Beta Adrenergic Receptors on Cultured Human Retinal Pigment Epithelium

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Cultured fetal human retinal pigment epithelium (RPE) was grown on a permeable substrate and sealed in an Ussing chamber. The average electrical resistance (R) was 330 ohm-cm², the average transepithelial voltage (Ve) was 3.0 mV (apical side positive), and the average short circuit current (Isc) was 9.1 μA/cm². When these RPE preparations were exposed to isoproterenol (a β-adrenergic agonist), the Isc increased by 88%, R was reduced by 6%, and Ve increased by 85%. The effect of isoproterenol was blocked by propranolol (a β-adrenergic antagonist). When cultured human RPE was exposed to isoproterenol, intracellular cyclic adenosine monophosphate (AMP) levels rose more than threefold. The effect of isoproterenol on cyclic AMP levels was blocked by propranolol. When the cultured RPE was exposed to dibutyryl cyclic AMP, both Ve and Isc rose by 47% with a time course similar to that which occurred when the cells were exposed to isoproterenol. Preparations treated with dibutyryl cyclic AMP did not respond to subsequently applied isoproterenol. These results indicate that cultured human RPE possesses a β-adrenergic receptor and that stimulation of this receptor produces a change in cyclic AMP concentration which affects RPE electrical activity. Invest Ophthalmol Vis Sci 31:1767-1772, 1990

The transport properties of the retinal pigment epithelium (RPE) are a crucial part of the mechanisms that maintain the extracellular environment necessary for normal photoreceptor function. The transepithelial transport properties of RPE from a number of species have been studied in vitro in several laboratories. In addition to determining the components of RPE transport, it is important also to understand how this transport is regulated. Adrenergic agents may regulate RPE transport. We grew fetal human RPE on a permeable substrate and sealed this tissue within a conventional Ussing chamber. These experiments show that cultured fetal human RPE possesses β-adrenergic receptors. Stimulation of these β adrenoceptors increases the intracellular cyclic adenosine monophosphate (AMP) concentration and alters RPE mediated trans-RPE electrical current and voltage.

Materials and Methods

Fetal human RPE cells were grown in culture using a modified protocol originally developed to grow postnatal human RPE in culture. Sheets of RPE, together with fragments of Bruch’s membrane, were obtained from the eyes of normal human abortuses of 17–24 weeks gestation. Bruch’s membrane was split during the dissection so that it was technically easy to separate sheets of RPE mechanically from choroid and thus obtain RPE free of choroidal contamination. These explants were centrifuged (100 × g for 2.5 min) and washed twice in calcium- and magnesium-free balanced salt solution (CMF-BSS) with centrifugation between washes. The dissociated cells were resuspended in low-calcium culture medium, plated onto a 100-mm diameter tissue culture dish, and incubated at 37°C in an atmosphere of 5% CO₂:95% air.

Low-calcium medium (a modification of CEM 2000; Scott, Fiskeville, RI) was used to initiate cultures. It was formulated without addition of CaCl₂ and contained 0.08 mM Ca, 1% heat-inactivated calf serum (Hazleton Biologies, Lenexa, KS), 1.4 × 10⁻⁸ M selenious acid, 2.8 × 10⁻⁸ M hydrocortisone, 3 × 10⁻⁷ M linoleic acid complexed with albumin, 8.3 × 10⁻⁷ M bovine insulin, 6.3 × 10⁻⁸ M human transferrin, 2.4 × 10⁻⁶ M putrescine, 2 × 10⁻³ M L-glutamine, 10⁻¹¹ M triiodothyronine, and bovine retina...
extract 1% by volume. Selenious acid and putrescine were obtained from Collaborative Research (Bedford, MA) and the remaining reagents from Sigma (St. Louis, MO). Retina extract was prepared by homogenizing 12 fresh bovine retinas per 100 ml CMF-BSS. The mixture was cleared by centrifugation (17,000 g for 20 min).

