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

by

KYUNGHEE KIM

B.S., Sookmyung Women’s University, Seoul, South Korea 1999-2003
M.S., KAIST (Korea Advanced Institute of Science and Technology), Daejeon, South Korea 2003-2005

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Anatomy and Physiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2010

Approved by:
Major Professor
Dr. Daniel C. Marcus
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.
Table of Contents

List of Figures…………………………………………………………………………………….iv
List of Tables………………………………………………………………………………………v
Acknowledgments………………………………………………………………………………vi
Dedication…………………………………………………………………………………………viii

CHAPTER 1 – Introduction……………………………………………………………………………1

CHAPTER 2 – Inward-rectifier chloride currents in Reissner’s membrane epithelial cells……5
(Reproduced by permission of Elsevier Publishers)

CHAPTER 3 – Conclusion and future direction…………………………………………………22

References…………………………………………………………………………………………26

Appendix: License to reproduce article from Elsevier………………………………………….32
List of Figures

CHAPTER 1: Introduction

Figure 1 - Schematic drawing of one cochlear turn............................................................3

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

Figure 1 - Strong inward-rectifier and smaller outward Cl⁻ currents...................................11
Figure 2 - Dependence of inward-rectifier Cl⁻ currents on pH..............................................12
Figure 3 - Dependence of inward-rectifier Cl⁻ currents on inhibition by Zn²⁺ and Cd²⁺..........13
Figure 4 - Inhibition of inward-rectifier Cl⁻ currents by ClC-2 antibody..............................14
Figure S1 - Electropherograms of PCR products for Cl⁻ channels in Reissner’s membrane.....17
List of Tables

CHAPTER 1: Introduction

Table 1 - Fluid composition of cochlear endolymph and perilymph……………………………..2

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

Table 1 - Primer sequences for RT-PCR and expression of gene transcripts………………………9
Table S1 - Gene array detection of Cl^- channels in Reissner’s membrane……………………….16
Acknowledgments

I would first like to thank Dr. Daniel C. Marcus, who invited me to join his laboratory, trained me to understand ion transport mechanisms in the inner ear, to find research topics, and to conduct experiments. Furthermore, I am grateful to him for introducing me to the topic of Reissner’s membrane in the inner ear. I have enjoyed exploring the properties of Reissner’s membrane via electrophysiology and molecular biology techniques.

I would also like to thank my committee members, Dr. Bruce Schultz and Dr. Peying Fong for their valuable advice, teachings, and kind assistance. I am grateful to Dr. Philine Wangemann for her teaching of confocal microscopy and helpful advice in every Monday meeting, to Dr. Michael Kenney for his teaching and help with my career, and to Dr. Jane Westfall for the Graduate Fellowship that allowed me to travel to the annual meeting, Experimental Biology. I would also like to thank Dr. Soonchil Lee at KAIST and Dr. In Hee Park at Ohio State University for their constant encouragement in my career.

I am thankful to all my colleagues at Kansas State University. I would especially like to mention Donald Harbidge, Joel Sanneman, Brian Willis, Dr. Kalidou Ndiaye, Nithya Raveendran, Dr. Hiromitsu Miyazaki, Dr. Takayuki Kudo, Dr. Kazuhiro Nakaya, Dr. Muneharu Yamazaki, Dr. Hyoungmi Kim, Xiangming Li, Sara Billings, Dr. Ruchira Singh, Christa Linsenmayer, Dr. Katrin Reimann, Dr. Gayathri Krishnamoorthy, Dr. Sung Huhn Kim, and Qian Wang in the College of Veterinary Medicine and Dr. Larry Weaver, Dr. Bharat Ratra, Cheng Jin, Yi Wu, Dr. Sampyo Hong, Jinkang Lim, and Hyounguk Jang in the Physics department. I thank Jenny Cain,
Dr. Barbara Lutjemeier, Marion Noble, Dani Goodband, Julie Hix and Bonnie Thompson for their administrative assistance.

