CFTR-Regulated Chloride Transport at the Ocular Surface in Living Mice Measured by Potential Differences

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PURPOSE. To define the role of the cystic fibrosis transmembrane conductance regulator (CFTR) in Cl⁻ secretion at the mouse ocular surface in vivo.

METHODS. Open-circuit potential differences (PDs) across the fluid-bathed ocular surface were measured in anesthetized wild-type and cystic fibrosis (CF) mice in response to Cl⁻ ion substitution and transport agonists and inhibitors.

RESULTS. Basal ocular surface PD was $-23 \pm 1$ mV (SE; 20 wild-type mice), depolarizing to $-16 \pm 2$ mV after amiloride, then hyperpolarizing to $-34 \pm 3$ mV after low Cl⁻. CFTR activation by forskolin or a selective activator caused further sustained hyperpolarization to $-50$ to $-60$ mV. UTP produced a comparable but transient hyperpolarization. The CFTR inhibitors CFTRinh-172 and GlyH-101 largely reversed agonist- but not low Cl⁻-induced hyperpolarizations. PD in CF mice hyperpolarized by $2.1$ mV after low Cl⁻ and was insensitive to CFTR activators or inhibitors.

CONCLUSIONS. CFTR provides a major pathway for mouse ocular surface Cl⁻ secretion, suggesting the application of CFTR activators as therapy for dry eye. Amiloride-sensitive Na⁺ transporters facilitate Na⁺ absorption. PD measurements provide a robust and reproducible means of assessing ocular surface ion transporting mechanisms. (Invest Ophthalmol Vis Sci. 2005; 46:1428–1434) DOI:10.1167/iovs.04-1314

The ocular surface, lined by corneal and conjunctival epithelia, plays an important role in regulating tear film volume and composition. Prior studies indicate that these "tight epithelia" are able to secrete chloride and absorb sodium (reviewed in Refs. 1, 2). Apical chloride secretion across ocular surface epithelia provides a primary driving force for near-isomolar fluid transport across cornea and conjunctiva into the tear film. Measurements of basal and agonist-induced chloride-driven fluid secretion in several species suggest corneal and conjunctival involvement in basal tear production as well as their possible role in tear film dysfunction.5–5 Dry eye syndrome, or keratoconjunctivitis sicca (KCS), represents a diverse group of conditions that manifest as inadequate ocular surface lubrication. Dysfunction of various ocular adnexa can alter tear lipid, aqueous, or mucin components, resulting in dry eye symptoms in 15% to 60% of the population. In the United States, pathologic KCS is diagnosed in 7% of women aged 55 and older.6

The cystic fibrosis transmembrane conductance regulator (CFTR) is a CAMP-dependent chloride channel expressed in many mammalian tissues performing fluid transport, including epithelia of the airway, intestine, and pancreas (reviewed in Refs. 7,8). CFTR is a voltage-independent anion channel and a regulator of other ion channels. CFTR activation requires nucleoside triphosphates and CAMP for phosphorylation by protein kinase A on a cytoplasmic-facing regulatory domain. In humans, CFTR mutations that alter its cellular processing, expression, and/or intrinsic transport function cause cystic fibrosis (CF), the most common lethal hereditary disease. In the eye, CFTR is present in corneal endothelium and retinal pigment epithelium, where it has been proposed to facilitate corneal deturgescence21,22 and to regulate subretinal extracellular volume and chemical composition,10,11 respectively. CFTR has also been localized to apical superficial cell membranes of the stratified conjunctival epithelium.12,13 suggesting a role for CFTR in tear film homeostasis under normal conditions and as a target for therapy of KCS. Patients with CF exhibit subtle hints of ocular surface derangement, such as decreased tear production and increased corneal fluorescein staining.14 Characteristics of dry eye, including corneal and conjunctival squamous metaplasia, persist in patients with CF, even after correcting the potentially confounding Vitamin A deficiency that arises from intestinal malabsorption.15,16

