Suppression of cAMP-Induced Pigment Granule Aggregation in RPE by Organic Anion Transport Inhibitors

Dana M. García and Beth Burnside

**Purpose.** To investigate the mechanism(s) by which intracellular cAMP levels are elevated to induce pigment granule aggregation in teleost retinal pigment epithelium (RPE).

**Methods.** Pigment granule migration was studied in vitro using RPE sheets isolated from dark-adapted green sunfish, *Lepomis cyanellus*. After preculture to allow pigment granule dispersion, RPE sheets were incubated with various agents to test their ability to induce pigment granule aggregation. RPE sheets were then fixed, and pigment granule position was assessed microscopically.

**Results.** Pigment granule aggregation was induced by nonderivatized cAMP. At maximally effective concentration (1 mM), cAMP was as effective as its more membrane-permeant analogs dbcAMP and 8-Br-cAMP. Forskolin (1 or 10 μM), a stimulator of adenylyl cyclase, was also effective at inducing pigment aggregation. Two inhibitors of organic anion transport, probenecid and sulfinpyrazone, inhibited cAMP-induced aggregation by ~80% but had no effect on forskolin-induced aggregation. Several agents shown to stimulate RPE adenylyl cyclase in other species failed to induce pigment aggregation in isolated RPE sheets.

**Conclusions.** Our observations strongly suggest that exogenously applied, nonderivatized cAMP can gain access to the cytoplasm of isolated RPE cells via organic anion transporters. Thus, if cAMP were secreted by retinal cells into the subretinal space, it could be taken up by RPE cells and subsequently act as an intracellular messenger to activate dark-adaptive physiological processes such as pigment granule aggregation.

been shown to stimulate cyclic adenosine monophosphate (cAMP) production in cultured chick,11 rat,12 and human13 RPE.

Because agents that elevate cAMP induce dark-adaptive retinomotor movements in both retina and isolated teleost RPE,4,14-16 and because cAMP levels have been reported to be elevated in the outer plexiform and outer nuclear layer of the retina in the dark in a number of species,17-20 agents known to stimulate adenylyl cyclase in RPE are potential dark signals. Examples include vasoactive intestinal peptide (VIP), glucagon, histamine,11 and adenosine agonists12,21 in addition to prostaglandins.11-13 Histamine and VIP have been shown to elevate cAMP in chick RPE.11,22 Receptors for adenosine have been reported to be present in rat RPE19 and in chick, human, porcine, and bovine RPE21 in which they are coupled to adenylyl cyclase.

We have previously reported that in green sunfish (Lepomis cyanellus), pigment granule aggregation is induced in isolated RPE not only by forskolin, a stimulator of adenylyl cyclase,23-25 but also by nonderivatized cAMP.4 Because nonderivatized cAMP is relatively membrane impermeant,26 the mechanism by which pigment granule aggregation in isolated RPE is triggered by exogenous cAMP is not clear. Several modes of action might be considered: Exogenous cAMP might act by binding to extracellular receptors, either adenosine receptors12,21 or more specialized cAMP-receptors analogous to those of Dictyostelium27 or cardiac myocytes;28 sufficient exogenous cAMP might diffuse across the lipid bilayer of the plasma membrane to activate processes in the RPE cytoplasm; or exogenous cAMP might be transported into the cell by means of organic anion transporter activity. Cyclic AMP transport via organic anion transporters has been demonstrated in erythrocytes,29 hepatocytes,30 glioma cells,31 fibroblasts,32 and aortic smooth muscle cells.33 In each of these studies, the movement of cAMP was inhibited by probenecid, an organic acid that acts as a competitive inhibitor of organic anion transport.30 Although they did not measure intracellular cAMP levels, Pilks et al30 found effects of exogenously applied cAMP on gluconeogenesis in isolated hepatocytes. This observation suggested that the plasma membrane was selectively permeable to cAMP.

