Pertussis Toxin-Sensitive Melatonin Receptors Negatively Coupled to Adenylate Cyclase Associated With Cultured Human and Rat Retinal Pigment Epithelial Cells

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Purpose. Studies were conducted to investigate the influence of melatonin on adenylate cyclase activity in cultured human and rat retinal pigment epithelial (RPE) cells.

Methods. Adenylate cyclase activity was assessed by measurement of cAMP levels in cultured RPE cells using a specific cAMP-binding protein isolated from bovine adrenal cortex to detect cellular cAMP by competition with a standard amount of tritiated cAMP. The effects of melatonin on basal cAMP levels and those induced by the direct activator of adenylate cyclase, forskolin, were studied.

Results. Exposure of human RPE cells to 100 μM of melatonin had no effect on basal cAMP levels, but it caused a 41% reduction in the forskolin (5 μM) stimulation of cAMP. This melatonin-induced reduction in forskolin-stimulated adenylate cyclase activity was dose dependent, with half-maximal (EC50) reduction at 4.2 × 10⁻¹⁰ M. 2-iodomelatonin, 6-chloromelatonin, and 6-hydroxymelatonin mimicked the melatonin effect with EC50 values of 3.5 × 10⁻¹⁰ M, 4.3 × 10⁻⁹ M, and 1.9 × 10⁻⁸ M, respectively. Preexposure of cells to pertussis toxin (100 ng/ml) for 18 hours completely attenuated the ability of melatonin to influence the forskolin stimulation of cAMP levels. Propranolol did not influence the action of melatonin but did antagonize the ability of serotonin to reduce the forskolin-elevated cAMP levels. Thus, melatonin receptors are distinct from serotonin receptors. Melatonin receptors negatively linked to cAMP metabolism are also associated with cultured hooded rat RPE cells. Melatonin and iodomelatonin caused dose-dependent reductions in forskolin-stimulated cAMP production with half-maximal values of 2.4 × 10⁻⁹ M and 3.28 × 10⁻⁹ M, respectively.


Melatonin (5-methoxy N-acetyltryptamine) is synthesized primarily in the pineal gland, where levels fluctuate daily and the highest levels are reached in the dark. Melatonin has been implicated in the regulation of numerous physiological processes exhibiting circadian rhythms, including sleep, seasonal affective disorder, and seasonal reproductive changes in some mammals. Melatonin and its biosynthetic enzymes, N-acetyltransferase and hydroxyindole-O-methyltransferase (HIOMT), have subsequently been identified in the retina of a number of species, and, as in the pineal gland, the levels of melatonin exhibit circadian rhythms. HIOMT mRNA and melatonin immunoreactivity have been identified in the photoreceptors, and melatonin levels increase in rat photoreceptors in the dark of a light-dark cycle, suggesting that these cells may be the primary source of melatonin biosynthesis in the retina. Studies from a number of laboratories have implicated melatonin in the control of the circadian processes of the outer retina. Melatonin has been suggested to modulate the rhythmic photoreceptor outer segment disk shedding in Xenopus laevis and rats, to inhibit the phagocytosis of rod outer segments by chick retinal pigment epithelial (RPE) cells, to influence pigment aggregation and the electrical properties of the RPE cells, and to control retinomotor movements.
Specific melatonin binding sites in the retina were first identified by Wiechmann et al., who showed saturable and displaceable binding of \( ^{3}H \)-melatonin to the outer plexiform layer of \( Ranna \) pipes retina and RPE–choroidal melanosomes. Further studies with \( { \left[ 2^{-3} H \right]} \)-iodomelatonin identified high-affinity melatonin binding sites in chick, rat, rabbit, and mouse retina. The \( { \left[ 2^{-3} H \right]} \)-iodomelatonin specific binding to chick RPE cells is modulated by guanine nucleotides, suggesting that the putative melatonin receptor is G-protein coupled. Work on the ovine pars tuberalis has also demonstrated that high-affinity melatonin binding sites are negatively coupled to \( \text{cAMP} \) metabolism by a pertussis toxin-sensitive G-protein. An inhibitory effect of melatonin on \( \text{cAMP} \) metabolism has been identified in numerous other tissues, including chick brain and retina, rat brain, and the rabbit iris–ciliary body. The aim of this study was to investigate whether melatonin influences \( \text{cAMP} \) metabolism in mammalian retinal pigment epithelium and thus provide further evidence for the existence of functional melatonin receptors.

