Characterization of adenylate cyclase activity in bovine and human corneal epithelium

Ronald J. Walkenbach, Roy D. LeGrand, and Ronald E. Barr

The properties of adenylate cyclase from bovine and human corneal epithelium were investigated. Adrenergic drugs were the most effective stimulatory agents tested in bovine tissue, causing greater activation than did fluoride. Isoproterenol was the most potent agonist, followed by epinephrine and norepinephrine. Phenylephrine and dopamine also stimulated adenylate cyclase through β-adrenergic receptors at relatively high concentrations. Enzyme stimulation by all the adrenergic drugs tested was completely inhibited by 1 μM propranolol or 0.1 μM timolol. The GTP analogue, GppNp, produced considerable activation and caused an augmented response when combined with isoproterenol, but not with fluoride. Prostaglandins E1, E2, or F2α produced a small but significant stimulation over control which was not sensitive to propranolol inhibition. Adenylate cyclase from human corneal epithelium exhibited qualitatively similar characteristics to those of the bovine enzyme. Fluoride was the most effective stimulatory agent, followed by isoproterenol, phenylephrine, and dopamine. Prostaglandins failed to stimulate adenylate cyclase activity in human corneal epithelial preparations.

Key words: adenylate cyclase, cyclic AMP, cornea, epithelium, isoproterenol, prostaglandins, phenylephrine

Cyclic AMP has been found to be an extremely important mediator of a wide variety of biological functions in many different tissues. Its role in the regulation of both synthesis and breakdown of glycogen has been firmly established and has been strongly implicated in the control of lipolysis. More recently, cyclic AMP has been suggested in the mediation of immunologic reaction mechanisms, inflammatory response mechanisms, collagenase activity, cell growth and mitotic rates, and glycosaminoglycan synthesis by fibroblasts. All these cellular processes play important roles in corneal physiology. In addition, several corneal cell functions have already been shown to be regulated by cyclic AMP. Zadunaisky and co-workers have described a chloride transport system in rabbit and frog corneal epithelium which is regulated by cyclic AMP. Epinephrine, prostaglandins, dibutyryl cyclic AMP, and phosphodiesterase inhibitors stimulated the transfer of chloride from the aqueous to tear side of the epithelium. This function has been associated with an increase in transparency and thinning of the cornea. Other investigators have demonstrated the chloride transport to be mediated through β-adrenergic receptors. The number of these receptor sites

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Adenylate cyclase activity in corneal epithelium

Adenylate cyclase activity in corneal epithelium has been shown to be affected by topical treatment of rabbit corneas with a \( \beta \)-adrenergic agonist or antagonist. Cyclic AMP also appears to be involved in the regulation of corneal epithelial cell division. Drugs such as isoproterenol caused a transient inhibition of cell division in vivo and altered the circadian rhythm of mitosis.

Despite the accumulation of information implicating cyclic nucleotides as an important mediator in corneal physiology, little attention has been given to the characterization of the key enzymes involved in cyclic AMP metabolism. In this paper we describe the properties of adenylate cyclase, the membrane-bound enzyme which synthesizes cyclic AMP from ATP, in bovine and human corneal epithelium.

Methods and materials

Bovine eyes were enucleated within 45 min after death at a local abattoir. Eyes were transported in ice-cold 0.9% NaCl and processed immediately. All preparative steps were performed in the cold. Corneal epithelial tissue was scraped from the intact eye and immersed in a solution of 0.25M sucrose, 10 mM tris (hydroxymethyl) aminomethane (Tris), and 10 mM EDTA, pH 7.5. This mixture was homogenized with six to eight passes in a Teflon-glass tissue grinder. Connective tissue was removed by filtration through a layer of surgical gauze before centrifugation at 100,000 \( \times \) g for 60 min. The supernatant, containing cytoplasmic proteins and other soluble materials, wasdecanted. The pellet was resuspended in fresh buffer to a protein concentration of 5 to 10 mg/ml. This suspension was divided into small aliquots and stored at \(-70^\circ\). Approximately 3 mg of epithelial particulate protein was recovered from each bovine cornea.

