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Morphological aspects of potassium flow in the semicircular canal ampulla of the pigeon

K. Helling¹ and H.J. Merker²

¹Department of Otorhinolaryngology and

²Institute of Anatomy, Charité, University Medicine Berlin, Benjamin Franklin Campus, Germany

Summary. Potassium ions are a prerequisite for the development and regulation of sensory cell stimulation in the inner ear. From the potassium-rich endolymph the ions flow into the sensory cells apically and are released basolaterally. After transport pathways of various lengths potassium is released again into the endolymph - in the cochlea by marginal cells of the stria vascularis, in the vestibular labyrinth by dark cells. While this long recycling pathway is relatively well-known in the cochlea, few studies have been conducted on the semicircular canal ampullae (SCCA) where its morphological basis is largely unknown.

According to the present electron microscopic findings, potassium ions are initially released into the extracellular space during stimulation of the sensory cells and then absorbed by supporting and light cells. Finally they are transported transcellularly over numerous very long gap junctions into the region of the dark cells. From here they move to an extracellular compartment, which is more or less completely sealed off basally by basal plates of the light cells. Apically the intercellular space between light and dark cells is sealed by junctional complexes. This newly identified space in the SCCA corresponds to the extracellular compartment between the marginal and intermediate cells in the stria vascularis. At both sites, the cochlea and the SCCA, this probably serves as a regulatory valve, reservoir or storage space, particularly for potassium ions. It is likely that the different morphology of the ion transport pathways is related to the different flow levels of potassium ions expressed by the different levels of the so-called endocochlear potential and concomitant movement of other ions in the cochlea and SCCA.

Key words: Ultrastructure, K⁺ cycling, Basal labyrinth, Transcellular and extracellular K⁺ transport

Introduction

The endolymph of the inner ear shows marked differences in its ion composition compared to other body fluids like e.g. perilymph and cerebro-spinal fluid (Smith et al., 1954; Bosher and Warren, 1968; Salt and Konishi, 1986). The high concentration of potassium ions in the endolymph is a prerequisite for the development and regulation of sensory-cell stimulation (Corey and Hudspeth, 1979; Sterkers et al., 1984; Marcus, 1986; Salt and Konishi, 1986; Johnstone et al., 1989; Wangemann et al., 1995; Crouch et al., 1997). During the mechanical stimulation of the cilia, potassium ions flow into the apices of these cells and are released basolaterally. To maintain a constant concentration of potassium ions in the endolymph, it can be supposed that ions are transported back into the endolymph. In Corti's organ it has been established that the potassium ions first flow into the extracellular space between sensory and supporting cells (Konishi et al., 1978; Sterkers et al., 1984; Salt and Konishi, 1986; Marcus and Marcus, 1987; Ikeda and Morizono, 1989). The tunnel system of Corti's organ is possibly the morphological expression of this pronounced flow of fluid and ions. Subsequently the ions flow out transcellularly either via the epithelial lining of the scala media or in an intermediate stage extracellularly into the subepithelial connective tissue and then transcellularly along the root-fibrocyte chain. The following compartment is characterized by the intra- or transcellular pathway via gap junctions (Wangemann, 2002). The cells involved here represent a functional syncytium (Kikuchi et al., 1995). It includes the long compartment of the epithelial cell lining in the scala media or the fibrocytes located in the spiral lamina, possibly including those in Reissner's membrane up to the stria vascularis, to end there in the basal and intermediate cells (Spicer and Schulte, 1991, 1998; Wangemann, 1995). According to previous findings, the intermediate cells finally release the potassium ions in an enclosed extracellular compartment underneath the marginal cells from where they are absorbed by the

Offprint requests to: Dr. Kai Helling, MD, Department of Otorhinolaryngology, Charité - University Medicine Berlin, Benjamin Franklin Campus, Hindenburgdamm 30, D-12203 Berlin, Germany. email: kai.helling@charite.de

marginal cells, and again secreted in the direction of the endolymph. The structures and enzymes involved in this process have already been described morphologically and histochemically (Kikuchi et al., 1995; Wangemann, 2002). The structure of the marginal cells is typical for ion transport.

