

Alpha-1-adrenergic Modulation of K and Cl Transport in Bovine Retinal Pigment Epithelium

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ABSTRACT Intracellular microelectrode techniques were used to characterize the electrical responses of the bovine retinal pigment epithelium (RPE)-choroid to epinephrine (EP) and several other catecholamines that are putative paracrine signals between the neural retina and the RPE. Nanomolar amounts of EP or norepinephrine (NEP), added to the apical bath, caused a series of conductance and voltage changes, first at the basolateral or choroid-facing membrane and then at the apical or retina-facing membrane. The relative potency of several adrenergic agonists and antagonists indicates that EP modulation of RPE transport begins with the activation of apical alpha-1-adrenergic receptors. The membrane-permeable calcium (Ca^{2+}) buffer, amyl-BAPTA (1,2-bis(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid) inhibited the EP-induced voltage and conductance changes by ~50–80%, implicating $[\text{Ca}^{2+}]_i$ as a second messenger. This conclusion is supported by experiments using the Ca^{2+} ionophore A23187, which mimics the effects of EP. The basolateral membrane voltage response to EP was blocked by lowering cell Cl, by the presence of DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid) in the basal bath, and by current clamping V_b to the Cl equilibrium potential. In the latter experiments the EP-induced conductance changes were unaltered, indicating that EP increases basolateral membrane Cl conductance independent of voltage. The EP-induced change in basolateral Cl conductance was followed by a secondary decrease in apical membrane K conductance (~50%) as measured by $\Delta[\text{K}]_o$ -induced diffusion potentials. Decreasing apical K from 5 to 2 mM in the presence of EP mimicked the effect of light on RPE apical and basolateral membrane voltage. These results indicate that EP may be an important paracrine signal that provides exquisite control of RPE physiology.

INTRODUCTION

The absorption of photons by the photoreceptor outer segments leads to alterations in photoreceptor and second-order neuron activity within the neural retina. The light-induced changes in retinal activity alter the chemical composition of the extracellular (subretinal) space between the photoreceptor outer segments and the

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apical processes of the retinal pigment epithelium (RPE) (Oakley and Green, 1976; Griff and Steinberg, 1984; Linsenmeier and Steinberg, 1983, 1984; Livsey et al., 1988; Borgula et al., 1989; Dearry and Burnside, 1989; Uehara et al., 1990; Gallemore and Steinberg, 1991; Li et al., 1991). These alterations modulate the transport of ions, fluid, and metabolites across the RPE (Miller and Steinberg, 1979, 1982; Immel and Steinberg, 1986; la Cour et al., 1986; Edelman et al., 1988; Fong et al., 1988; Miller and Edelman, 1990; Joseph and Miller, 1991).

It has been shown in several retina-RPE and intact eye preparations that light-induced decreases in subretinal $[K^+]_o$ are sufficient to alter membrane voltages and conductances at both the apical and basolateral membranes (Oakley, 1977; Steinberg et al., 1985; Gallemore and Steinberg, 1989, 1990). The specific apical membrane mechanisms that generate these changes include a relatively large potassium conductance, a bumetanide-sensitive Na(K)Cl cotransporter, and a ouabain-inhibitable electrogenic Na/K pump, all of which are sensitive to K concentration changes outside the apical membrane (Miller and Edelman, 1990; Joseph and Miller, 1991; Quinn et al., 1991). These apical $[K^+]_o$ changes significantly alter intracellular Cl activity, cell pH, and basolateral membrane electromotive force (EMF) (Joseph and Miller, 1991; Kenyon et al., 1991). In the intact eye, the light-induced decrease in subretinal $[K^+]_o$ generates the "fast oscillation" (FO), a clinically informative component of the electroretinogram (ERG) and the electro-oculogram (EOG; Weleber, 1989). The Cl transport pathway that generates the FO has been described (Joseph and Miller, 1991).

Recent evidence suggests the possibility that epinephrine (EP) and other catecholamines released by retinal neurons at light onset act as paracrine signals to the RPE by diffusing to the apical membrane. For example, dopamine secreted by the neural retina at light onset triggers morphological changes of the RPE in lower vertebrates (Dearry and Burnside, 1989). In addition, it has been reported that the RPE transepithelial electrical properties in several species respond to catecholamines (Dawis and Niemeyer, 1988; Frambach et al., 1988; Gallemore and Steinberg, 1990). In this study intracellular microelectrode techniques were used in the bovine RPE to analyze the specific membrane transport mechanisms that are altered after the agonist-induced activation of apical membrane alpha-1 receptors. These experiments combined with fluid transport and fluorescence microscopy (fura-2) measurements in the same preparation show that EP can exert exquisite control of RPE physiology (Edelman and Miller, 1991; Lin and Miller, 1991).

A preliminary account of some of this work has been presented in abstract form (Joseph and Miller, 1988, 1989).

METHODS

The eyes used in these experiments were obtained from a local slaughterhouse 15–40 min after death, placed in ice-cold Ringer solution, and transported to the lab. The techniques for isolating and mounting the bovine RPE-choroid and the design of the temperature-controlled perfusion chamber have been previously described in detail (Joseph and Miller, 1991).

The Ringer solution used in these experiments contained (in millimoles per liter): 120 NaCl, 5.0 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 23 NaHCO₃, and 10 glucose. In the low Cl Ringer, Cl was replaced with equimolar cyclamate or methylsulfate (MeSO₄). In experiments where $[K]_o$ was

changed, Na^+ was used as the replacement ion. All solutions had a final osmolarity of 295 ± 5 mosM.

The Ringer solutions were bubbled with a gas mixture containing 5% CO_2 and 10% O_2 (balance N_2) and titrated to a final pH of 7.5 ± 0.1 if necessary. 1 mM glutathione was added to all perfusion solutions at the beginning of each experiment to increase tissue longevity. The appropriate agonists, antagonists, or transport inhibitors were added to the bathing solutions as indicated.

Dopamine (DA), sulphiride, EP, norepinephrine (NEP), isoproterenol, phenylephrine, yohimbine, prazosin, propanolol, clonidine, cAMP (cyclic 3',5'-adenosine monophosphate), dbcAMP (dibutyryl-cAMP), DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid), and IBMX (isobutylmethylxanthine) were obtained from Sigma Chemical Co. (St. Louis, MO). Bumetanide was a generous gift from Hoffman-LaRoche Inc. (Nutley, NJ). The Ca^{2+} buffer amyl-BAPTA was obtained from Molecular Probes, Inc. (Eugene, OR). The Ca^{2+} ionophore A23187 and forskolin were obtained from Calbiochem Corp. (La Jolla, CA).

Forskolin, A23187, and amyl-BAPTA were prepared as stock solutions in DMSO (dimethylsulfoxide) or EtOH (ethyl alcohol) and, unless stated otherwise, diluted to a final concentration not more than 0.05% (vol/vol). DMSO or EtOH by themselves did not significantly affect the electrical properties of the bovine RPE at this concentration.

Electrophysiology

The tissues were mounted apical side up between two halves of a perfusion chamber designed to maximize the rate of solution turnover in the apical and basal baths (10–20 chamber volumes/min). The area of tissue exposed to the apical and basal bathing solutions was 0.07 cm^2 . The temperature of the Ringer was maintained at $37 \pm 1.0^\circ\text{C}$ using Peltier heat pumps located at the entrance port of each chamber. The flow rates of the bathing solutions that entered the apical or basolateral chamber were independently controlled by adjusting the height of a pair of small peripheral reservoirs (volume = $100 \mu\text{l}$). These low volume reservoirs were kept filled and overflowing by constant perfusion. Solution changes were made by Ringer substitution at the inflow to these small reservoirs. Since the solution changes were made at a distance 10–15 cm from the chambers, there was a 15–40-s delay, depending on flow rate, in the arrival of new solution to the apical or basal bath.

The recording apparatus is similar to that described in previous studies (Hughes et al., 1988). Calomel electrodes in series with Ringer-agar bridges were used to make electrical contact with each bathing solution. The membrane potentials were recorded with conventional microelectrodes made from fiber-filled borosilicate glass tubing with inside diameter 0.5 mm and outer diameter 1 mm (Omega Dot; Glass Co. of America, Bargaingtown, NJ). The most stable impalements were obtained with microelectrodes that had tip resistances of 120–175 $\text{M}\Omega$ when filled with 150 mM KCl. The apical and basolateral membrane potentials, V_A and V_B , were recorded by referencing the intracellular microelectrode to the apical and basal calomels, respectively. The difference between the apical and basal membrane potentials is equal to the transepithelial potential (TEP) ($\text{TEP} = V_A - V_B$). The transepithelial resistance, R_t , and the apparent ratio of the apical to basolateral membrane resistance, a , were obtained by passing 4- μA current pulses across the tissue and monitoring the resulting voltage changes in TEP and membrane potential ($a = \Delta V_A / \Delta V_B$), respectively. In the present experiments these voltage changes were used to help determine the location of the catecholamine-induced changes in membrane resistance (Miller and Steinberg, 1977).

The data were digitized (4 Hz) and stored on a microcomputer for analysis. In some experiments a and R_t were followed over time using 4- μA bipolar current pulses of 3 s duration applied at 12-s intervals (VCC 600; Physiologic Instruments, San Diego, CA). The current-induced voltage deflections were digitally subtracted from the records for clarity.

