Adenylyl Cyclase in Human and Bovine Trabecular Meshwork

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Purpose. To determine the basic characteristics and responses of adenylyl cyclase in trabecular tissues. Because the second messenger cyclic adenosine monophosphate can lower intraocular pressure by increasing outflow facility, it is of interest to know which signalling pathways stimulating adenylyl cyclase are involved.

Methods. Adenylyl cyclase activity of bovine and human trabecular meshwork membrane fractions and of whole tissue homogenates (bovine) to forskolin, manganese, fluoroaluminate, isoproterenol, prostaglandins (PGE₁, PGE₂, PGF₂α), and vasoactive intestinal peptide, were evaluated.

Results. In bovine trabecular meshwork particulate fractions, adenylyl cyclase was stimulated 3.3- and 2.6-fold over basal by 60 and 2 μM forskolin, respectively, 2.2-fold by fluoroaluminate, and 1.5-fold by PGE₁ and PGE₂, whereas no or a very week response was obtained with PGF₂α, isoproterenol, and vasoactive intestinal peptide. PGE₁-induced stimulation was dose-dependent and G-protein-dependent, which provides evidence for EP receptor-mediated activation. Whole tissue homogenates of bovine trabecular meshwork did not differ from the particulate fractions. In human trabecular meshwork membrane fractions adenylyl cyclase stimulation was more pronounced, 12.4- and 5.5-fold by 60 and 2 μM forskolin, respectively, 8.2-fold by fluoroaluminate, and 3-fold by PGE₁ and PGE₂. PGF₂α had no effect. Significant stimulation was obtained with isoproterenol (2.8-fold) and with vasoactive intestinal peptide (1.8-fold).

Conclusions. Human and bovine trabecular meshwork can be stimulated at all known activation levels of adenylyl cyclase. The human adenylyl cyclase system, especially receptor-coupled activity, is more sensitive than that of bovines. Beta-adrenoreceptor stimulation, PGE₂, and vasoactive intestinal peptide may have a local physiologic function by activating adenylyl cyclase in human trabecular meshwork. Invest Ophthalmol Vis Sci. 1993;34:3028-3034.

Stimulators and inhibitors of adenylyl cyclase (AC) can modulate aqueous humor secretion in rabbits¹⁻⁴ and primates.⁵⁻¹⁰ Several studies provide evidence that cyclic adenosine monophosphate (cAMP) increases outflow facility.¹¹⁻¹⁵

Drugs and agents that act on receptors coupled to AC, in particular catecholamines,⁷,¹⁶⁻¹⁹ may affect outflow facility. The importance of cAMP as second messenger in response to in vivo treatment with catecholamines has further been demonstrated by combination drug studies with isobutylmethylxanthine, a phosphodiesterase inhibitor; isobutylmethylxanthine enhanced the hypotensive effect of epinephrine²⁰⁻²² by increasing its effect on outflow facility in the rabbit eye.²³ Other agents such as prostaglandins have been suggested,²⁴ but also questioned,²⁵ to play a role in increasing outflow facility if applied topically, or as endogenous intermediates in the response to epinephrine.²⁶ It has been shown that PGF₂α primarily increases uveoscleral flow over trabecular outflow in monkeys.²⁷

In vitro studies report some drug responses of AC in trabecular meshwork (TM) explants from bovine,²⁶ monkey,²⁷ and in human²⁸ TM membrane frac-
tions and in cultured human TM cells. Moreover, β2-adrenoceptors have also been localized and characterized in human TM. Because activators of AC may increase outflow facility, it is of interest to evaluate the basic characteristics and stimulatory responses of AC in TM by agents with different activation levels. Therefore, in this study TM membrane fractions, in addition to whole homogenates, were prepared from bovine and the membrane fraction from human donor eyes to investigate stimulated responses to various drugs and agents. The responses of AC to some agents, for example, catecholamines, forskolin, prostanoids, and vasoactive intestinal peptide (VIP) were investigated.

MATERIALS AND METHODS

Chemicals and Reagents

Biochemical reagents were purchased from Sigma Chemical Company (St. Louis, MO). [3H]c-AMP was obtained from New England Nuclear Corp. (Boston, MA) and α-32P-ATP from New England Nuclear or from Amersham (Arlington Heights, IL). Synthetic porcine VIP was obtained from Cambridge Research Biochemicals (Atlantic Beach, NY) or Sigma. All other reagents and drugs were obtained from Sigma or from Fisher Scientific (Pittsburgh, PA). Forskolin (FSK) was made up as 10 mmol/l stock in dimethyl sulfoxide. The assay kit for protein determination by dye-binding was purchased from BioRad (Richmond, CA).

