 110 (1990), 348-356

Abstract

Structural changes in the cellular morphology of the rat stria vascularis from a standardized region of the basal region and from a standardized region in the apical region of the rat cochlear duct were measured using stereological methods after removal of endogenous levels of adrenal steroids by bilateral adrenalectomy. Although there were some inconsistent and insignificant alterations in the volume density of the intermediate and basal cells, a decreased volume density of marginal cells in both the basal region and in the apical region in adrenalectomized (ADX) animals as compared to sham animals was consistent with a concomitant significant increased ($p<0.05$) volume density of intercellular space as observed in both the basal and apical regions of the stria vascularis of ADX animals. Findings of this study indicate that the strial cells of the stria vascularis react differently and independently in response to the removal of adrenal steroids, and such strial responses occur uniform in both the base and the apex.

INTRODUCTION

It is generally assumed that the cellular distribution of Na^+/K^+-ATPase in the stria vascularis plays a fundamental role in the preservation of ion and fluid homeostasis of endolymph (Bosher, 1980; Ross, 1982; Offner et al., 1987; Melichar and Syka, 1986). Na^+/K^+-ATPase is a membrane-bound ATP hydrolyzing enzyme complex that translocates Na^+ and K^+ over the cellular plasma membrane, and is considered to be primarily regulated by the intracellular Na^+ concentration (Rossier et al., 1987; Skou, 1988). In the kidney, Na^+/K^+-ATPase activity has been demonstrated to decrease following adrenalectomy and has partially recovered after administration of mineralocorticoids and/or glucocorticoids (Rodriguez et al., 1981; Marver and Kokko, 1983; Garg et al., 1981; Garg et al., 1985; Jorgensen, 1986). Based on these findings, Na^+/K^+-ATPase activity has been hypothesized to be regulated in part by adrenal hormones by their actions at the nuclear level (Jorgensen, 1986; Feldman et al., 1972; Morell and Doucet, 1986).

Protein receptors for both mineralocorticoids and glucocorticoids have been demonstrated in inner ear tissues (Rarey and Luttge, 1989). Removal of endogenous levels of adrenal steroids by bilateral adrenalectomy was shown by fluorometric assay to cause a significant decrease of ATPase activity in rat inner ear tissues, e.g. stria vascularis (Rarey et al., 1989). Hence, it can be conjectured that circulating levels of adrenal hormones can influence strial cell function, thereby providing a means of regulating cells involved in the microhomeostatic environment of the cochlear duct.

The objective of this study was to determine how the rat stria vascularis structurally adapts to the removal of adrenal stimuli via bilateral adrenalectomy on a regional basis by applying stereological methods. The findings complement biochemical data found previously and contribute to the understanding of the effect of adrenocortical imbalances on strial cell function and the ionic microenvironment within the cochlear duct.

MATERIALS AND METHODS

Twelve Long Evans rats (Charles River, Wilmonton, Ma., 175-200 g), were divided into two groups: Animals in group 1 (n=6) were adrenalectomized (ADX). Animals in group 2 (n=6) received a Sham operation. All rats were provided with standard Purina laboratory chow. Animals in group 2 received tap water ad libitum, while animals in group 1 were sustained with 0.9% saline ad libitum.

Bilateral adrenalectomy

Rats were anesthetized with intramuscular injections of ketamine hydrochloride (100 mg/kg body weight) and xylazine (10 mg/kg body weight), and the adrenal glands were carefully removed through dorsolateral incisions. Each adrenal gland was inspected under a stereomicroscope to ensure complete extraction. The suprarenal area was dabbed with 95% ethanol to aid in suppression of possible regrowth of remaining capsular cells. Abdominal muscles were sutured with 4-0 silk and animals were allowed to recover. A Sham-operation consisted of exposure of adrenal glands in the manner described without excision of the adrenal glands.

Tissue processing

Fifteen days after surgery, rats in the two groups were anesthetized with ketamine hydrochloride and xylazine as previously described. The abdominal cavity was exposed, the diaphragm was reflected, and approximately 3 ml blood was collected by intracardiac puncture. The descending vessels were clamped and each animal was intracardially perfused with 24 ml phosphate buffered saline (PBS: 20 mM $NaH_2PO_4 \cdot H_2O$ and 42 mM Na_2HPO_4), pH 7.4, followed by 36 ml fixative (2.5% glutaraldehyde and 2.0% paraformaldehyde in PBS). Following decapitation, the temporal bones were quickly removed, and each bulla was opened. Each cochlea was perilymphatically perfused with fresh fixative after opening the oval and round windows, and creating a small hole in the otic capsule at the cochlear apex. Cochleas were stored in fresh fixative overnight at 4°C, postfixed for 2 hours in phosphate buffered 1% osmium tetroxide, pH 7.4, rinsed 3x in PBS, and placed in 5% EDTA at 4°C. After 5 days of decalcification, a midmodiolar cut through the apex and between the oval and round window was performed, and each half cochlea was dehydrated in graded concentrations of ethanol, and processed for epon/araldite (Polysciences, Warrington, Pa.) embedding using normal procedures. After epon polymerization, standardized stria vascularis tissues from the cochlear basal turn and from the apical cochlear turn were obtained as previously described (Lohuis et al., 1990). After reembedding, thick sections were used to verify proper alignment (i.e., perpendicular to the surface of the stria vascularis). Ultra-thin (70 nm) sections were then collected on Formvar/carbon coated slot copper grids (2x1 mm; Electron Microscopy Sciences, Ft. Washington, Pa.) using a Reichert-Jung ultramicrotome and a Diatome MT401 diamond knife. These sections were examined in a JOEL 100S electron microscope and a series of micrographs was taken along the length of the stria vascularis. Of each region a montage of the complete stria vascularis was created at a final enlargement of X5340, uninterrupted by grid bars. The following

parameters were determined using stereological methods: volume density of strial cells, capillary space and intercellular space, strial width, the number of marginal cells across the strial width and the radial area. Serum aldosterone values of the animals were determined by radioimmunoassay (ICN diagnostics) in our laboratory.

Analysis of the stria vascularis

Strial components in both the basal and apical regions of the stria vascularis were identified and the strial boundaries were outlined as previously described by Santi (1983) and modified by Lohuis (1990). The following morphological parameters were determined from a standardized region of the basal turn of the cochlear duct:

STRIAL WIDTH. Strial width was defined as the distance between the spiral prominence and Reissner's membrane along the endolymphatic surface of the marginal cells. Determination of strial width was accomplished with the aid of computer-assisted image analysis (Southern Micro Instruments, Atlanta, Ga.).

NUMBER OF MARGINAL CELLS OVER THE STRIAL WIDTH. The number of marginal cells along the endolymphatic surface was determined by identifying apical tight junctions between adjacent marginal cells.

VOLUME DENSITY OF STRIAL COMPONENTS. Volume density (V_v) of marginal cells, intermediate cells, basal cells, capillary space, and intercellular space was determined using standard point-counting methods (Weibel, 1979; Santi et al., 1983; Lohuis et al., 1990). These methods are based upon the assumption that mean surface proportions of strial components are equal to the mean volume proportions of these components methods (Weibel, 1979; Steer, 1981). The surface area of each strial component was determined by dividing the point counts falling on that component by the total counts on the entire stria image.

RADIAL AREA OF THE STRIA VASCULARIS. The radial area was defined as the surface area of individual strial cross sections, and was determined by multiplying the total number of point-counts falling on the stria vascularis by d^2, where d is the distance between adjacent lines on the point count grid, divided by the magnification (d = 1.92 µm). The carbon replica grid was required for an accurate determination of d.

Morphometry

Differentiation between the different strial components in both the apical and basal regions of the stria vascularis was possible because of contrasting electron densities. Marginal cells were easily distinguished from the less dense intermediate cells, yet more care was needed to differentiate among basal cells and intermediate cells, as

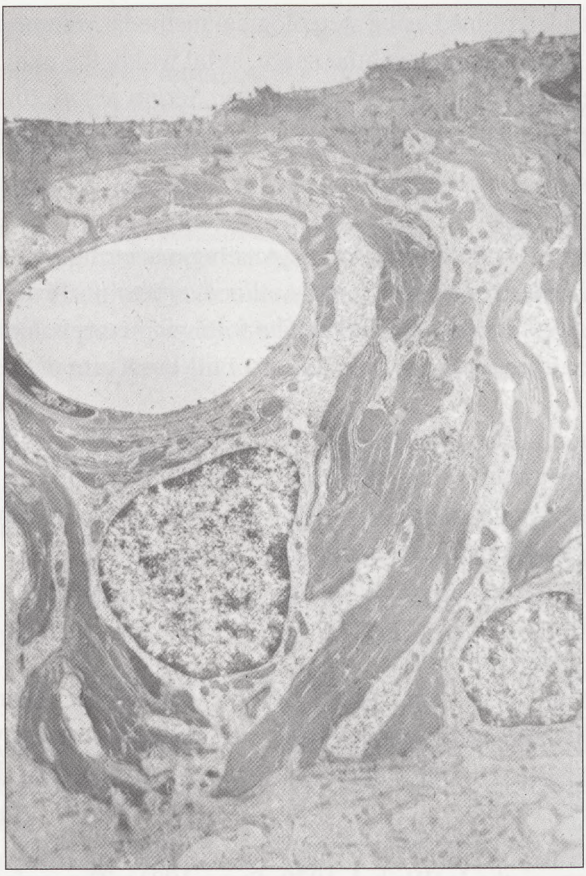

FIGURE 1. Transmission electron micrograph of the stria vascularis from a standardized basal region from a sham-operated animal. All strial components appear unremarkable. x4320. Osmium, uranylacetate and leadcitrate.

well as between basal cells and the type II fibroblasts of the adjacent spiral ligament. Endothelial cells, pericytes and the basal lamina of strial blood vessels were counted to be within the structure of the capillary space. Pigment-containing cells were included as intermediate cells.

Statistical methods

Adrenalectomized animals were compared to sham-operated animals using multiple t-tests. The basal and apical turns were analyzed separately. The probability level for accepting the null hypothesis was set at 0.05.

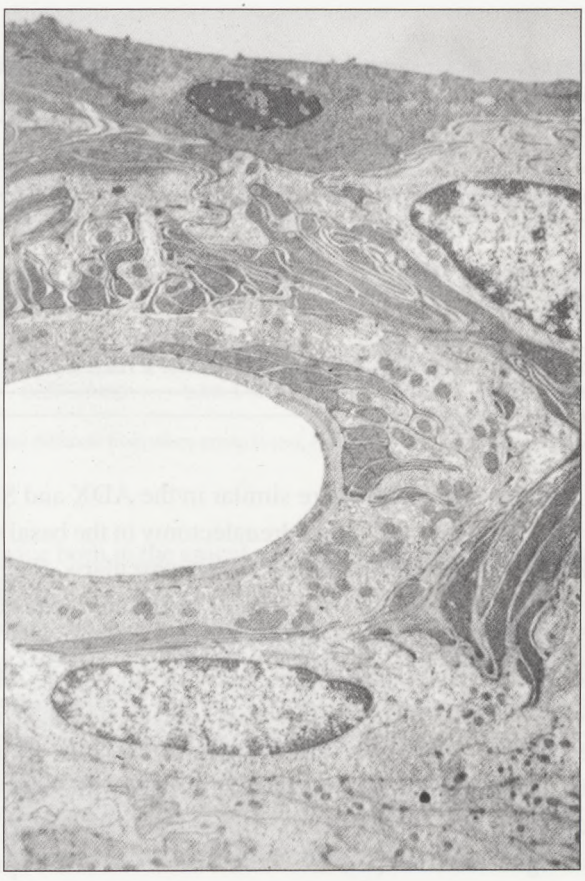

FIGURE 2. Representative transmission electron micrograph of the stria vascularis from a standardized basal region from an adrenalectomized animal. Note that the intercellular space between strial cells appears enlarged. x4410. Osmium, uranylacetate and leadcitrate.

RESULTS

The stria vascularis in both the basal and apical regions of the cochlear duct as seen by transmission electron microscopy appeared intact in both the adrenalectomized (ADX) animals and sham-operated (SHAM) animals (Figs. 1 and 2). The intercellular space within the stria vascularis appeared greater in ADX animals than in SHAM animals.

Means and standard deviations of marginal cell number, strial width and radial area from a standardized region in the basal turn and in the apical turn of the rat stria vascularis are presented in Table I. In each turn the number of marginal cells in the

TABLE 1

Comparison of morphological data from the standardized region in the basal turn and from a standardized region in the apical turn of the rat stria vascularis

	Marginal cell number ± SD	Strial width ± SD (μm)	Radial area ± SD (μm^2)
Basal turn			
- Sham (*n*=6)	22.8 ± 2.04	231.75 ± 10.18	5447.3 ± 376.0
- ADX (*n*=6)	23.2 ± 1.60	231.12 ± 12.57	4996.3 ± 510.3
Apical turn			
- Sham (*n*=6)	17.8 ± 3.5	158.1 ± 22.6	3088.9 ± 782.8
- ADX (*n*=6)	17.5 ± 2.0	169.6 ± 17.1	3429.1 ± 552.4

Values are means ± standard deviation

stria vascularis and the strial width were similar in the ADX and SHAM animals. The radial area decreased by 8.3% after adrenalectomy in the basal stria. However, the radial area increased by 11% in the apical stria vascularis. Such changes were not statistically significant. The increase apical radial area may reflect a greater variation in volume density (V_v) of strial components in the apex.

When the V_v of the different strial components was measured, distinct changes were observed (Figs. 3 and 4). The mean volume density of each strial component in a standardized region of the basal turn and that of the apical turn of the rat stria vascularis are presented in Table II. When ADX animals were compared to SHAM animals, the marginal cell V_v significantly decreased by 9% ($p<0.05$) in the basal turn, while a non-significant decrease of 7% was observed in the apical turn. There were non-significant changes in the volume density of intermediate cells, basal cells and capillary space in each standardized region examined of the different experimental groups. A significant increase ($p<0.05$) in the V_v of the intercellular space was observed after adrenalectomy when compared SHAM animals in both the basal and apical turns.

Mean serum aldosterone values and standard deviations for the two groups were: ADX, 27.5 (± 29.4 SD) pg/ml; SHAM, 433.0 (± 103.3 SD) pg/ml.

DISCUSSION

Findings of this investigation imply that removal of circulating adrenal steroids induced structural changes in the stria vascularis in both the basal and apical regions of the rat cochlear duct. The response of strial cells in the basal region of the cochlea was similar to that observed in the apical region. A decrease in the volume density of the marginal cells was consistently correlated with an increase of the

TABLE 2

Comparison of mean volume density (V_v) of each strial component in the standardized basal region and in the standardized region of the apical region of the rat stria vascularis

	Strial components				
Animal region	Marginal cell	Intermediate cell	Basal cell	Capillary space	Intercellular space
Basal turn					
- Sham (n=6)	0.422 ± 0.017	0.344 ± 0.026	0.149 ± 0.023	0.082 ± 0.010	0.001 ± 0.003
- ADX (n=6)	0.380 ± 0.020*	0.352 ± 0.039	0.159 ± 0.020	0.088 ± 0.021	0.021 ± 0.019*
Apical turn					
- Sham (n=6)	0.385 ± 0.051	0.374 ± 0.041	0.146 ± 0.031	0.092 ± 0.028	0.000 ± 0.000
- ADX (n=6)	0.357 ± 0.052	0.409 ± 0.047	0.130 ± 0.035	0.096 ± 0.037	0.003 ± 0.003*

* Statistical significant different from sham-group (t-test, $P \leq 0.05$). Values are means ± standard deviation.

intercellular space both in the apical and basal stria vascularis of adrenalectomized (ADX) animals.

The morphological changes detected in this study are concomitant with biochemical changes induced by a removal of endogenous circulating adrenal hormones. A significant decrease in Na^+/K^+-ATPase activity was previously found in the stria vascularis after bilateral adrenalectomy (Rarey et al., 1989). Collectively these data indirectly indicate that the cells of the stria vascularis adapt both structurally and biochemically in the absence of adrenal stimuli and strongly suggests a regulation of Na^+/K^+-ATPase activity by adrenal steroids in the metabolically active stria vascularis.

Perpetuation of cellular volume is a primary function of Na^+/K^+-ATPase (MacKnight and Leaf, 1977). This enzyme insures low intracellular Na^+ concentrations and prevents osmotic swelling and lysis. Because of its asymmetric distribution over the cellular surface of marginal cells, Na^+-K^+-ATPase is also thought to create the driving force for transepithelial movement of cations, solutes, and water, thereby controlling the ionic composition of the marginal cells and indirectly, that of endolymph (Ross, 1982; Offner, 1987; MacKnight and Leaf, 1977). Reduction of Na^+/K^+-ATPase activity in the stria vascularis should result in marginal cell accumulation of Na^+ and Cl^-, loss of K^+, and the osmotic uptake of water, concomitant with cellular swelling. This is in contrast with the observed marginal cell shrinkage. It might be hypothesized that reductions in Na^+/K^+-ATPase activity in the stria vascularis in the absence of adrenal stimuli, not only prevent marginal cells from volume maintenance, but also induce shifts in endolymphatic osmolarity, which would cause water retention via the apical surface of the marginal cells.

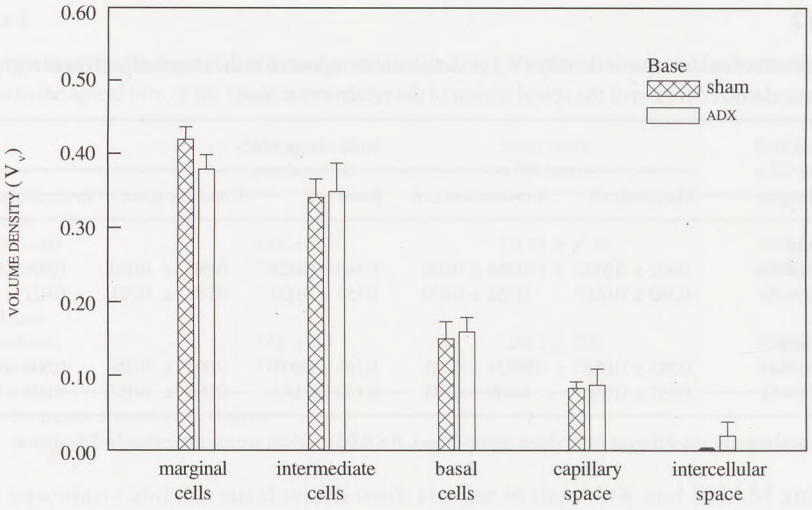

FIGURE 3. Histogram of the mean volume density (V_v) of each strial component in the basal stria vascularis from both the adrenalectomized and sham-operated animals. Low variability was observed within animal groups.

No significant changes were observed in the volume densities of the intermediate cells or basal cells of the apical and basal stria in the ADX animals as compared to SHAM animals. Intermediate cells and basal cells are thought to aid marginal cells by osmoregulating the stria vascularis (Salt et al., 1987). Possibly, these cells initially endured volume changes after removal of the adrenal steroids, but were able to adapt and compensate to such more than marginal cells. Alternatively, intermediate cells and basal cells may not readily respond to the presence or absence of adrenal stimuli, because they lack adrenocorticoid receptors and/or possess smaller concentrations of the Na^+/K^+-ATPase enzyme. Such findings indicate that different cells of the stria vascularis do react differently and can react independently.

The volume density of intercellular space in ADX animals was higher when compared to that of SHAM in both the apical and basal regions although it comprised only a very small portion of the total stria vascularis. Increased intercellular space has been used in the past as an indication of disturbed ionic homeostasis in the cellular environment of the stria vascularis and may be attributed to the disruption of ion transport into the cochlear duct. Intercellular space has been observed in the stria vascularis after administration of bumetanide (Santi and Lakhani, 1983), ethacrynic acid (Bosher et al., 1973), mannitol (Santi et al., 1985) and after perilymphatic perfusion with ouabain (Bosher, 1980).

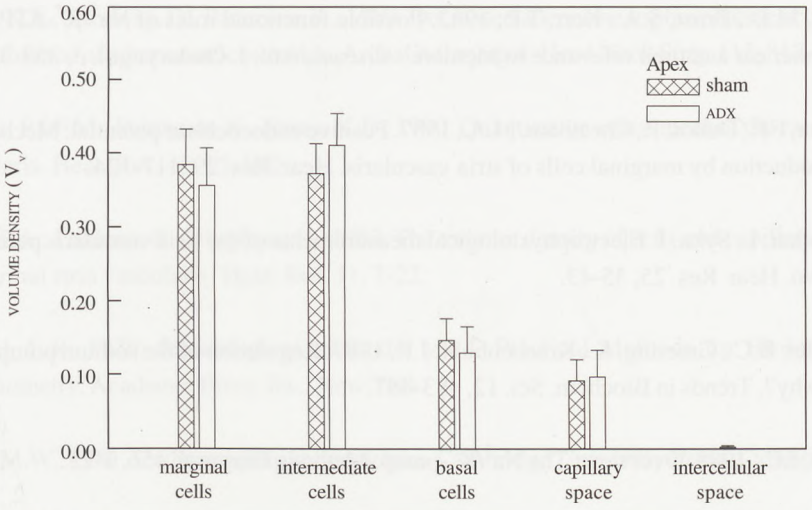

FIGURE 4. Histogram of the mean volume density (V_v) of each strial component in the apical stria vascularis from both the adrenalectomized and sham-operated animals. Unlike in the basal region, high variability was observed within animal groups.