Analysis

The equivalent circuit is shown schematically in Fig. 1. The apical membrane and the basolateral membranes of the RPE are both modeled as an electromotive force (EMF), E_A or E_B , in series with a resistor, R_A or R_B , respectively. The paracellular pathway is represented as a resistor, R_S , in series with a paracellular diffusion potential, E_S , which is assumed to be zero

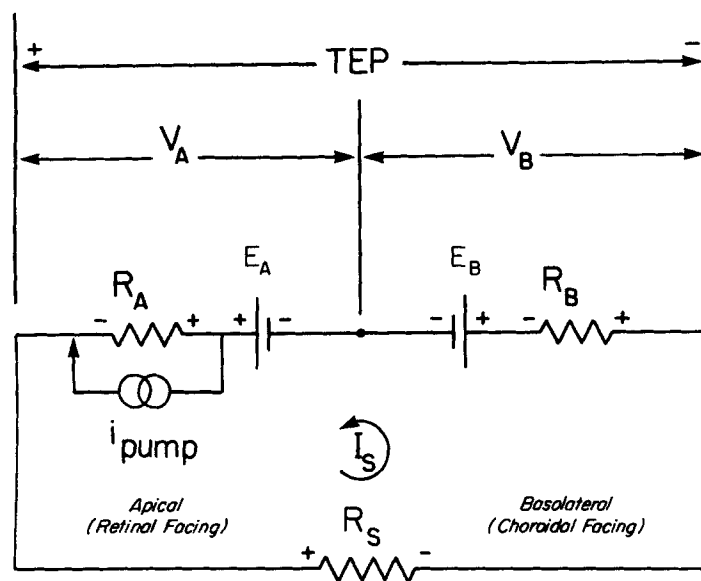


FIGURE 1. Equivalent circuit of the bovine RPE. R_A and R_B represent the resistances of the apical and basolateral membranes, respectively. R_S represents the parallel combination of the intercellular junctional complex resistance and the resistance of the damaged cells that form the mechanical seal at the circumference of the tissue. E_A and E_B represent the apical and basolateral membranes EMFs. Due to the difference between E_A and E_B a current (I_s) flows around this circuit that hyperpolarizes the basolateral membrane voltage (V_B) and depolarizes the apical membrane voltage (V_A) to the values measured by intracellular microelectrodes. The electrogenic Na/K pump at the apical membrane is modeled as a constant current source, i_{pump} . The TEP is the difference between V_A and V_B .

when both sides of the tissue are bathed with solutions of identical composition. The observed membrane potentials, V_A and V_B , are related to the EMFs as follows:

$$V_A = E_A - I_s R_A \quad (1)$$

$$V_B = E_B + I_s R_B \quad (2)$$

where I_s is the current that flows around the circuit driven by $E_A - E_B$. The effect of this current is to depolarize the apical membrane and hyperpolarize the basolateral membrane (Miller and Steinberg, 1977). Since $I_s = \text{TEP}/R_S$ (Fig. 1), E_A and E_B can be calculated from Eqs. 1 and 2 once the cell membrane and shunt resistances are specified (see Results, Fig. 3).

Since the apical and basolateral membranes are electrically coupled through R_S , an EMF or resistance change at either membrane affects both V_A and V_B . For a solution composition change that mainly alters E_A or R_A , the ratio of the measured membrane potential changes is

given by:

$$\Delta V_B/\Delta V_A = R_B/(R_B + R_S) < 1 \quad (3)$$

where most of the change in V_B (ΔV_B) is a passive consequence of current shunted from the apical membrane. Conversely, for a solution composition change that mainly alters E_B or R_B , this ratio is given by:

$$\Delta V_B/\Delta V_A = (R_A + R_S)/R_A > 1 \quad (4)$$

Since, using absolute values, $TEP = V_A - V_B$, it also follows that:

$$\Delta TEP = \Delta V_A[1 - (\Delta V_B/\Delta V_A)] \quad (5)$$

These equations can be used in analyzing the present data, especially Figs. 2 and 13. In Fig. 2, C–E, the basolateral membrane is initially depolarizing at a faster rate than the apical membrane, $\Delta V_B/\Delta V_A$ is greater than one, and the TEP is increasing (Eq. 5). At the dashed line both membranes continued to depolarize and the TEP reversed direction. As seen in Eq. 5, this reversal could only have occurred if the ratio $\Delta V_B/\Delta V_A$ also changed from greater than one to less than one. This reversal in ΔTEP is an easily detectable and convenient signal that the apical membrane is generating a voltage depolarization.

Isotopic Flux Measurements

The chamber design and the techniques used for the measurements of unidirectional mannitol fluxes across the bovine RPE were identical to those previously described (Miller and Edelman, 1990). In each experiment 10 μCi of [^3H]mannitol was added to the “hot” side and 100- μl samples were removed from the “cold” side and replaced with cold Ringer at 10-min intervals. The control Ringer contained 1 mM cold mannitol. Calomel electrodes, connected to the bathing solution via Ringer-agar bridges, were used to monitor TEP. Ag-AgCl₂ electrodes were used to pass current for measuring R_i .

Statistical Analysis

Unless stated otherwise, all values are reported as the mean \pm SEM, and statistical significance is determined at a P value less than 0.05.

RESULTS

Recent evidence suggests that the mammalian retina releases nanomolar amounts of EP after light onset (Hadjiconstantinou et al., 1983, 1984; Cohen and Hajiconstantinou, 1984). Like other, more abundant catecholamines released by the retina, EP may act as a paracrine signal that moves from the neural retina to its receptor on the apical surface of the RPE (Sato et al., 1987; Dawis and Niemeyer, 1988; Dearth and Burnside, 1989; Gallemore and Steinberg, 1990). The present experiments provide evidence for an EP-modulated signal transduction pathway that helps regulate K^+ and Cl^- transport at the RPE apical and basolateral membranes.

EP-induced Changes in Membrane Voltage and Resistance

Fig. 2, A–E, shows the electrical responses of a single tissue to a series of EP concentration changes in the apical bath, ranging over four orders of magnitude, from 1 nM to 10 μM . In each panel the top portion shows the EP-induced changes in TEP (solid line) and R_i (open squares). The lower portion of each panel shows the

concomitant changes in apical and basolateral membrane voltage (V_A , V_B) and the change in the ratio of apical to basolateral membrane resistance (open squares, a). In Fig. 2 B, for example, 10 nM apical EP depolarized both membranes and increased the TEP. Therefore the rate of depolarization, at every point in time was greater at

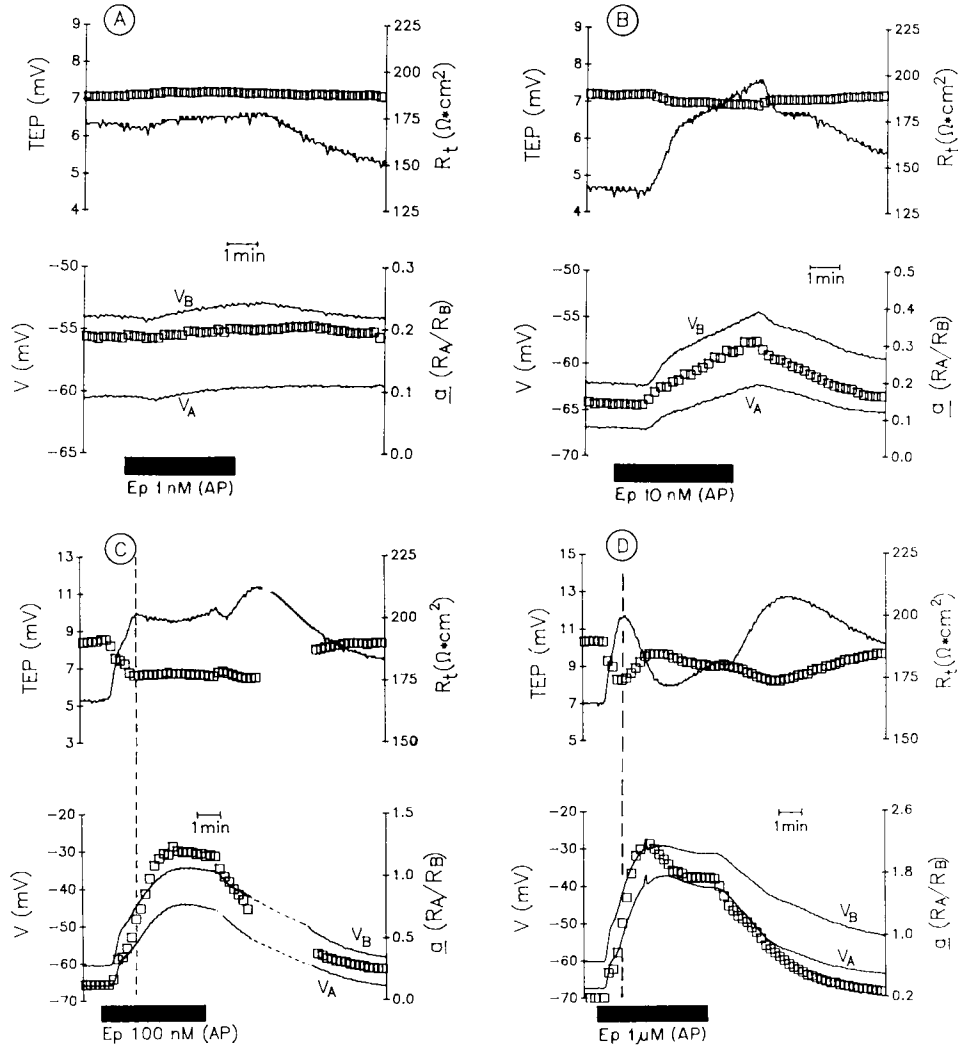


FIGURE 2

the basolateral than at the apical membrane. In addition, there was a small decrease in R_t and a larger increase in a (~ 0.2). Assuming that R_s was not appreciably altered (see below), this result implies that apical EP decreased R_B (Miller and Steinberg, 1977).

In 12 cells from 6 eyes, a increased 0.34 ± 0.10 , R_i decreased $15 \pm 5 \Omega \cdot \text{cm}^2$, V_B depolarized $11.3 \pm 1.3 \text{ mV}$, and TEP increased $3.8 \pm 0.8 \text{ mV}$ (mean \pm SEM) after the addition of 10 nM apical EP. These results indicate that apical EP increased a basolateral membrane conductance whose equilibrium potential is more positive than the resting membrane potential, V_B .

Fig. 2 also shows that the magnitude and time course of the electrical responses change with concentration. At concentrations higher than 10 nM the effect of EP on an apical transport mechanism could also be observed. In Fig. 2, C–E, the dashed lines indicate when the EP-induced TEP change ceased to increase and began to decrease. Since both membrane potentials continued to depolarize, this shows that

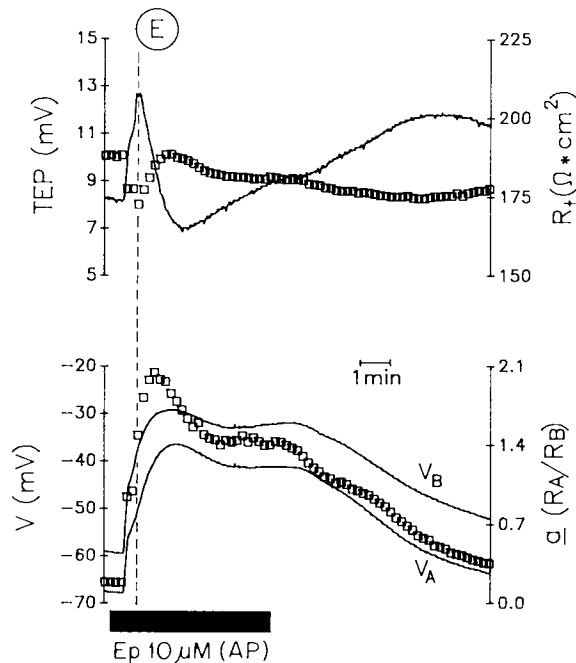


FIGURE 2. Voltage and resistance changes of the bovine RPE in response to varying concentrations of EP over the range 1.0 nM–10 μM . Upper trace, TEP (continuous line) and R_i (\square). Lower trace, V_A , V_B (continuous lines), and a (\square). The horizontal black bar indicates when EP was perfused into the apical chamber. The voltage response was saturated above 1.0 μM (compare D and E). In A and B the voltage response was initially generated by a basolateral membrane mechanism (see text). In C–E, after the first minute, the apical membrane component of the response became evident as indicated by the vertical dotted lines. At that time TEP began decreasing but V_A and V_B continued to depolarize. R_i first decreased and then increased during the apical phase of the response; in contrast, a increased throughout the response (see text). All responses were obtained from a single tissue.