The retinal pigment epithelium is a monolayer of mitotically inactive, cuboidal cells situated between the photoreceptors and the choriocapillaries, with the apical villous processes interdigitating with the outer segments of the rods and cones. Several RPE cell functions have been shown to be influenced by drugs associated with the \( \text{cAMP} \) second messenger system, including phagocytosis of rod outer segments and ion and fluid transport. To date, \( \beta_{2} \)-adrenergic and \( \text{A}_{2} \)-adenosine receptors have been shown to exist on human RPE cells. These receptors, when activated, lead to a stimulation of \( \text{cAMP} \). In this study, we showed the existence of melatonin receptors negatively coupled to \( \text{cAMP} \) in human and rat RPE cells.

MATERIALS AND METHODS

Materials

Foetal calf serum, Hams F10, fungazone [ampoteri cin B], glutamine, trypsin, and NUNC 24 multiwell plates were from Gibco (Paisley, UK), and 25-cm\(^2\) and 75-cm\(^2\) tissue culture flasks were obtained from Falcon (Oxford, UK). \( \left[ 2,8^{-3} H \right] \)-adenosine 3'5' cyclic monophosphate, 33 Ci/mmol obtained from Amersham International (Amersham, UK). \( \left[ 2^{-3} H \right] \)-iodomelatonin was first identified by Wiechmann et al., who showed saturable and displaceable binding of \( ^{3}H \)-melatonin to the outer plexiform layer of \( Ranna \) pipes retina and RPE–choroidal melanosomes. Further studies with \( { \left[ 2^{-3} H \right]} \)-iodomelatonin identified high-affinity melatonin binding sites in chick, rat, rabbit, and mouse retina. The \( { \left[ 2^{-3} H \right]} \)-iodomelatonin specific binding to chick RPE cells is modulated by guanine nucleotides, suggesting that the putative melatonin receptor is G-protein coupled. Work on the ovine pars tuberalis has also demonstrated that high-affinity melatonin binding sites are negatively coupled to \( \text{cAMP} \) metabolism by a pertussis toxin-sensitive G-protein. An inhibitory effect of melatonin on \( \text{cAMP} \) metabolism has been identified in numerous other tissues, including chick brain and retina, rat brain, and the rabbit iris–ciliary body. The aim of this study was to investigate whether melatonin influences \( \text{cAMP} \) metabolism in mammalian retinal pigment epithelium and thus provide further evidence for the existence of functional melatonin receptors.

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Human RPE Cell Culture

Human RPE cultures were prepared as described by Lui et al. Eyes were obtained from Bristol Eye Bank between 24 and 48 hours after enucleation and removal of the cornea for transplantation, and they were stored overnight at 4°C. After transfer to a sterile laminar flow hood, the anterior portion of the eye was removed by cutting just below the ora serrata. The lens, vitreous, and retina were extracted and replaced by phosphate-buffered saline (PBS; 137 mM NaCl, 5.4 mM KCl, 1.28 mM NaH\(_{2}\)PO\(_{4}\), 7 mM Na\(_{2}\)HPO\(_{4}\), pH 7.4). The PBS solution was aspirated off, and the eyecup was filled with 0.25% trypsin solution. Care was taken to cover only the exposed RPE cells to avoid contamination of the culture by other cell types. After a 60-minute incubation at 35.5°C in a moist 5% CO\(_{2}\) atmosphere, the loosely adherent RPE cells were detached by repeated aspiration of the trypsin solution with a Pasteur pipette. The cell suspension was transferred to a centrifuge tube, the eyecup was washed with culture medium (Hams F10 supplemented with 10% fetal calf serum, 0.4% glucose, 2 mM glutamine, 2.5 \( \mu \)g/ml amphotericin B, and 100 \( \mu \)g/ml gentamicin) and the medium was combined with the trypsin solution. The RPE cells were then centrifuged at 80g for 8 minutes at 4°C, and the pellet was resuspended in 5 ml culture medium and transferred to a 25 cm\(^2\) tissue culture flask. The primary cultures were grown to confluence (2 to 3 weeks) in an incubator (35.5°C, 5% CO\(_{2}/95%\) O\(_{2}\), moist environment) and passaged with a ratio of 1:3. Cells from passages 2 to 4 were used for experimentation. RPE cells were stored, when necessary, in liquid nitrogen, and the purity was routinely assessed using immunocytochemistry to detect RPE cell-specific cytokeratins.