The architecture of the stria vascularis is altered in the absence of circulating adrenal steroids. Because volume is a sensitive indicator of osmoequilibrium within a cell, changes in cellular volume within the stria vascularis may have been due to a disequilibrium in normal ion transport, which would cause disruption of the homeostasis in the cochlear duct, which may lead, in turn, to functional alterations in hearing. Further studies are necessary to evaluate the effect of adrenal steroids on the stria vascularis to provide more insight into the role of adrenal steroids in the regulation of inner ear homeostasis.

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CHAPTER IV

Response of the stria vascularis to corticosteroids

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Laryngoscope 101 (1991), 1081-1084

Abstract

Structural changes in the cellular architecture of the stria vascularis in adrenalectomized rats were quantitated by stereological methods after the administration of either aldosterone or dexamethasone. The volume densities of the differing strial components from steroid-administered animals were determined to approximate those of sham-adrenalectomized animals in general. The increased volume density of intercellular space as was observed following adrenalectomy, however, was only restored after the administration aldosterone. These data correlate a recovery of the cellular architecture of the stria vascularis in adrenalectomized animals with the restoration of endogenous levels of adrenal steroids. These findings provide further information with regard to the effects of varying serum levels of corticosteroids on inner ear morphology.

INTRODUCTION

Change in hearing acuity, vertigo and tinnitus have been reported in certain patients with hormonal imbalances (Goldman, 1962; Powers, 1972; Currier, 1971; Naftalin, 1975ab). Certain cases of Menière's syndrome, for example, are presumed to be related to hypoadrenocorticism (Goldman, 1962; Powers, 1972). Following the administration of whole adrenocortical extract to some patients with adrenal imbalances, vertigo was improved, tinnitus was relieved, and hearing was restored to varying degrees (Powers, 1972). Patients with adrenal cortical insufficiencies have been reported paradoxically to have a decreased auditory threshold concomitant with an increased sensitivity to taste and smell (Henkin et al., 1977). Based upon such clinical findings, fluctuating levels of endogenous adrenal steroid levels appear related to altered inner ear physiology.

Receptors for adrenal steroids were detected previously within cochlear and vestibular labyrinthine tissues by specific binding assays (Rarey and Luttge, 1989). To determine whether adrenal steroids directly impact upon inner ear microhomeostasis, select inner ear tissues were examined biochemically and morphologically after the removal of endogenous adrenal steroids in the rat experimental-animal model via bilateral adrenalectomy (Rarey et al., 1988; Rarey et al., 1989; Lohuis et al., 1990a; ten Cate et al., 1990). This animal model was created to mimic human adrenal insufficiency. A significant decline in the sodium- and potassium-activated adenosine triphosphate (Na^+/K^+-ATPase) activity in the stria vascularis, spiral ligament and ampullar dark cells was detected fluorometrically after bilateral adrenalectomy (Rarey et al., 1988; Rarey et al., 1989). In addition, morphological changes have been observed and quantitated in the stria vascularis (Lohuis et al., 1990a) and ampullar dark cells (ten Cate et al., 1990) after bilateral adrenalectomy.

Reestablishment of an endogenous level of only aldosterone after adrenalectomy has been shown recently to restore the more normal appearance of ampullar dark cell morphology (ten Cate and Rarey, 1991). The administration of dexamethasone after adrenalectomy had no significant effect on ampullar dark cell morphology. Based upon such studies, it can be conjectured that cells of the stria vascularis, particularly marginal cells, might respond in a similar manner as ampullar dark cells under identical experimental conditions. Therefore, the objective of this investigation was to examine morphometrically the stria vascularis tissues from postadrenalectomized animals that were administered either aldosterone or dexamethasone. The hypothesis was that the structural integrity of cells of the stria vascularis, which were demonstrated to be altered in the absence of adrenal steroids, would recover to within normal appearance upon the administration of the mineralocorticoid, aldosterone, and/or the synthetic glucocorticoid, dexamethasone.

MATERIALS AND METHODS

Twelve, healthy, Long Evans rats (Charles River, Wilmington, Mass.; male, 175-200 g) were divided into two groups. Animals in both group 1 and group 2 were bilaterally adrenalectomized as described previously (Lohuis et al., 1990a; ten Cate et al., 1990). On day eight postadrenalectomy, animals were anesthetized intramuscularly with ketamine hydrochloride (100 mg/kg body weight) and xylazine hydrochloride (10 mg/kg body weight). A mini-osmotic pump (Alzet model 2001, Alza Corp., Palo Alto, Calif.) was implanted subcutaneously above the lumbar spine. Before placement of the mini-osmotic pumps, they were placed in individual 0.9% saline baths for 4 hours to insure proper delivery of the given agents at the outset. Animals in group 1 (n = 6) were administered aldosterone (25 mg/100 g/day; Sigma #A-6628), and animals in group 2 (n = 6) were administered dexamethasone sodium phosphate (25 mg/100 g/day; Quad NDC 51309-405-05). Each pump delivered each given agent at a rate of 1 ml/hour for 7 days. All animals received 0.9% saline and Purina laboratory chow ad libitum, and were housed in the University of Florida Animal Facilities.

After 7 days of administering the described hormones (day 14 postadrenalectomy), animals in the individual groups were anesthetized in a similar manner as described previously. Blood samples for steroid levels determination (ICN diagnostics) were collected by cardiac puncture. Subsequently, each animal was intracardially perfused with 24 cc phosphate buffered saline (PBS: 20 mM $NaH_2PO_4 \times H_2O$ and 42 mM Na_2HPO_4), pH 7.4, followed by 36 cc fixative (2.5% glutaraldehyde and 2.0% paraformaldehyde in a phosphate buffer). Following decapitation, the temporal bones were quickly removed, and each bulla was opened. Each cochlea was perilymphatically perfused with fresh fixative. Cochleas were stored in fresh fixative overnight at 4°C, postfixed for 2 hours in phosphate buffered 1% osmium tetroxide, pH 7.4, rinsed 3x in PBS, and placed in 5% EDTA at 4°C. After 5 days of decalcification, a midmodiolar cut was performed, and each half cochlea was dehydrated in graded concentrations of ethanol, and processed for Epon/Araldite (Polysciences, Warrington, Pa.) embedding, using normal procedures.

A standardized basal region the stria vascularis at 80% of the distance from the apical end of the basilar membrane was reembedded in Epon/Araldite. Ultra-thin sections of the stria vascularis perpendicular to the endolymphatic surface of the stria vascularis were cut on a Reichert-Jung ultramicrotome with a Diatome MT401 diamond knife. Sections were collected on Formvar film-coated slot grids (2x1 mm; Electron Microscopy Sciences, Ft. Washington, Pa.). Tissues were examined in a JEOL 100S electron microscope. A montage at a final magnification of X 5338 was formed from a series of micrographs of a cross section of the basal stria vascularis. Strial width,

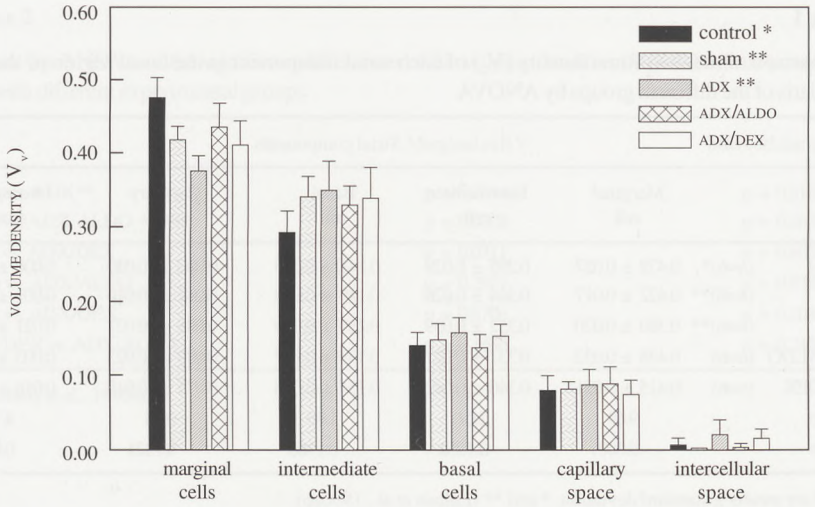

FIGURE 1. Histogram of volume densities (V_v) of individual components of stria vascularis from adrenalectomized animals that were administered aldosterone (ADX/ALDO) or dexamethasone (ADX/DEX) compared to those from previously examined control, sham- operated and adrenalectomized animals (Lohuis et al., 1990ab).

the number of marginal cells across the strial width, the volume density of the different strial components, and the radial area of the stria vascularis were determined for each of the stria regions examined as described previously (Lohuis et al., 1990). An analysis of variance (ANOVA) was used to statistically compare the different groups.

RESULTS

The stria vascularis in the standardized basal region of the cochlear duct of adrenalectomized animals, which were subsequently administered either aldosterone (group ADX/ALDO) or dexamethasone (group ADX/DEX), showed no remarkable morphological alterations compared to similar previously examined regions from control, sham, and ADX groups (Lohuis et al., 1990ab).

Volume densities of each strial component of the standardized basal region of the stria vascularis from animals of the ADX/ALDO and ADX/DEX groups are presented in Table I and Fig. 1, as well as those previously obtained for control, sham, and ADX groups for purpose of comparison. The volume densities of marginal cells of animals of the ADX/ALDO group, the ADX/DEX group, and the sham group were similar (Table II). The volume density of marginal cells increased by 15% in animals of the ADX/ALDO group, and by 9% in animals of the ADX/DEX group compared

TABLE 1

Comparison of mean volume density (V_v) of each strial component in the basal region of the stria vascularis of the different groups by ANOVA

		Strial components				
		Marginal cell	Intermediate cell	Basal cell	Capillary space	Intercellular space
Control	(*n*=6)*	0.478 ± 0.027	0.295 ± 0.029	0.141 ± 0.019	0.080 ± 0.020	0.006 ± 0.011
Sham	(*n*=6)**	0.422 ± 0.017	0.344 ± 0.026	0.149 ± 0.023	0.082 ± 0.010	0.001 ± 0.003
ADX	(*n*=6)**	0.380 ± 0.020	0.352 ± 0.039	0.159 ± 0.020	0.088 ± 0.021	0.021 ± 0.019
ADX/ALDO	(*n*=6)	0.438 ± 0.032	0.331 ± 0.027	0.139 ± 0.017	0.089 ± 0.023	0.003 ± 0.005
ADX/DEX	(*n*=6)	0.415 ± 0.031	0.340 ± 0.042	0.154 ± 0.016	0.075 ± 0.018	0.016 ± 0.012
F value		9.69	2.04	1.60	0.56	4.76
P value		0.0001	0.1056	0.1966	0.7321	0.0032

Values are means ± standard deviation. * and ** (Lohuis et al., 1990ab).

to animals of the ADX group. These increases were statistically significant (ANOVA, $p<0.05$, Table II). The volume density of intercellular space of animals of the ADX/ALDO group was similar to that observed in animals of the sham and control group. The volume density of intercellular space of animals of the ADX/ALDO group decreased by 86% compared to animals of the ADX group; this was significant at the $p = 0.1$ significance level (Table II). The volume density of intercellular space in animals of the ADX/DEX group was not significantly different from that observed in animals of the ADX/ALDO group (ANOVA, $p<0.05$, Table II). The volume densities of intermediate cells, basal cells, and capillary space were similar between all treated groups.

Means and standard deviation of the number of marginal cells, strial width (mm), and radial area (mm^2) of the standardized region in the basal turn of the stria vascularis of animals from the ADX/ALDO and ADX/DEX groups were: 22.0 ± 1.3, 21.8 ± 2.2; 220.4 ± 14.5, 235.6 ± 14.2; 5107.5 ± 502.2, 5704.0 ± 830.1, respectively. These data were similar to previously measured values from animals of the control, sham and ADX groups (Lohuis et al., 1990ab).

In order to determine whether the impact of surgery in the creation of the animal model was an important factor, volume densities from both the sham-operated animals and control (non-operated) animals were incorporated into the analysis of variance. Although the volume density of intermediate cells was significantly lower in animals of the control group and the volume density of marginal cells was significantly higher in the control group compared to the other groups (ANOVA, $p<0.05$), the effects of adrenalectomy, plus the administration of the corticosteroids, were lucid.

TABLE 2

Follow-up ANOVA of the significant differences of the volume densities (V_v) of the strial components between different experimental groups

	Marginal cell V_v	Intercellular space V_v
Sham vs. ADX**	p = 0.0044	p = 0.0180
ADX vs. ADX/ALDO	p = 0.0008	p = 0.563
ADX vs. ADX/DEX	p = 0.0311	p = 0.6055
Sham vs. ADX/ALDO	p = 0.2921	p = 0.8388
Sham vs. ADX/DEX	p = 0.6706	p = 0.1048
ADX/DEX vs. ADX/ALDO	p = 0.3430	p = 0.2420

** (Lohuis et al., 1990ab).

Mean serum aldosterone value and standard deviation for the animals of the ADX/ALDO group were 486± 332 pg/ml. Mean serum dexamethasone and aldosterone values of animals from the ADX/DEX group were 27 ± 12 ng/ml and 14 ± 5 pg/ml, respectively. Normal rat serum levels for aldosterone were determined to be 30 to 700 pg/ml.

DISCUSSION

A striking, distinctive correlation was observed between the reestablishment of circulating corticosteroids and the structural integrity of the highly metabolic region of the cochlear duct. The volume densities of marginal cells and intercellular space in the basal stria vascularis in adrenalectomized animals were shown to approximate normal values after administration of the mineralocorticoid, aldosterone. Administration of the synthetic glucocorticoid, dexamethasone, at a comparable concentration with that of aldosterone also was found to have a similar effect as that of aldosterone on the volume density of marginal cells. However, the volume density of the intercellular space of animals that were administered dexamethasone did not approximate normal values. The response of the strial marginal cells to the corticosteroids appears similar to that of other epithelial cells which have been shown to be responsive both morphologically and biochemically to such steroids (Rodriguez et al., 1981; Stanton et al., 1985a; El Mernissi et al., 1984; Mujais et al., 1984). In addition, modulation of ampullar dark cells to their normal architecture has been measured quantitatively after administration of aldosterone to adrenalectomized rats in a parallel study (ten Cate and Rarey, 1991).

A direct or indirect action of corticosteroids on Na^+/K^+-ATPase in all transporting tissues, e.g., kidney, in general has been difficult to determine. For example, while the variation of steroid levels has been shown to directly regulate Na^+/K^+-ATPase activity, corticosteroids can also indirectly alter Na^+/K^+-ATPase activity by mediating changes in serum sodium and potassium concentrations (Kaissling, 1982; Katz, 1982; Garg et al., 1986; Stanton et al., 1985b). Sodium and potassium concentrations can independently regulate Na^+/K^+-ATPase activity (Mujais et al., 1986; Le Hir et al., 1982). Thus, the concise role of adrenal steroids on Na^+/K^+-ATPase activity remains unclear. A direct physiological effect of adrenal steroids upon inner ear microhomeostasis that is not mediated by serum sodium and serum potassium concentrations changes may be conjectured, since receptors for them have been identified within cochlear and vestibular tissues (Rarey and Luttge, 1989). Studies of the effects of altered sodium and potassium dietary intake may provide a means to delineate primary or secondary actions of adrenal steroids on inner ear Na^+/K^+-ATPase. Corticosteroids (e.g., dexamethasone, prednisone, Decadron®) have been prescribed empirically at times for certain disorders of both the middle and inner ear (Lambert et al., 1986; Wilkins et al., 1987; Moskowitz et al., 1984). Morphological changes in sensory cells of vestibular end organs have been described following the experimental administration of such steroids (Trevisi et al., 1980). The observed consistent, although small, changes identified in this study suggest that differing levels of corticosteroids may also modulate the structural integrity of the stria vascularis. Future studies, especially the functional effects of such steroids on hearing and equilibrium, appear warranted to further delineate the association between adrenocortical hormones and inner ear physiology.

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CHAPTER V

Electrophysiological and morphological effects of deprivation of circulating adrenal hormones in the rat cochlea

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Submitted

Abstract

Circulating adrenal hormones affect strial function. Removal of endogenous levels of adrenal steroids by bilateral adrenalectomy (ADX) in rats causes a decrease of Na^+/K^+-ATPase activity in the cochlear lateral wall (Rarey et al., 1989) and a decrease of the volume of the marginal cells in the stria vascularis (Lohuis et al., 1990). To further study the effect of absence of circulating adrenocorticosteroids on cochlear function, eighteen male Long Evans rats underwent either an ADX or a SHAM operation. Electrocochleography was performed one week after surgery for tone bursts in a frequency range of 1-16 kHz. Thereafter the cochleas were harvested and examined histologically. No significant changes in the amplitude growth curves of the summating potential (SP), the compound action potential (CAP) and the cochlear microphonics (CM) were detected after ADX. However, visually, there appeared to be a decrease of endolymphatic volume (tentatively called imdrops). Reissner's membrane extended less into scala vestibuli in ADX animals than in sham-operated animals. The ratio between the length of Reissner's membrane and the straight distance between the medial and lateral attachment points of Reissner's membrane was used as an objective measure to quantify this effect in each subapical half turn of the cochlea. The decrease in length of Reissner's membrane was statistically significant.
Thus, circulating adrenal hormones appear to be necessary for normal cochlear fluid homeostasis. Absence of one or more of these hormones leads to shrinkage of the scala media (imdrops). However, the absence of adrenal hormones does not affect the gross cochlear potentials. Apparently, the cochlea is capable of compensating for the absence of circulating adrenal hormones to sustain the conditions necessary for proper cochlear transduction.

INTRODUCTION

Na^+/K^+-ATPase is a ubiquitous plasma membrane enzyme that catalyzes the movement of K^+ into cells in exchange for Na^+. In epithelial cells, Na^+/K^+-ATPase is capable of vectorial transport across the epithelial barrier as a result of the asymmetric distribution of the Na^+/K^+-ATPase, which is present mainly in the basolateral membrane of these cells (Ewart and Klip, 1995). In the cochlea Na^+/K^+-ATPase is found in high concentrations along the basolateral membrane of the marginal cells of the stria vascularis (Kerr et al., 1982; Schulte and Adams, 1989). This configuration is essential for the capacity of the stria to actively maintain the endocochlear potential (EP) and the K^+ gradient, which both are necessary for the mechano-electrical transduction process in the cochlea (Dallos, 1996). Inhibition of the strial Na^+/K^+-ATPase by perilymphatic perfusion with ouabain was shown to decrease both the EP and the endolymphatic K^+ concentration (Bosher et al., 1980), which emphasizes the importance of maintaining the strial Na^+/K^+-ATPase activity at a certain level.

In general, the Na^+/K^+-ATPase activity is directly regulated by the intracellular Na^+ concentration. Since the enzyme usually works below its maximal capacity, an increase of intracellular Na^+ concentration immediately activates the enzyme. In addition, in various tissues the Na^+/K^+-ATPase activity is indirectly regulated by inducing the synthesis of new active pump-units at the nuclear level by adrenal hormones (Ewart and Klip, 1995). Supported by several investigations it has been hypothesized that the latter also occurs in the stria vascularis. First, the presence of protein receptors for both mineralocorticoids and glucocorticoids has been demonstrated in inner ear tissues using different techniques (Rarey and Luttge, 1989; Pitovski et al., 1993a; Furuta et al., 1994; Yao and Rarey, 1996). Second, injection of aldosteron was reported to increase the binding of [^{3}H]ouabain in the cochlear lateral wall, indicative of an increase of the number of Na^+/K^+-ATPase sites (Pitovski et al., 1993b). Third, removal of endogenous levels of adrenocorticoids by bilateral adrenalectomy (ADX) was shown by fluorometric assay to cause a significant decrease of ATPase activity in the cochlear lateral wall (Rarey et al., 1989).

In view of the studies quoted above, we may hypothesize that circulating adrenocorticoids influence strial Na^+/K^+-ATPase activity and thereby affect the ionic homeostasis in the cochlear duct. In line with this hypothesis, a decrease of the volume of the marginal cells was detected in the stria vascularis of adrenalectomized rats as a sign of disturbed ion transport (Lohuis et al., 1990).

