Ion Transport Mechanisms in Native Human Retinal Pigment Epithelium

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Electrophysiologic techniques were used to characterize the electrical properties and the ion transport mechanisms at the apical and basolateral membranes of the human retinal pigment epithelium (RPE). These experiments used fresh native tissue from adult donor and fetal eyes. In the upper range, adult donor RPE had an apical membrane resting potential (Vₐ) of ≈−60 mV and a transepithelial potential (TEP) and resistance (Rₑ) of 3.5 mV and 148 Ω·cm², respectively. The means were at least 50% of these values. In RPE from fetuses of gestational age 19–23 wk, Vₐ was −56 ± 4 mV, TEP was 2.2 ± 1.5 mV, Rₑ was 206 ± 151 Ω·cm², and the ratio of apical to basolateral resistance was 0.70 ± 0.50 (mean ± standard deviation; n = 15). The apical membrane of the adult donor and fetal RPE contains a large relative K⁺ conductance (TK > 0.3) that is barium blockable. In fetal RPE, there is evidence for separate K⁺ and Cl⁻ conductive mechanisms at the basolateral membrane. However, the evidence for the Cl⁻ conductance is indirect. The fetal RPE apical membrane, but not the basolateral membrane, contains a ouabain-sensitive mechanism that exhibits two distinct phases of apical depolarization. The first, rapid phase suggests that the pump is electrogenic. The apical membrane of fetal RPE contains a bumetanide-sensitive mechanism and a receptor activated by nanomolar amounts of epinephrine. In fetal RPE, step changes in apical [K⁺]₀ between 5 and 2 mmol/l produced a delayed basolateral membrane hyperpolarization that in situ generates the fast oscillation trough of the electroretinogram. Invest Ophthalmol Vis Sci 33:3513–3527, 1992

The cell polarity of the human RPE overlaps in many ways with its counterparts from the bullfrog and bovine eyes. In all three species, there are a variety of anion- and cation-dependent conductances, pumps, cotransporters, and exchangers on both membranes (Edelman JL, Lin H, and Miller SS, in preparation). These individual plasma membrane mechanisms are further organized into a variety of transport pathways that allow ion and fluid transport across the epithelium and help mediate the ionic changes that occur on the retinal and the choroidal sides of the tissue during transitions between light and dark (la Cour M, Lin H, and Miller SS, in preparation). In vitro, apical [K⁺]₀ changes between 5 and 2 mmol/l have been used in frog and bovine RPE to approximate the light-induced [K⁺]₀ changes that occur in the subretinal space of the intact eye. It has been shown that these [K⁺]₀ changes significantly alter RPE physiology by: (1) changing the apical and basolateral membrane potentials and resistances; (2) altering intracellular pH; and (3) reversing the direction of active Cl⁻ transport from net absorption to net secretion with a resulting decrease in net fluid absorption (Edelman JL, Lin H, and Miller SS, in preparation). One consequence of these transport changes (frog data) is that apical (retinal) HCO₃⁻ is exchanged for basal (choroidal) Cl⁻. In the intact

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eye, this could provide a way to remove metabolically produced CO$_2$ from the retina.$^{14}$ After a similar strategy, the lactate transport mechanisms that have been identified at the apical and basolateral membranes of the bovine and human RPE could move lactate into or out of the subretinal space in response to light-induced changes in retinal metabolism (la Cour M, Lin H, and Miller SS, in preparation).$^{20}$

A preliminary account of this work has been reported in abstract form.$^{22}$

**Methods and Materials**

Protocols were approved by the University of California Committee for the Protection of Human Subjects. Eyes from three adult human donors were obtained from the Northern California Transplant Eye Bank (San Francisco, CA). The donors' ages ranged from 30–69 yr. In all three cases, the eyes were enucleated immediately after elective abortion of fetuses with gestational ages that ranged from 19–23 wk. After enucleation, eyes were placed in containers with Dextrisol Ringer's (Chiron, Irvine, CA) packed in ice and were received at the lab within approximately 2 hr. Upon receipt, eyes were immediately transferred to ice cold Ringer's solution that was gassed with a mixture of 5% CO$_2$, 10% O$_2$, and 85% N$_2$. Oxygen was restricted to 10% because higher concentrations were found to be toxic in bovine RPE.$^{6,5}$ In the intact cat eye, the O$_2$ tension across the RPE is $\approx$65–85 mmHg ($\approx$10%).$^{23}$

The retinal pigment epithelium (RPE) of adult eyes was isolated by first cutting the globe behind the ora serrata and removing the anterior portion of the eye. The posterior pole then was cut into four sections of approximately equal size and returned to cold control Ringer's solution. A trephine was used to punch out a circular 0.38 cm$^2$ piece of retina and RPE choroid, which was separated from the sclera by blunt dissection. The retina then was slowly peeled away from the RPE choroid. The dissection of the fetal eyes was similar except that the posterior pole first was bisected, and then relatively circular pieces were obtained by trimming the corners of each half.

**Chamber and Flow System**

The RPE choroid was mounted on a nylon mesh support and clamped into a modified Ussing chamber, as previously described.$^{11}$ Solution reservoirs were maintained at 40–45$^\circ$C using heat lamps and were kept at pH 7.4 with a mixture of 8% CO$_2$, 10% O$_2$, and the balance N$_2$. The solutions were delivered to the chamber through CO$_2$ impermeable Saran tubing (Clarkson Controls & Equipment Co, Detroit, MI). At the basal and apical entry ports, Peltier heat pumps (Cambion Thermoelectric Products, Santa Ana, CA) maintained the solutions entering the chamber baths at 37$^\circ$C.$^{11}$ Solution changes were made at open manifolds designed to maintain a constant hydrostatic head at the chamber. The responses at the apical and basolateral membranes were obtained $\approx$1.25 and 2.5 min after the solution changes were made at the manifold. This includes the time to clear the dead space in the tubing, mix in the chamber, and cross unstirred layers. In all Figures, the horizontal bars indicate when the solution changes were made at the manifold.