Rat RPE Cell Culture

This research adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rat RPE cells were cultured from 10- to 12-day-old hooded rats using a method adapted from Edwards. Eyes were enucleated from 8 to 10 rats and were incubated overnight in the dark at room temperature in 10 ml of a balanced salt solution (BSS; 136.9 mM NaCl, 5.4 mM KCl, 4.2 mM NaH\(_{2}\)PO\(_{4}\), 0.34 mM Na\(_{2}\)HPO\(_{4}\), 5.55 mM glucose, 0.5 mM Ca\(_{3}\)Al\(_{2}\), and 10 mg/ml gentamicin, pH 8.0). The BSS solution was then decanted, and the eyes were incubated for 45 minutes at 37°C in 1 mg/ml trypsin and 70 U/ml collagenase in calcium-free BSS containing 0.1 mM ethylenediaminetetraacetic acid, pH 8.0. After enzyme incubation, the eyes were transferred to a petri dish containing culture medium (as used for human RPE). Cornea, lens, vitreous, and retina were dissected, and the eyecups were washed with fresh medium. RPE cells were isolated by gentle scraping with a flame-tipped Pasteur pipette into approximately 3 ml culture medium. The cell suspension was centrifuged at 80g for 8 minutes at 4°C and washed by resuspension in calcium-free BSS and
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FIGURE 1. Phase-contrast micrographs showing a human RPE cell culture derived from a 30-year-old donor (A) and a primary hooded rat RPE cell culture (B). Note the typical "crazy paving" morphology and the presence of pigment granules within the cells. (A, B) Original magnification, X100. (C) Localization of RPE-specific cytokeratins (Kc8.13) in cultured human RPE cells isolated from a 16-year-old donor at passage 3 by immunocytochemistry. Original magnification, X250. RPE = retinal pigment epithelial.

centrifugation. The RPE cells were then incubated for 5 minutes at 37°C in 2 ml of 1 mg/ml trypsin in calcium-free BSS. The enzyme reaction was halted with culture medium, and the cell suspension was recentrifuged. Cells were resuspended in 5 ml of culture medium, rapidly tritutated with a pasteur pipette to separate individual RPE cells, and transferred to a 25-cm² tissue culture flask. Primary cultures reached confluency after 7 days and were treated as described for human RPE cell culture thereafter. For experiments conducted on primary cultures, the number of isolated RPE cells was counted using an improved Neubauer hemocytometer, and the cell suspension was diluted to 5 x 10⁶ cells/ml. After that, 500-μl aliquots were grown until confluent (7 days) in 24 multiwell plates.

Determination of Cellular cAMP

Cultured RPE cells at passages 2 to 4 were grown to confluence (approximately 4 to 7 days) in 24 multiwell plates. The cells were washed free of serum containing medium by incubation with serum-free Hams F10 at 35.5°C/5% CO₂ for 2 to 4 hours. Then, the medium was replaced with 200 μl of assay buffer (Hams F10, 20 mM HEPES, pH 7.4) and incubated at 37°C for 5 minutes. The incubation buffer was adjusted to maintain a final reaction volume of 210 μl, allowing for the addition of 10 μl of test drugs. In experiments involving melatonin and its analogues, 10 μl of drug was added, and cells were given a 5-minute preincubation before the addition of 10 μl forskolin (5 μM) and a further incubation of 5 minutes. In experiments using Bordetella pertussis toxin (PTX), RPE cells were incubated in 250 μl culture medium containing 100 ng/ml PTX for 18 hours, and 100 ng/ml PTX was added to the assay buffer during the experiment. When propranolol was used, cells were given an extra 5-minute incubation with the antagonist.

The reaction was terminated by transferring the multiwell plate to a boiling water bath for 3 minutes. A 50-μl aliquot of buffer was removed and stored at −20°C for subsequent analysis. cAMP content was determined using the method of Brown et al.,42 which uses the competition between a standard amount of tritiated cAMP and cellular cAMP for sites on a binding protein prepared from bovine adrenal cortex.

