**Adrenergic Receptor Activated Ion Transport in Human Fetal Retinal Pigment Epithelium**

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**Purpose.** To identify the apical and basolateral membrane mechanisms and intracellular signaling pathways in human fetal retinal pigment epithelium (HRPE) that mediate membrane voltage and resistance changes caused by apical membrane adrenergic receptor activation.

**Methods.** Intact sheets of RPE-choroid from human fetal eyes were mounted in a modified Ussing chamber. Ringer's solution composition changes on the retina-facing and choroid-facing sides of the tissue were separately controlled. Intracellular microelectrodes recorded the membrane voltage and resistance changes after the addition of pharmacologic agents to the apical or basal baths.

**Results.** Apical adrenergic agonists, isoproterenol and epinephrine (10−8 M), depolarized the basolateral membrane, decreased total tissue resistance (Rt) and increased the ratio of apical-to-basolateral membrane resistance (Rap/Rbas). Experiments using antagonists for α1 and β adrenergic receptors, prazosin and propranolol, respectively, indicated that both receptor types were present. The epinephrine responses were inhibited by apical bumetanide and basal 4,4′-disothiocyanostilbene-2,2′-disulfonic acid (DIDS). A cocktail of cyclic adenosine monophosphate (cAMP)-elevating agents produced basolateral membrane voltage and resistance changes very similar to the isoproterenol responses. The cAMP-induced electrical responses were strongly inhibited by basal 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB). Ionomycin (to elevate intracellular Ca2+, [Ca2+]i) produced electrical responses similar to those caused by epinephrine. The Ca2+ responses were unaffected by NPPB but were inhibited by 3 mM DIDS in the basal bath.

**Conclusions.** The results provide evidence for two apical membrane adrenergic receptors, α1 and β, activated by epinephrine and isoproterenol, respectively. The membrane voltage and resistance changes produced by these two agonists mimic those produced by elevating [Ca2+]i and [cAMP], suggesting that these ubiquitous signaling molecules activate separate basolateral membrane Cl channels inhibited by DIDS and NPPB, respectively. These two receptors, the apical membrane NaK2Cl cotransporters and the basolateral membrane Cl channels form a complex of proteins that help mediate fluid absorption across human RPE. (Invest Ophthalmol Vis Sci. 2001;42:255–264)

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The retinal pigment epithelium (RPE) is remarkable in its ability to absorb or secrete fluid. For example, NaCl-coupled fluid absorption across bovine and fetal human RPE is mediated by apical membrane NaK2Cl cotransporters and basolateral membrane Cl channels.1–5 Fluid secretion has been demonstrated in bovine and human RPE.5,6 P2Y2, α1, and β receptors have been identified in cultured human RPE.7–9 The apical membrane of native bovine and human RPE have been shown to contain α1 adrenergic and P2Y2 purinergic receptors that when activated increase cell calcium and KCl-coupled fluid absorption.4,8–10

In the subretinal space of the intact eye, the RPE apical membrane is in close physical proximity to the distal ends of the photoreceptors, whereas the basolateral membrane is closely apposed to Bruch’s membrane and the choroidal blood supply. The RPE is thus critically positioned to maintain and regulate the chemical composition of the subretinal and choroidal extracellular spaces on the apical and basal sides of the tissue. In vivo, active, solute-linked fluid transport across the RPE is determined by the integrated activity of paracrine, autocrine, and hormonal signals that continuously impinge on both membrane surfaces during the light and dark. These extracellular signals activate a myriad of receptors coupled to a wide variety of intracellular second messengers, such as calcium and cyclic adenosine monophosphate (cAMP) whose signal transduction pathways can be coupled at various sites within the cell.11–13 There is considerable evidence for these two ubiquitous pathways in cultured human and rat RPE, as summarized by Nash and Osborn.14 In the present in vitro experiments, apical epinephrine and isoproterenol produced membrane voltage and resistance changes at the basolateral membrane of native fetal human RPE. The results helped to identify the apical membrane receptors, intracellular second messengers, and membrane transport proteins activated by these putative retinal paracrine signals.

**Materials and Methods**

**Solutions**

The experimental control Ringer’s solution consisted of the following reagent grade chemicals (in millimolar): 5 KCl, 0.8 MgSO4, 113.4 NaCl, 26.2 NaHCO3, 1 NaH2PO4, 5.6 glucose, and 1.8 CaCl2. In a few of the experiments an older formula was used that contained MgCl2 instead of MgSO4.15 In some experiments the solution also contained 5 mM taurine. There was no difference between results from experiments using Ringer’s with or without MgSO4 or taurine. These additions were one of several manipulations intended to maintain consistently healthier tissues.

Supplemented Ringer’s solution contained the same salt composition as the control Ringer’s with the addition of the following reagent grade vitamins and l-amino acids (in grams per liter): 0.110 pyruvic acid, 0.001 t-panthenolic acid, 0.001 folic acid, 0.002 myoinositol, 0.001 niacinamide, 0.001 pyridoxal, 0.001 riboflavin, 0.001 thiamin, 0.001 choline chloride, 0.042 histidine, 0.052 leucine, 0.052 isoleucine, 0.015 methionine, 0.032 phenylalanine, 0.048 threonine, 0.010 tryptophan, 0.046 valine, 0.073 lysine, 0.126 arginine, 0.024 cystine, 0.292 glutamine, and 0.036 tyrosine. The transport and dissection of eyes in supplemented Ringer’s solution resulted in healthier tissues as...
evidenced by consistently larger values of transepithelial potential (TEP) and total tissue resistance (Rt). If dissected in supplemented Ringer's and then mounted in the chamber at 37°C and perfused with control Ringer's, tissues could be maintained for several hours.