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an extra depolarization was being generated at the apical membrane (Eqs. 3–5). During this period of time R_i either remained constant (Fig. 2 C) or increased (Fig. 2, D and E) and the a values continued to increase. This secondary response to 100 nM EP at the apical membrane was observed in 21 of 33 cells ($n = 12$ eyes). The average voltage and resistance ratio changes of the secondary response, relative to the peak of the TEP response, are as follows: V_A depolarized by $9.2 \pm 1.5 \text{ mV}$, TEP decreased by $1.1 \pm 0.2 \text{ mV}$, a increased by 0.42 ± 0.09 , and R_i increased by $2 \pm 1 \Omega \cdot \text{cm}^2$. These results indicate that EP decreased an apical membrane conductance whose equilibrium potential is more negative than V_A .

Does EP alter R_s ? The effect of EP on R_s was examined by measuring [^3H]mannitol unidirectional fluxes in the absence (control) and presence of 100 nM EP. Mean steady-state unidirectional mannitol fluxes (J^{man}) were measured in the apical to basal ($[J^{\text{man}}]_{\text{AB}}$) and basal to apical ($[J^{\text{man}}]_{\text{BA}}$) directions. That [^3H]mannitol is a hydrophilic molecule and probably restricted to the paracellular path has been shown most clearly by Dawson (1977) in turtle colon where the paracellular component (R_s) of the tissue conductance is highly correlated with unidirectional mannitol flux.

In RPE the shunt conductance is 80–90% of total tissue conductance (Joseph and Miller, 1991) and therefore the EP-induced change in R_t should provide a reasonably accurate estimate of the change in R_s , if, in fact, R_s was altered by EP. Although electrically well-matched tissues did not exhibit statistically different unidirectional fluxes, regardless of direction (Table I), the fluxes did vary as a function of R_t . In 22 tissues J^{man} increased by 7.6 nmol/cm²·h, from 5.1 to 12.7 nmol/cm²·h as R_t ranged from 195 to 100 Ω ·cm². In these tissues mannitol flux and total tissue conductance (G_t) were highly correlated ($R \approx 0.81$) and therefore an EP-induced decrease in R_s of

TABLE I
Effect of EP on Transepithelial Mannitol Flux

	Control	+EP	n
$[J^{\text{man}}]_{\text{AB}}$ (nmol/cm ² · h)	8.2 ± 0.8	7.9 ± 0.9	6
$[J^{\text{man}}]_{\text{BA}}$ (nmol/cm ² · h)	8.7 ± 1.2	9.0 ± 1.4	5

Summary data for A to B (apical to basal or retina to choroid) and B to A (basal to apical or choroid to retina) mannitol fluxes before (control) and after the addition of 100 nM EP (+EP). Each entry shows the mean ± SEM of n steady-state flux measurements (J^{man}). The differences are not significant ($P > 0.2$). In control Ringer the mean values of R_t and TEP in the A to B and B to A directions are: 137 ± 9.7 and 130 ± 10 Ω · cm² and 8.0 ± 1.3 and 7.1 ± 1.5 mV, respectively.

10–20 Ω ·cm², produced by 100 nM EP, should have increased J^{man} by ~1.0–2.0 nmol/cm²·h. Table I shows, however, that in electrically well-matched tissues, EP had no significant effect on either of the unidirectional mannitol fluxes (see legend). The mean EP-induced change in J^{man} , for all 11 tissues, taken together, is: $\Delta = -0.05 \pm 0.20$ nmol/cm²·h, which is not distinguishable from zero ($P = 0.79$). This result provides strong support for the assumption that EP does not appreciably alter R_s .

The time course of membrane resistance and EMF changes at the apical and basolateral membranes were calculated (Fig. 3) by using Eqs. 1 and 2 and the observed changes in a and R_t that are shown in Fig. 2 C. R_s was calculated from a previously obtained relationship, $R_t \approx 0.85 R_s$ (Joseph and Miller, 1991), and the measured value of R_t before the addition of EP (Fig. 2). In Fig. 3, the upper panel shows that the EP-induced changes in a and R_t are accompanied by a rapid decrease in R_B (open symbols) and a slower, secondary increase in R_A . The lower panel shows that these changes are also accompanied by a relatively rapid and large change in E_B (closed symbols) followed by a smaller, delayed change in E_A . Similar time courses (not shown) are obtained even if one assumes that R_s changed by ±20 Ω ·cm² or if one uses the 10-nM data in Fig. 2 B.

Apical membrane K conductance. Since the apical membrane of the bovine RPE is dominated by a large relative K conductance (~ 0.9 ; Joseph and Miller, 1991), the results summarized in Fig. 2, C–E, and the calculations in Fig. 3 suggest that the secondary depolarization of the EP response, delineated by the dashed lines, was generated in part by a decrease in apical membrane K conductance.

This notion was tested by comparing the magnitude of a K diffusion potential across the apical membrane in the absence and presence of 100 nM EP. The experiment summarized in Fig. 4A (control) shows that the apical membrane depolarized by ~ 30 mV after an increase in apical $[K]_o$ from 5 to 20 mM and that the response was reversible. In Fig. 4B (same cell) 100 nM EP was added and the membrane potentials were allowed to approach a new steady state. After V_A and V_B leveled off, apical $[K]_o$ was increased from 5 to 20 mM, and V_A depolarized by ~ 10 mV. The following control in Fig. 4C shows that the effect of EP was reversible. In 15 cells (5 eyes) EP inhibited the $[K^+]_o$ -induced diffusion potential by $\sim 44\%$ (Table II).

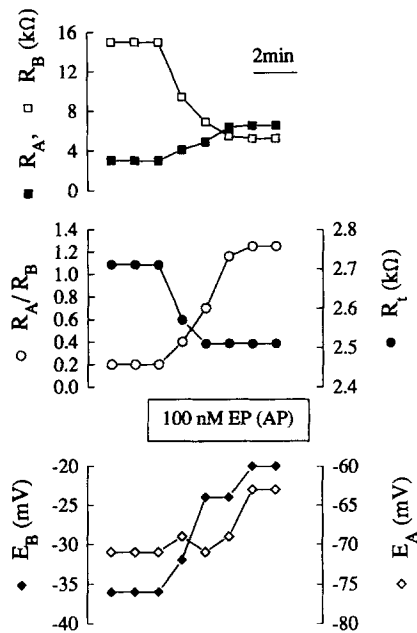


FIGURE 3. Effect of 100 nM EP on the apical and basolateral membrane resistances and EMFs. R_A , R_B , E_A , and E_B were calculated from the data of Fig. 2C (replotted in the middle panel), by assuming R_s is constant (see text), and by using equations 1 and 2 and the resistance ratios, $a = R_A/R_B$ and $R_t = R_s(R_A + R_B)/(R_A + R_B + R_s)$. Upper panel shows the time course of the calculated changes in R_A (closed symbols) and R_B (open symbols) after the addition of EP. The bottom panel shows the time course of the calculated changes in E_A (open symbols) and E_B (closed symbols).

Basolateral membrane Cl conductance. In a previous electrophysiological analysis of the bovine RPE it was demonstrated that the EMF of the basolateral membrane is ~ -24 mV and that most of this EMF is determined by a DIDS-inhibitable, Cl-conductive mechanism, probably a Cl channel (Joseph and Miller, 1991). This suggests that the initial EP-induced voltage and resistance changes shown in Figs. 2 and 3 were generated by an increase in basolateral membrane Cl conductance.

Several different tests of this hypothesis were carried out, beginning with the measurement of Cl diffusion potentials at the basolateral membrane. In the absence and presence of apical EP, a step change in basolateral $[Cl]_o$, between 131 and 13 mM, depolarized V_B by 8.0 ± 0.6 and 11.0 ± 0.8 mV, respectively ($n = 8$ cells, 4

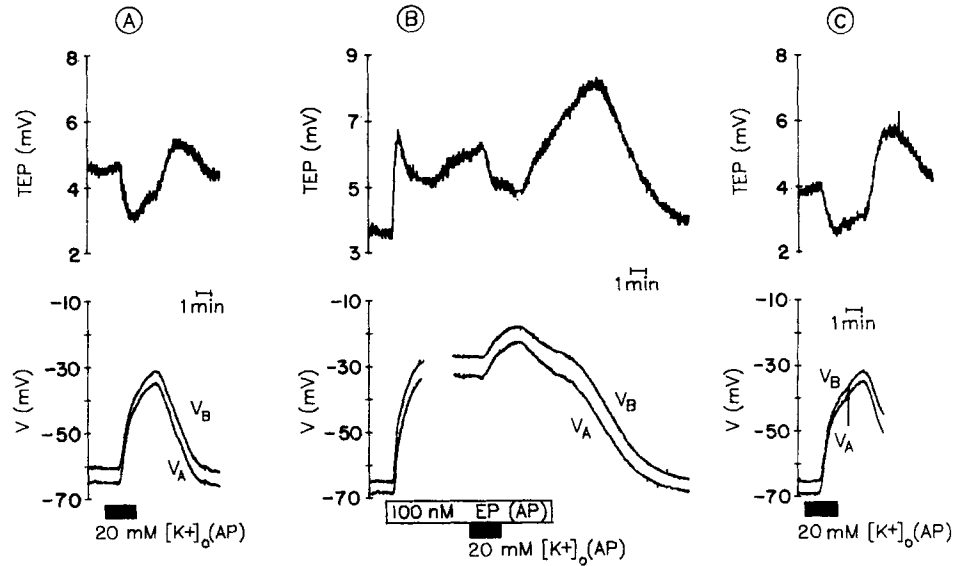


FIGURE 4. Effect of EP on the apical membrane response to a step increase in apical $[K^+]_o$ from 5 to 20 mM (black bars). (A) Control response. (B) Increase of apical $[K^+]_o$ in the presence of apical EP (horizontal bar). (C) Following control. EP decreased the $[K^+]_o$ -induced response of V_A by $\sim 65\%$.

eyes). This difference is significant ($P < 0.05$, paired t test) and suggests that apical EP increased the relative basolateral membrane Cl conductance. However, EP could have altered a conductive mechanism other than the Cl channel. This possibility was tested by measuring the effect of basal DIDS on the EP-induced basolateral membrane depolarization.

