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Na^+ absorption by Claudius' cells is regulated by purinergic signaling in the cochlea

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Running title: **Na^+ absorption and purinergic regulation in Claudius' cells**

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Abstract

Conclusion: Claudius' cells absorb Na^+ through the amiloride-sensitive epithelial sodium channel (ENaC). Transepithelial ion transport through ENaC and possibly a Cl^- secretory pathway is regulated by P2Y purinergic signaling.

Objectives: The purpose of this study was to investigate the ion transport in the Claudius' cells and its purinergic regulation.

Method: Young adult Sprague–Dawley rats and gerbils were studied. The Claudius' cell layer on the basilar membrane was dissected from the basal turn of the cochlea. The voltage-sensitive vibrating probe was used to measure transepithelial short circuit current (I_{sc}). The baseline I_{sc} of Claudius' cells was measured in the perilymph-like control solution and the change of I_{sc} after application of amiloride (10 μM) or uridine triphosphate (UTP, 100 μM).

Results: A negative baseline I_{sc} was observed in the control solution (-12.50 ± 3.95 μA/cm^2, n=8) and the addition of amiloride resulted in a decrease of I_{sc} by 75.8%. The application of UTP, an agonist for P2Y purinergic receptors, led to a partial inhibition of I_{sc} (by 38.2 ± 3.2%, n=5), and subsequent addition of amiloride abolished the remaining I_{sc}.

Keywords : epithelial sodium channel, purinergic receptor, voltage-sensitive vibrating probe, rat, gerbil

Introduction

Endolymph maintains a unique ion concentration that presents high-K^+ (approximately 150 mM) and low Na^+ (approximately 1 mM). This unusual extracellular fluid composition of the endolymph is important for proper auditory transduction. K^+ transport mechanisms in the cochlea have been elucidated [1] and the processes involved in Na^+ transport in the cochlea have also been reported recently [2]. Epithelial cells of the Reissner's membrane (RM) [3-5] and outer sulcus cells [6] were reported to contribute to endolymphatic homeostasis by absorbing Na^+.

Claudius' cells form an epithelial sheet on the basilar membrane and continue partially up the cochlear lateral wall [7]. Claudius' cells form a functional syncytium through formation of gap junctions and are in the middle of a putative lateral K^+ recycling pathway [7]. On the other hand, it was reported that the strongest mRNA expressions of amiloride-sensitive epithelial Na^+ channel (ENaC) subunits were observed in the Claudius' cells of rats by *in situ* hybridization [8]. Thus Claudius' cells may also have capability to absorb Na^+ from the endolymph.

Variations in the intensity and duration of acoustic stimuli would cause fluctuations in endolymph cation composition if there were no regulation of the rates of secretion and/or absorption. Secretion and absorption in the cochlea are known to be regulated by purinergic signaling [9,10]. Taken together we investigated the possibilities of Na^+ absorption through ENaC and its purinergic regulation in the Claudius' cells using voltage-sensitive vibrating probe technique, which is suitable for the study of transepithelial vectorial ion transport in small epithelial domains.

Materials and methods

Tissue preparation

Sprague–Dawley rats at postnatal 3 weeks were studied. Rats were anesthetized with sodium pentobarbital (50–100mg/kg intraperitoneally) and were killed under a protocol approved by the Institutional Animal Care and Use Committee of Seoul National University (IACUC No. 10-0056 issued on May 19, 2010) to remove the temporal bones. Temporal bones from gerbils were similarly obtained under a protocol approved by the Institutional Animal Care and Use Committee of Kansas State University (IACUC No. 2925). The temporal bones were removed from rats and cochleae were dissected. After removal of the bony portion of cochlea, the lateral wall of the basal turn cochlea was isolated. The stria vascularis and the remaining Reissner's membrane were removed from the lateral wall using a fine forceps. The Claudius' cell layer remained on the basilar membrane attached to the medial part of the lateral wall. The Claudius' cell layer including the lateral wall was folded with the apical membrane of the Claudius' cells facing outward and mounted in a perfusion chamber on the stage of an inverted microscope (Fig. 1) (Olympus IX70, Olympus, Pennsylvania, USA). The mounted tissue was continuously perfused at 37°C at an exchange rate of 1.1 times/s.

Voltage-sensitive vibrating probe

The voltage-sensitive vibrating probe technique was used to measure transepithelial currents under short circuit conditions. The current density (I_{sc}) was monitored by vibrating a platinum–iridium wire microelectrode that was insulated with parylene-C (Micro Electrodes, Gaithersburg, Maryland, USA), which had been coated with platinum black on the exposed tip. The vibration was approximately 20 μm along both horizontal (x) and vertical (z) axes. The x-axis was

perpendicular to the face of the epithelium. The probe was positioned 20 μm from the apical surface of the folded Claudius' cell layer with computer-controlled, stepper-motor manipulators (Applicable Electronics, Forest Dale, Massachusetts, USA) and specialized probe software (ASET version 1.05, Science Wares, East Falmouth, Massachusetts, USA). The bath references were 26-gauge platinum-black electrodes.

