INWARD-RECTIFIER CHLORIDE CURRENTS IN REISSNER'S MEMBRANE EPITHELIAL CELLS

by

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Abstract

Sensory transduction in the cochlea depends on regulated ion secretion and absorption. Results of whole-organ experiments suggested that Reissner's membrane may play a role in the control of luminal Cl^-. We tested for the presence of Cl^- transport pathways in isolated mouse Reissner's membrane using whole-cell patch clamp recordings and gene transcript analyses using RT-PCR. The current-voltage (I-V) relationship in the presence of symmetrical NMDG-Cl was strongly inward-rectifying at negative voltages, with a small outward current at positive voltages. The inward-rectifying component of the I-V curve had several properties similar to those of the ClC-2 Cl^- channel. It was stimulated by extracellular acidity and inhibited by extracellular Cd^{2+}, Zn^{2+}, and intracellular ClC-2 antibody. Channel transcripts expressed in Reissner's membrane include ClC-2, Slc26a7 and ClC-Ka, but not Cftr, ClC-1, ClCa1, ClCa2, ClCa3, ClCa4, Slc26a9, ClC-Kb, Best1, Best2, Best3 or the beta-subunit of ClC-K, barttin. ClC-2 is the only molecularly-identified channel present that is a strong inward rectifier. This thesis incorporates the publication by KX Kim and DC Marcus, Inward-rectifier chloride currents in Reissner's membrane epithelial cells, *Biochem. Biophys. Res. Commun.*, doi:10.1016/j.bbrc.2010.03.048, 2010 (in press) with permission of the publisher Elsevier, and is the first report of conductive Cl^- transport in epithelial cells of Reissner's membrane and is consistent with an important role in endolymph anion homeostasis.

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Dedication

This dissertation is dedicated to my father Yongnam Kim and my mother Kyungja Lee.

CHAPTER 1 - Introduction

Hearing depends on proper transduction of sound to nerve impulses in the cochlea, the peripheral hearing organ. Transduction depends on a carefully controlled ionic composition of the fluid, endolymph, in the cochlear lumen. The work presented in this thesis is a contribution toward understanding the processes that control endolymph composition and that thereby support normal hearing.

Cochlear structure: Knowing the cochlear structure is important for understanding ion movement mechanisms in the cochlea because the cochlear structure provides us the clues of ion transport pathways. Although there are differences of cochlear structure (such as number of turns) among different species, many similarities have been observed between human specimens and rodents [31]. In this study, we used C57BL/6 mice.

The cochlea has a spiral structure. One cochlear turn is illustrated in Figure 1. The light pink region indicates endolymph, which fills the luminal compartment, scala media (SM). The endolymph has a high K^+ concentration (Table 1) with high endocochlear potential (EP; 80-100 mV) with respect to other "grounded" body fluids, such as blood (the EP is generated by stria vascularis (StV) [22]), whereas the scala vestibuli (SV) and scala tympani (ST) are filled with perilymph, which is characterized by a high Na^+ concentration (Table 1). Main sources to maintain and drive the scala media ion composition can be identified in epithelial cells that bound this structure. These include Reissner's membrane epithelial cells, strial marginal cells of stria vascularis, spiral prominence, outer sulcus, sensory epithelium of organ of Corti, and inner

sulcus. Net ion transport via epithelial cells surrounding endolymph will contribute to cochlear homeostasis [21].

Table 1. Fluid composition of cochlear endolymph and perilymph [22]

	Unit	Cochlear Endolymph	Cochlear Perilymph
Na^+	mM	1.3	148
K^+	mM	157	4.2
Cl^-	mM	132	119
HCO_3^-	mM	31	21
Ca^{2+}	mM	0.023	1.3
pH		7.5	7.3