Finally, I would like to thank my whole family for sharing in my happiness and sadness and for their support through all my years at Kansas. I am also thankful for the support given to me by members of the Korean Church of Manhattan.

This work was supported by grants to Daniel C. Marcus from the National Institutes of Health R01-DC00212 and P20-RR017686.
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]

<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>Cochlear Endolymph</th>
<th>Cochlear Perilymph</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>mM</td>
<td>1.3</td>
<td>148</td>
</tr>
<tr>
<td>K⁺</td>
<td>mM</td>
<td>157</td>
<td>4.2</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>mM</td>
<td>132</td>
<td>119</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>mM</td>
<td>31</td>
<td>21</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>mM</td>
<td>0.023</td>
<td>1.3</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>7.5</td>
<td>7.3</td>
</tr>
</tbody>
</table>
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:


This chapter is reproduced with permission by the publisher Elsevier (see Appendix).
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 MgCl₂, 0.273 CaCl₂, 1 EGTA, 10 Hepes, pH 7.3, ~300 mOsm, and bath 150 NMDG-Cl, 1 MgCl₂, 0.7 CaCl₂, 10 Hepes, 5 glucose, pH 7.3, ~300 mOsm. The pH was adjusted at room temperature (~25 °C) and expected to be about 7.2 at 37 °C. The free Ca²⁺ 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 °C was followed by 15 min at 95 °C and 40 PCR cycles. Each PCR cycle consisted of 94 °C for 20 sec, 56 °C for 30 sec, and 72 °C for 30 sec and readings of fluorescence were made at 78 °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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
<th>Product Size (bp) Exp. /Meas.&quot;</th>
<th>GenBank</th>
<th>P / A &quot;b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slc26a7</td>
<td>S GAAAAAAGAGAAGCGTGCTG 309 / 317 NM_145947 P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS AGGATGTCAGGCAAGGGCTG 317</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slc26a9</td>
<td>S CCTGACTGCTGTCATCCAGA 324/323 NM_177243 A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS GTAGGGATGGGGAAGTTGGAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIC-1</td>
<td>S CTGGTGACCTCTCCACTTA 292 / 284 NM_013491 A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS TGGCTGTCATAGACACCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIC-2</td>
<td>S CTGGATGTCTGCTGACTGCTA 271 / 271 NM_009900 P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS AGGCAGAATGTCAGGATCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIC-Ka</td>
<td>S ACTCCCAGAGCTGAAAGACCA 337 / 337 NM_024412 P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS CCAGACGGAGAAGTGAAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barttin</td>
<td>S CAGAGCCTCACCAGACTTCAC 399 / 387 NM_080458 A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS TGTAGGGGTGTGTCATTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Best1</td>
<td>S TACAAGCTGTTCCCCTCTTC 366/376 NM_011913 A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS CATCTGATGGGATGGGTAGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Best3</td>
<td>S GCTGCCGACTGACCTTACAC 368/362 NM_001007583 A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS GTTCACCGATGGGTGACAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CICa1</td>