**Electrophysiology**

The electrophysiologic methods have been described in detail previously.$^{8,11,12}$ Briefly, electrical connections to the apical and basal chambers were made by Ringer-agar bridges in series with calomel electrodes. Intracellular potentials were recorded through conventional microelectrodes pulled from borosilicate glass and filled with 150 mmol/l KCl. The apical and basolateral membrane potentials ($V_a$ and $V_b$) were obtained by referencing the intracellular electrode to the apical and basal bath electrodes, respectively. The transepithelial potential ($TEP = V_a - V_b$) is the difference between the apical and basal bath electrodes. These signals were amplified and digitized (4Hz) for analysis on a microcomputer. The transepithelial resistance ($R_t$) and the apparent ratio of apical to basolateral membrane resistances ($R_a/R_b$) were obtained by passing bipolar current pulses ranging from 2–8 $\mu$A across the tissue and monitoring the current-induced ($\Delta I$) changes in TEP ($R_t = \Delta TEP/\Delta I$) and membrane potential ($R_a/R_b = \Delta V_a/\Delta V_b$). For presentation, these voltage deflections were digitally subtracted from the traces.

**Solutions**

Control Ringer's consisted of the following reagent grade chemicals (in mmol/l): 5 KCl, 0.8 MgCl$_2$, 113.4 NaCl, 26.2 NaHCO$_3$, 1 NaH$_2$PO$_4$, 5.6 glucose, and 1.8 CaCl$_2$. Solutions that had different amounts of K$^+$ were made by substituting equimolar amounts of KCl for NaCl. For Ba$^{2+}$ containing solutions, 1 mmol/l BaCl$_2$ was added to the solution without substituting for other salts. The osmolality of the above solutions was 284 ± 3 mosm. All solutions contained 1 mmol/l glutathione as an antioxidant.$^{13}$ Bumetanide, epinephrine, and ouabain were obtained from Sigma Chemical Co. (St. Louis, MO).

**Circuit Analysis**

The RPE, like all transporting epithelia, consists of two functionally distinct membranes that are electri-
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Fig. 1. Equivalent circuit of the RPE. Each membrane is modeled as a battery ($E_A$, $E_B$) in series with a resistor ($R_A$, $R_B$). The two membranes are electrically coupled by the cytoplasm and by a shunt pathway ($R_s$), which consists of the paracellular resistance pathway in parallel with a resistance pathway at the edge of the tissue. $E_A$ is more hyperpolarized than $E_B$, and this difference drives a current ($I_s$) through $R_s$. The Na-K-ATPase at the apical membrane contributes an additional hyperpolarizing current ($I_{pump}$) to the circuit and contributes to the transepithelial potential, TEP = $V_A - V_B$.

The observed membrane potentials are related to the EMFs as follows:

$$V_A = E_A - I_s R_A$$

(1)

$$V_B = E_B + I_s R_B$$

(2)

where $I_s$ acts to depolarize the apical membrane and hyperpolarize the basolateral membrane. Because the apical and basolateral membranes are electrically coupled via $R_s$, a solution composition change in the apical bath that hyperpolarizes or depolarizes $V_A$ also will hyperpolarize or depolarize $V_B$. For an apical solution composition change that only alters $E_A$ or $R_A$, the ratio of the measured membrane potential changes is given by:$$\frac{\Delta V_B}{\Delta V_A} = \frac{R_B}{R_B + R_s} \leq 1$$

(3)

Conversely, for a basal solution composition change that alters only $E_B$ or $R_B$, this ratio is given by:

$$\frac{\Delta V_B}{\Delta V_A} = \frac{R_A + R_s}{R_A} \geq 1$$

(4)

The transepithelial resistance, $R_t$, is determined by the parallel combination of ($R_A + R_B$) and $R_s$. Thus, if $R_t$ is small compared to $R_A$ or $R_B$, $R_t \approx R_A$ and $R_B$ will also be small. In contrast, if $R_t$ is large compared to $R_A$ or $R_B$, $R_t$ will be relatively large. In practice, $R_A$ and $R_B$ are relatively small or large depending on the degree of edge damage that inevitably occurs when the RPE choroid is mechanically mounted in the chamber. Based on these considerations, equations 3 and 4 can be used to help analyze the data summarized in Figures 2 and 3. For example, during an apical solution change (equation 3), if $R_t$ is small, because $R_t \ll R_A$, most of the change in apical EMF will be shunted to the basolateral membrane, $\Delta V_A \approx \Delta V_B$, and $\Delta TEP$ will be small ($\Delta TEP \approx 0.6$ mV; Fig. 2). Figure 3 illustrates a basolateral solution change (equation 4) in which $R_t$ is large, probably because the tissue is well sealed and $R_s$ is large. In that case, less of the change in basolateral EMF is shunted to the apical membrane and $\Delta TEP$ is relatively large ($\Delta TEP \approx 5$ mV; Fig. 3).

The ratio $R_A/R_B (\alpha)$ is a measure of the relative change in the conductances of these two membranes. For example, an increase in $\alpha$ indicates an increase in $R_A$ relative to $R_B$ or a decrease in $R_B$ relative to $R_A$. A change in $R_t$, concurrent with a change in $\alpha$, can be used to indicate which membrane resistance changed if $R_s$ is constant. For example, an increase in $\alpha$ and a decrease in $R_t$ suggests a decrease in $R_B$. Thus, the resistance parameters $\alpha$ and $R_t$ help localize the resistance and voltage changes to one membrane or the other.

