“Reversed polarization” of Na/K-ATPase—a sign of inverted transport in the human endolymphatic sac: a super-resolution structured illumination microscopy (SR-SIM) study

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Abstract
The human endolymphatic sac (ES) is believed to regulate inner ear fluid homeostasis and to be associated with Meniere’s disease (MD). We analyzed the ion transport protein sodium/potassium-ATPase (Na/K-ATPase) and its isoforms in the human ES using super-resolution structured illumination microscopy (SR-SIM). Human vestibular aqueducts were collected during translabyrinthine vestibular schwannoma surgery after obtaining ethical permission. Antibodies against various isoforms of Na/K-ATPase and additional solute-transporting proteins, believed to be essential for ion and fluid transport, were used for immunohistochemistry. A population of epithelial cells of the human ES strongly expressed Na/K-ATPase α1, β1, and β3 subunit isoforms in either the lateral/basolateral or apical plasma membrane domains. The β1 isoform was expressed in the lateral/basolateral plasma membranes in mostly large cylindrical cells, while β3 and α1 both were expressed with “reversed polarity” in the apical cell membrane in lower epithelial cells. The heterogeneous expression of Na/K-ATPase subunits substantiates earlier notions that the ES is a dynamic structure where epithelial cells show inverted epithelial transport. Dual absorption and secretion processes may regulate and maintain inner ear fluid homeostasis. These findings may shed new light on the etiology of endolymphatic hydrops and MD.

Keywords Human · Endolymphatic sac · Na/K-ATPase · Reversed polarity · SIM

Abbreviations
ED Endolymphatic duct
ES Endolymphatic sac
E Endolymph
EDTA Ethylene-diamine-tetra-acetic acid
Na/K-ATPase Sodium/potassium-ATPase
NKCC2 Sodium/potassium/chloride cotransporter 2
VA Vestibular aqueduct
ENaC Epithelial sodium channel
MD Meniere’s disease
TJ Tight junctions
SR-SIM Super-resolution structured illumination microscopy
MRC Mitochondria-rich cell
RRC Ribosome-rich cell
PBS Phosphate buffered saline
BSA Bovine serum albumin
CF Cerebrospinal fluid

Introduction
The function of the human endolymphatic sac (ES) remains an enigma. This intriguing part of the inner ear is believed to be...
involved in several activities, such as endolymph resorption, pressure regulation, and immune reactions (Lundquist et al. 1964; Rask-Andersen et al. 1991; Jansson et al. 1992; Salt and DeMott 2000). The ES develops early (Raft et al. 2014), suggesting a role in the maturation of the inner ear fluid compartments.

The ES is linked to Meniére’s disease (MD), as interruption from the rest of the inner ear leads to endolymphatic hydrops, an equivalent of MD (Kimura and Schuknecht 1965; Kimura 1967). Pendrin, an anion exchanger encoded by the SLC26A4 gene, may play a crucial role in the development of the inner ear fluid spaces (Honda et al. 2017). A lack of pendrin reduces endolymph resorption and leads to congenital enlargement of the ES (Schuknecht 1965; Kimura 1967). Pendrin, an anion exchanger encoded by the SLC26A4 gene, may play a crucial role in the development of the inner ear fluid spaces (Honda et al. 2017). A lack of pendrin reduces endolymph resorption and leads to congenital enlargement of the ES and vestibular aqueduct (EVA) which is associated with childhood deafness (Park 2003).