Since the strial Na^+/K^+-ATPase is thought to be involved in endolymph production and the marginal cells are immediately adjacent to the endolymph, we may also expect disturbed endolymph homeostasis after adrenalectomy.
In this study, the consequences of bilateral adrenalectomy in Long Evans rats were further investigated with respect to endolymph homeostasis. Electrophysiologically we measured the growth curves of the compound action potential (CAP), cochlear microphonics (CM) and the summating potential (SP). Especially the SP is a sensitive indicator of disturbances in endolymphatic volume, at least in guinea pigs (Klis et al., 1999). In addition we also determined the length of Reissner's membrane (RM) in response to adrenalectomy. The length of RM can be used as an indicator for endolymphatic volume (before fixation).

MATERIALS AND METHODS

Experimental design

The experiments included 18 male Long Evans rats with a positive Preyer reflex. The rats were divided in two groups: animals in group one (n=10) were adrenalectomized (ADX), animals in group two (n=8) received a Sham operation (SHAM). All animals were provided with standard laboratory food. ADX animals were sustained with 0.9% saline ad libitum, the other animals received tap water ad libitum. The care and use of the animals reported on in this paper were approved by the Animal Care and Use Committee of the Faculty of Medicine, Utrecht University, under number FDC-89007 and GDL-20008.

Bilateral adrenalectomy

First the rats were anesthetized with intramuscular injections of ketamine hydrochloride (100 mg per kg body weight) and xylazine (10 mg per kg body weight). Then the adrenal glands were carefully removed through dorsolateral incisions. Each adrenal gland was inspected under a stereomicroscope to ensure complete extraction. The effectiveness of the adrenalectomy was checked when the animals were sacrificed after the electrophysiological experiments by collecting 3 ml of blood via intracardiac puncture. The serum aldosterone levels were determined using radioimmunoassays (ICN Biomedicals). The incision was closed in two layers and animals were allowed to recover. A Sham-operation consisted of exposure of both adrenal glands in the manner described without excision of the adrenal glands.

Electrophysiological measurements

Seven days after the first surgical procedure, the rats were anesthetized as described in the previous paragraph. Rectal temperature was kept at around 38 °C by means of a thermostatically controlled heating pad. Heart rate was monitored continuously. The right cochlea was exposed for electrophysiological measurements through a ventrolateral approach.
Stimulus generation and data acquisition were controlled by a PC using a CED 1401-plus laboratory interface. Tone bursts (probes) were calculated and stored in a revolving memory consisting of 2,500 points with 12-bit resolution. Trains of 8 ms probes of 1, 2, 4, 8, 12 and 16 kHz were used. The probes had cosine-shaped rise-and-fall times of 2 ms at 1 kHz, of 1.5 ms at 2 kHz and 1 ms at the higher frequencies. Consecutive tone bursts were presented with alternating polarity at 99-ms intervals from onset to onset. The stimuli were led via a computer controlled attenuator to a Beyer DT48 dynamic transducer, which was connected to a hollow ear bar that was fitted into the exposed outer ear canal.

Sound-evoked potentials were measured differentially with a silver-ball electrode placed on the apical surface of the right cochlea. A surgical clamp connected to the neck musculature served as the reference electrode. Signals were led to a computer-controlled amplifier and were band-pass filtered between 1 Hz and 10 kHz before AD conversion and averaging (max. 250 times). The averaged responses to the tone bursts of opposite polarity were stored separately for off-line analysis. The SP and the CAP were obtained by addition of the responses to the opposite polarity tone bursts, the CM by subtraction. The SP was measured as the difference between the pre-stimulus DC level and the DC level approximately 6 ms after the onset of the 8 ms probe. The CAP was measured relative to the SP, because it is superimposed on the SP. Thus, the distance between the the first negative peak (N_1) and the steady-state level of the SP was taken as CAP amplitude. Moreover, this method of measurement of the CAP has the advantage over the more common N_1-P_1 method that it is less sensitive to changes in the waveform of the CAP. CM was determined as the peak-to-peak value in the temporal middle of the AC response.

Tissue processing

Directly after the last recording the abdominal cavity was exposed and the diaphragm was reflected. The descending aorta was clamped, the right atrium opened and each animal was intracardially perfused with phosphate buffered saline (PBS, pH 7.4), followed by fixative (2.5% glutaraldehyde and 2% paraformaldehyde in PBS).

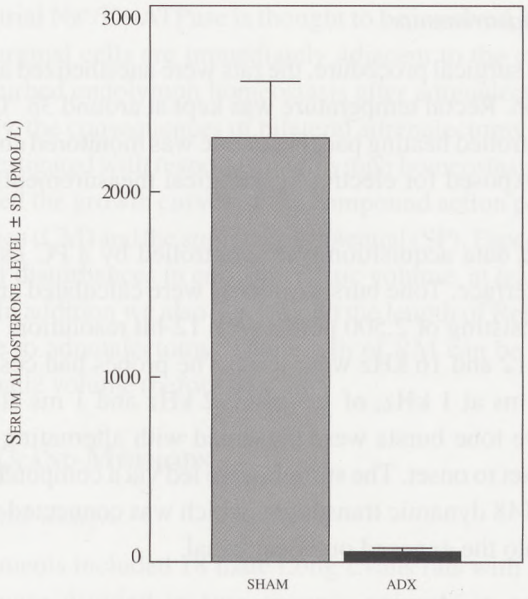

FIGURE 1. Decrease in serum aldosterone level seven days after adrenalectomy (ADX) as compared to the Sham-operated animals (SHAM). The result confirms the success of the surgery.

Following decapitation, the temporal bones were quickly removed and each bulla was opened. Cochleas were stored in fresh fixative overnight at 4°C, postfixed in 1% $Os0_4$, rinsed 3 times in PBS, placed in 10% EDTA.2Na (pH 7.4) for 4-5 days, dehydrated in a graded ethanol series and embedded in toto in Spurr's low-viscosity resin. After dividing the cochleas along a midmodiolar plane, they were ready for semi-thin (1mm) sectioning and light microscopic evaluation.

Morphometry

We used a Quantimet 500 Image Analysis system connected to a Leica microscope to quantify the extension of Reissner's membrane (RM) into scala vestibuli in each sub-apical half turn of the cochlea. The length, l_R, of RM and the straight distance, d, between the medial and lateral attachment points of Reissner's membrane were measured as described in an earlier paper on guinea pigs (Bouman et al., 1998). The ratio, l_R / d, was used as an objective measure to assess the degree of extension of RM into scala vestibuli. This method has the advantage that in case of folding of RM it reflects the area of scala media which was presumably present previously (before fixation), when RM was still under tension from endolymphatic pressure.

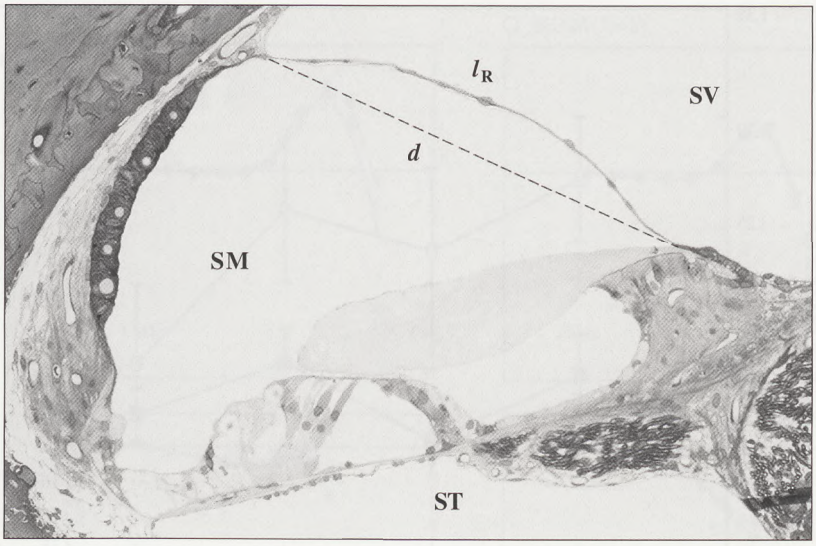

FIGURE 2. Example of the determination of l_R / d in a sham-operated animal. The length, l_R, of Reissner's membrane and the straight distance, d, between the medial and lateral attachment points of Reissner's membrane are indicated.

Statistics

Our electrophysiological data were evaluated by means of analysis of variance (ANOVA). CAP and CM were logarithmically transformed before analysis to improve homogeneity of variance. In the analysis, ADX treatment was taken as a between animals factor. Since the amplitude of the SP is extremely level-dependent and virtually absent at the lower levels of stimulation in rats, the statistical evaluation was performed on the potentials evoked by the highest three stimulus levels depicted in the figures. Concerning the CAP and the CM, statistical evaluation was performed on all relevant data, defined here as all CAP and CM data above 5 μV. For the morphological data ANOVA was used to compare l_R / d between groups. Statistica® software was used in all analyses.

RESULTS

Serum aldosterone levels were determined in both groups to assess the success of the surgery (Fig. 1). Aldosterone levels were almost undetectable in the ADX animals, which confirms the success.

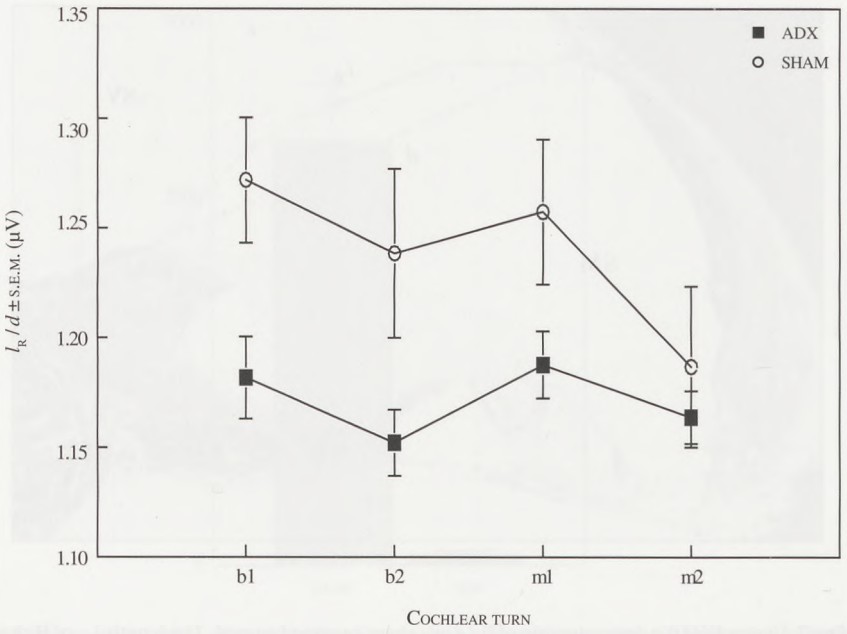

FIGURE 3. The l_R / d ratio as determined in every sub-apical half turn of the cochlea comparing the adrenalectomized (ADX) and sham-operated (SHAM) animals. b_1, b_2, m_1, m_2: inferior and superior transection of the basal and middle turn, respectively.

The micrograph (Fig. 2) shows the length, l_R , of Reissner's membrane (RM) and the straight distance, d, between the medial and lateral attachment points of RM in a SHAM animal. The ratio, l_R / d, at specific locations, was used as an objective manner to assess the effect of ADX. In all ADX and SHAM animals l_R / d was measured in the first four sub-apical half turns as observed in a midmodiolar transection of the right cochlea. The results are presented in Fig 3. The average of the measured l_R / d values in the SHAM animals was 1.24, the average in the ADX animals was 1.17. ANOVA shows that this difference represents a significant main effect of adrenalectomy. The figure suggests that the decrease of l_R / d depends on the place along the cochlear turns: the decrease seems more prominent in the basal half turns. However, statistical analysis showed no interaction between the factors adrenalectomy and cochlear turn ($p > 0.1$).

Fig. 4 shows the SP growth curves for probes at 1-16 kHz for both the adrenalectomized (ADX) and the sham-operated (SHAM) rats. Measurements were performed one week after surgery. With the electrode placed on the apical surface of the cochlea, the SP was negative at stimulus frequencies of 4-16 kHz. At 1 and 2

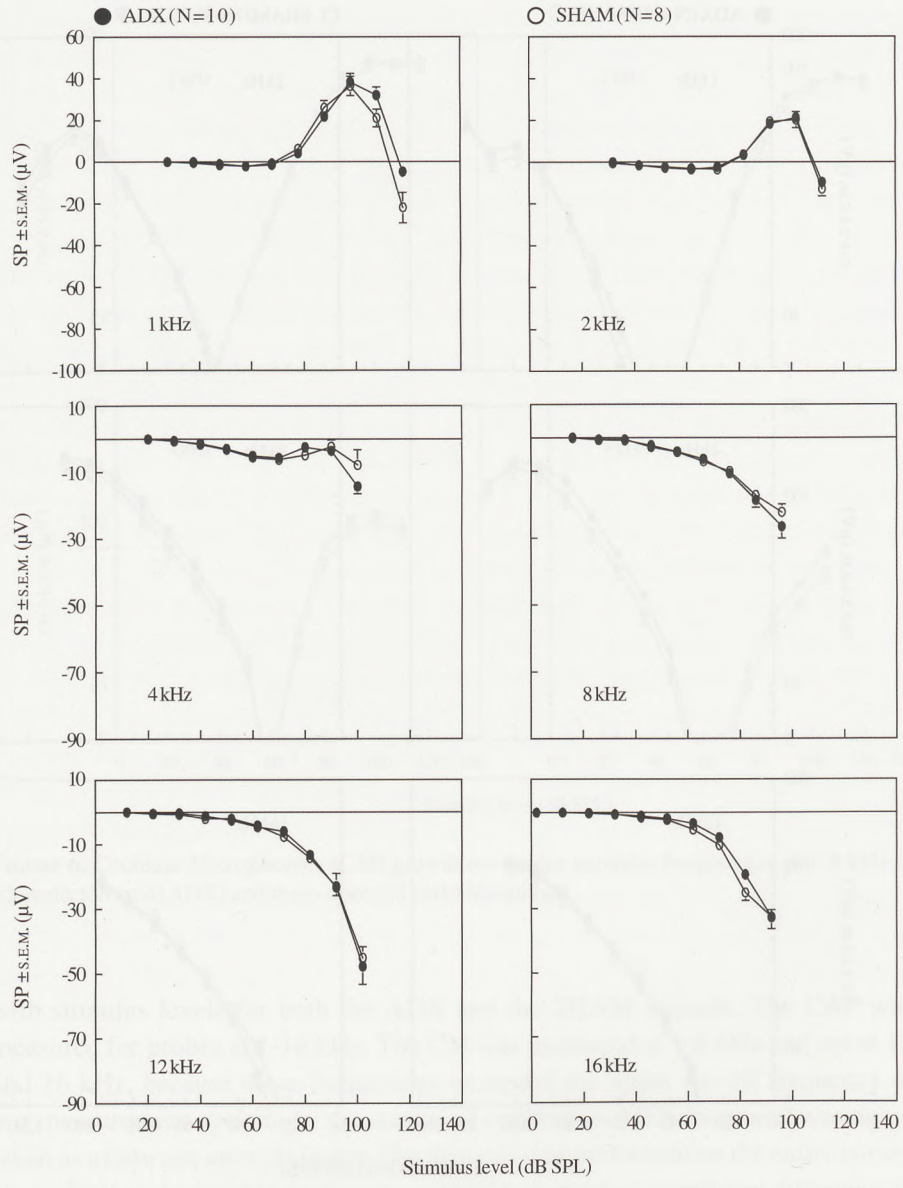

FIGURE 4. Summating Potential (SP) growth curves for stimulus frequencies of 1-16 kHz in adrenalectomized (ADX) and sham-operated (SHAM) animals.

kHz the SP shows a small deflection toward positive values, before turning negative at higher levels. The statistical analysis of the SP was performed on the potentials evoked by the highest three stimulus levels depicted in the figures with the ADX

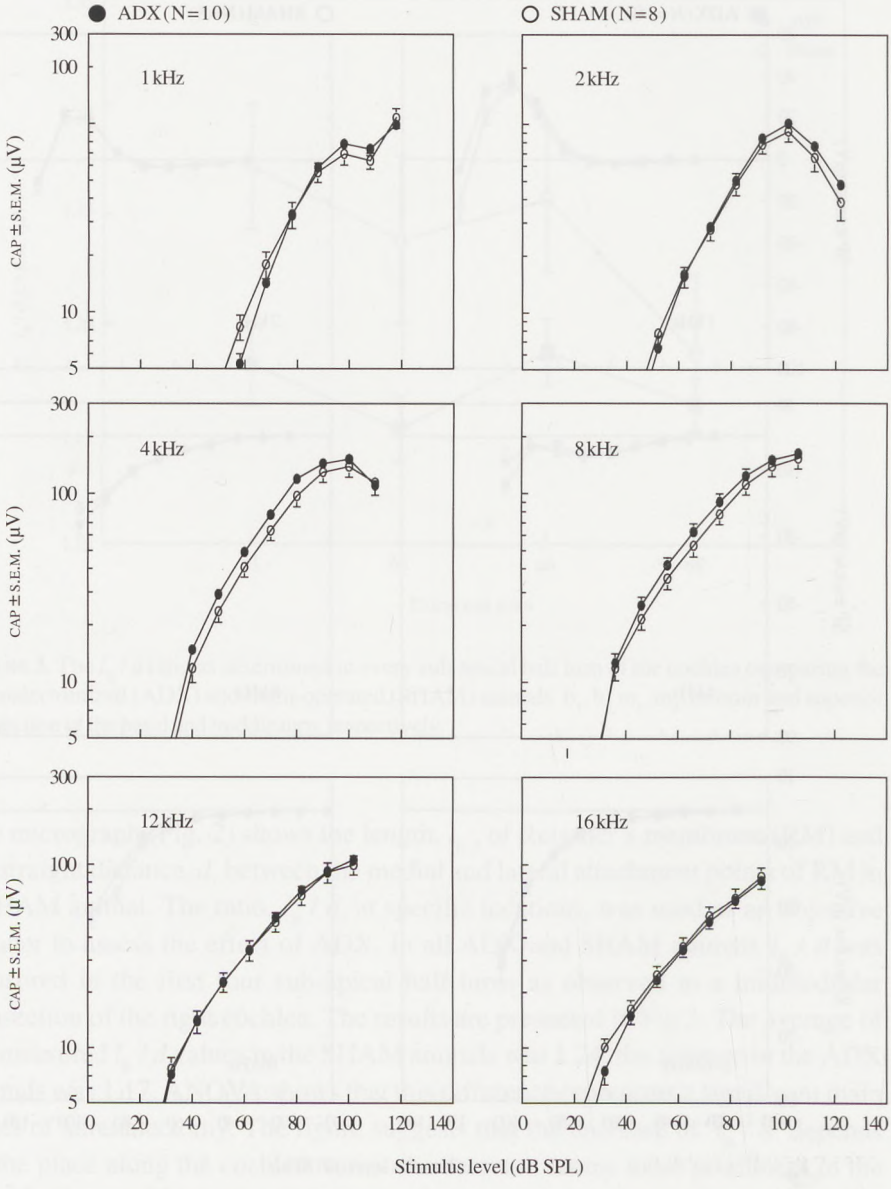

FIGURE 5. Compound Action Potential (CAP) growth curves for stimulus frequencies of 1-16 kHz in adrenalectomized (ADX) and sham-operated (SHAM) animals.

treatment taken as a between animals factor. There was no significant difference in SP between the two groups at all stimulus frequencies.

