Regional differences in rat conjunctival ion transport activities

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Yu D, Thelin WR, Rogers TD, Stutts MJ, Randell SH, Grubb BR, Boucher RC. Regional differences in rat conjunctival ion transport activities. Am J Physiol Cell Physiol 303: C767–C780, 2012. First published July 18, 2012; doi:10.1152/ajpcell.00195.2012.—Active ion transport and coupled osmotic water flow are essential to maintain ocular surface health. We investigated regional differences in the ion transport activities of the rat conjunctivae and compared these activities with those of cornea and lacrimal gland. The epithelial sodium channel (ENaC), sodium/glucose cotransporter 1 (Slc5a1), transmembrane protein 16 (Tmem16a, b, f, and g), cystic fibrosis transmembrane conductance regulator (Cftr), and mucin (Muc4, 5ac, and 5b) mRNA expression was characterized by RT-PCR. ENaC proteins were measured by Western blot. Prespecified regions (palpebral, fornical, and bulbar) of freshly isolated conjunctival tissues and cell cultures were studied electrophysiologically with Ussing chambers. The transepithelial electrical potential difference (PD) of the ocular surface was also measured in vivo. The effect of amiloride and UTP on the tear volume was evaluated in lacrimal gland excised rats. All selected genes were detected but with different expression patterns. We detected αENaC protein in all tissues, βENaC in palpebral and fornical conjunctiva, and γENaC in all tissues except lacrimal glands. Electrophysiological studies of conjunctival tissues and cell cultures identified functional ENaC, Slc5a1, Cftr, and Tmem16. Fornical conjunctiva exhibited the most active ion transport under basal conditions amongst conjunctival regions. PD measurements confirmed functional ENaC-mediated Na+ transport on the ocular surface. Amiloride and UTP increased tear volume in lacrimal gland excised rats. This study demonstrated that the different regions of the conjunctiva exhibited a spectrum of ion transport activities. Understanding the specific functions of distinct regions of the conjunctiva may foster a better understanding of the physiology maintaining hydration of the ocular surface.

conunctiva; cornea; ion transport; lacrimal gland

THE TEAR FILM OCCUPIES THE INTERFACE BETWEEN THE OCULAR SURFACE AND THE EXTERNAL ENVIRONMENT. It serves a fundamental role in corneal and conjunctival health, protecting the ocular surface from invasion by microorganisms in the external environment, providing a smooth refractive surface for light transmission, and maintaining a moist ocular surface microenvironment (8, 16, 32). The normal tear film is maintained through an integrated system, the lacrimal functional unit, consisting of the ocular surface epithelium (cornea and conjunctiva), the lacrimal and Meibomian glands, and the interconnecting innervation (31, 46). In this unit, the Meibomian glands provide the outermost hydrophobic lipid layer that slows tear film evaporation (6, 30). Secretions from the cornea, conjunctiva, and lacrimal gland may all contribute to the aqueous/mucous layer, which is composed of water, mucins, ions, amino acids, growth factors, neurotransmitters, and other proteins (17, 52). The neural input to these tissues regulates secretion rates (9). Compromise of any component of this system can lead to tear film disorders, most commonly dry eye (22, 40).

In current models of ocular surface fluid balance, the lacrimal glands provide the bulk of aqueous tears during reflex stimulation, whereas the epithelial cells of the conjunctiva and cornea are key mediators of basal tear volume and composition. Similar to the epithelia of the lungs, gastrointestinal tract, and kidneys, the ocular epithelia regulate water (volume) transport through active electrolyte transport (3–5, 42, 50). Epithelial chloride secretion provides the primary driving force for increasing tear volume, whereas sodium absorption provides an opposing osmotic gradient for water absorption. Previous studies (10) have characterized the capacity of the conjunctiva to secrete chloride and water in response to agents that increase intracellular calcium or cAMP. However, the specific chloride channels that provide the secretory activity on the ocular surface are not clearly defined. Similarly, neither the contribution of sodium channel-mediated fluid absorption to ocular surface fluid balance, nor the molecular identity of conjunctival sodium channels, is well understood.

Among the elements of the lacrimal functional unit, the ion transport characteristics of the conjunctiva and its different regions are perhaps least understood. The conjunctiva is a thin, mucin-secreting tissue that lines the posterior eyelid surface (palpebral conjunctiva) and reflects forward onto the anterior surface of the sclera where it merges with the corneal epithelium at the limbus (bulbar conjunctiva). The intermediate fornical conjunctiva resides in the recess joining the palpebral and bulbar regions. Each conjunctival region exhibits distinct morphological features, mainly reflected in the depth of the stratified epithelial cell layer and goblet cell densities. In humans and rabbits, the conjunctival surface area is ~17 and 9 times larger than the corneal surface area, respectively (51), making it a potentially significant regulator of the tear film. However, the study of conjunctival ion transport is challenging due to the fragility of this tissue and a lack of well-characterized cell culture models that retain the ion transport properties of the native tissue.

To characterize the role of the different conjunctival regions to ocular fluid balance, we developed experimental systems using rat tissues to characterize the expression of conjunctival ion channels. Specifically, we evaluated the expression and activity of sodium and chloride channels in the three regions of the conjunctiva (i.e., the palpebral, fornical, and bulbar regions) and compared those data to cornea and lacrimal glands. To assess the activity of various chloride and sodium channels in the conjunctiva, we utilized a comprehensive series of pharmacologic studies that evaluated the bioelec-
The transcription properties of 1) the three regions of freshly excised conjunctivae; 2) primary conjunctival cell cultures; and 3) the intact ocular surface in vivo. Finally, we confirmed that activation of chloride efflux or inhibition of sodium absorption increases tear volume in vivo. This combination of approaches helps to define the role of the conjunctiva as a mediator of tear volume and identify the major ion channels capable of regulating tear volume.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Charles River, Wilmington, MA), weighing 250–300 g, were studied at age 10–12 wk. Animals were treated in accordance with the Association for Research in Vision and Ophthalmalogy statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Xenopus laevis frogs (Xenopus Express, Brooksville, FL) were maintained and studied under protocols approved by the University of North Carolina Institutional Animal Care and Use Committee.

