A₃ adenosine receptors regulate Cl⁻ channels of nonpigmented ciliary epithelial cells

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The aqueous humor is formed by the ciliary epithelium, which comprises two cell layers: the outer pigmented cells facing the stroma and the inner nonpigmented (NPE) cells in contact with the aqueous humor. Secretion is thought to reflect a primary transfer of solute, principally NaCl, from the stroma into the aqueous humor, with the secondary transfer of water down its chemical gradient. One major factor governing the rate of secretion is the rate of Cl⁻ release from the NPE cells into the aqueous humor (3).

Recently, adenosine has been found to activate NPE Cl⁻ channels that subserve this release (2). Adenosine triggered isotonic shrinkage of cultured human cells from the human ciliary epithelial (HCE) cell line. The contribution of Cl⁻ channels to this shrinkage was identified by performing the experiments in the presence of the cationic ionophore gramicidin. In addition, adenosine produced a Cl⁻-dependent increase in short-circuit current across rabbit iris-ciliary body while the nonmetabolizable adenosine analog 2-Cl-adenosine was shown to activate Cl⁻ currents in HCE cells using the whole cell patch-clamp technique. Although this study clearly established that adenosine could activate Cl⁻ channels on NPE cells, the concentrations of agonist used were capable of stimulating all four known adenosine receptor subtypes: A₁, A₂A, A₂B, and A₃ (12, 13, 25). Ciliary epithelial cells are known to possess A₁, A₂A, and A₂B adenosine receptors (27, 35, 36). Although stimulation of these receptors can be associated with specific changes in the levels of second messengers CaM (6, 35, 36) and Ca²⁺ (11), the effect of these receptors on Cl⁻ channels of NPE cells is unknown.

The aim of the present study was to determine which receptor mediates the activation of Cl⁻ channels by adenosine. We now report that A₃ receptors are present on human and rabbit NPE cells and underlie the activation of NPE Cl⁻ channels by adenosine.

**MATERIALS AND METHODS**

Cultured cells. We have continued to study the HCE cell line (2), an immortalized NPE cell line developed by one of us (M. Coca-Prados) from primary cultures of adult human epithelium. Cells were grown in DMEM (no. 11965-027; Gibco BRL, Grand Island, NY) with 10% FBS (A-1115-L; HyClone Laboratories, Logan, UT) and 50 µg/ml gentamycin (no. 15750-011, Gibco BRL), at 37°C in 5% CO₂ (36). The growth medium had an osmolality of 328 mosmol. Cells were passaged every 6–7 days and were studied 8–13 days after passage, after reaching confluence.

Measurement of cell volume in isosmotic solution. A 0.5-ml aliquot of the cell suspension in DMEM was added to 20 ml of each test solution, which contained (in mM): 110.0 NaCl, 15.0 HEPES, 2.5 CaCl₂, 1.2 MgCl₂, 4.7 KCl, 1.2 KH₂PO₄, 30.0 NaHCO₃, and 10.0 glucose, at a pH of 7.4 and osmolality of 298–305 mosmol. Parallel aliquots of cells were studied on the same day. One aliquot usually served as a control, and the others were exposed to different experimental conditions at the time of suspension. The same amount of solvent vehicle (dimethylformamide, DMSO, or ethanol) was always added to the control and experimental aliquots. The sequence of studying the suspensions was varied to preclude systematic time-dependent artifacts (4).

Cell volumes of isosmotic suspensions were measured with a Coulter Counter (model ZBI-Channelyzer III), using a
100-µm aperture (4). As previously described (37), the cell volume \( V_c \) of the suspension was taken as the peak of the distribution function. Cell shrinkage was fit as a function of time (t) to a monoeponential function

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V_c = V_o + (V_0 - V_o) \cdot (\frac{1}{1 + e^{-t/t^*}})
\]

where \( V_o \) is the steady-state cell volume, \( V_0 \) is the cell volume at the first point \( t_0 \) of the time course to be fit, and \( t^* \) is the time constant of the shrinkage. For purposes of data reduction, the data were normalized to the first time point, taken to be 100% isotonic volume. Fits were obtained by nonlinear least-squares regression analysis, permitting both \( V_o \) and \( t^* \) to be variables.

Transpalpebral measurements. Adult male Dutch belted rabbits weighing 1.8–2.4 kg (Ace Animals, Boyertown, PA) were anesthetized with pentobarbital sodium and killed (1). After enucleation, the iris-ciliary body was isolated as previously described (1). The experiments were in accordance with the Resolution on the Use of Animals in Research of the Association for Research in Vision and Ophthalmology.