Fig. 5 and Fig. 6 show respectively the growth of the CAP and the CM amplitude

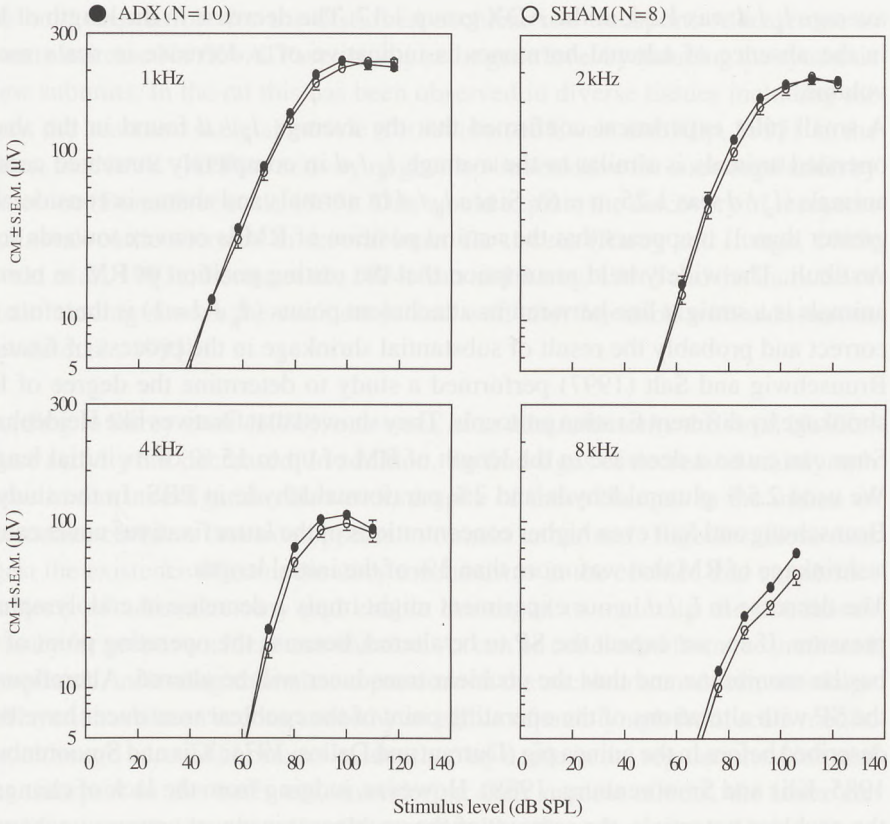

FIGURE 6. Cochlear Microphonics (CM) growth curves for stimulus frequencies of 1-8 kHz in adrenalectomized (ADX) and sham-operated (SHAM) animals.

with stimulus levels for both the ADX and the SHAM animals. The CAP was measured for probes at 1-16 kHz. The CM was measured at 1-8 kHz and not at 12 and 16 kHz, because these frequencies exceeded the upper cut-off frequency of our measurement system. In the statistical evaluation ADX treatment was again taken as a between animals factor. The analysis was performed on the entire curves above 5 μV as depicted in the figures. ANOVA showed no significant difference in CAP or CM between the two groups at any of the tested stimulus frequencies.

DISCUSSION

In the present study we found that removal of endogenous adrenal hormones in rats induced a significant decrease of l_R / d in the cochlea. In the SHAM group the

average l_R / d was 1.24, in the ADX group 1.17. The decrease in the length of RM in the absence of adrenal hormones is indicative of a decrease in scala media volume.

A small pilot experiment confirmed that the average l_R / d found in the sham-operated animals is similar to the average l_R / d in completely untreated control animals (l_R / d was 1.25; n = 6). Since l_R / d in normals and shams is considerably greater than 1, it appears that the natural position of RM is convex towards scala vestibuli. The widely held assumption that the resting position of RM in normal animals is a straight line between its attachment points ($l_R / d = 1$) is therefore not correct and probably the result of substantial shrinkage in the process of fixation. Brunschwig and Salt (1997) performed a study to determine the degree of RM shrinkage by different fixation protocols. They showed that fixatives like Heidenhain-Susa can cause a decrease in the length of RM of up to 15 % of its initial length. We used 2.5% glutaraldehyde and 2% paraformaldehyde in PBS. In the study of Brunschwig and Salt even higher concentrations of the latter fixatives never caused a shrinkage of RM that was more than 3% of the initial length.

The decrease in l_R / d in our experiment might imply a decrease in endolymphatic pressure. If so, we expect the SP to be altered, because the operating point of the basilar membrane and thus the cochlear transducer will be altered. Alterations of the SP with alterations of the operating point of the cochlear transducer have been described before in the guinea pig (Durrant and Dallos, 1974; Klis and Smoorenburg, 1985, Klis and Smoorenburg, 1988). However, judging from the lack of change in the cochlear potentials, the setpoint of the cochlear transducer appears unchanged when comparing the ADX group and the SHAM group one week after the adrenalectomy. This may be due to a temporal factor. Postoperatively the length of RM might have decreased slowly in combination with an efflux of endolymph from scala media such that the pressure in scala media did not change. As a result of this slowly, adaptive process there was no displacement of the position of the basilar membrane at the time the electrocochleography was performed. For the decrease in endolymphatic volume we coin the term 'endolymphatic imdrops' as opposed to 'endolymphatic hydrops'.

The combination of the unaltered SP and the decrease in the length of RM implies that removal of endogenous adrenal hormones via ADX results in a decrease of endolymphatic volume. Previously, ADX had already been shown to decrease strial Na^+/K^+-ATPase activity (Rarey et al., 1989) and the volume of the strial marginal cells (Lohuis et al., 1990). Together, these findings are indicative of disturbed ion and fluid transport in the cochlea in the absence of circulating adrenal hormones. Out of the diversity of adrenal hormones, aldosterone is the hormone most likely

to be held responsible for the disturbed cochlear ion transport. Aldosterone is known to increase Na^+/K^+-ATPase activity at the gene level by inducing the synthesis of new subunits. In the rat this has been observed in diverse tissues including the kidney, the heart and skeletal muscle (for review see Ewart and Klip, 1995). In the cochlea, the Na^+/K^+-ATPase activity might also be sensitive for such regulation by aldosterone (Dunnebier et al., 1997). This would explain the discovery of receptors for mineralocorticosteroids in several inner ear tissues (Rarey and Luttge, 1989; Pitovski et al., 1993a; Furuta et al., 1994; Yao and Rarey, 1996) and the increase of ouabain binding sites in the cochlear lateral wall after injection with aldosterone (Pitovski et al., 1993b).

According to Rarey et al. (1989), one week after adrenalectomy the strial Na^+/K^+-ATPase activity has decreased. In addition, we find signs of decreased endolymph volume in our investigation. However, despite of these changes in the milieu of scala media, cochlear function appears to remain unharmed. Together, these data suggest the existence of compensatory mechanisms in the cochlea that counteract the effects of adrenalectomy and enable the organ to maintain the conditions necessary for proper cochlear transduction. As can be concluded from the unaltered SP amplitude, indicating an unaltered pressure on the cochlear transducer, shrinkage of RM must have been accompanied by an efflux of endolymph from scala media. Thus, although adrenal hormones evidently play a role in the regulation of ion and water transport in the inner ear, considering their modest effects, the inner ear epithelium is probably not primarily regulated by these hormones. Their effects may even be due to cross talk on receptors which normally interact with more specific inner ear ligands. Dysregulation in the activity of such ligands, affecting Na^+/K^+-ATPase activity in the cochlea could account for disorders such as observed in Menière's disease.

Acknowledgements

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CHAPTER VI

Signs of endolymphatic hydrops after perilymphatic perfusion of the guinea pig cochlea with cholera toxin; a pharmacological model of acute endolymphatic hydrops

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Hearing Research (in press)

Abstract

There are indications that endolymph homeostasis is controlled by intracellular cAMP levels in cells surrounding the scala media. Cholera toxin is a potent stimulator of adenylate cyclase, i.e., it increases cAMP levels. We hypothesized that perilymphatic perfusion of cholera toxin might increase endolymph volume by stimulating adenylate cyclase activity, providing us with a pharmacological model of acute endolymphatic hydrops (EH). Guinea pig cochleas were perfused with artificial perilymph (15 min), with or without cholera toxin (10 μg/ml). The endocochlear potential (EP) was measured during and after perfusion. The summating potential (SP), evoked by 2, 4 and 8 kHz tone bursts, was measured via an apically placed electrode 0, 1, 2, 3 and 4 hours after perfusion. Thereafter, the cochleas were fixed to enable measurement of the length of Reissner's membrane, reflecting endolymphatic hydrops. After perfusion the EP increased significantly over time in the cholera toxin group as compared to the controls. Also, the SP increased gradually at all frequencies in the cholera toxin group. Comparison within animals showed that the increase in SP became significant after 2 hours at 4 kHz , after 3 hours at 2 kHz and after 4 hours at 8 kHz. In the control group the SP did not change significantly. The compound action potential (CAP) amplitude decreased monotonically over time at all frequencies in both the cholera toxin group and the control group, but it decreased faster in the cholera toxin group. Also, the cochlear microphonics (CM) amplitude decreased over time at all frequencies in both groups, but the decrease was significant only in the cholera toxin group after 3 hours at 2 and 4 kHz. Quantification of the length of Reissner's membrane showed a small but insignificant enlargement in the cholera toxin treated animals compared to controls. These results are in accord with our view that EH is accompanied by an increase in SP and a decrease in CAP. Our results partially confirm previous results of Feldman and Brusilow (Proc. Nat. Acad. Sci. USA (1973); 73, 1761-1764). New aspects in relation to that study are the significantly increased EP and SP. In the classical EH model, based on obstruction of the absorptive function of the endolymphatic sac, increased SP's are accompanied by decreased EP's. In this cholera toxin model of EH, it is unlikely that the endolymphatic sac is involved. Apparently, EH can be based on mechanisms located in the cochlea itself as opposed to mechanisms located in the endolymphatic sac.

INTRODUCTION

In 1938 endolymphatic hydrops (EH) was detected for the first time in temporal bones of patients suffering from Menière's disease (Hallpike and Cairns, 1938; Yamakawa, 1938). Since then, several experimental animal models have been developed to study the etiology of EH as well as the associated physiological alterations. Of these animal models, the one originally described by Kimura and Schuknecht (1965) has been studied most extensively. In this model, based on obstruction of endolymph absorption, EH is experimentally induced by obliteration of the endolymphatic sac. Several investigators have studied the morphological and electrophysiological changes that occur in the cochlea at different time intervals after obliteration of the endolymphatic sac. The most distinct morphological effects in the first few months after endolymphatic obliteration include distention of Reissner's membrane (RM), degenerative changes in outer hair cells and degeneration of the stria vascularis (for review see Horner, 1993). In later stages, even more extensive degeneration of cochlear structures is found such as missing outer hair cells (Albers et al., 1987) and missing endothelial cells of RM (Ruding et al., 1987). Salient electrophysiological changes in the cochlea after induction of EH include a decreased endocochlear potential (EP) (Cohen and Morizono, 1984; Kusakari et al., 1986), a decrease in the compound action potential (CAP) (Horner and Cazals, 1986), a decrease in the low-frequency cochlear microphonics (CM) (Morizono et al., 1985; Klis and Smoorenburg, 1988) and an increase of the summating potential (SP) amplitude (Van Deelen et al., 1987; Bouman et al., 1998). The enhanced SP was mainly found in animals with EH not accompanied by any further light-microscopically determined cochlear disorders (Van Deelen et al., 1988). Although the 'obstruction of endolymph absorption' model has provided us with important information about the pathophysiology of EH, this model seems to have reached its limits. On the one hand the extent, the degree and the speed of inner ear degeneration in this animal model are much larger than in most Menière's patients (Merchant et al., 1995). On the other hand, considering the scientific basis of this model, it is not at all clear that EH is caused by disturbed absorption of endolymph. Instead, EH could very well be based on increased production of endolymph.

The most convincing evidence to support the latter hypothesis comes from an experiment by Feldman and Brusilow (1976). Their experiment involved endolymphatic application of cholera toxin. Cholera toxin binds to GM1, a ganglioside that is found in most epithelial cells lining the endolymphatic surface of scala media, including those of the stria vascularis (Santi et al., 1994). After binding to GM1 the A-subunit of cholera toxin penetrates the cell membrane, where it

irreversibly activates adenylate cyclase (AC), which is abundantly present in the stria vascularis and along the endolymphatic side of Reissner's membrane (Schacht, 1982; Zajic et al., 1983). AC subsequently increases the intracellular concentration of cAMP. The endolymphatic injection of cholera toxin gradually decreased the concentration of a simultaneously injected isotope (inuline). By inference, Feldman and Brusilow (1976) concluded that the dilution was caused by developing EH. Other studies also support the hypothesis that EH can be caused by increased endolymph production. Perilymphatic perfusion of forskolin, another AC stimulant, was shown to increase the EP (Doi et al., 1990). This increase in EP, which appeared almost directly after starting the perfusion, was later found to be correlated to an enhancement of the endolymphatic Cl^- activity suggesting that AC-activity might regulate the Cl^- permeability of the endolymph-perilymph barrier (Doi et al, 1992a). It was suggested that stimulation of the AC system would lead to a Cl^- influx, followed by an increase of the endolymphatic volume due to an increase of the osmolarity of the endolymph.

Although both experiments support the concept that EH is based on an increased production of endolymph, both experiments lack electrophysiological and morphological support. Particularly the SP is of importance, since it has proven to be a sensitive indicator of acute EH in both humans (Gibson et al., 1977; Goin et al., 1982; Mori et al., 1987) and experimental animals (Kumagami and Miyazaki, 1983; Aran et al., 1984; Van Deelen et al., 1987; van Benthem et al., 1993; Bouman et al., 1998).

In this work the cholera toxin model for acute EH was further explored. We decided to apply the toxin via the perilymphatic space to keep the endolymphatic boundary of scala media intact. Electrophysiological data including SP, CAP, CM and EP were gathered as a function of time after cholera toxin perfusion. Further, the cochleas were harvested and subjected to morphometric analysis.

MATERIALS AND METHODS

Experiments were performed on 18 albino female guinea pigs (250-400 g), Dunkin Hartley strain. The left cochleas of ten of these guinea pigs were perfused with artificial perilymph supplemented with (n=5) or without (n=5) cholera toxin. The cochleas were subjected to electrocochleography in a time series and subsequently harvested for morphometrical analysis. Eight additional animals underwent a similar perfusion with artificial perilymph supplemented with (n=4) or without (n=4) cholera

toxin. In this group we measured the EP continuously during and after perfusion. Care and use of the animals reported on in this paper were approved by the Animal Care and Use Committee of the Faculty of Medicine, Utrecht University, under number FDC-89007 and GDL-20008.

The animals were anaesthetised by preoperative intramuscular injections of Thalamonal (a mixture of 2.5 mg/ml of droperidol and 0.05 mg/ml of fentanyl; 0.1 ml/100 g body weight), followed by artificial ventilation through a cannula in the exposed trachea with a gas mixture containing 33 % O_2, 66 % N_2O and 1 % Halothane. Rectal temperature was kept at around 38 °C by means of a thermostatically controlled heating pad. Heart rate was monitored continuously. The cochlea was exposed through a ventrolateral approach. To enable perfusion of the cochlea two 0.2-mm holes were drilled. One hole opened into the scala tympani, the other one into the scala vestibuli of the basal turn. In order to measure the endocochlear potential (EP) an extra 0.1-mm hole was drilled in the bony wall overlying the scala media of the second turn.

Stimulus generation and data acquisition were controlled by a pc using a Cambridge Electronic Design 1401-plus laboratory interface. Tone bursts (probes) were calculated and stored in a revolving memory consisting of 2,500 points with 12-bit resolution. Trains of probes of 2, 4 and 8 kHz were used. The probes lasted 8 ms and had cosine-shaped rise-and-fall times of 1.5 ms at 2 kHz and 1 ms at 4 and 8 kHz. Consecutive tone bursts were presented with alternating polarity at 99-ms intervals from burst onset to onset. The stimuli were led through a computer-controlled attenuator to a Beyer DT48 dynamic transducer, which was connected to a hollow ear bar that was fitted into the exposed outer ear canal.

Sound-evoked potentials were measured differentially with a silver-ball electrode placed in the apical region of the cochlea. A surgical clamp connected to the neck musculature served as the reference electrode. Signals were led to a computer-controlled amplifier and band-pass filtered between 1 Hz and 10 kHz before AD conversion and averaging (max. 250 times). The averaged responses to the tone bursts of opposite polarity were stored separately for off-line analysis. The SP and the CAP were obtained by addition of the responses to the opposite polarity tone bursts, the CM by subtraction. The SP was measured as the difference between the pre-stimulus DC level and the DC level approximately 6 ms after the onset of the 8 ms tone burst. The CAP was measured relative to the SP, i.e. the distance

between the the first negative peak (N_1) and the steady-state level of the SP was taken as CAP amplitude. In principle, the CAP is superimposed on the SP. Moreover, this method of measurement of the CAP has the advantage over the more common N_1-P_1 method that it is less sensitive to changes in the waveform of the CAP. CM was determined as the peak-to-peak value in the middle of the AC response.

The endocochlear potential (EP) was measured with a glass micropipette filled with 0.15 M KCl (impedance 10-20 MW), which was connected to a World Precision Instruments 705 preamplifier through an Ag-AgCl pellet. The electrode was inserted through the spiral ligament into the scala media of the second turn. The EP was measured relative to a chlorided silver pellet in the neck musculature. AD-converted samples of the EP were taken every two seconds. The EP was monitored during and after perfusion of the cochlea. Only those preparations where a stable recording of initially more than 70 mV for 10 min had been achieved were used to study the effect of cholera toxin on the EP.

For perfusions of the cochlea a blunt glass micropipette was positioned in the hole drilled in scala tympani. The perfusate was introduced at a rate of 2.5 ml/min for 15 min. The fluid flowing out of the hole drilled in scala vestibuli was removed from the bulla by suction with a paper wick. Two perfusions were performed in all experimental animals. The first perfusion with normal artificial perilymph (ArP) was the same in all animals and served to make sure that the cochlea was not damaged and that the perfusion pathway was patent. ArP contained (mM): 137 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 11 glucose, 1 NaH_2PO_4 and 12 $NaHCO_3$; pH 7.2-7.4. The second perfusion was performed with ArP containing 0.5% bovine serum albumin with or without cholera toxin (complete cholera toxin; Sigma, St Louis; 10 µg/ml). Sound-evoked potentials were measured directly after the first perfusion and additionally at 0, 1, 2, 3 and 4 hours after the second one.

Directly after the last recording the cochleas were fixed by perilymphatic perfusion with tri-aldehyde fixative (3% glutaraldehyde, 2% formaldehyde, 1% acrolein and 2.5% dimethylsulfoxide in 0.08 M sodium cacodylate buffer; pH 7.4). After fixation, both temporal bones were removed and decalcified in 10% EDTA.2Na (pH 7.4) for 4-5 days, postfixed in 1% OsO_4, dehydrated in a graded ethanol series and embedded in toto in Spurr's low-viscosity resin. After dividing the cochleas along a midmodiolar plane, they were ready for semi-thin (1mm) sectioning and light-microscopic evaluation.

We used a Quantimet 500 Image Analysis system connected to a Leica microscope to quantify the dimensions of the scala media in each sub-apical half turn of the cochlea. The length, l_R ,of Reissner's membrane and the straight distance, d, between the medial and lateral attachment points of Reissner's membrane were measured as described in a previous paper (Bouman et al., 1998). The ratio l_R / d was postulated to be a suitable and objective measure to assess the degree of endolymphatic hydrops in histological preparations of the inner ear. This method has the advantage that in case of folding of Reissner's membrane it reflects the area which was presumably present previously, when it was still under tension from endolymphatic pressure.

Our data were evaluated by means of analysis of variance (ANOVA). CAP and CM were logarithmically transformed before analysis to improve homogeneity of variance. Treatment with cholera toxin was tested as a between-subjects factor. For the SP, the CAP and the CM within-subjects tests were performed on post-treatment time interval, frequency and level of stimulation. Because there was a significant interaction between post-treatment time interval and frequency, the data will be presented for each frequency seperately. Significance of difference between mean values was tested post hoc using Tukey's HSD (honestly significant differences) test.
ANOVA was performed on the difference between the EP measured just before perfusion and the EP measured 4 hours later. In the analysis cholera toxin treatment was taken as a between-animals factor and time after perfusion as a within-animals factor.
Finally, for the morphological data ANOVA was used to compare l_R / d between groups over all turns and in each individual turn. Statistica® software was used in all analyses.