RT-PCR analysis. Rats were killed by CO₂ asphyxiation. Under a dissecting microscope, the palpebral, fornical, and bulbar conjunctiva were separately removed according to their anatomic distributions, the width of each section being 1.5–2 mm. In addition, the whole cornea and a piece of lacrimal glands were also excised for analysis. Total RNA was isolated from tissues with RNeasy Plus Mini kit (Qiagen, Valencia, CA) in accordance with the manufacturer’s instructions. Complementary DNA (cDNA) was prepared from total RNA with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s instructions. PCR master mix (Fermentas, Glen Burnie, MD) was processed for protein concentration measurements with the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Forty-five s, 55°C 45 s, 72°C 1 min, and 72°C 7 min. The amplified products were separated and visualized on ethidium bromide-stained agarose gels. Selected genes of interest, including the rat epithelial sodium channel (ENaCα, β, and γ subunits), the sodium/glucose cotransporter 1 (Slc5a1), the cystic fibrosis transmembrane conductance regulator (Cftr), members of the transmembrane protein 16 (Tmem16a, f, and g), and mucins (Muc4, 5ac, and 5b) were determined with the primers listed in Table 1. Primers for 18S rRNA standard were obtained from Ambion (Carlsbad, CA). Reactions substituting reverse transcriptase with water were used as negative controls.

Real-time RT-PCR. cDNA was prepared as described above and further analyzed using Fast SYBR Green Master Mix in the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster, CA). The PCR reaction was performed in a 96-well optical plate. The thermal cycler profile started with 2 min at 95°C, followed by 40 cycles of 95°C 3 s and 60°C 30 s, and ended up with the dissociation stage of 95°C 15 s, 60°C 15 s, 95°C 15 s, and 60°C 15 s. The relative mRNA levels were analyzed based on the comparative Ct of each gene against 18S rRNA which served as a control. The purity of amplified products was determined with the single peak of the dissociation curve. The specificity of each pair of primers was confirmed with a water control replacing the sample in the reaction. Information on primers included is shown in Table 2.

Western blot analysis. Xenopus laevis oocytes were isolated; injected with cRNAs encoding rat α, β, and γENaC subunits; and harvested for protein, as described previously (13). Ocular tissue isolation was as described above. Harvested tissues were homogenized in ice-cold RIPA buffer (Sigma), supplemented with phosphatase and protease inhibitors (Roche Diagnostic). Lysates were centrifuged at 14,000 rpm for 10 min. Supernatants were collected and processed for protein concentration measurements with the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Forty-five micrograms of protein were separated by electrophoresis on NuPAGE

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### Table 1. Oligonucleotide primers for RT-PCR

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4–12% Bis-Tris precast gels (Invitrogen) and transferred to PVDF membranes. Antibodies against αENaC (1:600), βENaC (1:1,000), and γENaC (1:1,000) were obtained from StressMarq Biosciences Incorporated (Canada). Antibody against β-actin (Cell Signaling Technology, Danvers, MA) was used at a 1:1,000 dilution. Immunoreactive proteins were visualized with Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA).

**Ussing chamber measurement of rat conjunctiva tissue.** Rats were killed by CO2 asphyxiation. To retain the integrity of the epithelium and minimize the dissection time, the palpebral conjunctiva was dissected along with underlying Meibomian gland tissue. Segments of dissected conjunctival tissue were sent for histology and stained with hematoxylin and eosin and Alcian blue-periodic acid Schiff. Fornical conjunctival tissue were stained with hematoxylin and eosin and Alcian blue-periodic acid Schiff. For immunohistochemistry, tissues were fixed in 10% formalin for 24 h, dehydrated through graded ethanol, and embedded in paraffin. 6 μm-thick sections were cut and stained with hematoxylin and eosin. Immunohistochemistry was performed with antibodies against αENaC (StressMarq Biosciences, Canada), γENaC (StressMarq Biosciences, Canada), Muc5ac, Muc5b, Muc5b, Muc5c, Slc5a1, Tmem16a, Tmem16b, Tmem16f, and Tmem16g, as well as β-actin (Cell Signaling Technology, Danvers, MA).

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**Fig. 1.** Gene expression in rat ocular tissues measured by conventional RT-PCR. Lane 1: Pal conj, palpebral conjunctiva; lane 2: For conj, fornical conjunctiva; lane 3: Bul conj, bulbar conjunctiva; lane 4: Cor, cornea; lane 5: LG, lacrimal gland. mRNA expression of selected genes, including sodium channels, chloride channels, and mucins, were detected in conjunctival tissues. α-ENaC, γ-ENaC, sodium/gluconate cotransporter 1, and mucin 4 were abundantly detected in all tissues. Tmem16a and Tmem16b mRNA were barely detectable in cornea. Tmem16b mRNA was weakly detected in the palpebral and fornical conjunctiva, and cornea. βENaC, Tmem16g, Cffr, Muc5ac, and Muc5b mRNA were weakly detectable in lacrimal glands.
Forc
LG
Cornea
LG
LG
LG