Fig. 5A shows the membrane voltage and TEP response to 100 nM apical EP. After

TABLE II
Effect of 100 nM EP On Apical K^+ -induced Voltage Changes

$\Delta[K^+]_o$	Phase	ΔV_A	ΔTEP	Isolated ΔV_b	N(n)
		mV	mV	mV	
5.0 to 2.0 mM control	1	-17 ± 0.5	0.8 ± 0.1		5 (2)
	2	-2.0 ± 0.5	-0.6 ± 0.2	-0.8 ± 0.1	
5.0 to 2.0 mM (+100 nM EP)	1	$-10 \pm 2.2^*$	$2.0 \pm 0.5^*$		4 (2)
	2	$-13 \pm 4.2^*$	$-3.8 \pm 1.0^*$	$-6.9 \pm 2.0^*$	
5.0 to 20 mM control		30 ± 1.2	-1.0 ± 0.3		15 (5)
5.0 to 20 mM (+100 nM EP)		$17 \pm 2.2^*$	$-2.5 \pm 0.3^*$		12 (5)

All values are given as mean \pm SEM. The response to decreasing $[K^+]_o$ in the apical bath from 5.0 to 2.0 mM has two phases (see text). N(n) is the number of cells (eyes) examined. * $P < 0.05$.

the tissue was returned to control Ringer, the electrical parameters reached steady state and then 5 mM DIDS was added to the basal bathing solution (not shown). Over the next 10 min V_B hyperpolarized by ~ 8.0 mV, the TEP decreased by 1.5 mV, a decreased by 0.18, and R_t decreased by $\sim 10 \Omega \cdot \text{cm}^2$. Fig. 5 B shows that the EP-induced voltage response was almost completely abolished in the presence of 5 mM basal DIDS. In two other tissues (two eyes) pretreatment with 5 mM basal DIDS caused an 80 and 93% inhibition of the EP response.

Since it has been shown that basal DIDS blocks net ^{36}Cl efflux across the basolateral membrane and $\Delta[\text{Cl}]_o$ -induced basolateral membrane diffusion potentials (Miller and Edelman, 1990; Joseph and Miller, 1991), it seems likely that EP is having its effect on a Cl-conductive mechanism at the basolateral membrane.

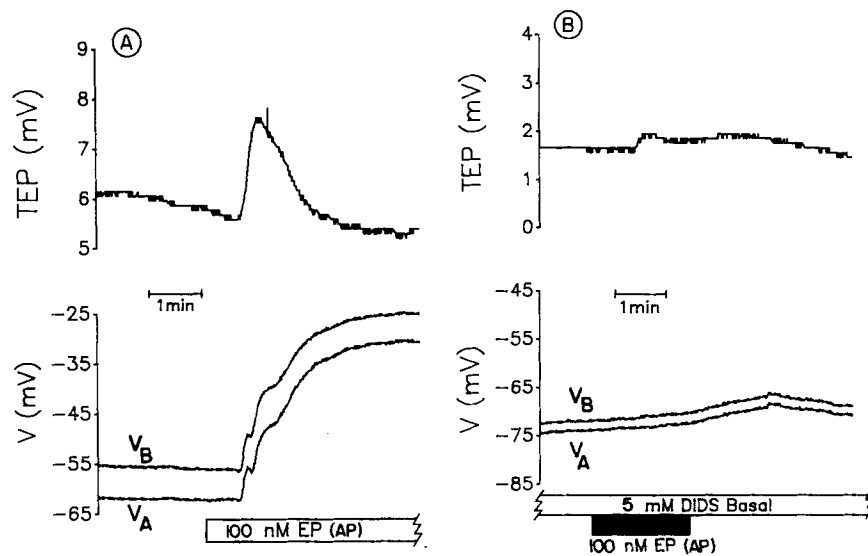


FIGURE 5. Effect of 5 mM basal DIDS on the EP response. (A) Control EP response. (B) Pretreatment of the tissue with basal DIDS (open bar) almost completely inhibited the EP response.

If the main effect of EP is to increase basolateral membrane Cl conductance, then the voltage response at that membrane should be significantly reduced as V_B is depolarized toward the Cl equilibrium potential ($E_{\text{Cl}} \approx -13.5$ mV; Joseph and Miller, 1991). To test this notion we current clamped the RPE and depolarized the basolateral membrane potential toward or beyond E_{Cl} .

Fig. 6 A shows the control response to 100 nM EP. The TEP and basolateral membrane voltage changes are shown in the upper and lower traces, respectively. In Fig. 6 B a constant current ($-22 \mu\text{A}$) was used to depolarize the basolateral membrane from an initial value of -65 mV to a final value of ~ -25 mV. 1 min after the tissue was current clamped, 100 nM EP was perfused into the apical chamber and the EP-induced voltage response was recorded. It was almost completely abolished. In Fig. 6 C the basolateral membrane was depolarized even further, to ~ -10 mV,

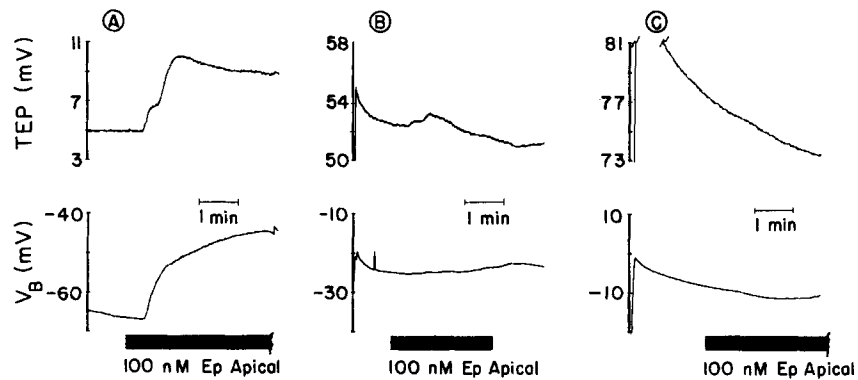


FIGURE 6. Effect of basolateral membrane voltage depolarization on the EP response (filled bars). The upper trace shows TEP and the lower trace V_B . (A) Control response. (B) Inhibition of the EP response by current clamping ($\sim -22 \mu\text{A}$) V_B to a value near the basolateral EMF ($\sim -24 \text{ mV}$). (C) Complete inhibition of the EP response by depolarizing V_B to the Cl equilibrium potential (-13 mV).

and again the response was abolished. Larger currents that exceeded the Cl reversal potential also inhibited but did not reverse the EP response.

Fig. 7 illustrates the effect of the current clamp on the EP-induced changes in a and R_t . The control responses in the upper and lower traces of Fig. 7A show that the

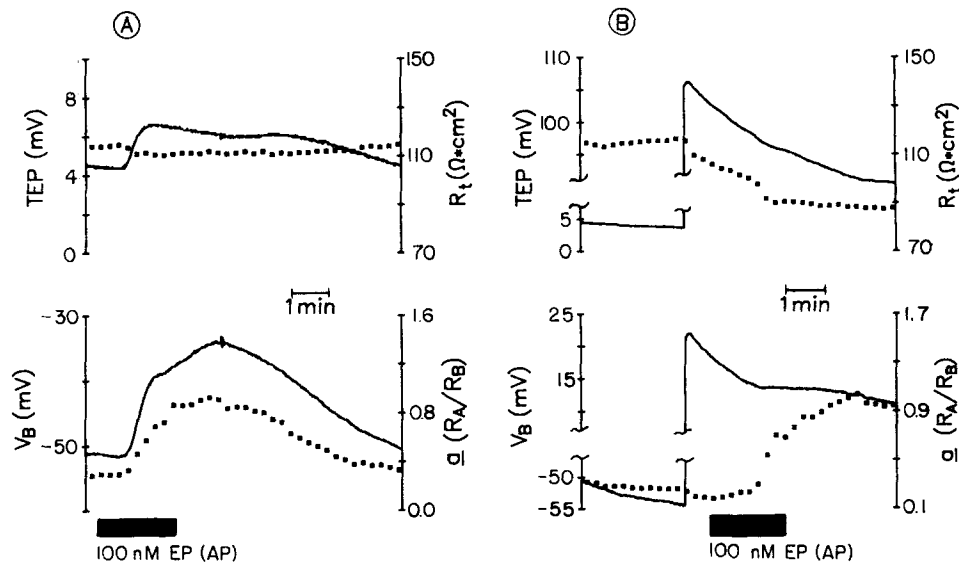


FIGURE 7. EP-induced conductance changes are not inhibited by depolarization of the basolateral membrane potential. Upper trace shows TEP (continuous line) and R_t (\square). Lower trace shows V_B (continuous line) and a (\square). (A) Control response. (B) At $\sim 3 \text{ min}$, constant current ($-62 \mu\text{A}$) was passed across the tissue to depolarize the basolateral membrane potential from -55 to $\sim +20 \text{ mV}$. R_t and a decreased $5 \Omega \cdot \text{cm}^2$ and 0.1 , respectively. When EP was added (filled bar) to the current-clamped tissue the resistance changes were almost identical in time course and magnitude to the control.

time course of the EP-induced voltage and resistance changes were practically identical. V_B was then current clamped to ~ 15 mV and EP was perfused into the apical bath (Fig. 7 B). The voltage response was almost completely abolished, but the a and R_i changes were practically identical to control. In seven cells from four eyes we altered V_B from its resting membrane level to values ranging between -25 and $+16$ mV and inhibited the EP-induced voltage response by $98 \pm 1.8\%$. In contrast, there was no significant difference in the EP-induced changes of a and R_i in the presence ($\Delta a = 0.28 \pm 0.09$; $\Delta R_i = 7 \pm 3 \Omega \cdot \text{cm}^2$) or absence ($\Delta a = 0.44 \pm 0.12$; $\Delta R_i = 12 \pm 3 \Omega \cdot \text{cm}^2$) of the current clamp. These data suggest that the EP-induced changes in conductance are not voltage dependent.