To examine the mode of action of cAMP in inducing pigment granule aggregation, we compared cAMP’s effectiveness at inducing pigment granule aggregation with the effectiveness of its membrane permeant analogs, adenosine and adenosine triphosphate (ATP). We also tested the effects of organic anion transport inhibitors on cAMP-induced pigment aggregation. Here we present evidence that cAMP enters the retinal pigment epithelium via a probenecid- and sulfinpyrazone-sensitive organic anion transporter and acts intracellularly to stimulate dark-adaptive pigment granule aggregation. We further report that adenosine, ATP, melatonin, histamine, VIP, and prostaglandins fail to produce dark-adaptive pigment granule aggregation in isolated RPE sheets. We propose that cAMP itself may act as an extracellular messenger secreted by the retina to signal darkness to the RPE.

MATERIALS AND METHODS

Animals

The breeding, housing, and use of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Green sunfish, L. cyanellus, ranging from 6 to 15 cm in length were obtained from Fender Fish Hatchery (Baltic, OH). Animals were kept in indoor aquaria with aerated, dechlorinated tap water on a 14-hour light/10-hour dark cycle for a minimum of 2 weeks before use.

Solutions

Modified low-calcium Earle’s Ringer solution (MLCER) prepared on the day of each experiment was used for dissection and culture. MLCER contained 116 mM NaCl, 5 mM KCl, 1 mM NaH2PO4, 26 mM glucose, 24 mM NaHCO3, 3 mM HEPES, 1 mM ascorbic acid, 0.8 mM MgSO4, 1 mM EGTA, and 0.9 mM CaCl2. Calculated free calcium was 2 ^M.36 The pH of MLCER was titrated to 7.4; after gassing with 95% O2/5% CO2, pH level dropped to 7.2. Although pigment migration responses are similar in RPE cultured in normal-calcium (2 mM) and low-calcium (2 ^M) Ringer, cell viability is improved in low-calcium Ringer (unpublished observation, 1991). Therefore, for the studies reported here, we have used low-calcium Ringer. Drugs were prepared on the day of use from dry powder (cAMP, dbcAMP, 8-Br-cAMP, IBMX), from refrigerated 10 mM stocks (probenecid in 100% dimethyl sulfoxide [DMSO] and sulfinpyrazone in alkaline MLCER), or frozen 10 mM stocks (forskolin in 100% DMSO). Cyclic AMP and its analogs were purchased from Sigma (St. Louis, MO); and forskolin was purchased from Calbiochem (San Diego, CA). When drugs were prepared in DMSO, they were diluted with MLCER so that the concentration of DMSO was 1%, a concentration found to have no effect on pigment migration.

Isolation of RPE Sheets

RPE were prepared as previously described,3 with some modifications. Briefly, to facilitate separation of RPE from retina, fish were dark adapted in aerated...
aquaria for 30 to 90 minutes in the early afternoon before dissection.

Dissections were carried out in dim, incandescent light (0.2-foot candles) or under infrared illumination of wavelength $> 880$ nm (Wratten filter 87A; Eastman Kodak, Rochester, NY) using an image converter (FJW Industries, Mt. Prospect, IL). Dark-adapted fish were killed by spinal section and pithing. Enucleated eyes were hemisected, and retinas were removed and discarded. RPE were dislodged from the eyecup by a stream of MLCER pipetted from a pasteur pipette.

Fragments of RPE ranging from 0.5 to 5 mm in diameter (hereafter referred to as “RPE sheets”) were collected from both eyes and pooled. Generally, one to six samples were generated from each fish, and RPE from different fish were not pooled.

**Culture and Fixation of RPE Sheets**

RPE sheets were cultured in MLCER in 24 well plates or $10 \times 35$ mm plastic petri plates (Falcon, NJ) in a humidified culture chamber gassed with 95% O$_2$/5% CO$_2$. Drugs were added from 10X stocks, except for IBMX, which was made up as a 4 mM stock. After culture, RPE sheets were fixed overnight at room temperature in 0.5% glutaraldehyde, 0.5% paraformaldehyde, and 0.8% K$_2$Fe(CN)$_6$. Fixative was prepared in 0.1 M phosphate buffer on the day of use.