*Co

Gel loading pipette tip filled with 3% agar in 0.9% NaCl. PE 10 tubing, temperature of 37°C. The exploring electrode was constructed from a thermistor was inserted which was connected to a heat lamp interfaced to a feedback system (Physiotemp, Clifton, NJ) set to maintain a body temperature of 37°C. The exploring electrode was constructed from a gel loading pipette tip filled with 3% agar in 0.9% NaCl. PE 10 tubing, through which the perfusate solution was delivered via a perfusion pump, was glued to the tip of the exploring electrode. The exploring electrode was placed vertically in a micromanipulator and positioned directly over the cornea. The exploring electrode perfusate flowed over the ocular surface at a rate of 50 μl/min, with excess fluid being allowed to drip off of the eye. The agar bridge of the exploring electrode was interfaced to a calomel electrode connected to a voltmeter (Iso-Millivolt Meter, WPI, Sarasota, Fl), which in turn was connected to a chart recorder. The reference electrode was agar filled 3% agar in 0.9% NaCl PE 60 tubing with a needle on one end, which was placed subcutaneously in the rat abdomen. This electrode was also interfaced via a calomel electrode to the voltmeter. The perfusate solution was phosphate buffered saline adjusted to an osmolality of 320 mosM with NaCl with or without 100 μM amiloride.

**Using chamber measurement of rat conjunctival cell cultures.** Seven days after ALI culture was initiated, cells growing on Snapwells were mounted in Ussing chambers for bioelectric measurements. Cells were bathed on both sides with KBR at 37°C, bubbled with 95% O2-5% CO2 to maintain the pH at 7.4. The transepithelial voltage (VT) was clamped to zero and pulsed to 5 mV for 0.2-s duration every 20 s to calculate transepithelial resistance (RT). The RT and Is were continuously recorded on a computer and analyzed with Acquire and Analysis software (Physiologic Instruments, San Diego, CA). Similar series of drug additions as cited above plus 50 μM N-(4-trifluoromethylphenyl)anthranilic acid (TFMP, a TMEM16 inhibitor, Ref. 37) were applied to the apical bath of chambers.

In **vivo transepithelial electrical potential difference measurements of the ocular surface.** Male Sprague-Dawley rats were anesthetized with isoflurane (2%) via a nose mask. After anesthesia, a rectal thermometer was inserted which was connected to a heat lamp interfaced to a feedback system (Physiotemp, Clifton, NJ) set to maintain a body temperature of 37°C. The exploring electrode was constructed from a gel loading pipette tip filled with 3% agar in 0.9% NaCl. PE 10 tubing, with 95% O2-5% CO2 to maintain the pH at 7.4. The transepithelial voltage (VT) was clamped to zero and pulsed to 5 mV for 0.2-s duration every 20 s to calculate transepithelial resistance (RT). The RT and Is were continuously recorded on a computer and analyzed with Acquire and Analysis software (Physiologic Instruments, San Diego, CA). Similar series of drug additions as cited above plus 50 μM N-(4-trifluoromethylphenyl)anthranilic acid (TFMP, a TMEM16 inhibitor, Ref. 37) were applied to the apical bath of chambers.

**Tear volume studies in a rat model of dry eye.** A dry eye rat model (ExLac) was generated by removing the main lacrimal gland of anesthetized male Sprague-Dawley rats (36). Two weeks after the lacrimal excision, tear production was measured on unanesthetized rat using phenol red impregnated cotton threads (Zone-Quick, Lacrimedics). The threads were held with fine forceps and applied to the ocular surface, on both eyes, in the lateral canthus for 10 s. Wetting of the

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**Fig. 2.** Quantitative mRNA analysis by real-time RT-PCR. Relative mRNA level of each gene was calculated based on 18S rRNA and plotted as means ± SE (n = 3). Comparative analyses revealed higher level of αENaC mRNA in palpebral conjunctiva than in cornea and lacrimal glands; higher βENaC mRNA level in palpebral conjunctiva than in bulbar conjunctiva, cornea, and lacrimal glands; and uniform γENaC mRNA levels in all tissues. The mRNA level of Slc5a1 in palpebral and fornical conjunctiva was higher than that in cornea and lacrimal glands. Cfr mRNA level in palpebral conjunctiva was higher than in bulbar conjunctiva, cornea and lacrimal glands. Tmem16a and Tmem16f mRNA levels were significantly higher in lacrimal glands than other tissues, amongst which no further differences were revealed. Tmem16g mRNA was found at higher level in bulbar conjunctiva than cornea. Muc5ac mRNA level was found to be statistically higher in palpebral conjunctiva than in cornea and lacrimal glands. No statistical differences were found regarding to the Muc4 and Muc5b mRNA level amongst those tissues. *P < 0.05.

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thread (which turns red in contact with tears) was measured in millimeters under a dissecting microscope. All tested compounds were dissolved in a buffer that mimics the composition of tears, containing the following (in mM): 106.5 NaCl, 26.1 NaHCO3, 18.7 KCl, 1.0 MgCl2, 0.5 K2HPO4, 1.1 CaCl2, and 10 HEPES pH 7.4. Tears output was measured at baseline and at various time points after application of vehicle (tears buffer) or the tested compounds in a 5-μl drop to both eyes.

Statistical analysis. Data were described as means ± SE. Paired t-tests were used to compare the $I_{sc}$ changes caused by drug treatments. One-way ANOVA combined with Tukey posttest was used to compare the differences among groups. $P < 0.05$ was considered significant.

RESULTS

Expression of sodium and chloride channels in rat cornea, conjunctiva, and lacrimal gland tissues. The expression of the major epithelial sodium channels (ENaC α, β, and γ subunits), sodium/glucose cotransporter 1 (Slc5a1), transmembrane protein 16 (Tmem16a, b, f, and g), cystic fibrosis transmembrane conductance regulator (Cftr), and mucin (Muc4, 5ac, and 5b) was evaluated in rat conjunctiva, cornea, and lacrimal glands by RT-PCR and real-time PCR (Fig. 1). To evaluate gene expression in the individual conjunctival segments, mRNA was prepared from conjunctiva dissected into the fornical, bulbar, and palpebral regions. Relative mRNA levels of each gene were obtained by normalizing to the 18S rRNA gene (Fig. 2).