The pupil and central iris were occluded with a Lucite disc, and the iris-ciliary body was mounted between the two halves of a Lucite chamber (1). The annulus of exposed tissue provided a projected surface area of 0.93 cm². Preparations were continuously bubbled with 95% O₂-5% CO₂ for maintenance of pH 7.4 in a Ringer solution comprising (in mM): 110.0 NaCl, 10.0 HEPES (acid), 4.0 HEPES (Na⁺), 30.0 NaHCO₃, 2.5 CaCl₂, 1.2 MgCl₂, 5.9 KCl, and 10.0 glucose, at an osmolality of 305 mosmol. BaCl₂ (5 mM) was added to the solution to block \( K^+ \) currents. The transepithelial potential difference was monitored on a chart recorder.

Measurements of intracellular Ca²⁺. HCE cells grown on coverslips for 24–48 h were loaded with 1–5 µM fura 2-AM for 30–45 min at room temperature. The cells were subject to a postincubation interval of 20–40 min at room temperature before recording began. The coverslips were mounted on a Nikon Diaphot microscope and visualized with a ×40 oil-immersion fluorescence objective. The emitted fluorescence (510 nm) from 10–12 confluent cells was acquired at a temperature ranging from 58 to 48°C. The resulting PCR product was size-fractionated by electrophoresis on 1% agarose gel. To sequence the PCR product, a band of the expected size (462 bp) was extracted from low-melting point agarose gel using a Qiaex II Agarose Gel Extraction kit. The purified PCR product was reamplified using the touchdown PCR method with fresh primers and Taq polymerase, using an annealing temperature ranging from 58 to 48°C. The resulting PCR product was sequenced from an ABI/100 sequencer by the DNA Sequencing Facility at the Cell Center of the University of Pennsylvania and compared with the predicted sequence using a DNASTAR program.

The RT-PCR assay of rabbit A3 receptor message was conducted in the same way with the following changes. RNA was obtained from the tips of New Zealand White rabbit ciliary processes using TRIzol reagent and was reverse transcribed using 3–6 µg total RNA, MuLV RT, and oligo(dT) primers. The reaction was carried out at 42°C for 30 min, followed by 5 min at 95°C. The PCR reaction was conducted for 35 cycles, each cycle comprising 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. The final extension was prolonged by 7 min at 72°C. The PCR reamplification was performed using the touchdown PCR method with fresh primers and Taq polymerase, using an annealing temperature ranging from 58 to 48°C. The resulting PCR product was size-fractionated by electrophoresis on 1% agarose gel. To sequence the PCR product, a band of the expected size (462 bp) was extracted from low-melting point agarose gel using a Qiaex II Agarose Gel Extraction kit. The purified PCR product was directly sequenced on an ABI/100 sequencer by the DNA Sequencing Facility at the Cell Center of the University of Pennsylvania and compared with the predicted sequence using a DNASTAR program.

The product of the PCR reamplification of rabbit tissue was cloned into the PCR-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer’s directions. After transformation, plasmids were isolated using the Wizard Plus Miniprep DNA Purification System (Promega, Madison, WI). The cloned plasmid was cut with EcoRI restriction nuclease, and a band of approximately the expected size (479 bp) was identified by running the cut product on an agarose gel. The plasmid was sequenced from the S6 promoter site 80 bp proximal to the PCR product. The sequence was compared with the expected rabbit A3 sequence using a DNASTAR program.
Chemicals. All chemicals were reagent grade. Gramicidin and adenosine were purchased from Sigma Chemical (St. Louis, MO). N\textsuperscript{6}-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (IB-MECA), and 2-chloro-1B-MECA (Cl-IB-MECA) were obtained from Research Biochemicals International (Natick, MA). Fura 2-AM was bought from Molecular Probes (Eugene, OR). MRS-1097, MRS-1191, and MRS-1235 were graciously gifts from Drs. Kenneth A. Jacobson (National Institutes of Health) and Bruce L. Liang (University of Pennsylvania).

Data reduction. Values are presented as means ± SE. The null hypothesis, that the experimental and baseline measurements shared the same mean and distribution, was tested with Student's t-test and by the upper significance limits of the F-distribution, as indicated. The t-test was applied to compare the significance between single means or single fit parameters. The F-distribution was applied to test whether the time course of volume measurements in different suspensions could reflect a single population of data points.