The normal calcium medium used to maintain established cultures consisted of a 1:1 ratio of CEM 2000 and modified Eagle's medium (Sigma). It also contained 2.5 × 10⁻⁷ M ZnSO₄, 5.0 × 10⁻¹⁰ M CuSO₄, 2.5 × 10⁻¹⁰ M MnCl₂, L-ascorbic acid (1.3 × 10⁻⁴ M), and bovine retinal extract (0.5%) in addition to all of the other additives in the low-calcium medium.

Complete culture media were adjusted to pH 7.3 with NaOH if necessary and sterilized by filtration through a 0.22-μm Millipore filter (Bedford, MA).

After initiation, primary cultures were incubated for 7–10 days without a change of medium. Thereafter, the medium was replaced with fresh low-calcium medium at 3–4-day intervals. The RPE cells proliferated rapidly, and successful cultures usually reached confluency within 3 weeks of initiation. In addition to attached cells, the cultures contained numerous single spheric cells that had been released into the medium. These nonattached cells (floaters) were collected at 1–3-day intervals up to 1 month after initiation of the primary cultures and were used to seed additional dishes.

Millicell-HA culture wells (Millipore) of 12-mm diameter and 0.45-μm porosity were used to grow the RPE on a permeable substrate. To enhance attachment of the seeded cells, Millicell membranes were pretreated with mouse laminin or human Biomatrix, both obtained from Collaborative Research. The RPE floaters, suspended in normal calcium medium, were seeded at a density of 1.7 × 10⁵ cells/cm². The Millicell culture wells were incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air. The cultures were maintained for 2 months and then screened for transepithelial resistance with an epithelial volt-ohmmeter (World Precision Instruments, New Haven, CT). Cultures were typically used 3–8 months after seeding.

The entire Millicell culture well was scaled within the conventional Ussing chamber that has been described previously, except that the area of exposed RPE was 0.60 cm². The chamber held 4 ml of fluid on each side of the tissue and was water jacketed to keep the temperature within the chamber at 37°C. Using a DVC-1000 voltage/current clamp (World Precision Instruments) under microcomputer control (International Business Machines PC-AT, Boca Raton, FL), we were able to measure the open-circuit voltage and short-circuit current (Isc) directly. The transepithelial resistance was measured by passing 4–6-μA current pulses across the epithelium and recording the resulting voltage drop.

The cells were maintained in their culture medium for all experiments. Since the culture medium contained retinal extract, its exact ionic composition was not known. However, analysis with an automated colorimeter and ion-specific electrodes calibrated for clinical use (Adam 717 737, Hitachi, Danbury, CT) revealed that the medium contained (in mM): 130 Na⁺, 5.3 K⁺, 1.9 Ca²⁺, 1.0 Mg²⁺, 108 Cl⁻, 21 HCO₃⁻, 1.0 PO₄³⁻, and 15 glucose. Total protein was 298 g/l. The pharmacologic agents tested were isoproterenol, propranolol, 3-isobutyl-1-methyl-xanthine (IBMX), and N⁶,²⁻O-dibutyryladenosine 3':5'-cyclic monophosphate (dibutryl cyclic AMP). Except for propranolol (Inderal; Ayerst, New York, NY), these agents were obtained from Sigma. Isoproterenol and propranolol were racemic mixtures. Experiments with the Ussing chamber consisted of sealing the RPE within the chamber and taking measurements until a stable baseline was obtained. This typically took about 30 min. The desired drug, in 100-μl volumes, was then injected into one (or both) sides of the chamber and its effect upon transepithelial voltage (Ve), resistance (R), and Isc determined. Except where specifically noted, agents were added to both sides of the RPE without perfusion or medium exchange. The medium on both sides of the tissue was gently circulated with magnetic stirring bars to ensure prompt dispersal of the injected drugs.

Experiments to determine intracellular cyclic AMP concentration changes in response to β-adrenergic stimulation were done in a manner designed to duplicate as closely as possible the conditions in the Ussing chamber. The RPE on its supporting matrix was removed from the Millicell culture well and cut into three or four pieces so that tissue from each culture well was exposed to all experimental conditions. These pieces were placed in culture medium maintained at 37°C. After 30 min, the medium was gently exchanged with medium containing the desired pharmacologic agents. Tissue exposed to more than one agent was incubated with each agent for at least 5 min before another drug was added. Samples were placed in 0.1 M HCl 15 min after exposure to isoproterenol.