As shown in Figs. 1 and 2, sodium channels were detected in all rat ocular surface tissues. Comparative analyses revealed more αENaC mRNA in palpebral conjunctiva than in cornea or lacrimal glands ($P < 0.05$); more βENaC mRNA in palpebral conjunctiva than in bulbar conjunctiva, cornea, and lacrimal glands ($P < 0.05$); and uniform γENaC mRNA levels amongst all tissues. Slc5a1 mRNA was also detectable in all tissues, and its expression level was higher in palpebral and fornical conjunctiva than in cornea and lacrimal glands ($P < 0.05$).

Different expression patterns of Tmem16 family members were also revealed in this study (Figs. 1 and 2). Tmem16a mRNA was modestly expressed in conjunctiva, abundantly in lacrimal glands, but was barely detectable in cornea. Tmem16b mRNA was very weakly detected in the palpebral and fornical conjunctiva, cornea, and not in the bulbar conjunctiva and lacrimal glands. Tmem16f mRNA was detected in all tissues. Lacrimal glands again exhibited significantly higher levels of Tmem16f mRNA than the other tissue types. Tmem16g transcripts were also found to be present in all tissues, with higher levels in bulbar conjunctiva than in cornea ($P < 0.05$).

Cftr transcripts were abundant in conjunctival tissues, barely detectable in cornea, and weakly detectable in lacrimal glands. The Cftr mRNA expression level in palpebral conjunctiva was significantly higher than that in bulbar conjunctiva, cornea, and lacrimal glands ($P < 0.05$).

The membrane associated mucin Muc4 was intensely expressed in conjunctiva and cornea, and weakly expressed in lacrimal glands (Figs. 1 and 2). In contrast, the gel-forming mucins Muc5ac and Muc5b were abundantly expressed in conjunctiva, weakly expressed in cornea, and barely detectable in lacrimal glands. Although they appeared different, relatively low values and high variability precluded statistical comparison of Muc4 and Muc5b expression amongst tissues. Relative Muc5ac mRNA expression was significantly higher in palpebral conjunctiva than in cornea and lacrimal glands ($P < 0.05$). Identification of ENaC proteins by Western blot. Affinity purified antibodies were used to detect the presence of α, β, and γENaC protein in rat conjunctiva, cornea, and lacrimal gland tissues. lysates of Xenopus laevis oocytes with and without rat ENaC cRNA over expression served as antibody specificity controls. As shown in Fig. 3, αENaC protein was detected in oocytes lysate as three bands of 95, 32, and 18 kDa, respectively. As reported previously, the 95-kDa band is the full-length of αENaC, whereas the 32- and 18-kDa fragments are αENaC products resulting from cleavage at a furin consensus site and another site just distal to the first transmembrane segment, respectively (13). The full-length 95-kDa αENaC band was detectable only in conjunctival tissues, while the lower 18-kDa fragment was detected in all tissues. The 32-kDa band was not detected in any ocular tissue. This difference may be due to tissue-specific cleavage of αENaC. βENaC protein was detected as a band at 95 kDa in oocytes, palpebral and fornical conjunctiva, but not in the rest of the tissues. In contrast, γENaC protein was detected with different sizes in oocytes controls and ocular tissue samples. In oocytes with rat γENaC cRNA injection, there was an intense ∼72-kDa band. However, we detected a band at 105 kDa in conjunctiva and cornea, but not in lacrimal glands.

In vitro electrophysiological properties measurement of rat conjunctiva. To investigate potential regional differences in conjunctival ion transport, freshly excised rat palpebral, fornical, and bulbar conjunctiva were mounted on Ussing chambers followed by a series of drug applications. The morphologic properties of each conjunctival region are shown in Fig. 4. Drugs targeting specific ion channels were applied to the apical bath of the chamber to determine the contribution of each apical membrane channel to the $I_{sc}$. Trypsin causes the cleavage of ENaC subunits (α, β, and γ) protein expression in rat ocular tissues. Oocytes without and with rat ENaC cRNA injection were used as controls. The identity of tissue is marked at top of each lane (lane 1: Pal conj; lane 2: For conj; lane 3: Bul conj; lane 4: Cor, cornea; lane 5: LG). In oocyte lysates, αENaC was detected as 3 bands at 95, 32, and 18 kDa, respectively. The 95-kDa band was detected only in conjunctival tissue, whereas the 18-kDa band was detected in all tissues. βENaC was only detected in palpebral and fornical conjunctiva as a 95-kDa band. γENaC protein was detected as a 105-kDa band in all examined tissues except lacrimal glands. The β-actin was used as an endogenous control.
age of ENaC and activates ENaC. Amiloride is an inhibitor of ENaC. Phloridzin is a sodium/glucose cotransporter blocker. Forskolin, an activator of adenylate cyclase, activates the CFTR through increasing cell cAMP. CFTRinh172 is a selective CFTR blocker that alters channel gating. UTP activates calcium-regulated chloride channels primarily through P2Y2 and P2Y4 receptors (15).

