Stimulation of A_2 Adenosine Receptors Inhibits the Ingestion of Photoreceptor Outer Segments by Retinal Pigment Epithelium

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Purpose. Recent studies have shown that A_2 adenosine receptors are present in retinal pigment epithelium (RPE). In this study, the effect of adenosine and adenosine analogues on photoreceptor outer segment (ROS) phagocytosis by RPE was investigated.

Methods. Primary cultures of RPE cells were incubated with isolated outer segments in the presence of various adenosine derivatives. Changes in adenylyl cyclase activity was measured by cyclic adenosine monophosphate (cAMP) production using a radioimmunoassay detection system.

Results. Adenosine inhibited the ingestion phase of phagocytosis (IC_{50} = 50 μM), and this effect was potentiated 80-fold in the presence of dipyridamole (IC_{50} = 0.6 μM). In the presence of 10 μM 8-phenyltheophylline, the inhibitory effect of 100 μM adenosine was reduced from 80% inhibition of ROS ingestion to 33% inhibition. The rank order of potency of adenosine analogues to inhibit ROS ingestion by RPE was N^6-cyclohexyladenosine/5'-[N-ethylcarboxamido]-adenosine (NECA) = NECA > adenosine >> [R]-N^6-[2-phenylisopropyl]-adenosine. The greatest stimulation of cAMP production was observed with 33.3 μM NECA. The production of cAMP reached its maximum level after 2 minutes of incubation, and after 10 minutes the levels of cAMP were back to basal.

Conclusions. These results suggest that adenosine and adenosine analogues modulate ROS ingestion by RPE via activation of adenosine A_{2b} receptors, possibly through the cAMP intracellular signaling pathway. Invest Ophthalmol Vis Sci. 1994;35:819–825.

Recent biochemical studies on the retinal pigment epithelium (RPE) and its physiologic functioning have led to the identification of a number of pharmacologic receptors on the apical and basal surfaces of the RPE. Such receptors include adrenergic receptors,[1–5] dopaminergic receptors,[2] cholinergic receptors,[6] prostaglandin receptors,[7] and benzodiazepine receptors.[8] Recently, the RPE was shown to possess another receptor, the A_2 adenosine receptor,[9–10] which is generally associated with modulation of neurotransmission in both central and peripheral nervous systems. Pharmacologic studies have led to the classification of a number of subtypes of adenosine receptors.[11–13] High-affinity A_1 receptors are coupled to adenylyl cyclase via the inhibitory guanine nucleotide binding protein. A_2 receptors are linked to adenylyl cyclase via the stimulatory guanine nucleotide binding protein (Gs), and are of two subclasses, an A_{2a} high-affinity receptor present in the striatum and limbic structures, and an A_{2b} low-affinity receptor present throughout the brain.[14] More recently, an A_3 receptor has been proposed that inhibits neurotransmitter release.[15] Finally, there is an intracellular regulatory site on adenylyl cyclase called the "P-site" that inhibits enzyme activity.[11]

Activation of A_2 receptors stimulates the production of cyclic adenosine monophosphate (cAMP) via adenylyl cyclase. This second messenger has been shown to be involved in the regulation of a number of RPE cell functions such as fluid transport,[16] dark-adaptive pigment migration in teleost fish,[2] cell migra-

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tion,[17] and electrical activity of the RPE.[18] Photoreceptor outer segment (ROS) phagocytosis by the RPE[19] is also reduced by cAMP and phosphodiesterase inhibitors.[20–22] In the retina, A_1 receptors have been implicated in the modulation of acetylcholine release,[23] and in other systems, adenosine is known to be an intercellular messenger.[24–27] Although the role of the A_2 receptor in RPE cells has not yet been determined, it may be involved in intercellular cross-talk between the retina and the RPE.

In this study, we have tested the hypothesis that stimulation of A_2 adenosine receptors modulates ROS phagocytosis by the RPE. Initial studies suggested that ROS phagocytosis could be inhibited by adenosine receptor agonists,[28] and we have now further characterized this relationship.