Relatively little has been reported about the morphological principles underlying the movement of potassium ions in the vestibular organ. Even if the tasks are somewhat similar (Kimura, 1969; Spicer et al., 1990; Wangemann, 1995), the morphological conditions clearly differ from those in the cochlea, especially in the stria vascularis (Spicer and Schulte, 1998; Villegas et al., 2001). Stimulation of the sensory cells of the SCCA crista also leads to a potassium inflow and subsequent basolateral outflow, followed by transport to the dark cells near the crista ampullaris and finally to secretion from these cells into the endolymph (Wangemann, 1995, 2002; Coppens et al., 2004). While the ion channels and gap junctions involved in the potassium flow have been identified (Wangemann, 2002), the precise morphological basis of the pathway between sensory and dark cells and the associated mechanisms have not yet been defined clearly.

This problem is discussed on the basis of the present findings in the pigeon (Villegas et al., 2001) with reference to conditions in the cochlea already described in part by other authors.

Materials and methods

The SCC ampullae of four pigeons were provided by the Institute of Applied Zoology (Free University of Berlin). The pigeons were not uniformly of a specific breed. Experiments were performed according to the general animal protection guidelines (animal experiment registration Berlin G 0349/98). The animals were decapitated after anaesthesia with Ketanest[®].

The skin was detached from the skull and the laterooccipital bone around the inner ear was removed with a microraspatory. Thereafter, the bony labyrinthine capsule was further exposed by removing the surrounding trabicular bone material, the vestibulum of the bony labyrinth was opened, and an in situ fixation with Karnowsky's solution (3% glutaraldehyde, 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2) was performed. After 30 minutes, the fixed membranous labyrinth was extracted completely from the bony labyrinth and the membranous ampulla separated. The preparation was either transferred to Karnowsky's solution or further fixed in an alternative medium (1% glutaraldehyde, 0.5 tannic acid in phosphate buffer) for 2-4 hours.

After washing in buffer solution, refixation was also done with buffered 1% OsO₄ solution. The preparations were washed and dehydrated using an alcohol series. After embedding in epon, semithick sections (0.5-1.0 mm) were prepared, stained with toluidine blue and examined under a light microscope. Selected segments were further processed as 50nm-thin slice sections, obtained using a LEITZ Ultracut E. These were then contrast-enhanced with uranyl acetate and lead citrate and examined under a ZEISS EM10 transmission electron microscope.

Results

The following description only considers the morphology of those structures located between the sensory cells on the crista ampullaris and the dark cells in the SCCA, where the flow of ions presumably takes place (Fig. 1). The surfaces of the hair cells, except for the apical area with stereocilia, are delimited by sensitive end structures, typical of sensory cells, or by supporting cells with relatively smooth cell membranes (Figs. 2A, 3A, 4A). The supporting cell membranes attach over wide areas in an almost parallel fashion. Obviously, the intercellular space is relatively narrow (25-80 nm). No special cell contacts were found between the basal part of the hair cells and the supporting or light cells (Fig. 3A). The intercellular space is sealed at the apex of the cell, i.e. towards the endolymph space, by junctional complexes with desmosome-like contacts,



Fig. 1. Schema illustrating potassium flow (arrows) in the SCCA: During stimulation of the haircells, the potassium ions, originally transferred from the endolymphatic space (es) into the hair cells, are subsequently released into the supporting cells. There follows a transcellular transport via the gap junctions (g) of the light cells (l) to the region of the dark cells (d). The potassium ions are then temporarily stored in the extracellular space of the basal labyrinth formed by the dark cells (see enlarged image). This basal labyrinth is connected to the connective tissue through the porelike orifices, which result from the basal plates (p) of the neighbouring light cells. The precise function of these pores remains unclear (?). Finally the ions are returned from the dark cells into the endolymphatic space (es). ca: Crista ampullaris; jc: junctional complex.