RESULTS

Cholera toxin effects on SP

Fig. 1 shows the SP growth curves at the stimulus frequencies of 2, 4, and 8 kHz in both the cholera toxin treated group and the control group. For clarity, only the measurements at 0, 2 and 4 hours after perfusion are shown. Data obtained just after the first control perfusion and at 1 and 3 hours were in line with the data presented. However, the 1 and 3 hours data were included in the statistical analysis. In the cholera toxin treated group the SP monotonically increased over time at all frequencies. ANOVA was performed on the SP evoked by the highest three stimulus

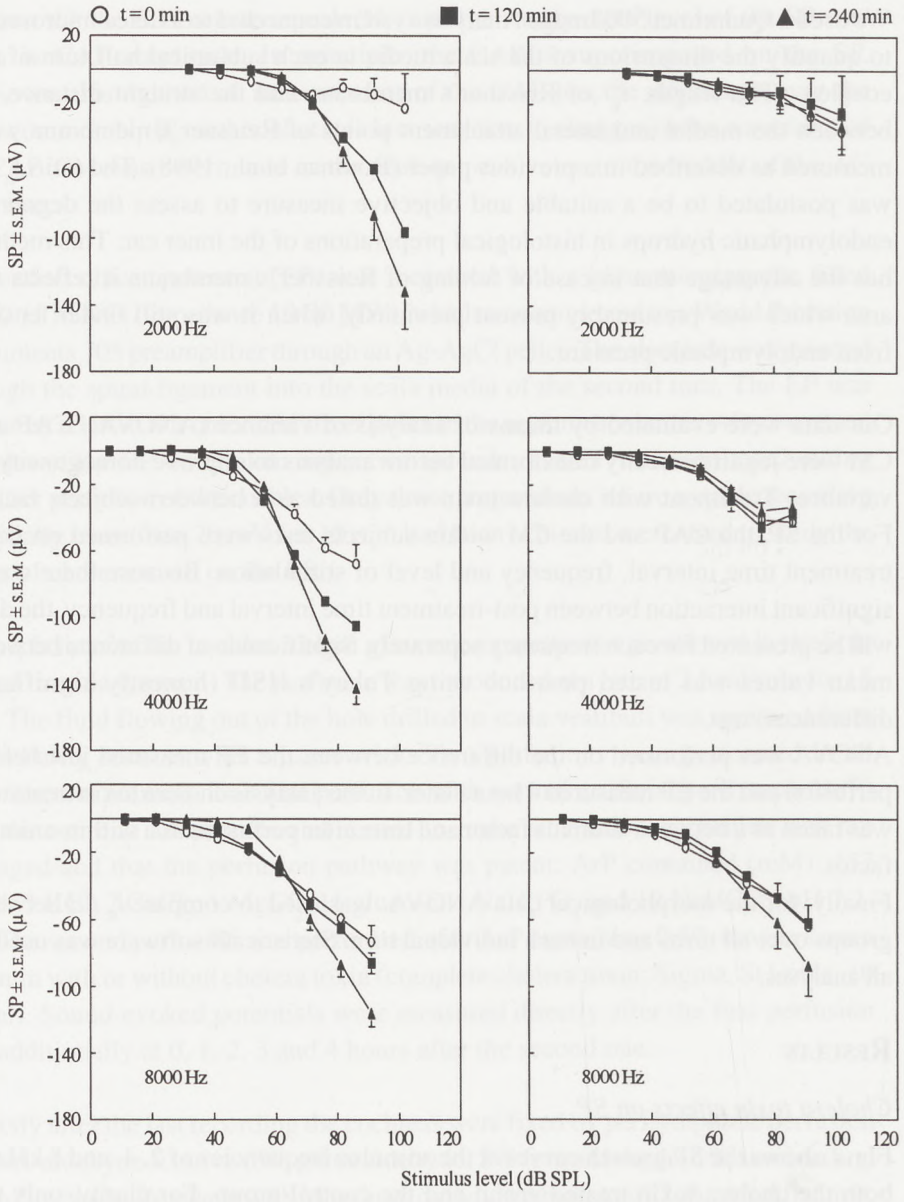

FIGURE 1. Summating Potential (SP) growth curves at time intervals of 0, 2 and 4 hours after perfusion with artificial perilymph with cholera toxin (left) or without cholera toxin (right). Panels arranged by stimulus frequency.

levels depicted in Fig. 1 and a comparison within animals was made. We decided to use only the SP at the highest three stimulus levels, because at lower levels the SP was either absent or very small and variable. The increase in SP became significant after 2 hours at 4 kHz, after 3 hours at 2 kHz and after 4 hours at 8 kHz ($p<0.01$). In the control group the SP did not change significantly over time.

Cholera toxin effects on CAP

Fig. 2 shows the growth of CAP amplitude with stimulus level at the stimulus frequencies of 2, 4, and 8 kHz in both the cholera toxin treated group and the control group. Over time, a clear decrease of the CAP amplitude was noticed at all frequencies, both in the cholera toxin group and in the control group, but the decrease was more pronounced in the cholera toxin group. ANOVA was performed on the CAP curves as depicted, per frequency and including all stimulus levels. We did restrict ourselves to the highest stimulus levels, as we did for the SP, because the effects on the CAP seemed more pronounced at lower stimulus levels. In the analysis per frequency, time after perfusion was taken as a within animals factor, cholera toxin treatment as a between animals factor. The interaction term, cholera toxin treatment versus time, was significant at each and every frequency ($p<0.01$). A significant decrease in CAP amplitude ($p<0.01$) was reached 4 hours after perfusion in the cholera toxin group at all frequencies. Some deterioration was also seen in the control group. However, this deterioration was not significant at all frequencies (2 kHz: $p<0.05$; 4 kHz: $p<0.05$; 8 kHz: ns). Inevitably, the CAP amplitude deteriorates with time, because of deterioration of the preparation, but the differences in the statistical analysis strongly suggest that the CAP amplitude decreases faster in the cholera toxin treated group.

Cholera toxin effects on CM

Fig. 3 shows the CM amplitude growth with stimulus level for the probes at 2, 4, and 8 kHz in both the cholera toxin treated group and the control group. In time, the CM-amplitude showed a small decrease at all frequencies in both groups. ANOVA was performed on the CM evoked by the highest three stimulus levels depicted in Fig. 3. The lower levels were not used, because especially at 8 kHz, CM is virtually absent at lower stimulus levels, which affects the statistical efficacy. In the cholera toxin group the decrease in CM-amplitude became significant at the 5% level 3 hours after perfusion at 2 and 4 kHz. In this group there was no significant decrease of CM at 8 kHz. In the control group the CM did not change significantly at any frequency.

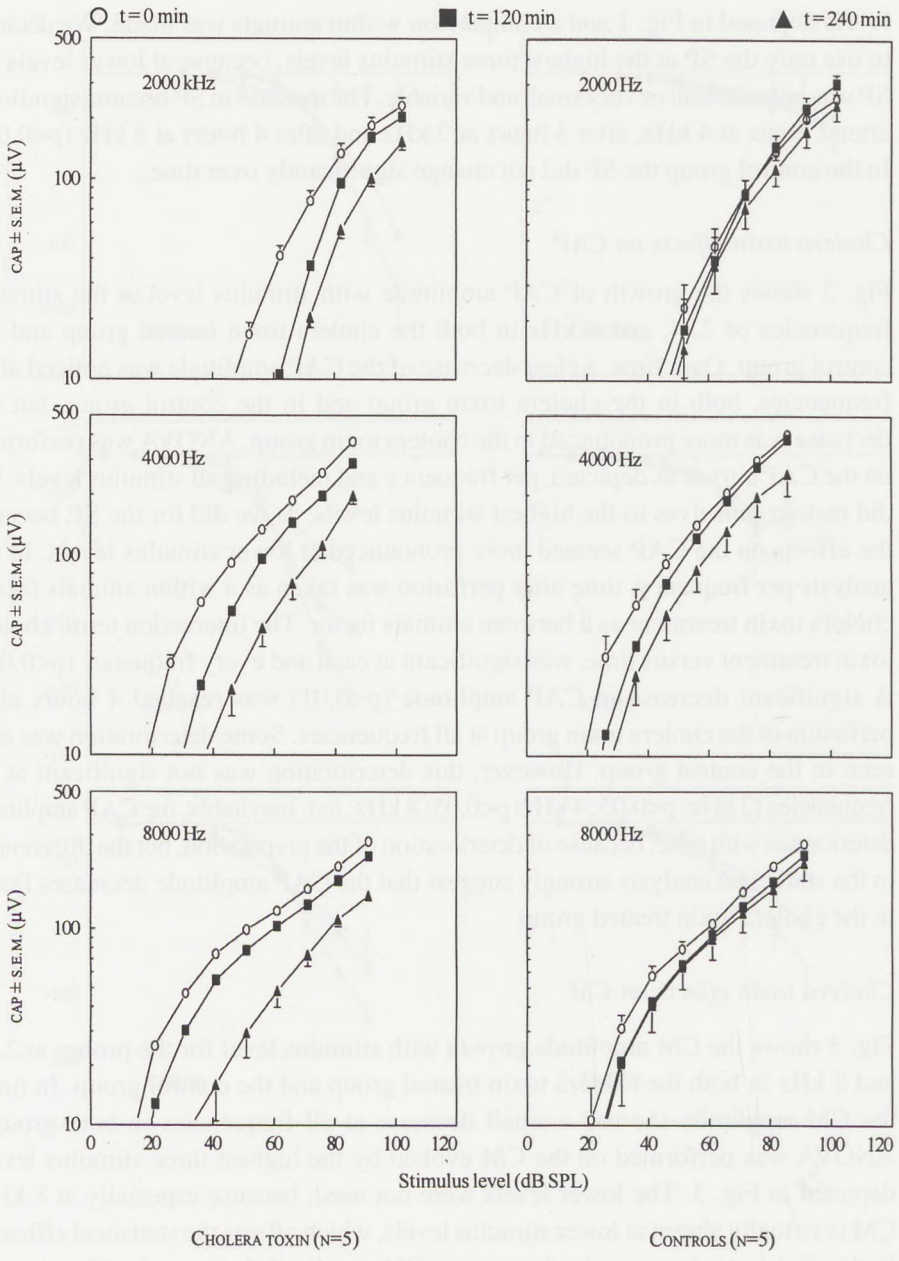

FIGURE 2. Compound Action Potential (CAP) growth curves at time intervals of 0, 2 and 4 hours after perfusion with artificial perilymph with cholera toxin (left) or without cholera toxin (right). Panels arranged by stimulus frequency.

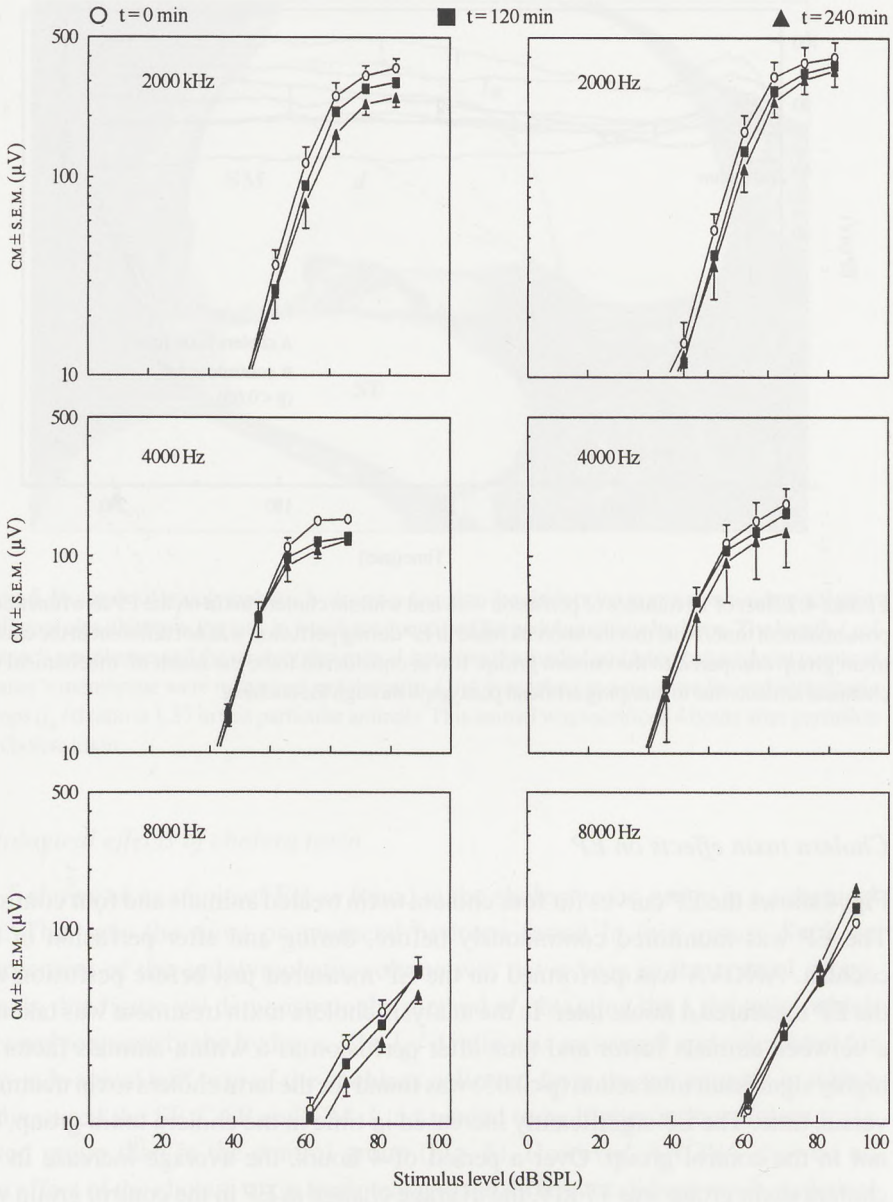

FIGURE 3. Cochlear Microphonics (CM) growth curves at time intervals of 0, 2 and 4 hours after perfusion with artificial perilymph with cholera toxin (left) or without cholera toxin (right). Panels arranged by stimulus frequency.

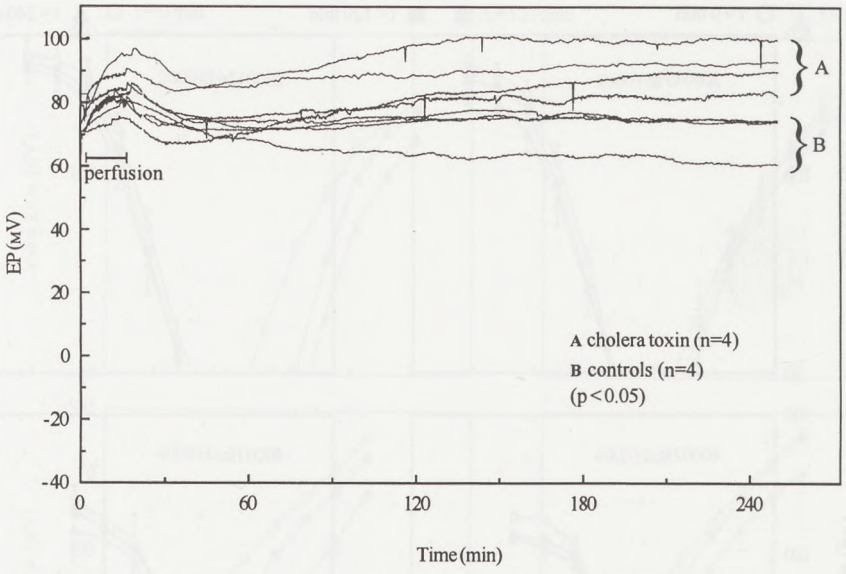

FIGURE 4. Effect of 15 minutes of perfusion with and without cholera toxin on the EP as a function of post-treatment time. Note that the short increase in EP during perfusion was not different in the cholera toxin group compared to the control group. It was considered to be the result of mechanical and chemical artifacts due to pumping artificial perilymph through the cochlea.

Cholera toxin effects on EP

Fig. 4 shows the EP curves for four cholera toxin treated animals and four controls. The EP was monitored continuously before, during and after perfusion of the cochlea. ANOVA was performed on the EP measured just before perfusion and the EP measured 4 hours later. In the analysis cholera toxin treatment was taken as a between animals factor and time after perfusion as a within-animals factor. A highly significant interaction ($p<0.05$) was found for the term cholera toxin treatment versus time. The EP significantly increased in time in the cholera toxin group, but not in the control group. Over a period of 4 hours, the average increase in the cholera toxin group was 17 mV, the average change in EP in the control group was less than 1 mV. There was no significant difference between the short increase in EP *during* perfusion with and without cholera toxin. This short increase was considered to be the result of mechanical and chemical artifacts occuring while pumping artificial perilymph through the cochlea.

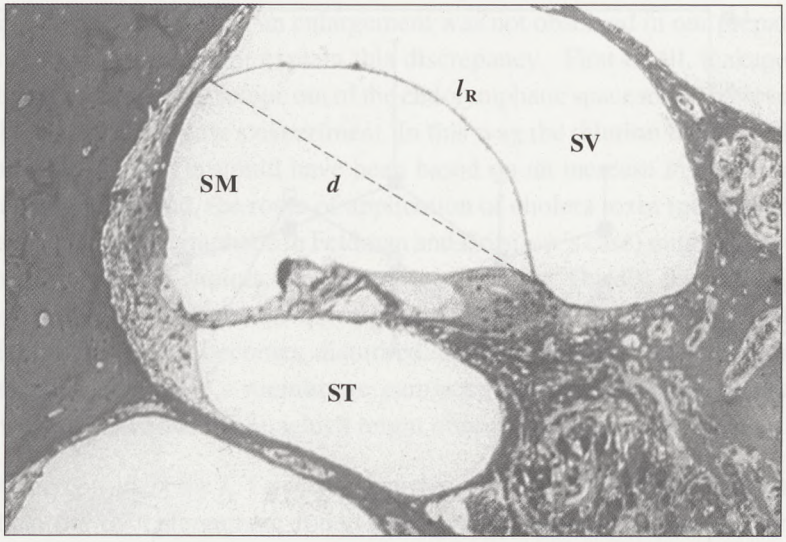

FIGURE 5. Example of endolymphatic hydrops as found in the cholera toxin group in a subapical turn. This Figure also illustrates the way in which we quantified the endolymphatic hydrops. The length, l_R, of Reissner's membrane and the straight distance, d, between the medial and lateral attachment points of Reissner's membrane were measured and the ratio, l_R / d, was taken as a measure for endolymphatic hydrops (l_R / d ratio is 1.37 in this particular animal). This animal was sacrificed 4 hours after perfusion with cholera toxin.

Histological effects of cholera toxin

Fig. 5 shows an example of EH as found in the cholera toxin group in a subapical turn. This was the most pronounced hydrops found in this group. Such an enlargement of the endolymphatic volume was never seen in the control group. Also, in this figure we demonstrate the method of obtaining the l_R / d ratio, which was used to quantify the hydrops. The l_R / d ratio was measured and calculated for every sub-apical half turn of the cochleas collected from the ten animals in which we measured the SP, CAP and CM. l_R / d tended to be higher in the cholera toxin treated group than in the control group (Fig. 6). However, ANOVA showed no main effect of the cholera toxin treatment and the difference did not reach statistical significance in t-tests comparing the data in each individual half turn. Also, we did not detect an obvious correlation between the l_R / d and the increase of SP amplitude.

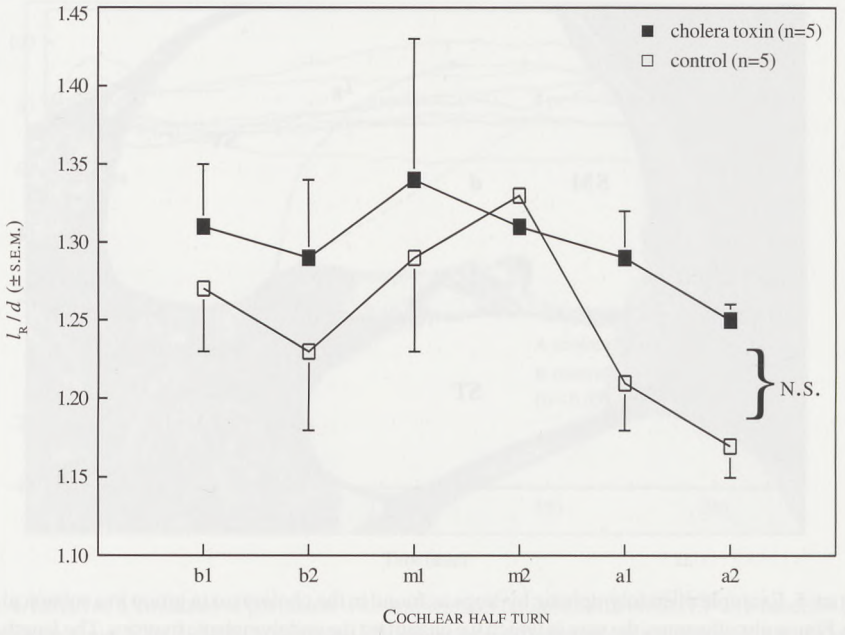

FIGURE 6. The l_R / d ratio as measured and calculated in every sub-apical half turn of the cochlea comparing the cholera toxin treated group with the control group. Cochlea's were fixed 4 hours after perfusion with cholera toxin. Bars indicate the standard errors of the means. b1, b2 m1, m2, a1, a2 indicate samples from the inferior and superior half of the basal, middle and apical turn respectively.