All amino acids, salts, and vitamins, as well as epinephrine, prazosin, forskolin, isobutylmethylxanthine (IBMX), 8-(4-chlorophenylthio)-adenosine 3’,5’ cyclic monophosphate (CPT-cAMP), 4’,4’-diisothiocyanostilbene-2,2’ disulfonic acid (DIDS), isoproterenol, t-phenylephrine, propranolol, and indomethacin were obtained from Sigma (St. Louis, MO). The compounds were obtained from other suppliers: ionomycin (Calbiochem, La Jolla, CA), bis-(o-aminophenoxy-N,N,N’,N’-tetramethylacetoxymethyl ester (BAPTA-AM; Molecular Probes, Eugene, OR), r-phenylephrine (Aldrich, Milwaukee, WI), and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB; a generous gift from Alan Verkman at the University of California, San Francisco and RBI/Sigma, Natick, MA).

Human Fetal Tissue

The research protocols were approved by the University of California Committee for the Protection of Human Subjects. Fetal eyes were obtained by an independent procurer (Advanced Bioscience Resources, Alameda, CA). The eyes were enucleated immediately after elective abortion of fetuses of gestational age 16 to 24 weeks, placed in supplemented Ringer's solution, packed in ice, and delivered to the laboratory within approximately 2 hours. To isolate the RPE-choroid, the eye was first placed in a dissection dish containing cold supplemented Ringer's solution. The anterior portion of the eye was removed by cutting just posterior to the ora serrata. The posterior pole was then bisected and cut into approximate circles of 0.38 cm². The retina was peeled away from the RPE, and the RPE-choroid was then separated from the sclera by blunt dissection.

Chamber and Flow System

The RPE-choroid was mounted on a nylon mesh support and clamped into a modified Ussing chamber. The methods for maintaining the temperature and pH of the solutions have been described previously. 10,15 Briefly, solutions were maintained at 40°C to 45°C in reservoirs and kept at pH 7.4 by bubbling with a mixture of 8% CO₂, 10% O₂, and 82% N₂. The temperature of solutions entering the chamber was maintained at 37°C by Peltier heat pumps.

Electrophysiology

The equivalent circuit analysis and electrophysiological methods have been described in detail previously. 10,15,16 Briefly, electrical connections to the apical and basal chambers were made with Ringer-agar bridges in series with calomel electrodes. Intracellular potentials were recorded with conventional microelectrodes filled with 150 mM KCl. These signals were amplified, digitized, and sampled at 4 Hz for analysis on a computer. The apical and basolateral membrane potentials (Vₐ and Vₜ) are the voltage differences between the intracellular microelectrode and the apical and basal bath electrodes, respectively. The resistances of the apical and basolateral membranes (Rₐ and Rₜ), and the paracellular shunt (Rₛ) determine the total transepithelial resistance (Rₑ = Rₐ + Rₛ). The transepithelial potential (TEP = Vₐ - Vₜ) is the voltage difference between the apical and basal bath electrodes. Epithelial resistance parameters were obtained by passing 1- to 8-μA bipolar current pulses (i) using Ag-AgCl electrodes, one located in the apical chamber and another located in the basal chamber. Rₛ was then calculated from the current-induced changes in TEP (Rₛ = ΔTEP/i). The apparent membrane resistance ratio (Rₜ/Rₐ) was calculated from the change in Vₐ and Vₜ (Rₜ/Rₐ = ΔVₐ/ΔVₜ). These voltage deflections have been digitally subtracted from the figures.

Statistical analysis data are reported as mean ± SEM, unless otherwise specified. Statistical comparisons were made using Student’s t-test. Differences were considered statistically significant at P < 0.05.

RESULTS

Preliminary experiments on native human fetal and adult RPE indicate that the addition of nanomolar amounts of apical epinephrine or isoproterenol can produce significant changes in basolateral membrane voltage and resistance. In the present experiments we sought to identify the apical membrane receptors, second-messenger pathways, and membrane transport proteins that are activated by these putative retinal paracrine signals.