Preparation of the Tissues

Fresh bovine eyes were immediately dissected on ice, on arrival from a nearby slaughterhouse. TM tissue was prepared according to a method described by Anderson, Wang, and Epstein. The anterior segment of the eye was cut off between the limbus and the equator. Choroidal tissue was severed from the sclera; the remaining corneoscleral ring was dissected in 8-10 equal radial segments; the scleral spur was identified by its ridgelike configuration. In each segment a partial thickness cut was made anterior to the scleral spur and a second parallel cut was made posterior to the line of Schwalbe. TM tissue was lifted away with a sharp forceps. Histologic sections confirmed the presence of predominantly trabecular-tissue (not shown). Trabecular tissue from four human eyes was prepared for one experiment, hand-homogenized in 1.2 ml of the same isotonic divalent ion chelating buffer containing 0.1 M indomethacin and 10 μg/ml leupeptin in a small Dounce glass/glass homogenizer (Contes, Vineland, NY) using 35 strokes of the pestle, passed through a mesh and centrifuged (15 minutes, 27,000 g). The supernatant was removed by decanting and the particulate fraction resuspended in 1.2 ml homogenizing buffer containing 0.1 M indomethacin and 10 μg/ml leupeptin. The resuspended-particulate fraction had a protein content of approximately 0.10–0.30 mg/ml. Tissue was only frozen and thawed once.

Adenylyl Cyclase Assay

Enzyme activity was determined in glass test tubes in a total volume of 250 μl for bovine tissue and 125 μl for human tissue, containing 60 M sucrose, 80 M Tris buffer pH 7.6, 3 M MgCl2 (or MnCl2, as indicated), 1 M ethylenediaminetetraacetic acid, 1 mmol/l EGTA, 5 mmol/l dithiothreitol) in a Teflon/glass homogenizer using 25 strokes of the pestle. The centrifuged particulate fraction (15 minutes, 27,000 g) was washed once in the homogenizing buffer (6 ml), recentrifuged (15 minutes, 27,000 g), decanted, resuspended in the same buffer (3 ml), containing 0.1 mmol/l indomethacin and 10 μg/ml leupeptin, and passed through a 0.5 mm nylon mesh. If whole tissue homogenate was used the thawed tissue was homogenized in 3 ml of the homogenizing buffer containing 0.1 mmol/l indomethacin and 10 μg/ml leupeptin with 25 strokes of the pestle and passed through the mesh. The protein content of the preparations usually ranged between 1 and 1.5 mg/ml.

Human donor eyes were obtained from the Eurotransplant Eye Bank of the Netherlands Ophthalmic Research Institute. During the procedure to preserve corneas for transplantation purposes, eyes are enucleated as soon as possible and delivered on ice by courier. The donors’ ages ranged between 20 and 80 years. The anterior part of the eyes was cut off at the ora serrata, quickly frozen at −80°C and stored for up to 4 weeks before use. The postmortem time before freezing the tissues was usually less than 8 hours. The tissue was thawed to 4°C and the TM on ice was surgically prepared under an operating microscope according to a procedure described by Tripathi et al. The lens was removed after being severed from the zonular attachment. The iris/ciliary body was pulled away from the sclera; the remaining corneoscleral ring was dissected in 8–10 equal radial segments; the scleral spur was identified by its ridgelike configuration. In each segment a partial thickness cut was made anterior to the scleral spur and a second parallel cut was made posterior to the line of Schwalbe. TM tissue was lifted away with a sharp forceps. Histologic sections confirmed the presence of predominantly trabecular-tissue (not shown). Trabecular tissue from four human eyes was prepared for one experiment, hand-homogenized in 1.2 ml of the same isotonic divalent ion chelating buffer containing 0.1 M indomethacin and 10 μg/ml leupeptin in a small Dounce glass/glass homogenizer (Contes, Vineland, NY) using 35 strokes of the pestle, passed through a mesh and centrifuged (15 minutes, 27,000 g). The supernatant was removed by decanting and the particulate fraction resuspended in 1.2 ml homogenizing buffer containing 0.1 M indomethacin and 10 μg/ml leupeptin. The resuspended-particulate fraction had a protein content of approximately 0.10–0.30 mg/ml. Tissue was only frozen and thawed once.
for human tissue), 1 M cAMP, and $^3$H-cAMP $(1 \times 10^4$ cpm). The tubes were preincubated at 30°C to achieve equilibration. To start the assay, 50-μl aliquot portions of the membrane or full tissue suspension were added to triplicate (bovine) or quadruplicate/quintuplicate (human) tubes containing a premix of all other incubation ingredients and drugs as indicated. The assay was terminated after 5 (bovine) or 10 (human) minutes by the addition of sodium dodecyl sulfate, placement of the tubes in boiling water (5 minutes) and isolation of the $^{32}$P-cAMP with $^3$H-cAMP tracer by the double-column method of Salomon et al. $^{34}$ When FSK was used, the control assay tubes contained dimethyl sulphoxide (<1%) equivalent to the amount used to dissolve FSK. GDPβS, if used, was added to the bovine cell homogenate before the assay and incubated 10 minutes to allow equilibration with the tissue.