Apical membrane Na,K,2Cl cotransporter. The driving force for chloride exit across the basolateral membrane Cl channel can also be reduced by decreasing $[\text{Cl}]_i$. This

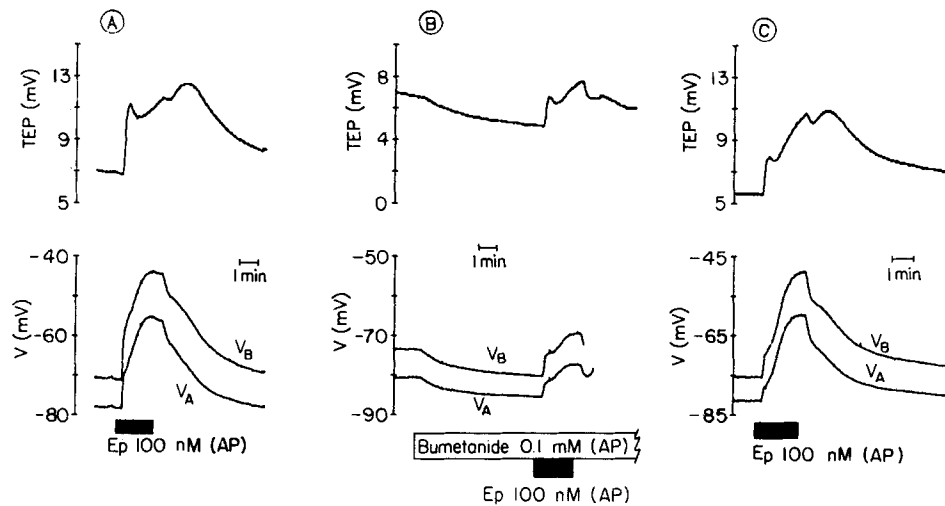


FIGURE 8. Effect of apical bumetanide on the voltage response to EP (filled bar). (A) Control response. (B) At $t = 1$ min, 0.1 mM bumetanide was added to the apical bath. After 5 min V_A and V_B hyperpolarized to a new steady state, and 100 nM EP was added to the apical bath. (C) Following control. Pretreatment with bumetanide reduced the EP response by 65%.

was accomplished in two ways: (a) by removing $[\text{Cl}]$ from the apical bath; and (b) by inhibiting the apical membrane Na,K,2Cl cotransporter with bumetanide (Miller and Edelman, 1990; Joseph and Miller, 1991). It has been shown that apical bumetanide decreased $[\text{Cl}]_i$ by 20–30 mM ($n = 5$) and hyperpolarized the basolateral membrane (5–10 mV); in addition, the resistance ratio a decreased by 0.16 ± 0.03 and R_i increased by $6.0 \pm 1.2 \Omega \cdot \text{cm}^2$ (Joseph and Miller, 1991; Bialek, S., D. Joseph, and S. Miller, unpublished observations). Therefore, lowering $[\text{Cl}]_i$ by any means should significantly block the EP-induced changes in basolateral membrane resistance and voltage.

Fig. 8 shows that the magnitude of the EP response was significantly reduced by apical bumetanide (0.1 mM). In the control response (Fig. 8 A), 100 nM EP depolarized V_B by 28 mV. When 0.1 mM bumetanide was added to the apical

perfusate (Fig. 8 *B*), a 7-mV hyperpolarization of the basolateral membrane potential was observed as expected for a decrease in $[Cl]_i$ expressed across the basolateral membrane Cl conductance. After the membrane potentials reached steady state, 100 nM EP was added to the apical bathing solution. In the presence of bumetanide the EP-induced depolarization of V_B was inhibited by 65%. The control response was restored after the washout of bumetanide (Fig. 8 *C*).

In four tissues (four eyes), pretreatment with apical bumetanide inhibited the EP-induced depolarization of V_B and the increase of TEP by 50 ± 6 and $54 \pm 8\%$, respectively. The EP-induced changes in a and R_i were also inhibited, by 72 ± 2 and $92 \pm 22\%$, respectively.

The dependence of the EP response on external $[Cl^-]$ was examined by decreasing the Cl^- concentration from 131 to 13 mM in the apical and basal bathing solutions. Decreasing $[Cl]$ in the basal bathing solution alone had a small inhibitory effect on

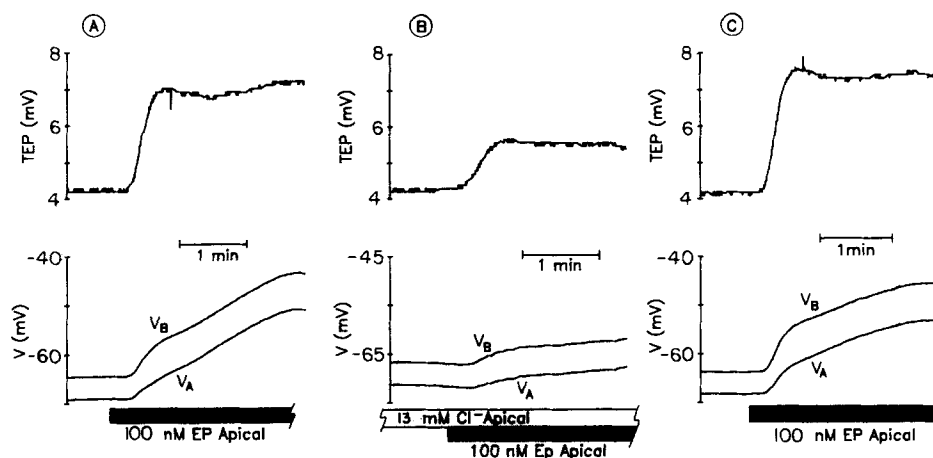


FIGURE 9. Effect of decreasing apical $[Cl]_o$ from 131 to 13 mM (open bar) on the EP response (filled bar). (A) Control response. (B) EP response after pretreatment with low $[Cl]_o$. (C) Following control. Low apical $[Cl]_o$ inhibited the EP response by 90%.

the EP response ($10 \pm 5\%$, $n = 3$). However, decreasing $[Cl]$ in the apical bathing solution (or both the apical and basal bathing solutions) inhibited the EP-induced change in V_B and a by 70 ± 12 and $61 \pm 17\%$, respectively ($n = 5$ cells, 3 eyes).

The results of an apical $[Cl]$ removal experiment are summarized in Fig. 9. *A* shows a control voltage response to the addition of 100 nM EP. In the time interval between the recordings shown in *A* and *B* low $[Cl]$ Ringer was perfused into the apical bathing solution. Several minutes were allowed for the membrane potentials to reach steady state. Fig. 9 *B* shows that the EP response was significantly reduced in apical low Cl (equimolar replacement with $MeSO_4$; see Methods). The following control, in Fig. 9 *C*, showed that the EP response was almost completely recovered once chloride was returned to the apical bath.

Taken together, these results provide strong evidence that the EP-induced depo-

larization at the basolateral membrane requires a functioning Cl absorption pathway and is due to an increase in Cl conductance.

Pharmacology of the EP Response

Since dopamine is the predominant catecholamine in the vertebrate retina (Ehinger, 1983) and since NEP may also occur in the bovine retina (Osborne and Ghazi, 1988), we next carried out a series of dose-response experiments using DA, NEP, and EP. The relative potency of each agonist was operationally defined by the peak change in basolateral membrane depolarization (ΔV_B).

Fig. 10 shows the dose-response curves for DA, EP, and NEP obtained by plotting the magnitude of ΔV_B as a function of the log [catecholamine]. Each point on the

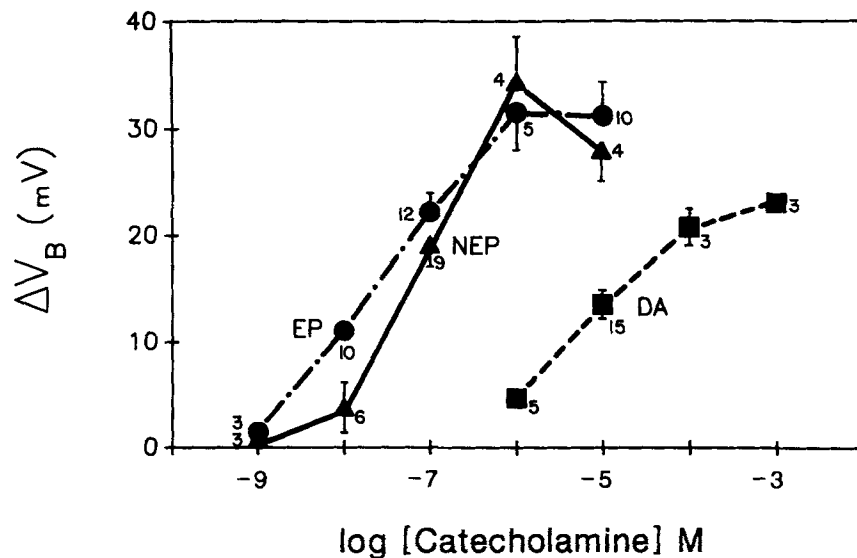


FIGURE 10. Catecholamine concentration, EP (●), NEP (▲), and DA (■), as a function of basolateral membrane voltage. Each data point represents an average obtained from the indicated number of experiments. The SEM for each data point is given by the error bars. The SEM of the points without error bars are less than half the height of the symbols.

graph represents an average value of ΔV_B obtained from several different experiments. The graph shows that the RPE responds to nanomolar concentrations of EP (circles) and NEP (triangles) and micromolar concentrations of DA (squares). The error bars represent the SEM and are not shown when they are smaller than the height of the symbol. From these results it is clear that the receptors that mediate the electrical response to catecholamines have a much higher affinity for the adrenergic agonists EP and NEP, compared with DA.

The effects of relatively specific adrenergic agonists, isoproterenol, clonidine, and phenylephrine, were examined to help determine whether the adrenergic response was generated by beta- or alpha-adrenergic receptors. The alpha-adrenergic agonists, clonidine and phenylephrine, were both effective at nanomolar concentrations, with

phenylephrine being ~ 10 -fold more potent than clonidine. Electrical responses to 10 nM phenylephrine were observed in two of four tissues tested. The average basolateral membrane depolarization was 1.8 ± 1.4 mV. Electrical responses to 100 nM phenylephrine were observed in all tissues tested ($\Delta V_B = 9.3 \pm 2.1$ mV; $n = 8$). The average ΔV_B in 100 nM clonidine was 2.0 mV ($n = 2$). No effect of clonidine was observed at lower concentrations. The average depolarization of V_B in response to 10 μ M isoproterenol was 5.7 ± 4.1 mV ($n = 3$ eyes). Isoproterenol, a beta-adrenergic agonist, was only effective at concentrations greater than 5 μ M. The order of effectiveness of the adrenergic agonists is as follows: EP \approx NEP $>$ phenylephrine $>$ clonidine \gg isoproterenol. This sequence suggests that the adrenergic response is mediated by alpha-1-adrenergic receptors (Minneman, 1988).