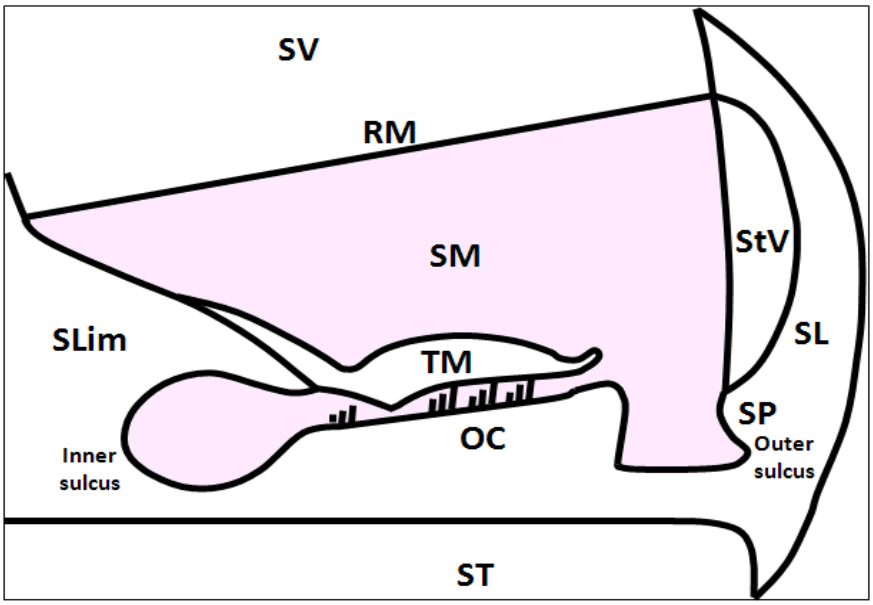

Figure 1 - Schematic drawing of one cochlear turn. The scala vestibuli (SV) and scala tympani (ST) are filled with a high-sodium solution called the perilymph. The scala media (SM) is filled with a high-potassium solution called the endolymph and is surrounded by the stria vascularis (StV), Reissner's membrane (RM), inner sulcus, the organ of Corti (OC), outer sulcus, and spiral prominence (SP). The lateral wall contains the stria vascularis (StV) and the spiral ligament (SL). The tectorial membrane (TM) is an acellular structure that couples the mechanical auditory stimulus to the hair cells in the organ of Corti. Individual cells are not shown.

Reissner's membrane: The scala vestibuli and scala media are separated by Reissner's membrane, which consists of two cell layers: a continuous epithelial cell layer faces the endolymph and a discontinuous mesothelial cell layer faces the perilymph. In addition, Reissner's membrane is attached to insertions of both spiral limbus and spiral ligament. These distinct tissues at the insertion regions require careful exclusion from experimental measurements. For example, possible contamination in RNA isolation procedures can occur, but care was taken to exclude the adjacent tissues and we changed the dissection solution twice during the isolation procedure to reduce cross contamination from other tissues.

Several studies have been performed to determine ion transport mechanisms in Reissner's membrane. Na^+ absorption in the epithelial cells of Reissner's membrane was demonstrated by pharmacological agents with electrophysiological measurements of transepithelial current using a vibrating probe [17;20]. An ATP-gated ion channel was observed in the apical side of Reissner's membrane epithelial cells [18]. Cochlear perfusion studies suggested that the primary function of Reissner's membrane may be to transport Cl^- [19].

Although Cl^- ion concentrations are similar between endolymph and perilymph (Table 1), the presence of the high endocochlear potential suggests that there is likely significant Cl^- transport by the bounding epithelium. Because Reissner's membrane forms much of the boundary between these two fluids, and because previous studies implicated Reissner's membrane in Cl^- transport (see Chapter 2), it became important to investigate Cl^- ion transport in Reissner's membrane in order to increase our understanding of ion homeostasis in the cochlea.

CHAPTER 2 - Inward-rectifier chloride currents in Reissner's membrane epithelial cells

These data have been published in the following refereed journal article:

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Introduction

The transduction of sound into neural activity depends on the creation and maintenance of a luminal fluid, endolymph, in the inner ear that is high in K^+ concentration ($[K^+]$) and low in both $[Na^+]$ and $[Ca^{2+}]$ [22]. However, there is little difference in $[Cl^-]$ (~120 to 130 mM) between endolymph and the basolateral fluid, perilymph, in spite of the large transepithelial endocochlear potential (EP) of +80 to +100 mV [22]. The EP and perilymphatic $[Cl^-]$ predict (via the Nernst equation) an extremely high endolymphatic $[Cl^-]$ of ~2600 mM based on simple passive electrochemical diffusion. Dysfunction of Cl^- regulation would be expected to lead to large osmotic disturbances that would result in luminal volume changes and the consequent disruption of normal hearing. Gross volume changes have been associated with pathological states such as Meniere's syndrome (swelling) and Schiebe's deformity (shrinking).

On that basis, it has long been thought that some epithelial cells lining the cochlear duct may actively absorb Cl^- from endolymph to maintain its $[Cl^-]$ near that of perilymph, and radiotracer experiments in the intact cochlea point to Reissner's membrane as a mediator of Cl^- transport [19]. Reissner's membrane is an epithelial monolayer (with a discontinuous mesothelial layer on the basolateral side) that forms much of the boundary of the cochlear lumen. The present study was undertaken to resolve at the single cell level whether there are significant Cl^- conductive pathways in Reissner's membrane epithelial cells that could support its putative role in endolymph Cl^- homeostasis.

Methods

Tissues were obtained for RNA isolation and for electrophysiology following protocols approved by the Institutional Animal Care and Use Committee of Kansas State University as described earlier [17]. The compositions of the solutions for electrophysiological recordings were (in mM): pipette 150 NMDG-Cl, 1 $MgCl_2$, 0.273 $CaCl_2$, 1 EGTA, 10 Hepes, pH 7.3, ~300 mOsm, and bath 150 NMDG-Cl, 1 $MgCl_2$, 0.7 $CaCl_2$, 10 Hepes, 5 glucose, pH 7.3, ~300 mOsm. The pH was adjusted at room temperature (~25 ^oC) and expected to be about 7.2 at 37 ^oC. The free Ca^{2+} at this temperature and pH is predicted to be 100 nM [30]. All solutions for patch clamp were passed through 0.22 μm cellulose acetate filters (Corning). ClC-2 antibody against an intracellular domain was obtained from Alomone Labs. Other chemicals were purchased from Sigma Chemical Co. (St Louis, MO).