Human eye tissue (donor ages 65 to 82) was obtained through the Lions Eye Tissue Bank, Columbia, Mo. Corneal epithelium was scraped from each intact eye within 16 hr after death, immersed in 250 \( \mu \)l of the above buffer, and stored at \(-70^\circ\) until analyzed. On the day of assay, tissue from 20 to 25 corneas was thawed, pooled, and homogenized, and the particulate fraction isolated as described for the bovine tissue. Each human cornea yielded approximately 0.3 mg of epithelial particulate protein.

Adenylate cyclase activity was measured by use of the method of Salomon et al. Final assay concentrations were 40 mM N-2-hydroxyethyl-piperazine-N'2-ethanesulfonic acid (HEPES), 1.2 mM \( \alpha^{32}\text{P}-\text{ATP} \) (5 to 10 cpm/pmol), 10 mM MgCl\(_2\), 0.5 mM 3-isobutyl-1-methylxanthine, 2 mM cyclic AMP, 0.25 mg/ml bovine serum albumin, 25 mM creatine phosphate, and 0.01 U of creatine phosphokinase. Unless otherwise indicated, 75 to 100

![Fig. 1. Stimulation of bovine corneal epithelial adenylate cyclase by various drugs. Activity was measured with varying concentrations of isoproterenol (•), epinephrine (○), norepinephrine (△), GppNp (●), phenylephrine (□), dopamine (△), and NaF (○).](image-url)
Activation of bovine corneal epithelial adenylate cyclase by prostaglandins. Activity was measured with varying concentrations of prostaglandins E₁ (●), E₂ (○), and F₂α (▲). Values represent mean ± S.E. of six determinations.

μg of protein of enzyme preparation was assayed for 40 min at 30°, pH 7.5, in a 75 μl volume. Cyclic 32P-AMP was isolated by sequential chromatography on Dowex 50 and neutral alumina columns before measurement of radioactivity by standard liquid scintillation techniques.

Protein was measured by the method of Lowry et al. 26

Radiolabeled ATP was purchased from ICN Pharmaceuticals, Irvine, Calif. Timolol used was Timoptic sterile ophthalmic solution, Merck, Sharp & Dohme, West Point, Pa. Other drugs and biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Chemicals used were at least reagent grade.

Results

Bovine corneal epithelial adenylate cyclase. Preliminary experiments showed that bovine corneal epithelial adenylate cyclase exhibited kinetic properties similar to those of the enzyme from other tissues; that is, enzyme activity was proportional to protein concentration between 25 to 100 μg per assay and to assay time for at least 40 min under basal conditions and when stimulated by NaF or isoproterenol. Activity was proportional to substrate concentrations up to 1 mM ATP, with half-maximal activity (Km) exhibited at 0.2 mM under basal or stimulated conditions. Enzyme activation occurred through an increase in the number of active enzyme sites (Vmax), with no change in substrate affinity. Maximal enzyme activity required the presence of 5 to 10 mM MgCl₂. Manganese ion was partially effective, but calcium ion inhibited activity at all levels tested (data not presented).

The pharmacological regulation of adenylate cyclase in bovine corneal epithelium is summarized in Fig. 1. This graph shows the potency and efficacy of various drugs on enzyme activation. Isoproterenol, a β-adrenergic agonist, was the most potent stimulatory agent tested. Epinephrine and norepinephrine, which exhibit both α- and β-adrenergic agonist properties, also stimulated adenylate cyclase at relatively low concentrations. Phenylephrine and dopamine activated adenylate cyclase at higher drug levels. The nonmetabolizable analogue of guanosine triphosphate, 5' guanylylimidodiphosphate (GppNp), produced considerable enzyme stimulation between 0.05 and 10 μM. Sodium fluoride stimulated activity between 1 and 5 mM but did not produce as great a maximal response as did the β-adrenergic agonists.

The following drugs failed to affect adenylate cyclase activity: carbachol (0.1 to 100 μM), parathormone (0.1 to 10 μM), vasopressin (0.1 to 10 μM), histamine (0.1 to 100 mM), or adenosine (0.1 to 100 μM).