Apical Membrane Adrenergic Receptors

Figure 1A illustrates the voltage and resistance changes produced by isoproterenol, a β-receptor agonist. In Figure 1A, 10⁻⁶ M apical isoproterenol depolarized Vₐ (and Vₜ) by approximately 15 mV, increased the membrane resistance ratio (Rₛ/Rₐ) from 0.26 to 1.45, and increased the TEP approximately 0.9 mV. The depolarization was biphasic, as were the majority of responses to isoproterenol, suggesting the activation of more than one mechanism or pathway. The changes in total tissue resistance, Rₜ, were small and difficult to quantify, perhaps because they were caused by a combination of shunt and cell membrane resistances changes. In contrast, the changes in membrane voltage and Rₛ/Rₐ value were large, reversible, and consistent with a conductance increase in a basolateral membrane channel, probably a Cl channel as found in bovine and human fetal RPE. 1,4,6,10

In Figure 1B, 10⁻⁶ M isoproterenol was again added to the apical bath, but this time in the presence of 10⁻⁷ M propranolol, a β-receptor antagonist. In this case, Vₐ and Vₜ depolarized much less, approximately 2 mV, whereas Rₛ/Rₐ and TEP barely changed. This experiment was repeated in three tissues, and the isoproterenol-induced changes in Vₐ, Rₛ/Rₐ, and TEP were 11 ± 3, 0.98 ± 0.13, and 1.6 ± 0.6 mV, respectively. In the presence of 10⁻⁷ M propranolol, the isoproterenol-induced changes in Vₐ, Rₛ/Rₐ, and TEP were reduced by more than 80%, to 2.0 ± 1.0, 0.16 ± 0.12, and 0.2 ± 0.03 mV; the agonist-induced changes in Rₛ were similarly reduced, by 83% and 100% (n = 2) in the presence of propranolol. In two other tissues, all the electrical responses to 10⁻⁶ M isoproterenol were completely blocked by 10⁻⁴ M propranolol. Figure 1C shows that the effects of propranolol were partially reversible. These results, taken together, indicate the presence of β-adrenergic receptors on the apical membrane of native human fetal RPE.

In a set of preliminary experiments, the α₁ adrenergic agonist phenylephrine (10⁻⁶ M, apical bath) depolarized the basolateral membrane in five of nine tissues (6 ± 2 mV; range, 1-12 mV); 10⁻⁶ M apical phenylephrine produced similar voltage responses in four of five tissues. In four tissues the effect of prazosin, an α₂ antagonist, was tested on the phenylephrine-induced depolarization but microelectrode impedance for the complete set of solution changes, including prazosin, could be obtained in only one of the tissues. In addition, the concentration of phenylephrine required to produce TEP responses of approximately the same size was variable: One tissue required 10⁻⁷ M, whereas two others required 10⁻⁶ M phenylephrine to produce an approximately 1-mV increase in TEP (1.0 ± 0.2 mV). In each of these three experiments, an equal concentration of prazosin was then added to the apical bath and, in the presence of prazosin, the phenylephrine-induced increase in TEP was significantly reduced to 0.3 ± 0.1 mV. In the fourth experiment, the TEP response (0.8 mV) to 10⁻⁶ M phenylephrine was completely blocked by 10⁻⁷ M apical prazosin. The phenylephrine-induced decrease in Rₛ, 4.8 ± 1.3 Ω·cm² (n = 4), was completely inhibited in the presence of prazosin (n = 2). These responses, blocked by
Epinephrine is a nonspecific adrenergic agonist that could activate both α and β adrenergic receptors. Figure 2A shows the control responses after the addition of 10^{-6} M epinephrine to the apical bath: $V_h$ depolarized by approximately 22 mV, TEP increased by 4 mV, $R_b$ decreased by approximately 12 Ω·cm², and there was a concomitant increase in $R_b/R_a$ from 0.2 to 3.1. Figure 2B illustrates the electrical responses to epinephrine in the presence of 10^{-6} M propranolol and 10^{-7} M prazosin. In the presence of both antagonists, 10^{-6} M apical epinephrine depolarized $V_h$ by approximately 6 mV and increased TEP by 1.5 mV. In addition, $R_b/R_a$ increased from 0.20 to 0.42 and $R_b$ decreased by approximately 13 Ω·cm². Prazosin was then removed from the apical bath and, in the presence of 10^{-6} M propranolol alone (Fig. 2C), the epinephrine-induced changes in membrane voltage and resistance were again measured. Epinephrine depolarized $V_h$ by approximately 13 mV, increased TEP by approximately 2 mV, increased $R_b/R_a$ from 0.15 to 0.92, and decreased $R_b$ approximately 16 Ω·cm². In four experiments with the same design as that shown in Figure 2, the epinephrine-induced changes in $V_h$ and TEP were 22 ± 1 and 4.5 ± 0.8 mV, respectively; at the same time, $R_b/R_a$ increased by a factor of 3.4 ± 0.80, and $R_b$ decreased by 20 ± 10 Ω·cm². In the combined presence of prazosin and propranolol, the epinephrine-induced changes in $V_h$, TEP, and $R_b/R_a$ were significantly inhibited, to $8 ± 2$, $2.1 ± 0.3$, and $1.1 ± 0.60$ mV, respectively, but the $R_b$ decrease $(16 ± 8$ Ω·cm²) was not significantly different from the epinephrine control. In the presence of 10^{-6} M propranolol alone, the epinephrine-induced changes in $V_h$ and TEP were $12 ± 3$ and $3.0 ± 0.8$ mV, respectively, and the resistance ratio changes were $1.3 ± 0.6$ and $19 ± 10$ Ω·cm², for $R_b/R_a$ and $R_b$, respectively.