Statistical Analysis

Statistical significance was assessed by Student’s t-test, and a P value less than 0.05 was considered significant. Half-maximal (EC₅₀) values were calculated by determination of the concentration of agonist required to achieve half the maximum observed reduction.

RESULTS

Human and rat RPE cells in primary culture adopted the typical polygonal morphology associated with the intact RPE and possessed pigment granules (Figs. 1A, 1B). The pigment is lost from cells at later passage as a result of dilution by cell division. Immunohistochemistry using the monoclonal antibody Kc8.13 (Sigma)
TABLE 1. Effect of Melatonin and a Number of Analogues (100 μM) on Basal and Forskolin-Stimulated Levels of cAMP in Cultured Human RPE Cells

<table>
<thead>
<tr>
<th>Drugs Added</th>
<th>% Stimulation of cAMP Production Relative to Basal Levels</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin (5 μM)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>+Melatonin</td>
<td>59.0 ± 4.2*</td>
<td>10</td>
</tr>
<tr>
<td>+2-Iodomelatonin</td>
<td>53.4 ± 8.0†</td>
<td>9</td>
</tr>
<tr>
<td>+6-Chloromelatonin</td>
<td>70.3 ± 3.9*</td>
<td>3</td>
</tr>
<tr>
<td>+6-Hydroxymelatonin</td>
<td>62.1 ± 4.5†</td>
<td>7</td>
</tr>
<tr>
<td>+Tryptamine</td>
<td>64.2 ± 3.9†</td>
<td>7</td>
</tr>
<tr>
<td>+N-acetylserotonin</td>
<td>93.2 ± 10.3</td>
<td>5</td>
</tr>
<tr>
<td>+5-Methoxytryptamine</td>
<td>91.4 ± 8.2</td>
<td>6</td>
</tr>
<tr>
<td>Melatonin</td>
<td>-2.4 ± 8.2</td>
<td>3</td>
</tr>
<tr>
<td>2-Iodomelatonin</td>
<td>-29.1 ± 8.7</td>
<td>4</td>
</tr>
<tr>
<td>6-Chloromelatonin</td>
<td>-18.4 ± 3.9</td>
<td>3</td>
</tr>
<tr>
<td>6-Hydroxymelatonin</td>
<td>-5.2 ± 10.3</td>
<td>3</td>
</tr>
</tbody>
</table>

Cells were either pretreated with 100 μM agonist for 5 minutes before a 5-minute incubation with forskolin or just with agonist for 5 minutes. Results are expressed as mean ± SEM from n separate experiments carried out in triplicate. *P < 0.05, †P < 0.02 when agonist addition is compared to forskolin addition alone by Student's paired t-test.

Effect of Melatonin on cAMP Production

Human RPE Cells. Cultures used for experimentation were derived from four different human donors 18, 23, 26, and 41 years of age. No obvious differences in responsiveness between the cell lines were apparent. Melatonin at 100 μM did not influence the basal level of cAMP (Table 1). However, a 5-minute preincubation with melatonin (100 μM) attenuated the forskolin (5 μM)-induced stimulation of cAMP production by 41%. This effect of melatonin was dose dependent, with half-maximal reduction of elevated cAMP levels occurring at 4.2 × 10^-10 M (Fig. 2). Numerous melatonergic analogues also attenuated forskolin action (Table 1). The order of effectiveness was 2-iodomelatonin (46.6%) > melatonin (41.0%) > 6-hydroxymelatonin (37.9%) > tryptamine (35.8%) > 6-chloromelatonin (29.7%). N-acetylserotonin and 5-methoxytryptamine were without effect. Iodomelatonin and chloromelatonin reduced basal cAMP levels (Table 1), although these influences were statistically insignificant. The reduction in forskolin-stimulated cAMP production by 2-iodomelatonin, 6-chloromelatonin, and 6-hydroxymelatonin was concentration dependent with EC50 values of 3.5 × 10^-10 M, 4.3 × 10^-9 M, and 1.9 × 10^-7 M, respectively (Fig. 3). Pretreatment of cultured human RPE cells with...
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100 ng/ml pertussis toxin for 18 hours resulted in a slight increase in basal cAMP levels (Fig. 4) and a slight, though insignificant, reduction in forskolin-stimulated adenylate cyclase activity. The 52.3% decrease in forskolin-elevated cAMP induced by melatonin in these experiments was, however, abolished by preexposure of the cells to pertussis toxin (Fig. 4).