4. DISCUSSION

In this investigation signs were found that activation of the cochlear AC system can produce an acute EH. Electrophysiological data were measured as a function of time after cholera toxin treatment. An increase of the SP was found, accompanied by a pronounced decrease in CAP amplitude. These findings are in line with previous findings in animals with EH, brought about by obliteration of the endolymphatic duct and sac (Kumagami and Miyazaki, 1983; Aran et al., 1984; Horner and Cazals, 1986; Van Deelen et al., 1987; van Benthem et al., 1993; Bouman et al., 1998). We found an increase of the EP, which is not in line with findings in obliterated animals (Cohen and Morizono, 1984; Kusakari et al., 1986). Finally, we used the l_R / d method to assess the degree of endolymphatic hydrops and found it to be increased in cholera toxin treated cochleas, though not statistically significant at the 5% level. The latter result is in general agreement with the study of Feldman and Brusilow (1976). The enlargement of the endolymphatic space inferred by Feldman and Brusilow from the decreased isotope concentration was estimated to be up to a

factor of four. Clearly, such an enlargement was not observed in our preparations. Several mechanisms might explain this discrepancy. First of all, leakage of the simultaneously injected isotope out of the endolymphatic space might have occurred in Feldman and Brusilow's experiment. In this way the dilution factor could have appeared larger than it would have been based on an increase in endolymphatic volume alone. Second, the route of application of cholera toxin (perilymphatic in our case versus endolymphatic in Feldman and Brusilow's case) might have resulted in a smaller effect of cholera toxin in our experiments. Thirdly, the tension in RM might force it back towards its original position during fixation, when the endolymphatic lining becomes disturbed. Finally, it is known that substantial shrinkage of Reissner's membrane can occur during histologic processing (Brunschwig and Salt, 1997), which might obscure the hydrops.

When one considers the l_R / d increase in this study to be the result of a very mild EH, then the SP enlargement found after perfusion with cholera toxin may be regarded as remarkable. At a stimulus level of around 90 dB SPL the SP at 2, 4 and 8 kHz increased by a factor of 6.0, 2.5 and 2.0, respectively, in the four hours following perfusion. Considering the magnitude of these SP alterations it is highly unlikely that the relatively small change in EP (around 25 %) could be responsible for this effect. The data suggest that the SP enlargement occurs predominantly at the lower frequencies. When EH develops, Reissner's membrane distends into the scala vestibuli and the basilar membrane, in view of its limited stiffness, will distend somewhat into scala tympani. The latter distention would change the setpoint of the cochlear transducer resulting in an increase of the SP amplitude (Durrant and Dallos, 1974; Klis and Smoorenburg, 1988). The degree of distention of the basilar membrane, and thus the change of setpoint of the cochlear transducer, is probably correlated more strongly to the stiffness of RM than to the increase of endolymphatic volume *per se* (Klis et al., 1998). Only when RM has sufficient stiffness it will be able to produce a counterforce of a magnitude that can displace the basilar membrane towards scala tympani, suggesting that an increase of the SP would typically occur in the first stage of endolymphatic hydrops. In our study the EH develops in 4 hours. Therefore it may be assumed that in that period RM is still so stiff, that a small EH is capable of causing a relatively large distention of the basilar membrane. The difference in the mechanical properties of the basilar membrane along the cochlear duct might then explain the predominantly low-frequency effect. Due to the normal decrease in stiffness of the basilar membrane in the apical direction the same increase of endolymphatic pressure will cause a larger distention of the basilar membrane apically, which might result in a larger increase of SP at lower test frequencies.

Feldman and Brusilow (1976) made an attempt to measure the effects of cholera toxin on the EP. If anything, they found the EP to decrease after endolymphatic injection of cholera toxin. However, their initial values of the EP were about 50 mV. This suggests strial damage, possibly caused by insertion of the micropipette. In our study we found the EP to increase monotonically in time, concomitant with an increase of the SP and a decrease of the CAP. An increase of SP and a decrease of CAP were also found after obliteration of the endolymphatic duct. As mentioned before, the EP enhancement was not. In the 'obstruction of endolymph absorption' model the EP decreases, which might be explained by a decreased strial activity in reaction to a overpressure. In turn, the increase of the EP in our 'increased production of endolymph' model might be caused by increased strial activity. According to the 'single-pump model' explaining the generation of the electrogenic K^+ potential (Offner et al., 1987), the EP can be enlarged by activation of the Na^+/ K^+-ATPase and/or an increase of the K^+-conductance of the apical membrane of the marginal cells of the stria vascularis. Based on experiments in the kidney, there are indications that Na^+/ K^+-ATPase activity might be regulated by intracellular levels of cAMP (Ewart and Klip, 1995). Also, using patch-clamp techniques, recent investigation by Sunose et al. (1997) suggests an important role of the cAMP pathway in the generation of the EP by its influence on the I_{sK} channel current in the apical membrane of the marginal cells.

However, combined perfusion of forskolin, an adenylate cyclase stimulator, and ouabain, a specific Na^+/K^+-ATPase inhibitor, demonstrated that adenylate cyclase can enhance the EP in the absence of Na^+/K^+-ATPase activity (Doi et al., 1992). This suggests that the EP enhancement is not solely related to Na^+/K^+-ATPase activity, at least not for forskolin, which might imply that the same holds for cholera toxin.

From another perspective, focusing on Reissner's membrane (RM), it was found that perilymphatic application of cholera toxin in combination with IBMX, a specific phosphodiesterase inhibitor, caused a dramatic increase (20x-80x) of cAMP in RM (Thalmann et al., 1982). The physiological consequences of this increase are unknown, but activation of the AC system by forskolin increased the Cl^- activity in SM from 105 to 125 mEq (Kitano et al., 1995). One might hypothesize that the increase in Cl^- concentration is responsible for EP enhancement. However, even if Cl^- was the sole ion responsible for the potential in scala media, which it is certainly not, the increase in Cl^- concentration would only lead to an EP increase of around 5 mV, because the negative Cl^- equilibrium potential would decrease, which would make the EP (which is the sum of the equilibrium potential and an electrogenic

potential) larger. Instead of the increase of 5 mV which this mechanism would maximally provide, we measured 17 mV (Fig. 4).
As an alternative hypothesis for the EP increase, it was suggested that activation of cAMP-regulated Cl^- channels on the endolymphatic side of RM would lead to an influx of Cl^--ions into the endolymph which would subsequently lead to an increase of the osmolarity of scala media followed by EH (Doi et al., 1992a). In this case the increase of the EP would be secondary to a distention of the basilar membrane towards scala tympani, which would decrease the number of open transduction channels in the apical membrane of the OHC. However, our group has previously estimated that closing all transduction channels in the OHCs, which would enlarge the shunt resistance of scala media, would increase the EP by only a few mV (van Emst, 1998), not enough to explain the increase of 17 mV we have found here.
In summary, the Cl^- permeability hypothesis does not seem to be able to explain the full 17 mV EP increase we found. However, the fact remains that the stria is not the only site where cholera toxin exerts its effects (Thalmann et al., 1982). Thus, based on our experiments, it is difficult to favor one of the two sites, stria or RM, in trying to explain the origin of the EP enlargement. After a short perfusion with cholera toxin (15 min), we observed a monotonical and virtually coinciding increase of both the EP and SP, which makes it impossible to make a choice.

In the present study a model of EH was described based on increased production of endolymph. In contrast to the classical model of EH, based on decreased absorption of endolymph by obstruction of the endolymphatic sac, EH can apparently be based on mechanisms located within the cochlea itself. It remains unclear wether this EH is a result of increased strial activity or the result of a change in the ion-permeability of the cellular boundary of scala media. However, this model of acute EH provides us with a tool to further explore both the etiology of acute EH and the physiological alterations that are caused by it. Since AC-activation by drugs can produce EH, other drugs may in turn prevent this. Using the SP as indicator of endolymphatic pressure, such drugs may be tried out by simultaneous perfusion with cholera toxin.

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CHAPTER VII

The summating potential in the guinea pig cochlea after perilymphatic perfusion with arginine-vasopressin

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Submitted

Abstract

We hypothesized that arginine-vasopressine (AVP), the natural vasopressin in the guinea pig, might increase endolymphatic volume by activation of the cochlear adenylate cyclase (AC) system. Guinea pig cochleas were perfused with artificial perilymph (15 min), with or without arginine-vasopressin ($2x10^{-6}$ M). In one group of animals summating potentials (SP), compound action potentials (CAP) and cochlear microphonics (CM), evoked by 2, 4 and 8 kHz tone bursts, were measured with an apically placed electrode at 15 minutes, 1 and 2 hours after perilymphatic perfusion. In another group of animals the SP and the endocochlear potential (EP) were measured simultaneously in scala media during and after perfusion. In both groups the SP had increased significantly at 15 minutes after perfusion with AVP and this increase appeared reversible. With the concentration of AVP used ($2x\ 10^{-6}$ M) the SP increase was not related to EP alterations. In histological preparations of the cochlea no evident increase of scala media volume could be detected.
The increase of the SP (a sensitive indicator of acute endolymphatic hydrops) after perfusion with AVP, suggests that this neuropeptide plays a role in the regulation of the ion and fluid balance in the cochlea.

INTRODUCTION

Adenylate cyclase (AC) is a membrane-bound enzyme that is part of a second-messenger system which mediates the effect of hormones and neurotransmitters on a wide variety of cellular processes by increasing the intracellular cyclic adenosine monophosphate (cAMP) concentration after being activated by extracellular signals (Schacht, 1982). Several biochemical and histochemical studies detected the presence of AC in the cochlea, notably in the stria vascularis and Reissner's membrane (Zajic et al., 1983; Schacht, 1985). Consequently, it was hypothesized that this enzyme can play a regulatory role in the cochlear ion and fluid homeostasis. This hypothesis was supported by a study of Feldman and Brusilow (1976) in the guinea pig. The endolymphatic injection of cholera-toxin, a potent stimulator of the AC-system, gradually decreased the concentration of a simultaneously injected, presumably non-permeant isotope (inuline). Recently, this issue was further explored in our department. Perilymphatic perfusion of cholera toxin resulted in an increase of the summating potential (SP), a sensitive indicator of endolymphatic hydrops (van Deelen et al., 1987; van Benthem et al., 1993; Bouman et al., 1998), concomitant with an increase of the endocochlear potential (Lohuis et al., submitted). Both these studies with cholera toxin suggest an important role for AC in the development of endolymphatic hydrops (EH).

Hence, the logical next step would be to determine the role of a more specific, endogenous AC stimulant in the regulation of cochlear ion and fluid balance. Vasopressin, also known as antidiuretic hormone (ADH), might perform such a regulatory role. It stimulates water permeability in the principal cells of the mammalian kidney by the activation of AC (for a review, see Knepper, 1997). With regard to the inner ear, an *in vitro* study by Zenner and Zenner (1979) showed that vasopressin increased AC-activity in enzyme samples collected from the whole cochlea. In addition, a clinical study revealed that plasma ADH levels were significantly higher in patients suffering from Menière's disease as compared to controls and that this increase was closely linked to vertigo and/or dizziness attacks (Takeda et al., 1995). Menière's disease is probably related to disturbed fluid and ion homeostasis in the auditory-vestibular system. The recent discovery of receptor mRNA's for vasopressin in the rat cochlea might form a pharmacological base for these findings (Kitano et al., 1997).

Assuming that vasopressin is capable of activating the cochlear AC-system, cochlear perfusion of this neuropeptide may result in similar effects as found after perfusion with cholera toxin. This was shown in an investigation by Mori et al. (1986; 1989).

Perilymphatic perfusion with vasopressin at a concentration of $6x10^{-5}$M produced a decrease in the compound action potential (CAP) and an increase in the negative summating potential (SP). However, using arginine-vasopressin (AVP) in this concentration, the EP showed a reversible decrease, which is in contrast to our perfusion experiments with cholera toxin. In another experiment, Mori et al. (1989) showed that this effects of AVP were mediated by V_2 receptors. Activation of V_2 receptors results in AC-activation and is known to have an antidiuretic effect in the kidney (Jard, 1983).

In the present study, the effect of perilymphatic perfusion of AVP, the natural vasopressin in the guinea pig, is further explored focusing on the SP. After perfusion with AVP, in one group of guinea pigs SP growth curves were measured extracochlearly. In another group the SP was measured in scala media in relation to the EP. In these experiments AVP was used at a concentration of $2x10^{-6}$M. Using this concentration, the EP remains unaffected by AVP (Mori et al., 1986; 1989). This is important, because changes in the EP are known to influence the SP amplitude (Tilanus et al., 1992; van Emst et al., 1998). Finally, at the end of the perfusion experiment, the cochleas were harvested for histological analysis.

MATERIALS AND METHODS

Experimental design

Experiments were performed on 15 female albino guinea pigs (250-400 g), Dunkin Hartley strain. These animals were divided between two separate experiments.

The first experiment (A) involved 9 animals. Within this group the cochlea of each animal was either perfused twice with artificial perilymph (n=5) or once with artificial perilymph followed by a perfusion with AVP (n=4). SP, CAP and CM amplitude growth curves were measured with an electrode at the apex of the cochlea 15 minutes after the first perfusion and 15 minutes, 2 and 3 hours after the second perfusion.

The second experiment (B) involved a group of 6 animals. The right cochlea of each animal was perfused with artificial perilymph followed by a perfusion with AVP 30 min later. The EP and the SP were measured repeatedly in the second turn of scala media during and after the perfusions. In this group of animals the cochleas were fixed for histiological analysis at approximately 20 minutes after the second perfusion.

Surgery

The animals were anaesthetised with preoperative intramuscular injections of Thalamonal (a mixture of 2.5 mg/ml of droperidol and 0.05 mg/ml of fentanyl; 0.1 ml/100 g body weight), followed by artificial ventilation through a cannula in the exposed trachea with a gas mixture containing 33 % O_2, 66 % N_2O and 1 % Halothane. Rectal temperature was kept at around 38 °C by means of a thermostatically controlled heating pad. Heart rate was monitored continuously. The cochlea was exposed through a ventrolateral approach. To enable perfusion of the cochlea two 0.2-mm holes were drilled. One hole opened into the scala tympani, the other one into the scala vestibuli of the basal turn. For measuring the endocochlear potential (EP) an extra 0.1-mm hole was drilled in the bony wall overlying the scala media of the second turn. The care and use of the animals reported on in this paper were approved by the Animal Care and Use Committee of the Faculty of Medicine, Utrecht University, under number FDC-89007 and GDL-20008.

General stimulus generation

Stimulus generation and data acquisition were controlled by a pc using a Cambridge Electronic Design 1401-plus laboratory interface. Tone bursts (probes) were calculated and stored in a revolving memory consisting of 2,500 points with 12-bit resolution. Trains of probes of 2, 4 and 8 kHz were used. The probes lasted 8 ms and had cosine-shaped rise-and-fall times of 1.5 ms at 2 kHz and 1 ms at 4 and 8 kHz. Consecutive tone bursts were presented with alternating polarity at 99-ms intervals from onset to onset. The stimuli were led through a computer controlled attenuator to a Beyer DT48 dynamic transducer, which was connected to a hollow ear bar that was fitted into the exposed outer ear canal.

Electrophysiology of experiment A (measurements at the apex of the cochlea)

Sound-evoked potentials were measured differentially with a silver-ball electrode placed at the apex of the cochlea. A surgical clamp connected to the neck musculature served as a reference electrode. Signals were led to a computer-controlled amplifier and were band-pass filtered between 1 Hz and 10 kHz before AD conversion and averaging (max. 250 times). The averaged responses to the tone bursts of opposite polarity were stored separately for off-line analysis. The SP and the CAP were obtained by addition of the responses to the opposite polarity tone bursts, the CM by subtraction. The SP was measured as the difference between

the pre-stimulus DC level and the DC level approximately 6 ms after the onset of the 8 ms probe. The CAP was measured relative to the SP, i.e. the distance between the first negative peak (N_1) and the steady-state level of the SP was taken as CAP amplitude. This method was used, because, in principle, the CAP is superimposed on the SP. Moreover, our method of measurement of the CAP has the advantage over the more common N_1-P_1 method that it is less sensitive to changes in the waveform of the CAP. CM was determined as the peak-to-peak value in the middle of the AC response.

Electrophysiology of experiment B (measurements in scala media)

EP and SP were measured repeatedly in the second turn of scala media with a glass micropipette filled with 0.15 M KCL (impedance 10-20 MΩ), which was connected to a World Precision Instruments 705 preamplifier through an Ag-AgCl pellet. The EP was measured relative to a chlorided silver pellet in the neck musculature. AD-converted samples of the EP were taken once every minute. To measure the SP in scala media the method as described above was used. 4 kHz tone bursts presented at 80 dB SPL were used to record the SP.

Cochlear perfusion

For perfusions of the cochlea a blunt glass micropipette was positioned in the hole drilled in scala tympani. The perfusate was introduced at a rate of 2.5 µl/min for 15 min. The fluid flowing out of the hole drilled in scala vestibuli was removed from the bulla by suction with a paper wick. Artificial perilymph contained (mM): 137 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 11 glucose, $1NaH_2PO_4$ and 12 $NaHCO_3$; pH 7.2-7.4. Arginine-vasopressine (Sigma, St Louis) was dissolved in artificial perilymph at a concentration of $2x\ 10^{-6}$ M.

Histology of scala media

Cochleas were collected from the animals which participated in experiment B, approximately 20 minutes after the second perfusion. The cochleas were fixed by perilymphatic perfusion with tri-aldehyde fixative (3% glutaraldehyde, 2% formaldehyde, 1% acrolein and 2.5% dimethylsulfoxide in 0.08 M sodium cacodylate buffer; pH 7.4). After fixation the right temporal bone was removed and decalcified in 10% EDTA.2Na (pH 7.4) for 4-5 days, postfixed in 1% $Os0_4$, dehydrated in a graded ethanol series and embedded in toto in Spurr's low-viscosity resin. After dividing the cochleas along a midmodiolar plane, they were ready for semi-thin (1mm) sectioning and light microscopic evaluation.

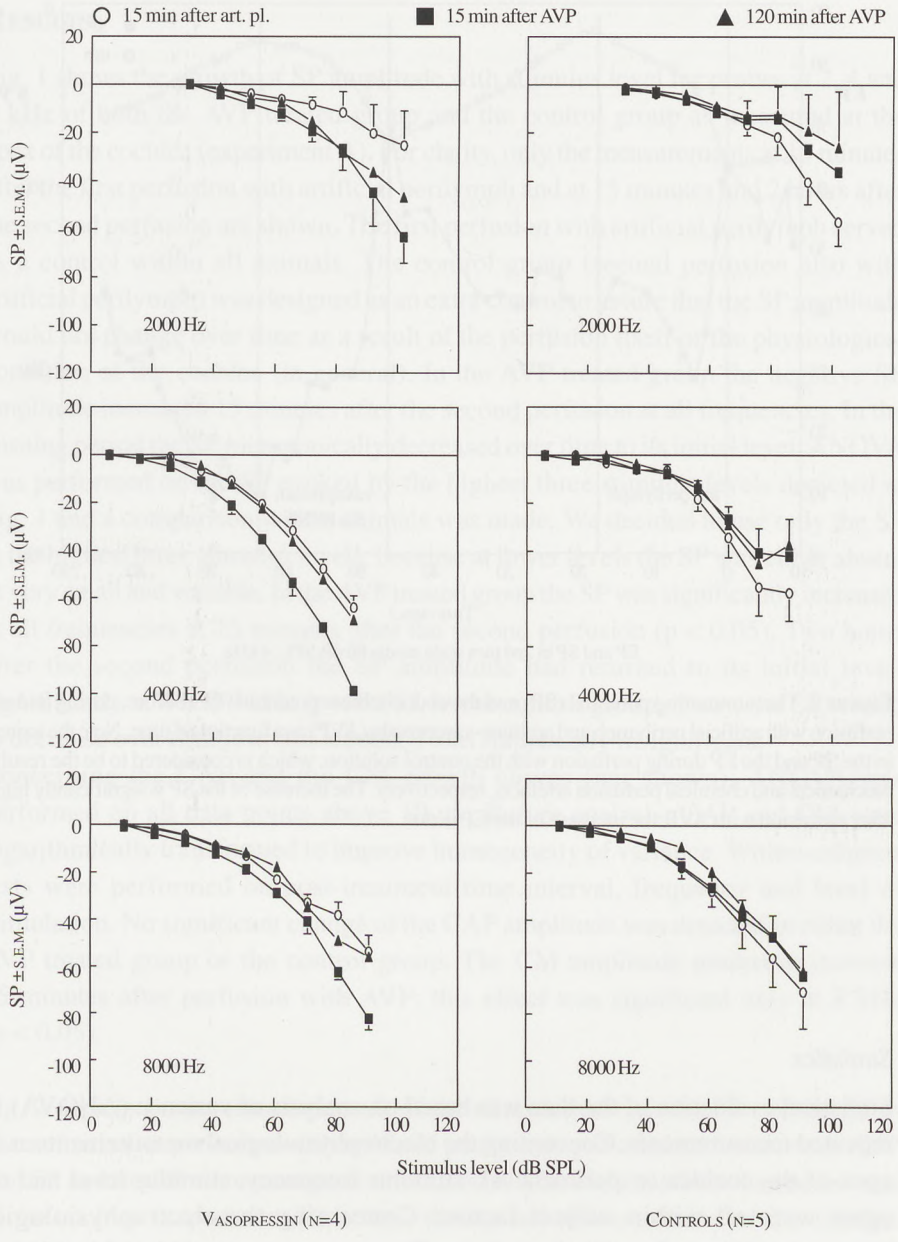
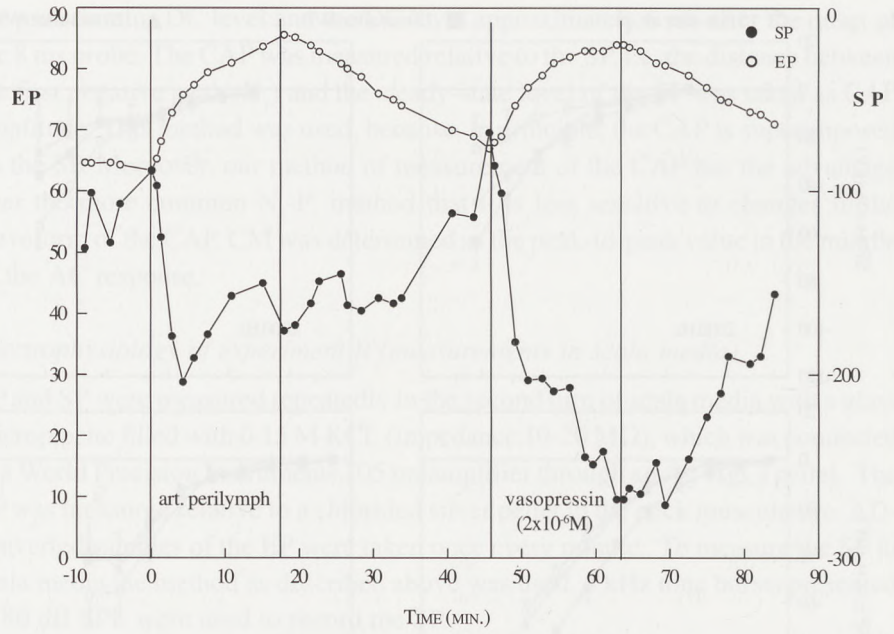


FIGURE 1. Summating potential (SP) growth curves after perfusion with artificial perilymph with arginine-vasopressin (left) or without arginine-vasopressin (right). Measurements were taken directly after perfusions and 120 minutes after the second perfusion. Panels arranged by stimulus frequency.