MATERIALS AND METHODS

All investigations conformed to the ARVO Resolution on the Use of Animals in Research.

RPE Cell Culture

RPE cells were isolated from 9- to 11-day-old normal (Long Evans) rats as previously described.[29] Primary cultures of RPE cells were cultured at 37°C in 5% CO_2/95% air on 18-mm glass coverslip discs in growth medium, which consisted of Earle's minimum essential medium (MEM; Mediatech, Washington, DC) containing 10% fetal bovine serum, 40 μg/ml gentamycin, 40 μg/ml kanamycin, and 2 mM L-glutamine. After 7 days in culture, the confluent RPE cells were used in experiments.

ROS Phagocytosis Assay

ROS were isolated from adult Long Evans rat according to published methods,[30] and then the purified ROS were suspended in growth medium at a concentration of 2×10^7 OS/ml (2× ROS). Each disc of RPE cells was preincubated for 15 minutes with 1 ml of test drugs (see below), and then 1 ml of 2× ROS was added (final concentration, 1×10^7 OS/ml) for 1 hour at 37°C, 5% CO_2. After incubation, the RPE cells were washed twice in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 145 mM NaCl, pH 7.2), containing 1.27 mM $CaCl_2$ and 0.81 mM $MgSO_4$ (PBS-Ca^{2+}/Mg^{2+}), and then fixed in 3.5% formaldehyde. ROS that were bound and ingested by the RPE cells were stained using a double immunofluorescent technique.[30] The number of bound and ingested ROS was counted on duplicate discs according to previously described criteria.[30]

Experimental Drug Treatment of RPE Cells

All drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Phagocytosis of ROS or cAMP dose–response analyses were carried out after incubating RPE cells with the following drugs: adenosine, 5′-[N-ethylcarboxamido]-adenosine(NECA), [R]-N^6-[2-phenylisopropyl]-adenosine (R-PIA), N^6-cyclohexyladenosine (CHA), 8-phenyltheophylline (8-PT), and dipyridamole. In studies using 8-PT and dipyridamole, cells were preincubated with these drugs for 15 minutes, then the test agonist was added to the incubation medium for a further 15 minutes before the addition of ROS. All drugs were dissolved in DMSO (final concentration less than 0.05%), and then serially diluted with MEM to the required concentration.

Adenylyl Cyclase Assay

Adenylyl cyclase activity was measured by the production of cAMP. Discs of RPE cells were incubated with various drugs, and then the cells were washed for 2×5 seconds in PBS-Ca^{2+}/Mg^{2+}. The cells were then quickly scraped off the discs into microfuge homogenization tubes (Kontes, Vineland, NJ) containing 100 μl of 0.1 N HCl on ice. The samples were homogenized with a micropestle (Kontes) for 10 seconds at 3500 rpm. Aliquots (2×10 μl) were removed from each sample for protein determination using the BCA protein assay kit according to manufacturer's instructions (Pierce, Rockford, IL). The remainder of each sample was centrifuged for 4 minutes at 12,000 rpm at 4°C. The supernatants were removed to fresh tubes and the samples were neutralized to pH 6.0 using the Assay Buffer provided with the Rianen cAMP [^{125}I] radioimmunoassay kit (NEN-DuPont, Billerica, MA). cAMP production was measured using the acetylated (plasma) protocol according to manufacturer's specifications.

RESULTS

Effect of Adenosine and Adenosine Analogues on ROS Phagocytosis

To determine whether a drug acts on the binding or ingestion phase of ROS phagocytosis, the number of ROS bound is compared to the number of ROS ingested. In steady-state phagocytosis (controls), we observed that 22% of the total number of ROS were bound and 78% of the ROS were ingested (Fig. 1, treatment C). In the presence of 100 μM adenosine, 80% of the total number of ROS were bound and only 20% of the ROS were ingested (Fig. 1, treatment A). This indicated that the effect of adenosine was to inhibit the ingestion phase of ROS phagocytosis. The concomitant increase in the number of ROS bound was expected, since the ROS binding sites become saturated due to bound ROS, which are not ingested. One of the characteristics of adenosine A_1 and A_2 receptors is that activation of these receptors can be in-