<td>S CTACAAGTGCCGACGCTGCTTCC 367/358 NM_009899 A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS GCAGTAGCCAGGAGTGGTTC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S, sense primer; AS, antisense primer; Product Size, length in the base pairs (bp) of RT-PCR product including primers. "Expected/Measured product size; "Present/Absent.
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, Cd2+, Zn2+ 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 Zn2+ (Fig. 3A) and Cd2+ (Fig. 3B) at concentrations known to inhibit ClC-2 channels [12;38]. I−100 was reversibly decreased by 50 µM Zn2+ by 45.6 ± 7.5 % (from -248 ± 24 pA to -132 ± 15 pA, n=4) and by 500 µM Cd2+ 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 \(\mu\)M Zn\(^{2+}\). B, The inhibition of the current at -100 mV by 500 \(\mu\)M 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 detected include 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Affymetrix Probe Set ID</th>
<th>Average Signal Mean</th>
<th>Ratio (RM/SV)</th>
<th>Present (P) Marginal(M) Absent (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RM^a</td>
<td>SV^b</td>
<td></td>
<td>AAA/APA^d</td>
</tr>
<tr>
<td>Cftr</td>
<td>1420579_s_at</td>
<td>8</td>
<td>0.2</td>
<td>AAA/APA^d</td>
</tr>
<tr>
<td>Cftr</td>
<td>1427767_a_at</td>
<td>9</td>
<td>0.3</td>
<td>AAA/AAA_d</td>
</tr>
<tr>
<td>Slc26a7</td>
<td>1425841_at</td>
<td>1909</td>
<td>25</td>
<td>PPP/AAA</td>
</tr>
<tr>
<td>ClC-1</td>
<td>1427591_at</td>
<td>17</td>
<td>0.2</td>
<td>AAA/AAA</td>
</tr>
<tr>
<td>ClC-2</td>
<td>1449248_at</td>
<td>296</td>
<td>3.1</td>
<td>PPP/AAA</td>
</tr>
<tr>
<td>ClC-Ka</td>
<td>1450182_at</td>
<td>362</td>
<td>0.2</td>
<td>PPP/PPP</td>
</tr>
<tr>
<td>ClC-Ka</td>
<td>1455677_s_at</td>
<td>372</td>
<td>0.2</td>
<td>PPP/PPP</td>
</tr>
<tr>
<td>ClC-Kb</td>
<td>1450340_a_at</td>
<td>46</td>
<td>0.1</td>
<td>AAA/PAA_c</td>
</tr>
<tr>
<td>Barttin</td>
<td>1421482_at</td>
<td>52</td>
<td>0.1</td>
<td>AAA/AAA_c</td>
</tr>
<tr>
<td>ClCa1</td>
<td>1417852_x_at</td>
<td>66</td>
<td>0.6</td>
<td>PPP/PPM</td>
</tr>
<tr>
<td>ClCa1</td>
<td>1417853_at</td>
<td>28</td>
<td>0.9</td>
<td>PPA/AAA</td>
</tr>
<tr>
<td>ClCa2</td>
<td>1419463_at</td>
<td>2</td>
<td>0.0</td>
<td>AAA/PPA</td>
</tr>
<tr>
<td>ClCa2</td>
<td>1437578_at</td>
<td>7</td>
<td>0.7</td>
<td>AAA/AAA</td>
</tr>
<tr>
<td>ClCa3</td>
<td>1416306_at</td>
<td>5</td>
<td>0.8</td>
<td>AAA/AAA</td>
</tr>
<tr>
<td>ClCa3</td>
<td>1459889_at</td>
<td>32</td>
<td>0.3</td>
<td>AAA/AAA</td>
</tr>
<tr>
<td>ClCa4</td>
<td>1451823_at</td>
<td>28</td>
<td>0.3</td>
<td>AAA/AAA</td>
</tr>
<tr>
<td>Best 1</td>
<td>1428841_at</td>
<td>366</td>
<td>2.1</td>
<td>PPP/AAA</td>
</tr>
<tr>
<td>Best 2</td>
<td>1425729_at</td>
<td>12</td>
<td>0.2</td>
<td>AAA/AAA</td>
</tr>
<tr>
<td>Ano1/tmem16a</td>
<td>1426571_at</td>
<td>5</td>
<td>0.1</td>
<td>AAA/AAA</td>
</tr>
<tr>
<td>Ano1/tmem16a</td>
<td>1459713_s_at</td>
<td>46</td>
<td>0.1</td>
<td>AAA/PPP</td>
</tr>
<tr>
<td>Clns1a</td>
<td>1423181_s_at</td>
<td>2463</td>
<td>1.3</td>
<td>PPP/PPP</td>
</tr>
<tr>
<td>Clns1a</td>
<td>1427548_a_at</td>
<td>145</td>
<td>0.6</td>
<td>PPP/PAA</td>
</tr>
<tr>
<td>Clns1a</td>
<td>1436935_x_at</td>
<td>10426</td>
<td>0.9</td>
<td>PPP/PPP</td>
</tr>
</tbody>
</table>