Bovine eyes are relatively abundant, so tissues with low TEP and $R_t$ can be discarded. In contrast, the very limited availability of human tissues demands that we use almost all tissues, even those with relatively low values for $V_m$, TEP, and $R_t$. In some cases, particularly involving tissues with a relatively low value for the resting membrane potential, post-mortem trauma may have been a contributing factor. A major difference between the adult and fetal tissues is that the fetal eyes were enucleated immediately, whereas adult eyes were enucleated several hours after death. This differ-

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Fig. 2. Fetal RPE. Ba²⁺ inhibitable K⁺ conductance. (A) Apical [K⁺]₀ was increased from 5 mmol/l to 20 mmol/l (dark bar), resulting in 20 mV apical depolarization. (B) After recovering in 5 mmol/l [K⁺]₀, the apical bath was perfused with 1 mmol/l Ba²⁺ (open bar). Once the membrane potentials reached a new steady state in Ba²⁺, [K⁺]₀ again was increased to 20 mmol/l (dark bar), resulting in only 4 mV depolarization. (C) After recovery in 5 mmol/l K⁺ and washout of the Ba²⁺, [K⁺]₀ again was increased to 20 mmol/l (dark bar), resulting in 17 mV apical depolarization. Traces A and B were recorded from the same cell. This impalement was lost before the following control could be obtained. Therefore, the trace in C was recorded from a different cell (same tissue).

ence in procedure may explain the lower voltage and resistance values obtained for adult RPE. The electrical parameters could be maintained for hours once a tissue was mounted in the chamber and stabilized (~30 min). The electrical values presented in this report are not completely comparable to those presented in our other report because of differences in the type of chamber used.¹

**Results**

The membrane transport mechanisms and receptors that help regulate ion and fluid transport across the in vitro RPE choroid preparation have been most thoroughly analyzed using fresh explant tissue from amphibians (bullfrog, toad) and bovine eye.²,⁴-⁷,⁹,¹²,¹³,¹⁵-¹⁷ Important information also has been obtained using several other preparations from the mammalian, chick, and human eye.¹⁶,¹⁷,¹⁹,²³,²⁴ The present experiments summarize our initial attempts to characterize the ion transport mechanisms at the apical and basolateral membranes of human RPE. All of these in vitro experiments were performed using fresh native tissue from adult donor or fetal eyes. In each experiment, the RPE choroid was removed from the eye and mounted in a modified Ussing chamber, and the cells were impaled with microelectrodes.

**Adult RPE**

For adult RPE in normal Ringer’s solution, the mean apical membrane potential was -49 ± 7 mV, the TEP was 1.9 ± 0.6 mV, and Rₑ was 79 ± 48 Ω · cm² (mean ± standard deviation; n = 4). The ranges for these parameters are listed in Table 1. In one tissue, we also obtained the ratio of apical to basolateral membrane resistance (a; ≈ 0.29 ± 0.03, mean ± SD; 10 samples).

**Apical Membrane K⁺ Conductance**

Apical K⁺ conductance was estimated by measuring the changes in membrane potential (ΔVₐ) after a
Fig. 3. Fetal RPE. Basolateral K+ response. Basal [K+]o was increased from 5 mmol/l to 25 mmol/l (dark bar). This depolarized VA and VB, increased the transepithelial potential and $R_t$, and decreased $R_c$. The size of the liquid junction potential produced by the [K+]o change at the basal salt bridge was small (0.5 mV) compared to the response.

Step change in apical [K+]o. The data summarized in Figure 4 show that the resting membrane potentials were relatively depolarized ($\approx -33$ mV) and that a fourfold increase in apical [K+]o, from 5 to 20 mmol/l, depolarized $V_A$ and $V_B$ by $\approx 13$ mV. It has been shown in bovine RPE that the apical membrane K+ conductance is Ba2+ inhibitable. The intracellular recording in Figure 4 shows that Ba2+ had an almost identical effect in the human RPE: It depolarized $V_A$ by $\approx 17$ mV, consistent with the blockade of a K+ conductive mechanism, presumably the K+ channel. This was corroborated by increasing [K+]o from 5 to 20 mmol/l in the presence of Ba2+, which produced no detectable change in $V_A$. After Ba2+ was removed (8 min), both membranes repolarized to their approximate control levels and the $\Delta$[K+]o-induced diffusion potential was remeasured (far right) and found to be $\approx 70\%$ of its original size.

Apical Membrane Adrenergic Receptor

The apical membrane of the bovine RPE has an $\alpha$-adrenergic receptor that produces an increase in basolateral membrane Cl− conductance and a basolateral membrane depolarization when activated by epinephrine. Figure 5 shows that adding 1 μmol/l epinephrine to the apical bath caused both membranes to depolarize, $V_A$ by $\geq 3$ mV and $V_B$ by $\geq 6$ mV. Because the TEP increased, the rate of basolateral membrane depolarization (d$V_B$/dt) must have exceeded the rate of apical membrane depolarization (d$V_A$/dt). There also was a concomitant decrease in $R_c$, from 152 to 144 $\Omega \cdot$ cm², and an increase in the ratio of apical to basolateral membrane resistance, $a$, from 0.30 to 0.44. Assuming that epinephrine had little or no effect on the shunt resistance, $R_s$, these voltage and resistance changes indicate that apical epinephrine caused an increase in a basolateral membrane conductive mechanism whose equilibrium potential is depolarized with respect to the resting membrane potential.

Fetal RPE

In RPE from fetuses of gestational age 19–23 wk, the apical membrane potential was $-56 \pm 4$ mV, the TEP was $2.2 \pm 1.5$ mV, $R_t$ was 206 ± 151 $\Omega \cdot$ cm², and the ratio of apical to basolateral membrane resistance, $a$, was 0.70 ± 0.50 (n = 15). The ranges for $V_A$, TEP, and $R_t$ are listed in Table 1.

Apical Membrane K+ Conductance

In Figure 2A, increasing apical [K+]o from 5 to 20 mmol/l depolarized $V_A$ by $\approx 20$ mV, increased the membrane resistance ratio ($a$) from 0.30 to 1.05 (lower panel), and decreased the TEP $\approx 0.6$ mV (upper panel). $R_t$ (upper panel, open squares) increased steadily, except for a plateau that coincided with the increase in $a$. Both membranes depolarized by approx-