Currents were recorded using the whole-cell configuration of the patch clamp technique, similar to our previous study [3]. Patch pipettes were made from borosilicate glass capillaries (1B150F; World Precision Instruments, Sarasota, FL), pulled in three stages. Inner diameter of the tip was approximately 2 μm and after heat polishing the pipettes had resistances of 3.6 – 5.2 MΩ (n=46) in NMDG-Cl solutions.

Currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and low-pass filtered at 1 kHz. Current signals were digitized at 5 kHz using a computer with a Digidata 1322A (Axon Instruments) and pCLAMP 9 software (clampex9, Axon Instruments). In addition, AxoScope software (Axon Instruments) with MiniDigi 1A (Axon Instruments) data acquisition hardware was simultaneously used for continuous trace recordings and current signals were digitized at 1 kHz. The temperature was maintained at 37°C on a glass-

bottomed bath chamber by a continuous, warmed perfusion with supplemental chamber heater. Liquid junction potentials in symmetrical NMDG-Cl were near zero. Voltage protocols were used as described in the figures. Data were plotted with Origin software, version 7 (OriginLab Software, Northampton, MA).

Real-time RT-PCR experiments were performed on total RNA using QuantiTect SYBR Green RT-PCR Kit (Qiagen) and an iQ5 Real-Time PCR Detection System (Bio-Rad). Primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3) and produced by Integrated DNA Technologies (Table 1). Reverse transcription for 30 min at 50 ºC was followed by 15 min at 95 ºC and 40 PCR cycles. Each PCR cycle consisted of 94 ºC for 20 sec, 56 ºC for 30 sec, and 72 ºC for 30 sec and readings of fluorescence were made at 78 ^{o}C. PCR products were analyzed by Bioanalyzer, purified with a PCR purification kit (Qiagen) and sequenced to validate the identity of the RT-PCR products.

Data were expressed as the mean ± S.E.M. (n=number of whole cell patches). Increases and decreases in current and conductance were determined by Student's paired or unpaired t-test and correlation coefficients were calculated and tested for significance. Differences were considered statistically significant at a level of $P < 0.05$.

Table 1. Primer sequences for RT-PCR and expression of gene transcripts.

Gene		Sequence (5´-3´)	Product Size (bp) Exp. /Meas.[a]	GenBank	P / A[b]
Slc26a7	S	GGAAAAAGAGAAGCGTGCTG	309 / 317	NM_145947	P
	AS	AGGATGTCAAGGCAAAGGTG			
Slc26a9	S	CCTGACTGCTGTCATCCAGA	324/323	NM_177243	A
	AS	GTAGGGATGGGGAAGTGGAT			
ClC-1	S	CTGGGTCACCTTCCCACTTA	292 / 284	NM_013491	A
	AS	TGGCTGCTCATAGACACCAG			
ClC-2	S	CTGGATGTCTGCACTGGCTA	271 / 271	NM_009900	P
	AS	AGGCAGAATGTGAGCGATCT			
ClC-Ka	S	ACTCCCAGAGCTGAAGACCA	337 / 337	NM_024412	P
	AS	CCAGACGGAGAAGTGAGAGG			
Barttin	S	CAGAGCCTCCCAGACTTCAC	399 / 387	NM_080458	A
	AS	TGTAGGGGTGTCGTCAATCA			
Best1	S	TACAAGCGCTTTCCCACTCT	366/376	NM_011913	A
	AS	CATCTCATGGCCTGGGTAGT			
Best3	S	GCTGCCGACTACTGCATACC	368/362	NM_001007583	A
	AS	GTCTCCCTGATGGTGGACAG			
ClCa1	S	CTACAAGTGGCAGCGTCTCC	367/358	NM_009899	A
	AS	GCAGTAGCCAGGAGTGGTTC			

S, sense primer; AS, antisense primer; Product Size, length in the base pairs (bp) of RT-PCR product including primers. [a]Expected/Measured product size; [b]**P**resent/**A**bsent.

Results

Whole cell patch clamp recordings from Reissner's membrane epithelial cells were made under conditions where Cl^- was the only major permeating ion. The Cl^- currents were characterized by a) strong inward-rectification with slow activation at negative voltages and b) weak outward-rectification (Fig. 1). The prominent inwardly-rectifying currents were similar to those described for ClC-2 and were investigated in more detail.

We tested the effects of agents (external pH, Cd^{2+}, Zn^{2+} and intracellular ClC-2 antibody) known to stimulate and inhibit ClC-2 Cl^- channels on the Cl^- currents in Reissner's membrane epithelial cells (Figs. 2, 3, 4).