Human ES contains a heterogeneous population of epithelial cells, while the rodent ES contains two distinguishable cell types, namely, the mitochondria-rich cells (MRCs) and ribosome-rich cells (RRCs) (Friberg et al. 1984a; Rask-Andersen et al. 1991; Dahlmann and von Düring 1995; Qvortrup et al. 1999; Peters et al. 2001). In the guinea pig, these cells are represented by the “light” cells (Lundquist et al. 1964) and in man by the cylindrical cells (Friberg et al. 1985). The MRCs are believed to absorb sodium and fluid from the ES lumen, while the RRCs may be involved in secretion and immune activity (Erwall et al. 1988). MRCs express SLC26A4, Na+-permeable channels, and Na/K-ATPase (Miyashita et al. 2007). Na/K-ATPase is localized in the basolateral cell membrane of the MCRs and is thought to expel sodium ions into the extracellular space along with fluid. Sodium absorption may be driven by the Na/K-ATPase pump in combination with selective ion channels and a positive ES potential (ESP) in the lumen (Amano et al. 1983; Mori et al. 2017).

Studies of the human ES are motivated because of species differences. Recently, several reports on ion transporters in the human ES were presented using cDNA micro-array, reverse transcription polymerase chain reaction (RT-PCR), and immunohistochemistry (Kumagami et al. 2004; Kim et al. 2009; Kakigi et al. 2009; Lee et al. 2012; Möller et al. 2015).

We used super-resolution structured illumination microscopy (SR-SIM) to analyze the Na/K-ATPase protein and its isoforms in the human ES. The technique allows immune histochemical investigation of proteins with high resolution beyond the diffraction (Hell and Wichmann 1994; Gustafsson et al. 2008). The goal was to expand our knowledge of endolymph resorption in the human ES and its role in MD. An SR-SIM study of Na/K-ATPase activity in the human cochlea was recently presented (Liu et al. 2017).

Material and methods

The human vestibular aqueduct

The technique used to collect the ES was recently described by the authors (Kämpfe Nordström et al. 2018). After obtaining ethical permission, human ESs were collected during trans-labyrinthine vestibular schwannoma removal. The ES was drilled out with a thin rim of surrounding bone to preserve the epithelium. The endolymphatic duct ED and the ES overlying the sigmoid sinus (SS) were not analyzed (Fig. 1). The tissue was fixed in 4% paraformaldehyde diluted with 0.1 M phosphate buffered saline (PBS) at pH 7.4 in the operating room. After 24 h, the specimens were washed in PBS and then placed in 0.5 M Na-ethylene-diamine-tetra-acetic acid (EDTA) solution (Medicago AB, Uppsala, Sweden) buffered to pH 7.2 for decalcification. The decalcification process lasted for approximately 3 weeks. The ESs were embedded in Tissue-Tek O.C.T.™ compound (Sakura® Finetek, Torrance, CA, USA) in preparation for freezing. They were rapidly frozen and sectioned at 8–10 μm using a cryostat microtome (Leica CM 1860, Leica Microsystems GmbH, Wetzlar, Germany). The frozen sections were collected onto glass slides (SuperFrost® Plus, Menzel-Gläzer, Braunschweig, Germany) and stored below −70 °C before immunohistochemistry was conducted.

Antibodies and immunohistochemistry

Table 1 shows the series of antibodies used in the present study. The specificity of the antibodies was proven by the following:

- Na/K-ATPase β1: western blot (Wyckelsma et al. 2017)
- Na/K-ATPase β2: western blot (www.alomone.com)
- Na/K-ATPase β3: western blot (www.abbrevx.com)
- Na/K-ATPase α1*: western blot (Ren et al. 2019)
- Na/K-ATPase α1": western blot (Yamashita et al. 2018)
- Na/K-ATPase α2: western blot (www.avivaysbio.com)
- Na/K-ATPase α3: western blot (Ivakine et al. 2013)
- Carbonic anhydrase II: western blot (www.atalasantibodies.com)
- NKCC2: western blot (www.avivaysbio.com)
- Mineralocorticoid receptor*: western blot (Bordin et al. 2016)
- Mineralocorticoid receptor": western blot (www.thermofisher.com)
- Vasopressin receptor type 2: western blot (www.abbrevx.com)
- ENaC: western blot (Li et al. 2017)
- Aquaporin 2: western blot (Yang et al. 2015)
- Aquaporin 4: western blot (Ruiz-Ederra et al. 2007)
- Pendrin: western blot (Jia et al. 2016)
The immunohistochemistry procedures performed on the sections have been described in previous publications (Liu et al. 2009, 2017). Briefly, the slide-mounted sections were incubated with an antibody solution under a humidified atmosphere at 4 °C for 20 h. After rinsing with PBS three times for 5 min each, the sections were incubated with secondary antibodies conjugated to Alexa Fluor 488 and 555 (Molecular Probes, Carlsbad, CA, USA) for 2 h at room temperature. The primary and secondary antibodies were diluted in 2% bovine serum albumin (BSA) dissolved in PBS. The sections were then counter-stained with the nuclear stain DAPI (4′,6-diamidino-2-phenylindole dihydro-chloride) for 5 min, rinsed with PBS (3 × 5 min), and mounted with ProLong® Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA) as well as the specified cover glass required for optically matching the microscope objectives. Primary and secondary antibody controls and labeling controls were used to exclude endogenous labeling or reaction products (Burry 2011). Control sections were incubated with 2% bovine serum albumin (BSA), omitting the primary antibodies. The control experiment revealed no visible staining in any structure of the inner ear sections. Both wide-field and confocal fluorescence imaging software exhibited sensitive fluorescent saturation indications, thereby avoiding overexposure. In addition, archival sections of guinea pig (Harlan Sprague Dawley Inc., Indianapolis, Indiana, USA) choroid plexus were analyzed according to methods described by Liu et al. (Kämpfe Nordström et al. 2018).

**Imaging and photography**

Microscopy was performed in an inverted fluorescence microscope (Nikon TE2000; Nikon, Tokyo, Japan) with three filters (for emission spectra maxima at 358, 461, and 555 nm). The microscope is equipped with a three-channel laser emission system for confocal microscopy. SR-SIM was performed using a Zeiss Elyra S.1 SIM system and a 63×/1.4 oil Plan-Apochromat objective (Zeiss, Oberkochen, Germany), a sCMOS camera (PCO Edge), and the ZEN 2012 software (Zeiss). Multicolor SR-SIM imaging was achieved with laser and filter setup: 1st channel—405-nm laser excitation and BP 420–480 + LP 750 filter; 2nd channel—488-nm laser excitation and BP 495–550 + LP750 filter; and 3rd channel—561-nm laser excitation and BP 570–620 + LP 750 filter. Five grid rotations and five phases were used for each image plane and channel. SR-SIM images were processed with ZEN software using automatic settings and theoretical point spread function (PSF) calculations. 3D reconstruction was performed with Imaris 8.2 (Bitplane AG, Zürich, Switzerland). A lateral (X-Y) resolution of ≈100 nm and an axial (Z) resolution of ≈

- NKCC1: western blot (Hampel et al. 2018)
- NCC: western blot (www.thermofisher.com)
300 nm is achieved (Gustafsson et al. 2008). The resolution of the SIM system in BioVis (Uppsala, Sweden) was analyzed using fluorescent beads (40 nm, Zeiss) in the green channel (BP 495–550 + LP750). The resolution of the system was found to be 107 nm in the X-Y plane and 394 nm in the Z plane. The principle of structured illumination microscopy is based on a wide-field technique using the moire effect of interfering fine striped patterns of excitation. This contrasts to the confocal technique using detected fluorescence light at the focal plane.

**Numerical analysis of staining characteristics**

As the expression of Na/K-ATPase \( \beta_1 \) and Na/K-ATPase \( \alpha_1 \) may represent two different cell types, a quantification of these subunit expressing cells was made. Five sections that were stained for Na/K-ATPase \( \beta_1 \) and five sections that were stained for Na/K-ATPase \( \alpha_1 \) were randomly chosen. Only sections where the entire ES epithelium was visible were selected. Cells were counted manually, and staining was categorized in two groups (positive staining or negative staining). A total number of 7936 cells were counted (Table 2).