<table>
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<th>Range</th>
<th>Human adult</th>
<th>Human fetal</th>
<th>Cat</th>
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<tr>
<td>$V_A$ (mV)</td>
<td>-35 to -63</td>
<td>-46 to -64</td>
<td>-60 to -76</td>
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<tr>
<td>TEP (mV)</td>
<td>0.9 to 3.5</td>
<td>0.3 to 4.8</td>
<td>4.6 to 15.0</td>
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<tr>
<td>$R_t$ ($\Omega \cdot$ cm²)</td>
<td>36 to 148</td>
<td>18 to 486</td>
<td>280 to 420</td>
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<tr>
<th>Mean ± standard deviation</th>
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<tr>
<td>Bovine</td>
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<tr>
<td>$V_A$ (mV)</td>
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<td>TEP (mV)</td>
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* Human and bovine tissues were in 5 mmol/l [K+]o, cat was in 4 mmol/l [K+]o, and dog was in 0.8 mmol/l [K+]o. Ringer's solution. No intracellular data are available for dog.
Fig. 4. Adult RPE. Ba\(^{2+}\) inhibitable K\(^+\) conductance.
Apical [K\(^+\)]\(_o\) was first increased from 5 mmol/l to 20 mmol/l (dark bar) and V\(_A\) depolarized by \(\pm 13\) mV. After V\(_A\) recovered in 5 mmol/l [K\(^+\)]\(_o\), the apical bath was perfused with 1 mmol/l Ba\(^{2+}\) Ringer’s solution (open bar). Once the membrane potentials reached a new steady state in Ba\(^{2+}\), [K\(^+\)]\(_o\) again was increased to 20 mmol/l (dark bar). After Ba\(^{2+}\) removal, [K\(^+\)]\(_o\) again was increased to 20 mmol/l. Donor was a 30-year-old female who died of aspiration, leading to cardiac arrest and renal failure.

Fig. 5. Adult RPE. Epinephrine-induced changes in membrane voltage and resistance. 10\(^{-6}\) mol/l epinephrine was perfused through the apical chamber (dark bar). This depolarized V\(_A\) by \(\pm 14\) mV, decreased the TEP by \(\pm 0.4\) mV, and increased \(a\) from 0.27 to 1.51. In the presence of Ba\(^{2+}\), increasing apical [K\(^+\)]\(_o\) from 5 to 20 mmol/l produced a much smaller response, \(\approx 20\%\) of the control. The TEP decreased slightly, \(a\) increased from 1.51 to 1.88, and there was no significant effect on R\(_t\). Thus, Ba\(^{2+}\) significantly inhibited the voltage and resistance changes induced by high [K\(^+\)]\(_o\). After Ba\(^{2+}\) removal and recovery in control Ringer’s solution, the impalement was lost and a nearby cell with a resting potential \(\approx 15\) mV more depolarized was impaled. In this cell, the [K\(^+\)]\(_o\) induced voltage and resistance changes were comparable to those in the previous control, showing that the Ba\(^{2+}\) effects are reversible.

In three cells from two tissues, increasing apical [K\(^+\)]\(_o\) from 5 to 20 mmol/l depolarized V\(_A\) by 16 ± 5 mV. By comparing this \(\Delta[K^+]_o\) induced voltage change to the predicted Nernstian response (61 mV/decade at 37°C), the apparent relative conductance of the apical membrane to K\(^+\) (T\(_K\)) was estimated to be >0.44. T\(_K\) is defined as \(g_K/(g_K + g_i)\), where \(g_K\) is the (apical) membrane K\(^+\) conductance and \(g_i\) is the sum of all other (apical) membrane conductances. The \(\Delta[K^+]_o\) induced change in V\(_A\) is always smaller than the change in E\(_A\) because of shunting (equation 1). Thus, the actual value of T\(_K\) is larger than the estimate. The increase in [K\(^+\)]\(_o\) also decreased the TEP by 0.4 ± 0.3 mV and increased \(a\) from 0.37 ± 0.05 to 0.95 ± 0.31. In these tissues, Ba\(^{2+}\) inhibited the [K\(^+\)]\(_o\) induced change in V\(_A\) by \(\approx 78\%\) and inhibited the changes in TEP and \(a\) by 67% and 81% of control, respectively.

Because Ba\(^{2+}\) depolarized the apical membrane, it is possible that the reduced response to high apical [K\(^+\)]\(_o\) resulted from the depolarized membrane potential rather than from direct blockade of the K\(^+\) channels by Ba\(^{2+}\). To examine this possibility, the tissue...
was current clamped and $V_A$ depolarized by $\approx 20 \text{ mV}$, which simulated the depolarizing effect of $\text{Ba}^{2+}$ (not shown). Then $[K^+]_b$ was increased from 5 to 20 mmol/l, which resulted in an additional 20 mV depolarization. This value was within 1 mV of the control high $[K^+]_o$ response from the same cell, suggesting that the $K^+$ conductance is not voltage sensitive, at least for depolarized potentials, and that $\text{Ba}^{2+}$ directly blocks the $K^+$ channels.

**Basolateral Membrane $K^+$ Conductance**

Figure 3 shows a response of human fetal RPE to an increase in basal $[K^+]_o$ from 5 to 25 mmol/l. $V_B$ depolarized by 21 mV, indicating that $T_K$ at the basolateral membrane was $>0.49$. Again, $\Delta V_B < \Delta E_K$ because of the shunt (equation 2), resulting in an underestimate in the size of $T_K$. The TEP increased by $\approx 5 \text{ mV}$ because the basolateral membrane depolarized more rapidly than the apical membrane. $R_I$ decreased from 490 to 450 $\Omega \cdot \text{cm}^2$, while $a$ increased from 0.4 to 1.9. These results are consistent with a basal $K^+$ induced increase in basolateral membrane $K^+$ conductance.

In a series of similar experiments, increasing basal $[K^+]_o$ from 5 to 25 mmol/l depolarized $V_B$ by $6 \pm 0.7 \text{ mV}$ and increased the TEP by $1.0 \pm 0.7 \text{ mV}$ ($n = 3$). These responses are small compared to the one shown in Figure 3, perhaps because these tissues had a smaller shunt resistance, indicated by the relatively small size of $R_I$ (85 to 120 $\Omega \cdot \text{cm}^2$). In these tissues, the change in basal $[K^+]_o$ did not significantly change the membrane resistance ratio $a$ (0.38 $\pm$ 0.10 to 0.34 $\pm$ 0.11, mean $\pm$ standard error of the mean; $n = 3$). Also, $R_I$ increased throughout each experiment and $\Delta [K^+]_o$ had no effect ($n = 2$) or caused a plateau ($n = 1$). A $[K^+]_o$ induced plateau in a rising $R_I$ probably is equivalent to the decrease in $R_I$ shown in Figure 3.

