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Ocular Surface Potential Difference Measured in Human Subjects to Study Ocular Surface Ion Transport

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Received: June 11, 2020
Accepted: September 8, 2020
Published: October 15, 2020

Keywords: corneal epithelium; conjunctiva; membrane transport; chloride channel; sodium transport

Citation: Pasricha ND, Smith AJ, Levin MH, Schallhorn JM, Verkman AS. Ocular surface potential difference measured in human subjects to study ocular surface ion transport. Trans Vis Sci Tech. 2020;9(11):20, https://doi.org/10.1167/tvst.9.11.20

Purpose: The epithelium lining the ocular surface, which includes corneal and conjunctival epithelia, expresses the prosecretory chloride channel cystic fibrosis transmembrane conductance regulator (CFTR) and the proabsorptive epithelial sodium channel (ENaC). Here, methodology was established to measure the millivolt (mV) potential differences at the ocular surface, called ocular surface potential difference (OSPD), in human subjects produced by ion transport.

Methods: OSPD was measured in human subjects in which a fluid-filled measuring electrode contacted a fluid pool created by eversion of the lateral lower eyelid, with a reference electrode placed subcutaneously in the forearm. Through the use of a high-impedance voltmeter, OSPD was measured continuously over 10 to 15 minutes in response to a series of perfusate fluid exchanges.

Results: Baseline OSPD (± SEM) in six normal human subjects was –21.3 ± 3.6 mV. OSPD depolarized by 1.7 ± 0.6 mV following the addition of the ENaC inhibitor amiloride, hyperpolarized by 6.8 ± 1.5 mV with a zero chloride solution, and further hyperpolarized by 15.9 ± 1.6 mV following CFTR activation by isoproterenol. The isoproterenol-induced hyperpolarization was absent in two cystic fibrosis subjects lacking functional CFTR. OSPD measurement produced minimal epithelial injury.

Conclusions: Our results establish the feasibility and safety of OSPD measurement in humans and demonstrate robust CFTR activity, albeit minimal ENaC activity, at the ocular surface. OSPD measurement may be broadly applicable to investigate fluid transport mechanisms and test drug candidates to treat ocular surface disorders.

Translational Relevance: To the best of our knowledge, this is the first measurement of the electrical potential generated by the ocular surface epithelium in human subjects, offering a new approach to study ocular surface function and health.

Introduction

The cornea and conjunctiva are lined by stratified epithelial cell layers in contact with the tear film. As in other organs, epithelial cells lining the ocular surface express ion transport proteins that can facilitate active fluid secretion or absorption to regulate tear fluid volume and osmolality. Major ion channels that are functionally expressed in ocular surface epithelial cells include the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel and the epithelial sodium channel (ENaC), which are thought to facilitate fluid secretion and absorption, respectively.[1–5] The ocular surface epithelium is subject to injury in various infectious and inflammatory conditions, such as bacterial keratitis and Sjögren's syndrome, and by various types of trauma, including desiccation and abrasion.

Clinical evaluation of ocular surface health typically involves slit-lamp examination of the fluorescein-stained cornea and the lissamine green-stained conjunctiva, as well as measurement of tear breakup

Table 1. Perfusate Solution Compositions

Solution No.	Solution Name	Solution Contents
1	High Cl^-	Buffered Ringer's solution[a]
2	Amiloride	Buffered Ringer's solution[a] + 100 μM amiloride
3	Zero Cl^-	Buffered zero chloride solution[b] + 100 μM amiloride
4	Isoproterenol	Buffered zero chloride solution[b] + 100 μM amiloride + 10 μM isoproterenol
5	ATP	Buffered zero chloride solution[b] + 100 μM amiloride + 10 μM isoproterenol + 100 μM ATP

[a]Buffered Ringer's solution: 1 L Ringer's injection (containing 147.16-mM NaCl, 2.24-mM $CaCl_2{\cdot}2H_2O$, and 4.02-mM KCl), 2.41-mM K_2HPO_4, 0.37-mM KH_2PO_4, and 1.18-mM $MgCl_2{\cdot}6H_2O$.