The RPE samples in each tube were separated from the filter with a Kontes disposable pestle and homogenized. Two 10-μl aliquots were separated for the determination of protein by the method of Lowry et al. The rest of the homogenate was boiled for 1 min, centrifuged at 4000 g for 10 min, and the supernatant fraction removed. Depending on the protein concent-
centration and agent added to the tubes, the sample used for the measurement of cyclic AMP varied from 10–40 µl. Each sample was diluted to 100 µl with 50 mM sodium acetate, pH 6.2, containing 21 mM CaCl₂, and was acetylated with 5 µl of a 1:2 mixture of acetic anhydride:triethylamine before assay. The succinyltyrosine I²I methyl ester derivative of cyclic AMP and the antibodies against the cyclic nucleotide were purchased from New England Nuclear (Boston, MA). After completion of the radioimmunoassay as described by Farber and Lolley,¹² the samples were counted, and nonspecific binding was subtracted. Total cpm/bound in the standards was plotted against increasing fmol of standard added, and the cyclic nucleotide concentration in the tissue samples was read from the linear plot. The results were expressed in pmol cyclic AMP/mg protein.

Values are presented as arithmetic mean and standard deviation. Statistical significance of differences between means were determined using the student’s two-sample t-test (adjusted for unequal variances when necessary) with a Bonferroni adjustment for α.

Results

For 54 preparations, the average spontaneous Ve was 3.0 mV (apical side positive) (+/- 1.6 mV standard deviation [SD]), the average R was 330 ohm-cm² (±80 ohm-cm², SD), and the average Isc was 9.1 µA/cm² (±3.1 µA/cm² SD). The tissue behaved as an ohmic resistor (over Ve values of ±5 mV) since Isc measured under short-circuit conditions was identical, within experimental error, to that calculated from measured Ve and R values.

When the preparations were exposed to 10⁻⁷ M isoproterenol, an adrenergic agonist with predominantly β-adrenergic activity, Ve and Isc abruptly rose by 85% (±47% SD, 9 preparations) and by 88% (±42% SD) respectively while R was reduced by 6% (±3% SD). This small reduction in measured resistance was statistically significant (P < 0.05) and was consistently seen. A typical experiment is illustrated in Figure 1. This effect lasted for approximately 15 min, after which Isc and Ve returned to baseline values even though the isoproterenol was not removed from the bathing medium. The effect was seen if the apical or basolateral (or both) membranes were exposed to isoproterenol, and the effect of isoproterenol could be repeated several times (Fig. 2).

The effect of 10⁻⁷ M isoproterenol was blocked by 93% (±8% SD, 6 preparations) if the preparations were first exposed to 10⁻⁶ M propranolol, a β-adrenergic antagonist. The effect of propranolol seemed to be competitive because it could be overridden by the addition of 10⁻⁵ M isoproterenol (Fig. 3). The effect of 10⁻⁷ M isoproterenol applied to only the basal side of the RPE was blocked when 10⁻⁶ M propranolol had been previously only applied to the basal side of the RPE but not when 10⁻⁶ M propranolol had been applied to only the apical side of the RPE (two preparations). Conversely, the effect of 10⁻⁷ M isoproterenol applied to only the apical side of the RPE was blocked when 10⁻⁶ M propranolol had been previously applied to only the apical side of the RPE but not when 10⁻⁶ M propranolol had been previously

![Fig. 1. Cultured fetal human RPE sealed in an Ussing chamber. Measured values of the short circuit current (Isc), transepithelial voltage (Ve), and transepithelial resistance (R) versus time. Isoproterenol at 10⁻⁷ M increased Isc and Ve of cultured fetal human RPE by an average of 85% and 88%, respectively. Isc and Ve returned to baseline even though the isoproterenol was not removed. Isoproterenol has predominantly β-adrenergic activity.](image1)

![Fig. 2. Isoproterenol at 10⁻⁷ M was effective when applied to the apical or to the basal side of the RPE and the response could be elicited several times on the same preparation even though the previously applied isoproterenol was not removed from the chamber.](image2)
propranolol, cyclic AMP levels rose more than threefold over the level present when incubated in IBMX alone. The effect of isoproterenol was also blocked by propranolol in the presence of IBMX. These measured effects are statistically significant with $P < 0.01$. There was no significant difference in total protein between control and drug-treated tissues.