Fig. 2. A. Entry site of a sensory axon (a) into the epithelium (asterisk) with many neurotubuli. Basal demarcation of the epithelium by a basal lamina (cross). Open intercellular space (ICS) of 25 nm in width (arrow) between the cell membrane of the light cells (I) and that of the axon. ct: connective tissue. Tannic acid (TA) fixation. x 30000. **B.** Cell contacts between hair (h) and light cells (I) with open (arrow) and narrowed ICS, gap junctions between light cells (open arrow). No TA fixation. x 61000



Fig. 3. A. Narrow basal hair cell (h) process between light cells (l). In between open ICS of about 25 nm (arrows) without special cell contacts. Basal lamina (cross). TA fixation. x 76000. B. Contact between two light cells (l) with gap junctions (arrow), zonulae adherentes (open arrow) and oblique intersections through the cell membrane. TA fixation. x 61000. C. Border between epithelium and connective tissue at the basal lamina (cross), basal plates (p) of the light cells, connected via gap junctions (arrow), pore-like openings (open arrow) in the basal laminae of the light cells (l) with direct contact between dark cells (d) and the basal lamina. Connective tissue (ct), myelin figure (m), extracellular compartment (asterisk) in the basal labyrinth of the dark cells. TA fixation. x 18000 (see enlargement of marked region in Fig. 7B).



Fig. 4. A. Intersection of a hair cell (h) and a light cell (l) without tannic acid fixation, in between a 25 nm open ICS without special contacts (thick arrow). A narrowed ICS (arrow) with a gap junction between two light cells (l). No TA fixation. x 61000. B. Basal part of a dark cell (d). Basal plates (p) of light cells (l) constrict the contact of dark cells with connective tissue (ct) in a pore-like manner (arrow). ICS between dark and light cells (asterisk), basal lamina (cross), gap junctions between light cells (thick arrow). TA fixation. x 8000

tight junctions and interlocking (see overview of the different types of cell contacts in Fig. 7).

The supporting, connecting and light cells between the sensory and dark cells in the SCCA can only be reliably differentiated by location but not by morphology. They can be clearly identified and referred to as supporting cells at those locations directly adjacent to hair cells or sensitive end structures. Thus, in the following they are collectively referred to as light cells. The light cells are interconnected by numerous long gap junctions (Fig. 3B,C). In regions showing this type of contact, cell membranes may be straight or curved, and in some cases with indentations (Fig. 7B,C,E,F). The number and size of gap junctions are surprisingly large in these areas. Subsequently the ions are transported through the extracellular space between these cells and the dark cells (Figs. 3A, 4A, 5, 6). The size of this space varies considerably, depending on the spacing between the plate-like processes of the basal labyrinth (this term is adopted from its usage for the similar structure in the tubular cells of the kidney) of the dark cells, which can range from 300 to 1000 nm (Figs. 3C, 4A, 5, 6). The variable capacity of the extracellular compartment is essentially determined by the packing density of the dark cell processes (Figs. 4B, 5, 6). Its variable capacity facilitates adaptation to varying fluid volumes. The fine structure of the dark cells, which constitute the final intracellular compartment before secretion of potassium ions, is not dealt with in the present report. This topic is discussed in detail in a previous publication (Villegas et al., 2001). The junctional complexes (also described in Villegas et al., 2001) are located in the apical part between both cell types (Figs. 5, 7A).

In the basal part of the epithelial layer, the basal plates of the light cells penetrate between the basal lamina and the dark cells. This results in the pore-like openings of varying diameter (300-1000 nm) between neighboring plate-like processes (Figs. 4B, 5). Frequently, pores cannot be detected at all (Fig. 6). However, it must be taken into consideration that detecting such pores depends on the direction and thickness of the section. Only very rarely are the basal plates missing below the dark cells, in which case they border the basal lamina over large areas. The varying size of the pores may indicate their dynamic behavior, e.g. a function-dependent change in the extent of free communication with the connective tissue. The rare presence of microfilament bundles in the basal plates of the light cells can be interpreted in terms of a capacity for active movement and thus for regulation of pore size. This is supported by the observed changes in pore width (Figs. 5, 6).