Like melatonin, the indoleamine serotonin (5-hydroxytryptamine [5-HT]) is known to decrease forskolin-stimulated adenylate cyclase activity in cultured human RPE cells. However, as shown in Table 2, 1 μM propranolol antagonized the serotonin effect on forskolin-elevated cAMP levels by 32.2% but was without influence on the melatonin response.

**TABLE 2. Effect of Propranolol on the Serotonin- and Melatonin-Induced Reduction of Forskolin-Stimulated cAMP Levels in Cultured Human RPE Cells**

<table>
<thead>
<tr>
<th>Drugs Added</th>
<th>% Reduction of cAMP (5 μM)-Elevated cAMP Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Serotonin (100 μM)</td>
<td>40.7 ± 6.5</td>
</tr>
<tr>
<td>Melatonin (100 μM)</td>
<td>34.9 ± 9.7</td>
</tr>
</tbody>
</table>

Data are mean ± SEM from 3 separate experiments performed in triplicate.

*P < 0.02 when compared to serotonin effect in the absence of propranolol.

**DISCUSSION**

Numerous studies have demonstrated that a subpopulation of melatonin receptors, identified by radioligand-binding studies, is negatively coupled to the activation of adenylate cyclase. The present study shows that melatonin receptors negatively coupled to adenylate cyclase activation exist in human RPE cells. Receptors linked to adenylate cyclase activity belong to the seven transmembrane domain containing family of proteins which modulate its influence on cAMP production by coupling to GTP-binding proteins (G-
Log [Agonist] M

**Figure 5.** Dose-response curves for the effect of melatonin (•) and 2-iodomelatonin (°) on the stimulation of cAMP production by forskolin (5 μM) in cultured hooded rat RPE cells. Data are mean ± SEM from five and six separate experiments performed in triplicate, respectively. RPE = retinal pigment epithelial.

The inhibitory G-proteins (Gi) act to reduce adenylate cyclase activity and are sensitive to the PTX, which catalyzes the ADP-ribosylation of the α subunit. The presence of a pertussis toxin-sensitive G-protein in human RPE cells was postulated by a study demonstrating potentiated action of drugs stimulating adenylate cyclase activity when cells were preexposed to pertussis toxin. The results presented here suggest that this inhibitory G-protein may couple to a melatonin receptor.

The pharmacologic data indicate that melatonin has a high affinity for the receptor site in human RPE cells, with maximal inhibition of forskolin stimulation of cAMP production at approximately 10 to 100 nM and an EC50 value of 422 pM. Serotonin also decreases cAMP levels (Table 2) by a receptor-mediated process that is antagonized by propranolol (see ref. 43 for details). However, the melatonin effect is unchanged by propranolol. Thus, melatonin acts through a different receptor process from serotonin to decrease cAMP levels. The maximal inhibitory concentration is in good agreement with other studies, although the observed EC50 value is approximately 10-fold higher than the half-maximal effect demonstrated in cultured ovine pars tuberalis (19.1 pM), and chick brain (22 pM) and retinal (30 pM) membranes, but it is significantly lower than that in iris–ciliary body homogenates (200 nM). Work with other receptor types has demonstrated numerous different subtypes with different pharmacologic profiles and affinities for their agonists. Whether such a situation exists for the melatonin receptor must await molecular biologic studies and the identification of specific agonists and antagonists. Two subtypes, however, have been suggested, although this classification has been questioned. An alternative explanation for the differences in affinity might lie in recent work by Hazlerigg et al., which demonstrated that prolonged exposure to low concentrations of melatonin caused a 10-fold shift in the half-maximal value without affecting the maximal effective concentration. It is possible that the serum used for the cell culture contains low levels of melatonin that might lead to such a downregulation, as has been observed for other receptors. To further clarify this point, radioimmunoassay will be required to demonstrate the presence of melatonin in the growth medium. The observed effect of pertussis toxin is strongly indicative of coupling of the melatonin receptor to a G-protein; further studies will be required to confirm the ADP-ribosylation of the α subunit as shown by others.