Calibration was performed in the physiologic saline (see below) using a glass microelectrode (tip <1 mm outer diameter) filled with 3M KCl as a point source of current. The frequencies of vibration were in the range of 200–400 Hz and were well separated for the two orthogonal directions. The signals from the oscillators driving the probe were also fed to a dual-channel phase-sensitive detector. Asymmetry of the probe design yielded different resonant frequencies for the two directions of vibration. The signals of the x and z detectors were connected to a 16-bit analog-to-digital converter (CIO-DAS1602/16, Computer Boards, Mansfield, Massachusetts, USA) in a Pentium IV, 700MHz computer and sampled at an interval of 0.6 s. The electrode was positioned where I_{sc} showed a maximum x-value and minimum z-value; data are expressed as the x-value of the current density and plotted with Origin 6.1 software (Origin Lab Software, Northampton, Massachusetts, USA).

Solutions and chemicals

In all experiments, both sides of the epithelium were perfused with a perilymph-like physiologic saline containing (in mM) 150 NaCl, 3.6 KCl, 1 $MgCl_2$, 0.7 $CaCl_2$, 5 glucose, 10 HEPES at pH 7.4. All drugs were purchased from Sigma (St. Louis, Missouri, USA). Amiloride was dissolved in dimethyl sulfoxide (DMSO) and then diluted to 0.1% DMSO in the control solution before application. DMSO at this concentration had no effect on the short circuit current.

Data presentation and statistics

Data were expressed as mean ± SEM (*n*=number of tissues) of the I_{sc}. Increases or decreases in I_{sc} were considered significant at a *P* value of less than 0.05. Paired *t*-test was used for statistical analysis.

Results

We measured baseline I_{sc} of Claudius' cells in the control solution and the change of I_{sc} after application of amiloride (10 μM). Negative baseline I_{sc} values of -12.50 ± 3.95 μA/cm^2 (*n*=8, Fig. 2) were observed in the control solution, demonstrating net cation absorption and/or net anion secretion.

Administration of amiloride led to a significant inhibition of the baseline I_{sc} (75.8 ± 12.6%) which was changed to -3.03 ± 2.93 μA/cm^2 (*n*=8, $p < 0.05$, Fig. 2). We also measured the change of I_{sc} after administration of uridine triphosphate (UTP, 100 μM). The application of UTP led to a partial inhibition of I_{sc} (by 38.2 ± 3.2%, *n*=5, $p < 0.05$), and subsequent addition of amiloride abolished the remaining I_{sc} (Fig. 3, Table 1). In contrast, the application of UTP after pretreatment of amiloride, which abolished most of baseline I_{sc}, showed no change of I_{sc} (*n*=5, Fig. 4, Table 1).

Similar inhibitions of I_{sc} by amiloride and the purinergic agonist adenosine triphophate (ATP) were observed (Fig. 5). Taken together, these results indicate that purinergic agonists partially inhibit ENaC-mediated Na^+ absorption in the Claudius'cells of rat and gerbil.

Discussion

Claudius' cells are cuboid cells that cover the endolymphatic side of the basilar membrane and extend along the width of the basilar membrane from Hensen's cells to the spiral prominence of the basal turn [7].

They form a tight cellular border between endolymph of the scala media and perilymph of the scala tympani. Their apical surface contains short microvilli, and their borders are sealed on their endolymphatic surface by tight junctions [11]. Claudius' cells were regarded as a pathway for K^+ recycling [7]. However, our results showed for the first time that the Claudius' cells absorb Na^+ through ENaC and they are regulated by UTP-responsive P2Y purinergic signaling. This functional scheme was also observed in gerbil Claudius' cells and is in exact accordance with the process in the epithelial cells of the Reissner's membrane [10].

The majority of the baseline I_{sc} was reduced by application of 10 μM amiloride, which indicates that Na^+ absorption via ENaC is a major ion transport in the Claudius' cells. The electrical driving force across the apical membrane *in vivo* is the sum of the endocochlear potential of about +80 mV and the likely basolateral membrane potential of about -100 mV [7,12] or a total of 180 mV. If intracellular Na^+ is maintained at about 10 mM, there will be a chemical driving force of -60 mV for Na^+ since endolymphatic Na^+ is about 1 mM in the cochlea. It is conceivable that the inward net electrochemical driving force for Na^+ across the apical membrane (180-60=120 mV) would therefore strongly drive Na^+ absorption and consequently contribute to maintain Na^+ low in the endolymph. This Na^+ movement might be associated with endolymphatic homeostasis via aquaporin 4 which was expressed in the Claudius' cells of human temporal bone demonstrated by immunohistochemistry [13]. Interestingly, aquaporin 4 knockout mice were reported to exhibit an elevation in the auditory brainstem threshold [14].