Acidifying the bath pH from 7.2 to 6.7 caused a reversible increase in I_{-100} by 79.4 ± 11.1 % (from -104 ± 26 pA to -181 ± 37 pA, n=5) (Fig. 2A). By contrast, alkalinizing the bath pH from 7.2 to 7.7 caused a reversible decrease in I_{-100} by 37.9 ± 3.7 % (from -106 ± 37 pA to -69 ± 28 pA, n=4) (Fig. 2B). These pH changes are in the monophasic pH response region of inward-rectifier Cl^- channels in mouse parotid acinar cells [1].

Similar experiments were performed with Zn^{2+} (Fig. 3A) and Cd^{2+} (Fig. 3B) at concentrations known to inhibit ClC-2 channels [12;38]. I_{-100} was reversibly decreased by 50 µM Zn^{2+} by 45.6 ± 7.5 % (from -248 ± 24 pA to -132 ± 15 pA, n=4) and by 500 µM Cd^{2+} by 45.3 ± 7.1 % (from -138 ± 28 pA to -79 ± 23 pA, n=5).

Antibodies against intracellular epitopes of ClC-2 have been reported to block inward-rectifier Cl^- currents in native cells [9;27]. Intracellular ClC-2 antibody (3 µg/ml) [9] significantly reduced the conductance at -120 mV from 11.5 ± 2.5 nS (control with heat-inactivated antibody) to 3.8 ± 1.1 nS, n=5 (Fig. 4).

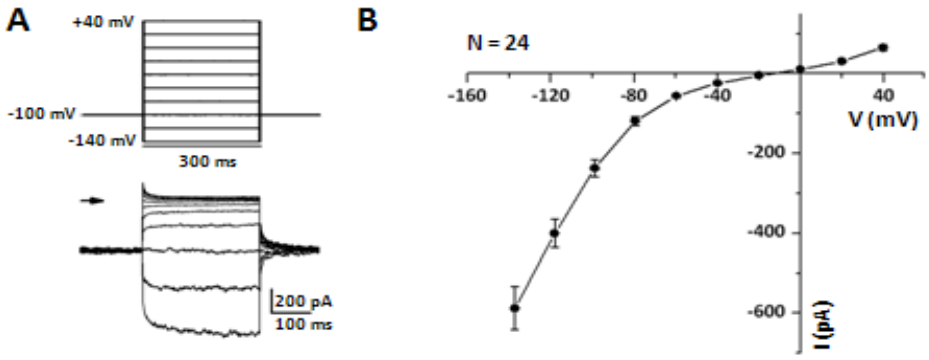

Figure 1. Strong inward-rectifier and smaller outward Cl^- currents. *A*, Step pulses were applied from -140 mV to +40 mV, returning to the holding voltage -100 mV. *B*, The mean current voltage relationship was obtained from 24 cells.

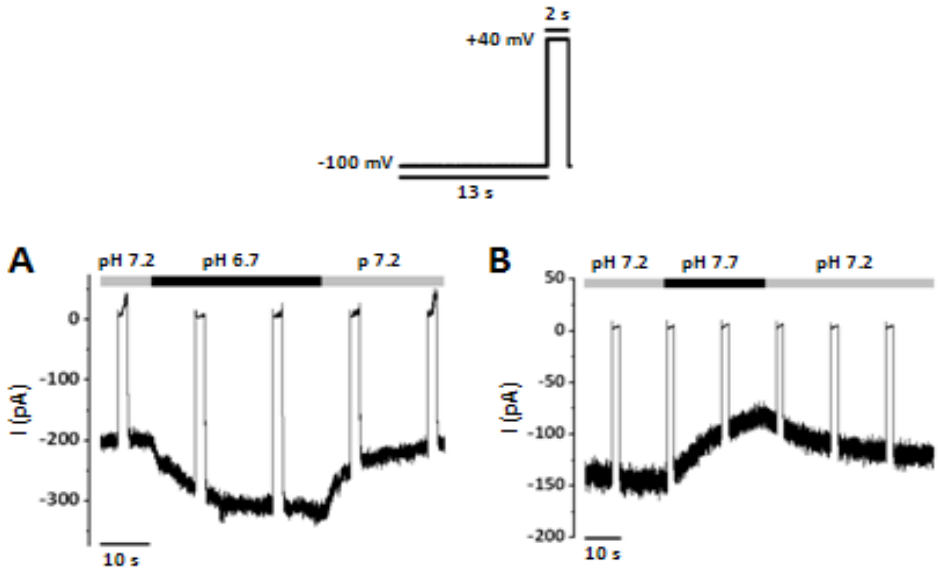

Figure 2. Dependence of inward-rectifier Cl^- currents on pH. The voltage protocol consisted of holding at -100 mV for 13 s with a 2 s pulse at +40 mV. All effects of pH were reversible. *A*, The activation of the current at -100 mV by external acidification from pH 7.2 to pH 6.7. *B*, The inhibition of the current at -100 mV by external alkalinization from pH 7.2 to pH 7.7.