Finally, the electron microscopic picture of the subepithelial connective tissue will be briefly discussed. Underneath the crista ampullaris, the nerve fibers naturally dominate (Fig. 2A). The few irregularly distributed fibrocytes do not show any contacts with adjacent cells. They are plump and contain only a few cell organelles and some cavities of rough endoplasmic

reticulum. There are fewer of these cells towards the edges of the crista ampullaris, i.e., underneath the region containing dark cells. Irregularly running filaments with a mean thickness of 12 nm are dominant in the extracellular space (Fig. 2A). Cross striation cannot be reliably detected; however immunohistological findings indicate that this is collagen type II (unpublished results). The filaments are occasionally arranged in parallel bundles of varying thickness. No specific directional preference of the filaments and bundles can be determined. After tannic acid fixation, granular structures (50-500 nm) can be observed on and between the filaments. According to comparable studies on different tissue, these are morphologically equivalent to glycoprotein and proteoglycan macromolecules. In addition to nerve fibers and fibrocytes, capillary-like structures also occur in the connective tissue space. These structures are characterized by an interesting morphology. The outer sheath consists very flat bipolar cells enveloping the vessel with 2 to 6 layers.

Discussion

According to current knowledge, both morphological and functional similarities exist between the marginal cells of the stria vascularis and the dark cells in the SCCA (Kimura et al., 1964; Kimura, 1969, 1984; Santi, 1986; Igarashi, 1989; Sakagami et al., 1993; Wangemann, 1995, 2002; Villegas et al., 2001). Both types of cells subserve the transport and secretion of potassium ions (Marcus and Shipley, 1994), but there are also obvious differences. The dark cells are additionally responsible for the secretion of other ions such as Na⁺, Cl⁻ and H⁺ and are thus not completely comparable, at least in a quantitative sense. In the dark cells, pronounced H⁺ ion secretion must be assumed due to the existence of a tubulovesicular organ (Marcus et al., 1993; Villegas et al., 2001; Helling et al., 2004). On stimulation of sensory hair cells, potassium is thought to be absorbed apically, released basolaterally (Corey and Hudspeth, 1979), and transported to the marginal cells in the stria vascularis or to the dark cells in the SCCA. If this is true, then different pathways must exist in these two locations.

In the cochlea, many compartments and various cell types have to be passed on the long pathway from the sensory hair cells in Corti's organ to the marginal cells in the stria vascularis (Kimura, 1975; Santi, 1986; Kikuchi et al., 1995; Spicer and Schulte, 1996, 1998). In the cochlea the pathway begins (1) with the cytoplasm of the sensory hair cells, followed by (2) an extracellular compartment between the hair cells and either the light cells, or the subepithelial fibrocytes including the tunnel system of Corti's organ. The epithelial compartment is sealed against the endolymph space by junctional complexes, and is open basally towards the connective tissue. The third – intracellular - compartment (3) includes the epithelial fibroblasts underlying Corti's organ and



Fig. 5. A. Low magnification of a dark cell (d) with apical microvilli towards the endolymph space (es) and fissured infranuclear cell structure, pore-like contact with the basal lamina (arrow), constricted by basal plates (p) of the light cells (I). TA fixation. x 6000. **B.** Basal parts of dark cells (d), demarcated from connective tissue (ct) by basal plates (p) of the light cells (I). In between, pore-like openings of dark cells towards the connective tissue (arrow). Extracellular spaces (asterisk) between the fissured part of the dark cells, the basal labyrinth. TA fixation. x 8000



Fig. 6. Basal part of dark cells (d), demarcated from the basal lamina (cross) by basal plates (p) of light cells (l), which are connected via gap junctions (arrow). Extracellular spaces (asterisk)in the so-called basal labyrinth. m: myelin figures (m). TA fixation. x 8000