The effect of prostaglandins E₁, E₂, and F₂α on adenylate cyclase activity is shown in Fig. 2. Each prostaglandin tested significantly increased activity over basal levels in a dose-dependent manner, but the magnitude of stimulation was far less than that of the adrenergic drugs, GppNp, or fluoride.

Stimulation of β-adrenergic receptor-mediated adenylate cyclase activity can be specifically inhibited by antagonists such as propranolol. Fig. 3 shows the effect of propranolol and timolol (Timoptic) on isoproterenol-stimulated adenylate cyclase. Both drugs decreased activity to near basal levels. Propranolol exhibited half-maximal inhibition at 50 nM. Timolol was approximately 10-fold more potent than propranolol.
Adenylate cyclase activity in corneal epithelium

Fig. 3. Effect of β-adrenergic antagonists on isoproterenol-stimulated bovine corneal epithelial adenylate cyclase. All assays contained 0.2 μM isoproterenol and varying concentrations of propranolol (•) or timolol (○). Data are presented as a percent of the activity expressed in the absence of antagonist.

Table I. Effect of propranolol on bovine corneal epithelial adenylate cyclase stimulation by various agents

<table>
<thead>
<tr>
<th>Agents added</th>
<th>Enzyme activity (pmol/mg/min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>− Propranolol</td>
</tr>
<tr>
<td>None (basal)</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>NaF, 5 mM</td>
<td>29.3 ± 1.4</td>
</tr>
<tr>
<td>GppNp, 10 μM</td>
<td>25.7 ± 1.9</td>
</tr>
<tr>
<td>PGE₂, 5 μM</td>
<td>7.7 ± 0.6</td>
</tr>
<tr>
<td>Isoproterenol, 0.2 μM</td>
<td>33.4 ± 1.5</td>
</tr>
<tr>
<td>Epinephrine, 1 μM</td>
<td>32.2 ± 1.7</td>
</tr>
<tr>
<td>Norepinephrine, 10 μM</td>
<td>32.9 ± 2.1</td>
</tr>
<tr>
<td>Phenylephrine, 100 μM</td>
<td>30.9 ± 1.8</td>
</tr>
<tr>
<td>Dopamine, 100 μM</td>
<td>31.5 ± 1.4</td>
</tr>
<tr>
<td>NaF + isoproterenol</td>
<td>32.8 ± 1.4</td>
</tr>
<tr>
<td>NaF + PGE₂</td>
<td>30.2 ± 0.9</td>
</tr>
<tr>
<td>NaF + GppNp</td>
<td>29.3 ± 1.3</td>
</tr>
<tr>
<td>GppNp + PGE₂</td>
<td>26.0 ± 0.7</td>
</tr>
<tr>
<td>Isoproterenol + GppNp</td>
<td>39.7 ± 1.6</td>
</tr>
<tr>
<td>Isoproterenol + PGE₂</td>
<td>37.9 ± 1.7</td>
</tr>
<tr>
<td>Isoproterenol + phenylephrine</td>
<td>32.9 ± 1.1</td>
</tr>
</tbody>
</table>

PGE₂ = prostaglandin E₂.
*Values are mean ± S.E. of triplicate assays on four enzyme preparations.

The mechanisms of bovine corneal epithelial adenylate cyclase activation were investigated by observing the effect of propranolol on the level of enzyme activation produced by the various stimulatory agents seen in Fig. 1. Table I shows that propranolol (1 μM) had little or no effect on basal enzyme activity or on activity stimulated by F−, GppNp, or prostaglandin E₂. In contrast, propranolol caused essentially complete inhibition of adenylate cyclase activation by isoproterenol, epinephrine, norepinephrine, phenylephrine, or dopamine.