Short-circuit current measurements on rabbit cornea and conjunctiva17,18 and patch-clamp studies on cultured corneal epithelial cells19 demonstrated a CAMP-dependent outward apical chloride current, possibly due to activation of CFTR. Calcium and phospholipase C signaling stimulate transient chloride secretion across conjunctiva through an as yet unidentified channel.20 INS365, a long-acting UTP agonist that stimulates calcium-sensitive chloride–fluid secretion,21,22 is currently in phase III clinical trials for treatment of dry eye. Our laboratory has identified potent CFTR-selective activators by high-throughput screening,23,24 with the potential for use in dry eye syndrome if CFTR is found to provide a major route for ocular surface chloride secretion. A potential advantage of agonists of CFTR versus calcium-activated chloride channels is the sustained versus transient activation kinetics.

The purpose of this study was to identify the major transport pathways for Cl⁻ at the ocular surface, focusing on the potential role of CFTR. Experiments were performed on mice based on the availability of transgenic mice lacking functional CFTR and on our prior work establishing optical methods to characterize aquaporin-dependent water transporting mechanisms at the mouse ocular surface.25 In addition to comparative measurements in CF mice, potent CFTR inhibitors26,27 and an activator24 identified by our laboratory were used to define the role of CFTR in ocular surface Cl⁻ transport. To study conductive Cl⁻ transport in vivo, we introduce a novel, minimally invasive potential difference (PD) measurement approach, which was motivated by the successful application of open-circuit PD measurements to study ion transport mechanisms in
nasal and rectal epithelia. We found large, robust, and reproducible PDs at the mouse ocular surface that were sensitive to ion substitution and pharmacologic maneuvers, providing direct evidence for a major role of CFTR in ocular surface Cl⁻ secretion.

Materials and Methods

Mice

Wild-type mice and CF mice (homozygous G551D-CFTR mutant mice) in a CD1 genetic background were bred at the University of California, San Francisco Animal Facility. G551D heterozygous mice were bred to generate homozygous CF mice. Wild-type mice were fed a standard diet, and CF mice were fed Peptamen (Nestle Nutrition, Vevey, Switzerland). Mice weighing 25 to 30 g were studied at age 8 to 12 weeks. Protocols were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of California at San Francisco Committee on Animal Research.

Mouse Preparation for PD Measurements

Mice were anesthetized with 2,2,2-tribromoethanol (avertin, 125 mg/kg intraperitoneal; Sigma-Aldrich, St. Louis, MO), with additional avertin injected during experiments to maintain anesthesia. Mice were immobilized with a custom-built stereotaxic device equipped with a rotating jaw clamp to orient the eye under study to face upward. Corneas were kept hydrated with PBS supplemented with NaCl to 320 mOsm to match mouse serum osmolarity. A heating pad and rectal temperature probe were used to maintain body temperature at 37 ± 1°C. The cornea and bulbar conjunctiva were exposed by gently retracting the loose facial skin by hand (Fig. 1A, inset). A probe catheter fashioned from PE-90 polyethylene tubing beveled at a 45° angle was positioned ∼1 mm above the ocular surface using a microprositioner and was not adjusted during experiments. Solutions were perfused through plastic tubing (0.16 cm inner diameter) using a multireservoir gravity pinch valve system (ALA Scientific), with the flow rate set at 6 mL/min using a variable-flow peristaltic pump (mechanical pump, Fisher). A −50-μL fluid reservoir was maintained around the probe tip on the ocular surface throughout the experiment by surface tension, with a suction cannula positioned ∼3 mm from the orbit to drain excess fluid. This setup permitted rapid solution changes (dye-exchange experiments indicated a solution exchange time of <3 seconds) with minimal electrical noise.