One set of preparations (n = 6) was tested using the trypsin-amiloride-phloridzin-forskolin-CFTRinh172-UTP drug treatment order (paradigm 1; representative traces are shown in Fig. 5A). The basal $I_{sc}$ (uA/cm²) of palpebral, fornical, and bulbar conjunctiva bathed in regular KBR was 26.1 ± 7.43 (means ± SE), 123.7 ± 7.81, and 44.0 ± 24.99, respectively (plotted in Fig. 5B). The basal $I_{sc}$ of fornical conjunctiva was statistically higher than that of palpebral or bulbar conjunctiva ($P < 0.05$), with no significant difference between the latter two regions. The $V_T$ (mV) of palpebral, fornical, and bulbar conjunctiva was 2.2 ± 0.69, 4.9 ± 0.81, and 4.5 ± 1.42, respectively. The $R_T$ (Ω·cm²) of palpebral, fornical, and bulbar conjunctiva was 87.3 ± 9.94, 38.4 ± 4.26, and 91.4 ± 18.0, respectively.

Changes in $I_{sc}$ ($\Delta I_{sc}$) following drug application per paradigm 1 are summarized in Fig. 5C. No significant changes in $I_{sc}$ were caused by the addition of trypsin. The decrease in $I_{sc}$ caused by amiloride was small but statistically significant in fornical conjunctiva ($-3.8 ± 0.95$ uA/cm²) and bulbar conjunctiva ($-3.1 ± 0.91$ uA/cm²; $P < 0.05$) but not in palpebral conjunctiva ($-0.2 ± 0.09$ uA/cm²; $P > 0.05$). Phloridzin significantly inhibited $I_{sc}$ in palpebral conjunctiva ($-1.9 ± 0.44$ uA/cm²), fornical conjunctiva ($-4.3 ± 0.50$ uA/cm²), and bulbar conjunctiva ($-6.0 ± 1.13$ uA/cm²; $P < 0.05$). The bulbar conjunctiva exhibited a larger response to phloridzin compared with palpebral conjunctiva ($P < 0.05$). $I_{sc}$ increased following forskolin addition in palpebral ($1.9 ± 0.61$ uA/cm²), fornical ($21.0 ± 4.86$ uA/cm²), and bulbar conjunctiva ($8.9 ± 2.80$ uA/cm²; $P < 0.05$). CFTRinh172 significantly reduced $I_{sc}$ in palpebral ($-13.0 ± 3.1$ uA/cm²), fornical ($-68.7 ± 9.63$ uA/cm²), and bulbar conjunctiva ($-34.4 ± 4.82$ uA/cm²; $P < 0.05$).
The forskolin and CFTRinh172 ∆I_{sc} were greater in fornal than in palpebral and bulbar conjunctiva (P < 0.05). Lastly, UTP modestly increased I_{sc} in palpebral (2.2 ± 0.32 µA/cm²), fornal (3.27 ± 1.07 µA/cm²), and bulbar conjunctiva (7.3 ± 3.26 µA/cm²; P < 0.05), but there were no statistical differences amongst the three conjunctival regions.

To determine whether the order of drug addition affected the I_{sc} results, we evaluated a second paradigm with the drug treatment as follows: CFTRinh172-trypsin-amiloride-phloridzin-forskolin-UTP (paradigm 2; representative traces are shown in Fig. 6A). As in paradigm 1, the basal I_{sc} was markedly higher in fornal than in palpebral or bulbar conjunctiva (P < 0.05; see Fig. 6B). Changes in I_{sc} following drug applications are summarized in Fig. 6C. The results were very similar to paradigm 1 except for the following points. First, amiloride produced a significant decrease in I_{sc} not only in fornal and bulbar conjunctiva, but also in palpebral conjunctiva. The decrease in I_{sc} caused by amiloride and phloridzin were significantly larger in fornal than in palpebral conjunctiva (P < 0.05). Second, forskolin administration elicited a statistically significant, but much smaller increase in I_{sc} in all regions of conjunctiva (P < 0.05), with no differences amongst groups. This difference likely was due to pretreatment with the CFTR inhibitor (CFTRinh172).

A significant residual current was identified in paradigm 1 and 2 following cumulative application of both Na⁺ and Cl⁻ transport inhibitors. Accordingly, in third paradigm, we studied the tissues in Cl⁻-free KBR to test the hypothesis that Cl⁻ transport dominated the residual I_{sc} of conjunctiva. The basal I_{sc} of all three regions of conjunctival tissues were smaller in Cl⁻-free KBR than regular KBR (P < 0.05; Fig. 7). Indeed, the basal I_{sc} of palpebral, fornal, and bulbar conjunctiva in Cl⁻-free KBR compared with regular KBR decreased by 76, 75, and 48%, respectively. In addition, the resistance of all conjunctival tissues in Cl⁻-free KBR were higher than in regular KBR (P < 0.05). As shown in Fig. 8, A and B, the application of the Na⁺ transport inhibitors phloridzin and amiloride and the Na⁺-K⁺ ATPase inhibitor ouabain significantly reduced the I_{sc} in all conjunctival tissues bathed in Cl⁻-free KBR (P < 0.05), with the bulbar and fornal conjunctiva exhibiting larger ∆I_{sc} than palpebral conjunctiva.

**Figure 5.** Ussing chamber studies of freshly excised rat conjunctival tissues to measure ex vivo bioelectric properties in regular Krebs-bicarbonate Ringer (KBR; paradigm 1). Drugs including trypsin, amiloride, phloridzin, forskolin, CFTRinh172, and UTP were applied sequentially to the apical bath of chamber. A: representative recorded short circuit current (I_{sc}) traces following the paradigm 1 serial drug treatments. Amiloride, phloridzin, and CFTRinh172 inhibited I_{sc}, whereas forskolin and UTP increased I_{sc}. B: average basal I_{sc} of 6 preparations of each region of conjunctiva. Fornical conjunctiva exhibited higher I_{sc} than palpebral and bulbar conjunctiva. C: I_{sc} changes (∆I_{sc}) caused by addition of each drug. Amiloride addition resulted in slight but significant decrease in I_{sc} in fornal and bulbar conjunctiva but not in palpebral conjunctiva. Phloridzin treatment caused smaller I_{sc} changes in palpebral than bulbar conjunctiva. Forskolin elicited a greater I_{sc} increase in fornal conjunctiva than in palpebral and bulbar conjunctiva, whereas CFTRinh172 resulted in a greater I_{sc} decrease in fornal conjunctiva than in palpebral and bulbar conjunctiva. *P < 0.05.
All tissues in Cl–bulbar conjunctiva, the residual secretion reflected active ion transport, ouabain was added to tissues as Zn inhibited the three regions of conjunctiva. For example, amiloride and phloridzin inhibited chloride secretion exceeded sodium absorption in all modes of active ion transport across freshly isolated rat conjunctiva. Chloride secretion exceeded sodium absorption in all regions of conjunctiva. Fornical conjunctiva exhibited higher Isc than palpebral and bulbar conjunctiva. *P < 0.05.

