Adenosine Promotes Regulation of Corneal Hydration Through Cyclic Adenosine Monophosphate

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Purpose. To investigate the cellular mechanisms whereby adenosine increases net transendothelial fluid transport by the endothelial cells of the cornea.

Methods. Rabbit corneas were isolated and the endothelial surface was superfused while thickness was measured with the specular microscope. Cyclic adenosine monophosphate (cAMP) was measured in endothelia from fresh and incubated corneas, and adenylyl cyclase and phosphodiesterase activities were measured in homogenates or the particulate fraction of endothelia from bovine or rabbit. Adenosine, adenosine-receptor agonists, dibutyryl cAMP, forskolin, and phosphodiesterase inhibitors were used to modulate physiological and biochemical parameters.

Results. Adenosine, N-ethyl(carboxamido)adenosine, dibutyryl cAMP, forskolin, and phosphodiesterase inhibitors all promoted deturgescence of swollen corneas and maintained fresh corneas at lower steady state thicknesses than in controls. These effects were abolished in the presence of ouabain or 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid or after complete removal of HCO₃⁻ from the media. Intracellular cAMP was significantly increased by forskolin and phosphodiesterase inhibitors and, to a lesser extent, by agonists. Increases in cAMP concentration declined rapidly with time. Cyclase activity in the bovine tissue was enhanced by agonists and by G-protein activators. Dose-response curves of corneal swelling indicated a greater sensitivity to N-ethyl(carboxamido)adenosine than to the A₂ₐ specific agonist CGS 21680.

Conclusions. Adenosine increases net endothelial fluid transport through an increase in cAMP. The effects are mediated by stimulation of adenylyl cyclase through a G-protein coupled to an adenosine receptor, which is most probably of the A₂ₐ subtype. Results suggest that the regulation of corneal hydration by adenosine is more probably through stimulation of active transport than through a change in permeability, involving either transmembrane fluxes of Na⁺ or HCO₃⁻ or another step tightly coupled to these primary events in fluid movement. Invest Ophthalmol Vis Sci. 1996;37:1-10.

The transparency of the mammalian cornea depends on the maintenance of a constant hydration of the stromal matrix, a function governed by the permeability and active transport characteristics of the endothelial cells bordering its posterior surface. This single layer of cells allows access of nutrients to the stroma and epithelium through a leak of fluid drawn from the aqueous humor by the imbibition pressure of the glycosaminoglycans in which the collagen fibrils of the stroma are embedded. Hydration of the tissue is maintained at a constant level by an equivalent active extrusion of fluid driven by the Na⁺-K⁺ ATPase on the basolateral membranes of the endothelial cells. Isolated corneas can be maintained in vitro at a constant thickness (determined by the degree of hydration) when superfused at the endothelial surface with an appropriate HCO₃⁻-Ringer and, if swollen by cold treatment or by temporary exposure of the denuded outer stromal surface to fluid, will recover their normal thickness by transport of the excess fluid across the endothelium.

Several studies have shown that adenosine promotes this deturgescence of swollen corneas and stim-
ulates and prolongs net transeptional fluid movement in isolated stroma–endothelial preparations, but the mechanism whereby these effects are achieved has not been elucidated. The initial assumption that adenosine provided ribose as a substrate for the generation of adenosine triphosphate through glycolysis, as in erythrocytes, was abandoned on finding that the nonmetabolizable chloroadenosine was equally effective and that no lactate was derived from adenosine even under anaerobic conditions. An alternative hypothesis, that adenosine acts through a cyclic adenosine monophosphate (cAMP)-mediated second-messenger system, was tested, but it produced negative results in terms of physiological response (no improvement in deturgescence with dibutyl cAMP or theophylline) and biochemical response (no significant change in cAMP content of the endothelial cells). However, a subsequent study by Walkenbach showed that adenosine does increase the cAMP content of cultured bovine corneal endothelial cells. Moreover, Walkenbach has demonstrated that two key enzymes of a cAMP second-messenger system are active in the corneal endothelium, namely adenyl cyclase and cAMP-dependent protein kinase.