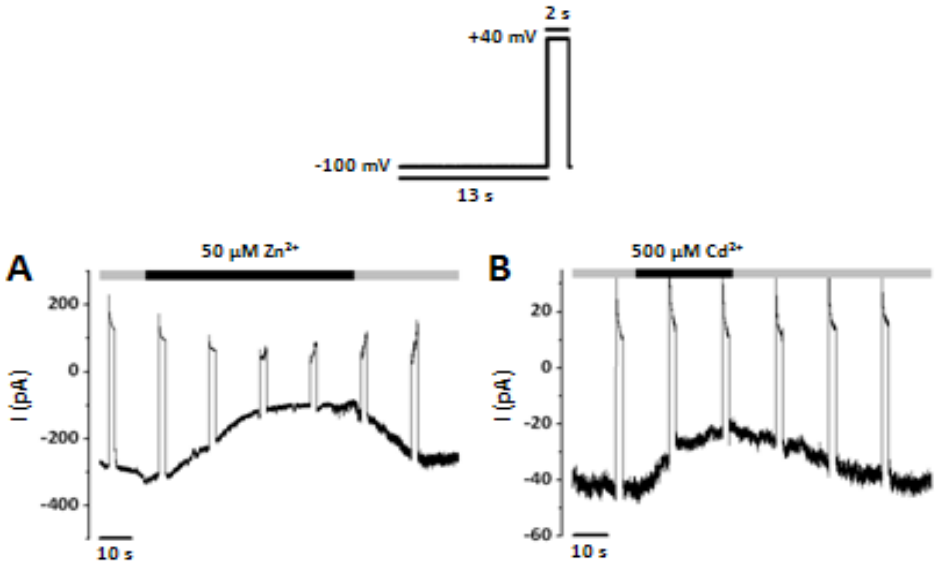

Figure 3. Dependence of inward-rectifier Cl^- currents on inhibition by Zn^{2+} and Cd^{2+}. Representative recordings; voltage protocol as in Figure 2. All effects of Zn^{2+} and Cd^{2+} were reversible. *A*, The inhibition of the current at -100 mV by 50 µM Zn^{2+}. *B*, The inhibition of the current at -100 mV by 500 µM Cd^{2+}.

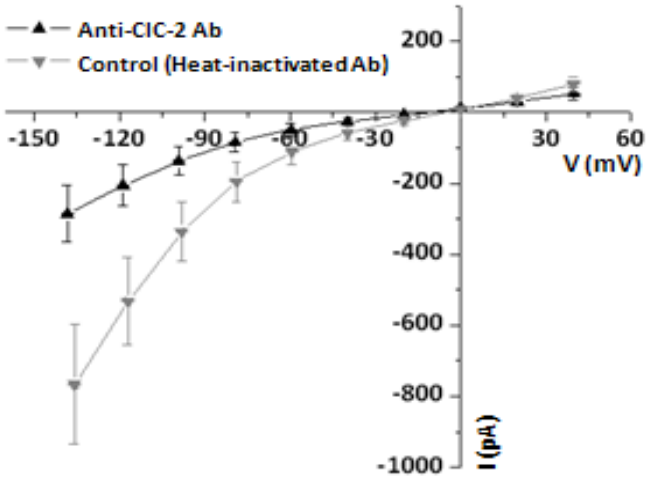

Figure 4. Inhibition of inward-rectifier Cl^- currents by ClC-2 antibody. Summary I-V relationships; voltage protocol as in Figure 1. Currents recorded with antibody (3 μg/ml) raised against an intracellular epitope of ClC-2 added to the pipette solution (Anti-ClC-2 Ab; *up triangles*) were significantly reduced at negative membrane voltages compared to those serving as "Control" with heat-inactivated antibody (*down triangles*).

Candidate anion channel genes were determined by their presence call in our gene array database (GEO accession number GSE6196 [17]), compared to expression levels in the neighboring tissue, stria vascularis (GSE4749 [11]). Genes related to Na^+ absorption and its regulation in Reissner's membrane were reported previously [17]. Several Cl^- channels were found to be present (Table S1). ClCa1 was called 'present' by the gene array, but the signal strength was less than twice the background level of the chips.

On the basis of those results, RT-PCR experiments were conducted to validate the presence or absence of selected genes (Table 1, Fig. S1). Cl^- channels for which mRNA was present and which are known to be located in the plasma membrane include ClC-2, Slc26a7 and ClC-Ka. Interestingly, the beta-subunit of ClC-K (barttin) was not expressed in Reissner's membrane. Cl^- channels for which mRNA was not detectedinclude Cftr, ClC-1, ClCa1, ClCa2, ClCa3, ClCa4, Slc26a9, ClC-Kb, Best1, Best2, Best3.

These results are not specific to the epithelial cells since Reissner's membrane also consists of a discontinuous subepithelial layer of mesothelial cells. Whole cell currents, however, originated solely from the epithelial cells.

Table S1. Gene array detection of Cl^- channels in Reissner's membrane.