**Results**

SR-SIM showed rich expression of Na/K-ATPase isoform subunits \( \alpha_1 \), \( \beta_1 \), and \( \beta_3 \) in the epithelium of the human ES. There was no or only sparse staining for the isoform subunits \( \alpha_2 \), \( \alpha_3 \), and \( \beta_2 \). Na/K-ATPase was expressed in the basolateral cell membrane in cubic and cylindrical cells (Figs. 2, 3, and 4) and in the apical cell membrane with “reversed polarity” in cubic and low epithelial cells (Figs. 5, 6, and 7). The cylindrical cells, which are believed to represent the MRC cell in rodents (Dahlmann and von Düring 1995) strongly expressed the isoform \( \beta_1 \) in the basolateral cell membrane together with carbonic anhydrase (CA) (Fig. 4). A corresponding \( \alpha_1 \) subunit could not be established, but it was not \( \alpha_1 \). Both the intra- and extra-osseous parts of the ES expressed the isoform \( \beta_1 \) in the basolateral cell membranes. The cylindrical cells were mostly located in the intermediate portion of the ES (Fig. 2). Some lower or cubic cells also strongly expressed Na/K-ATPase in the basolateral plasma membrane. Some epithelial cells, particularly in the distal sac, did not express the \( \beta_1 \) subunit to a major extent. Quantification of cells showed that 78% of the ES epithelial cells expressed \( \beta_1 \) in the basolateral cell membrane. The \( \alpha_1 \) subunit was expressed in the apical cell membrane in 40% of the cells. At high magnification, Na/K-ATPase protein was seen to translocate from organelles near the cell nucleus to the cell nucleus.

**Table 1** Antibodies used in the study

| Antibody       | Type       | Dilution | Host       | Catalog number | Producer                          |
|----------------|------------|----------|------------|----------------|-----------------------------------|
| Na/K-ATPase \( \beta_1 \) | Monoclonal | 1:100    | Mouse      | MA3-930        | Thermo Fisher, Waltham, USA       |
| Na/K-ATPase \( \beta_2 \) | Polyclonal | 1:50     | Rabbit     | ANP-012        | Alomone Labs, Jerusalem, Israel   |
| Na/K-ATPase \( \beta_3 \) | Polyclonal | 1:200    | Rabbit     | Abx111158      | Abbexa, Cambridge, UK             |
| Na/K-ATPase \( \alpha_1 \) | Monoclonal | 1:50     | Mouse      | SC 21712       | Santa Cruz Biotechnology, Dallas, USA |
| Na/K-ATPase \( \alpha_1^* \) | Monoclonal | 1:50     | Mouse      | NB300-146      | Novus, Littleton, USA             |
| Na/K-ATPase \( \alpha_2 \) | Polyclonal | 1:100    | Rabbit     | OAAB03232      | Aviva Systems Biology, San Diego, USA |
| Na/K-ATPase \( \alpha_3 \) | Monoclonal | 1:50     | Mouse      | NB300-540      | Novus, Littleton, USA             |
| Carbonic anhydrase II | Polyclonal | 1:50     | Rabbit     | HPA001550      | Atlas Antibodies, Stockholm, Sweden |
| NKCC2          | Polyclonal | 1:200    | Rabbit     | OABB01331      | Aviva Systems Biology, San Diego, USA |
| Mineralocorticoid receptor' | Monoclonal | 1:50     | Mouse      | MA1-620        | Thermo Fisher, Waltham, USA       |
| Mineralocorticoid receptor" | Polyclonal | 1:200    | Rabbit     | PAS-81527      | Thermo Fisher, Waltham, USA       |
| Vasopressin receptor type 2 | Polyclonal | 1:50     | Rabbit     | Abx133128      | Abbexa, Cambridge, UK             |
| ENaC           | Polyclonal | 1:100    | Rabbit     | PA1-920A       | Thermo Fisher, Waltham, USA       |
| Aquaporin-2    | Polyclonal | 1:50     | Rabbit     | NB110-74682    | Novus, Littleton, USA             |
| Aquaporin-4    | Polyclonal | 1:50     | Rabbit     | AQP-004        | Alomone Labs, Jerusalem, Israel   |
| Pendrin        | Polyclonal | 1:50     | Rabbit     | NBPI-60106     | Novus, Littleton, USA             |
| NKCC1          | Polyclonal | 1:100    | Rabbit     | AB59791        | Abcam, Cambridge, UK              |
| NCC            | Polyclonal | 1:100    | Rabbit     | PA5-77816      | Thermo Fisher, Waltham, USA       |