When the RPE was exposed to dibutyryl cyclic AMP at $10^{-4}$ M, both Ve and Isc abruptly rose by 47% ($\pm 25\%$ SD), while R did not change (seven preparations). Isoproterenol had no effect on preparations that had been previously treated with cyclic AMP (Fig. 5).

**Discussion**

The results of these experiments clearly indicate that cultured human RPE has a receptor for isoproterenol that can influence RPE-generated trans-RPE electrical current. This isoproterenol receptor is a $\beta$-adrenergic receptor because isoproterenol has predominantly $\beta$-adrenergic activity; the effect of isoproterenol can be blocked by the $\beta$ antagonist, propranolol; and isoproterenol was 100-times more effective at eliciting the RPE's electrical response than propranolol, cyclic AMP levels rose more than threefold over the level present when incubated in IBMX alone. The effect of isoproterenol was also blocked by propranolol in the presence of IBMX. These measured effects are statistically significant with $P < 0.01$. There was no significant difference in total protein between control and drug-treated tissues.

When the RPE was exposed to dibutyryl cyclic AMP at $10^{-4}$ M, both Ve and Isc abruptly rose by 47% ($\pm 25\%$ SD), while R did not change (seven preparations). Isoproterenol had no effect on preparations that had been previously treated with cyclic AMP (Fig. 5).

**Table 1. Effect of isoproterenol and propranolol on intracellular cyclic AMP concentration**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Isoproterenol</th>
<th>Propranolol, isoproterenol</th>
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<tr>
<td></td>
<td>$2.51^*$</td>
<td>$8.24$</td>
<td>$2.73$</td>
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<td></td>
<td>($\pm 0.55$; $n = 10$)</td>
<td>($\pm 2.22$; $n = 11$)</td>
<td>($\pm 0.94$; $n = 5$)</td>
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</tbody>
</table>

Differences:

- Control vs. isoproterenol = 5.73; $P < 0.01$
- Isoproterenol vs. isoproterenol + propranolol = 5.51; $P < 0.01$
- Control vs. isoproterenol + propranolol = 0.22; NS $\dagger$

* pmoles/mg total protein.

† NS = not significant ($P > 0.05$).

**Table 2. Effect of isoproterenol and propranolol on intracellular cyclic AMP concentration in the presence of IBMX**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IBMX</th>
<th>IBMX, isoproterenol</th>
<th>IBMX, propranolol, isoproterenol</th>
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<tbody>
<tr>
<td></td>
<td>$2.72^*$</td>
<td>$5.93$</td>
<td>$18.9$ ($\pm 4.35$)</td>
<td>$6.39$ ($\pm 1.32$)</td>
</tr>
<tr>
<td></td>
<td>($n = 5$)</td>
<td>($n = 5$)</td>
<td>($n = 5$)</td>
<td>($n = 5$)</td>
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</table>

Differences:

- Control vs. IBMX = 3.21; $P < 0.01$
- IBMX vs. IBMX + isoproterenol = 12.97; $P < 0.01$
- IBMX + isoproterenol vs. IBMX + propranolol + isoproterenol = 12.51; $P < 0.01$
- IBMX vs. IBMX + propranolol + isoproterenol = 0.46; NS $\dagger$

* pmoles/mg total protein.

† NS = not significant ($P > 0.05$).
Fig. 5. Cyclic AMP (dibutyryl form) affected $V_e$ and $I_{sc}$ in a manner similar to isoproterenol. Isoproterenol had no effect upon $V_e$ and $I_{sc}$ in the presence of cyclic AMP.

than was the $\alpha$ agonist, phenylephrine. However, our experiments do not exclude the possibility that there are $\alpha$-adrenergic receptors present on the cultured human RPE cells as well as $\beta$ adrenoreceptors.