The observation that adenosine increases cAMP in cultured endothelial cells suggests that an A2 type adenosine receptor is involved in the reactions. A similar mechanism was demonstrated in the β-adrenergic-stimulated increase of cAMP in corneal endothelial cells, and adenosine A2 receptors have been identified in both bovine retina and retinal pigment epithelial cells, also acting through the G-protein mechanism. In view of these findings, we have attempted to modulate several elements of this signaling system (ligand, receptor, G-protein, cyclase, cAMP, phosphodiesterase) to reexamine their physiological effects on control of corneal hydration and the effects on the cAMP concentration in the endothelial cells of the treated corneas.

METHODS

Cornea Preparation

New Zealand white rabbits, each weighing 1.8 to 2.5 kg and maintained on laboratory chow and water ad libitum, were killed by intracardiac injection of Euthanasia-5 solution (Veterinary Laboratories, Lenexa, KS), and eyes were enucleated with the lids. All experiments were conducted in accordance with the Guide to the Care and Use of Laboratory Animals (DHHS Publication No. NIH 85-23, revised 1985) and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Corneas (in some cases, after removal of the epithelium by gentle scraping with a scalpel blade) were mounted for superfusion as previously described. Measurements of corneal thickness, directly proportional to the degree of hydration of the tissue, were made with the specular microscope to assess the ability of the endothelial cell layer to maintain normal thickness or to deturgescence a previously swollen cornea to its original thickness. Other corneas were used for short-term incubations and for preparation of tissue homogenates of endothelial cells as described below. Bovine eyes were obtained from a local abattoir, and a suspension of cells was prepared by scraping endothelium from the isolated corneas into ice-cold buffer.

Superfusion

The control medium was a Krebs–Ringer bicarbonate solution containing NaHCO3 at either 43 mM or 25 mM and the following compounds (in mmol/l): NaCl 104 (or 122), KCl 5, CaCl2 1, MgSO4 1, NaHPO4 0.8, glucose 5.5, and 40 mg/l gentamycin. The pH was 7.6 (or 7.4) after bubbling with 7% O2, 5% CO2, and 88% N2, and the osmolarity was 292 to 296 mOsm/kg (Advanced Instruments, Needham Heights, MA). A nominally bicarbonate-free medium was obtained by using Balanced Salt Solution (BSS; Alcon Laboratories, Fort Worth, TX) with 5.5 mM glucose and adjusted to pH 7.4. Complete elimination of HCO3− was achieved by the addition to BSS of 0.2 mM ethoxzolamide (Upjohn, Kalamazoo, MI) and 5 µM antimycin A (Sigma, St. Louis, MO), which required inclusion of 0.5% and 0.1% ethanol, respectively.

Additions were made from stock solutions that caused no more than a 0.5% dilution of these media. All stocks were in water, except in the case of the forskolins and dipivaloylxylo-methylgriseolate (di POM-GA, a membrane-permeable ester of griseolic acid), which were in ethanol; for these, the vehicle was added to controls. Superfusion was at 35°C and 3 ml/hour using syringe pumps (Sage; Orion Research, Cambridge, MA). Changes of the superfusion media were made by flushing 1 ml of the new solution through the chamber (four turnovers) in 1 minute. Preswelling of corneas was performed as described previously, using the appropriate Ringer, without additions, on the bare stromal surface. After 3 to 5 hours of superfusion, corneas were removed from the chamber and rinsed; buttons (1 cm² area) were cut, and endothelium with Descemet’s membrane was scraped off and placed in 0.2 ml 0.5 N HCl for cAMP analysis.

Incubations

Isolated corneas, with epithelium intact and a 2-mm scleral rim, were bisected, and the halves were immersed in 7 ml Ringer medium, with additions as described above, at 37°C for different periods. They were
then rinsed, and the endothelium and Descemet's were removed for cAMP analysis.