**Table 2** Evaluation of staining

| Na/K-ATPase subunit      | Negative | Positive |
|--------------------------|----------|----------|
| \( \beta_1 \) (basolateral expression) | 22%       | 78%      |
| \( \alpha_1^* \) (apical expression) | 60%       | 40%      |

\( \alpha_1 \) subunit mostly co-expressed with \( \beta_3 \) subunit
the lateral plasma membrane (Fig. 4, insets). Na/K-ATPase expression in the basolateral plasma membrane ended apically at the tight junctions. In a few cells, parts of the apical cell membrane also expressed the ion transporter (Fig. 4). The apical cell membrane (small arrow) expresses the α1 subunit, while the β3 isoform is strongly expressed in the perinuclear zone (long arrow). An ES was labeled with antibodies against both β1 and β3 isoforms from different species (c). In the proximal part of the sac (left), the β1 isoform was more expressed and in the basolateral cell membrane of the epithelium. In the distal part (right), the β3 subunit was more expressed and in the apical cell membranes in the epithelium. Here, only a few cells express the β1 isoform (inset). Left frame is magnified in Fig. 3.

Many epithelial cells in the intermediate portion expressed the mineralocorticoid receptor (MR) (Fig. 8). The MR was expressed both in the cytoplasm and cell nuclei. Several of these cells expressed Na/K-ATPase β1 isoform (Fig. 8, inset). Pendrin and vasopressin receptor (V2) were diffusely expressed in the cytoplasm in some epithelial cells (not shown). Antibodies against the Na/K-ATPase α1 and β3 isoforms were used to label (positive control) the secretory epithelium of the guinea pig choroid plexus. Both isoforms stained positive for the isoforms (Fig. 2a, b). The α1 expression was “polarized” and restricted to the apical plasma membrane. Positive controls also showed Na/K-ATPase β1 and NKCC1 co-expression in marginal cells of the cochlea, but with separate expressions in the basolateral membrane in the outer sulcus (Fig. 9). Aquaporin 2 and 4 were moderately expressed in epithelial cells of the intermediate sac (not shown).
Discussion

This is the first study to analyze ion channels and transporters in the human ES using SR-SIM. We focused on Na/K-ATPase and its possible role in endolymph resorption. The human ES is unique compared with the rest of the inner ear because of its variable morphology and mixed cell types (Bauwens et al. 1991; Lee et al. 2012) reflecting multiple functions (Bagger-Sjöbäck et al. 1988). Five types of epithelial cells were earlier observed in the human ES (Bagger-Sjöbäck et al. 1988).

We found expression of α1, β1, and β3 isoforms in the epithelial cells of the human ES. Various Na/K-ATPase isozymes are known to have an exclusive affinity for ions serving specialized cell functions. Na/K-ATPase consists of four catalytic α isoforms, three regulatory β, and seven FXYD subunits with ATP hydrolysis (Crambert et al. 2000; Blanco 2005; Geering 2008; Xie et al. 2013). The α1 subunit is expressed in most tissues, and the α2 and α3 isoforms are expressed in skeletal muscle, neuronal, and cardiac tissues, while the α4 isoform is restricted to the testis. The α1β1 heterodimer is the exclusive Na/K-ATPase complex expressed in kidney tubules (Feraille and Dizin 2016), but any α isoform can be expressed with any beta (Clausen et al. 2017). The β association is necessary for adequate folding of synthesized subunits (Hasler et al. 1998), while the FXYD proteins modulate Na/K-ATPase (Blanco 2005; Clausen et al. 2017).