**Quantitative Analysis**

Fixed RPE sheets were minced on a glass slide with a single-edge razor blade. Minced RPE sheets contained pieces ranging from clumps of many cells to single cells. Slices of single-cell thickness and individual cells were examined at $\times400$ magnification using phase-contrast optics (Zeiss, Thornwood, NY). The total apico-basal length of the RPE cell and the distance from the base to the apical-most pigment granules were measured using an ocular micrometer (Fig. 1).

Pigment position is reported as pigment index (PI), the ratio between the length of the cell occupied by pigment and the total length of the cell.$^4$ A PI $> 0.85$ is dispersed, whereas a PI $< 0.75$ is aggregated. Only cells 50 $\mu$m in length that retained apical projections were measured. Thirty cells were measured per sample, and their mean PI determined. $n$ refers to the number of samples.

**RESULTS**

**Dispersion and Aggregation of Pigment Granules in Isolated RPE Sheets**

When RPE sheets were isolated from dark-adapted fish in dim, incandescent light (0.2 footcandle) or under infrared lighting, pigment granules spontaneously and completely dispersed into the apical projections within 15 minutes (Fig. 2). Slightly higher levels of dispersion were achieved in RPE isolated under in-
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Infrared illumination (Fig. 2). Even more extensive dispersion was consistently observed when dopamine (100 μM) was included in the medium for 30 minutes (PI = 0.99 ± 0.00, n = 11). The spontaneous dispersion of pigment granules facilitated our study of pigment aggregation in isolated RPE sheets. Dark adapting the fish was necessary to separate the RPE from the retina, but we then could obtain RPE sheets with dispersed pigment granules without pharmacologic manipulation simply by culturing for 15 to 30 minutes in low-calcium Ringer (MLCER). Various agents could then be tested for their ability to induce pigment granule aggregation. In previous studies using RPE sheets in this laboratory, spontaneous pigment granule dispersion was less extensive than we describe here. We have not been able to identify the source of this change in response. Because we first observed spontaneous dispersion when our fish were moved to their present quarters in a new building, it may reflect some altered physiological state of the fish under the new conditions.

Characterization of cAMP-Induced Pigment Aggregation

When dispersed RPE sheets were treated with cAMP or its analogs, 80% to 100% of the maximal aggregation attained under a given treatment occurred within 30 to 45 minutes (Fig. 3). Nondervatized cAMP was remarkably effective at activating pigment aggregation in isolated RPE sheets (Fig. 3). Because cAMP is a charged molecule and would not be expected to cross the lipid bilayer passively, the effectiveness of nondervatized cAMP at triggering pigment aggregation suggested that cAMP might be activating surface receptors or perhaps gaining entry to the cell through transporters.

To examine the mechanism of cAMP action further, we compared the dose response characteristics of nondervatized cAMP and the more membrane-permeant analogs, dibutyryl cAMP (dbcAMP) and 8-Br-cAMP (Fig. 4). At submillimolar concentrations, dbcAMP and 8-Br-cAMP were slightly more effective than cAMP at inducing aggregation; however, at millimolar concentrations, cAMP was as effective as its analogs. n = 3, except as noted. Error bars indicate the standard error of the mean.

![Figure 3](image3.png)

**Figure 3.** Time course of pigment granule aggregation in RPE sheets induced by cAMP or its analogs. Isolated RPE sheets were precultured 15 or 30 minutes to allow pigment granules to disperse to indicated t₀ PI, then sheets were treated with 1 mM cAMP, dbcAMP, or 8-Br-cAMP. n = 3, except as noted. Error bars indicate the standard error of the mean.