To further define the receptor type that mediates the adrenergic electrical response, we measured the effects of the alpha-1- and alpha-2-adrenergic receptor antagonists, prazosin and yohimbine, respectively. Fig. 11 shows the effect of the alpha-1 antagonist prazosin on the electrical response to 10 nM EP. The control

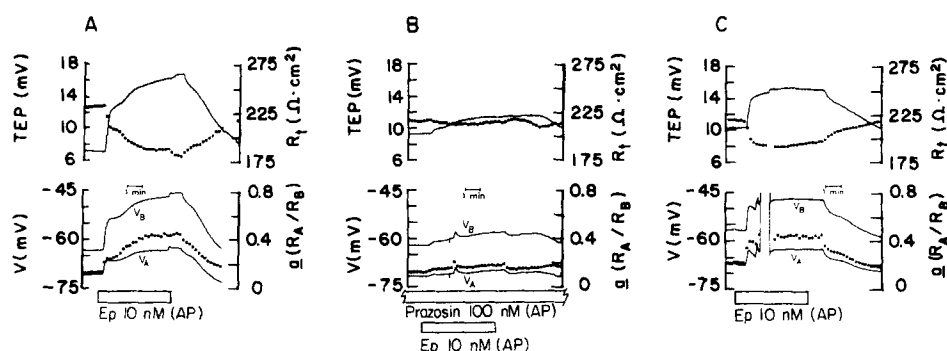


FIGURE 11. Effect of prazosin on the EP response. (A) Control response. (B) EP response after pretreatment of tissue with 100 nM apical prazosin. (C) Following control. The EP response was almost completely inhibited by prazosin.

response to 10 nM EP is shown in Fig. 11 A. When the tissue was pretreated with 100 nM prazosin the response to 10 nM EP was almost completely abolished (Fig. 11 B). Fig. 11 C shows that the EP response was restored after prazosin was removed from the apical bathing solution. These results suggest that prazosin effectively inhibits the entire EP response. This conclusion was corroborated in a series of similar experiments, using seven cells from seven eyes. In the presence of prazosin the average depolarization of V_B and increase of TEP were 26 ± 4 and $28 \pm 16\%$ of control. The average increase of a and decrease of R_t were 13 ± 5 and $40 \pm 15\%$ of control, respectively. In two tissues equimolar concentration of prazosin (100 nM) inhibited the EP response $> 50\%$ (not shown).

Paired t tests were performed on the electrical response during both components of the EP response. Prazosin inhibited both components of the EP response ($P < 0.05$); however, the inhibition of the a value increase during the secondary response may not be significant ($P < 0.1$).

Similar experiments were repeated in seven cells (four eyes). In the presence of yohimbine, the depolarization of V_B averaged $59 \pm 12\%$ of control and the TEP increase was $97 \pm 25\%$ of control. The a increase and R_i decrease were 48 ± 19 and $103 \pm 58\%$ of control, respectively. The first and second phases of the EP response were also analyzed separately using paired t tests; yohimbine significantly ($P < 0.05$) inhibited the membrane voltage but not the conductance changes during the initial phase of the response. Yohimbine had no effect on any component of the secondary response to EP.

We have not ruled out the possibility that DA receptors are present in the bovine RPE. However, there are data to indicate that the electrical responses to DA are mediated, at least in part, by alpha-adrenergic receptors. First, the addition of relatively selective D1 and D2 agonists SKF-383934 and LY-17555 at concentrations between 10 μM and 1 mM did not elicit significant electrical responses ($n = 4$). Second, 0.1 μM prazosin almost completely abolished the electrical response to 10 μM DA ($n = 4$). Third, 1.0 μM sulpiride, a DA antagonist, did not significantly inhibit the electrical response to 100 nM EP ($n = 2$).

These results indicate that the RPE electrical responses produced by EP are mainly mediated by alpha-1 receptors located at the RPE apical membrane.

Intracellular Messengers

The signal transduction pathway that is activated by the binding of EP to alpha-1-adrenergic receptors is thought to include an elevation of cell calcium (Exton, 1981; Bylund and U'Prichard, 1983; Minneman, 1988). It has also become clear that $[\text{Ca}^{2+}]_i$ can regulate the enzymes responsible for the synthesis and degradation of intracellular cAMP and vice versa (Rassmusen and Barrett, 1984; Cotecchia et al., 1990). These observations suggest that both cAMP and Ca^{2+} can serve as intracellular messengers in the generation of the EP response.

Cyclic AMP. Evidence supporting the involvement of $[\text{cAMP}]_i$ was obtained in the course of a previous collaborative study with Dr. Debora Farber (Hughes et al., 1987). In bovine RPE she measured the EP- and IBMX-induced elevation of cAMP in cells that were scraped free of the choroid. The basal activity level of cAMP in these cells was 8.2 ± 1.9 pmol/mg protein (mean \pm SEM, $n = 5$ eyes). Treatment of these cells with 100 nM EP also significantly increased $[\text{cAMP}]_i$, by a factor of three, to 25.7 ± 4.0 pmol/mg protein. The phosphodiesterase inhibitor IBMX increased cAMP to 32.5 ± 5.0 pmol/mg protein.

In frog RPE it has been shown that dbcAMP or IBMX elevated cell cAMP (Hughes et al., 1987, 1988) and caused basolateral membrane voltage and resistance changes qualitatively similar to those produced in the present study by EP. Similar results have been obtained in the bovine RPE. Table III (rows 1 and 2) shows that apical addition of dbcAMP plus IBMX or IBMX alone significantly depolarized the basolateral membrane, by ~ 11 and 7 mV, respectively. The TEP and the ratio of apical to basolateral membrane resistance were also increased, but the decrease in R_i was not statistically significant. These results suggest that the elevation of cell cAMP could contribute to the EP-induced electrical responses.

Calcium. The evidence for Ca^{2+} as the second messenger after receptor activation by EP was also examined in several ways. In bovine RPE the ionophore

TABLE III
Effects Of Apical cAMP, IBMX, and Forskolin on Membrane Voltages and Resistances

	<i>N</i> (<i>n</i>)	ΔV_b	ΔTEP	Δa	ΔR_i
		<i>mV</i>	<i>mV</i>		<i>ohm · cm²</i>
cAMP (or dbcAMP)	7 (4)	$1.5 \pm 0.4^*$	0.4 ± 0.5	0.03 ± 0.02	0.5 ± 1.3
IBMX	4 (3)	$6.7 \pm 1.3^*$	$1.9 \pm 0.5^*$	$0.1 \pm 0.03^*$	-4.2 ± 2.1
dbcAMP + IBMX	3 (2)	$11 \pm 1.4^*$	$3.2 \pm 0.7^*$	$0.17 \pm 0.02^*$	-2.3 ± 2.8
Forskolin	3 (2)	2.3 ± 2.3	0.2 ± 0.6	0.06 ± 0.04	2.3 ± 1.4

Values are given as mean \pm SEM. *N* is the number of cells and *n* is the number of eyes. **P* < 0.05.

A23187 increases intracellular Ca^{2+} (Lin and Miller, 1991). When the ionophore (1 μ M) was added to the apical bathing solution, the basolateral membrane depolarized by 8.2 ± 2.6 mV and the TEP increased by 2.2 ± 0.7 mV. The ratio of apical to basolateral membrane resistance, *a*, also increased by 0.15 ± 0.09 , and *R_i* decreased by $5.0 \pm 3.0 \Omega \cdot cm^2$ (*n* = 4 cells, 4 eyes).

In another class of experiments we examined the effects of the cell-permeable Ca^{2+} buffer, aml-BAPTA, on the EP response. If BAPTA is able to enter the cell it should block the increase of intracellular Ca^{2+} that follows receptor activation by EP. Fig. 12 shows a tissue in which aml-BAPTA inhibited the EP response by $\sim 80\%$. In the control response (Fig. 12 A), 10 nM apical EP depolarized the basolateral membrane by ~ 15 mV and caused the TEP to increase by 5 mV. After the removal of EP the membrane voltages returned to their resting values. Before the record shown in Fig. 12 B, the tissue was bathed in 0.1 mM aml-BAPTA for a period of 25 min, and then washed in control Ringer for an additional 20 min. This concentration of BAPTA did

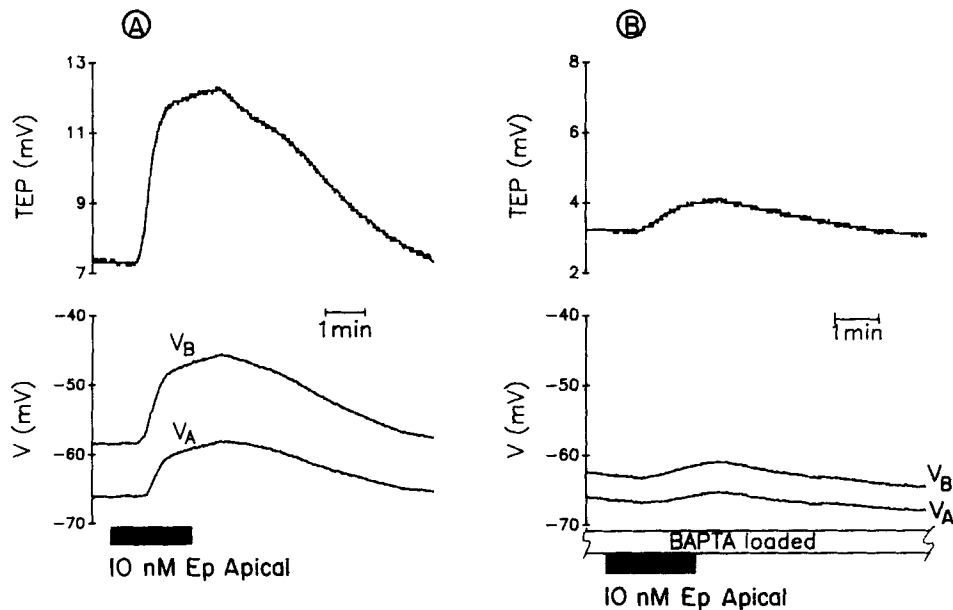


FIGURE 12. Effect of aml-BAPTA on the EP response. (A) Control response. (B) EP response after the cells were loaded with aml-BAPTA (open bar).

not alter the resting membrane voltages and resistances observed in control Ringer, indicating that it was not toxic to the cells. Lower concentrations of BAPTA were tried without apparent effect. Fig. 12 *B* shows that the response to 10 nM EP was almost completely abolished after the treatment with BAPTA. This result was corroborated in two other impalements from the same tissue.