**Apical Membrane Na/K Pump**

In the present experiments, ouabain was used first at the basolateral membrane and then at the apical membrane to localize the Na/K ATPase. In one such experiment summarized in Figure 6A, exposure of experiment summarized in Figure 6A, exposure of time. These results show that an Na/K pump (probable electrogenic) is present on the apical but not the basolateral membrane of human RPE.

In bovine RPE, after prolonged exposure to ouabain, there was a significant decrease in the apical voltage response to a step change in $[K^+]_o$. A similar response was observed in human fetal RPE by reducing apical $[K^+]_o$ from 5 to 2 mmol/l after a 28 min exposure to apical ouabain (Fig. 6A). The apical membrane hyperpolarized 3 mV, which is smaller than the response recorded from the same cell before ouabain exposure (12 mV). This 75% inhibition indicates that ouabain caused a significant reduction in the relative $K^+$ conductance of the apical membrane.

Solution changes in the basolateral bath must diffuse across the choroid to reach the basolateral membrane. This raised the question about whether sufficient time was given for ouabain to penetrate the chorial barrier and reach the basolateral membrane. In another tissue, a step change in basal $[K^+]_o$ produced a response by $\approx 2 \text{ min}$ (see inset, Fig. 6B). This was followed by the basal perfusion of ouabain, which had no discernible effect on $V_A$, $V_B$, or TEP, even after 15 min, which is much longer than the diffusion time required to reach the basolateral membrane. Subsequent exposure of the apical membrane to $10^{-4} \text{ mol/l}$ ouabain (Fig. 6B, lower panel) produced a biphasic depolarization and a decrease in the TEP similar to that shown in Figure 6A.

**Apical Membrane Bumetanide-Sensitive Mechanism**

The bovine RPE has an apical membrane bumetanide-sensitive mechanism that mediates transepithelial KCl and fluid transport and intracellular $Cl^-$ activity (Edelman JL and Miller SS, in preparation). Figure 7 shows that apical bumetanide slowly hyperpolarized $V_A$ and $V_B$ by $\approx 6 \text{ mV}$. The TEP decreased by 0.8 mV because $V_B$ hyperpolarized at a greater rate than $V_A$. As $V_A$ and $V_B$ hyperpolarized, $R_I$ increased from 244 to 249 $\Omega \cdot \text{cm}^2$, and $a$ decreased from 2.1 to 1.3, consistent with a decrease in basolateral membrane conductance. Similar voltage and resistance changes were recorded from three cells (tissues from two eyes). These electrical changes suggest that in the human fetal RPE, apical bumetanide caused a basolateral membrane conductance decrease of a mechanism with an equilibrium potential that is depolarized relative to the membrane potential. In bovine RPE, this mechanism is a $Cl^-$ conductance.

**Apical Membrane Adrenergic Receptor**

Figure 8 shows that 1 nM apical epinephrine significantly altered the RPE membrane voltages and resistances. $V_A$ and $V_B$ depolarized $\approx 8 \text{ mV}$, and as the membranes depolarized, the TEP increased by 0.5 mV, indicating that $V_A$ depolarized faster than $V_A$. This suggests that activation of an adrenergic receptor
at the apical membrane caused a voltage response at the basolateral membrane. \( R_e \) in this particular tissue increased steadily, before and after treatment with epinephrine. Epinephrine increased \( a \) from 0.87 to 1.98, and \( R_e \) leveled off during the sharp rise in \( a \). This plateau is consistent with an epinephrine-induced decrease in one of the parallel components of \( R_e \). In two other experiments, \( R_e \) was level before treatment and \( 10^{-8} \) mol/l epinephrine decreased \( R_e \) by \( \approx 4 \) \( \Omega \cdot \text{cm}^2 \). This effect was reversible. The decrease in \( R_e \) coupled with an increase in \( a \) suggests that apical epinephrine increased the conductance of a basolateral membrane mechanism with an equilibrium potential that is depolarized with respect to \( V_B \). In bovine RPE, this mechanism is a \( \text{Cl}^- \) conductance.\(^{12}\)

### Apical Low \([K^+]_o\) Response

The concentration of \( K^+ \) in the subretinal space of the vertebrate eye transiently decreases from \( \approx 5 \) to 2 mmol/l with light onset.\(^{16,17,36-39}\) In cat, light onset causes a series of RPE apical and basolateral membrane voltage changes that generate the c-wave, fast oscillation, and light peak of the electroretinogram (ERG).\(^{16,40}\) These changes also have been studied in vitro using gecko, chick, bovine, and toad RPE.\(^{11,12,13,26,33,41}\) The waveforms obtained from human DC-ERG and electro-oculogram (EOG) are very similar to those recorded from cat, indicating that the same sequence of membrane responses occurs at the apical and basolateral membranes of the human RPE after light onset.\(^{17,42}\)

Figure 9 shows the changes in membrane voltage and resistance in human fetal RPE that occurred after a small change in apical \([K^+]_o\). A step change in apical \([K^+]_o\) from 5 to 2 mmol/l (solid bar) hyperpolarized the apical and basolateral membranes by \( \approx 20 \) mV. During this hyperpolarization, the change in TEP had two phases indicated by the dashed lines. During phase 1, the TEP increased by 0.6 mV, showing that
3.2 -i 260
-60

0.5 mM bumetanide
apical

Fig. 7. Fetal RPE. Response to apical bumetanide. 0.5 mmol/l bumetanide was perfused into the apical bath (dark bar). It hyperpolarized both membranes, decreased transepithelial potential, and $a$, and increased $R_t$.