**Phosphodiesterase Activity**

Endothelium with Descemet's membrane was removed from isolated rabbit corneas and sonicated for 45 seconds in 50 mM Tris buffer, pH 7.5 (four to five endothelia per milliliter) with 5 mM MgCl₂ and 1 mM dithiothreitol. The sonicate (15 μl) was preincubated at 30°C for 10 minutes with 2 μl of 3-isobutylmethyl-xanthine (IBMX) or griseolic acid (GA) or H₂O, and then the reaction was started by adding 25 μl of the buffer containing 0.1 μCi ³H-cAMP (INC, Costa Mesa, CA) and 0.1, 1.0, or 10 μM cAMP. After 5, 10, or 20 minutes, the reaction was stopped by the addition of 20 μl 150 mM EDTA containing 2 mM adenosine, 2 mM cAMP, and 2 mM 5’ AMP, and heating to 85°C for 5 minutes. Adenosine compounds were separated by spotting 4 μl of the supernatants on polyethylene-imine cellulose (Aldrich Chemical, Milwaukee, WI) and developing for 1.5 hour in 0.15 M LiCl. Spots visualized under ultraviolet light were cut out, eluted in 1 ml 2 M KCl, and counted after the addition of 10 ml scintillation fluid.

**Cyclic Adenosine Monophosphate Assay**

Aliquots of the hydrogen chloride extracts of endothelia from superfused or incubated corneas (see above) were dried and assayed in duplicate using a nonisotopic, colorimetric, immunoassay kit (Oxford Biomedical, Auburn Hills, MI). Duplicate standards in the range 0.02 to 2.0 ng/ml were run with every group of assays.

**Adenylyl Cyclase Assay**

Bovine or rabbit endothelia were homogenized or sonicated, respectively, in 20 mM Tris buffer, pH 7.6, with 0.3 mM sucrose, 5 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol, and 1 μg/ml of the protease inhibitors leupeptin, pepstatin, and phenylmethylsulfonyl fluoride (all from Sigma). After centrifugation at 25,000g for 45 minutes at 4°C, the pellets were resuspended in 2 ml of the same buffer and allowed to stand at 20°C to 22°C for 10 minutes with 5 U of adenosine deaminase (Sigma). Five milliliters of buffer without protease inhibitors was added after recentrifugation and resuspension of the pellet to give protein concentrations of approximately 0.1 to 0.2 mg/ml. Fifty-microliter aliquots were added to the assay medium containing, in a final volume of 200 μl, 50 mM Tris HCl at pH 7.6, 4 mM Mg Cl₂, 20 μM GTP, 1 mM ATP, and 4 μM GA. Incubation was for 10 minutes at 37°C, and the reaction was stopped by the addition of 0.5 ml ice-cold 0.5 N HCl. Cyclic adenosine monophosphate was measured in the supernatant as described above.

**Chemicals**

Griseolic acid and diPOM-GA were gifts from Dr. Masakatsu Kaneko (Sankyo, Tokyo, Japan). CGS 21680, N-methyladenosine (NMA), 5’-N-ethylcarboxamidoadenosine (NECA), and rolipram were obtained from Research Biochemicals International (Natick, MA); adenosine, cAMP, dibutyryl-cAMP (dBcAMP), dBcGMP, forskolin, IBMX, guanosine 5’-O-(3-thiotriphosphate), (GTPγS), and 4,4’-disothiocyanostilbene-2,2’-disulfonic acid (DIDS) were obtained from Sigma.

**RESULTS**

**Superfusion**

Freshly isolated corneas superfused with 43 mM HCO₃⁻ Ringer maintained a constant thickness or swelled at a slow rate of 1 μm/hour to 4 μm/hour during a 3-hour period (Fig 1). When 10 μM adenosine was added to the medium, corneas thinned at 5 to 10 μm/hour for a period of 1 to 2 hours and then maintained a constant hydration or started to swell at approximately the same slow rate as the controls. In 25 mM HCO₃⁻ Ringer, corneas without adenosine swelled to reach a new steady state thickness 20 to 25 μm above their initial value, but, with the inclusion of adenosine in the medium, the swelling was eliminated and the initial constant hydration was maintained. The swelling of the control in 25 mM HCO₃⁻ could be reversed with the later addition of adenosine (arrow).