The electrical responses to 10 nM EP in tissues pretreated with 100 μ M aml-BAPTA were as follows: V_B depolarized by 5.2 ± 0.6 mV; TEP increased by 1.6 ± 0.3 mV; a increased by 0.08 ± 0.02 ; and R_t decreased by $5 \pm 1 \Omega \cdot \text{cm}^2$ ($n = 7$ cells, 5 eyes). The control responses to EP were as follows: V_B depolarized by 11 ± 1.1 mV; TEP increased by 3.8 ± 0.8 mV; a increased by 0.34 ± 0.1 ; and R_t decreased $15 \pm 5.0 \Omega \cdot \text{cm}^2$. Therefore aml-BAPTA inhibited the EP-induced electrical voltage and resistance changes by 50–80%.

These results taken together indicate that the EP-induced changes in RPE membrane voltage and resistance could be mediated by at least two intracellular messengers, Ca^{2+} and cAMP. However, the BAPTA experiments imply that $[\text{Ca}^{2+}]_i$ is an important determinant of the EP response.

EP and the Low $[\text{K}]_o$ Response

In bovine and other RPE preparations it was previously demonstrated that a step decrease in apical $[\text{K}]_o$ from 5 to 2 mM had at least two effects: (a) a relatively rapid hyperpolarization of the apical membrane due to the increase in E_K ; and (b) a delayed basolateral membrane hyperpolarization (Griff and Steinberg, 1984; Joseph and Miller, 1991). The latter response is generated by a decrease in $[\text{Cl}]_i$ and the subsequent intracellular diffusion potential at the basolateral membrane Cl channel (Joseph and Miller, 1991). Similar RPE voltage changes are produced in the intact eye after light onset. They are caused by the changes in $[\text{K}]_o$ that occur in the extracellular space between the retinal photoreceptors and the RPE apical membrane (Oakley and Green, 1976; Oakley, 1977; Linsenmeier and Steinberg, 1983, 1984). Since EP is a possible retinal paracrine signal to the RPE, we examined its effect on both components of the low $[\text{K}]_o$ response.

Fig. 13 *A* shows the control response to a step decrease in $[\text{K}]_o$. During the first part of the response (labeled 1) the apical membrane hyperpolarized at a greater rate than the basolateral membrane and TEP increased. During the second phase of the response (labeled 2) the basolateral membrane hyperpolarized at a greater rate and therefore TEP decreased. In the intact eye these two TEP responses correspond, approximately, to the C-wave and the fast oscillation (FO) of the DC-recorded ERG (Steinberg et al., 1985).

In the upper trace of each panel we have calculated the isolated basolateral membrane voltage response (Linsenmeier and Steinberg, 1984). This is the extra hyperpolarization that originated at the basolateral membrane during phase 2. It was calculated by first estimating the fraction of the apical membrane hyperpolarization that was shunted to the basolateral membrane. This voltage was then subtracted from the observed basolateral membrane hyperpolarization in the bottom trace and plotted above as the isolated V_B (Joseph and Miller, 1991).

In Fig. 13, *B* shows that the presence of 100 nM EP significantly altered both phases of the low $[\text{K}]_o$ response. The apical hyperpolarization during phase 1 was reduced from 20 to 10 mV, suggesting that EP decreased apical membrane K

conductance. EP had an even larger effect on the basolateral membrane. The isolated basolateral membrane hyperpolarization was increased by more than a factor of 10, from ~ 0.8 to ~ 11 mV, suggesting that EP increased basolateral membrane Cl conductance. Table II summarizes a series of similar results from five cells (two eyes) which strengthen our conclusion that apical membrane receptor activation by EP leads to an increase in basolateral membrane Cl conductance and a decrease in apical membrane K conductance. The implications for retinal and RPE physiology are discussed below.

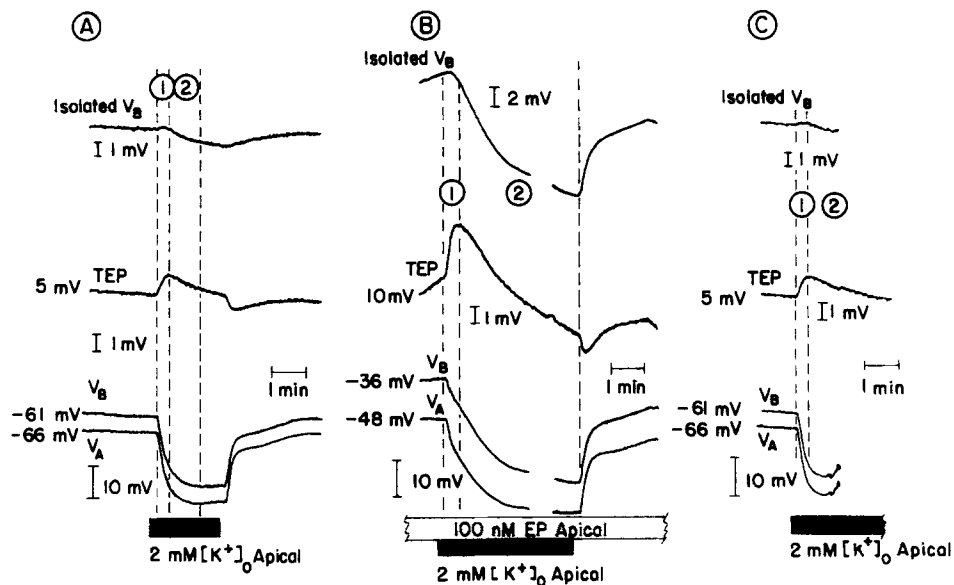


FIGURE 13. Effect of EP on reduction of $[K^+]_o$ in the apical bath from 5.0 to 2.0 mM (filled bar). In all three panels, the isolated basal membrane response is shown in the top trace. The TEP, V_b , and V_a are shown below. (A) Control response to apical $[K^+]_o$ reduction. The vertical lines indicate the two phases of the response (see text). (B) Apical $[K^+]_o$ reduction in a tissue pretreated with 100 nM EP. (C) Following control.

DISCUSSION

The transport properties of the bovine RPE are exquisitely sensitive to the presence of EP (NEP) in the apical bathing solution. These experiments show that the basolateral membrane voltage and resistance are altered by EP concentrations as low as 1 nM (Figs. 2A, 3, and 10). At somewhat higher concentrations (> 10 nM) the initial basolateral membrane changes were followed by voltage and resistance changes at the apical membrane (Figs. 2, C-E, 3, 4, 5A, and 13; Table II).

Modulation of Basolateral Membrane Cl Conductance

Several lines of evidence indicate that apical EP caused an increase in basolateral membrane Cl conductance. For example, $\Delta[Cl]_o$ -induced diffusion potentials, produced by solution composition changes in the basal bath, were significantly increased

(~63%) in the presence of apical EP. Conversely, decreasing the driving force for Cl efflux across the basolateral membrane, either by depolarizing V_B toward E_{Cl} or by decreasing $[Cl]_i$, inhibited the EP-induced changes in ΔV_B (Figs. 6–9). However, it was not possible to reverse the EP-induced voltage response (Fig. 7), perhaps because the Cl channel is outwardly rectifying (Tabcharani et al., 1989). Fig. 7 also shows that depolarizing V_B toward E_{Cl} did not alter the EP-induced changes in a and R_i .

In contrast, apical bumetanide or lowering Cl in the apical bath both inhibited the EP-induced changes in a and R_i by 50–70%. Since cell Cl is normally high (~60 mM) and significantly reduced by apical bumetanide or the reduction of apical Cl, these observations suggest that basolateral membrane Cl conductance is $[Cl]_i$ dependent (Fong et al., 1988; Joseph and Miller, 1991; Bialek, S., D. Joseph, and S. Miller, unpublished observations). This notion was corroborated in the course of the present experiments by the observation that the EP-induced voltage and resistance changes were inhibited by ~70% ($n = 2$) after a $\Delta[K]_o$ (5 to 2 mM)-induced reduction in $[Cl]_i$ (~30 mM; Joseph and Miller, 1991).

Apical Membrane K Conductance Changes

It has recently been demonstrated in cardiac cells that EP or NEP activate alpha-1 receptors and subsequently decrease K conductance (Fedida et al., 1989; Rosen et al., 1991). In these experiments, after the initial increase of basolateral g_{Cl} , V_B and V_A continued to depolarize while the TEP either leveled off or began to decrease. Concomitantly, a continued to increase and R_i usually increased. These electrical responses are consistent with a decrease in the apical membrane K conductance (Fig. 2, C–E, right of dotted lines). This conclusion was corroborated by the calculations shown in Fig. 3 and by making step changes in $[K^+]_o$ from 5 to 20 mM or from 5 to 2 mM in the presence and absence of EP (Figs. 4 and 13; Table II). In both cases, apical EP decreased the ΔK -induced diffusion potential by ~40%.

There is no evidence that the apical membrane response comes in only at 100 nM EP and only after the TEP starts to decrease. On the contrary, Fig. 3 shows that R_A starts to change during the initial basolateral membrane depolarization, but the voltage change is probably masked by the larger effect of decreasing R_B . A similar calculation for an electrical response to 10 nM EP (not shown) also indicates that small increases in R_A are occurring during the larger basolateral membrane response. This could be seen in some tissues where 10 nM EP induced a TEP response that initially increased and then leveled off while V_A and V_B continued to depolarize. A response where V_A and V_B depolarize at the same rate supports the notion that the EP response is generated by mechanisms located at both the apical and basolateral membranes.

Pharmacology and Intracellular Signaling

The receptors that respond to apical EP are 1,000-fold more selective for EP and NEP than DA (Fig. 10), where selectivity is assessed by the ability of a given concentration of agonist to depolarize V_B . The receptor type was characterized by measuring the relative potency of a variety of adrenergic agonists. These agonists had the following order of potency: EP \approx NEP > phenylephrine > clonidine \gg isoproterenol \approx DA. This selectivity sequence (Minneman, 1988) is consistent with

the presence of alpha-1-adrenergic receptors on the bovine RPE apical membrane, a conclusion that was strengthened by the use of relatively specific alpha-adrenergic receptor antagonists prazosin (alpha-1) and yohimbine (alpha-2). These experiments showed that prazosin was a more effective antagonist of the EP response than yohimbine (Fig. 11).

In other systems alpha-1 receptors mediate cellular responses by altering intracellular concentrations of calcium and cAMP (Exton, 1981; Bylund and U'Prichard, 1983; Cotecchia et al., 1990; Smith et al., 1990). In the bovine RPE, elevating intracellular Ca^{2+} with A23187 depolarized the basolateral membrane and increased the basolateral conductance. Pretreating these cells with the intracellular Ca^{2+} buffer aml-BAPTA inhibited the EP response (Fig. 12). Transient elevations of intracellular Ca^{2+} (200–400 nM) that can be inhibited by prazosin are observed after the application of apical EP (Lin and Miller, 1991). These results strongly support the notion that Ca^{2+} acts as a second messenger after the activation of apical membrane alpha-1 receptors.