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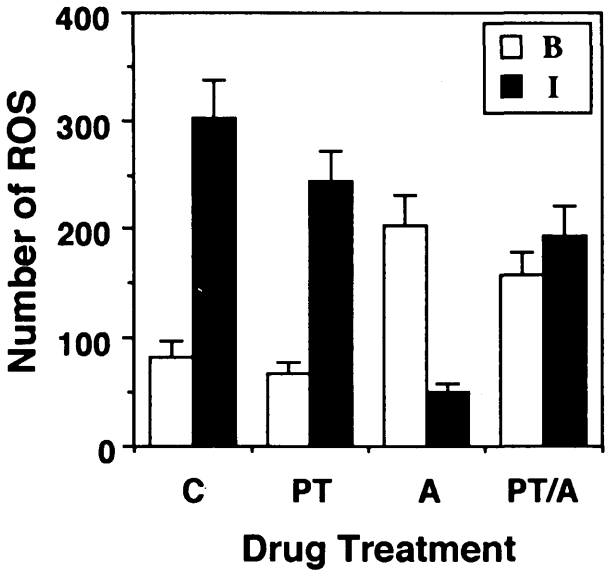

FIGURE 1. The effect of adenosine on ROS phagocytosis. Cultured RPE cells were incubated for 15 minutes with drugs before the addition of ROS for 1 hour. Bound (B) and ingested (I) ROS were distinguished using a double immunofluorescent assay. C = control cells; PT = 10 μM 8-phenyltheophylline; A = 100 μM adenosine; PT/A = 100 μM adenosine in the presence of 10 μM 8-phenyltheophylline. Bars show the mean of four determinations ± SD.

hibited by methylxanthine antagonism.[31] We used 8-PT because it is a potent adenosine receptor antagonist with little effect on cAMP phosphodiesterase activity.[32] Ten micromolar 8-PT alone had no effect on ROS binding or ingestion within one standard deviation of error (Fig. 1, treatment PT). When RPE cells were pretreated with 8-PT, however, the inhibitory effect of adenosine on ROS ingestion was reduced (Fig. 1, treatment PT/A). Adenosine, in the presence of 8-PT, inhibited ROS ingestion by only 33%, compared to 80% inhibition of ROS ingestion when RPE cells were incubated with adenosine alone. 8-PT partially antagonizes the effect of adenosine by slowing the rate of ingestion of ROS. Because ROS binding is unaffected by either drug, the number of bound ROS increases in proportion to the decrease in ROS ingestion. This suggested that adenosine inhibited ROS ingestion via adenosine receptors.

To characterize further this adenosine-mediated inhibition of ROS ingestion, dose–response curves were constructed. We observed that ROS ingestion was inhibited by adenosine in a dose-dependent manner, with an IC_{50} of 50 μM (Fig. 2). Dipyridamole alone (50 μM) had no effect on binding (data not shown) or ingestion of ROS (Fig. 2C). Dipyridamole is known to inhibit the active uptake system for adenosine in cells.[33] In the presence of 50 μM dipyridamole, the IC_{50} for inhibition of OS ingestion by adenosine was potentiated approximately 80-fold, to a value of 0.6 μM (Fig. 2). This suggests that more adenosine was available to bind to extracellular receptors, thereby increasing the inhibitory effect of the drug.