RESULTS

Human NPE cells. In previous studies demonstrating that adenosine causes isotonic cell shrinkage by activating Cl\textsuperscript{−} channels in NPE cells (2), the levels of adenosine used were sufficiently high to activate A\textsubscript{1}, A\textsubscript{2A}, A\textsubscript{2B}, or A\textsubscript{3} adenosine receptor subtypes (12, 13, 25). To differentiate among these receptors, the experiments were repeated in the present study using a series of agonists and antagonists selective for these receptors. Because we wished to identify the effects of these receptors specifically on Cl\textsuperscript{−} channels, 5 \( \mu \text{M} \) gramicidin D was included in all solutions to eliminate any potential contribution from K\textsuperscript{+} channels. This ionophore readily partitions into plasma membranes to form a cation-selective pore and is widely used for studying volume regulation (16). Under these conditions, release of cell Cl\textsuperscript{−} becomes the rate-limiting factor in both hypo- (4) and isosmotic cell shrinkage (2).

In the presence of gramicidin, the A\textsubscript{3} agonist IB-MECA caused the cells to shrink in a concentration-dependent manner (Fig. 1, A and B). Least-squares analysis of the linearized Lineweaver-Burke plot generated from monoeponential fits of these data indicates that the apparent K\textsubscript{a} for the IB-MECA-induced shrinkage was 55 ± 10 \( \text{nM} \) (Fig. 1C). IB-MECA is specific for the A\textsubscript{3} receptor; the K\textsubscript{a} for the A\textsubscript{3} receptor is 50 times lower than it is for A\textsubscript{1} or A\textsubscript{2A} receptor (14, 20, 21). Cl-IB-MECA is even more specific for A\textsubscript{3} receptors, with a K\textsubscript{a} for A\textsubscript{3} receptors 2,500 times lower than for A\textsubscript{1} receptors and 1,400 times lower than for A\textsubscript{2A} receptors. The ability of Cl-IB-MECA to induce cell shrinkage (Fig. 1D) further strengthens the hypothesis that stimulation of A\textsubscript{3} receptors stimulates Cl\textsuperscript{−} channels.

We also tested whether we could use A\textsubscript{3}-selective antagonists to prevent the putative A\textsubscript{3}-mediated shrinkage produced by IB-MECA. We preincubated parallel aliquots of suspensions with MRS-1097, an A\textsubscript{3}-selective antagonist.
antagonist with $K_i$ values for the binding (in nM) to human $A_1, A_2,$ and $A_3$ receptors of 5,930, 4,770, and 108, respectively (22). Preincubation for 2 min with 300 nM MRS-1097 blocked the isosmotic shrinkage characteristically triggered by 100 nM IB-MECA (Fig. 2A). We also used a second highly selective $A_3$ antagonist, MRS-1191 (24), with $K_i$ values for the binding (in nM) to human $A_1, A_2,$ and $A_3$ receptors of 40,100, >100,000, and 31.4, respectively (22). Preincubation for 2 min with 100 nM MRS-1191 also prevented the subsequent response to 100 nM IB-MECA (Fig. 2B). There was an indication in the results of Fig. 2B that MRS-1191 might actually produce a small amount of cell swelling. This was not a constant finding (Fig. 3B), and may have reflected variations in the background level of $A_3$-receptor occupancy.

The physiological agonist reaching the adenosine receptors is likely to be the nucleoside adenosine itself, arising from release of ATP by the ciliary epithelial cells and ecto-enzyme activity (30). We have previously found that adenosine triggers isosmotic shrinkage of cultured human NPE cells with an EC$_{50}$ of 3–10 µM (2). In this concentration range, adenosine acts as a nonselective agonist of all four subtypes of the adenosine receptor (12, 13). As illustrated by Fig. 3, 2-min preincubation with either 100 nM of the $A_3$-selective antagonist MRS-1191 (Fig. 3B) or 300 nM of the $A_3$-selective antagonist MRS-1097 (Fig. 3A) all prevented the characteristic shrinkage triggered by nonselective activation of adenosine receptors with 10 µM adenosine ($P < 0.01$, F-distribution).