With epithelia removed, corneas swollen by brief exposure of the anterior stromal surface to Ringer medium, on replacement of that fluid with silicone oil, deturgesced and approached their original thickness. The extent to which this deturgescence could be completed was influenced by the composition of the medium superfusing the endothelium in a manner analogous to that of Figure 1. Figure 2 shows that in 25 mM HCO₃⁻ Ringer, corneas without adenosine swelled to reach a new steady state thickness 20 to 25 μm above their initial value, but, with the inclusion of adenosine in the medium, the swelling was eliminated and the initial constant hydration was maintained. The swelling of the control in 25 mM HCO₃⁻ could be reversed with the later addition of adenosine (arrow).
of cAMP in the endothelium: addition of a membrane-permeable analog, a direct activator of adenylyl cyclase, or inhibitors of the cAMP phosphodiesterase. Enhanced deturgescence of corneas in 25 mM HCO₃-Ringer was seen with addition to the superfusion medium of 1 mM dBcAMP, but not with dBcGMP, the guanosine cyclic nucleotide (Fig. 3A); 50 μM forskolin, but not with dideoxyforskolin, the cyclase-inactive derivative (Fig. 3B); 0.1 mM IBMX (Fig. 3C); and 4 μM diPOM-GA (Fig. 3D). The presence of ethanol, required as a solvent for the forskolins and diPOM-GA, decreased the rate and extent of deturgescence in control medium.

Effects on corneal thickness of other adenosine receptor agonists are shown in Figure 4. NECA, an agonist with broadly equivalent affinity for A₁ and A₂ receptors, was more potent than adenosine, entirely eliminating swelling in 25 mM HCO₃ at concentrations of 3 μM and above. CGS 21680, a specific agonist of the A₂a receptor, was less potent than adenosine at all concentrations and restricted swelling to less than 20% of controls only above 10 μM, some 1000-fold higher than its Kᵣ for binding to the A₂a site.²²

Cyclic Adenosine Monophosphate Content of Endothelium

Table 1 shows the quantity of cAMP measured in the endothelial cells scraped from a button of tissue punched from corneas after superfusion in their respective media for 4 hours. Each of the compounds that improved deturgescence in Figure 3 significantly increased the cAMP content of the endothelium. In contrast, the adenosine receptor agonists, which also improved deturgescence, produced only small, insignificant changes in endothelial cAMP concentration. When tested in combination with 0.5 mM IBMX, the agonists yielded cAMP values comparable to those found with the PDE inhibitor alone.

Subsequently, half-corneas were incubated in the HCO₃-Ringer for shorter time periods. Figure 5 shows that there was a rapid decline of cAMP content after the initial high levels measured after 15 minutes of treatment with diPOM-GA, with and without different...
Concentrations of adenosine. With 4 μM diPOM-GA at 60 minutes, there was less than a twofold stimulation above the base (~9 pmol/mg protein in fresh and incubated corneas) and only a limited response to adding adenosine. By 120 minutes, all responses were smaller than twofold. Qualitatively similar results were obtained when 20 μM diPOM-GA was used to inhibit the PDE activity. With 0.1 mM rolipram as the PDE inhibitor, the response to 0.1 mM adenosine was enhanced significantly (from 75 ± 8 to 148 ± 14 pmol/mg protein), but again it was short-lived.

In other incubations of half-corneas, 50 μM forskolin increased the cAMP content of the endothelium in a time-dependent manner. At 5 and 15 minutes, the control value of 9 pmol/mg protein was increased to 207 and 222 pmol, respectively, but after 60 minutes, it fell to 36 pmol (n = 3 in each case). When PDE was inhibited with 4 μM diPOM-GA, 50 μM forskolin increased the cAMP content over controls by 80- to 100-fold, reaching 700 to 900 pmol/mg protein at 15 minutes.

**Phosphodiesterase and Cyclase**

The activity of PDE in the endothelial sonicates was found to be in the range of 110 to 150 pmol cAMP hydrolyzed/mg protein per minute. The effectiveness of the inhibitors IBMX and GA is shown in Figure 6. It can be calculated that the entire cAMP content of the endothelium, approximately 1.4 pmol (approximately 3 μM in cell water) can be turned over 12 to 15 times per minute by these cells. Even with the enzyme inhibited by IBMX or GA, turnover would still be in the range of 1/minute.