Measurement of Ocular Surface PD

PD at the ocular surface was measured using a protocol adapted from nasal PD studies, with the experimental apparatus depicted in Figure 1A. Ocular surface PD was measured continuously with the ocular surface perfused serially with up to eight different solutions. PD was insensitive to exact catheter probe orientation and was unaffected by switching between identical solutions. The probe was connected through a 1-M KCl agar bridge to an Ag/AgCl electrode and high-impedance digital voltmeter (Isomilivolt Meter; World Precision Instruments, Sarasota, FL). Voltmeter input and system electrical resistances were 10¹⁵ and 1.1 × 10⁸ Ω, respectively. PD was recorded at 5 Hz with a 14-bit analog-to-digital converter. The reference electrode consisted of a second Ag/AgCl electrode with 1-M KCl agar bridge and was connected to a winged 21-gauge needle filled with 320 mOsm saline (PBS + NaCl). The needle was inserted in the subcutaneous tissue at the back of the neck, though PD was insensitive to reference electrode placement (<1 mV difference with subcutaneous placement in neck versus abdomen). Cl⁻ replacement produced a small positive junction potential (generally ∼1 mV) that was measured daily and used to correct PDs measured with low Cl⁻ perfusates.

Compounds and Solutions

All perfusion solutions were isosmolar to mouse serum (320 ± 5 mOsm) as measured using a freezing point-depression osmometer (Precision Systems, Natick, MA). The compositions of each solution are provided in Table 1. Solution 1 (control solution) consisted of phosphate buffered-saline supplemented with 15 mOsm NaCl. In Solution 2 (low-Cl⁻ solution; 4.7 mM Cl⁻), most Cl⁻ was replaced by gluconate. In some experiments, NaCl was replaced isosmotically with 25 mM NaHCO₃ (solution 3). Unless noted otherwise, all chemicals were purchased from Sigma-Aldrich and prepared as 1000 molar stock solutions in dimethyl sulfoxide (DMSO). All perfusion solutions contained 10 μM indomethacin (see the Results section). In most experiments, amiloride (100 μM) was present in all solutions after baseline measurement. Other activators and inhibitors used in the perfusates included forskolin (2 or 10 μM); UTP (10 μM, freshly dissolved in PBS); DIDS (4-[N-4(3-(3-fluoromethyl)phenyl)-5(3-carboxyphe-nyl)-2-thiazolidinone; 10 μM); GlyH-101 (N-(2-naphthalenyl)-[1-(5,5-dibromo-2,4-dihydroxyphenyl)methylene]glycine hydrazide; 20 μM); and

Table 1. Composition of Perfusate Solutions (mM)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Name</th>
<th>Cl⁻</th>
<th>Gluconate</th>
<th>HCO₃⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>160.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Low Cl⁻</td>
<td>4.7</td>
<td>155</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Bicarbonate</td>
<td>135.0</td>
<td>0</td>
<td>25</td>
</tr>
</tbody>
</table>

All solutions contained (in mM): 170 Na⁺, 4.2 K⁺, 9.5 phosphate, 1 Ca²⁺, 0.5 Mg²⁺ (pH 7.4).
solutions to minimize the influence of Na
be subsequently reversed by a Cl
independent Cl
(solution 2) to give a sustained hyperpolarization related to cAMP-
then largely replaced by the relatively impermeant anion gluconate
The baseline PD of approximately
solutions that could be switched without interruption of flow.
ocular surface was perfused at constant flow (6 mL/min) with
used in nasal PD studies. As diagrammed in Figure 1A, the
Ocular surface PD was measured by modification of methods
RESULTS
Data are expressed as the mean ± SE of absolute PDs or PD differences
SE of absolute PDs or PD differences
channel function. Perfusate Cl
in many tissue electrophysiology studies, indomethacin was
or trauma-induced prostaglandin production. As has been done
attributed to variable basal cAMP stimulation related to stress-
within 4 mV of baseline value. Although minimal drift of PD in
these control experiments implies the maintenance of epithelial barrier function, return to baseline was not routinely assessed, because the various maneuvers performed in the study are expected to alter intracellular ionic activities over time, thus changing electrochemical driving forces. Moreover, some of the activators/inhibitors used may not be fully or rapidly reversible.

In preliminary experiments, the hyperpolarization due to Cl
substitution was somewhat variable and was often larger than the effects of subsequently added agonists, which was attributed to variable basal CAMP stimulation related to stress- or trauma-induced prostaglandin production. As has been done in many tissue electrophysiology studies, indomethacin was present in all solutions to block prostaglandin production. We found large and reproducible effects of CAMP agonists in the presence of indomethacin with reduced initial low Cl
induced hyperpolarization.