MRS-1191 (Fig. 3B) or 300 nM of the $A_3$-selective antagonist MRS-1097 (Fig. 3A) blocked the shrinkage characteristically produced by 10 µM adenosine. MRS-1523, an $A_3$ antagonist with $K_i$ of 43 nM (25), and CGS-21680, a widely used $A_2A$ agonist with an IC$_{50}$ value of 22 nM for the $A_2A$ receptor (17, 23), had no detectable effect at 100-nM concentration (Fig. 4B), but did trigger isosmotic shrinkage at a 30-fold higher concentration (3 µM) (Fig. 4C). However, there was no detectable effect at 100-nM concentration (Fig. 4B), but did trigger isosmotic shrinkage at a 30-fold higher concentration (3 µM) (Fig. 4C). However, the $K_i$ for the CGS-21680 at the $A_3$ receptor is 67 nM (25), and thus CGS-21680 could have been acting through either $A_2A$ receptors or $A_3$ receptors at the higher concentration. To distinguish
between these possibilities, we preincubated parallel aliquots of suspensions with 100 nM of the antagonist MRS-1191. MRS-1191 prevented the shrinkage produced by the high concentration of CGS-21680 (Fig. 4), indicating that the shrinkage observed was mediated by cross-reactivity with A3 receptors. Because there are presently no high-affinity A2B agonists, the contribution of A2B receptor stimulation was not pursued, although the ability of A3 antagonists to inhibit the response to 10 µM adenosine (Fig. 3) argues against a role for the A2B receptor.

In other cells, stimulation of the A3 receptor can lead to an elevation of intracellular Ca2+ (26), so we monitored intracellular Ca2+ in HCE cells to provide an additional physiological assay for the presence of A3 receptors. Superfusion of HCE cells with 100 nM IB-MECA produced a sustained, repeatable, and frequently reversible increase in the intracellular Ca2+ concentration (Fig. 5). The increase in Ca2+ was dependent on concentration, with 100 nM IB-MECA leading to a mean rise of 17 ± 5 nM Ca2+ (P < 0.01, n = 8) whereas 1 µM IB-MECA increased intracellular Ca2+ by 22 ± 6 nM (P < 0.05, n = 3). Although these changes were relatively small, they were sustained, suggesting that these increases in Ca2+ could be responsible for physiological effects occurring on a time scale of minutes to hours.

RT-PCR amplifications of RNA from the human NPE cells were conducted using primers for the human A3-type adenosine receptor. A fragment of the expected 462-bp size was obtained and was enhanced by direct PCR amplification of the product (Fig. 6). The sequence obtained from the reamplified product was compared with the sequences of known human adenosine receptors using the DNASTAR program. The results displayed a 97.4% similarity to the published base sequence for the A3 receptor, whereas the similarity indexes for the other known adenosine-receptor subtypes were all <40% [37.9% for A1 (accession no. C663A3 RECEPTORS REGULATE CL− CHANNELS] by 10.220.33.3 on October 20, 2017 http://ajpcell.physiology.org/ Downloaded from

Fig. 4. Effects of adenosine-receptor agonists on isosmotic volume of NPE cells. A: A3-selective agonist IB-MECA produced prompt shrinkage at 100 nM (n = 4, V = 95.6 ± 0.2%, t = 4.5 ± 0.6 min, P < 0.01 by F-distribution). In contrast, A1-selective agonist N6-cyclopentyladenosine (CPA) had little effect at 100 nM, and none at all at 3 µM (n = 4). B: at 100 nM, A2-selective agonist CGS-21680 exerted no effect, but A2-selective agonist IB-MECA again produced shrinkage (n = 4, P < 0.01 by F-distribution). C: at high concentration (3 µM), A2-selective agonist CGS-21680 also triggered isosmotic shrinkage. However, preincubation of the cells with the selective A3 receptor antagonist MRS-1191 (100 nM) abolished this effect (n = 4, P < 0.01, F-distribution).

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Fig. 5. Effects of IB-MECA on level of free intracellular calcium. Concentration of intracellular Ca2+ increased steadily after application of 100 nM IB-MECA and returned to baseline levels once IB-MECA was removed. Similar increases were observed in 7 other trials. Data were obtained at a sampling rate of 1 Hz and smoothed by 21 points. Box indicates the duration of the IB-MECA application.