To determine more specifically whether the adenosine-mediated inhibition of ROS ingestion was due to activation of A_1 or A_2 receptors, we compared the IC_{50} values (50% inhibition of ingestion) of specific adenosine receptor agonists from their dose–response curves (Fig. 3). NECA is a mixed A_1–A_2 agonist, and R-PIA and CHA are selective A_1 receptor agonists. The dose–response curve to NECA was repeated in the presence of CHA, to eliminate selectively the A_1 component of the response. We chose to use CHA rather than R-PIA to bind out A_1 activity, because CHA has a much lower affinity for A_2 receptors than R-PIA.[14] The rank order of potency (with IC_{50} values in parentheses) for these agonists to inhibit ROS ingestion by RPE was CHA/NECA (4 μM) = NECA (6 μM) > adenosine (60 μM) >> R-PIA (400 μM). This same rank order has been reported for numerous A_2 responses, including A_2 receptor activation in RPE[9] and in other systems,[14,32,34] and indicates that the inhibitory effect on ROS ingestion by adenosine is elicited via the A_2 adenosine receptor subtype.

A_2 Receptor Modulation of cAMP Levels in RPE

Because A_2 adenosine receptors stimulate the production of cAMP, through Gs-mediated activation of

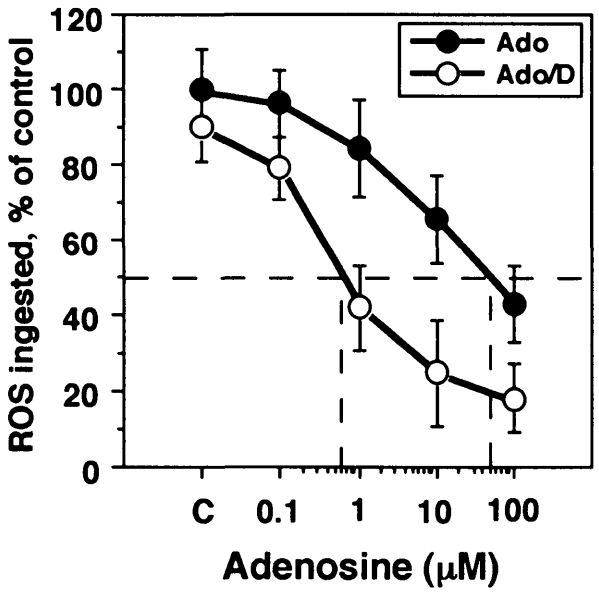

FIGURE 2. The effect of inhibition of adenosine uptake on ROS ingestion. Cultured RPE cells were preincubated (C) for 15 minutes with 50 μM dipyridamole (D) before addition of 0.1 to 100 μM adenosine (Ado) for 15 minutes, before ROS addition. Dashed lines indicate the IC_{50} values for Ado in the presence and absence of D. Results are presented as the mean of four determinations ± SD.

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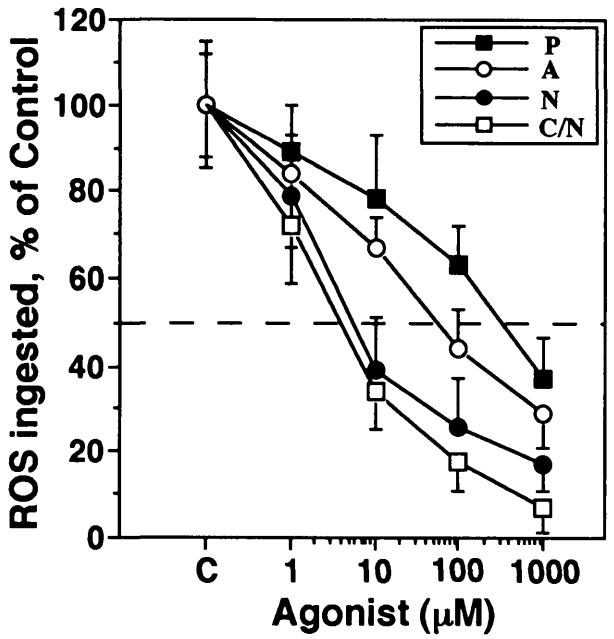

FIGURE 3. The effect of adenosine receptor agonists on ROS ingestion. RPE cells were preincubated for 15 minutes with test agonists, before addition of ROS for 1 hour. The number of ROS ingested was determined using a double immunofluorescent assay. The bars represent the mean of six determinations ± SD. P = R-PIA; A = adenosine; N = NECA; C/N = NECA in the presence of CHA. Dashed line indicates IC_{50} level.