The apical membrane hyperpolarized more rapidly than the basolateral membrane, or $dV_A/dt > dV_B/dt$. This indicates that phase 1 primarily was an apical membrane diffusion potential generated by the step change in $[K^+]_o$. The onset of phase 2 is operationally defined by the decrease in TEP. During this phase, the TEP decreased despite continued hyperpolarization of both membranes, showing that $dV_B/dt > dV_A/dt$ and that an additional delayed hyperpolarization was generated at the basolateral membrane. The membrane resistance ratio, $a$, decreased from 0.29 to 0.13 during phase 1, while $R_t$ increased from 485 to 497 $\Omega \cdot \text{cm}^2$. This is consistent with a decrease in basolateral membrane conductance. Based on the change in $a$, this conductance decrease began during phase 1 and reached its peak at the beginning of phase 2. During phase 2, the $a$ value was constant and $R_t$ continued to increase, suggesting that during phase 2 both $R_A$ and $R_B$ increased.

After the return to control Ringer’s solution, the membrane voltages, the TEP, and the resistance ratios went through an additional series of changes (dashed lines, phases 3 and 4). Immediately after the increase in $[K^+]_o$ from 2 to 5 mmol/l (phase 3), both membranes depolarized and the TEP decreased, as is expected for an EMF change ($\Delta E_K$) at the apical membrane ($dV_A/dt > dV_B/dt$; equation 3). During the second phase of recovery (phase 4), $V_A$ and $V_B$ continued to depolarize, but the TEP increased, indicating the generation of a delayed depolarization at the basolateral membrane ($dV_B/dt > dV_A/dt$, equation 4). The delayed basal depolarization previously was observed in cat (in vivo) and in gecko retina RPE choroid preparation at light offset and in the gecko and toad RPE choroid preparation in response to an increase in apical $[K^+]_o$. The membrane resistance ratio, $a$, increased throughout phases 3 and 4. In phase 4, $R_t$ decreased, consistent with a steady-state increase of conductance at the basolateral membrane.

In six tissues, the voltage and $a$ value changes after the change to 2 mmol/l $[K^+]_o$ were similar to that shown in Figure 9. $V_A$ and $V_B$ hyperpolarized by 20 ± 2 mV (mean ± SD), and the TEP first increased 0.60 ± 0.29 mV (phase 1) and then decreased (phase 2). The membrane resistance ratio, $a$, decreased from 0.27 ± 0.09 to 0.16 ± 0.07 during phase 1 and remained level during phase 2. During the recovery (2 to 5 mmol/l $[K^+]_o$), the TEP decrease during phase 3 and the increase in $a$ occurred in all six tissues, and 2.5

Fig. 8. Fetal RPE. Epinephrine-induced change in membrane voltage and resistance. $10^{-9}$ mol/l epinephrine was perfused into the apical bath (dark bar), depolarizing both membranes and increasing the transepithelial potential and $a$. $R_t$ increased throughout the record, except for a plateau corresponding to the peak in $a$. 

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Fig. 9. Fetal RPE. Low $[K^+]_o$ response. Apical $[K^+]_o$ was reduced from 5 mmol/l to 2 mmol/l, indicated by the dark bar. This increased $R_t$, both membranes hyperpolarized, and $a$ decreased. The transepithelial potential (TEP) first increased (phase 1) and then decreased (phase 2). Return to 5 mmol/l $[K^+]_o$ depolarized both membranes, increased $a$, and caused complex changes in $R_t$. The TEP decreased (phase 3) and then increased (phase 4). The dashed lines indicate distinct phases of the response.

the TEP increase during phase 4 occurred in four of the six tissues. The $R_t$ response during the step reduction in $[K^+]_o$ varied from tissue to tissue. For example, in one tissue, $R_t$ remained level during phase 1 and 2 and then increased after the return to 5 mmol/l $[K^+]_o$. In another tissue, the $R_t$ changes were similar to that shown in Figure 9. In no instance, however, did $R_t$ decrease during phases 1 or 2.

Discussion

The present results summarize our initial analysis of the membrane mechanisms that help regulate ion and fluid transport across the human RPE. Most of this work was carried out using fetal tissue of gestational age 19 to 23 wk, but some results were obtained from adult donor material. The adult and fetal responses are qualitatively similar, but a detailed comparison would require additional data from adult donor eyes. For comparison, Figure 10 summarizes many of the apical and basolateral membrane transport mechanisms that have been previously studied in frog, bovine, and now in the human RPE.

Ion and Fluid Transport Mechanisms in Frog and Bovine RPE

Frog: The apical membrane contains an electrogenic Na/K pump and a Ba$^{2+}$ inhibitable K$^+$ conductance. Absorption of Cl$^-$, net fluid absorption, and hypertonic regulatory volume increase all are inhibited by apical bumetanide, suggesting that all three of these functions are mediated at least in part by the apical membrane Na-K-2Cl cotransporter (Edelman JL, Lin H, and Miller SS, in preparation). In addition, the apical membrane contains a DIDS-inhibitable NaHCO$_3$ cotransporter that has a stoichiometry of $\approx 2$ HCO$_3$:1 Na and an amiloride-inhibitable Na-H exchanger. Most of the pH$_i$ regulation ($\approx 90\%$) is carried out by the NaHCO$_3$ cotransporter. The basolateral membrane contains a DIDS-inhibitable Cl$^-$ conductance and a Cl-HCO$_3$ exchanger (Edelman JL, Lin H, and Miller SS, in preparation). When bathed on both sides by control HCO$_3^-$-Ringer’s solution, the isolated frog RPE transports fluid in a retinal to choroidal direction (absorption) by mechanisms that require bicarbonate and are inhibited by elevating cyclic adenosine monophosphate (cAMP) in the cells. Increasing cAMP alters RPE membrane voltage and resistances as well as the vectorial transport of Na$^+$, K$^+$, and Cl$^-$. Increasing apical $[K^+]_o$ from 2 to 5 mmol/l approximates the changes that occur in vivo in the subretinal space during the transition from light to dark. In vitro, this $[K^+]_o$ change stimulates NaHCO$_3$ uptake at the apical membrane, alkalinizes pH$_i$, activates the basolateral membrane Cl/HCO$_3$ exchanger, and reverses the direction of active Cl transport from net absorption to net secretion (Edelman JL, Lin H, and Miller SS, in preparation). These $[K^+]_o$ induced changes allow the exchange of choroidal Cl$^-$ for subretinal HCO$_3^-$. Also, this exchange, in the intact eye, could help reduce the accumulation of CO$_2$ in the dark-adapted retina.