![Figure 4](image4.png)

**Figure 4.** Dose response studies of cAMP and cAMP analog effects on pigment granule aggregation in isolated RPE sheets in 30-minute cultures. RPE sheets were isolated and precultured as described in Figure 2. The t₀ PI was 0.93 ± 0.01 (n = 31). At submillimolar concentrations, dbcAMP and 8-Br-cAMP were slightly more effective than cAMP at inducing aggregation; however, at millimolar concentrations, cAMP was as effective as its analogs. n = 3, except as noted. Error bars indicate the standard error of the mean.
TABLE 1. Effects of IBMX on Pigment Position in Isolated RPE Sheets

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<tr>
<td>3</td>
<td>.87 ± .02</td>
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</table>

The slightly enhanced effectiveness of cAMP analogs compared to nonderivatized cAMP in producing pigment aggregation at submillimolar concentrations might reflect the higher membrane permeance of the analogs or the greater resistance of analogs to hydrolysis by phosphodiesterase (PDE). To examine the role of PDE in the differential effect of cAMP and its analogs, we conducted dose response studies for cAMP and dbcAMP in the presence of 0.1 mM IBMX, a PDE inhibitor. IBMX alone had no effect on pigment position at concentrations 2 mM (Table 1). In the presence of 0.1 mM IBMX, the dose response characteristics of cAMP and dbcAMP were nearly identical (Fig. 5). The dose response characteristics of dbcAMP were essentially identical in the presence and absence of IBMX (compare Figures 4 and 5), whereas the dose response curve for cAMP was shifted slightly to the left in the presence of IBMX. These results suggest that the difference in efficacy between cAMP and dbcAMP could be explained by the difference in their susceptibility to hydrolysis by PDE and suggests that both have access to the cell interior.

The effectiveness of 0.1 mM IBMX at enhancing the effects of cAMP on pigment aggregation further suggests that RPE sheets possess some endogenous PDE activity under the conditions of our experiments. However, the insensitivity of pigment migration to IBMX alone (no effect at 2 mM) (Table 1) suggests that under the conditions of our experiments, insufficient endogenous cAMP is being generated to produce pigment aggregation even when PDE is strongly inhib-

![Figure 5](image1)

**FIGURE 5.** Dose response studies of the effects on pigment granule aggregation in isolated RPE sheets of cAMP and dbcAMP in the presence of 0.1 mM IBMX. RPE sheets were precultured as described in Figure 2 and then cultured with dbcAMP and IBMX or cAMP and IBMX for 30 minutes. The to PI was 0.94 ± 0.01 (n = 28). IBMX controls (indicated as [cAMP or dbcAMP] = 0) contained 0.1 mM IBMX. Dose response characteristics of cAMP and dbcAMP were essentially identical in the presence of IBMX. Error bars indicate the standard error of the mean.

![Figure 6](image2)

**FIGURE 6.** Dose response study of the effects of forskolin and 1,9-dideoxyforskolin on pigment granule aggregation in isolated RPE sheets. RPE sheets were precultured as described in Figure 2, and then cultured with forskolin or 1,9-dideoxyforskolin for 30 minutes. t<sub>i</sub> PI was 0.94 ± 0.04 (n = 13). Carrier controls (indicated as [Forskolin] = 0) contained 0.5% DMSO. Forskolin stimulated full aggregation at micromolar concentrations, whereas 1,9-dideoxyforskolin was without effect. Error bars indicate the standard error of the mean.
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On the other hand, treating RPE sheets with forskolin, an activator of adenylyl cyclase, induces maximal aggregation, whereas the forskolin analog 1,9-dideoxyforskolin, which does not activate adenylyl cyclase, does not induce pigment granule aggregation. (Fig. 6). These observations, together with the insensitivity of pigment position to IBMX alone, suggest that although RPE cells possess an adenylyl cyclase capable of generating enough cAMP to trigger aggregation when stimulated by forskolin under the conditions of our experiments, the RPE cells' basal adenylyl cyclase activity is insufficient to elevate cAMP to levels adequate for stimulating pigment granule aggregation. It is also possible that RPE adenylyl cyclase does produce cAMP under control conditions but that the cAMP is transported out of the cells.