adenylyl cyclase, the ability of 10 μM NECA to stimulate cAMP production was measured. cAMP accumulation was very rapid for the first 2 minutes, whereupon it reached the maximum response (Fig. 4A). Levels of cAMP then began to fall, and after 10 minutes of incubation the levels of cAMP were almost back to basal levels. This is slightly different from the response seen in human RPE cells,[9] where a linear time course was observed for the first 5 minutes. However, in those experiments the endogenous levels of adenosine were decreased by pretreating cells with papaverine and adenosine deaminase, which may account for the difference.

We also investigated the production of cAMP by RPE cells using a range of NECA concentrations and a fixed incubation time of 3 minutes to give a maximal response. No significant increase in cAMP production was observed with 0.1 to 3.3 μM NECA (Fig. 4B). The maximum cAMP production was observed when RPE cells were stimulated by 33.3 μM NECA, which gave a tenfold increase over basal levels. A concentration of 100 μM NECA produced only a threefold increase in cAMP production, indicating that there is a narrow concentration window for maximal activation of A_2 receptors by NECA. The A_{2a} subclass of adenosine receptors exhibits an EC_{50} of about 0.5 μM in brain, compared to the A_{2b} subclass of receptors, which has an EC_{50} of 10 to 20 μM.[14] By these criteria, our results indicate that NECA is acting on the low-affinity A_{2b} adenosine receptor to inhibit the ingestion of outer segments.

cAMP levels were also measured in RPE cells that had been pretreated with NECA, and then incubated for 1 hour with ROS. At concentrations of NECA that inhibited ROS ingestion (1.0, 10, and 100 μM), only a small increase in the production of cAMP was observed (Fig. 5). However, Figure 4A shows that NECA treatment increases cAMP within the first 10 minutes