We next compared the data from paradigm 2 (KBR) and 3 (Cl–free KBR) to ask whether the residual current post CFTRinh172 and phloridzin/amiloride in paradigm 2 reflected residual Cl– secretion. Shown in Fig. 8C are the residual Isc from paradigm 2 (KBR) vs. the residual Isc from paradigm 3 (Cl–free KBR) post-Na+ transport inhibitors (phloridzin and amiloride). In both palpebral and fornical conjunctiva, but not bulbar conjunctiva, the residual Isc was significantly reduced in Cl–free KBR. To ascertain whether the residual current reflected active ion transport, ouabain was added to tissues as part of paradigm 3. Ouabain further reduced the residual Isc in all tissues in Cl–free KBR (Fig. 8C).

Taken together, our data revealed evidence of multiple modes of active ion transport across freshly isolated rat conjunctiva. Chloride secretion exceeded sodium absorption in all three regions of conjunctiva. For example, amiloride and phloridzin inhibited the Isc slightly, whereas the response to CFTRinh172 was large and the basal Isc decreased by 48–76% in Cl–free KBR compared with Cl–containing KBR.

Bioelectric properties of cultured rat conjunctival epithelial cells. Rat palpebral conjunctival epithelial cells were expanded on plastic culture dishes for several passages. As shown in Fig. 9A, conjunctival cells cultured on tissue culture plastic exhibited a cobblestone morphology typical of epithelial cells. Cells were then plated onto permeable Snapwell inserts (Fig. 9B) and were subjected to ALI culture after reaching confluence (Fig. 9C). Six days after ALI culture (Fig. 9D), Snapwells were mounted on Ussing chambers to study ion transport across the cells. Twelve Snapwells of cells from two different tissue isolations were analyzed.

Basal bioelectric parameters, including PD, RT, and Isc, were first measured. Cells were then separated into two groups and treated with a series of agonists/inhibitors in different orders, i.e., paradigm 1 and 2. In paradigm 1 experiments (Fig. 10, A and B), the cells exhibited a basal PD of −3.6 ± 0.43 mV, an Isc of 7.1 ± 0.56 µA/cm², and a RT of 432 ± 59 Ω·cm². Amiloride and subsequent phloridzin addition significantly inhibited Isc by −1.1 ± 0.20 and −1.4 ± 0.19 µA/cm², respectively (P < 0.05). Forskolin elicited a significant increase in Isc of 1.4 ± 0.14 µA/cm² (P < 0.05), which waned within several min. CFTRinh172 decreased the Isc slightly but statistically significantly by −0.6 ± 0.10 µA/cm² (P < 0.05). The Isc response to UTP was the largest amongst the tested compounds, with a statistically significant ΔIsc of 3.0 ± 0.26 µA/cm² (P < 0.05). The increased Isc waned within several minutes and remained stable at a level above UTP pretreat-
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Fig. 7. Comparison of bioelectric properties of rat conjunctival tissues in regular (Cl−-containing) and Cl−-free KBR. A: all 3 regions of conjunctiva exhibited statistically lower basal Isc in Cl−-free KBR compared with regular KBR (n = 6). B: resistance of each region of conjunctiva was larger in Cl−-free than regular KBR (n = 6). *P < 0.05.

DISCUSSION

Chloride secretion and sodium absorption provide the main driving forces for osmotically driven water movement across many epithelia throughout the body. Active sodium and chloride transport across the conjunctiva have been predominantly studied in tissues derived from rabbits and mice (24, 26). In Ussing chamber studies of freshly excised tissues, Kompalla et al. (24) reported active chloride transport accounted for 75% of the total Isc across rabbit conjunctiva. An N-phenylantranilic acid-sensitive Cl− channel was detected on the mucosal surface of conjunctiva, whereas the Na+−K+−ATPase, the Na+−(K+−2Cl−) cotransporter, and K+ conductive pathways were detected on the basolateral surface. However, amiloride-sensitive ENaC Na+ channels did not appear to be active at either surface of conjunctival epithelia (3, 24). Instead, a Na+−amino acid cotransporter (25) and a Na+−glucose cotransporter (20) on the mucosal surface accounted for the Na+ entry into the conjunctival epithelial cells that mediated fluid absorption.

In the present study, we first investigated the expression and function of ENaC in rat conjunctiva. ENaC, a multimeric channel comprised of α-, β-, and γ-subunits, plays a pivotal role in the regulation of transepithelial sodium transport.

Lastly, TFMP, a chloride channel inhibitor effective in inhibiting TMEM16A (37), reduced Isc significantly by 0.7 ± 0.19 μA/cm² (P < 0.05). The relative percentage change in basal Isc caused by each drug was as follows: CFTRinh172 −1 ± 0.8%, amiloride −18 ± 2.6%, phosphoridzin −29 ± 1.8%, forskolin +2 ± 1.0%, UTP +44 ± 2.7%, and TFMP −14 ± 2.7%. Again, a significant residual Isc was noted.