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: ^aGSE6196, ^bGSE4749. cThese proteins are known to be present in SV. dReported 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 CIC-1, barttin, Best1, Best3; ClCa1 was judged to be absent. NTC, no template controls.
DISCUSSION

The contribution of Cl\textsuperscript{−} 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\textsuperscript{−} pathways in Reissner’s membrane epithelium. We identified by means of gene array, RT-PCR and electrophysiology several channels that carry Cl\textsuperscript{−}. 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\textsuperscript{−} 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\textsuperscript{−} channel in Reissner’s membrane is ClC-2, although many Cl\textsuperscript{−} channels have been demonstrated functionally whose molecular identity remains unknown [12;38]. Few plasma membrane Cl\textsuperscript{−} 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\textsuperscript{2+} and Zn\textsuperscript{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 (Kyunghgee 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 Zn²⁺ and Cd²⁺. 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 (Kyounghee 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⁻/HCO₃⁻ exchanger. Gene array analyses revealed high levels of Slc26a7 expression in Reissner’s
membrane, which prompted us to investigate whether \textit{Slc26a7} is functional in Reissner’s membrane epithelial cells and whether \textit{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 (\textit{Slc26a7}^{+/+}) and in mice lacking \textit{Slc26a7} (\textit{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 \textit{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 \textit{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 \textit{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.
Reference List


[37] A.A. Zdebik, J.E. Cuffe, M. Bertog, C. Korbmacher, T.J. Jentsch, Additional disruption of the ClC-2 Cl− channel does not exacerbate the cystic fibrosis phenotype of cystic fibrosis

This is a License Agreement between Kansas State University ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

**All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.**

**Supplier**
Elsevier Limited  
The Boulevard, Langford Lane  
Kidlington, Oxford, OX5 1GB, UK

**Registered Company Number**
1982084

**Customer name**
Kansas State University

**Customer address**
Kansas State University  
Manhattan, KS 66506

**License Number**
2392550541872

**License date**
Mar 19, 2010

**Licensed content publisher**
Elsevier

**Licensed content publication**
Biochemical and Biophysical Research Communications

**Licensed content title**
Inward-rectifier chloride currents in Reissner’s membrane epithelial cells

**Licensed content author**
Kyunghhee X. Kim, Daniel C. Marcus

**Licensed content date**
10 March 2010

**Volume number**
n/a

**Issue number**
n/a

**Pages**
1

**Type of Use**
Thesis / Dissertation

**Portion**
Full article

**Format**
Electronic

**You are an author of the Elsevier article**
Yes

**Are you translating?**
No

**Order Reference Number**
YBBRC24704
INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

   “Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER].” Also Lancet special credit - “Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier.”

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevvoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable
to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website**: The following terms and conditions apply to electronic reserve and author websites:
   - **Electronic reserve**: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:
     - This license was made in connection with a course,
     - This permission is granted for 1 year only. You may obtain a license for future website posting,
     - All content posted to the web site must maintain the copyright information line on the bottom of each image,
     - A hyper-text must be included to the Homepage of the journal from which you are licensing at [http://www.sciencedirect.com/science/journal/xxxxx](http://www.sciencedirect.com/science/journal/xxxxx) or the Elsevier homepage for books at [http://www.elsevier.com](http://www.elsevier.com), and
     - Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

   All content posted to the web site must maintain the copyright information line on the bottom of each image, and
   - the permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version,
   - A hyper-text must be included to the Homepage of the journal from which you are licensing at [http://www.sciencedirect.com/science/journal/xxxxx](http://www.sciencedirect.com/science/journal/xxxxx). As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier’s online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article’s
Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at http://www.elsevier.com

All content posted to the web site must maintain the copyright information line on the bottom of each image
You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx. or for books to the Elsevier homepage at http://www.elsevier.com

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**: None

v1.6

**Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.**

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10753875.

Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.
Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2392550541872 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.