Na/K-ATPase plays a crucial role in ion and fluid transportation in the inner ear that contains systems responsible for secretion of K⁺ into endolymph and generation of the endocochlear potential. The α1β1 heterodimer is the predominant combination in epithelial cells to maintain ion gradients (Weber et al. 2001). In a recent study, the dense accretion of Na/K-ATPase αβ heterodimer complexes was exposed in the basolateral cell membrane of the marginal cells and outer sulcus in the human cochlea (Liu et al. 2017). A strong basolateral distribution of Na/K-ATPase β1 isoform together with apical Na⁺-selective channels suggests an active sodium uptake from the ES lumen with excretion into the subcellular space. The findings are consistent with earlier studies showing Na/K-ATPase in the adult ES (Yamane and Nakai 1988;...
Ichimiya et al. 1994; ten Cate et al. 1994; Peters et al. 2001; Akiyama et al. 2010; Miyashita et al. 2012). ten Cate et al. (1994) found $\alpha_1$ and $\beta_2$ subunit isoforms and, to a lesser extent, the $\beta_1$ subunit in the guinea pig ES. They concluded that, since the combination is different from typical ion and fluid transporting tissues, for example, kidney and colon, it may reflect distinctive characteristics of inner ear Na/K-ATPase. Fina and Ryan (1994) examined the mRNA encoding isoforms and found only weak expression of Na/K-ATPase, limited to $\alpha_1$, $\beta_1$, and $\beta_2$ in the rat ES. They suggested that the ES is not a major site for ion exchange. Indirect studies of epithelial transport by the ESP (endolymphatic sac potential), recorded after injection of ouabain, were also unchanged (Couloigner et al. 1998; Teixeira et al. 1999). No specific enzyme activity was found in the guinea pig ES using a cytochemical technique (Albers et al. 1991). Likewise, low levels of Na/K-ATPase with co-existence of Ca$^{2+}$-ATPase and calcium-binding proteins were restricted to the intermediate and distal portions of the ES with no $\beta_2$ subunits, compared with the rest of the inner ear (Ichimiya et al. 1994). A low Na/K-ATPase activity could be consistent with the decrease in mRNA ENaC subunits found early, suggesting that Na$^+$ reabsorption in the ES occurs mostly during development (Gründer et al. 2001). However, laser capture microdissection, RT-PCR, and immunohistochemistry verified Na$^+$-selective channels in mature ES epithelia (Mori and Wu 1996) that co-localized apically with the Cl$^-$ channel cystic fibrosis transmembrane conductance regulator (CFTR) (Matsubara et al. 2014).

Additional ion transport systems identified in the mature ES, such as K$^+$ conductance channels (Wu and Mori 1996), vacuolar-type H$^+$-ATPase (Stanković et al. 1997), Na$^+$/H$^+$-exchanger (NHE) (Wu and Mori 1998), pendrin (SLC26A4) (Dou et al. 2004), Cl$^-$-HCO3 exchanger (SLC4A2) (Stanković et al. 1997), and CA (Takumida et al. 1988, 1989; Tsujikawa et al. 1993), suggested that CA activity is involved in the formation of ES fluid. Na/K/2Cl cotransporter 2 (NKCC2) (Akiyama et al. 2007; Nishimura et al. 2009),
thiazide-sensitive Na/Cl cotransporter (NCC) (Akiyama et al. 2008; Møllere et al. 2015), and aquaporin have also been identified (Couloigner et al. 2004; Taguchi et al. 2007). Aldosterone may regulate Na⁺ transport via apical sodium channels, and Na/K-ATPase may also influence the effect of salt-reduced diet treatment in Meniere’s disease. Couloigner injected diuretics locally, such as Na/K/2CI and Na-Cl cotransporter inhibitors which failed to alter the ESP (Couloigner et al. 1998). They found that locally applied diuretics, such as acetazolamide and amiloride, acting in acid-base transports, are essential for the ESP. They recommended further studies on their influence on endolymph homeostasis for possible future treatments of Meniere’s disease (Couloigner et al. 1998).