**Data Analysis**

AC stimulation is expressed as the ratio of corrected $^{32}$P-cAMP cpm for a drug effect to the corrected cpm for the control (baseline) assay tubes. Correction was based on recovery of the $^3$H-cAMP tracer, which was in the range of 55% to 80%. This yields a relative value, that is, stimulation index. Mean basal AC specific activities were calculated for bovine and human tissue preparations in triplicate/quadruplicates and are based on membrane protein solubilized with NaOH, as determined by the Bradford Dye-binding method with bovine gamma globulin as the reference protein. Stimulation indices multiplied by the basal specific activity yield the approximate specific activities obtained for various drugs. Experiments, consisting of triplicate/quadruplicate measurements and illustrated by bar graphs were done three to eight times (n) using different membrane/full tissue preparations. Error bars represent the standard error of the mean and significance (discrimination at $P < 0.05$) were determined by the paired t-test.

The AC activation dose-response curve is fitted by nonlinear regression analysis using the formula:

$$ \text{Effect} = \frac{E_{\text{max}} \times c^\eta}{E_{50} + c^\eta} $$

$E_{\text{max}}$ is maximal response, $E_{50}$ is drug concentration that yields 50% of the maximal effect, and $\eta$ is Hill’s slope coefficient, all being calculated values.

**RESULTS**

The divalent cations magnesium (Mg$^{2+}$) or manganese (Mn$^{2+}$) are required cofactors for AC activity. $^{35,36}$ Therefore, routine assay conditions included Mg$^{2+}$ at the 3 M concentration, which provides approximately 1.8 M free Mg$^{2+}$ after correction for the presence of ethylenediaminetetraacetic acid and EGTA, chelating agents for divalent ions. $^{37}$

Basal AC specific activity in bovine TM preparations was 31.1 ± 10.7 pmol cAMP/min/mg protein (n = 4).
Stimulation of the AC system in bovine TM particulate fractions (Fig. 1A) and whole tissue homogenates (Fig. 1B) was obtained with FSK, which acts directly on the AC enzyme; AlF₄⁻, a direct stimulator of G-protein, and Mn²⁺, which has been shown to be more effective than Mg²⁺ to activate the catalytic unit of the AC enzyme in rabbit ciliary process membranes.

FSK, 2 and 60 µM, activated AC in bovine particulate fractions by 2.6-fold (n = 4) and 3.3-fold (n = 7), respectively, indicating that FSK-sensitive AC is present in TM membranes. Mn²⁺ activated basal TM membrane AC activity 2.7-fold (n = 4) at a 3 M concentration.

G-protein-dependent AC activity was stimulated with AlF₄⁻ (2M fluoride + 0.1 M Al³⁺) in TM membranes 2.2-fold (n = 5). In whole bovine tissue homogenate 60 µM FSK and 3 mmol/l Mn²⁺ activates 60 µM FSK and 3 M Mn²⁺ activated basal TM AC activity 3.6-fold (n = 4) and 1.9-fold (n = 3), respectively (Fig. 1B).

AlF₄⁻-stimulated AC activity (2.2-fold) in bovine TM. This indicates the presence of the stimulatory G-protein. Some receptors, especially β-adrenoceptors, are linked to the AC system via activation of the G-protein. Surprisingly, isoproterenol (ISO; 10⁻⁵M, n = 6) did not stimulate bovine TM AC (Fig. 1A). Attempts to augment potential small effects of ISO by combining ISO with 2 µM FSK and/or by using Mn²⁺ instead of Mg²⁺ were similarly negative (data not shown). Positive controls using ISO were obtained with particulate fractions of bovine ciliary processes prepared from the same eyes as the TM (not shown) and with human TM tissue particulate fractions (see later). This may indicate that in bovine TM β-adrenoceptors are not present or, if present, not coupled to AC.