In vivo measurement of ocular surface transepithelial electrical PD. PD measurements were used to study ion transport across the ocular epithelial surface in vivo. Due to their anatomical continuity in vivo, both conjunctival and corneal epithelia likely contributed to the measured PD. Baseline PD stabilized ~15 min after initial measurement. As shown in Fig. 11, the baseline PD (performed on 6 eyes of 4 rats) was −10.6 ± 1.52 mV (tear film negative with respect to the subcutaneous tissue reference electrode). Perfusion with an amiloride solution induced ocular surface depolarization. After 3–5 min, the PD reached a new stable level of to −2.8 ± 1.16 mV. The depolarization caused by amiloride (−7.8 ± 0.70 mV) accounted for 78 ± 8.5% of basal PD, indicating the involvement of amiloride-sensitive ENaC in ocular surface ion transport in vivo.

Effects of amiloride and UTP on tear volume. Our bioelectric data suggest that both Na+ and Cl− transport pathways are expressed in rat conjunctiva and may contribute to ocular surface volume regulation. Accordingly, we evaluated the effects of a Na+ channel blocker, amiloride, and a Cl− secretagogue, UTP, relative to vehicle treatments, on tear volume in a dry eye rat model (ExLac rats; Ref. 36). Following lacrimal glands excision, ExLac rats exhibited significantly reduced basal tear volumes (47.5% lower than normal rats; n = 4; P < 0.05) as assessed by the wetting distance measured by insertion of a phenol red thread into the lateral canthus for 10 s (Fig. 12A). Application of a single 5-μl instillation of amiloride (1 mM) or UTP (62 mM) produced statistically significant and similar increases in tear volume 15 min post-dosing relative to vehicle treated animals (P < 0.05). Tear output levels returned to approximate baseline levels after 60–120 min post-dosing for both amiloride and UTP treatment groups (Fig. 12B).

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physiological role in Na$^+$ absorption across airway and renal epithelia (12, 29). We detected ENaC subunit mRNAs and protein in rat conjunctiva (Figs. 1–3). ENaC mRNA levels were significantly higher in palpebral than bulbar conjunctiva. ENaC is activated by proteolytic cleavage at multiple sites. These include the degenerin site close to the first transmembrane segment and the furin-consensus site of the ENaC subunit, reported to be cleaved upon catalytic activation (13, 41). We found biochemical correlates of ENaC activation/cleavage in conjunctiva by Western blot, which revealed an ENaC band at 95 kDa and a lower band at 18 kDa (Fig. 3). The exact proteolytic activation mechanism of ENaC in ocular tissues, however, remains to be elucidated. Amiloride application to the Ussing chambers produced a small but significant inhibition on $I_{sc}$ of freshly isolated rat conjunctiva tissues, which suggests ENaC is indeed active in rat conjunctiva (Figs. 5, 6, and 8).

There was also evidence for a second Na$^+$ absorptive process, i.e., phloridzin sensitive Na$^+$-glucose absorption. Slc5a1 mRNA was uniformly expressed in the conjunctiva (Fig. 2). The magnitude of phloridzin-sensitive, electrogenic Na$^+$-dependent glucose transport was equal to ENaC-mediated Na$^+$ absorption and showed only modest regional differences in the three different conjunctival preparations.

We next investigated Cl$^-$ transport paths in the rat conjunctiva. CFTR is a cAMP activated chloride channel and potential regulator of other ion channels, including ENaC (14). CFTR has been identified in corneal endothelium (47), corneal epithelium (1), conjunctival epithelium (48), and lacrimal glands (11, 49). Ocular surface PD measurements in mice demonstrated active Cl$^-$ transport mediated via CFTR (27). The importance of CFTR in ocular surface health of humans has been proposed in several clinical studies. For example, CF patients exhibit ocular abnormalities, including dry eye symp-
Fig. 9. Phase contrast micrographs of primary rat conjunctival epithelial cells. A: conjunctival epithelial cells growing on plastic dishes exhibited typical cobblestone like cell morphology. B: conjunctival epithelial cells growing on Snapwell with media on both apical and basolateral compartment. C: cell culture on Snapwell with apical surface media removed and air-liquid interface culture initiated. D: differentiated conjunctival epithelial cells under air-liquid interface culture for 6 days.

Fig. 10. Ussing chamber studies of conjunctival epithelial cell cultures. A: Representative trace of recorded $I_{sc}$ under paradigm 1 series of drug treatments. B: $I_{sc}$ changes ($\Delta I_{sc}$) caused by each drug application ($n = 6$). C: representative trace of $I_{sc}$ performed with paradigm 2 drug treatments. Amiloride, phloridzin, CFTRinh172, and $N$-(4-trifluoromethylphenyl)anthranilic acid (TFMP) addition resulted in significant decreases of $I_{sc}$, whereas forskolin and UTP treatment elicited an increase of $I_{sc}$. D: $\Delta I_{sc}$ caused by each drug application ($n = 6$). In contrast to paradigm 1, CFTRinh172 addition first caused a slight but not statistically significant inhibition on $I_{sc}$. In addition, this treatment almost abolished the $I_{sc}$ response to forskolin. Other compounds, including amiloride, phloridzin, UTP, and TFMP, evoked a similar response as seen in paradigm 1. *$P < 0.05$. 

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and cornea as well as lacrimal glands (Fig. 1 and 2). The lacrimal glands expressed much higher levels of Tmem16a and f mRNA than conjunctiva or cornea, raising the possibility that Tmem16a and f play roles in lacrimal gland secretion. These data are paralleled by the relatively small UTP functional responses in freshly excised conjunctival tissues (Figs. 5 and 6).