Bovine: The apical membrane contains an electrogenic Na/K pump, a barium-inhibitable K$^+$ conductance, and a bumetanide-sensitive mechanism, the putative Na-K-2Cl cotransporter. At the basolateral membrane, K$^+$ and Cl$^-$ exit the cell conductively, driven by favorable electrochemical gradients. The basolateral membrane Cl$^-$ conductance is DIDS inhibitable. The apical membrane contains $\alpha$-1 receptors that are activated by epinephrine to transiently elevate [Ca$^{2+}$], and probably increase basolateral membrane Cl$^-$ conductance. In addition, apical epinephrine stimulates net solute (KCl) and fluid absorption that is bumetanide inhibitable and presumably is mediated by the Na-K-2Cl cotransporter. Decreasing apical $[K^+]_o$, from 5 to 2 mmol/l hyperpolar-
Fig. 10. Transport model of the RPE. The mechanisms analyzed in the present study are shaded. See companion paper for pH regulating mechanisms. Other mechanisms have been studied using frog and bovine RPE.

izes the apical and basolateral membranes and inhibits the Na/K pump and the Na-K-2Cl cotransporter. This reduction in apical [K+], significantly decreases [Cl-], which causes a delayed hyperpolarization of the basolateral membrane (S. Bialek, D. Joseph, and S. Miller, unpublished results). The [K+]o response has two operationally distinct phases, the second of which generates the "fast oscillation" component of the clinically recorded EOG or the DC-ERG.11,15-17-26-35

Human RPE Voltage and Resistance Values

Because the present study is the first to use fresh human RPE, it is important to consider whether the experimentally obtained voltage and resistance values indicate a healthy tissue. One approach is to compare the present values with those obtained from other mammalian RPE. Table 1 shows that the upper ranges obtained for adult and fetal human tissues are comparable to other in vitro mammalian preparations.11,13,30 Resting membrane potentials in cat RPE in situ are -65 to -80 mV (Roy Steinberg, UCSF, April 1992, oral communication). From this, we can conclude that the upper range of values from the present data represent tissues that are healthy, based on other mammalian preparations.

As a measure of viability, transepithelial voltage and resistance values from fresh fetal human RPE also could be compared to the values obtained using cultured fetal human RPE. In one report, the mean TEP and R of cultured human fetal RPE was 3.0 ± 1.6 mV and 330 ± 80 Ω·cm², respectively (mean ± SD),31 which is similar to the present data that is based on fresh fetal tissue.

Ion Transport Mechanisms in Human RPE: Na/K Pump

In most cell types, including epithelia, the Na/K pump is electrogenic. The RPE is no exception.4 The RPE is exceptional because the pump is located on the apical (retinal facing) membrane. In all other epithelia, except for the choroid plexus, this pump is located on the basolateral membrane.51-53 Examination of the transport model shown in Figure 10 suggests why this configuration is functionally important. In the photoreceptors, light decreases an inward Na+ current in the outer segments that is maintained by a high level of [Na+]o in the extracellular (subretinal) space.54 The TEP, the Na/H exchanger, and the NaHCO3 and Na/K/2Cl cotransporters all move Na+ out of the subretinal space. Only the Na/K pump on the apical membrane is positioned to return Na+ to the subretinal space and thus help maintain the Na+ gradient needed for the photoreceptors to function properly. Of course, this pump also provides the chemical gradient necessary to energize the Na+ dependent mechanisms at the apical and basolateral membranes of the RPE.

Inhibiting the pump with ouabain depolarized both membranes by shutting down the pump current and
by allowing the K⁺ gradient to run down. Extended exposure to ouabain caused a decrease in relative apical membrane K⁺ conductance (Fig. 6A), which also could have contributed to the ouabain-induced membrane depolarization. In frog, the ouabain-induced reduction of electrogenic current has been shown to be rapid, occurring as soon as the ouabain molecules bind to the pump sites. Depolarization associated with the K⁺ leak would be slower because of a decrease in [K⁺], or a decrease in K⁺ conductance over time.

The absence of a voltage response during basal exposure to ouabain indicates that the basolateral membrane does not contain the Na/K ATPase. This conclusion sharply contrasts the results obtained using cultured RPE where the pump sites appear on both sides of the cell. In view of this difference, we considered the possibility that the choroid (a large unstirred layer made up of blood vessels, melanocytes, and connective tissue) could have effectively increased the diffusion path for ouabain. Miller et al showed that 10⁻⁴ mol/l ouabain took only a few (2) seconds longer than a step change in K⁺ to produce a voltage response at the apical membrane. Most of the response time was determined by the velocity of new solution as it moved from the manifold to the chamber. In Figure 6B (inset), the basal K⁺ response started 2 min after the solution change at the manifold. Thus, the solution front was able to reach the basal chamber, diffuse across the unstirred layer, and produce a detectable change in voltage during that time. Given the same flow rate, the solution front for ouabain reached the basal chamber in the same amount of time as K⁺ (≈2 min). Even if we estimate the basolateral unstirred layer to be 10 times the size of the apical layer, the diffusion time for ouabain would be only ≈20 sec longer than K⁺. Clearly, the 15 min exposure time was more than sufficient for ouabain to produce a response, had the Na/K pump been present.

Cl⁻ Transport Pathway

The voltage and resistance changes produced by apical bumetanide are consistent with a basolateral hyperpolarization (ΔV_B > ΔV_A; equation 4) and a basolateral conductance decrease. This finding suggests that an apical membrane cotransport mechanism indirectly causes a conductance change at the basolateral membrane. More direct evidence has been presented using bovine RPE. The membrane voltage and resistance changes after epinephrine was added to the apical bath essentially were the same in human RPE as in bovine RPE, where it has been shown that epinephrine activates an apical membrane α₁ adrenergic receptor and increases basolateral membrane Cl⁻ conductance. Therefore, the present results suggest that in human RPE, apical epinephrine similarly opens a Cl⁻ channel at the basolateral membrane and that [Cl⁻] is above electrochemical equilibrium. Evidence for a basolateral Cl⁻ conductance in human RPE is indirect. In bovine RPE, the electrical responses to apical epinephrine, bumetanide, and low [K⁺]o all involve a basolateral Cl⁻ conductance and are the same in human and bovine RPE.