Gene	Affymetrix Probe Set ID	Average Signal		Ratio (RM/SV)	Present (P) Marginal(M) Absent (A)
		RM[a] Mean	SV[b] Mean		
Cftr	1420579_s_at	8	52	0.2	AAA/APA[d]
Cftr	1427767_a_at	9	30	0.3	AAA/AAA[d]
Slc26a7	1425841_at	1909	76	25	PPP/AAA
ClC-1	1427591_at	17	95	0.2	AAA/AAA
ClC-2	1449248_at	296	97	3.1	PPP/PAA
ClC-Ka	1450182_at	362	1458	0.2	PPP/PPP
ClC-Ka	1455677_s_at	372	2030	0.2	PPP/PPP
ClC-Kb	1450340_a_at	46	837	0.1	AAA/PAA[c]
Barttin	1421482_at	52	550	0.1	AAA/AAA[c]
ClCa1	1417852_x_at	66	104	0.6	PPP/PPM
ClCa1	1417853_at	28	31	0.9	PPA/AAA
ClCa2	1419463_at	2	107	0.0	AAA/PPA
ClCa2	1437578_at	7	10	0.7	AAA/AAA
ClCa3	1416306_at	5	6	0.8	AAA/AAA
ClCa3	1459889_at	32	95	0.3	AAA/AAA
ClCa4	1451823_at	28	94	0.3	AAA/AAA
Best 1	1428841_at	366	177	2.1	PPP/AAA
Best 2	1425729_at	12	77	0.2	AAA/AAA
Ano1/tmem16a	1426571_at	5	62	0.1	AAA/AAA
Ano1/tmem16a	1459713_s_at	46	414	0.1	AAA/PPP
Clns1a	1423181_s_at	2463	1913	1.3	PPP/PPP
Clns1a	1427548_a_at	145	234	0.6	PPP/PAA
Clns1a	1436935_x_at	10426	11084	0.9	PPP/PPP

Affymetrix annotation date is July 13, 2009; results are from three gene chips for each tissue; mean signal background for RM= 57, SV=100; GEO accession numbers: [a]GSE6196, [b]GSE4749. [c]These proteins are known to be present in SV. [d]Reported earlier [17].

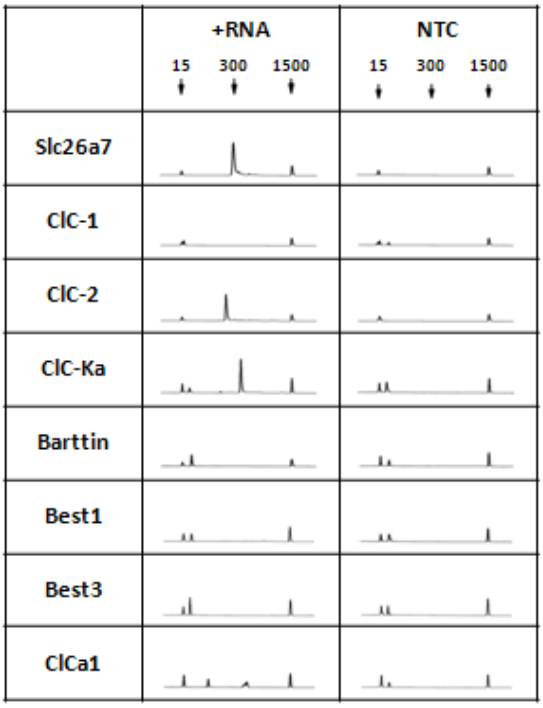

Figure S1. Electropherograms of PCR products for Cl^- channels in Reissner's membrane. Analyses of DNA products of RT-PCR by Agilent Bioanalyzer. Transcripts for all genes tested in column "+RNA" were present except for ClC-1, barttin, Best1, Best3; ClCa1 was judged to be absent. NTC, no template controls.

DISCUSSION

The contribution of Cl^- transporters to the support of auditory and vestibular neural processes has been reviewed recently [22]. However, the present paper is the first report of a significant involvement of conductive Cl^- pathways in Reissner's membrane epithelium. We identified by means of gene array, RT-PCR and electrophysiology several channels that carry Cl^-. The molecular identities of the channels that carry the observed currents were not unambiguously determined, but candidate genes were identified.

The voltage-dependence of the current under symmetrical Cl^- conditions has some similarities to channels reported in the literature. The strong inward rectification has been observed in expression systems and native cells. The strongest candidate for a molecularly-identified, inward-rectifier Cl^- channel in Reissner's membrane is ClC-2, although many Cl^- channels have been demonstrated functionally whose molecular identity remains unknown [12;38]. Few plasma membrane Cl^- channels are known to be inwardly-rectifying [12] and of those that are molecularly identified, the only candidate channel transcript in Reissner's membrane was ClC-2.