Also no response to the peptide VIP was found for this tissue preparation (Fig. 1A). Prostaglandin E₂ (PGE₂) can increase AC activity in rabbit iris/ciliary body preparations, although not in homogenates of rabbit ciliary processes in vitro, indicating that PGE₂ is linked to the AC system in the intact iris/ciliary body. To determine whether prostaglandins are linked to the AC system in TM, the effects of PGE₁, PGE₂, and PGF₂α were evaluated. PGE₁, 10⁻⁴ M, stimulated bovine particulate fractions 1.5-fold (n = 5, Fig. 1A). Similar PGE₁ activation was observed in whole tissue homogenates (n = 5, Fig. 1B), which included the cytosol.

Some AC activity was also found for the collected supernatant, that is, 0.75 ± 0.07 (n = 4) compared to the basal activity of corresponding centrifuged membranes normalized to 1.00. PGE₁(10⁻⁵M) stimulation was 1.24 ± 0.02 in the membranes (n = 9) and 1.25 ± 0.06 in the supernatant (n = 3) relative to their controls. This finding probably indicates that small particulate membrane fractions remain in the 27000 x g supernatant. Among other prostaglandins tested, PGE₂ activated bovine TM AC to a similar extent as PGE₁, whereas PGF₂α had no effect (Fig. 1B). The stimulation achieved with various concentrations of PGE₁ is dose dependent (Fig. 2). To test the receptor-mediated activity of PGE₁ and its G-protein dependence, the effect of GDP/βS on the AC response to PGE₁ in TM tissue homogenate was evaluated. GDP/βS irreversibly binds to G-proteins in the inactive form and thus inhibits their activation.

GTP dependence of the AC response to PGE₁ is shown by adding GDP/βS to the tissue homogenate and leaving GTP out of the buffer. In two experiments (n = 8) GDP/βS significantly reduced basal activity to a stimulation index of less than 1, that is, 0.58 ± 0.04 (P < 0.05); the PGE₁ stimulation index was reduced from 1.5 ± 0.1 in the presence of GTP to 0.58 ± 0.05 (P < 0.05). Thus inhibition of G-protein with GDP/βS resulted in an apparent down-regulation of PGE₁-stimulated AC activity, which is probably related to the decrease of the basal response. This further supports the presence of an EP receptor-mediated AC activity in bovine TM tissue homogenates.

A time course experiment (n = 4) in full tissue homogenates showed near linearity for both basal AC activity and the PGE₁-induced activity with assay times ranging from 5 to 30 minutes. Basal activity was 1, 0.99, 1.00, and 1.04 in 5-, 10-, 20-, and 30-minute assays. The corresponding PGE₁ (10⁻⁵M) stimulation was 1.31, 1.26, 1.27, and 1.22, indicating minimal loss of stimulatory capacity (i.e., no short-term desensitization).

The results of experiments with human TM tissue are shown in Figure 3. The basal specific activity in human TM particulate fractions was 38.8 ± 8.8 pmol/
The results of this study show that the AC system of DISCUSSION
bovine and human TM can be stimulated at different activation levels, like other mammalian cells including ciliary processes, that bind ISO-ac-
ceptors using ISO commonly occurs in most tissues
and prostaglandins was evaluated. AlF₄⁻, a direct stimulator of Gₛ-protein, stimulates human basal TM AC activity 8.2-fold (n = 5), 1.8-fold (n = 3), and 2.1-fold (n = 3), respectively, but PGF₂α (10⁻⁵M, n = 3) was inactive. Thus, the human TM AC system is relatively sensitive to stimulation at the hormone-receptor level (VIP, ISO, prostaglandins), stimulation at the level of the Gₛ-protein (AlF₄⁻) and to direct stimulation of the catalytic unit with FSK than that of bovine TM.

**DISCUSSION**

The results of this study show that the AC system of bovine and human TM can be stimulated at different activation levels, like other mammalian cells including ciliary processes. Responses at the hormone receptor level were mediated by VIP, ISO, and PGE₂ as well as PGE₁, AlF₄⁻, and PGF₂α in bovine TM. AlF₄⁻, an activator of the Gₛ-protein, which bypasses the receptor level, stimulates both bovine and human AC as does FSK (a direct stimulator of the catalytic unit). Divalent cations such as Mg²⁺ or Mn²⁺ are required as cofactors for AC activity.