Basolateral Membrane K⁺ Conductance

The evidence for a basolateral K⁺ conductance is more direct. It stems from the basolateral membrane voltage and resistance changes produced by a step change in basal [K⁺]. The large variation observed in basal [K⁺]o response also has been observed in bovine RPE. This is most likely due to differences in diffusion across the choroid and in the effect of the shunt current on ΔV_B (equation 2).

In Vitro Approximation of Light Onset:
Low Apical [K⁺]

Phases 1 and 2 in the response of human fetal RPE to reduced apical [K⁺]o indicate there is an apical hyperpolarization followed by a delayed basolateral hyperpolarization. The apical hyperpolarization results from the change in E_K expressed across the apical K⁺ conductance. The decrease in the apical to basolateral membrane resistance ratio, α, concurrent with the increase in R_B during phase 1 (Fig. 9) is consistent with a decrease in basolateral membrane conductance that begins during phase 1. The results in bovine RPE indicate that this conductance change is most likely due to a [Cl⁻] induced decrease in basolateral membrane Cl⁻ conductance. This suggests that the basolateral membrane starts to hyperpolarize during phase 1 when dV_B/dt > dV_B/dt and increases until dV_B/dt > dV_A/dt, resulting in phase 2. The continued increase in R_B during phase 2, when α is constant, suggests that an additional decrease in apical membrane conductance occurred after the basolateral conductance decrease began. One possibility is that low apical [K⁺]o inhibited the Na/K pump and led to a decrease in [K⁺], and apical membrane K⁺ conductance.

The return from 2 to 5 mmol/l [K⁺]o resulted in a sequence of voltage and resistance changes that essentially were the opposite of those produced by lowering [K⁺]o—an apical depolarization followed by a delayed basolateral depolarization. The simplest explanation for this sequence of events is that the increase
in apical \([K^+]_o\) rapidly depolarized the apical membrane because of the apical K\(^+\) conductance and also increased the inward transport of Cl\(^-\) across the apical membrane Na-K-2Cl cotransporter. The increased Cl\(^-\) influx gradually increased \([Cl^-]_i\), causing an additional, delayed depolarization across the basolateral membrane because of a relatively large Cl\(^-\) conductance. The increase in \(a\) and the decrease in \(R_t\) during phase 4 (Fig. 9) are consistent with an increase in basolateral membrane conductance and the explanation for the delayed depolarization. The variation in the low apical \([K^+]_o\) induced \(R_t\) changes between different tissues could result from differences in \(R_t\) and \(\Delta[K^+]_o\) induced changes in \(R_t\). The change in ratio of apical to basolateral membrane resistance \((a)\), which is independent of \(R_t\), was very similar from tissue to tissue, further suggesting that \(R_t\) is the variable factor.

The mechanisms for which we now have evidence in human RPE are summarized in Figure 10. The apical membrane of adult and fetal RPE contains a Ba\(^{2+}\) inhibitable K\(^+\) conductance (Figs. 2 and 4) and an adrenergic receptor that causes changes in basolateral membrane voltage and resistance in response to epinephrine (Figs. 5 and 8). The apical membrane of fetal RPE also contains a ouabain-sensitive mechanism, the Na/K pump, which is absent in the basolateral membrane (Fig. 6). Also located on the apical membrane is a bumetanide-sensitive mechanism (Fig. 7), presumably the Na-K-2Cl cotransporter that has been studied in cultured human RPE cells. The basolateral membrane of fetal RPE has a K\(^+\) conductance (Fig. 3) and most likely a Cl\(^-\) conductance.

Physiologic and Clinical Significance

It has previously been shown that the voltage changes produced in vitro by a step change in apical \([K^+]_o\) approximate the RPE voltage responses produced in the intact eye (cat, monkey, and human) after a transition between light and dark.\(^{17,42,57}\) In the intact eye, these RPE responses underlie the slow potential changes clinically recorded in the EOG or DC-ERG. In particular, the apical hyperpolarization during phase 1 corresponds to the c-wave,\(^{40}\) and the delayed basal hyperpolarization corresponds to the fast oscillation.\(^{15-17}\)

A comparison of the bovine\(^{11,12}\) and human (Figs. 4, 6, 8, and 9) RPE responses indicate that the same ion transport mechanisms are present in both preparations and produce the same sequence of events in response to step changes in \([K^+]_o\) that are produced in the subretinal space after the transition between dark and light. The concomitant measurements of c-wave, fast oscillation, and light peak,\(^{18}\) with a detailed understanding of the underlying apical and basolateral membrane transport proteins, should help define the pathophysiology associated with specific RPE/retinal diseases.

The ability of the RPE to absorb fluid out of the subretinal space may be an important factor in maintaining retinal adhesion.\(^{59-61}\) In bovine RPE, it has been shown that apical epinephrine stimulates fluid absorption and that apical bumetanide, an inhibitor of Na-K-2Cl cotransport, inhibits the epinephrine-stimulated absorption.\(^{13}\) In addition, electrophysiologic experiments indicate that apical epinephrine stimulates a Cl\(^-\) transport pathway that consists of the Na-K-2Cl cotransporter and a basolateral Cl\(^-\) conductance.\(^{12,49}\) Together, these results provide strong evidence for at least one pathway that controls fluid absorption across the bovine RPE. The present data make it seem likely that similar transport mechanisms exist in the human RPE.\(^{34}\) Therefore, the Cl\(^-\) transport pathway may play a central role in human RPE physiology and provide an important locus for therapeutic intervention in diseases that cause fluid accumulation in the subretinal space.

Key words: adrenergic receptor, Cl channel, human RPE, K channel, Na/K ATPase, Na-K-2Cl cotransporter

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