Ocular Surface Cl
Transport
The first series of experiments was designed to identify CFTR Cl
channel function using the CAMP agonist forskolin and two classes of high-affinity CFTR inhibitors. Thiazolidinone (CFTR
172) and glycine hydrazide (GlyH-101) CFTR inhibitors have low micromolar or better inhibitory potencies, and their application in vivo has been demonstrated in mouse models of secretory diarrhea and nasal epithelial CFTR Cl
secretion. Representative ocular surface PD recordings in Figure 2A show a small amiloride-induced depolarization. The amiloride effect at 10 μM was ~90% of maximum response at 100 μM (not shown). Exchange to a low Cl
perfusion resulted in a hyperpolarization, and subsequent activation of CFTR (and possibly other CAMP-dependent ion transporters)
by forskolin produced a further hyperpolarization. CFTRinh-172 or GlyH-101 added after forskolin produced a depolarization. Figure 2B summarizes absolute PDs for a series of experiments as in Figure 2A. The forskolin-induced hyperpolarization was reversed by CFTRinh-172 and to a lesser extent by GlyH-101. Higher concentrations of GlyH-101 were not used to avoid nonspecific inhibition of calcium-activated chloride channels. The forskolin-induced hyperpolarization in the presence of low Cl⁻ and its reversal by CFTR inhibitors provide evidence for functional CFTR Cl⁻ channels at the ocular surface.

UTP is a Cl⁻ channel agonist that stimulates ocular surface Cl⁻ secretion by a calcium-dependent process. UTP in the low-Cl⁻ perfusate consistently produced a large, negative spike in PD, followed by a slower, transient hyperpolarization (Figure 3A). Addition of the disulfonic stilbene inhibitor DIDS, but not CFTRinh-172 (not shown), reversed the UTP-dependent hyperpolarization when added at any time during the transient hyperpolarization. A substantial forskolin response occurred after the transient UTP-induced hyperpolarization. To determine the contribution of HCO₃⁻ transport to PDs, a small set of studies was performed in which 25 mM HCO₃⁻ was added to the perfusate. PD did not change when HCO₃⁻ was added to the physiological Cl⁻-containing solution, but depolarized reversibly by 1.5 ± 0.2 mV (SE) when HCO₃⁻ was added to the low-Cl⁻ perfusate after forskolin (six eyes studied); CFTRinh-172 did not prevent the small HCO₃⁻-induced depolarization.

To investigate whether the low Cl⁻-induced hyperpolarization in the absence of agonists involves DIDS- or CFTRinh-172-sensitive Cl⁻ channels, inhibitors were added without prior addition of the agonist. In some experiments, as shown in Figure 3B, DIDS and CFTRinh-172 partially reversed the low-Cl⁻-induced hyperpolarization. Significant variability was noted from mouse to mouse, though the combined depolarizations from DIDS and CFTRinh-172 were always <30% of the low-Cl⁻-induced hyperpolarization.

Two additional strategies were applied to investigate the involvement of CFTR in ocular surface Cl⁻ secretion: use of a CFTR-selective agonist that activates CFTR without elevating cAMP (CFTRact-04) and measurements on transgenic mutant (CF) mice lacking functional CFTR. Figure 4A (left) shows a representative PD measurement as in Figure 2A, except that CFTRact-04 was added before forskolin. CFTRact-04 produced a prompt hyperpolarization that was increased relatively little by forskolin, and was reversed by CFTRinh-172. Agonist-induced hyperpolarization was absent when CFTRinh-172 was added with CFTRact-04 (not shown), and in some cases a small depolarization was noted, resembling the depolarization that occurred when CFTRinh-172 was added after low Cl⁻ in the absence of agonists. Figure 4A (right) shows a study on CF mice. Although substantial amiloride-sensitive depolarization occurred, there was little effect of low Cl⁻ or CFTR agonists or inhibitors. Baseline ocular surface PD was similar in wild-type and CF mice, but the depolarization due to amiloride was significantly greater in CF mice. Absolute PDs are summarized in Figure 4B (left), and a paired analysis of PD changes after specific maneuvers (ΔPD) is given in Figure 4B (right). The CFTR agonist and CF mouse studies provide direct evidence for the involvement of CFTR in ocular surface Cl⁻ transport.