Fig. 6. Amplified RT-PCR product. Products of PCR reamplification using A3 primers on total RNA from human NPE (HCE) cells were run on a 1% agarose gel. Lanes A and B: duplicate 40-µl aliquots of the PCR reamplification product. Band seen in lanes A and B corresponds to predicted product of 462 bp. Lane C: 40 µl of PCR reamplification product obtained when RT was omitted from initial reaction. Extreme left lane contains a 100-bp DNA ladder molecular weight marker; numbers correspond to base pairs.
Rabbit iris-ciliary body. Adenosine in high concentration (100 μM) has been found to increase the short-circuit current across the rabbit ciliary body (2). We therefore tested whether a high concentration (30 μM) of the A3 agonist IB-MECA also affected short-circuit current. At this concentration, the vehicle (dimethylfor-mamide) itself exerts significant effects (Fig. 7, lowest trajectory). We corrected for the solvent effect in the following way. Solvent alone was initially introduced (to 0.1%), followed by the same volume of solvent (to 0.2%) containing agonist, and ending with addition of a third identical volume of solvent alone (to a final concentration of 0.3%). The reduction in short-circuit current following the first addition of solvent was always greater than the third. In each of four experiments, we averaged the time courses of the first and third additions to estimate the effect of raising the solvent concentration without agonist from 0.1 to 0.2% during the experimental period.

Figure 7 presents the mean trajectory for the averaged solvent effect, the uncorrected mean time course following exposure to IB-MECA, and the mean trajectory ± SE for the solvent-corrected response. The experiments were performed in the presence of 5 mM Ba2+ to minimize the contribution of K+ currents. IB-MECA produced a significant increase in the short-circuit current; an increase in short-circuit current in the presence of Ba2+ suggests that the effect is mediated by activating a Cl− conductance on the basolateral membrane of the NPE cells. The sustained nature of the stimulation is consistent with the time course of the cell shrinkage in response to A3 stimulation.

In view of the short-circuit response to IB-MECA, RT-PCR amplification was also conducted with rabbit ciliary processes, using primers for the rabbit A3-type adenosine receptor. The RT-PCR product was reamplified, cloned, and sequenced. The sequence displayed a 97.4% similarity with the published base sequence for the rabbit A3 receptor. There was only 27.9% homology between rabbit A3 (accession no. L01700) and A3 receptors. Sequences are not yet available for the remaining A2A and A2B subtypes of adenosine receptors in the rabbit. Our rabbit product also displayed 75.1% similarity to the human A3 receptor but only <30% similarity indexes for the other human adenosine-receptor subtypes (28.2% for A1, 27.7% for A2A, and 29.5% for A2B). No product was detected when RT was excluded from the reaction mixture.

**DISCUSSION**

Measurements of short-circuit current across intact rabbit ciliary epithelium, of cell volume in suspended cultured human NPE cells, and of whole cell currents from patch-clamped cultured human and fresh bovine NPE cells have indicated that adenosine-receptor occupancy stimulates Cl− secretion in mammalian NPE cells (2). The following evidence strongly suggests that these effects are mediated by A3 receptors. A1 receptors are present in both human HCE cells and rabbit ciliary body. The A3-selective agonist IB-MECA increased the short-circuit current across rabbit iris-ciliary body in the presence of Ba2+, a change consistent with an increased efflux of Cl− from NPE cells. In the presence of gramicidin to isolate the Cl− conductance, IB-MECA caused human HCE cells to shrink in a dose-dependent manner; the Kd of ~55 nM is consistent with a maximal stimulation of A3 receptors in cardiac myocytes at 100 nM IB-MECA (34). The highly specific A3 agonist Cl-IB-MECA also produced shrinkage of HCE cells in the presence of gramicidin. The A3 antagonists MRS-1097 and MRS-1191 were able to prevent the shrinkage induced by IB-MECA at concentrations far below their Ki for A1 and A2A receptors. The A1 agonist CPA did not have a consistent effect on cell volume. The A2B agonist CGS-21668 had no effect at low concentrations. The effect of CGS-21668 on shrinkage was only detected at a concentration 500-fold higher than the Ki values for the A3 receptor, and this effect was blocked by the A3 agonist MRS-1191. The A1 antagonists MRS-1097, MRS-1191, and MRS-1523 blocked the shrinkage produced by 10 μM adenosine; at the concentrations used, <20% of the A1 and A2B receptors could have been occupied by MRS-1097 and <1% of those receptors could have been blocked by MRS-1191 and MRS-1523. Together, these observations lead us to conclude that the adenosine-stimulated activation of Cl− release by the HCE line of human NPE cells is primarily mediated by occupancy of an A3-subtype adenosine receptor.