Using 4 μM GA to inhibit PDE by approximately 97%, the activity of adenylyl cyclase was measured at 94 ± 3.1 pmol/mg protein per minute in the particulate fraction of rabbit endothelial cells. This activity was not changed by the addition of 10 μM adenosine to...
the assay medium, but it was increased 50% to 75% by the addition of 20 μM GTPyS, the nonhydrolyzable analog of GTP (data not shown). However, in the bovine particulate fraction (Table 2), marked stimulation of the basal cyclase activity was seen with adenosine and also with the addition of the G-protein activators Mn²⁺ and F⁻. Similar activation was found with NMA and GTPyS, whereas forskolin caused a sevenfold increase in cyclase activity.

### Inhibitors of Transport

To examine which regulatory component of corneal hydration might be modulated by adenosine or other promoters of cAMP, their effects on thickness were examined in the presence of inhibitors of active transport. The addition of ouabain (10⁻⁴M) to the superfusion medium caused corneas to swell at rates that were unaltered by the addition of adenosine, forskolin, or IBMX (Fig. 7A). The addition of DIDS (0.2 mM), which inhibits HCO₃⁻-exchange processes, had a similar effect, swelling rates with and without adenosine being indistinguishable within the limits of detection (Fig. 7B). Surprisingly, when corneal thickness was measured during superfusion with BSS solution, which is nominally HCO₃⁻ free, the addition of adenosine caused a marked change in the swelling pattern compared to that seen in BSS alone (Fig. 8A). However, this effect was abolished when trace sources of HCO₃⁻ were eliminated (see ref. 39) by the addition of 0.2 mM ethoxzolamide and 5 μM antimycin A (Fig. 8B).

### DISCUSSION

Previous examinations of the mechanism by which adenosine promotes deturgescence of the swollen cornea have led to conflicting conclusions. Dikstein, considering a cyclic nucleotide-regulated mechanism, reported that neither cAMP nor dBcAMP improved corneal deturgescence and that the positive effect of adenosine was inhibited by 0.1 μM cAMP. He concluded that adenosine acted by an A₁ receptor mechanism to maintain intracellular cAMP at a low concentration by the inhibition of adenylyl cyclase. However, when this concept was examined by direct measurement of the cAMP content of cultured bovine endothelial cells, Walkenbach found that adenosine actually increased the cAMP level.

Current results clearly show a positive effect of dBcAMP on corneal deturgescence, in contrast to the null effect reported previously that may have resulted from an absence of glucose in the medium (which leads to a failure of the fluid pump after 3 to 4 hours). Comparable positive effects on deturgescence of the cyclase activator, forskolin, and the PDE inhibitors, IBMX or DiPOM-GA, which were shown to produce significant increases in the cAMP content.

### TABLE 1. Cyclic Adenosine Monophosphate Content of Superfused Endothelia*

<table>
<thead>
<tr>
<th>Control</th>
<th>Forskolin (50 μM)</th>
<th>IBMX (0.5 mM)</th>
<th>DiPOM-GA (4 μM)</th>
<th>Adenosine (10 μM)</th>
<th>NECA (5 μM)</th>
<th>NMA (1 mM)</th>
<th>CGS 21680 (50 μM)</th>
<th>Adenosine + IBMX</th>
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<tr>
<td>Controls</td>
<td>5.4 ± 2.7</td>
<td>132.0 ± 81</td>
<td>30.6 ± 2.4</td>
<td>31.8 ± 9.9</td>
<td>7.2 ± 1.8</td>
<td>7.8 ± 1.5</td>
<td>9.6 ± 3.6</td>
<td>8.4 ± 1.2</td>
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<tr>
<td>Forskolin (50 μM)</td>
<td>132.0 ± 81</td>
<td>30.6 ± 2.4</td>
<td>31.8 ± 9.9</td>
<td>7.2 ± 1.8</td>
<td>7.8 ± 1.5</td>
<td>9.6 ± 3.6</td>
<td>8.4 ± 1.2</td>
<td>29.4 ± 2.1</td>
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<td>IBMX (0.5 mM)</td>
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<td>DiPOM-GA (4 μM)</td>
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<td>Adenosine (10 μM)</td>
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<td>NECA (5 μM)</td>
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<td>NMA (1 mM)</td>
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<td>CGS 21680 (50 μM)</td>
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<td>Adenosine + IBMX</td>
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* Endothelia scraped from buttons of corneas superfused for 4 to 5 hours with 25 mM HCO₃⁻-Ringer and additions. Values are mean ± SD pmol/mg protein, n values in parentheses.