These results suggest that epinephrine simultaneously activates apical membrane α₁ and β adrenergic receptors to produce a synergistic response, one that exceeds the membrane voltage and resistance changes produced by the activation of either adrenergic receptor alone. If the electrical responses to epinephrine were due only to activation of β receptors then the epinephrine-induced changes in $V_h$, TEP, $R_b/R_a$, and $R_b$ should be completely blocked by a 100-fold excess concentration of the β antagonist propranolol. This was clearly not the case, in that a 100-fold excess of propranolol decreased $ΔV_h$, $Δ$TEP by only 45% and 33%, respectively. In contrast, a 10-fold excess of propranolol (Fig. 1 and summary data) was sufficient to reduce the isoproterenol-induced changes in $ΔV_h$ and $Δ$TEP by 78% and 88%, respectively. This comparison indicates that the electrical responses produced by apical epinephrine are not primarily mediated by the activation of β receptors.

Membrane Transport Mechanisms Involved in the Adrenergic Response

Net CI and fluid transport across bovine RPE is mediated in part by bumetanide-inhibitable apical membrane NaK2Cl cotransporters and DIDS-inhibitable basolateral membrane CI channels. Figure 3A shows the typical control responses to
apical epinephrine ($10^{-8}$ M), and Figures 3B and 3C show that apical bumetanide completely blocked the epinephrine-induced electrical responses and that this blockade was reversible. In four similar experiments, bumetanide inhibited the mean epinephrine-induced changes in $V_B$, $R_A/R_B$, and $R_t$ by 60% to 80%. The control changes were $19 \pm 4$ and $2.1 \pm 0.4$ mV, and $1.63 \pm 0.54$ and $11 \pm 3 \Omega \cdot \text{cm}^2$, respectively. In the presence of apical bumetanide, the changes in $V_B$ and TEP were reduced to $8 \pm 6$ and $0.6 \pm 0.5$ mV, respectively, whereas the changes in $R_A/R_B$ and $R_t$ were reduced to $0.35 \pm 0.32$ and $3 \pm 2 \Omega \cdot \text{cm}^2$, respectively.

NPPB and DIDS can be used (with appropriate caution) to block cAMP and Ca$^{2+}$-dependent Cl channel currents, respectively. We first compared the epinephrine-induced changes in TEP and $R_t$ in the presence and absence of NPPB ($100 \mu M$). In five experiments, the epinephrine-induced ($10^{-8}$ M) changes in TEP and $R_t$ were significantly decreased from $2.9 \pm 1.2$ mV and $33.6 \pm 18.9 \Omega \cdot \text{cm}^2$ in control samples to

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933218/)  
**Figure 2.** Additive effect of prazosin and propranolol on the epinephrine response. (A) Epinephrine ($10^{-8}$ M) was perfused into the apical bath (filled bar), depolarizing both membranes, increasing TEP and $R_A/R_B$, and decreasing $R_t$. (B) In the presence of both $10^{-6}$ M propranolol and $10^{-7}$ M prazosin (open bar), $10^{-8}$ M epinephrine produced responses approximately 30% of the control in (A). (C) With $10^{-6}$ M propranolol alone (open bar), $10^{-8}$ M epinephrine gave responses approximately 60% of the control in (A). The traces were sequentially recorded from a single tissue.

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933218/)  
**Figure 3.** Effect of apical bumetanide on the epinephrine response. (A) Control response: $10^{-8}$ M epinephrine apical (filled bar) depolarized $V_A$ and $V_B$ approximately 14 mV. (B) Apical bumetanide (0.5 mM; open bar) blocked the epinephrine response. (C) After the control was added, epinephrine depolarized $V_A$ and $V_B$ approximately 15 mV, showing that the bumetanide effects were completely reversible. The traces were sequentially recorded from a single tissue.
and bovine RPE. 5,22 These observations suggest that at least part of the epinephrine response in human RPE is mediated by an NPPB-sensitive Ca channel that is defective in cystic fibrosis (CF).6,22 The data summarized in Figures 4A and 4B also indicate the significant involvement of a basolateral membrane DIDS-sensitive mechanism. Typical control responses (Fig. 4A) indicate an intercellular Ca\(^{2+}\) [Ca\(^{2+}\)]\(_{i}\)-induced increase of basolateral membrane Cl conductance as previously shown in bovine RPE.4,10 The decrease in \(R_t\), usually small in these experiments, could not be measured, because the baseline increased over time. Figure 4B shows that the prior addition of basal DIDS, a specific RPE Cl channel blocker,1,19 completely inhibited the epinephrine-induced electrical responses. In three tissues, DIDS (3 mM) inhibited the mean epinephrine-induced changes in \(V_R\), \(R_s/R_b\), and TEP by 70% to 93%, from 16 ± 1, 1.27 ± 0.40, and 1.27 ± 0.46 mV to 5 ± 2, 0.14 ± 0.14, and 0.15 ± 0.15 mV, respectively. In two other tissues, 1 mM DIDS inhibited the 10\(^{-8}\) M epinephrine-induced changes in \(V_R\), \(R_s/R_b\), and TEP by approximately 60% and the \(R_t\) changes by approximately 80%. These observations, combined with previous work on human and bovine RPE,5 suggest the presence in human fetal RPE of two basolateral membrane Cl channels, one activated by cAMP and the other activated by Ca\(^{2+}\).