No additional stimulation was observed when NaF was combined with isoproterenol, prostaglandin E₂, or GppNp. Combinations of GppNp and prostaglandin E₂ showed no augmented response over that with GppNp alone, but GppNp and isoproterenol produced further enzyme activation. The com-
Table II. Human corneal epithelial adenylate cyclase

<table>
<thead>
<tr>
<th>Agents added</th>
<th>Enzyme activity (pmol/mg/min) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (basal)</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>NaF, 5 mM</td>
<td>35.6 ± 4.2</td>
</tr>
<tr>
<td>GppNp, 10 μM</td>
<td>17.6 ± 0.4</td>
</tr>
<tr>
<td>Isoproterenol, 0.2 μM</td>
<td>16.3 ± 1.3</td>
</tr>
<tr>
<td>Epinephrine, 1 μM</td>
<td>17.5 ± 0.8</td>
</tr>
<tr>
<td>Epinephrine + propranolol (1 μM)</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>Norepinephrine, 10 μM</td>
<td>16.0 ± 1.7</td>
</tr>
<tr>
<td>Phenylephrine, 100 μM</td>
<td>14.6 ± 1.3</td>
</tr>
<tr>
<td>Phenylephrine + propranolol</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>Dopamine, 100 μM</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>PGE1, 10 μM</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>PGE2, 10 μM</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>PGF2α, 10 μM</td>
<td>2.6 ± 0.1</td>
</tr>
</tbody>
</table>

PGE = prostaglandin E; PGF = prostaglandin F.
*Values are mean ± S.E. for six determinations.

Particulate fractions of both bovine and human corneal epithelium exhibit relatively high adenylate cyclase activity. For example, isoproterenol-stimulated activity from either tissue is approximately twice that of antidiuretic hormone-sensitive adenylate cyclase from similarly prepared porcine kidney. 28 Adenylate cyclase from the two corneal sources exhibited properties generally similar to enzymes from other tissues in terms of their kinetic properties, substrate-affinity characteristics, divalent cation dependency, and fluoride activation. Maximal β-adrenergic stimulation of the bovine enzyme resulted in activity greater than that produced by fluoride. This finding is unusual, but has been observed previously in several other tissues. 29, 30 Adenylate cyclase in both bovine and human corneal epithelium appears to be regulated predominantly by β-adrenergic receptors. As expected, isoproterenol and epinephrine were the most potent agonists. Interestingly, both phenylephrine and dopamine were effective at relatively high concentrations (Fig. 1, Table I). Although these drugs are not considered β-adrenergic agonists, each have been shown to activate β-adrenergic-mediated adenylate cyclase at similar concentrations in other systems. 31, 32 Stimulation of corneal epithelial adenylate cyclase by phenylephrine may be clinically significant, in view of the widespread use of 10% (0.49M) phenylephrine (Neo-Synephrine) to produce maximal mydriasis. This concentration is 5000 times greater than the level needed to substantially activate human corneal epithelial adenylate cyclase in vitro (Table II), suggesting that clinical applications of topical phenylephrine could significantly elevate cyclic AMP in the cornea. It also seems plausible that β-adrenergic influences may play a role in the untoward effects of high doses of topical phenylephrine on corneal epithelium. 33, 34

Bovine corneal epithelial adenylate cyclase showed small but significant increases in activity with all of the prostaglandins tested. Typically, activity was increased 60% to 70% over control (Fig. 2). Although this level of stimulation may seem small in comparison to the fivefold to sixfold increase produced by
β-adrenergic agonists, it nevertheless could be physiologically significant. Corneal epithelial chloride transport was stimulated by prostaglandins E₁, E₂, or F₂α in frog corneal epithelium, but this species may possess greater prostaglandin-sensitive adenylate cyclase than the bovine tissue. It remains unclear whether the lack of prostaglandin response in human corneal epithelial adenylate cyclase represents a species difference or a lack of prostaglandin receptor stability during the time between enucleation and enzyme preparation. The former seems more probable since bovine corneas do not lose any prostaglandin-sensitive adenylate cyclase if eyes are stored at 4°C for 16 hr in a moist chamber before enzyme preparation (data not presented).

These data indicate that cyclic AMP synthesis in bovine and human corneal epithelium is controlled primarily by β-adrenergic-mediated adenylate cyclase, which is consistent with previous reports of increased cyclic AMP content in rabbit corneas by epinephrine. Bovine tissue also contains separate prostaglandin receptors. Activation of adenylate cyclase appears to be the initial step in the stimulation of chloride transport, inhibition of collagenase, and alterations in mitotic activity in corneal tissue.

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