[b]Buffered zero chloride solution: 1 L ddH_2O, 2.41-mM K_2HPO_4, 0.37-mM KH_2PO_4, 147.71-mM sodium gluconate, 1.22-mM $MgSO_4{\cdot}7H_2O$, 4.06-mM potassium gluconate, and 2.26-mM calcium gluconate.

time, Schirmer test of tear fluid volume, and corneal sensation.[6,7] Determinations of tear fluid osmolality and cytokine levels and the cellular composition of ocular surface tissues may also provide useful data for the evaluation of ocular surface disease.[8] Here, we reasoned that electrophysiological measurement of the potential difference (PD) across the ocular surface, termed OSPD, could provide unique functional data on the physiology of the human ocular surface in health and disease. We originally introduced the idea of OSPD measurement in mice and showed that the millivolt (mV) potentials were dependent on CFTR and ENaC activity, enabling mathematical modeling of individual ion transporter activities.[3,9] We subsequently applied OSPD measurement in mice and rabbits to test the efficacy of a prosecretory drug candidate targeting CFTR.[10,11]

The purpose of this study was to establish and validate the methodology to measure OSPD in human subjects and, in doing so, to investigate for the first time, to the best of our knowledge, the in vivo function of CFTR and ENaC at the human ocular surface. This work was inspired by nasal PD measurements in humans, which is an established experimental approach to study CFTR function in cystic fibrosis (CF) subjects that lack functional CFTR.[12,13] We show here that OSPD can be reliably and safely measured in human subjects and that the measured millivolt potentials provide a robust functional assessment of the ocular surface with potentially broad applications in ocular health and disease.

Methods

Human Subjects

This study was Health Insurance Portability and Accountability Act compliant, was approved by the University of California San Francisco (UCSF) Institutional Review Board, and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all study subjects. Six non-CF subjects were healthcare personnel recruited through the UCSF Department of Ophthalmology, and two CF subjects with non-functional CFTR mutations (N1303K/Q1100P and W1282X/W1282X) not on CFTR modulator therapy were recruited from the UCSF Cystic Fibrosis Clinic. Exclusion criteria included pediatric age, presence of ocular surface disease on slit-lamp examination, history of ocular surgery, current topical eyedrop use, or clinically significant allergic rhinitis, ocular allergies, or upper respiratory infection within 30 days. All subjects were given the Ocular Surface Disease Index (OSDI) questionnaire, a validated 12-item scale graded 0 to 100 to assess for symptoms related to dry eye disease and their effect on vision.[14]

Perfusion Solutions

The compositions of the perfusion solutions (Table 1) follow the solutions used in the standardized human nasal potential difference protocol.[13] Solutions 1 to 3 were made in 1-L batches, pH balanced to 7.4, and filtered in a sterile environment prior to refrigeration (stable for 3 months). Solutions 4 and 5 (containing isoproterenol or adenosine triphosphate [ATP]) were made within 2 hours of OSPD measurement. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

OSPD Instrumentation

The electrical components of the instrumentation include measuring and reference electrodes connected to an ISO-Z Head Stage and BMA-200

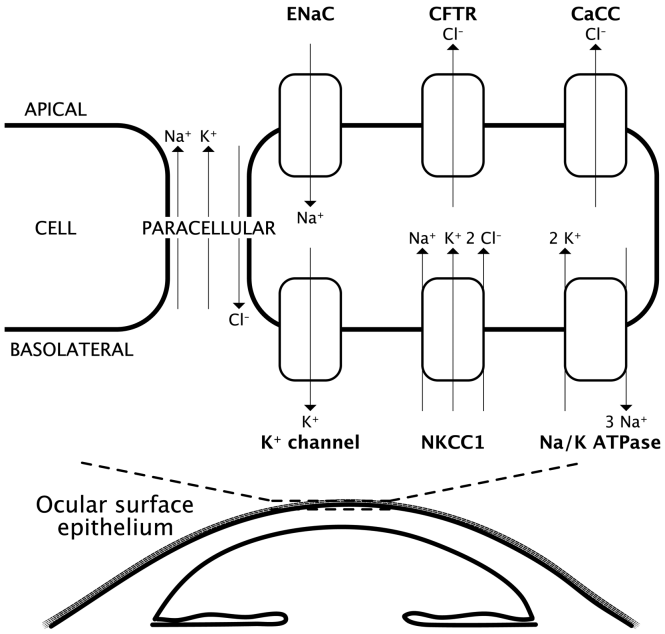

Figure 1. Schematic of major ion transporters in human ocular surface epithelia. Transepithelial chloride secretion onto the ocular surface requires an electrochemical driving force to transport chloride from cell cytoplasm onto the ocular surface through chloride channels CFTR and/or CaCC. The electrochemical driving force is established by the concerted action of K^+ channels, NKCC1, and Na/K ATPase.

high-impedance amplifier/voltmeter (CWE Inc., Ardmore, PA) and a PowerLab analog-to-digital converter (ADInstruments Inc., Colorado Springs, CO) connected to a computer. A perfusion system delivered specified solutions to a perfusion catheter (Thermo Fisher Scientific, Waltham, MA), the tip of which was positioned in a fluid pool at the ocular surface.