Inhibition of cAMP-Induced Aggregation by Two Inhibitors of Organic Anion Transport

We investigated a possible role for organic anion transporters in carrying extracellular cAMP into the RPE cells, leading to pigment aggregation. We tested the effects of the organic anion transport inhibitors, probenecid and sulfinpyrazone, on cAMP-induced pigment aggregation. Little effect on pigment position in previously dispersed RPE sheets was produced by submillimolar concentrations of probenecid or sulfinpyrazone alone (Fig. 7a). However, cAMP-induced aggregation was inhibited by either probenecid or sulfinpyrazone in a dose-dependent manner with maximal inhibition (70% and 80%, respectively) at 100 µM (Fig. 7b).

To control for possible nonspecific side effects of probenecid or sulfinpyrazone on the ability of RPE cells to aggregate pigment, we tested whether these drugs inhibited forskolin- and dbcAMP-induced aggregation. Forskolin should circumvent the need for transport of cAMP across the plasma membrane by stimulating the RPE cells' own adenylyl cyclase to elevate intracellular cAMP, and dbcAMP should be able to pass through the lipid bilayer despite the inhibition of organic anion transporters. Both probenecid and sulfinpyrazone strongly inhibited cAMP-induced aggregation but had no effect on either forskolin or dbcAMP.

**FIGURE 7.** Dose response studies of the effects of the organic anion transport inhibitors probenecid and sulfinpyrazone on pigment granule migration in isolated RPE sheet. Isolated RPE sheets were precultured as described in Figure 2 and then incubated with probenecid or sulfinpyrazone in the absence (a) or presence (b) of 1 mM cAMP for 30 minutes. In (a), the to PI was 0.94 ± 0.02 (n = 6), and in (b) the to PI was 0.93 ± 0.01 (n = 14). The carrier controls (indicated as [inhibitor] = 0) were 1% DMSO for probenecid and MLCER alone for sulfinpyrazone. In the absence of cAMP, probenecid (n = 2 at all points) or sulfinpyrazone (n = 4 at all points) had little effect on pigment granule position; however, both drugs inhibited cAMP-induced pigment aggregation at concentrations as low as 10 µM, with maximal inhibition at 100 µM (for probenecid n = 4 and for sulfinpyrazone n = 9, except as noted). Error bars indicate the standard error of the mean.
dbcAMP-induced aggregation (Fig. 8). Thus, cells retained the ability to aggregate pigment granules despite the presence of the drugs, suggesting that inhibition of cAMP-induced aggregation by the drugs was not due to nonspecific damage to the RPE cells’ ability to aggregate pigment. The suppression of cAMP-induced but not forskolin- or dbcAMP-induced pigment aggregation by organic anion transport inhibitors suggests that exogenous cAMP gains entry into RPE cells and triggers pigment aggregation intracellularly.

Effects of Extracellular Messengers on RPE Pigment Granule Aggregation

To identify possible extracellular messengers that might represent a signal from the retina to RPE for darkness, we tested several agents for the ability to induce pigment granule aggregation in isolated RPE sheets (Table 2). All the agents tested were reported to be present in retina and to be associated with cAMP elevation or dark-adaptive retinomotor movements; they included melatonin, prostaglandins E1 and E2, histamine, VIP, adenosine, and ATP. None of these agents induced marked pigment granule aggregation in isolated RPE sheets, even when 0.25 mM of IBMX was included in the medium to inhibit phosphodiesterase activity (Table 2). At this concentration, IBMX alone had little effect on pigment position in isolated RPE sheets (Table 1). In contrast, exogenous cAMP was highly effective at inducing aggregation under these conditions. These results suggest that under the conditions of our experiments, none of the tested agents act directly on RPE cells to stimulate them to undergo pigment granule aggregation. Because adenosine and ATP might be expected to activate purinergic or nucleotide receptors, these results further suggest that cAMP activation of pigment aggregation is highly specific and does not involve other purinergic or nucleotide receptors.