The EP-induced membrane depolarizations were generated by separate conductance changes at the apical and basolateral membranes. These changes could have been generated by one or more second messengers or by the activation of one or more adrenergic receptors at the apical membrane (Welsh, 1986; Liedtke, 1989; Apkon and Nerbonne, 1988). If EP activated two separate receptors at the apical membrane, then one might expect to observe an enlarged EP-induced apical membrane depolarization in the presence of the alpha-1 antagonist prazosin. This was not the case. In two experiments 100 nM prazosin was added equimolar with EP and inhibited the basolateral membrane voltage response by >50%; in both cases the apical membrane response was completely abolished. In addition, the agonists DA, isoproterenol, or clonidine, which might be expected to activate dopaminergic, beta-adrenergic, or alpha-2 receptors, respectively, did not invoke an apical membrane depolarization at the lowest effective concentrations. At higher concentrations the basolateral membrane depolarization was followed by an apical membrane event. These results argue against, but do not eliminate, the possibility that EP activated multiple receptor types at the apical membrane.

It seems more likely that the EP-induced decrease in apical K conductance is produced after intracellular elevation of a second messenger, generated during the IP_3 or cAMP cascade, or by a tertiary signal associated with the increase in basolateral membrane Cl conductance. Support for the latter possibility comes from the observations that both the apical and the basolateral components of the EP response were blocked by: (a) Cl removal from the apical bath; (b) addition of bumetanide to the apical bath; and (c) addition of DIDS to the basal bath (Figs. 5, 8, and 9).

Physiological Implications

In the intact eye, the physiological interactions of the retina and RPE are coordinated by paracrine and hormonal signals that are generated continuously in the light or dark, or altered as result of light/dark transitions. Apical and basolateral membrane receptors convert these extracellular signals into a variety of intracellular or transepithelial responses. For example, RPE phagocytosis of photoreceptor outer segments takes place on a diurnal basis (Bok, 1985). Light onset also initiates the transfer of

visual pigment chromophore (retinal) from the photoreceptors to the RPE, a process that is reversed after the transition from dark to light (Saari, 1990; Rando et al., 1991). There is still relatively little understanding of the signals, receptors, or transport mechanisms that help mediate these functionally crucial activities.

Although DA is the dominant catecholamine in the vertebrate retina (Ehinger, 1983), it has been also reported that EP is localized to specific retinal neurons and released following light onset (Haggendal and Malmfors, 1965; Hadjiconstantinou et al., 1983, 1984; Cohen and Hadjiconstantinou, 1984; Dacey, 1988; Dearry and Burnside, 1989). These results implicate EP as a possible paracrine signal whose release from the neural retina after light onset could trigger a variety of RPE physiological responses.

Signaling light onset. An alpha-1 receptor is also located on the apical membrane of the human RPE (Quinn et al., 1991) and could underlie several RPE electrical responses that are used clinically in the differential diagnosis of retina/RPE diseases (Weleber, 1989). In the intact eye, three RPE voltage responses are produced after the absorption of light by the photoreceptors. These three responses, the C-wave, the fast oscillation (FO), and the light peak are relatively slow voltage changes produced at the apical or basolateral membranes (Steinberg et al., 1985).

The C-wave occurs ~5 s after light onset and is caused mainly by a decrease in subretinal K and a subsequent $[K]_o$ -induced hyperpolarization of the apical membrane (Steinberg et al., 1970; Oakley and Green, 1976). In vitro, this response corresponds approximately to phase 1 of the low K response (Fig. 13). The FO occurs ~20 s after light onset, and is a delayed basolateral membrane hyperpolarization (Griff and Steinberg, 1984; Linsenmeier and Steinberg, 1984) that is also produced in the isolated RPE preparation by reducing apical $[K]_o$ from 5 to 2 mM (Fig. 13, phase 2). The decrease in apical K leads to a subsequent decrease in cell Cl activity that is expressed across the basolateral membrane Cl conductance and causes the delayed basolateral membrane hyperpolarization during phase 2 (Joseph and Miller, 1991). The light peak occurs ~5 min after a step increase in light and is probably due to an increase in basolateral membrane Cl conductance (Gallemore and Steinberg, 1991). The paracrine signal(s), the RPE receptor(s), and the intracellular signal(s) that generate this later response are not known.

In the intact eye, the C-wave and FO both increase in amplitude during the light peak (Nilsson and Skoog, 1975; Linsenmeier and Steinberg, 1983; Gallemore and Steinberg, 1990). If EP is the paracrine signal that helps initiate the RPE light peak, then in vitro it should mimic light in at least two ways: (a) by increasing basolateral membrane Cl conductance; and (b) by increasing both phases of the TEP response to a step change in apical $[K]_o$ (5 to 2 mM). The present data show that both of these expectations are fulfilled. Fig. 13 (middle) shows that EP enlarged both phases of the TEP response. Phase 2 increased because of the increase in basolateral membrane Cl conductance, and this conductance increase, which is plotted in Fig. 3 (top) as a decrease in R_b , also caused an increase in the TEP response during phase 1 (Eqs. 3 and 5). Therefore EP satisfies some of the necessary conditions required of a light peak substance.

These results and the observation that EP produces a prazosin-inhibitable rise in $[Ca^{2+}]_i$ (Lin and Miller, 1991) suggest a straightforward model for the sequence of

events that produce the light peak in the intact eye. After light onset EP is slowly released from the retina (over several minutes) and diffuses to the RPE apical membrane to activate the alpha-1 receptors that are coupled through an appropriate G protein and the IP_3 pathway (Minneman, 1988) to an increase in $[Ca^{2+}]_i$ -activated Cl conductance and the observed basolateral membrane depolarization.

In support of this model are the observations using the arterially perfused cat eye, which show that elevated extracellular Ca^{2+} abolishes the light peak (Hofmann and Niemeyer, 1985). One interpretation of these experiments is that an elevation of

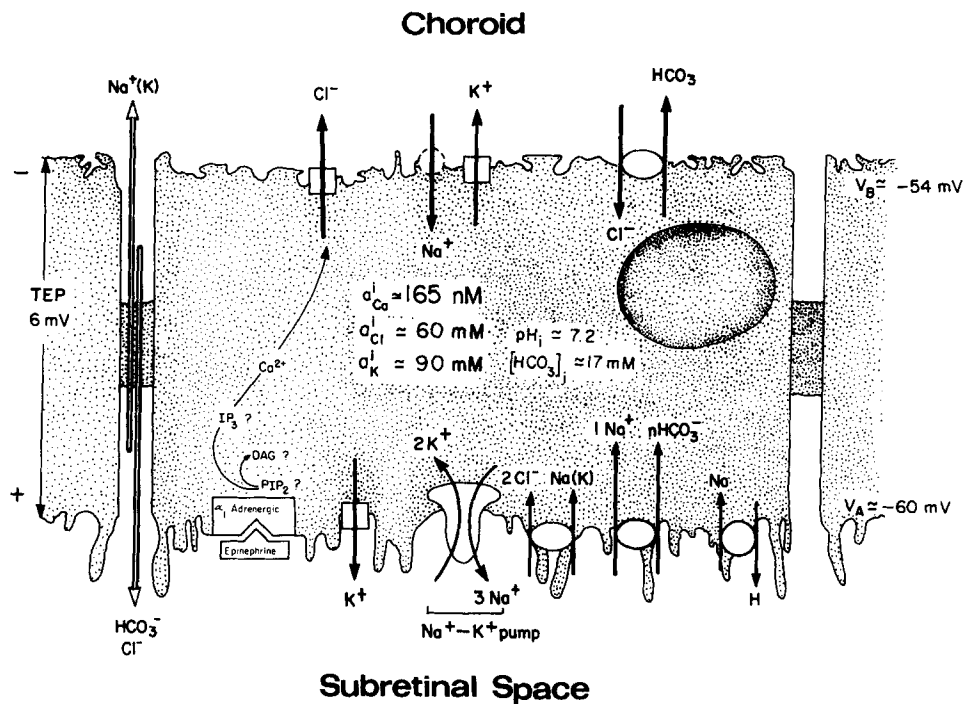


FIGURE 14. Model of RPE transport including the alpha-1 receptor and its putative signaling pathway (see text).

$[Ca^{2+}]_i$ increased basolateral membrane Cl^- conductance, as in bovine RPE, and therefore light had no further effect. In the chick retina-RPE-choroid preparation there is evidence to indicate a photoreceptor origin for the light peak substance (Gallemore et al., 1988). In any case, further more direct experiments will be required to determine if there is a light-induced release of EP from the retina that elevates RPE $[Ca^{2+}]_i$.

Fluid transport. The functional significance of the Cl^- transport pathway makes it an important focus of RPE/retina pathophysiology (Bird, 1989; Weleber, 1989; Miller and Edelman, 1990; Edelman and Miller, 1991; Joseph and Miller, 1991). It has been shown that nanomolar amounts of apical EP more than double the rate of fluid absorption (J_v) across the in vitro bovine RPE; this increase in J_v is completely

blocked by apical bumetanide or prazosin (Edelman and Miller, 1991). The present results, in combination with previous tracer flux studies, indicate that the EP-induced stimulation of J_v is mediated by activation of a Cl transport pathway, which consists of an apical membrane Na,K,2Cl cotransporter and basolateral membrane K and Cl efflux mechanisms; the latter are conductive, but other possibilities (KCl cotransport) are not excluded.

At the apical membrane of bovine RPE, K is taken up by two separate mechanisms, a bumetanide-sensitive cotransporter and an electrogenic Na/K pump (Joseph and Miller, 1991; Fig. 14). Most of this K is returned to the apical bath via the apical membrane K channel (relative conductance ≈ 0.9). Although net K absorption is normally zero, it can be significantly increased by blocking apical membrane K conductance with barium (Miller and Edelman, 1990; Joseph and Miller, 1991). Furthermore, this increased net K flux is partially blocked by apical bumetanide. These experiments show that EP mimics the effect of barium on apical membrane K conductance, but further tracer flux studies will be required to determine if EP increases the steady-state absorption of K and Cl.

Previous experiments showed that net Na transport is secretory in the short circuit and absorptive in the open circuit, and that net absorption increases with TEP (Hughes et al., 1984; Miller and Farber, 1984; Miller and Edelman, 1990), suggesting a paracellular pathway. Therefore, the EP-induced increase in TEP could contribute indirectly to an increase in net (Na + K)Cl and fluid transport. More important, the apical membrane alpha-1 receptor could play a significant role in determining the pathophysiology associated with disease-induced accumulation of fluid in the subretinal space (Bird, 1989).

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