As a general rule, cyclic AMP is the "second messenger" for $\beta$ adrenoreceptors. In most tissues, stimulation of $\beta$-adrenergic receptors activates adenylate cyclase and thereby increases cellular levels of cyclic AMP, while stimulation of $\alpha$ adrenoreceptors either reduces or does not affect intracellular cyclic AMP concentration. In the current study, isoproterenol increased intracellular cyclic AMP concentration in the presence and in the absence of the phosphodiesterase inhibitor, IBMX. The effect of isoproterenol on intracellular cyclic AMP concentration was blocked by propranolol. Furthermore, exogenously applied dibutyryl cyclic AMP produced an effect on $V_e$ and $I_{sc}$ that was very similar to that produced by isoproterenol, and isoproterenol had no effect upon cells that had been previously exposed to cyclic AMP. Isoproterenol produced similar effects upon the RPE $V_e$, $R$, and $I_{sc}$ when applied to the apical or to the basal side of the RPE. Since the effect of isoproterenol applied to only one side of the RPE was blocked when propranolol was applied to the same side of the RPE but not when propranolol was applied to the opposite side of the RPE, this suggests that $\beta$-adrenergic receptors are present on both the apical and basolateral membranes of cultured fetal human RPE and that isoproterenol did not simply diffuse across the RPE. It is unclear whether $\beta$ adrenoreceptors are present on both sides of the RPE in vivo because the distribution of receptors in cultured tissue may not be the same as in intact RPE.

It was interesting to note that the $V_e$ and $I_{sc}$ returned toward baseline values even though the isoproterenol was not removed from the chamber. The $V_e$ and $I_{sc}$ are functions of the difference of voltages across the apical and basolateral membranes, so that $V_e$ and $I_{sc}$ can return toward baseline values even though the voltages across the apical and basolateral membranes do not. Although intracellular recordings were not made to confirm this, it appears that a major portion of the applied isoproterenol became inactive, and the voltages across the apical and basolateral membranes returned to baseline values because a nearly identical response was elicited when freshly prepared isoproterenol was reapplied to the RPE (Fig. 2). Isoproterenol is easily inactivated by oxidation and probably became oxidized in the Ussing chamber. It is unlikely that the RPE internalized $\beta$-adrenergic receptors to any significant degree as has been shown to occur in the ciliary body and other tissues because the "inactivation" was too rapid and subsequent stimulation with isoproterenol produced equally large effects upon $V_e$ and $I_{sc}$ (Fig. 2).

Adrenergic receptors have been previously demonstrated on a number of RPE preparations. The $\beta$-adrenergic receptors have been detected biochemically on cultured embryonic chicken RPE, on freshly dissected embryonic chicken RPE, and on cultured human RPE. Application of isoproterenol to bullfrog RPE elevates intracellular cyclic AMP levels, presumably by stimulating $\beta$ adrenoreceptors. Exogenously applied cyclic AMP reduces RPE-mediated transepithelial fluid movement in bullfrogs. Although adrenergic receptors have been found on rabbit and bovine RPE, $\alpha$-adrenoreceptors do not employ cyclic nucleotides as their second messenger. Furthermore, exogenously applied cyclic AMP and isoproterenol had no effect upon rabbit RPE $V_e$, $R$, and $I_{sc}$.

If $\beta$ adrenoreceptors are present on adult human RPE in vivo, this may have important clinical implications, since these receptors might provide a way to modify human RPE transport for therapeutic goals. The need for doing so is well established. For example, central serous chorioretinopathy (CSR), a disease caused by alterations in RPE transport and barrier function, is thought to be most common in patients with excess endogenous adrenergic stimulation. Furthermore, an animal model of CSR has been produced by administering exogenous epinephrine (an adrenergic agonist) to monkeys. It may be possible to treat CSR and other diseases of the RPE by blocking or by stimulating the RPE's adrenoreceptors.

Key words: retinal pigment epithelium, cell culture, human, adrenoreceptor, isoproterenol

References