![Comparison of the effects of probenecid and sulfinpyrazone on pigment granule aggregation induced by forskolin, dbcAMP, or cAMP.](image-url)
DISCUSSION

We have shown that in the presence of 0.1 mM IBMX, nonderivatized cAMP is as effective as dbcAMP at inducing pigment granule aggregation in isolated green sunfish RPE sheets. This observation suggests that nonderivatized cAMP either gains access to the RPE cytoplasm or acts via a cAMP receptor at the cell surface. A possible mechanism by which cAMP could gain access to the RPE cytoplasm is influx via organic anion transporters.

Possible roles of organic anion transporters have been examined in many cell types by the use of two relatively specific inhibitors, probenecid and sulfinpyrazone.38-40 Probenecid is a lipid-soluble, organic acid that acts as a competitive inhibitor of and substrate for anion transporters.35 Although very different in structure, sulfinpyrazone, too, inhibits organic anion transport.35 Both drugs also have been used to inhibit transport of fluorescent, ion-indicator dyes such as FURA.41,42

We report here that both probenecid and sulfinpyrazone inhibit the induction of pigment granule aggregation in teleost RPE by nonderivatized, exogenous cAMP. These findings suggest that cAMP gains access to the RPE cytoplasm via an organic anion transporter. This conclusion is supported by our observation that probenecid fails to block forskolin-induced pigment granule aggregation. Because forskolin stimulates the cell's own adenylyl cyclase to generate endogenous cAMP, it circumvents the need for cAMP import across the plasma membrane.

Further evidence that some cells import cAMP is provided by a study using adrenal tumor cells,43 which showed that regulatory subunits of cytosolic protein kinase A (PKA) were labeled by externally applied [32P]-N3-cAMP. Probenecid reduced the amount of label bound to intracellular PKA subunits but had no effect on binding of [3H]-cAMP to surface cAMP-binding proteins. These observations are consistent with our interpretation that probenecid and sulfinpyrazone inhibit cAMP-induced pigment granule aggregation by reducing transporter-mediated import of cAMP into the RPE cells, rather than by interfering with a surface cAMP receptor. Furthermore, the failure of adenosine or ATP to trigger pigment migration is inconsistent with cAMP crossover effects on surface adenosine or nucleotide receptors.

Nondonerivatized cAMP has been observed to affect numerous physiological processes in isolated RPE in addition to pigment granule aggregation, including hyperpolarization of the RPE basal membrane, increased basal membrane resistance, and decreased ERG c-wave in chick RPE,44 modulation of trans-RPE fluid45 and ion46 transport, inhibition of phagocytosis,47 and pigment dispersion5 in frog RPE. Together these observations suggest that if cAMP were released into the subretinal space, it could have significant effects on RPE cell physiology.

We hypothesize that changes in cAMP levels in the subretinal space could be produced by dark/light fluctuations in cAMP efflux from photoreceptors. Many observations are consistent with a model in which photoreceptor cAMP levels are elevated in the dark and fluctuate on a diurnal or circadian cycle: cAMP stimulates melatonin synthesis,48 and melatonin synthesis is elevated in the dark,49 depolarization of photoreceptors in vitro increases intracellular cAMP levels50 and photoreceptors are more depolarized in the dark;51 and treatments that elevate intracellular cAMP induce both rods and cones to assume the retinomotor posi-

### TABLE 2. Effects of Extracellular Messengers on Pigment Granule Position

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<th>Agent</th>
<th>Concentration</th>
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<td></td>
<td>1 µM</td>
<td>.92 ± .03</td>
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<tr>
<td></td>
<td>100 µM</td>
<td>.92 ± .02</td>
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<td></td>
<td>1 µM</td>
<td>.79 ± .01</td>
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* % EtOH control; † 0.01% EtOH/0.25 mM IBMX control; ‡ 0.25 mM IBMX control.