**Second-Messenger Pathways that Involve Ca\(^{2+}\) or cAMP**

In bovine RPE we demonstrated that \(\alpha\) adrenergic receptor activation causes membrane voltage and resistance changes that are accompanied by an elevation in cell Ca and cell cAMP.5,10,25 If these measured second-messenger changes directly cause the observed membrane voltage and resistance changes, then elevation of cell Ca or cAMP per se should produce very similar responses.

**Calcium**

BAPTA, a calcium chelator, lowered cell calcium and blocked epinephrine-induced [Ca\(^{2+}\)]\(_{i}\) transients in bovine RPE.5,6 In human fetal RPE (10 experiments), BAPTA-AM increased \(R_t\) (21.2 ± 7.0 Ω cm\(^{-2}\)) and decreased \(R_s/R_b\) (0.36 ± 0.20); at the same time, \(V_R\) hyperpolarized by 5.4 ± 0.2 mV, and TEP decreased by −1.1 ± 0.3 mV. All these membrane voltage and resistance changes are consistent with a BAPTA-induced decrease in basolateral membrane conductance of a mechanism with an equilibrium potential that is depolarized relative to the resting membrane potential. [Ca\(^{2+}\)]\(_{i}\) was elevated by adding ionomycin (500 nM), a Ca\(^{2+}\) ionophore, to the apical bath. In Figure 5, apical ionomycin depolarized \(V_R\) by approximately 14 mV, increased \(R_s/R_b\) by a factor of four from 0.21 to 0.85, increased TEP by 2 mV, and decreased \(R_t\) by 12 Ω cm\(^{-2}\). The magnitude of these changes provide strong evidence for an increase in basolateral membrane conductance (see the Discussion section). The ionophore-induced changes in TEP and \(R_t\) were also measured in the presence and absence of basal DIDS, a Cl channel blocker and other epithelia.1,10,19,22 In control tissues, ionomycin (500 nM) increased TEP by 1.7 ± 0.3 mV and decreased \(R_t\) by 0.7 ± 1.4 Ω cm\(^{-2}\) (mean ± SD; n = 3). In the presence of 3 mM basal DIDS these [Ca\(^{2+}\)]\(_{i}\) responses were completely abolished (n = 3, not shown), suggesting the presence of DIDS-inhibitable Ca\(^{2+}\)-activated Cl channels in human fetal RPE.

**Cyclic AMP**

In the experiment summarized in Figure 6, a cAMP cocktail consisting of 10 μM forskolin, 0.5 mM IBMX, and 0.1 mM

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933218/)

**Figure 4.** Effect of basal DIDS on the epinephrine response. (A) Control response to 10\(^{-8}\) M epinephrine (filled bar): \(V_A\) and \(V_B\) depolarized 19 mV. (B) Basal DIDS (3 mM; open bar) blocked the epinephrine response. In the following control response (not shown), epinephrine depolarized \(V_A\) and \(V_B\) by approximately 4 mV, indicating that the effect of DIDS were poorly reversible. The traces were sequentially recorded from a single tissue.

- 1.8 ± 1.1 mV and 12.7 ± 3.8 Ω cm\(^{-2}\) in the presence of basal NPPB. These observations suggest that at least part of the epinephrine response in human RPE is mediated by an NPPB-inhibitable mechanism at the basolateral membrane, perhaps the Cl channel that is defective in cystic fibrosis (CF).6,22
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- These observations, combined with previous work on human and bovine RPE,5 suggest the presence in human fetal RPE of two basolateral membrane Cl channels, one activated by cAMP and the other activated by Ca\(^{2+}\).

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933218/)

**Figure 5.** Ionomycin-induced changes in membrane voltage and resistance. Perfusion of 500 nM ionomycin into the apical bath (filled bar) depolarized \(V_A\) and \(V_B\) by approximately 15 mV. TEP and \(R_s/R_b\) increased, and \(R_t\) decreased.
CPT-cAMP was added to the apical bath. The elevation of cell cAMP caused $V_B$ (and $V_A$) to depolarize by approximately 20 mV, and $R_{A}/R_{B}$ to increase by a factor of more than seven, from 0.4 to 3.0. $TEP$ increased by more than 1 mV showing that $V_B$ depolarized at a faster rate than $V_A$, but there was little change in $R_t$, $V_t$, $TEP$, and $R_{A}/R_{B}$ changed in several discrete phases: The first phase (or sometimes two phases) occurred during the cAMP pulse, and the second phase began after the cAMP cocktail was removed from the apical bath. Eventually, all these parameters returned to pre-cAMP control levels. This pattern of responses was clearly observed in most but not all the present experiments (compare Figs. 6 and 7), suggesting that cAMP may activate multiple mechanisms.5

In six similar experiments, the addition of cAMP cocktail to the apical bath depolarized $V_B$ by $20 \pm 1.2$ mV and increased $R_{A}/R_{B}$ by a factor of almost seven, from $0.40 \pm 0.1$ to $2.7 \pm 0.5$ ($n = 6$). In addition, $TEP$ increased by $2.8 \pm 0.6$ mV and $R_t$ decreased by $40 \pm 7 \Omega \cdot \text{cm}^2$ ($n = 8$; mean $\pm$ SEM). All these concomitant changes are qualitatively similar to those produced by the adrenergic agonists in Figures 1 through 4 and confirm the existence of a large cAMP-dependent increase in basolateral membrane conductance.