Fig. 7. A. Apical junction complex between two light cells after tannic acid fixation. Area with tight junctions without tannic acid penetration (between thick arrows) towards the endolymph space (e). In a basal direction, small gap junctions (arrow) and interlockings (i). Endocytotic structures (en), endoplasmic reticulum (asterisk). TA fixation. x 37000 (modified after Villegas et al. 2001). **B.** Gap junctions (arrow) between basal plates (p) of the light cells. Basal lamina (cross), basal labyrinth of the dark cells (d), extracellular spaces (asterisk) in between. TA fixation. x 76000 (enlargement from Fig. 3C). **C.** Cell contacts between hair (h) and light cells (l) with open ICS (thick arrow) and between supporting cells with gap junctions (arrow). TA fixation. x 97000. **D.** Demarcation of dark cells (d) from the basal lamina (cross) by basal plates (asterisk) of light cells (l) with gap junctions at the contact sites, TA fixation. x 18000. **E.** Contacts between light cells with gap junctions (arrow) and defined extensions of ICS (i). TA fixation. x 96000. **F.** Undulating course of a contact zone between light cells (l) with gap junctions and flat sections through the cell membrane (thick arrow). TA fixation. x 76000

in the spiral lamina, possibly also in Reissner's membrane, and finally the basal and intermediate cells of the stria vascularis. Along this pathway, the ions flow transcellularly via gap junctions. This involves specific cell connections via small canals (gap junctions) that enable a transcellular exchange of small molecules (< 1000 Da) (Nadol, 1978; Spray and Bennett, 1985; Bennett et al., 1991; Kikushi et al., 1995; Hama and Saito, 1977). This is followed by (4) a further shift of potassium into an extracellular space of varying width, which is located between the intermediate cells and the processes of the marginal cells. The potassium is then absorbed (5) into the marginal cells and finally expelled into the endolymph.

In the SCCA, on the other hand, the morphology appears to be simpler (Kimura, 1969; Villegas et al., 2001). Here, potassium ions also flow basolaterally from the sensory hair cells into the extracellular space, where they are absorbed by the adjacent light or supporting cells. The functions and nomenclature of the cells located between the sensory and dark cells are not entirely clear. They are variously referred to as supporting, transitional, connecting or light cells in the area around dark cells. It is difficult to differentiate these cells by their morphology alone, so it is possible that they belong to the same cell type. Their mutual interconnection via numerous very long gap junctions indicates their similarity, since normally only homogeneous cells are connected by such contacts. There are only a few sporadic fibrocytes in the subepithelial space. The existence of intracytoplasmic, fibrocytic compartments with gap junctions, as is the case in Corti's organ, cannot be assumed. However, it must be noted that the number and possibly also the function of fibrocytes could well be species-specific, as is the case with melanocytes (unpublished results; Villegas et al., 2001). After transport through the light cells, the potassium ions enter a further extracellular compartment in the region of the dark cells. Due to the fissured basolateral surface of the dark cells, which is similar to the epithelial cells in the tubular system of the kidney (Greger, 1996), the ions are finally absorbed and secreted into the endolymph. This hypothesis is supported by a number of findings obtained by enzymatic (Yoshihara and Igarashi, 1987), electrophysiological (Marcus et al., 1993; Wangemann, 1995, 2002; Wangemann et al., 1995, 1996) and immunohisto-chemical techniques (Spicer and Schulte, 1998). Cells involved in this pathway manifest all of the enzymes required for ion transport such as Na⁺/K⁺ ATPase, H⁺ ATPase, other ATPases, carboanhydrase, Na⁺/K⁺/Cl⁻ cotransporters and Cl⁻-HCO₃ exchanger (Yoshihara and Igarashi, 1987; Iwano et al., 1989; Carlisle et al., 1990; Spicer et al., 1990; Sakagami et al., 1993; McGuirt and Schulte, 1994; Schulte and Steel, 1994; Kikuchi et al., 1995; Wangemann, 1995, 2002; Crouch et al., 1997; Stankovic et al., 1997; Bond et al., 1998; Spicer and Schulte, 1998).