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tions induced by darkness. Cyclic AMP efflux has been described in many cell types and has been shown to be a first-order process related to intracellular cAMP accumulation. Diurnal cycles of cAMP efflux have been observed in cultured pineal cells in which elevated cAMP levels and elevated melatonin release occur at night. Orr et al. showed that the dark-associated increase in retinal cAMP levels is most marked in the outer nuclear and outer plexiform layers. Accumulation of intracellular cAMP in photoreceptors or in the outer plexiform layer could lead to efflux of cAMP into the extracellular space.

If efflux of cAMP from the photoreceptors occurred, the ongoing vitreous-to-choroid movement of fluid in vivo would tend to carry the cAMP toward the RPE. Increased cAMP in the subretinal space could lead to entry of cAMP into RPE cells via organic anion transporters with consequent triggering of dark-specific physiological responses. Thus, cAMP could act as a retina-to-RPE first messenger to signal darkness. If cAMP acts as a dark retina-to-RPE signal as we suggest here, and if dopamine acts as a light signal as we proposed earlier, then both these signaling mechanisms might participate in diurnal or circadian regulation, or both, of retinomotor movements in vivo. The existence of multiple signaling pathways might explain the recent observation that after destruction of dopaminergic interplexiform cells by 6-hydroxydopamine, RPE continues to undergo light- and dark-induced retinomotor movements.

Our proposed role for cAMP as an intercellular messenger in the subretinal space is consistent with recent observations suggesting a role for extracellular cAMP in intercellular signaling in the vertebrate circulatory system. Vascular endothelial cells respond to adrenergic stimulation by secreting cAMP, which stimulates phosphorylation of pyruvate kinase in red blood cells and causes a decrease in the adherence of polymorphonuclear lymphocytes, thus producing the increased numbers of circulating polymorphonuclear lymphocytes associated with the "fight or flight" response. Cardiac myocytes have also been shown to have a cell-surface cAMP receptor that regulates Na+ conductivity. Thus, cAMP may play important roles in extracellular signaling in metazoans as well as in the extensively studied slime mold Dictyostelium.

Because forskolin triggers pigment granule aggregation in RPE sheets, the absence of effects by numerous agents shown to activate RPE adenyl cyclase in other species is surprising. We did not determine whether any of the agonists we tested actually raised intracellular cAMP in fish RPE sheets. It is possible that fish RPE have receptors for the tested agonists but that induced increases in cAMP were subthreshold for stimulating pigment granule aggregation. It is also possible that the low Ca2+ concentration in our Ringer compromised signaling mechanisms requiring calcium. Because forskolin induces maximal pigment aggregation in isolated fish RPE sheets, these cells clearly have a functional adenyl cyclase capable of generating sufficient cAMP to induce pigment aggregation, even in the absence of IBMX. However, a low basal activity is suggested by the failure of high concentrations of IBMX to stimulate pigment aggregation. The enhancement of cAMP-induced pigment granule aggregation by 0.1 mM IBMX suggests that RPE cells do have PDE activity that can be inhibited by IBMX.

We were unable to demonstrate an effect of melatonin and its less labile analog 2-iodomelatonin on pigment position in RPE sheets isolated from green sunfish. This result suggests that fish RPE do not respond directly to melatonin, and it is consistent with previous observations from our laboratory that melatonin has no effect on RPE or cone retinomotor movements either in isolated fish RPE/retinas or in intraocular injection experiments.

In conclusion, we have shown that nonderivatized cAMP can activate pigment granule aggregation in isolated RPE sheets and that this activation is inhibited by the organic anion transport inhibitors probenecid and sulfipyrazone. We suggest that extracellular cAMP may be elevated in the subretinal space in the dark and that cAMP could enter RPE cells via organic anion transporters to influence RPE physiological processes. Thus, we suggest that extracellular cAMP could act as a signal for darkness from retina to RPE.

Key Words
RPE, cAMP, pigment granule aggregation, organic anion transport inhibitors, dark adaptation

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