EP and SP in 2nd turn scale media 80 dB SPL; 4 kHz

FIGURE 2. The summating potential (SP) and the endocochlear potential (EP) before, during and after perfusion with artificial perilymph and arginine-vasopressin (AVP) as a function of time. Note the increase in the SP and the EP during perfusion with the control solution, which is considered to be the result of mechanical and chemical perfusion artefacts, respectively. The increase of the SP is significantly higher after perfusion with AVP, the increase of the EP is not.

Statistics

Statistical evaluation of the data was based on analysis of variance (ANOVA) for repeated measurements. Concerning the electrophysiological measurements at the apex of the cochlea (experiment A), stimulus frequency, stimulus level and test agent were all within subject factors. Concerning the electrophysiological measurements in scala media (experiment B), test agent was a within subject factors. The EP and the SP values at 15 minutes after perfusion with perilymph were compared with those at 15 minutes after perfusion with vasopressin. Statistica® software was used in all analyses.

RESULTS

Fig. 1 shows the growth of SP amplitude with stimulus level for probes at 2, 4 and 8 kHz of both the AVP treated group and the control group as measured at the apex of the cochlea (experiment A). For clarity, only the measurements at 15 minutes after the first perfusion with artificial perilymph and at 15 minutes and 2 hours after the second perfusion are shown. The first perfusion with artificial perilymph served as a control within all animals. The control group (second perfusion also with artificial perilymph) was designed as an extra control to assure that the SP amplitude would not change over time as a result of the perfusion itself or the physiological condition of the cochlea (in general). In the AVP treated group the negative SP amplitude increased 15 minutes after the second perfusion at all frequencies. In the ensuing period the SP monotonically decreased over time to its initial level. ANOVA was performed on the SP evoked by the highest three stimulus levels depicted in Fig. 1 and a comparison within animals was made. We decided to use only the SP at the highest three stimulus levels, because at lower levels the SP was either absent or very small and variable. In the AVP treated group the SP was significantly increased at all frequencies at 15 minutes after the second perfusion ($p < 0.05$). Two hours after the second perfusion the SP amplitude had returned to its initial level, significantly at 4 and 8 kHz ($p < 0.05$). In the control group, if any, the SP tended to decrease over time, but this tendency was statistically insignificant.
Concerning the CAP and the CM growth curves (not shown), ANOVA was performed on all data points above 10 μV. Before analysis, CAP and CM were logarithmically transformed to improve homogeneity of variance. Within-subjects tests were performed on post-treatment time interval, frequency and level of stimulation. No significant change of the CAP amplitude was detected in either the AVP treated group or the control group. The CM amplitude tended to increase 15 minutes after perfusion with AVP; this effect was significant only at 8 kHz ($p < 0.05$).

Fig. 2 depicts a representative result of the relation between the SP and the EP in one animal after perfusion with artificial perilymph and after perfusion with AVP. The SP and the EP were measured simultaneously in scala media in the second turn of the cochlea (experiment B) before, during and after perfusion of the cochlea. ANOVA within-subject tests were performed on the SP and the EP values at 10 minutes after the end of each perfusion. There was a significant increase of the SP 10 minutes after perfusion with AVP (2×10^{-6} M) compared to 10 minutes after perfusion without AVP. There was no difference between the EP's at these moments.

The figure clearly illustrates the effect of the perfusion itself on the SP and the EP amplitude. Perfusion with a control solution resulted in a rapid increase of the SP (4 kHz, 80 dB SPL) and the EP amplitude. In general, these values returned to their initial levels within 15 to 30 minutes after the end of the perfusion. The implications of this phenomenon will be discussed below.
The cochleas were fixed for histological examination at the end of experiment B, 20 minutes after the perfusion with AVP had stopped. Although at this time of the experiment the SP amplitude had increased significantly, no evident increase of scala media volume could be detected.

DISCUSSION

In this study study we found that stimulation of the cochlear AC activity by perilymphatic perfusion with AVP resulted in an increase of the negative SP amplitude. This result corresponds with the result that we obtained after perfusion with another AC stimulant, i.e. cholera toxin (Lohuis et al., submitted). After perilymphatic perfusion with cholera toxin both the SP and the EP increased monotonically over time. Using a morphometrical analysis technique we also found a small, although not significant increase of the length of Reissner's membrane. It was concluded that stimulation of the cochlear AC-system results in an increase of endolymphatic volume based on an increased production of endolymph. The increase in SP amplitude was thought to be the result of a small endolymphatic hydrops (EH), which caused a change in the setpoint of the nonlinear apical hair cell transducer by displacement of the basilar membrane towards scala tympani (Klis et al., 1985; Durrant and Dallos, 1974).
In the present experiments we found the SP to be significantly increased almost directly after perfusion with AVP. Over time the SP returned to its initial level. In experiment A (extracochlear SP measurements) as well as in experiment B (SP measurements in scala media in relation to the EP) the only experimental manipulation consisted of the presence of AVP in the perfusion fluid. Therefore, we may conclude that AVP is capable of enlarging the SP amplitude, indicative of increased endolymphatic volume. Experiment B shows how important it is to include control experiments, because the SP measurements can be masked by unwanted effects of the perfusion itself. Fig. 2 illustrates that perilymphatic perfusion with a *control* solution can also cause an increase of the SP and the EP amplitude. Recently, Ma et al. (1998) and DeMott and Salt (1998) found the increase of the SP and the EP amplitude to correlate with the speed of perilymphatic perfusion and the K^+ concentration in artificial perilymph, respectively. The mechanical effect of the

perfusion results in a pressure-induced displacement of the basilar membrane, followed by a change in the setpoint of the cochlear transducer and an increase of the SP. The EP enlargement, which is equally present during both perfusions (Fig. 2) and therefore not considered to be an effect of AVP, might be related to the composition of the artificial perilymph that we used in our experiments. So far, we used artificial perilymph containing 5 mM KCl expecting to stabilize the CAP. However, the introduction of this relatively high concentration of K^+ in the perilymphatic space probably increased the EP as a result of a low equilibrium potential of K^+ between scala media and scala tympani. In turn, the increased EP might then increase the SP as a result of depolarization of the OHC (Frank and Kössl, 1997).
In our experiment the SP enlargement after perilymphatic perfusion with AVP is the result of a combination of factors: the mechanic effect of the perfusion, the increased EP (indirectly the result of the increased K^+ concentration) and the effect of AVP itself. After the end of the perfusion with the control solution the EP and the SP returned to their former levels within 20 minutes. Thus, when measuring the SP after a short-term cochlear perfusion, it is important to determine the effect of the perfusate after a period of recovery in order to minimize the mechanical effect of the perfusion. In addition, our results suggest that a lower perilymphatic K^+ concentration should be used to eliminate the indirect effect on the SP due to an EP enlargement. However, control perfusions should always be included.

Considering the possibility that the increase of the SP amplitude is the result of an increase of the endolymphatic volume and pressure, the question arises by which pathway AVP is inducing this increase in amplitude. From *in vitro* studies in the kidney it has been concluded that, after binding to a V2 receptor, the AVP-induced rise in intracellular cAMP triggers a redistribution of water channels to the apical plasma membrane by exocytosis of the aquaporin-2 containing intracellular vesicles (Knepper, 1997). The addition of water channels to the apical membrane increases the water permeability of the cell. When similar water channels would exist in the cochlea, e.g. in Reissner's membrane, AVP could induce a flow of H_2O between cochlear compartments. However, although recently a water channel protein (AQP-CHIP) has been found in some cochlear structures of the guinea pig, others (among which aquaporin 2) still remain to be detected (Stankovic et al., 1995).
Since AC is also found in high concentrations in strial tissue (Schacht, 1985) another origin of the increase in endolymphatic volume could be the AC stimulated influx of K^+ (Sunose et al., 1997), which would increase the osmolarity of scala media.

However, in this case one would also expect an increase of the EP like in our experiments with cholera toxin (Lohuis et al., 1999). In contrast, using higher concentrations of AVP (> 10^{-5} M) than we used in our experiment, Mori et al. (1989) found the EP to decrease after perfusion with AVP. In addition, in an *in vitro* study Schacht (1985) did not find the AC activity to increase in isolated stria vascularis tissue after stimulation with AVP. Hence, the possibility exists that, similar as to what happens in the kidney, AVP acts on the H_2O permeability of RM. Further investigation is needed to confirm the presence and the localization of aquaporin water channels and V2 receptors by immunohistochemistry or in situ hybridization to support this hypothesis.
The present study shows that perilymphatic perfusion of AVP leads to an increase of SP amplitude as a sign of increased scala media volume and that this effect is reversible. This finding contributes to the hypothesis that vasopressin might play a role in the regulation of the cochlear ion and fluid balance. It remains to be elucidated whether or not disorders of such a mechanism underlay EH such as found in patients suffering from Menière's disease.

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— CHAPTER VIII —

Summary and conclusions

The maintenance of cochlear ion and fluid homeostasis is basic to the process of mechano-electrical transduction. Especially the homeostasis of endolymph volume is important. There are indications that the ion and fluid homeostasis in the cochlea, to some extent, is regulated by hormones. This thesis presents a series of *in vivo* experiments, in which we tried to affect the cochlear ion and fluid homeostasis by interfering with the action of signaling molecules that are assumed to be capable of controlling mechanisms responsible for the maintenance of endolymph homeostasis. In these experiments we used histological and electrophysiological techniques in rats and guinea pigs.

Chapter 1, the introduction, describes the process of hearing and the structures that are involved. The relation between cochlear ion and fluid homeostasis and the process of mechano-electrical transduction is explained.
The components needed essentially required in controlling endolymph volume are discussed by using a reference frame work, which has been designed as a feedback system. This feedback system consists of three building blocks: (1) a monitor that keeps track of the volume of scala media, (2) a comparator that compares the actual endolymph volume with the desired one and (3) three effector organs (the stria vascularis, the endolymphatic sac and Reissner's membrane) that are involved in endolymph production and endolymph absorption. In order for this feedback system to be effective a continuous flow of information must be present between the building blocks at all times. This flow of information is thought to be of a chemical nature, probably hormones. Hormonal regulation of endolymph volume might form the link between the discovery that several hormone receptors are present in the cochlea and the physiology and pathophysiology of inner ear fluids. Identification of these putative inner ear hormones might therefore be the key in understanding and treating several inner ear disorders, including Menière's disease. In search of the nature of the signaling molecules in the cochlea that are involved in the maintenance of ion and fluid homeostasis of endolymph, we focused on four substances: aldosterone, dexamethasone, cholera toxin and arginine-vasopressin. Their working mechanisms are discussed. Hypothetically, adding or removing each of these compounds in the cochlea can interfere with endolymph homeostasis by their action on potential effectors such as the stria vascularis or Reissner's membrane.

Chapter 2 focuses on the morphological analysis of structures in the stria vascularis. Composition photographs of the stria vascularis were formed using Transmission Electron Microscopy (TEM). Stria vascularis tissues from standardized regions in

the basal, middle and apical turns of the rat cochlear duct were assessed quantitatively by stereological methods. Strial width, number of marginal cells across the strial width, radial area, as well as the volume density of the different components of the stria vascularis were determined for each standardized region. Strial width, number of marginal cells across the strial width and the radial area were greatest in the basal region and smallest in the apical region of the cochlea. The volume densities of the intermediate cells and of the capillary space in the three examined regions of the stria vascularis were statistically identical. However, the volume density of the marginal cells and that of the basal cells differed between regions. The volume density of the marginal cells was highest in the basal turn while the volume density of the basal cells was highest in the apical turn.
It was concluded that the technique, as described in chapter 2, can serve as a basis for the quantitative determination of the effect of different agents on the metabolically active stria vascularis.

In **Chapter 3** the stereological method, described in chapter 2, was used to measure structural changes in the cellular morphology of the rat stria vascularis from a standardized region in the basal region and from a standardized region in the apical region of the rat cochlear duct one week after removal of endogenous adrenal hormones by bilateral adrenalectomy. A decreased volume density of the marginal cells in both the basal and the apical region was found in adrenalectomized animals as compared to sham-operated animals. This decrease in the volume density of the marginal cells was consistently correlated with an increase of the intercellular space in the basal and apical stria vascularis of adrenalectomized animals.
This study shows that the architecture of the stria vascularis is altered in the chronic absence of circulating adrenal hormones. Because volume is a sensitive indicator of osmo-equilibrium within a cell, changes in cellular volume within the stria vascularis may have been due to a desequilibrium in normal ion transport. These data provide a basis for the hypothesis that adrenal hormones play a role in the regulation of the ion and fluid homeostasis in the cochlea.

Chapter 4 presents a series of experiments, which were based on the hypothesis that the structural integrity of the cells of the stria vascularis, which was demonstrated to be altered in the chronic absence of adrenal hormones in chapter 3, would recover to within normal limits by the administration of the mineralocorticoid, aldosterone, and/or the synthetic glucocorticoid, dexamethasone. On day eight post-adrenalectomy, a mini-osmotic pump was implanted subcutaneously that administered the latter hormones at a constant rate. The volume densities of marginal

cells and intercellular space in the basal stria vascularis in adrenalectomized animals were shown to approximate normal values after administration of aldosterone. Administration of dexamethasone at a comparable concentration with that of aldosterone was found to have a similar effect as that of aldosterone on the volume density of the marginal cells. However, the volume density of the intercellular space did not return to normal values after administration of dexamethasone.
Out of the diversity of adrenal hormones, aldosterone or a derivative seems the hormone most likely to be responsible for the disturbed cochlear ion transport. This hypothesis is in line with the discovery of receptors for mineralocorticoids in several inner ear tissues.

In **Chapter 5**, the consequences of bilateral adrenalectomy in Long Evans rats were further investigated with respect to endolymph homeostasis. In chapter 3 we described that the volume of the marginal cells of the stria vascularis decreased after bilateral adrenalectomy as a sign of disturbed equilibrium in normal ion transport. In other investigations, removal of endogenous levels of adrenal hormones by bilateral adrenalectomy has been shown to cause a significant decrease of Na^+/K^+-ATPase activity in the cochlear lateral wall (Rarey et al., 1989). Na^+/K^+-ATPase is found in high concentrations along the basolateral membrane of the marginal cells. The presence of Na^+/K^+-ATPase is essential for the capacity of the stria to actively maintain the endocochlear potential (EP) and the K^+ gradient. Since the strial Na^+/K^+-ATPase is thought to be involved in endolymph production and the marginal cells are immediately adjacent to the endolymph, we may also expect disturbed endolymph homeostasis after adrenalectomy and, subsequently, functional alterations in hearing.
To study the effect of the absence of circulating adrenocorticosteroids on cochlear function in rats, electrocochleography was performed one week after adrenalectomy. At the end of the measurements, the cochleas were harvested and examined morphologically. No significant changes in the amplitude growth curves of the summating potential (SP), the compound action potential (CAP) and the cochlear microphonics (CM) were detected after adrenalectomy compared to sham-operated animals. However, there appeared to be a decrease of endolymphatic volume. Reissner's membrane extended less into scala vestibuli. The ratio between the length of Reissner's membrane, l_R, and the straight distance between the medial and lateral attachment points of Reissner's membrane, d, was used as an objective measure to quantify this decrease of endolymphatic volume in the first four sub-apical half turns as observed in a midmodiolar transection of the cochlea. The decrease was statistically significant.

Thus, both the studies described in chapter 3 and 4 as well as this study indicate that circulating adrenal hormones are necessary for normal cochlear fluid homeostasis. Absence of one or more of these hormones leads to shrinkage of the scala media (imdrops). However, the absence of adrenal hormones does not affect the gross cochlear potentials. Apparently, the cochlea is capable of compensating for the absence of circulating adrenal hormones to sustain the conditions necessary for proper cochlear transduction.

We already mentioned that, out of the diversity of adrenal hormones, aldosterone seems the hormone most likely to be involved with the regulation of the ion and fluid homeostasis in the cochlea. It was hypothesized that aldosterone could influence the Na^+/K^+-ATPase activity in the stria vascularis by inducing the synthesis of new active pump-units at the nuclear level. However, more subcellular pathways seem to play a role in endolymph homeostasis. Adenylate cyclase is another membrane-bound enzyme that is abundantly present in the stria vascularis. Activation of adenylate cyclase increases intracellular levels of the second messenger cAMP, and finally alters cell function. Cholera toxin is a potent stimulator of adenylate cyclase.

In **Chapter 6**, a perfusion technique was used in guinea pigs to replace the natural perilymph by artificial perilymph supplemented with cholera toxin. We hypothesized that perilymphatic perfusion of cholera toxin might increase endolymph volume by stimulating adenylate cyclase activity in cells surrounding the scala media, possibly providing us with a pharmacological model of *acute* endolymphatic hydrops (EH). Guinea pig cochleas were perfused with artificial perilymph (15 min.), with or without cholera toxin (10 µg/ml). The endocochlear potential (EP) was measured during and after perfusion. SP, CAP and CM amplitude growth curves, evoked by 2, 4 and 8 kHz tone bursts, were measured via an apically placed electrode 0, 1, 2, 3 and 4 hours after perfusion. At the end of the experiment, the cochleas were fixed to enable quantification of changes in endolymphatic volume, using the l_R / d method. After perfusion the EP increased significantly over time in the cholera toxin group, as compared to the controls, as a sign of increased endolymph production after perfusion with cholera toxin. The SP increased gradually at all frequencies in the cholera toxin group and increased predominantly at the lower frequencies. Although the SP has been shown to be a sensitive indicator of EH, quantification of light microscopical sections only showed a small, statistically nonsignificant, enlargement of the volume of scala media in the cholera toxin treated animals, as compared to controls. It was concluded that in the *acute* phase of EH the degree of distention

of the basilar membrane, and thus the change of the setpoint of the cochlear transducer, is probably correlated more strongly to the stiffness of Reissner's membrane than to the increase of endolymphatic volume *per se*. Only when Reissner's membrane has sufficient stiffness, it will produce a counterforce of a magnitude that can displace the basilar membrane towards scala tympani. This suggests that an increase of the SP would typically occur in the first stage of endolymphatic hydrops.
In the classical EH model, based on obstruction of the absorptive function of the endolymphatic sac, increased SP's are accompanied by decreased EP's. In this cholera toxin model of *acute* EH the EP increased. Therefore, it is more likely that the stria vascularis is involved than the endolymphatic sac. Apparently, EH can be based on mechanisms located in the cochlea itself (increased endolymph production) as opposed to mechanisms located in the endolymphatic sac (decreased endolymph absorption).

Cholera toxin evoked large effects on endolymph homeostasis, which is an indication of the significance of the adenylate cyclase/cAMP pathway. Thus, the question arose, which agonist would be the *natural* ligand for this signaling route. An obvious candidate was the antidiuretic hormone vasopressine.