To create the reference and measuring electrodes, 3% agar in Ringer's solution was melted and poured into the calomel electrodes. The melted agar–Ringer's mixture was also injected into the Luer lock end of a 23-gauge butterfly needle to create the subcutaneous agar bridge, which was stored in sterile Ringer's solution at room temperature for up to 24 hours. Just prior to testing, offset zeroing was done in a bath containing solution 1 with the reference electrode connected to the agar bridge and the measuring electrode connected to the perfusion catheter.

For solution perfusion, a set of five 60-mL syringes, each with stopcocks, was connected via a multiport tubing system to deliver solutions to a single perfusion catheter. The syringe set was positioned on a height-adjustable column for gravity perfusion at a rate of 5 to 10 mL/min. Each syringe contained a different perfusion solution at room temperature, which has been shown to produce reliable results in nasal potential difference studies.[15] A three-way stopcock was used to connect the perfusion, measuring electrode, and multiport tubing. The perfusion system was flushed in reverse, starting with solution 5 and ending with solution 1.

OSPD Measurement

The subject was comfortably positioned in front of a slit lamp with their head stabilized on a chin rest. Absorbent gauze pads were secured with paper tape to the subject's cheek to absorb perfusate overflow. The 23-gauge butterfly needle agar bridge connected to the reference electrode was inserted subcutaneously in the forearm. One drop of 0.5% proparacaine was instilled into the test (left) eye for anesthesia. Steri-Strips (3M, St. Paul, MN) were used to evert the lateral lower eyelid to create an ~200-µL fluid pool. Using a three-axis micromanipulator (Thorlabs, Inc., Newton, NJ) fixed to the slit lamp, the perfusion catheter tip was guided under direct slit-lamp visualization into the inferior fornix and viewed during the OSPD measurement to ensure adequate contact with the fluid pool without contacting the ocular surface. Each solution was perfused onto the ocular surface for 1 to 3 minutes until a stable OSPD reading was obtained.

Safety

At the end of the OSPD measurement, lissamine green and fluorescein were applied topically to generate an ocular staining score (OSS) ranging from 0 to 12.[6] For one (non-CF) subject, best-corrected visual acuity (BCVA) and intraocular pressure (IOP) were measured before and just after the session. All subjects received lubricating ophthalmic ointment at the end of the session and were asked to report any subjective ocular surface discomfort at that time and again 24 hours later.

Data Analysis

OSPD values after each perfusion solution were calculated as the mean value of a 10-second interval at the end of the solution perfusion, as standardized in human nasal potential difference measurements.[16] Data are expressed as mean ± SEM. Statistical comparisons were made using two-tailed Student's *t*-test in Excel (Microsoft Corp., Redmond, WA).

Results

Determinants of the OSPD

The OSPD is created by the actions of the primary ion transporters expressed in the ocular surface

epithelium (Fig. 1), which are major determinants of tear fluid balance and corneal hydration. The apical membrane (in contact with tear fluid) expresses the prosecretory cyclic adenosine monophosphate (cAMP)-activated chloride channel CFTR and calcium-activated chloride channels (CaCCs). The basolateral membrane (facing the corneal stroma) contains potassium channels, an electroneutral sodium–potassium–chloride cotransporter (NKCC1), and a sodium–potassium pump (Na/K ATPase), the latter providing the energy to drive fluid secretion. There is paracellular ion transport, as well. To create the electrochemical driving force for apical chloride secretion, and hence fluid secretion, the basolateral membrane transporters act in concert to maintain a cell interior-negative membrane potential and, in cytoplasm, a high concentration of potassium, a low concentration of sodium, and a concentration of chloride that is above its electrochemical equilibrium potential for its transport onto the ocular surface when CFTR or CaCCs are open. The OSPD is negative at the ocular surface as referenced to the corneal stroma.