IBMX = 3-isobutylmethylxanthine; DiPOM-GA = dipivaloyloxymethylgriseolate; NECA = N-ethylcarboxamidoadenosine; NMA = N-methyladenosine.

FIGURE 4. Dose-response curves for corneas superfused with 25 mM HCO₃⁻ and different adenosine receptor agonists. The difference between the initial thickness of the corneas and steady state thickness reached after 1 to 2 hours of superfusion (see Fig. 1) is plotted against the respective concentrations of the agonists in the medium. ■ = adenosine; ● = CGS 21680; ▲ = NECA. Values are mean ± SD. n = 3 to 5. For abbreviations see text. Steady state thickness of control corneas in 25 mM HCO₃⁻ was 22.5 ± 2.9 μm above initial values (n = 10).
Adenosine, cAMP, and Corneal Hydration

Adenosine

- 1 mM
- 100 μM
- 10 μM
- 0

(cpm/ml protein)

Time (min)

FIGURE 5. Cyclic adenosine monophosphate content of endothelial cells of one-half corneas incubated with the phosphodiesterase inhibitor, diPOM-GA (4 μM), and different concentrations of adenosine. Error bars indicate SD, n = 3. cAMP = cyclic adenosine monophosphate.

of the superfused endothelia, strongly suggest that cAMP mediates the stimulation of fluid transport. Although adenosine and other receptor agonists did not cause significant increases of cAMP when assayed after 4-hour superfusions, this can be attributed to the rapid turnover of the endothelial cAMP that results from the high activity of the phosphodiesterases in these cells. Data in Figure 5 show that, with this activity substantially inhibited, adenosine does cause marked dose-dependent but short-term increases in endothelial cAMP of incubated corneas. Together with the increases seen in the presence of rolipram (see cAMP Content of Endothelium) and the data of Walkenbach,14,15 these results provide firm evidence that adenosine activates an A2 receptor pathway that stimulates adenylyl cyclase.

A2 receptors are of high-and low-affinity subtypes, α and β.17,18,25 The rank order in potency seen in Figure 4, NECA > adenosine > CGS 21680, is indicative of an A2β receptor, as reported in rat astrocytes24 and in a fibroblast cell line.25 Moreover, although the EC50 values cannot be determined accurately from the limited data of the figure, the approximate concentrations at which each agonist is effective in preventing at least 80% of the swelling found in 25 mM HCO3 is consistent with those seen in the same studies, i.e., NECA 1.0 μM, adenosine 3 μM, and CGS 21680 >10 μM. Respective EC50 values for these compounds acting at high-affinity A2β sites are as low as 16 nM, 0.1 – 1 μM, and 15 nM.22,25 Unfortunately, because receptor antagonists are virtually equipotent at A2α and A2β sites, studies with these compounds do not aid in their distinction. Further definition of the receptor subtype must await binding studies and more precise and complete evaluation of agonist potencies on the stimulation of cAMP production in the endothelium.

Although it is not yet possible to identify conclusively the subtype of adenosine receptor in the absence of A2 agonists or antagonists of high specificity and affinity,25 it is supported by the data on adenylyl cyclase activity of the bovine endothelium. In the particulate fraction of these cells from the bovine, receptor agonists and ionic activators of G-proteins increased the activity of the cyclase, as did GTPγS, the nonhydrolyzed form of GTP.26,27

Table 2. Stimulation of Adenylyl Cyclase Activity of Bovine Endothelial Particulate Fraction

<table>
<thead>
<tr>
<th>Agonist</th>
<th>EC50 μM</th>
<th>IC50 μM</th>
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<tbody>
<tr>
<td>Adenosine</td>
<td>10 μM</td>
<td>31 ± 10</td>
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<tr>
<td>Mn2+</td>
<td>2 mM</td>
<td>67 ± 11</td>
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<tr>
<td>F−</td>
<td>2 mM</td>
<td>65 ± 15</td>
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<tr>
<td>NMA</td>
<td>3 mM</td>
<td>67 ± 10</td>
</tr>
<tr>
<td>Forskolin</td>
<td>10 μM</td>
<td>710 ± 132</td>
</tr>
<tr>
<td>GTPγS</td>
<td>100 μM</td>
<td>57 ± 32</td>
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</table>

NMA = N-methyladenosine; GTPγS = guanosine 5'-O-3-thiotriphosphate.