In **Chapter 7**, we use the perilymphatic perfusion technique to introduce arginine-vasopressin (AVP) into the guinea pig cochlea. AVP, which is the natural vasopressin in the guinea pig, has been shown to increase adenylate cyclase activity in the cochlea in *in vitro* experiments. It was hypothesized that the endogenous AVP might increase endolymphatic volume by activation of adenylate cyclase in a similar fashion to our experiments with the toxic cholera toxin.
In one group of animals the SP was measured with an electrode at the apex of the cochlea at 15 minutes, 1 and 2 hours after perilymphatic perfusion with AVP. In another group of animals the SP and the EP were measured simultaneously in scala media during and after perfusion.
In both groups the SP had increased significantly at 15 minutes after perfusion with AVP (remarkably faster than cholera toxin) and this increase appeared reversible. The increase of the SP after perfusion with AVP, suggests that this neuropeptide plays a role in the regulation of the ion and fluid balance in the cochlea.

GENERAL CONCLUSION AND CLINICAL IMPLICATIONS

Mineralocorticoids, glucocorticoids and adenylate cyclase agonists all seem to have a clear, although different effect on the ion and fluid homeostasis in the cochlea.

The results from the *in vivo* experiments, presented in this thesis, strengthen our believe that in the cochlea the membrane incorporation of Na^+/K^+-ATPase, ion channels and water channels could, to some extent, be under hormonal control. Although it is emphasized that the regulation of the ion and fluid homeostasis is very complex, evidently because of the different pathways involved, the existence of these pathways creates possibilities for the pharmacological treatment of certain inner ear disorders, such as Menière's disease, which are associated with disturbances in cochlear ion and fluid homeostasis. The presented model of *acute* endolymphatic hydrops (EH), based on increased production of endolymph, can help to find such a treatment. Since adenylate cyclase activation by drugs can produce EH, other drugs may in turn prevent this. Using the SP as indicator of endolymphatic pressure, such drugs may be tried out by simultaneous perfusion with cholera-toxin or vasopressin.

Since aldosterone and vasopressin serve vital physiological effects in the kidney, it is not very likely that the inner ear epithelium is primarily regulated by these substances. To avoid effects on organs like the kidney, the inner ear probably works with its own, more specific ligands. The effects that we observed after perilymphatic perfusion with cholera toxin and vasopressin may be due to cross-talk on receptors that normally interact with these specific inner ear ligands. Fundamental research aimed at the identification of these specific inner ear ligands should continue, because dysregulation in the activity of such ligands may disturb the ion and fluid homeostasis in the cochlea and account for disorders such as found in Menière's disease.

Samenvatting en conclusies

Het handhaven van de ion- en vloeistofhomeostase in de cochlea is uitermate belangrijk voor het proces van mechano-elektrische transductie. Met name de homeostase van endolymfe-volume speelt hierbij een belangrijke rol. Er bestaan indicaties dat de ion- en vloeistofhomeostase in de cochlea, in ieder geval tot op zekere hoogte, gereguleerd wordt door hormonen. Echter, de details betreffende dit regulatiemechanisme zijn nog vrijwel onbekend. Dit proefschrift betreft een serie van experimenten, waarin werd geprobeerd om de ion- en vloeistofhomeostase in de cochlea te beïnvloeden door manipulatie met signaalmoleculen. Van deze signaalmoleculen werd verondersteld, dat ze een effect konden hebben op orgaanstructuren verantwoordelijk voor het handhaven van de endolymfe-homeostase in de cochlea. Histologische en elektrofysiologische technieken werden gebruikt bij *in vivo* experimenten in ratten en cavia's.

Hoofdstuk 1, de introductie, beschrijft de gehoorfunctie en de anatomische structuren die hierbij een rol spelen. De relatie tussen de ion- en vloeistofhomeostase in de cochlea en het proces van mechano-elektrische transductie wordt duidelijk gemaakt.
De minimaal vereiste componenten, die nodig zijn voor het reguleren van endolymfe-volume, worden besproken aan de hand van een referentiekader, dat is opgezet als een feedback-systeem (Fig. 1.2). Dit feedback-systeem bestaat uit drie bouwstenen: (1) een monitor, die het volume van scala media detecteert, (2) een comparator, die het gedetecteerde endolymfe-volume vergelijkt met het gewenste endolymfe-volume en (3) drie effectororganen (de stria vascularis, de saccus endolymphaticus en de membraan van Reissner), die bij de productie en absorptie van endolymfe betrokken te zijn. Een feedbacksysteem kan alleen effectief functioneren als er een continue informatiestroom tussen de bouwstenen rouleert. In de cochlea is deze informatie-stroom waarschijnlijk van een chemische aard, i.e., er zijn hormonen bij betrokken. Het karakteriseren van deze hormonen zou kunnen bijdragen tot het begrijpen en uiteindelijk behandelen van binnenoorafwijkingen, zoals de ziekte van Menière, aangezien de pathofysiologie van binnenoorvloeistoffen gerelateerd zou kunnen zijn aan een hormonale disregulatie.
In onze pogingen meer kennis te verzamelen over de signaalmoleculen die betrokken zouden kunnen zijn bij het handhaven van de ion- en vloeistofhomeostase in de cochlea, hebben we ons geconcentreerd op vier substanties: aldosteron, dexamethason, cholera-toxine en arginine-vasopressine. Het werkingsmechanisme van deze stoffen werd besproken. Hypothetisch zou in de cochlea het wegnemen of toevoegen van elk van deze stoffen de endolymfe-homeostase kunnen

beïnvloeden via een effect op effectoren zoals de stria vascularis of de membraan van Reissner.

Hoofdstuk 2 richt zich op de morfometrische analyse van de cellen in de stria vascularis. Compositiefoto's van de stria vascularis werden samengesteld met behulp van transmissie elektronen microscopie (TEM). Op gestandaardiseerde locaties in de basale, middelste en apicale winding van de scala media in de rat werden stukjes weefsel van de stria vascularis kwantitatief geanalyseerd met stereologische methoden. De breedte van de stria, het aantal "marginal cells" over de breedte van de stria, de oppervlakte van de loodrechte doorsnede door de stria en de "volume density" (V_v) van de verschillende componenten van de stria vascularis werden vastgelegd voor elk van deze gestandaardiseerde locaties. De V_v van de "intermediate cells" en de "capillary space" was statistisch identiek in de drie bestudeerde locaties van de stria vascularis. Echter, de V_v van de marginal cells en de "basal cells" was verschillend tussen de locaties. De V_v van de marginal cells was het hoogste in de basale winding, terwijl de V_v van de basal cells het hoogste was in de apicale winding. Er werd geconcludeerd, dat de techniek, zoals beschreven in dit hoofdstuk, als een basis kan dienen om het effect van verschillende stoffen op gestandaardiseerde locaties van de metabolisch actieve stria vascularis kwantitatief te bepalen.

In **hoofdstuk 3** werden de stereologische methoden, zoals beschreven in hoofdstuk 2, gebruikt om, een week na het verwijderen van circulerende bijnierhormonen via bilaterale bijnierextirpatie (ADX), de structurele veranderingen in de cellulaire morfologie van de stria vascularis vast te leggen in een gestandaardiseerde regio van de basale winding en in een gestandaardiseerde regio van de apicale winding van scala media. In zowel de basale als de apicale winding werd na ADX een verminderde V_v van de marginal cells gevonden in vergelijking met sham-geopereerde dieren. Deze vermindering in de V_v van de marginal cells was consistent gecorreleerd aan een toename van de intercellulaire ruimte in de basale en apicale stria vascularis in ADX-dieren. Deze studie toont aan dat de architectuur van de stria vascularis verandert bij chronische afwezigheid van circulerende bijnierhormonen. Omdat volume een gevoelige indicator is van het osmo-equilibrium in een cel, kunnen veranderingen in celvolume in de stria vascularis het gevolg zijn van een verstoord equilibrium in normaal iontransport. De data vormen een basis voor de hypothese dat bijnierhormonen een rol kunnen spelen in de regulatie van de ion- en vloeistofhomeostase in de cochlea.

Hoofdstuk 4 presenteert een serie experimenten, die gebaseerd zijn op de hypothese dat de structurele integriteit van de cellen in de stria vascularis verandert bij chronische afwezigheid van circulerende bijnierhormonen, zoals aangetoond in hoofdstuk 3, zou kunnen herstellen tot binnen normale afmetingen na toediening van het mineralocorticoïd, aldosteron, en/of het synthetische glucocorticoïd, dexamethason. Een week na het verwijderen van beide bijnieren werd een mini-osmotisch pompje subcutaan geimplanteerd, dat de laatst genoemde hormonen met een constante snelheid toediende. De V_v van de marginal cells en die van de "intercellular space" in de basale stria vascularis van ADX-dieren bleken normale waarden te benaderen na toedienen van aldosteron. Toedienen van dexamethason bleek een vergelijkbaar effect op de V_v van de marginal cells te hebben als aldosteron. De V_v van de intercellular space herstelde echter niet tot normale waarde na toedienen van dexamethason. Uit de verscheidenheid van bijnierhormonen leek aldosteron het hormoon te zijn, dat waarschijnlijk het meest verantwoordelijk kon worden geacht voor een verstoord iontransport in de cochlea na ADX. Deze hypothese werd ondersteund door de aanwezigheid van receptoren voor mineralocorticoïden in de verschillende binnenoorweefsels.

In **hoofdstuk 5** werden de gevolgen van ADX in Long Evans ratten verder onderzocht op het niveau van de endolymfe-homeostase. In hoofdstuk 3 beschreven we dat de V_v van de marginal cells na ADX verminderde ten teken van een verstoord equilibrium in normaal iontransport. In andere experimenten werd aangetoond dat het verwijderen van endogene bijnierhormonen via ADX een significante afname van de Na^+/K^+-ATPase activiteit in de stria vascularis veroorzaakte (Rarey et al., 1989). Na^+/K^+-ATPase wordt in hoge concentraties in de basolaterale membraan van de marginal cells gevonden. Deze configuratie is essentiëel voor het vermogen van de stria om de endocochleaire potentiaal (EP) en de K^+-gradiënt actief te handhaven. Omdat verondersteld wordt dat de Na^+/K^+-ATPase in de stria betrokken is bij de produktie van endolymfe en omdat de marginal cells direct aan de endolymfe grenzen, kan na ADX ook een verstoorde endolymfe-homeostase verwacht worden als gevolg van een afname van de Na^+/K^+-ATPase activiteit. Theoretisch zou dit moeten resulteren in een verminderde gehoorfunctie.
Om het effect van de afwezigheid van circulerende bijnierschorshormonen op de functie van de cochlea te bestuderen werd een week na ADX elektrocochleografie verricht. Aan het einde van de metingen werden de cochlea's bovendien gefixeerd en morfologisch onderzocht. In ADX-dieren werden geen significante veranderingen in de groeicurven van de sommatiepotentiaal (SP), de samengestelde actiepotentiaal

(CAP) en de cochleaire microfonie gedetecteerd in vergelijking met sham-geopereerde dieren. Echter, visueel leek het endolymfatisch volume in ADX-dieren verminderd te zijn. De membraan van Reissner bolde minder uit in scala-vestibuli. De ratio tussen de lengte van de membraan van Reissner, l_R, en de kortste afstand tussen de mediale en laterale aanhechtingspunten van de membraan van Reissner, *d*, werd als een objectieve maat gebruikt om deze afname van het endolymfatisch volume in de eerste vier sub-apicale halve windingen, zoals deze worden gezien in een midmodiolaire doorsnede van de cochlea, te kwantificeren. Met deze methode bleek de afname van het endolymfatisch volume statistisch significant te zijn.
Zowel de studies beschreven in hoofdstuk 3 en 4 als deze studie tonen aan, dat circulerende bijnierhormonen nodig zijn voor de normale vloeistofhomeostase in de cochlea. Afwezigheid van een of meer van deze hormonen leidt tot krimpen van scala media (imdrops). Echter, de chronische afwezigheid van bijnierhormonen heeft schijnbaar geen invloed op de cochleaire potentialen. Blijkbaar is de cochlea in staat om de afwezigheid van circulerende bijnierhormonen te compenseren en zodoende de condities, die nodig zijn voor normale cochleaire transductie, te handhaven.

We bespraken reeds dat, uit de verscheidenheid van bijnierhormonen, aldosteron waarschijnlijk het meest verantwoordelijk kan worden geacht voor een verstoord iontransport in de cochlea na ADX. We hypothetiseerden dat aldosteron de Na^+/K^+-ATPase activiteit in de stria vascularis zou kunnen beïnvloeden via inductie van de synthese van nieuwe actieve pomp-elementen op nucleair niveau. Andere subcellulaire mechanismen lijken ook een rol te kunnen spelen bij de homeostase van endolymfe. Adenylaat-cyclase is een membraangebonden enzym dat net als Na^+/K^+-ATPase in grote hoeveelheden in de stria vascularis aanwezig is en tevens in de membraan van Reissner wordt gevonden. Activatie van adenylaat-cyclase verhoogt de intracellulaire concentratie van het second messenger cAMP, wat uiteindelijk resulteert in een verandering van celfunctie. Cholera-toxine is een potente stimulator van adenylaat-cyclase.

In **hoofdstuk 6** werd in cavia's een perfusietechniek toegepast waarbij de natuurlijk aanwezige perilymfe vervangen werd door artificiële perilymfe, waaraan cholera-toxine was toegevoegd. We hypothetiseerden dat perilymfatische perfusie met cholera-toxine het endolymfe-volume kon vergroten door stimulatie van de adenylaat-cyclase-activiteit in cellen rondom scala media. Dit zou ons een farmacologisch model van *acute* endolymfatische hydrops (EH) verschaffen. Cochlea's van cavia's

werden geperfundeerd met artificiële perilymfe (15 min) met of zonder cholera-toxine (10 µg/ml). De endocochleaire pontentiaal (EP) werd tijdens en na perfusie continu gemeten. SP, CAP en CM groeicurven, opgewekt door 2, 4 en 8 kHz toonstootjes, werden via een elektrode op de apex gemeten op 0, 1, 2, 3, en 4 uur na de perfusie. Aan het einde van het experiment werden de cochlea's gefixeerd, zodat veranderingen in het endolymfatische volume gekwantificeerd konden worden met de l_R/d-methode.
Na de perfusie nam in de loop van de tijd de EP significant toe in de cholera-toxine groep in vergelijking met de controlegroep, ten teken van een verhoogde endolymfe produktie na perfusie met cholera-toxine. De SP nam in de cholera-toxine groep bij alle frequenties gradueel toe, maar was met name verhoogd bij de lage frequenties. Hoewel de SP een gevoelige indicator van EH is, bleek er, na l_R/d kwantificatie van lichtmicroscopische secties, slechts een kleine toename van het volume van scala media te bestaan in de cholera-toxine-groep in vergelijking met de controle-groep. Blijkbaar is in de *acute* fase van EH de mate van afbuiging van de basilaire membraan, en dus de verandering van het instelpunt van de cochleaire transducer, waarschijnlijk meer gecorreleerd aan de stijfheid van de membraan van Reissner dan aan toename van het endolymfatisch volume *per se*. Alleen wanneer de membraan van Reissner stijf genoeg is, zal deze een tegendruk kunnen produceren die voldoende groot is om de basilaire membraan in de richting van scala tympani te verplaatsen. Hiermee wordt gesuggereerd dat een toename van de SP typisch plaatsvindt in het eerste stadium van endolymfatische hydrops.
In het klassieke EH-model, gebaseerd op obstructie van de absorptieve functie van de saccus endolymphaticus, worden vergrote SP's begeleid door afgenomen EP's. In dit cholera-toxine model van *acute* EH is de EP vergroot en is het derhalve waarschijnlijker dat de stria vascularis een rol speelt in plaats van de saccus endolymphaticus. Blijkbaar kan EH veroorzaakt worden door mechanismen, die in de cochlea zelf gelokaliseerd zijn (verhoogde endolymfe-produktie) in tegenstelling tot mechanismen gelokaliseerd in de saccus endolymphaticus (verlaagde endolymfe-absorptie).

Cholera-toxine bleek grote effecten te hebben op de endolymfe-homeostase, hetgeen een indicatie geeft van de belangrijke rol van het adenylaat-cyclase/cAMP mechanisme in de cochlea. Vervolgens ontstond de vraag welke agonist dan de *natuurlijke* ligand van dit mechanisme zou kunnen zijn. Een voor de hand liggende kandidaat was het antidiuretisch hormoon vasopressine.

In **hoofdstuk 7** gebruiken we de perilymfatische perfusietechniek om arginine-vasopressine (AVP) in de cavia cochlea in te brengen. Van AVP, de natuurlijke vasopressine in de cavia, is aangetoond dat het de adenylaat-cyclase activiteit in de cochlea in *in vitro* experimenten vergroot. We veronderstelden dat AVP het endolymfatische volume zou kunnen vergroten door activatie van het adenylaat-cyclase, op een vergelijkbare manier zoals tijdens onze experimenten met cholera-toxine. In een groep dieren werd de SP gemeten met een elektrode op de apex van de cochlea op 15, 60 en 120 minuten na perilymfatische perfusie met AVP. In een andere groep dieren werd gedurende en na de perfusie de SP en de EP tegelijkertijd en continu gemeten in scala media. In beide groepen nam de SP significant toe op 15 minuten na perfusie met AVP (aanmerkelijk sneller dan met cholera-toxine). Deze toename bleek reversibel. De toename van de SP na perfusie met AVP suggereert dat dit neuropeptide een rol speelt bij de regulatie van de ion- en vochtbalans in de cochlea.

Algemene conclusie en klinische relevantie

Mineralocorticoïden, glucocorticoïden en adenylaat-cyclase agonisten lijken allemaal een duidelijk, hoewel verschillend effect te hebben op de ion- en vocht homeostase in de cochlea. De resultaten van de *in vivo* experimenten, gepresenteerd in dit proefschrift, versterken onze hypothese dat de membraan incorporatie van Na^+/K^+-ATPase, ionkanalen en waterkanalen in de cochlea tot op zekere hoogte door hormonen gereguleerd zou kunnen worden. We benadrukken dat de regulatie van de ion- en vloeistofhomeostase in de cochlea erg complex moet zijn, gezien de verschillende mechanismen die hierbij een rol spelen. Echter, een regulatie door hormonen creëert mogelijkheden voor de farmacologische behandeling van bepaalde binnenoorafwijkingen, die geassociëerd lijken te zijn met verstoringen van de ion- en vloeistofhomeostase in de cochlea (zoals de ziekte van Menière). Het gepresenteerde model van *acute* EH, gebaseerd op een toegenomen productie van endolymfe, kan bij het vinden van een dergelijke farmacologische behandeling behulpzaam zijn. Omdat verhoging van de adenylaat-cyclase activiteit door bepaalde substanties EH kan produceren, moeten andere stoffen dit juist ook weer kunnen voorkomen. Met de SP als indicator van endolymfatische druk kunnen deze farmacologische substanties worden uitgeprobeerd via simultane perfusie met cholera-toxine of vasopressine.
Omdat aldosteron en vasopressine betrokken zijn bij vitale fysiologische processen in de nier, lijkt het niet erg waarschijnlijk dat het binnenoorepitheel primair gereguleerd wordt door deze substanties. Om effecten op organen zoals de nier te voorkomen,

werkt het binnenoor vermoedelijk via zijn eigen, meer specifieke liganden. De effecten, die we observeerden na perilymfatische perfusie met cholera-toxine en vasopressine zouden het gevolg kunnen zijn van kruisreacties op receptoren in de cochlea die normaalgesproken binden met deze specifieke binnenoorliganden. Fundamenteel onderzoek gericht op het identificeren van deze specifieke binnenoorliganden moet een vervolg vinden, omdat disregulatie in de activiteit van dergelijke liganden de ion- en vloeistofhomeostase in de cochlea kan verstoren en daarmee verantwoordelijk kan zijn voor afwijkingen zoals gevonden bij de ziekte van Menière.

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Dankwoord

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Curriculum Vitae

Peter J.F.M. Lohuis was born on September 7th of 1967 in Montfoort. He finished gymnasium β at the St. Bonifatius College in Utrecht in 1985. In that year he also started his medical study at the Utrecht University. In 1988 he was selected for an exchange program with the University of Florida (SHANDS Hospital). There, he spent 14 months in the Laboratory of Otopathophysiology (Prof. Dr. Kyle E. Rarey), where the first three papers of this thesis were completed. In January 1994 he obtained his Medical Degree. In April 1994 he started his residency at the department of Otorhinolaryngology / Head & Neck surgery at the University Hospital Utrecht (Prof. Dr. G.J. Hordijk and Prof. Dr. E.H. Huizing), in part at the Hospital Center Apeldoorn (J.B. Antvelink). During the residency, which will finish in July 2000, this thesis was completed at the Utrecht Hearing Research Laboratories (Prof. Dr. G.F. Smoorenburg).

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