The absolute values for basal AC specific activity obtained in bovine and human TM tissue preparations were similar. However, stimulation at all levels of the AC system that were tested revealed higher responses in human TM than bovine TM, indicating species-specific differences in sensitivity of AC systems, especially in receptor-coupled activity. It is known that AC activation via β-adrenergic receptors using ISO commonly occurs in most tissues containing AC, but in this study an ISO response was absent in bovine TM. Bartels previously reported that epinephrine added to intact calf TM tissue incu-

bations did not stimulate cAMP levels and suggested that β-adrenoceptors were not present, but could not exclude α-adrenergic inhibition by Gₛ-protein. In this study the lack of stimulatory responses to ISO, which is highly selective for β-adrenoceptors, confirms that β-receptors are not present or, if present, are not coupled to AC. However, there is no known example of β-adrenergic receptors that are not connected to AC. It is noteworthy that the ciliary processes of the bovine eye by contrast have high levels of β-adrenoceptor-stimulated AC activity.

ISO and epinephrine have been shown to stimulate cAMP production in human scleral–trabecular rings. Human TM cells, grown in primary cultures, increase cAMP synthesis in response to β-receptor agonists. Furthermore, selective β₂-adrenoceptors have been characterized by autoradiography in sections of human TM and by radiolabeled ligand binding techniques in human cultured TM cells and human trabecular tissue. Recently, FSK-, ISO-, and EPI-stimulated AC has been reported in monkey TM membranes. In view of these studies and the current results showing an ISO response in human TM membranes, a β-receptor AC system is present in the primate TM.

Combining FSK with ISO induced an additive response only and did not show potentiation. FSK is known to elicit a potentiated activity of the AC catalytic unit if complexed to activated Gₛ alpha subunit. The lack of potentiation after FSK and ISO in this study and their additive effect may indicate the presence of different types of AC in TM, that bind ISO-activated Gₛ and FSK independently.

Several compounds that are known to activate receptor-coupled AC in other tissues were tested on the bovine TM, because no ISO response was present. AlF₄⁻-stimulated AC responses are indicative of the presence of a stimulatory Gₛ-protein, but do not indicate the receptors coupled to Gₛ. VIP has been shown to bind to VIP binding sites in membranes of rabbit ciliary processes and markedly stimulates AC activity in that tissue. In cultured monkey TM cells VIP increased cAMP production in a dose-dependent manner. In this study a VIP-sensitive AC was observed in human TM particulate fractions, which was not present in bovine TM particulate fractions. This observation is in accordance with the frequent coexistence of VIP and β₂-adrenoceptors on cells.

In rabbit scleral trabecular rings it was shown that PGE₁ stimulated cAMP production. In this study AC was also activated by PGE₁ in bovine TM tissue. The dose-response relationship and GTP dependence provide strong evidence for a receptor-mediated effect. The Hill coefficient of 0.92 is indicative of a single set of noninteractive ligand binding sites. The order of stimulation by the prostaglandins tested...
was \( \text{PGE}_1 > \text{PGE}_2 > \text{PGF}_2\alpha \), which also suggests involvement of EP receptors. Discrimination among three currently distinguished EP receptor subtypes remains to be established with sulprostone and EP\(_1\)-antagonists. It is reasonable to interpret the mechanism of PGE stimulation in human TM tissue also as EP-receptor-mediated, based on analogizing the bovine results. Moreover, most of the immediate postreceptor consequences of prostaglandins are not known except for the prostaglandins of the E-series, which in most tissues stimulate, but can also inhibit AC.\(^{48}\) In vitro studies of cultured human TM cells have shown that the cells are capable of synthesizing prostaglandins; quantitatively the order found was \( \text{PGE}_2 > \text{PGF}_{2\alpha} > 6\)-keto-PGF\(_{1\alpha}\).\(^{49}\) This together with the current results suggests a local physiologic role for PGE and EP receptors in the stimulation of AC in TM.

In conclusion, this study showed that the AC system of human and bovine TM can be stimulated at all activation levels, the human TM AC system being far more sensitive than that of bovines, especially receptor-coupled activity.

PGE\(_1\) and PGE\(_2\) stimulated both bovine and human TM AC. This suggests the presence of an EP receptor. Human TM membranes contained AC, which is responsive to ISO and VIP, whereas bovine TM membrane AC failed to respond to these hormones. These results provide evidence of a second messenger role for cAMP in response to stimulation of \( \beta_2\) adrenoceptors, and EP and VIP receptor sites in the human TM.

**Key Words**

adenylyl cyclase, trabecular meshwork, human, bovine, prostaglandins, catecholamines.

**References**


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