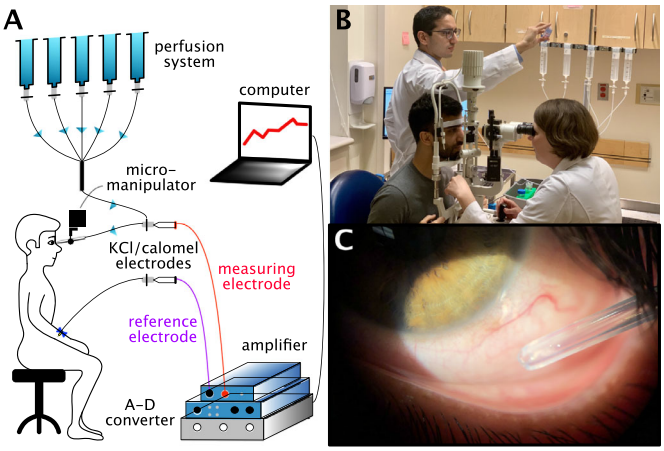

Figure 2. Method for measurement of OSPD in human subjects. (**A**) Schematic showing the multi-syringe perfusion system that delivers fluid to bathe a portion of the ocular surface and the electrical system with measuring electrode in contact with the ocular surface (through the perfusate), subcutaneous reference electrode, and high-impedance amplifier. The subject's head is stabilized using a slit lamp, and the tip of the perfusion catheter is positioned in a fluid pool near the ocular surface using a three-axis micromanipulator during slit-lamp visualization. (**B**) Photograph of an OSPD measurement study showing an operator positioning the tip of the perfusion catheter and an assistant operating the perfusion system. (**C**) Photograph of perfusion catheter in a fluid pool created by eversion of the lateral lower eyelid.

OSPD Measurement in Humans

A high-impedance voltmeter measures the electrical potential generated by the ocular surface epithelium, with the measuring electrode immersed in fluid contacting the ocular surface and the reference electrode inserted subcutaneously in the forearm (Fig. 2A). The measuring electrode makes electrical contact with the ocular surface via a perfusion catheter whose tip is inserted into a small fluid pocket created by eversion of the lateral lower eyelid (Fig. 2B). Solution exchange is accomplished using a gravity perfusion system. The subject's head is stabilized using a slit lamp, with the tip of the perfusion catheter positioned under direct visualization in the fluid pocket without contacting ocular surface tissue (Fig. 2C). Supplementary Movie S1 shows the perfused ocular surface in real time.

Table 2. Clinical Characteristics of Study Subjects

Subjects	Age (y)	Sex	Race	Ethnicity	OSDI	LG OSS	F OSS	Total OSS	Baseline OSPD (mV)	Isoproterenol ΔOSPD (mV)
Non-CF										
1	38.3	M	White	Other	0	2	0	2	−23.4	−11.9
2	66.0	F	White	Other	2.5	0	0	0	−15.3	−13.2
3	31.2	F	Black	Other	0	0	1	1	−11.7	−15.7
4	28.0	M	Other	Other	2.1	0	2	2	−25.7	−20.4
5	30.0	M	Other	Other	6.3	0	1	1	−16.4	−21.0
6	74.5	M	White	Other	0	5	4	9[a]	−35.6	−13.0
CF										
1	55.1	F	White	Other	14.6	3	0	3	18.0	−1.3
2	32.2	F	Other	Hispanic	0	0	0	0	−7.4	−2.4

LG, lissamine green; F, fluorescein; total OSS = LG + F + extra points.
[a]Patient was asymptomatic; total OSS was 0 the day after initial examination.

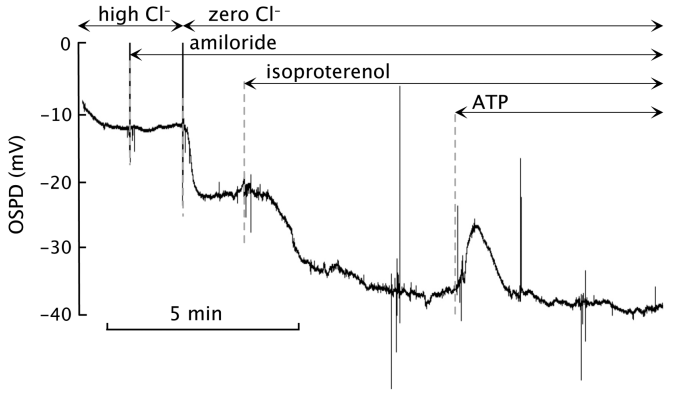

Figure 3. OSPD measurement in a non-CF subject. Original recording of OSPD from subject 3 showing OSPD over time in response to serial perfusate solution exchanges as indicated. See text for further explanation.