In the present study, Na/K/2Cl cotransporter 2 was expressed together with the thiazide-sensitive Na/Cl cotransporter (NCC) as well as ENaC. Notably, valuable information has been provided from embryological studies and endolymphatic space development. Single-cell RNA analyses demonstrated transcriptomes in the MRCs, suggesting their role in “vectorial” ion transport (Honda et al. 2017). Cells showed a high activity of Na/K-ATPase and Na⁺ permeability. Absorption depended on SLC26A4 with the combined action of Na/K-ATPase and K⁺ channels in the basolateral membrane and Na⁺ permeable channels in the apical membrane (Akiyama et al. 2008; Kim and Wangemann 2010; Li et al. 2013; Honda et al. 2017).

“Reversed polarity” of Na/K-ATPase expression

Remarkably, Na/K-ATPase α1β3 heterodimers were localized in the apical cell membrane in some epithelial cells. Such “reversed polarity” has been described previously in the eye, choroid plexus, prostate, lacrimal gland, umbilical endothelial cells, and malignant cells with an isoform combination of α1β3 and is associated with a reversed fluid transfer (Wood and Mircheff 1986; Gundersen et al. 1991; Blanco 2005; Pierre and Xie 2006). The choroid plexus secretes cerebrospinal fluid (CSF) with Na/K-ATPase, K⁺ channels, Cl⁻/HCO3⁻ exchangers, Na/2Cl/K cotransporters, and a variety of Na⁺-coupled HCO3⁻ transporters involved (Brown et al. 2004). We also found antibody labeling of the α1 subunit in the apical cell membrane in the guinea pig choroid plexus. The α1β3 polarity in the human ES seems to validate earlier outcomes indicating that the ES also has a secretory capability (Erwall et al. 1988, 1989; Jansson and Rask-Andersen 1992). The α1 subunit was expressed in 40% of the epithelial cells suggesting that the ES may have a considerable secretory capability. However, the ubiquitous β1 subunit was expressed basolateral in 78% of the cells. Some of these cells did not have the typical cylindrical shape. Nonetheless, the findings may be indicative of a considerable absorptive potential of many ES epithelial cells.

Can surgery initiate ES secretion?

SR-SIM showed, surprisingly, that some cells expressed only the β3 subunit in the apical plasma membrane without the α1 subunit. Both proteins appeared individually in the cytoplasm, suggesting an independent transfer to the plasma membrane. The β subunit is believed to serve as a molecular chaperone for the α subunit and is required for its folding and processing in the Golgi to avoid degradation (Geering 2001). Subunits are synthesized by ribosomes on the endoplasmic reticulum membrane and undergo packing by disulfide bonds. Both subunits may be inserted independently into the endoplasmic reticulum and associate after glycosylation of the β subunit (Gundersen et al. 1991). The Na/K-ATPase α subunit exits from the endoplasmic reticulum and passes to the cell surface under the regulation of the β subunit. The time required for Na/K-ATPase to arrive at the cell surface after synthesis has been
estimated to be approximately 50 min with mandatory processing for its catalytic function before its arrival at the cell surface (Caplan et al. 1990). The β subunit may reach the plasma membrane without interacting with the α subunit. The reversal of the ankyrin/fodrin sub-membrane cytoskeleton is thought to be responsible for the polarized distribution of the Na/K-ATPase (Gundersen et al. 1991) via an interaction with the α subunit of Na/K-ATPase protein (Nelson and Veshnock 1987; Morrow et al. 1989; Wetzel et al. 1999). An intriguing explanation for the solitary β3 incorporation in the apical plasma membrane (Fig. 5a) may be the swift transcription and migration of β subunit mRNAs triggered by inner ear surgery. Labyrinth ablation reduces fluid pressure 1–2 h before collecting the tissue, and in the meantime, the expression of pre-existing Na+ pumps via aldosterone or other instruments (Feraille and Dizin 2016) could increase in an attempt to restore fluid pressure.