**DISCUSSION**

In this study, we sought to clarify the involvement of CFTR in transepithelial chloride conductance at the mouse ocular surface. An ocular surface open-circuit potential difference approach was introduced, which is based on the generation of PDs across tight epithelia from asymmetries in apical and basal membrane ion conductances. Maurice et al. and Klyce measured electrical potentials between the body and tear film in rabbit of −15 to −30 mV (tears negative to blood). This original observation of the ability of the ocular surface to generate a PD was expanded on in the present study to characterize electrogenic Cl⁻ transport processes in living mice by perfusion of the ocular surface with solutions that created ionic gradients and contained transport activators or inhibitors. In contrast to short-circuit current and single-cell microelectrode approaches, which require tissue and cell isolation in vitro, the ocular surface PD approach is technically simple and permits minimally invasive in vivo measurements under physiological open-circuit conditions. Also, ocular PDs produce robust voltage changes that are readily interpreted in terms of specific ion transport processes. HCO₃⁻ transport contributed little to ocular surface PDs compared with Cl⁻, demonstrating the relative importance of chloride channels in ocular surface anion flux.

Several lines of evidence implicate the functional involvement of CFTR in ocular surface Cl⁻ secretion, including activation of Cl⁻ secretion by CFTR agonists and reversal of agonist effects by CFTR inhibitors. The incomplete reversal by GlyH-101 may be related to its external pore-blocking mechanism, in which reduced extracellular Cl⁻ concentration and plasma membrane depolarization are predicted to reduce its potency. While agonists were shown to stimulate CFTR conductance, experiments where Cl⁻ channel inhibitors were added under low Cl⁻ but in the absence of agonists indicate that CFTR does not mediate most cAMP-independent Cl⁻ secretion at the ocular surface. Because DIDS is a relatively nonspecific Cl⁻ channel inhibitor, the partial DIDS effect in these experiments cannot be interpreted to implicate the activity of specific Cl⁻ channels.

The absence of CFTR agonist and inhibitor effects in CF mice provides additional evidence for CFTR involvement in ocular surface Cl⁻ transport. One interesting observation was the near-zero hyperpolarization in CF mice after low Cl⁻ in the absence of CFTR agonists. This observation seems at variance...
with the conclusion based on CFTR inhibitor studies that only a small fraction of the low-Cl⁻–induced hyperpolarization involves CFTR-mediated transport. However, similar results were found in nasal PD measurements in CF mice,²⁸,²⁹ where it has been proposed that the low-Cl⁻ response results from Cl⁻ transport by a CFTR-regulated Cl⁻ channel, though not CFTR itself. An alternative possibility is that the non-CFTR Cl⁻ channel responsible for the low-Cl⁻ response is downregulated in CF mice.

Previous RT-PCR and immunocytochemical studies have detected CFTR in corneal and conjunctival epithelial cell cultures and tissue mounts from rabbit.¹²,¹³,¹⁹ However, functional studies on in vitro preparations relied exclusively on nonspecific channel activators (e.g., genistein) and inhibitors (e.g., glibenclamide), and cAMP-elevating agents (IBMX [3-isobutyl-1-methylxanthine] and forskolin). In our in vivo studies, we treat the ocular surface as a single functional unit as in the intact eye, so that ocular surface PDs represent the aggregate of corneal and conjunctival properties. Another reason that we performed measurements on the minimally perturbed whole ocular surface is that mechanical isolation of the two tissues in vivo disrupted epithelial cell integrity. A machined plastic chamber, beveled to create a seal at the limbus and permit selective perfusion and measurement of cornea or conjunctiva, results in a slow, progressive reduction in PD toward zero. Such contact also imposed a stress that may affect cellular transport processes. Further studies are needed to quantify the relative contributions of cornea versus conjunctiva to the ion channel-dependent PD responses recorded in the study and to determine whether increasing CFTR-dependent fluid transport across each tissue could supplement tear formation in states of aqueous deficiency.