In addition to CFTR and TMEM16A, the high residual currents of conjunctival tissue, which mainly reflect Cl⁻ transport based on the ion substitution (Cl⁻ free KBR) study, suggest other as yet unidentified Cl⁻ channels may participate in conjunctival Cl⁻ secretion. For example, it is possible that Tmem16g functions as Cl⁻ channel in conjunctiva based on its abundant expression level in this tissue. In contrast, Tmem16b’s low expression levels indicate that it may not be an important ion channel in ocular surface. Further investigations utilizing genetically modified mice may provide key insights regarding the clinical relevance of these channels to conjunctival transport (39).

It is noteworthy that the palpebral conjunctival preparations detected a much higher basal I_c in fornical compared with palpebral and bulbar conjunctiva (Fig. 5 and 6). Furthermore, the fornical conjunctiva responded more robustly to forskolin or CFTRinh172 addition than palpebral or bulbar conjunctiva, which suggests that fornical conjunctiva exhibits the highest capacity for CFTR-mediated chloride secretion in the conjunctiva. These data suggest that the fornical conjunctiva has a large capacity for increasing the tear volume through Cl⁻ and fluid secretion.

It is noteworthy that the palpebral conjunctival preparations utilized for Ussing chamber studies included the underlying Meibomian gland tissue, which increased the tissue thickness and may have decreased the metabolic health of the tissue. Hence, the transport activity of those tissues could have been underestimated and may explain the discrepancy between CFTR mRNA and functional studies in palpebral conjunctiva.

The capacity of UTP and other nucleotide analogs to accelerate the rate of conjunctival chloride and fluid secretion (21, 28, 44) and conjunctival goblet cell mucin secretion has been previously reported (23). TMEM16A and TMEM16B, also known as anoctamin 1 and 2, have been recently identified as Ca²⁺-activated chloride channels that may, in addition to CFTR, mediate UTP-activated Cl⁻ currents (7, 45). It is possible that additional members of this family have similar functions, particularly TMEM16F (2). We identified, for the first time, Tmem16a, b, f, and g mRNA in rat conjunctiva...
As part of this study, we established a rat palpebral conjunctival epithelial cell culture system suitable for Ussing chamber studies (Figs. 5, 6, and 10). Similar to freshly excised tissue, cultured cells exhibited functional apical membrane sodium and chloride channels. However, the ion transport features of the cultured epithelia were significantly different from freshly excised tissues. First, the basal Isc was markedly lower in cultures than in freshly excised tissues. Second, the relative magnitude of response to several inhibitors and agonists differed. For example, the effects of amiloride and phloridzin on Isc of cultured cells were greater than in freshly excised tissues. Conversely, forskolin-induced increases in Isc were relatively large and prolonged in freshly excised tissues whereas those of cultured cells were small and waned within several min (Figs. 5 and 10). Importantly, CFTRinh172 did not inhibit the basal Isc of cultured cells, but abolished forskolin-stimulated currents, indicating a smaller CFTR contribution to basal ion transport than observed in freshly excised tissues (Figs. 6 and 10). Finally, the calcium-activated chloride channel agonist UTP caused a transient Isc increase (2.2–13.8 uA/cm²) that relaxed back to pretreatment levels within s in freshly excised tissues, whereas in cultured cells UTP elicited a peak Isc increase (2.9–3.0 uA/cm²) that was sustained for several minutes.

It is well known that culture conditions can affect epithelial ion transport properties, and future studies will be needed to adjust culture conditions so that the transport properties of cultured conjunctival cells better mimic those of freshly isolated tissues. However, our current cell culture system offers advantages such as increased numbers of preparations per animal and preservation of regulated Na+ and Cl− transport, providing in vitro model for ion transport mechanistic studies and genetic manipulation.

The small Isc response to amiloride in freshly excised tissues (Figs. 5 and 6), compared with the robust ENaC expression (Fig. 2), was surprising and could reflect sensitivity of ENaC to metabolic disturbances incurred by tissues during the dissection procedure. Although we took special precaution to protect the integrity of the conjunctival epithelium, it is possible that tissue damage or hypoxia inhibited the ability to respond to amiloride. To reconcile differences between levels of ENaC expression and Ussing chamber function, we measured basal PD and the amiloride-sensitive PD in vivo (Fig. 11). Amiloride induced a large inhibition of the in vivo PD, which likely reflects ion transport across both cornea and conjunctiva. The rat in vivo PD data are consistent with a prior study (26) that demonstrated significant amiloride-sensitive Na+ transport in the mouse ocular surface utilizing PD measurements. Thus we conclude that ENaC is a major contributor to the bioelectric properties of rat corneal/conjunctival tissues in vivo.

Finally, the conjunctiva has been reported to regulate tear volume (10). Therefore, we tested the relative roles of Na+ absorption versus Cl− secretion on tear volume in vivo in a rat model of dry eye disease (ExLac, see MATERIALS AND METHODS). Based on previous reports (35, 38), UTP was tested as a prototypic Cl− secretagogue and was again found to increase tear volume (Fig. 12). Amiloride also increased tear volume, consistent with previous observations in normal rabbits (19). Importantly, the activity of each agent was similar, suggesting major roles for both pathways in controlling tear film volume. Taken together, our results suggest that both Na+ absorption and Cl− secretion are major contributors to ocular surface liquid homeostasis.

In summary, this study revealed the involvement of ENaC, SLC5A1, CFTR, and TMEM16 family members in the ion transport properties of rat conjunctiva. Cl− secretion was the dominant ion transport across freshly excised rat conjunctival tissues but varied as a function of different regions. However, studies of conjunctival transport in vivo and in cultured cells also suggest a key role of Na+ absorption. A better understanding of mechanisms underlying normal tear film maintenance may provide insights to develop therapeutic strategies for pathological conditions.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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