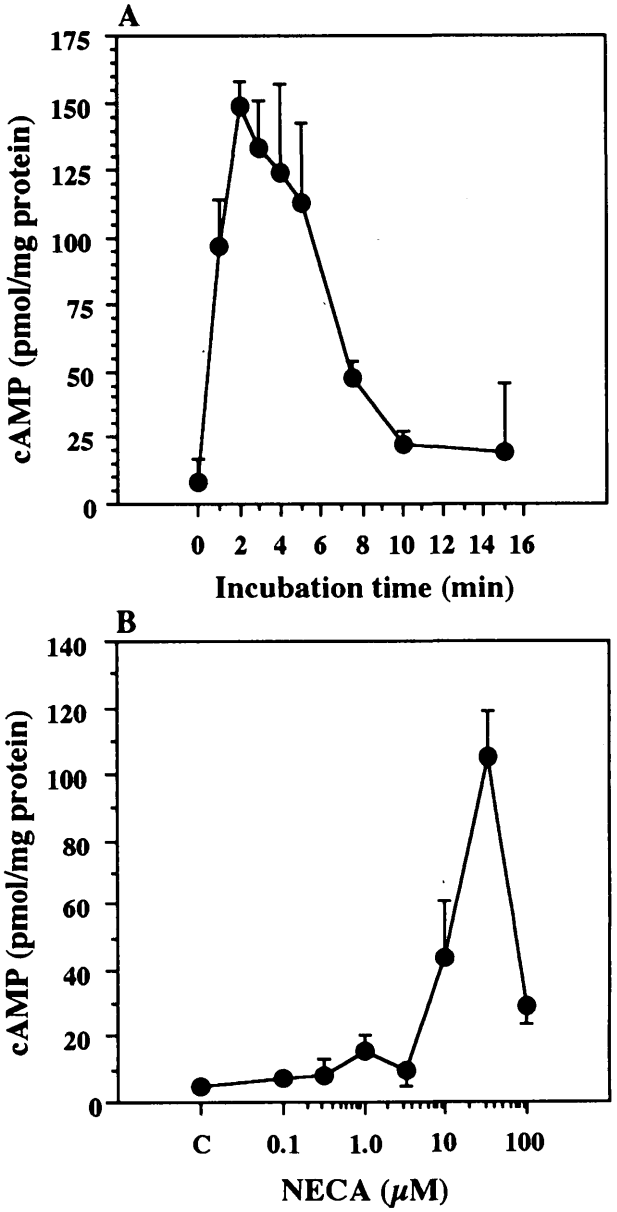

FIGURE 4. Dose–response and time–response analyses for NECA to stimulate cAMP production. cAMP was measured by radioimmunoassay. (**A**) RPE cells were incubated with 10 μM NECA for the indicated times. (**B**) RPE cells were incubated for 3 minutes with a range of concentrations of NECA. Each point is the mean of six determinations ± SD.

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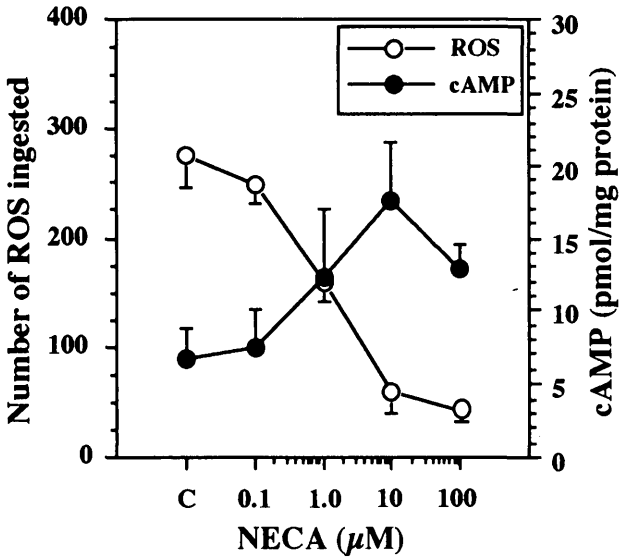

FIGURE 5. Effect of NECA on ROS ingestion compared to cAMP production. RPE cells were incubated for 15 minutes with NECA before addition of ROS. After a 1-hour incubation, ROS ingestion was measured by an immunofluorescent assay and cAMP was determined by a radioimmunoassay.

of incubation, and thereafter the levels return to basal. Due to the experimental parameters used to measure ROS ingestion (1 hour of incubation), it is not surprising that no significant stimulation of cAMP production is seen.

DISCUSSION

It has been shown, both biochemically[9] and immunohistochemically,[10] that RPE cells possess A_2 adenosine receptors. The evidence that A_2 receptors are present on RPE cells relies on the A_1/A_2 selectivity of agonists in dose–response studies.[11] In addition, the pharmacologic classification of adenosine receptors rests on their differential action on adenylyl cyclase. In this study, we tested the hypothesis that OS phagocytosis could be modulated by increased cAMP levels stimulated by A_2 receptor agonists.

We have shown that NECA stimulated the production of cAMP, which indicates an A_2 receptor response. Both the time–response and dose–response curves for cAMP production indicate that there are specific windows of activation of these adenosine receptors. Interestingly, cAMP production occurs rapidly after stimulation of the adenosine receptors (within the first 2 minutes), as might be expected for a signaling mechanism, yet the cellular response of inhibition of ROS ingestion is still in effect at the time of measurement, 75 minutes after presentation of the drug to the cells and at a time when cAMP levels have returned to basal. This, however, does not rule out cAMP mediating the observed inhibition of ROS ingestion by NECA, since cAMP can activate either protein kinase A and AII, which in turn phosphorylates various cellular proteins that may be associated with the control of ROS ingestion. These latter responses are likely to occur within a longer frame time than the initial cAMP response. cAMP production was maximally stimulated by 33.3 μM NECA, which suggests that the A_2 adenosine receptor present in RPE is the low-affinity A_{2b} subclass.[14] Bovine RPE membranes have been shown to exhibit the pharmacology associated with A_{2b} receptors,[35] and our studies in cultured RPE cells confirm the presence of this subtype of adenosine receptor.

