Measurement of Adenylate Cyclase Activity in the Minute Bovine Ciliary Epithelial Cells During the Pharmacological Stimulation of Adrenergic and Cholinergic Receptors

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Although essential to the secretion of aqueous humor, little is known about the signal transduction underlying postreceptor adrenergic and cholinergic processes in the ciliary epithelium. We adopted a highly sensitive fluorometric assay technique in order to examine adenylate cyclase activity in minute membrane preparations made from the bovine ciliary epithelial cells. The protein concentration of the preparation was 3–5 mg/ml. Norepinephrine (10^{-7} , 10^{-6} and 10^{-5} M) and carbachol (10^{-7} and 10^{-5} M) were incubated with 10 μ l of membrane preparation to analyze the extent of the receptor-coupled influences on the adenylate cyclase activity. Meanwhile, forskolin (10^{-5} M) was used to estimate the maximum adenylate cyclase activity. After the initial enzymatic destruction of noncyclic adenine nucleotides and phosphorylated metabolites, the diester linkage of cyclic AMP was cleaved and then

converted to ATP. The ATP was enzymatically amplified to about 10,000 times of fructose-6-phosphate. The NADPH, formed when the fructose-6-phosphate was converted to 6-phosphogluconolactone, was measured fluorometrically. Basal and forskolin-stimulated maximum adenylate cyclase activities (pmol/mg protein/min) were 29.6 ± 7.6 and 86.6 ± 7.2 (mean $\pm$ SE), respectively. Norepinephrine increased the adenylate cyclase activity in a dose-dependent manner, while carbachol hardly affected the activity. These results indicate that the adenylate cyclase activity can be measured in the minute ciliary epithelial cells and, moreover, that the current assay can be applied to assess the efficacy of newly available ophthalmic solutions or systemic drugs influencing adenylate cyclase activity in a discrete portion in the eye. *J. Clin. Lab. Anal.* 13:90–94, 1999. © 1999 Wiley-Liss, Inc.

Key words: adenylate cyclase; bovine ciliary epithelial cell; cyclicAMP; enzymatic fluorometric assay; nonradioactive

INTRODUCTION

Since intraocular pressure (IOP) is a function of the balance between fluid input and drainage out of the globe, the strategies for the treatment of open-angle glaucoma fall into two classes: reduction of aqueous humor secretion and enhancement of aqueous outflow (1–8). Cholinomimetics, α -agonists and β -blockers have been found to be useful in reducing IOP, and the clinical application of these agents has been well established in patients with glaucoma (1–8). However, little is known about the signal transduction underlying postreceptor adrenergic and cholinergic processes in a discrete portion in the eye, partly due to the lack of a versatile yet sensitive assay of adenylate cyclase activity for the minute tissue mass (9–11).

To begin to overcome this difficulty, we adopted a recently developed, highly sensitive, nonradioactive fluorometric assay technique to examine the effects of

pharmacological interventions on the adenylate cyclase activity in minute membrane preparation made from the bovine ciliary epithelial cells (12,13). In this study, norepinephrine and carbachol were incubated with the membrane preparations to analyze the extent of the receptor-coupled influences on the regional adenylate cyclase activity, while forskolin was used to estimate the maximum adenylate cyclase activity.

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MATERIALS AND METHODS

All experimentation was performed according to the rules and regulation of the Committee for Research at the Yamanashi Medical University.

Production of the Membrane Preparation

Ciliary epithelial cells were obtained from six different bovine eyes to prepare respective membrane preparations as previously described (14). Briefly, a circumferential incision was made in the sclera 8 mm behind the limbus. The anterior portion of the globe, including anterior sclera, lens, iris, and ciliary body were excised and placed on a culture dish. The ciliary body was excised and positioned with its posterior surface upward, and washed with Ca^{2+} - Mg^{2+} free-Hank's solution (D-Hank's, Gibco, Grand Island, NY). The ciliary body was separated from sclera, placed in another culture dish with its inner surface facing down, and immersed in 0.25% trypsin solution for 2 hr at 37°C. The ciliary epithelium was then separated from the ciliary body under the dissecting microscope. The epithelium was homogenized in 5 volume of ice-cold SET buffer (0.25 M sucrose, 0.1 mM EDTA, 5.0 mM Tris-HCl, pH 7.4) and centrifuged at 10,000g for 5 min (13). The pellet was resuspended in 5 volume of SET buffer and the mixture was centrifuged under the same setting. After this procedure was repeated twice, the pellet was finally suspended in 4 volume of SET buffer. The protein analysis was performed using commercially available protein assay reagent (Pierce, Rockford, IL). The membrane suspension in a concentration

of 3.0–5.0 mg of protein/ml was stored at -80°C until enzyme activity was measured.