**Blockade of cAMP-Activated Cl Channels**

Figure 7A shows that elevating cell cAMP depolarized $V_B$ by approximately 21 mV and increased $TEP$ by approximately 4 mV, whereas $R_{A}/R_{B}$ increased from 0.28 to 1.85, and $R_t$ decreased by 58 $\Omega \cdot \text{cm}^2$. After recovery in control Ringer (Fig. 7B), NPPB (0.5 mM) was perfused into the basal bath and hyperpolarized $V_A$ and $V_B$ approximately 10 mV and decreased $TEP$ approximately 4 mV. In addition, $R_{A}/R_{B}$ decreased from 0.47 to 0.17, and $R_t$ increased from the starting value of 559 $\Omega \cdot \text{cm}^2$ to 575 $\Omega \cdot \text{cm}^2$. All these changes are consistent with an NPPB-induced decrease in basolateral membrane conductance.

After the membrane voltages and resistances had reached a new steady state in NPPB (60 minutes), the cAMP cocktail was again perfused into the apical bath (Fig. 7C). In this case, $V_B$, depolarized by 10 mV, $TEP$ increased 1.6 mV, $R_{A}/R_{B}$ increased from 0.16 to 0.53, and $R_t$ decreased 16 $\Omega \cdot \text{cm}^2$. This 60% to 70% decrease, which took place over the course of 1 hour, was not observed in a control tissue experiment, in which cAMP cock-
tail was repeatedly perfused into the apical bath and the dimethyl sulfoxide (DMSO) carrier perfused into the basal bath (no NPPB) during the same period. Figure 7D shows the postcontrol readings indicating that the effects of NPPB were largely reversible.

The inhibitory effects of NPPB on the cAMP responses were confirmed in a larger series of experiments using either 500 μM (n = 2) or 100 μM (n = 3) NPPB. Except for \( R_0/ R_b \), all the membrane voltage and resistance measurements were combined, because they are practically indistinguishable. In the presence of NPPB, CAMP depolarized \( V_m \) by 12.2 ± 1.7 mV and increased \( TEP \) 1.6 ± 0.4 mV. At the same time, \( R_e \) decreased by 25.6 ± 9.0 Ω·cm² (n = 5). In 500 μM NPPB, the \( R_e/ R_0 \) value increased by 0.4 ± 0.04 (n = 3) and in 100 μM NPPB it increased by 1.1 ± 0.08 (mean ± SD; n = 2). These results show that basal NPPB reduced the CAMP-induced changes in membrane voltage and resistance by 40% or more, consistent with the presence of CAMP-dependent Cl channels, perhaps cystic fibrosis transmembrane conductance regulator (CFTR), at the basolateral membrane.5,6

**Discussion**

In the present experiments, nanomolar amounts of apical isoproterenol or epinephrine working through \( \beta \) and \( \alpha_1 \) adrenergic receptors produced qualitatively similar intracellular voltage and membrane resistance changes that mainly originated at the basolateral membrane of native fetal human RPE (16–24 weeks' gestation). Similar changes were produced by elevating cell cAMP or cell Ca\(^{2+}\).

**Secretagogue and Second Messenger–Induced Changes in Basolateral Membrane Conductance**

In each of the experiments summarized in Figures 1 through 3 or Figures 5 through 7, the initial response was an increase in \( TEP \). In each case, both membranes depolarized and \( TEP \) increased, and therefore the rate of basolateral membrane depolarization exceeded the rate of apical membrane depolarization, indicating that the voltage response mainly originated at the basolateral membrane. In addition, the concomitant increase in \( R_e/ R_b \) and decrease in \( R_e \) are consistent with a decrease in \( R_0 \), or equivalently, an increase in basolateral membrane conductance. Because the apical and basolateral membranes are electrically coupled by the paracellular shunt resistance (\( R_s \)), a significant fraction of \( \Delta V_m \) is passively shunted to the apical membrane and, in addition, secondary smaller voltage and resistance responses may be directly produced at the apical membrane, as previously demonstrated in bovine RPE.4,10

In the present experiments, an increase in basolateral membrane conductance can be inferred in part from the changes in resistance ratios, assuming that the agonist caused relatively little change in \( R_e \). This assumption may be incorrect, because there are only two independently measured membrane resistance parameters, \( R_e/ R_b \) and \( R_e \), but there are three unknown resistance parameters, \( R_e, R_0, \) and \( R_b \) (see the Methods section). Clearly, a direct experimental determination of \( R_0 \) would be helpful. For example, in bovine RPE, we performed an additional series of experiments to determine that \( R_0 \) is approximately 1.3 \( R_b \), thus allowing experimental determination of apical, basolateral, and paracellular (shunt) resistances.5 In preliminary experiments (unpublished data, 2000) similar results have been obtained using fetal human RPE (\( R_0 \approx 1.5 \) \( R_b \); n = 2). In addition, we have used a different strategy to estimate the secretagogue-induced changes in \( R_e \) and \( R_b \). From the equivalent circuit for this epithelium5,10 we know that

\[
\frac{R_0}{R_b} = \frac{R_e}{R_b} \cdot \left(1 + a \left( R_b - R_e \right) \right)^{-1},
\]

where \( a = R_e/ R_b \).