Is the Na/K-ATPase pump responsible for initiating longitudinal endolymph outflow?

Experimental studies provide little evidence of an endolymph "flow" (Salt and DeMott 1997), a situation possibly altered under stress and various physiological demands (Rask-
A trans-cellular transport of ions is consistent with observations of increased tight junction barriers in the distal ES as demonstrated by freeze fracturing, tracer studies, and morphological observations (Rask-Andersen et al. 1981; Friberg et al. 1984b; Hultgård-Ekwall et al. 2003, 2006). Active cellular pumps in the intraosseous ES could induce a longitudinal “syphon” of endolymph (Møller et al. 2013). There are wide intercellular spaces next to the MRCs in the murine ES, indicating an active bulk flow across the epithelium of the ES (Friberg et al. 1985). In man and guinea pig, a gradual para-cellular outflow may ensue already in the ED where tight junctions (TJs) are shallow and the epithelium is surrounded by specialized vascularity (Rask-Andersen et al. 1981; Bagger-Sjöbäck and Rask-Anderson 1986). Larger substances may accrue in the distal ES. This assumption is supported by immune histochemical studies (Ichimiya et al. 1994). The cylindrical and “light” cells may correspond to the MRCs (Erwall et al. 1988; Qvortrup et al. 1999; Møller et al. 2013) and are believed to be involved in fluid absorption (Bagger-Sjöbäck et al. 1986). The saccular endolymph is rich in potassium and low in sodium, and these solutes may equilibrate across the membrane system to the ES. Earlier studies showed that the ES may both absorb and secrete depending on the physiological demands (Rask-Andersen et al. 1999). The present results of a polarized expression of Na/K-ATPase in the human ES support such an action, and its interpretation is shown in Fig. 9. This may cast new light on the pathophysiology of MD since both a lowered absorption and a hypersecretion could be responsible for the accumulation of fluid and lead to endolymphatic hydrops.

A “polarized” expression of Na/K-ATPase consistent with both Na and fluid absorption and secretion in combination with hormone receptors may have implications for the etiology of MD. Intra-tympanic gentamicin is widely used in refractory MD. Notably, intra-tympanic methylprednisolone was recently shown to be as effective (Patel et al. 2016). Glucocorticoids also bind to the human mineralocorticoid receptor that regulates the transcription of the human Na/K-ATPase (Kolla and Litwack 2000). Furthermore, steroid treatment in MD may modulate the inflammatory response and/or restore endolymph ion balance. The anti-inflammatory effect of glucocorticoids is also linked to the suppression of the transcription factor nuclear factor-kappa beta (NF-κB) which plays a key role in regulating the immune response. In a recent case-control study (Kolla and Litwack 2000), the authors found a gene locus associated with bilateral MD suggesting that carriers may develop a NF-κB-mediated inflammatory response in MD. Friis et al. (2013) found that the ES of rats that were isoimmunized with an ES extract developed specific tissue alterations identical to those present in the ES of active MD. Those findings support the theory that an immune injury may be important in the pathogenesis in MD. Further studies, in combination with additional ion transporter systems, are necessary to fully understand the organization of this system at the molecular level and how it may relate to disease. Nonetheless, careful interpretation is needed since obtained tissue is derived from patients with benign tumors which could modify the inner ear steady-state conditions.
Ethical approval The study of human discarded materials was approved by the local ethics committee (Ettäpprovningssändningen Uppsala, no. 99398, 22/9 1999, cont. 2003, no. C254/4; no. C457/2 2007, Dnr. 2013/190), and informed consent was obtained. All procedures performed in this study involving human participants were in accordance with the ethical standards of the local research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Sections of archival guinea pig cochlea were also analyzed; the protocol was approved by the Regional Animal Review Board of Uppsala, Sweden (permit number C89/12 and C66/16). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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