Measurement of Adenylate Cyclase Activity

The adenylate cyclase activity of the membrane preparation made from the ciliary epithelium was measured using an enzymatic fluorometric assay technique (12,13). The assay consists of two parts: the production of cAMP by the adenylate cyclase (Part A), and the measurement of newly formed cAMP (Part B). Each reaction is summarized in Figure 1.

Part A

Ten μL of adenylate cyclase mix (100 mM Tris-acetate, pH 7.4; 20 mM KCl; 10 mM MgCl_2 ; 20 mM phosphoenolpyruvate; 2 mM ATP; 20 μM GTP; 2 mM dithiothreitol; 0.04% bovine serum albumin; 0.1 mM 3-isobutyl-1-methylxanthine (IBMX); 100 $\mu\text{g}/\text{mL}$ pyruvate kinase) was added to each microcentrifugation tube in duplicate with or without either of forskolin, carbachol or norepinephrine. Next, the membrane suspension in a volume of 10 μL was added to each tube. The reaction mixture and membrane suspension, both before and after being combined, must be maintained at 0°C to ensure the same starting time for all assay tubes. The reaction was initiated by placing the tubes in a water bath maintained at 37°C . After 20 min, the reaction was terminated by heating at 90°C for 5 min. The mixture was vortexed 3 times and centrifuged at 10,000g for 5 min. The supernatant solution was stored for the following cAMP assay.

Fig. 1. Diagram of reactions in the enzymatic fluorometric assay for cyclic AMP (cAMP). P_i : inorganic phosphate.

Part B

A volume of 5 μL of the supernatant was transferred to a 10 $\times$ 75 mm disposable assay tube (Iwaki Lab Ware, Tokyo, Japan) in duplicate. For the cAMP standard, 5 μL of known amount of cAMP was added to the tubes. The cAMP assay consists of the following four steps: (1) a volume of 25 μL of reaction mix A (100 mM Tris-HCl, pH 8.0; 2 mM MgCl_2 ; 2 units/mL apyrase; 2.5 units/mL 5'-nucleotidase; 10 units/mL adenosine deaminase; 20 units/mL alkaline phosphatase) was added to each tube. The mixture was incubated at 37°C for 1 hr. Enzymes were then destroyed by heating for 10 min at 90°C; (2) a volume of 25 μL of reaction mix B (100 mM Tris-HCl, pH 8.0; 2 mM MgCl_2 ; 0.01% bovine serum albumin; 12 units/mL phosphodiesterase; 150 mM KCl; 2 mM dithiothreitol; 80 nM ATP; 3 mM phosphoenolpyruvate; 4.5 units/mL myokinase; 80 units/mL pyruvatekinase) was added to each tube from step 1. The mixture was incubated for 8 hr at room temperature; (3) a volume of 25 μL of reaction mix C (100 mM Tris-HCl pH 8.0; 2 mM MgCl_2 ; 0.01% bovine serum albumin; 3 mM fructose; 60 units/mL hexokinase) was added to the tubes from step 2 on ice. The mixture was incubated for 2 hr at 37°C; and (4) a volume of 125 μL of reaction mix D (100 mM Tris-HCl, pH 8.0; 3.2 mM EDTA; 0.6 mM NADP^+ , 1 unit/mL phosphoglucose isomerase; 0.5 unit/mL glucose-6-phosphate dehydrogenase) was added to the tubes from step 3. After the mixture was incubated for 20 min at room temperature, a volume of 600 μL of distilled water was added to each tube. The concentration of NADPH was measured as an emission fluorescence of 460 nm by the 340 nm of excitation wave using fluorometric spectrophotometer (Hitachi, F3000, Tokyo, Japan). The sensitivity of the fluorometer was set such that a reading of 1 fluorometric unit was equivalent to 100 pmol of NADPH in 900 μL of Tris-HCl buffer (50 mM, pH 8.0).