In dose–response analyses for ROS phagocytosis, the ingestion phase of ROS phagocytosis was inhibited by adenosine agonists, and this inhibitory effect of adenosine could be potentiated 80-fold by inhibiting adenosine uptake into RPE cells. By ranking the order of potency of adenosine agonists to inhibit ROS ingestion, our data indicate that inhibition of ROS ingestion occurs via activation of A_2 adenosine receptors. The IC_{50} for NECA to inhibit ROS ingestion by RPE is 6 μM, again suggesting that the A_{2b} adenosine receptor is mediating the inhibition of ROS ingestion. At the protein level, the A_2 receptor is a glycoprotein with an apparent molecular mass of 45 kD.[34] We have previously shown that RPE cells contain a glycoprotein of this molecular mass,[36] and it is possible that this RPE protein is the adenosine receptor.

If these adenosine receptors are physiologically relevant in vivo, then one would expect the in vivo ligand (in this case adenosine) to possess a number of characteristics typical of neuromodulatory substances, such as specific cell localization, release by a specific stimulus, and a pathway of removal for excess extracellular ligand after release. For example, in the inner retina, adenosine has been shown to modulate the release of acetylcholine from the retina via A_1 receptors,[23] and this process has been characterized in some detail. A high-affinity uptake system for adenosine into ganglion cells[37,38] and displaced amacrine cells[39] has been demonstrated. The release of endogenous ^{3}H-purines from the retina has been reported in rabbit retina by potassium depolarization[40] and by light flashes.[41] Additionally, these light flashes also cause the release of acetylcholine.[42] Finally, the distribution of adenosine deaminase, which metabolizes adenosine into inosine, corresponds to the cells that accumulate adenosine.[43] All of these parameters support the role of adenosine as a neuromodulator at A_1 receptors.

There is a dearth of evidence available at present to support an in vivo neuromodulatory role for adenosine at the RPE A_2 receptor; however, the results from a few studies could lend support to this hypothesis. Adenosine uptake[44] and immunoreactivity[36] have been demonstrated in the ROS of photoreceptors. Because

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there is an intimate association between ROS and the apical villi of the RPE,[45] this could represent a source of adenosine. Alternatively, adenosine could be stored in the RPE cells themselves and then be released in some form of feedback regulation. The only evidence to support such a possibility comes from these studies, where we have shown that there is uptake of adenosine by RPE cells that can be inhibited by dipyridamole. In addition, the enzyme 5′-nucleotidase is involved in the synthesis of adenosine and has been isolated from bovine outer segments[46] and localized to RPE apical processes,[47] showing redistribution during shedding and phagocytosis.[48] As yet, there have been no studies to investigate the stimulatory release of adenosine from either the ROS or RPE; neither has any immunohistochemical analysis on adenosine deaminase localization been carried out. These parameters will be the subject of future studies. Evidence against adenosine being an in vivo ligand at the A_2 receptor stems from the observation that light flashes cause release of purines from the retina.[41] One might expect that during normal ROS shedding after the onset of light, the release of adenosine would inhibit phagocytosis of ROS according to our data. However, phagocytosis is occurring rapidly at this time.[49] It is possible that continuous light is required for release of sufficient adenosine to turn off the phagocytosis of ROS by RPE cells in vivo.

Although the specific role of adenosine receptors in RPE is unknown, these receptors effect responses via modulation of cAMP levels. cAMP is known to modulate a number of RPE cells functions in vitro, including ROS phagocytosis,[20–22] fluid and ion transport,[50] and alterations in membrane conductance.[51] We have shown in this study that activation of A_2 receptors can modulate ROS phagocytosis, possibly via the cAMP pathway; further studies, however, are required to ascertain whether this is the physiologic function of these receptors.

Key Words

A_2 receptors, adenosine, retinal pigment epithelium, phagocytosis, cyclic adenosine monophosphate

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