In this equation, we used the Ca\(^{2+}\)- and cAMP-induced changes in \( R_e \) and \( R_e/ R_b \) and the assumption that \( R_e \) either increases or decreases, to test our conclusion that increasing cell calcium or cAMP decreases \( R_0 \). This equation shows that if \( R_e \) increased, then basolateral membrane resistance must decrease, because both Ca\(^{2+}\) and cAMP decreased \( R_e \) and increased \( a \). However, if \( R_e \) decreased, it is possible that \( R_0 \) remained constant or even increased somewhat. In that case, the observed increase in \( a \) requires that there be a concomitant increase in \( R_e \) that significantly exceeds the change in \( R_b \). To examine that possibility, we calculated \( R_0 \) essentially over the entire range of possible agonist-induced decreases in \( R_e \).

Before the addition of secretagogue we assume that \( R_b = 1.3 \) \( R_e \) (as in bovine RPE), and after addition of secretagogue, we examined a wide range of possible \( R_b \) values: \( R_b = k R_e \) (where \( k > 1; k = 1 \) is physically impossible). Using this relationship for each of the experiments shown in Figures 4 to 7 we let \( k \) range from 1.009 to 30 and then calculated the secretagogue-induced changes in apical and basolateral membrane resistance. In each case, we calculated a striking decrease in \( R_b \). Two sample calculations are provided in Figure 8, but the same qualitative result is obtained for all \( k > 1 \) and is relatively unchanging for \( k > 10 \). The top portion of Figures 8A and 8B replots the ionomycin (Ca\(^{2+}\)) and cAMP-induced changes in \( R_e \) and \( R_e/ R_b \) from Figures 5 and 6. The lower portion of each panel is a calculation of the Ca\(^{2+}\) and cAMP-induced changes in \( R_e \) and \( R_b \), assuming that \( R_b = 1.3 \) \( R_e \). In these two examples and, more generally, for all the data, elevation of intracellular Ca\(^{2+}\) or cAMP increased basolateral membrane conductance and was accompanied by a decrease in apical membrane conductance. These calculations combined with the voltage and resistance measurements help corroborate our conclusion that epinephrine, isoproterenol, ionomycin, and cAMP all depolarized the basolateral membrane by increasing basolateral membrane Cl conductance.

**Receptors, Second Messengers, Chloride Transport, and the EOG**

In human fetal RPE, the epinephrine- or isoproterenol-stimulated \( \alpha_1 \) or \( \beta \) adrenergic–induced alterations in membrane voltage and resistance (Figs. 1, 2) are mediated mainly by basolateral membrane, DIDS or NPPB-sensitive mechanisms (Figs. 4, 7, and 9). The epinephrine-induced changes in \( TEP \) and \( R_e \) were also somewhat reduced by basal NPPB. In several epithelia, it has been shown that NPPB specifically blocks the CFTR, a CAMP-dependent Cl channel21,22,24 that resides in the apical membrane of most epithelia. In some of these epithelia, DIDS-inhibitable Ca\(^{2+}\)-dependent Cl channels are also present in the apical membrane.22,25

In bovine RPE, epinephrine increased net fluid absorption mediated by DIDS-inhibitable [Ca\(^{2+}\)]-activated Cl channels that reside in the basolateral membrane.1,4,2,25 The present human RPE show that the epinephrine and ionomycin-induced changes in membrane voltage and resistance were similar in magnitude and time course (Figs. 2, 5). In both cases, the responses were blocked by basal DIDS but the [Ca\(^{2+}\)]-induced changes were not affected by NPPB (250 μM) in the basal bath (n = 5; data not shown). In contrast, the CAMP responses (Fig. 6) were blocked by basal NPPB (Fig. 7). These results suggest that DIDS and NPPB mainly affect different Cl channels, [Ca\(^{2+}\)]; and CAMP-dependent, respectively (see Fig. 9), but further experiments are needed to confirm this conclusion.

The presence and basolateral membrane localization of CFTR in human fetal RPE has been demonstrated using reverse transcription–polymerase chain reaction (RT-PCR), Western blot analysis, and immunohistochemical techniques.19 That con-
clusion was tested in patients with CF by measuring two components of the clinical electrooculogram (EOG), the fast oscillation (FO) that occurs approximately 60 seconds after light onset, and the so-called light peak that reaches its maximum amplitude 6 to 9 minutes after light onset. On the basis of in vivo and in vitro experiments, it is thought that the FO and light peak voltage responses are generated by two different basolateral membrane Cl channels. The possibility that one of these channels is CFTR was tested by measuring EOG responses in eight patients with CF and eight volunteers without. In CF patients, the magnitude of the FO was significantly reduced in size (and altered in time course), whereas the concomitantly recorded light peaks were not significantly different from the those in the patients without CF or from the mean normal values reported in the literature. RT-PCR revealed that most of the patients with CF were homozygous for the most devastating form of the disease (the ΔF508 mutation) that in airway epithelia prevents the traffic of CFTR to the plasma membrane. These clinical results, combined with the present data suggest that the EOG light peak is generated by Ca$^{2+}$-dependent Cl channels, whereas the FO is generated by CFTR.