On the other hand, it should be noted that there was still remaining I_{sc} (about 24.2% of the baseline I_{sc}) after application of amiloride. It is unknown as yet which ion transport accounts for this remaining I_{sc}, although electrogenic Cl^- secretion would create an I_{sc} of the same polarity. This result is quite different from the previous report [10] which showed overshoot above the baseline (positive I_{sc} values) after application of amiloride at the same concentration in the epithelial cells of the Reissner's membrane.

Many cells in the endolymphatic duct do not have cell-to-cell gap junction communication [7] and therefore have to use another signal pathway to coordinate their activity. In this aspect extracellular nucleotides (ATP and UTP) and their purinergic receptors have an important role in paracrine and autocrine communication systems in the inner ear [9]. ATP is released from most cell types and acts as a natural agonist for purinergic receptors. UTP is also released from cultured cells of different tissues and may function as a natural agonist [15]. These extracellular nucleotides are coupled to purinergic receptors that are divided as ionotropic (P2X) and metabotropic (P1 and P2Y) receptors in several isoforms. The P2X channels are ATP-gated ion channels and metabotropic P2Y receptors are made of different subtypes of G protein-coupled nucleotide receptors. UTP used in this study is an agonist effective only to specific P2Y receptors [16]. Our results agree with previous reports in other epithelial cell types in the viewpoint that activation of P2Y receptors by 100 μM UTP resulted in a partial inhibition of amiloride-sensitive Na^+ transport (38.2%, Fig. 3). This partial inhibition at this concentration has been reported in other epithelia, i.e., 42.9% in gerbil Reissner's membrane [10], 76% in human bronchial epithelia [17], 40% in normal human airways[18], and 49.1% in mouse distal nephron [19]. It can be speculated that like in many other epithelia extracellular ATP or UTP decreases ENaC activity through P2Y purinergic signaling by depletion of phosphatidylinositol 4,5-bisphosphate

[$PI(4,5)P_2$] in the plasma membrane of Claudius' cells [10,18,20]. In addition, purinergic signaling in Claudius' cells may inhibit a putative Cl^- secretion.

From a physiologic point of view, it has been proposed that a nucleotide such as ATP reduces the sensitivity of sound transduction, especially under the condition of noise exposure. For example, if noise exposure elevates ATP levels in the scala media, then parasensory K^+ extrusion from endolymph via ionotropic P2X receptor would be increased and K^+ transport into scala media from the stria vascularis by activation of metabotropic P2Y4 receptor would be decreased [9]. Na^+ absorption via the Reissner's membrane epithelium [10] and Claudius' cells would be reduced by activation of P2Y receptors, leading to an increase of Na^+ concentration in the scala media. On the other hand, activation of P2X receptors in the apical membrane of many of the cells in scala media [9] by endolymphatic ATP would counteract the effect of P2Y receptors on Na^+ concentration. Therefore, the net movement of endolymphatic Na^+ would be regulated by a balance of P2X and P2Y purinergic signaling.

In conclusion, the Claudius' cells absorb Na^+ through ENaC. Transepithelial ion transport through ENaC, and possibly a Cl^- secretory pathway, is regulated by P2Y purinergic signaling.

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Figure Legends

Figure 1. Tissue preparation for Claudius' cell layer. The lateral wall of the basal turn of the cochlea from rat (*top panel*) and gerbil (*bottom panel*), along with a portion of basilar membrane where Claudius' cells reside, was dissected from the cochlea. The stria vascularis and Reissner's membrane were removed from the lateral wall and the Claudius' cell layer with the lateral wall was folded in a loop with the apical membrane of the Claudius' cells facing outwards. *SL*, spiral ligament; *VP*, vibrating probe.

Figure 2. Summary of effect of amiloride (10 μM) on I_{sc} of rat Claudius' cells. Administration of amiloride led to a 75.8% inhibition of the baseline I_{sc} (n=8). Data traces are averages and the error bars (SEM) are only shown at intervals for clarity.

Figure 3. Summary of effect of UTP (100 μM) on Isc of rat Claudius' cells in the absence of amiloride. The application of UTP led to a 38.2% inhibition of the baseline I_{sc}, and subsequent addition of amiloride (10 μM) inhibited the remaining I_{sc} (n=5). Data traces are averages and the error bars (SEM) are only shown at intervals for clarity.

Figure 4. Summary of effect of UTP (100 μM) on I_{sc} of rat Claudius' cells in the presence of amiloride (10 μM). The application of UTP after pretreatment of amiloride showed no change of the baseline I_{sc} (n=5). Data traces are averages and the error bars (SEM) are only shown at intervals for clarity.

Figure 5. Representative effects of ATP (300 μM) and amiloride (amil; 10 μM) on I_{sc} of gerbil Claudius' cells. The greater apparent electrical noise compared to figures 2 – 4 is due to the absence of averaging. These responses are typical of 2 (ATP) – 3 (amiloride) experiments.