Values are % increase ± SD. Incubation for 10 minutes with 2 mM ATP, 20 μM GTP, and 4 μM griseolic acid; n = 3.
able analog of GTP, which also stimulated activity in the rabbit preparations. These data indicate that the adenosine-mediated increase of cAMP in the endothelium of the incubated corneas is most probably effected through the well-recognized receptor-G-protein-cyclase system, as proposed initially for the corneal endothelium by Walkenbach and LeGrand. The difference between responses to agonists and ions in the bovine and rabbit preparations is not understood, particularly in view of the positive responses to agonists in the cAMP accumulation of the incubated rabbit corneas. It is possible that the rabbit preparation generates endogenous adenosine during the period of the assay, although both rabbit and bovine have adenosine deaminase during isolation of the particulate fraction. Alternatively, the structure of the plasma membrane might be altered in preparing the rabbit fraction so that the receptor-G-protein-cyclase association is disrupted.

We have not studied fully the mechanism whereby the increase in cAMP mediates the improvement of the fluid dynamics of the endothelium. Failure of adenosine to alter the swelling of ouabain-treated corneas indicates that its effect is eliminated when the sodium pump is inhibited. Moreover, the fact that these swelling patterns are congruent suggests that
endothelial permeability is unaltered by adenosine because ouabain itself does not change this parameter (i.e., paired corneas of equal thickness swelling at equal rates but with inhibited fluid transport may be presumed to have equally permeable endothelia). These findings support the conclusions of Fischbarg that adenosine stimulates stromal deturgescence by increasing active fluid transport rather than by altering permeability. Although direct confirmation of the role of endothelial barrier properties requires studies of passive ionic fluxes in the presence of adenosine, the prevention of an adenosine response on the elimination of \( \text{HCO}_3^- \) participation (by two different methods; Figs. 7B, 8B) also supports an action on active transport processes that are primary in the fluid transport mechanism of this tissue.²³,⁶⁰⁻⁶²

It is clear that the increase in cAMP concentration in the endothelium is short-lived, whereas the superfusion data suggest that the physiological effect is sustained. This is seen in Figure 1, in which immediate thinning indicates an immediate onset of the response to adenosine, and the maintenance of a lower steady state thickness for 3 hours indicates a sustained higher pump:leak ratio necessary to balance the greater imbibition pressure prevailing at this thickness. The same holds true in Figure 8A in which, even though the rates of swelling are equal (but not congruent; contrast Fig. 7A), a higher pump:leak ratio is required to sustain the consistently thinner state of the adenosine-treated cornea. Cessation of the physiological effect of adenosine would result in temporarily faster swelling until the control (BSS alone) curve was reached and the lower pump:leak ratio was again matched by a lower imbibition pressure.

Cyclic AMP could alter permeability or transport by its direct action on ion channels or, perhaps more likely because of its sustained effect, through activation of protein kinase A, which would then phosphorylate specific proteins in membrane systems.³⁵⁻³⁶ Indeed, cAMP has been reported to increase \( \text{Na}^+\text{-K}^+ \) ATPase activity in the sciatic nerve of diabetic rats,³⁷ a response that would fit well the data of this study. The suppression of adenosine's effects by elimination of \( \text{HCO}_3^- \) is especially interesting in view of the recent identification of an \( \text{HCO}_3^- \)-activated adenyl cyclase in the corneal endothelium³⁸ and the obligatory role of \( \text{HCO}_3^- \) in the maintenance of corneal thickness.³⁹⁻⁴⁰ A role for a \( \text{Cl}^- \) channel or exchanger is also possible because fluid transport continues only at a severely impaired rate in the absence of this ion.⁴¹ However, other components of the overall fluid regulatory system are candidates for cAMP action, whether direct or through protein kinase A, and further work is necessary to identify a specific site of action in the endothelial membranes.

**Key Words**

adenosine, adenosine \( A_2 \) receptor, corneal endothelium, cyclic adenosine monophosphate, fluid transport, G-protein

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**References**