The implications of this conclusion are subject to at least four caveats. First, our experiments were de-
signed to isolate the Cl⁻ component of both the volume and short-circuit current response. It is likely K⁺ channels may also be activated by an A3 receptor, for we previously reported that adenosine activates a Ba²⁺-sensitive component of short-circuit current across the rabbit ciliary epithelium (2). We would expect at least some component of this response to be mediated by an A3 Receptor, because the NPE cells possess KCa channels (10, 18) and our present study suggests that IB-MECA elevates intracellular Ca²⁺. Second, occupancy of A₁, A₂A, and A₂B receptors may well have physiologically important effects on transport mechanisms other than Cl⁻ channels. For example, occupancy of A₁ receptors by CPA alters intracellular cAMP (34), and cAMP activates K⁺ channels of these cells (4). Changes in K⁺-channel activity can alter membrane potential, thereby changing the electrical driving force for secretion. Depending on the baseline level of Cl⁻ and K⁺-channel activity, the actions on K⁺ channels could dominate the overall response to adenosine receptor stimulation. This possibility may be relevant to the reports that A₂B receptors stimulate and A₁ receptors inhibit aqueous humor secretion in rabbits (6, 7). Third, the effect of adenosine at concentrations other than those used here (Fig. 3) may alter the relative contribution of the adenosine receptor subtypes to the Cl⁻ channel response. It should be emphasized, however, that the concentration of adenosine used in this study (10 µM) is likely to be physiologically relevant; purine release from intracellular stores of ciliary epithelial cells is expected to raise adenosine to approximately this level (30). Fourth, the ability of IB-MECA to increase intracellular Ca²⁺ provides a physiological assay showing the existence of A₃ receptors on NPE cells attached to a substrate. Further work is required to show whether the elevation in Ca²⁺ is responsible for activating Cl⁻ channels or whether the A3 receptor acts synergistically with other stimuli to further increase Ca²⁺ as the A₁ receptor does (11).

The results of the present study add to a growing body of evidence suggesting that the A₃ receptors, the most recently identified subtype of adenosine receptors, may have multiple important physiological functions. Pharmacological identification of these receptors has been enormously facilitated by the recent and continuing introduction of highly selective A₃ receptor agonists (such as IB-MECA and CI-MECA) and antagonists (such as MRS-1097, MRS-1191, and MRS-1523). Despite these advances, a potential functional role of A₂B receptors cannot be as yet excluded, in the absence of A₂B-selective agonists and antagonists and without further information concerning the binding constants of A₃-selective antagonists to A₂B receptors. Because of this caveat, it was important to obtain molecular confirmation of the functional data, an approach that was facilitated by the availability of the sequences of the adenosine receptors for multiple species.

The need for two rounds of PCR amplification to establish identity of the A₃ message in both cultured human and fresh rabbit cells suggests that the message is present in low copy number. This appears to be a general characteristic of A₃ receptors. For example, Dixon et al. (8) detected A₃ message only in the testis with in situ hybridization, but found widespread distribution after amplification of the message using PCR. Indeed, a similar relationship seems to exist in the ciliary epithelium, for although message was undetected by in situ hybridization (27), we have clearly shown expression of A₃ message in both human and rabbit cells by amplifying with PCR. In our studies, we did not specifically address the levels of tissue protein expression. However, low binding of the A₃ receptor marker [¹²⁵I]-labeled N⁶-(4-amino-3-isobenzy)-adenosine-5’-N'-methyluronamide ([¹²⁵I]-AB-MECA) has been found in other tissues showing robust A₃ receptor-mediated responses (9, 21). Thus the possible functional importance of A₃ receptors cannot be directly correlated with copy number or binding density.

The full physiological implications of A₃ receptors are just now being clarified. A₃ receptors have also been localized to other epithelial tissues involved in Cl⁻ transport, such as the kidney and the lungs (8, 19). Among the effects on nonepithelial tissue, the potential therapeutic value of A₃ preconditioning to reduce ischemic damage is under consideration (19, 29, 34). Whether the protective effects of A₃ receptors during cardiac ischemia are also in part mediated by changes in Cl⁻ transport is unknown. In the case of the NPE cells, the present results provide additional information in the development of a paracrine/autocrine hypothesis of the regulation of aqueous humor secretion. Both nonpigmented and pigmented ciliary epithelial cells have been reported to store and release ATP, which can then be converted to adenosine through ecto-enzyme activity (30). An increase in the Cl⁻ conductance is expected to increase the rate of aqueous humor formation (3). Therefore, the activation of Cl⁻ channels by adenosine acting at A₃ receptors, as shown in the present study, provides a mechanism for adenosine to elevate the production of aqueous humor. The interactions of A₃-adenosine receptors with other adenosine and ATP receptors and their physiological significance in aqueous humor formation remains to be tested.

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