Biochemicals

Carbachol was obtained from Wako Chemical Industries (Osaka, Japan), while 5'-nucleotidase, apyrase, phosphoenolpyruvate, phosphodiesterase, IBMX, sucrose, fructose, dithiothreitol, norepinephrine, and forskolin were obtained from Sigma Chemical Company (St. Louis, MO). All other enzymes and substrates were obtained from Boehringer-Mannheim Company (Indianapolis, IN).

Statistical Analysis

The results of adenylate cyclase measurements from each individual experiment are expressed as mean $\pm$ SE. The statistical comparisons of mean values were evaluated by paired *t*-test or by one-way, repeated measures ANOVA followed by Contrasts. A *P*-value less than 0.05 was considered significant.

RESULTS

Sensitivity and Specificity of the Assay

A representative standard curve of cAMP is shown in Figure 2. The standard curve has a slope of 2.4 and fluorescence

Fig. 2. A representative standard curves of cAMP assayed in the presence (open circles) and absence (close circles) of phosphodiesterase in step 2. The equation for the cAMP standard curve is $y = 2.4x + 2.4$. Ten fluorescence units are equivalent to 1 nmol/tube of NADPH. Data are expressed as mean $\pm$ SE.

readings from 2.4 to 20.3 units over cAMP concentration range from 0 to 7.6 pmol/tube. When the step 2 was carried out without phosphodiesterase, the standard curve became almost flat with a slope of 0 and average fluorescence reading of 1.8 (closed circles), indicating that the increase of the fluorescence readings (open circles) specifically depends on the added cAMP. In addition, the ratio of the range for fluorescence readings over the background noise was about 10 ($n = 6$), as shown in Figure 2, indicating the high S/N ratio of the current assay.

Stability of Membrane Preparations

The stability of membrane preparations was initially assessed over 40 min. The linearity between the cAMP production and the incubation time is shown in Figure 3. Since the production rate of cAMP in the absence and presence of forskolin (10^{-5} M) was constant up to 20 min ($n = 6$), the incubation time of 20 min in the Part A was chosen to determine the adenylate cyclase activity (cAMP pmol/mg protein/min) in the following experiments.

Pharmacological Interventions of the Membrane Preparation

The results of the adenylate cyclase activities by each pharmacological intervention are summarized in Figure 4. The actual adenylate cyclase activities (cAMP pmol/mg protein/min, $n = 6$) in the basal and forskolin-stimulated membrane preparations were 29.6 ± 7.6 and 86.6 ± 7.2 , respectively. A significant difference was detected between these two values.

Norepinephrine increased the adenylate cyclase activity in a dose-dependent manner. The actual adenylate cyclase activities

Fig. 3. The stability of adenylate cyclase activity in the membrane preparation made from the bovine ciliary epithelial cells ($n = 6$). For this study a volume of 10 μ L of adenylate cyclase mix (100 mM Tris-acetate, pH 7.4; 20 mM KCl; 10 mM $MgCl_2$; 20 mM phosphoenolpyruvate; 2 mM ATP; 20 μ M GTP; 2 mM dithiothreitol; 0.04% bovine serum albumin; 0.1 mM 3-isobutyl-1-methylxanthine (IBMX); 100 μ g/mL pyruvate kinase) and 10 μ L of the membrane preparation were incubated without (closed circles), or with 10^{-5} M of forskolin (open circles). The reaction was linear for at least 20 min. Data are expressed as mean $\pm$ SE.

Fig. 4. Summary of the results of adenylate cyclase activity during pharmacological interventions ($n = 6$). The basal adenylate cyclase activity (meshed rectangle) and those with norepinephrine (closed rectangles), forskolin (open rectangle), and carbachol (hatched rectangles) are shown. Data are presented as mean $\pm$ SE. NE, norepinephrine; CCh, carbachol; #, $P < 0.05$ vs. basal value.