ClC-2 has an established electrophysiological and pharmacological fingerprint [13;33]. Salient features include whole-cell currents that a) are slowly activated by negative voltages; b) sensitive to extracellular pH (activated by acid); c) inhibited by Cd^{2+} and Zn^{2+} [4;28;38]; d) inhibited by antibodies directed against intracellular epitopes of the ClC-2 channel [9;27]. All of these characteristics were observed for currents in Reissner's membrane epithelial cells and transcripts for ClC-2 were present in the tissue.

ClC-2 was said earlier to be 'broadly' or 'ubiquitously expressed', although many studies have since shown a more specific distribution [12]. The view of cell-specific distribution is supported by our finding in the cochlea that Reissner's membrane expresses over 3 times as much transcript for ClC-2 as the neighboring tissue, the stria vascularis (Table S1). The stria is composed of numerous types of cells, including surface epithelial cells, intermediate cells of neural crest origin, basal cells, capillary endothelial cells and pericytes.

Nonetheless, Cl^- currents have been found in mouse choroid plexus epithelial cells that have many of the characteristics of ClC-2 but also display some differences, such as dependence on intracellular ATP [14]; in fact, it was found that those currents persisted in ClC-2 knockout mice, pointing to an unidentified channel with characteristics that overlap those of ClC-2 [32]. The lack of an antibody with convincing specificity for ClC-2 in fixed tissues [37] precluded localization of the protein to the apical or basolateral membrane in Reissner's membrane, although the effective inhibition of the inward current by ClC-2 antibody supports a similar epitope on the underlying channel or an associated protein.

Cellular functions ascribed to ClC-2 include Cl^- absorption in the colon, volume activation, volume inhibition, regulation of cardiac pacemaker activity and maintaining Cl^- homeostasis in rat rod bipolar cells of the retina [2;4;9;26], but the physiological function in mouse salivary gland epithelium is unknown [28]. The inward rectifier may participate in transepithelial Cl^- transport across Reissner's membrane, but a possible alternative or additional function includes regulation of cell volume [8].

Transcripts of additional Cl^- channels identified by gene array and/or RT-PCR in Reissner's membrane are Slc26a7 and ClC-Ka. Slc26a7 is a Cl^- channel with a nearly linear I-V relationship [16] that remains a candidate for the channel mediating the outward current in

Reissner's membrane. Studies of inward-rectifier currents in other native cells (e.g., rat parotid acinar cells and both rat and mouse choroid plexus epithelial cells) have also noted an additional minor outward current [14;15;25], even though heterologously expressed ClC-2 and the inward rectifier conductance of rat neocortical cultured astrocytes are nearly perfect inward-rectifiers [6;25]. ClC-K alpha-subunits require the presence of the beta-subunit, barttin, in order to be functional channels [5]. Barttin, however, was not detected by RT-PCR, suggesting that ClC-Ka does not form a functional channel in Reissner's membrane.

The Ca^{2+}-activated Cl^- channels (ClCa isoforms), bestrophin isoforms and Tmem16a were either absent or had weak gene array signal strength (Table S1). Clns1a is a putative Cl^- channel that was present at very low signal strength in the gene array. However, the protein is ubiquitously expressed and has been reported to have diverse functions that make it essential for cell viability, making it impossible to unambiguously determine whether it is indeed a Cl^- channel [7].

Previous reports of ion transport by Reissner's membrane epithelium have focused predominantly on cation transport. Observations include demonstrations of electrogenic transepithelial absorption of Na^+ from endolymph via Na^+-permeable, amiloride-sensitive channels in the apical membrane [17;20]. Na^+/K^+-ATPase in the basolateral membrane and Ca^{2+}-ATPase in the apical membrane [10;36] were found by histochemistry. Several patch-clamp studies have demonstrated the presence of ATP-gated cation channels [18], stretch and voltage-sensitive nonselective cation channels and potassium channels in the apical membrane [34;35]. Single-channel recordings of voltage-sensitive chloride channels were obtained from the apical membrane [35], but these channels had the opposite voltage sensitivity to those reported here and therefore may not play a significant role under physiological conditions.

Conclusion

In summary, we have identified a complex Cl^- current in Reissner's membrane epithelial cells that may be carried by multiple transport proteins. Cl^- is known to play a critical role in sensory outer hair cell tuning and amplification through its involvement with the motor protein, prestin [23;24;29], although the influence of luminal (endolymphatic) [Cl^-] is not known. Our findings support a possible role of Reissner's membrane in Cl^- homeostasis of endolymph in the support of hearing. Dysfunctions of Cl^- transport may contribute to pathological states such as Meniere's syndrome and Schiebe's deformity.

CHAPTER 3 - Conclusion and future directions

Our recent studies showed that Reissner's membrane epithelial cells participate in Cl^- ion transport, especially indicating characteristics of an inward-rectifier Cl^- channel. This finding is the first demonstration of electrogenic Cl^- transport pathways in Reissner's membrane epithelial cells using whole-cell patch-clamp experiment. The characteristics shown in Chapter 2 with strong inward rectification are consistent with ClC-2 Cl^- channel, whose transcript was confirmed to be present in Reissner's membrane. Furthermore, an ongoing project has been testing cyclic AMP-dependent Cl^- currents; preliminary findings were presented at a recent meeting (Kyunghee X. Kim and Daniel C. Marcus, "ClC-2 chloride channel in Reissner's Membrane", ARO Midwinter Research Meeting, Anaheim, California, USA, February 2010) and the abstract is reproduced below.