Robust CFTR Activity at the Human Ocular Surface

A total of six healthy non-CF subjects were studied, as well as two CF subjects as controls, for CFTR function (Table 2). Figure 3 shows a representative recording of OSPD in a non-CF human subject. At the start of the recording, there was an initial stabilization period, generally under 1 minute. There was less than 2-mV fluctuation in OSPD with no systematic electrical drift during continuous perfusion with solution 1, a physiological solution containing high chloride that approximates tear composition. The baseline OSPD in solution 1 was –21.3 ± 3.6 mV in the six non-CF subjects.

After determination of baseline OSPD, four solution exchanges were done to isolate ENaC, CFTR, and CaCC functions (Fig. 3). Solution 2, a high-chloride solution containing the ENaC inhibitor amiloride, produced minimal depolarization, suggesting minimal ENaC activity. Solution 3, a zero chloride solution that probes basal transcellular and paracellular chloride transport pathways, produced a rapid, modest hyperpolarization. Solution 4, containing the cAMP agonist isoproterenol, produced a more gradual but larger hyperpolarization due to activation of CFTR and potentially other cAMP-dependent ion channels. Solution 5, containing the calcium agonist ATP, produced a biphasic response due to complex actions of transient elevation in cytoplasmic calcium on CaCC and potassium channels.

Absolute OSPD values for the six non-CF subjects are summarized in Figure 4A, and the changes in OSPD (ΔOSPD) produced by the fluid exchanges from solution 1 to 2, from solution 2 to 3, and from solution 3 to 4 are summarized in Figure 4B. OSPD depolarized by 1.7 ± 0.6 mV following ENaC inhibition by

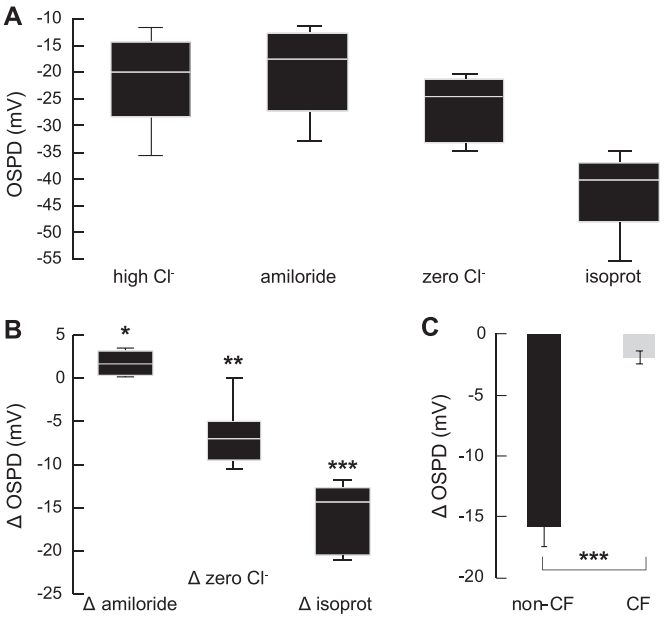

Figure 4. Summary of OSPD data. OSPD values were deduced from experiments as in Figure 3. (**A**) Absolute OSPD values from six non-CF subjects with normal physiological saline solution ("High Cl," solution 1), amiloride-containing normal physiological saline solution ("Amiloride," solution 2), zero chloride solution with amiloride ("Zero Cl^-," solution 3), and zero chloride solution with amiloride and isoproterenol ("Isoproterenol," solution 4). Data are shown as box-and-whisker plot. (**B**) Changes in OSPD (ΔOSPD) in response to indicated solution changes in non-CF subjects. (**C**) ΔOSPD in response to isoproterenol comparing non-CF subjects and two CF subjects. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by two-tailed Student's *t*-test.

amiloride (solution 1 to 2), hyperpolarized by 6.8 ± 1.5 mV following exchange from a high to zero chloride solution (solution 2 to 3), and further hyperpolarized by 15.9 ± 1.6 mV following CFTR activation by isoproterenol (solution 3 to 4). To confirm that the hyperpolarization induced by isoproterenol was due to CFTR activation, OSPD measurements were done on two CF subjects with CFTR mutations with predicted near-zero CFTR activity. Figure 4C shows that the large isoproterenol-induced hyperpolarization was largely absent in the CF subjects.