(cAMP pmol/mg protein/min, $n = 6$) with norepinephrine in concentrations of 10^{-7} M, 10^{-6} M, and 10^{-5} M were 31.3 ± 7.4 , 37.1 ± 7.8 , and 60.8 ± 9.5 , respectively. The adenylate cyclase activity with the highest concentration of norepinephrine was 70.3% of the maximum activity treated with forskolin.

The actual adenylate cyclase activities (cAMP pmol/mg protein/min, $n = 6$) with carbachol in concentrations of 10^{-7} M, 10^{-6} M, and 10^{-5} M were 29.7 ± 6.5 , 28.7 ± 6.1 , and 26.6 ± 7.3 , respectively. The adenylate cyclase activity tended to be decreased by carbachol in a dose related manner; however, no statistically significant difference was detected among the basal and any of the activities treated with carbachol.

DISCUSSION

Since the classical assay of adenylate cyclase activities including radioimmunoassay and Salomon method are not sensitive enough to examine a small amount of tissue like ciliary epithelial cells, we adopted a recently developed enzymatic fluorometric assay technique for this study (12,13). The fluorometric assay has been demonstrated to possess a number of potential advantages: the risks and costs associated with radioactivity are eliminated; expense on cost/assay basis are approximately 90% less than those associated with a radioimmunoassay; no overnight incubation or counting of radioactivity is necessary so the time required from start to finish is significantly shorter when compared with the Salomon assay; and the standard curve is linear and assay sensitivity can be easily varied over a wide range of cAMP (12,13). As demonstrated in the current results, absolute values of adenylate cyclase activities can be measured in a small amount of membrane preparation made from the bovine ciliary epithelial cells, which has not been reported elsewhere as far as we have examined. Thus, the assay can be considered to be applied for assessing the net function of the cAMP related signal transduction system in the ciliary epithelial cells. However, it should be noted that the results of this in vitro assay cannot always reflect the physiological regulation of autonomic nervous system in vivo.

As demonstrated in this study, norepinephrine increased the adenylate cyclase activity in a dose-dependent manner, while carbachol did not affect the activity. These results are in good accordance with the previous reports (15,16), in that the signal transduction system via β -adrenoceptors may play an important role for the production of aqueous humor, while cholinergic process cannot significantly affect the secretion in the ciliary epithelial cells. Moreover, our results with carbachol are at least in part in accordance with the previous knowledge (8) that cholinergic stimulation decreases the IOP via the increase of outflow of the aqueous humor through the trabecular meshwork rather than the inhibition of the inflow from the ciliary epithelial cells.

In the previous report using the cynomolgus monkey eyes (3), intracameral administration of norepinephrine or epineph-

rine in a dose of 10 μg , which was approximately corresponded to initial intracameral concentration of 10^{-4} M (3,17), significantly increased the total outflow facility of aqueous humor, while smaller doses of these drugs hardly affected it. Meanwhile, as shown in this study, the adenylate cyclase activities were stimulated by 10^{-5} M of norepinephrine in the membrane preparation made from the ciliary epithelial cells, which was about ten times lower than that could affect the outflow facility. Thus, it is quite important to clarify the signal transduction system in a discrete portion in the eye to better understand the mechanisms of the drug action. It is noteworthy that we have recently further modified the fluorometric adenylate cyclase assay (18,19). By coupling the assay with a microanalytical method, the volume of membrane preparation can be reduced to 1 μL for each experiment in the Part A.

In summary, the current study suggests that the absolute value of adenylate cyclase activity can be measured in the ciliary epithelial cells without using special cAMP antibodies or radioactivity, and that β -adrenoceptor-dependent pathway, rather than the cholinergic process, may play a significant role in the secretion of aqueous humor. The current assay may be used to assess the efficacy of newly available ophthalmic solutions or systemic drugs influencing β -adrenoceptors in the eye. Thus, the current approach promises to become a new and potentially important way to assess the mechanisms underlying the effects of catecholamine, adenosine, vasopressin, and other cAMP dependent processes on the anatomically complex tissues in the eye.

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