> "Sensory transduction in the cochlea depends on regulated ion secretion and absorption. Flux studies have provided evidence for Cl^- transport by Reissner's membrane (Konishi & Hamrick, 1978) and biochemical assays demonstrated a highly-active cAMP signal pathway (Thalmann & Thalmann, 1978). The present investigation utilized whole cell patch clamp, gene array and RT-PCR to determine the presence of Cl^- channels and transporters in mouse Reissner's membrane and to test for regulation by cAMP. Whole cell patch clamp recordings from epithelial cells under conditions where Cl^- was the only major permeant ion showed strong inward rectification. Channels expressed in the epithelial and/or mesothelial cells include ClC-2, Slc26a7 and ClC-Ka, but not ClC-1, ClCa1, ClCa2, ClCa3, ClCa4, Slc26a9, ClC-Kb, Best1, Best2, Best3 or the beta-subunit

of ClC-K, barttin. ClC-2 is the only channel present that is a strong inward rectifier. The inward currents matched additional key characteristics of ClC-2 Cl^- channels, including activation by lowered external pH and inhibition by the divalent cations Zn^{2+} and Cd^{2+}. Further, inward currents were stimulated by membrane-permeant analogs of cAMP. Electroneutral Cl^- transporters found to be expressed in Reissner's membrane include K^+/Cl^--cotransporter isoforms Kcc1, Kcc3, Kcc4, anion exchanger isoforms Ae2 and Ae3 but not Kcc2, Ae1, Ae4, Slc26a3 or Slc26a6. This is the first direct evidence that Reissner's membrane epithelial cells contain a transport pathway for Cl^- under control of cAMP mediated by ClC-2. Supported by NIH grants R01-DC000212 and P20-RR017686."

We also found that Cl^- currents have slight outward rectification (Chapter 2), which could be accounted for by the Cl^- anion transporter Slc26a7, whose expression in Reissner's membrane was also determined (Chapter 2). Preliminary findings on the location and importance of this transporter were presented at a recent meeting and the abstract (Kyunghee X. Kim, Joel D. Sanneman, Hyoung-Mi Kim, Donald G. Harbidge, Jie Xu, Daniel C. Marcus, Manoocher Soleimani, Philine Wangemann, " Loss of Slc26a7 in Reissner's membrane leads to hearing loss in mice", ARO Midwinter Research Meeting, Anaheim, California, USA, February 2010) is reproduced below.

"*Slc26a7* is a member of the Slc26 family that includes both pendrin (*Slc26a4*) and prestin (*Slc26a5*). Slc26a7 can function in two modes, as a Cl^- channel or as a Cl^-/HCO_3^- exchanger. Gene array analyses revealed high levels of *Slc26a7* expression in Reissner's

membrane, which prompted us to investigate whether *Slc26a7* is functional in Reissner's membrane epithelial cells and whether *Slc26a7* is essential for cochlear homeostasis, for hearing and, by extension, for balance. Cl^- currents were recorded in whole-cell patches of Reissner's membrane epithelial cells. Expression of Slc26a7 protein was localized by immunocytochemistry in developing and adult mice. Hearing and balance were evaluated by auditory brain stem recordings and RotaRod testing and cochlear morphology was assessed by immunocytochemistry in wild-type (*Slc26a7*$^{+/+}$) and in mice lacking *Slc26a7* (*Slc26a7*$^{-/-}$). Reissner's membrane epithelial cells expressed Slc26a7 protein in the basolateral membrane and carried Cl^- currents that carried NO_3^- significantly better than Cl^- and that were characterized by a slight outward rectification when studied with symmetrical NMDG-Cl solutions in whole-cell patches. The onset of protein expression was postnatal. At 10 month of age, two out of three *Slc26a7*$^{-/-}$ mice studied so far had a significant hearing loss at 16 and 32 kHz. No balance deficits were detected. Cochlear morphology was evaluated in one deaf *Slc26a7*$^{-/-}$ mouse. Reissner's membrane had a reduced number of nuclei and enlarged apical surface areas of the epithelial cells. Outer hair cell losses were found in the 16 and 32 kHz regions. In conclusion, the data demonstrate that Reissner's membrane epithelial cells express the Cl^- channel *Slc26a7* in the basolateral membrane. Based on a very limited dataset it appears that lack of this channel leads to a degeneration of Reissner's membrane, to a loss of outer hair cells and to a loss of hearing. Supported by NIH-R01-DC01098, NIH-R01-DC00212, NIH-P20-RR017686."

Future directions will include determining the first and second messenger pathways that regulate the Cl^- currents and determining the physiological significance of Slc26a7 in the cochlea.

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