Safety

Several types of studies were done to investigate whether the OSPD procedure caused injury to the cornea or conjunctiva. Total OSS determined immediately following the OSPD procedure was low (≤3 out of 12) in seven of the eight subjects. Most of the staining seen was at the inferotemporal ocular surface where the perfusion was done. One non-CF subject (subject 6) had a total OSS of 9, though he was asymptomatic and rechecked in the clinic the next day with a total

OSS of 0. Additionally, this subject had normal BCVA and IOP measure before (20/20-2 and 16 mm Hg) and just after (20/20 and 13 mm Hg) the OSPD procedure. No subjects reported ocular surface discomfort at the conclusion of the procedure or during the following 24 hours.

Discussion

We report here the first measurement, to the best of our knowledge, of the electrical potential generated by the ocular surface epithelium in human subjects, offering a new approach to study ocular surface function and health. This approach was motivated by the experimental use of nasal PD measurements to assess CFTR function in humans with CF[12,17,18] and the development of OSPD in our lab as applied to mice[3,9] and rabbits.[11] Measurement of OSPD in human subjects is technically straightforward. As discussed further below, the baseline OSPD provides a composite measure of the activities of membrane transport proteins in corneal and conjunctival epithelium. The responses to drugs and ion substitution isolate the activities of specific transport processes.

The technical methods used herein are based largely on prior nasal potential difference measurements in humans and OSPD measurements in small animals, although notable additional developments were necessary for OSPD measurement in human subjects. As done for nasal potential difference measurements in humans, an electrical recording system was used that produces accurate OSPD information without significant artifacts, such as junction potentials, and without causing electrical shock. Also, sterile perfusate solutions were used that contain clinical-grade compounds and approved drugs. Electrical contact with the ocular surface was accomplished by everting the lower eyelid to create a small fluid pool into which the tip of a soft, flexible perfusion catheter was immersed under direct slit-lamp visualization, as opposed to in nasal potential difference studies where the perfusion catheter is blindly inserted into the nostril, and the site of contact with the nasal epithelium cannot be directly visualized, thus causing variable electrical tracings. The perfusion catheter tip used herein both delivered specified perfusate solutions and maintained electrical contact with the ocular surface. Fluid overflow created by the continuous perfusion was collected using an absorbent gauze secured to the cheek. Various future adaptations and advances are possible, such as development of a custom perfused contact lens system to study cornea versus conjunctiva selectively and eliminate the need for external positioning of the perfusion catheter tip.

The design of OSPD experiments and the interpretation of data rely on an understanding of the origin of the PD. We previously reported a mathematical model to define quantitatively the influence of the various ion transport processes and paracellular conductance on the OSPD, as well as the effects of perfusate ion substitution maneuvers.[3] The baseline OSPD, which is exterior negative when referenced against the corneal stroma, is a consequence of the active Na/K ATPase at the basolateral membrane of ocular surface epithelial cells. The positive current from the cell interior to the corneal stroma (by exchange of three sodium ions for two potassium ions) produces, under open-circuit conditions, the exterior negative potential. The magnitude of the OSPD is affected by the various passive ion transport processes and paracellular resistance. Ion substitution creates a chemical driven force to bias OSPD values to focus on particular sets of ion transport pathways. For example, the low chloride maneuver used herein, together with ENaC inhibition, produces OSPD values that provide further information on chloride transport pathways, allowing interpretation of the isoproterenol effect in terms of CFTR activation. Although much can be learned by semiquantitative and comparative OSPD measurements, as has been done for nasal PD measurements, quantitative modeling of the OSPD can enhance data interpretation and identify mechanisms that may not be otherwise apparent.