Although CF is not generally associated with overt dry eye symptoms, the results of the current study suggest that CFTR provides an important pathway for agonist-stimulated fluid secretion across the ocular surface. Several factors may account for the mild ocular phenotype in CF. Basal CFTR activity may be very low under normal conditions, so that ocular surface hydration does not depend on CFTR function. Also, upregulation of alternative secretory pathways either in tear-producing glands, or within the cornea or conjunctiva, may compensate for impaired CFTR Cl⁻ channel function. Indeed, calcium-activated Cl⁻ channels have been found in some models to compensate for CFTR dysfunction.³⁷

Our results suggest the possibility of using CFTR activators for treatment of dry eye syndrome. Topical application of IBMX, a phosphodiesterase inhibitor/CFTR activator, has been found to reduce the osmolarity of collected tears in humans and rabbits with KCS.³⁸,³⁹ In our study, CFTR activation produced sustained, large increases in ocular surface Cl⁻ secretion, with quantitatively much greater time-integrated hyperpolarization than UTP, an activator of calcium-sensitive Cl⁻ channels. Currently, topical cyclosporin A, which suppresses downstream inflammatory cascades that ultimately compromise epithelial barrier function, is the only drug approved for treatment of dry eye, with UTP analogues currently under development.²¹,²²

Although this study was focused primarily on mechanisms of Cl⁻ secretion, ocular surface PD measurements in response to amiloride indicated the involvement of amiloride-sensitive Na⁺ channels, such as ENaC, in ocular surface Na⁺ absorption. Inhibitors of Na⁺ absorption may provide another therapy for dry eye syndrome. The evidence here for the involvement of amiloride-sensitive Na⁺ channels in mice contrasts with short-circuit experiments in rabbit tissue.¹⁷,⁴⁰ However, functional evidence for ENaC was reported in bovine cornea by Middelkamp,⁴¹,⁴² and ENaC protein has been found by immunocytochemistry in rat and human cornea.³⁵ The roles of individual ocular surface transporters are probably species specific; for example, CAMP activates basolateral K⁺ channels in frog corneal epithelium but inhibits them in rabbit.⁴³,⁴⁴ The amiloride-induced depolarization was greater in CF than wild-type mice.

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932933/) Reduced ocular surface Cl⁻ conductance in CF mice. (A) PD recordings in wild-type (left) and CF (right) mice in response to amiloride, low-Cl⁻, CFTRact-04 (10 μM), forskolin (2 μM), and CFTRinh-172 (10 μM). Inset: CFTRact-04 structure. Solutions used: 1 alone, 1+amiloride, 2+amiloride, 2+amiloride+CFTRact-04, 2+amiloride+CFTRact-04+forskolin, 2+amiloride+CFTRact-04+forskolin+CFTRinh-172. (B, left) PD (mean ± SE) measured after indicated maneuvers from experiments as in (A). (●) Wild-type mice (n = 7); (○) CF mice (n = 3). Right: Paired analysis of ΔPD for wild-type and CF mice for indicated maneuvers. *P < 0.01 comparing wild-type versus CF mice.
as has been reported in nasal PD measurements. The greater depolarization in nasal PDs in CF mice has been interpreted as increased ENaC activity in CF resulting from direct or indirect CFTR-ENaC interaction, though it remains controversial whether altered electrochemical driving forces for Na⁺ absorption in CF can account fully for the greater depolarization in CF.

In summary, measurements of ocular surface PD in mice indicated the involvement of CFTR in ocular surface Cl⁻ secretion. Based on evidence for Na⁺ absorption by corneal and conjunctival epithelia and the amiloride effects described herein, ocular surface PDs should also be useful in studying mechanisms of electrogenic Na⁺ transport at the ocular surface. The substantial and sustained activation of CFTR-mediated Cl⁻ secretion by CFTR agonists supports the evaluation of CFTR-selective activators as potential treatment for dry eye syndromes.

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References