Physiological Implications

In many epithelia, Ca$^{2+}$- and cAMP-dependent Cl channels help determine the rate of net transepithelial fluid transport. Any intracellular or plasma membrane proteins that help regulate second-messenger activity levels could in principle also help determine or maintain net fluid transport across the epithelium. For example, the endoplasmic reticulum (ER) contains several regulatory proteins that could serve as a locus of cross talk between the Ca$^{2+}$ and cAMP signaling pathways, and the molecular basis for cross talk between signal transduction pathways is beginning to emerge.

Phospholamban (PLB) is an ER protein that has been well studied in muscle, where it regulates stimulus-secretion coupling. In its unphosphorylated state PLB inhibits Ca$^{2+}$ uptake by the ER calcium adenosine triphosphatase (ATPase), a Ca$^{2+}$ pump. This inhibition is released after β adrenergic elevation of cell cAMP, which causes a protein kinase A-dependent increase of cell cAMP, stimulation of ER Ca$^{2+}$ ATPase activity, and a reduction in [Ca$^{2+}$]. Recently PLB has been detected by RT-PCR, Western blot analysis, and immunohisto-

FIGURE 8. Ca$^{2+}$ and cAMP-induced changes in cell membrane resistance ($R_1$ and $R_2$). The top portion of (A) and (B) replot the Ca$^{2+}$- and cAMP-induced changes in $R_1$ and $R_2/R_0$ from Figures 5 and 7, respectively. In each case, the lower portion of each panel shows that the calculated values of $R_0$ (and $R_2$) decrease after the elevation of cell calcium (A) and cell cAMP (B). This calculation assumes that $R_0 = 1.3 R_2$, but the conclusion is valid whether $R_0$ increases or decreases over a wide range.

FIGURE 9. Model of the human RPE showing identified membrane mechanisms and putative second messengers that contribute to the adrenergic responses. The apical membrane contains both α1 and β adrenergic receptors. Activation of either receptor type by epinephrine opens depolarizing conductances at the basolateral membrane. The effects of apical bumetanide and basal DIDS suggest that apical membrane NaK2Cl cotransporters and basolateral membrane Cl channels help mediate the electrical responses produced after activation of apical membrane adrenergic receptors. The epinephrine response can be mimicked by increasing either [Ca$^{2+}$], or [cAMP], cAMP activates an NPPB-inhibitable basolateral membrane conductance, most likely CFTR. The Ca$^{2+}$ responses were unaffected by NPPB but were blocked by basal DIDS, suggesting the presence of Ca$^{2+}$-activated Cl channels. light onset, and the so-called light peak that reaches its maximum amplitude 6 to 9 minutes after light onset. On the basis of in vivo and in vitro experiments, it is thought that the FO and light peak voltage responses are generated by two different basolateral membrane Cl channels.
chemical localization in intact sheets of bovine and human RPE.\textsuperscript{5–8} In bovine RPE, CAMP closes basolateral membrane Cl channels, hyperpolarizes $V_{m}$, and reverses the direction of fluid transport from absorption to secretion. These responses are blocked by pretreatment with pharmacologic inhibitors of Ca\textsuperscript{2+} ATPases, thapsigargin and cyclopiazonic acid,\textsuperscript{5,39,40} and are all consistent with the presence of PLB.

In contrast, CAMP had opposite effects in human fetal RPE. Basolateral membrane conductance increased, and $V_{m}$ depolarized. In the experiment summarized in Figure 6, CAMP depolarized the basolateral membrane in two distinct phases, and, more important, this depolarization increased further after nucleotide was removed from the apical bath. This observation was made in most but not all the CAMP experiments. But what of the putative cAMP-induced activation of PLB?

Once cell CAMP is elevated, CFTR is presumably activated, and the basolateral membrane begins to rapidly depolarize. We speculate that this is followed by a slower and smaller PLB-mediated, hyperpolarization of Ca\textsuperscript{2+}-activated Cl channels and that these two voltage changes are combined (algebraically summed) so that the dominant CFTR-induced depolarization rate is slowed and even flattened out (but not reversed) for a short period as seen in Figure 6. After this period of flattening, the hyperpolarization rate slows and therefore net depolarization continues, but at a slower rate of change, because of the putative smaller, concomitant hyperpolarization generated by Ca\textsuperscript{2+}-activated Cl channels. More important, on CAMP removal from the apical bath, the cycle is reversed and PLB again inhibits ER uptake of cytosolic Ca\textsuperscript{2+}. The subsequent increase in cell calcium, if dominant, could further activate and depolarize basolateral membrane Ca\textsuperscript{2+}-activated Cl channels and produce the overshoot seen in the CAMP experiments. Experimental verification of this hypothetical sequence of events would be a first step in the identification of PLB as a locus of signal integration for plasma membrane receptors,\textsuperscript{5–5} coupled to [Ca\textsuperscript{2+}], and [CAMP], that help determine net fluid transport across human RPE.

References

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