Cultured rat RPE cells were shown to possess a similar response to human RPE cells, but with a 10-fold higher EC50 value observed for melatonin and iodomelatonin. However, a comparison of the effect of melatonergic analogues showed some interesting differences (Tables 1 and 3). Melatonin, iodomelatonin, and 6-hydroxymelatonin all induced a reduction in forskolin stimulation of cAMP production. However, tryptamine also reduced cAMP levels in human RPE, and N-acetylserotonin and 5-methoxytryptamine had no effect (Table 1), whereas in rat RPE cells tryptamine and 5-methoxytryptamine significantly potentiated cAMP production (Table 3). Cultured rat RPE cells have previously been shown to possess serotonergic 5-HT2 receptors mediating phosphoinositide turnover and mobilization of intracellular calcium. Tryptamine and 5-methoxytryptamine were effective at mobilizing inositol phosphate accumulation through the serotonin receptor, and a recent report has shown that activation of protein kinase C by serotonin is able to potentiate the stimulation of adenylate cyclase by numerous compounds in cultured rat RPE cells. It seems likely, therefore, that in rat RPE tryptamine and 5-methoxytryptamine act at the 5-HT2 receptor to mediate the effect. Moreover, since little displacement of 125I-iodomelatonin from the melatonin receptor in the iris–ciliary body by tryptamine is observed (unpublished observation, 1994), suggesting low affinity for the melatonin receptor, it is likely that the observed reduction in forskolin-stimulated cAMP levels induced by tryptamine in human RPE cells (Table 1) is mediated by its interaction with the 5-HT2 receptor present in these cells. The possible presence of dopamine receptors on rat RPE...
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cells was also studied. Dopamine acts to reduce cAMP levels through D2-receptors and to increase levels through D1-receptors. Because neither dopamine nor the D2-receptor agonist, bromocriptine, influenced basal or forskolin-elevated levels (Table 3), the involvement of dopamine receptors in the RPE can be eliminated.

Because of the unavailability of sufficient tissue, the characterization of the melatonin effect on cAMP metabolism was conducted on later passage RPE cell cultures. To attempt to eliminate the possible expression of melatonin receptors only after extended culture, when dedifferentiation may have occurred, the effect of melatonin on the adenylate cyclase activity of primary rat RPE cell cultures was also studied. Melatonin reduced by 61.9% the forskolin-stimulated cAMP production in these cells, suggesting that the melatonin receptor is likely to be present in vivo.

The phagocytosis of shed photoreceptor outer segment disks represents one of the main functions of the retinal pigment epithelium, and a defect in the phagocytic ability in the Royal College of Surgeons rat is recognized as a major factor leading to the observed selective loss of photoreceptors found in these dystrophic animals. Increases in cAMP have been shown to have an inhibitory effect on the phagocytosis of rod outer segments (ROS) by cultured chick and rat RPE cells. More recently, activation of A2-adenosine receptors in cultured rat RPE cells was shown to increase cAMP levels and reduce ingestion of ROS. This study demonstrated for the first time the direct coupling of a cell surface receptor coupled to cAMP metabolism and the phagocytic machinery of the retinal pigment epithelium. The effect of reduced cAMP levels on phagocytosis is not known, but it is tempting to speculate that compounds negatively coupled to adenylate cyclase might stimulate phagocytosis of photoreceptor outer segments by the retinal pigment epithelium. The cyclic nature of melatonin synthesis and outer segment disk shedding and the negative influence of melatonin on cAMP metabolism lead to the exciting possibility that melatonin is the initiator of phagocytosis of outer segments by the RPE. In line with this, we are currently assessing the effect of melatonin on the phagocytosis of isolated ROS by cultured RPE cells.

In conclusion, we have demonstrated the existence of melatonin receptors negatively linked to cAMP production in cultured human and rat RPE cells.

Key Words
retinal pigment epithelial cells, melatonin receptors, adenylate cyclase, cAMP, pertussis toxin

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tion with melatonin on the c-wave of the direct current electroretinogram and on the standing potential of the eye in albino rabbits. Doc Ophthalmol. 1987;65:97–111.