According to the present electron microscopic

findings, the presence of a previously unknown extracellular compartment underlying the dark cells in the SCCA can be postulated, similar to that below the marginal cells in the stria vascularis. It is almost completely sealed against the connective tissue, i.e. towards the perilymph space, by the basal plates of the light cells. In the apical direction, towards the endolymph, it is sealed by the cell membranes of the dark cells and by junctional complexes. In contrast, it is very likely that the comparable extracellular space under the marginal cells of the stria vascularis is completely sealed against the endo- and perilymph. The marginal and intermediate cells are interconnected by tight junctions. Since there is an extracellular space related to both the marginal and to the dark cells, it can be assumed that this morphology is associated in both cases with potassium secretion. Its function is most probably involved in the collection of potassium-rich fluid for ion secretion into the endolymph. This hypothesis is supported by electrophysiological findings in the isolated SCCA of the frog. These suggest that the vestibular organs are endowed with K⁺ homeostatic mechanisms, which are able to efficiently buffer the concentration of K⁺ in both labyrinth fluid compartments around the SCCA, i.e. endolymph and perilymph (Valli et al., 1990).

The difference between the so-called endocochlear potential in the cochlea and the electrical potential in the SCCA may be due to the structural differences in their extracellular spaces. The incomplete sealing under the dark cells of the SCCA allows only a low potential to the build up (Marcus et al., 1994). If the extracellular space would serve as a potassium reservoir, then less potassium would be available in the SCCA for the buildup of the endocochlear potential.

The incomplete sealing of the extracellular space under the dark cells in the SCCA along the boundary to the connective tissue results from pore-like openings of varying size between the basal plates of the light cells. It is unlikely that these represent artifacts caused by shrinking of the basal plates during preparation. The alignment of the basal processes and invaginations of the dark cells (basal labyrinth) in the direction of these pores indicate rather their functional existence. An interesting aspect of these pores is the idea that their capacity can be changed actively by shortening their diameter, i.e. thickening and lengthening, and thus flattening of the basal plates. The amount of efferent or afferent fluid in the subepithelial space can thus be regulated. However, the presence of other ions, e.g. Na⁺, Cl⁻ and H⁺ and their possible influence must also be considered in the explanation of pore function (Wangemann et al., 1996; Stankovic et al., 1997).

In addition, capillaries are to be found below the crista. Their possible function must also be taken into consideration due to their characteristic multilayered wall structure. The present findings in the pigeon demonstrate that, in comparison to the stria vascularis, there are considerably fewer capillaries in the SCCA. In the stria vascularis many capillaries are situated very close to the epithelium; previously they have been ascribed to the intraepithelial capillaries. Currently, in the stria vascularis only the marginal cells are considered as real epithelial cells. The basal cells represent fibrocyte-like cells, whereas the intermediate cells are derived from the neural crest (Steel and Barkway, 1989). The fact that the function and origin of the characteristically multilayered membranes consisting of flat cells is still unknown makes any evaluation of the capillaries situated below the dark cells in the SCCA epithelium difficult. They are clearly not typical pericytes or smooth muscle cells (Villegas et al., 2001). These special capillaries may also be involved in the efferent and afferent flow of ions and their selection.

The findings indicate that potassium ions are transported from the hair cells of the Crista ampullaris to the neighbouring dark cells both transcellularly via gap junctions and extracellularly via the intercellular space. Furthermore, it is likely that the extracellular space between the light cells and the basal labyrinth of the dark cells, which exhibits variable width, is instrumental for the regulation of the K⁺ enriched fluid volume, respectively K⁺ concentration, prior to its release into the endolymph.

This demonstrates that K^+ transport pathways differ in the SCCA and the cochlea. In the SCCA, potassium ions are transported exclusively via epithelial layer, whereas in the cochlea both epithelial transport and rootfibrocyte transport occur.

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