The studies reported here represent assessment of the ion transport function at the ocular surface in live human subjects. Our OSPD measurements are open-circuit recordings of the physiological potential differences generated by ocular surface epithelial tissues. An alternative informative approach, although not possible in vivo, is measurement of short-circuit current across isolated epithelia. Short-circuit current reports the quantity of current that is exogenously driven across an epithelium in order to maintain a zero transepithelial potential difference. Short-circuit current has been measured in various preparations of isolated cornea[19,20] and conjunctiva[11,21,22] from rabbits and amphibia, as well as in corresponding epithelial cell cultures,[23] and has been informative in identifying sodium and chloride transporting pathways. Although electrophysiological measurements in isolated tissue allow precise specification of the composition of solutions bathing the apical (tear-facing) and basolateral surfaces of the epithelium, they do not preserve the in vivo architecture and hormonal/neural environment in live subjects.

The OSPD data implicate CFTR as a major prosecretory mechanism in human ocular surface

epithelia. A robust average hyperpolarization of 15.6 mV was seen in response to isoproterenol in a zero chloride solution, which was absent in two CF subjects lacking functional CFTR. This cAMP-dependent OSPD hyperpolarization is similar to that seen in human nasal potential difference measurements[24,25] and in OSPD studies in mice and rabbits.[9,11] In the animal studies, CFTR-selective inhibitors were also used to confirm that the OSPD hyperpolarization reflects CFTR function, although at present no CFTR inhibitor has been approved for human use. The significant role of CFTR as a prosecretory mechanism at the ocular surface supports the use of CFTR activators as potential therapy for dry eye disorders. A triazine small-molecule CFTR activator that is in preclinical development has been shown to prevent and reverse dry eye pathology in experimental animal models.[26,27]

An interesting and perhaps unexpected observation was the minimal effect of amiloride, a blocker of proabsorptive sodium channel ENaC, on OSPD, with only a 1.7-mV depolarization produced by a high concentration of amiloride. In similar nasal potential difference measurements in humans, amiloride generally produces a >10-mV depolarization,[16] and in mouse and rabbit OSPD measurements amiloride produced 6-mV and 5-mV depolarizations, respectively.[3,11] The simplest interpretation of this finding is that ENaC plays a minor role as a proabsorptive mechanism in human ocular surface, which would suggest that blockers of ENaC, which have been evaluated for dry eye disorders,[28] may have limited efficacy. However, the amiloride data should be interpreted with caution given our incomplete knowledge of the full repertoire of ion transporters in human cornea and conjunctiva.

Measurement of OSPD in human subjects has a number of potential applications in studying basic ocular physiology, evaluating disease status, monitoring epithelial health, and testing drug candidates. Changes in OSPD in response to selective modulators of transport and signaling mechanisms, together with ion substitution, are informative in defining transport mechanisms and their regulation, as done here for the investigation of ENaC and CFTR. Potassium channels, for example, might be investigated using selective channel modulators and studying effects of potassium ion substitution in the perfusate. OSPD measurements should be informative in quantifying the regulation of ion transport processes in response to disease conditions. For example, whether the expression or function of CFTR is altered in dry eye disorders can be studied, as can potential compensatory upregulation of other prosecretory mechanisms. An intriguing potential application of OSPD is following the recovery of corneal barrier disruption from a variety of conditions, including trauma, ocular prosthetic devices, infection, and neurotrophic keratopathy. Finally, measurement of OSPD can provide a quantitative surrogate measure of the efficacy and pharmacodynamics of drug candidates that target ion transport mechanisms, such as chloride or potassium channel activators and sodium channel inhibitors.

Acknowledgments

Supported by grants from the National Institutes of Health (EY13574, DK72517 and EY029881) and by the Cystic Fibrosis Foundation. This work was made possible in part by a Research to Prevent Blindness Unrestricted Grant to the University of California San Francisco, Department of Ophthalmology.

Disclosure: **N.D. Pasricha,** None; **A.J. Smith,** None; **M.H. Levin,** Vanda Pharmaceuticals, Inc. (C), University of California San Francisco (P); **J.M. Schallhorn,** Vanda Pharmaceuticals, Inc. (C); **A.S. Verkman,** Vanda Pharmaceuticals, Inc. (C), University of California San Francisco (P)

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Supplementary Material

Supplementary Movie S1. Slit-lamp view of solution perfusion onto the ocular surface at 10 mL/min during OSPD measurement. The perfusion catheter is immersed in the fluid pool without contacting the ocular surface.