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Rat Pineal Adenosine Cyclic 3′,5′-Monophosphate Phosphodiesterase Activity: Modulation *In Vivo* By a *Beta* Adrenergic Receptor

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SUMMARY

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Adenosine 3′,5′-monophosphate (cAMP) phosphodiesterase of rat pineal gland shows characteristic high- and low-K_m activities for cAMP. Treatment with *l*-isoproterenol (5 mg/kg intraperitoneally) increased the activity of the low-K_m phosphodiesterase of pineal by 40% within 1 hr, and the activity returned to normal within 5 hr. The gain of activity was accompanied by a change in V_{max}. Prior treatment of rats with *dl*-propranolol blocked the increase in phosphodiesterase activity while treatment with phentolamine did not, demonstrating modulation of enzyme activity through a *beta* adrenergic receptor. Activity was apparently induced at postsynaptic sites, as phosphodiesterase could still be increased by *l*-isoproterenol treatment after bilateral superior cervical ganglionectomy. Treatment of rats with cycloheximide prevented the response to *l*-isoproterenol, indicating that protein synthesis was required. Treatment with aminophylline, a phosphodiesterase inhibitor, produced a small but significant increase in the enzyme activity. Aminophylline in combination with a dose of *l*-isoproterenol below the threshold for phosphodiesterase activation resulted in greater activity than with aminophylline alone. These results suggest a direct relationship between cAMP concentrations and the increase in phosphodiesterase activity. Tolerance to repeated doses of *beta* receptor agonists might be explained by this mechanism.

INTRODUCTION

The intracellular concentration of adenosine cyclic 3′,5′-monophosphate is regulated in part by cAMP[1] phosphodiesterase (3′,5′-cAMP 5′-nucleotidohydrolase, EC 3.1.4.17) activity. Kinetically, the phosphodiesterases of many tissues consist of two activities with characteristic high and low K_m values for cAMP (1–3). The phosphodiesterase activities of several cell lines have been noted to increase under conditions which increase cAMP concentrations (4–10). *l*-Isoproterenol administration increases cAMP formation in rat pineal through a *beta* adrenergic receptor (11–13). We found that the low-K_m phosphodiesterase activity of rat pineal increased after *l*-isoproterenol treatment. The change was apparently mediated through a *beta* adre-

[1] The abbreviation used is: cAMP, adenosine cyclic 3′,5′-monophosphate.

nergic receptor and could be influenced by factors which modulate cAMP concentrations.

MATERIALS AND METHODS

Male Sprague-Dawley rats, 160–200 g (Zivic-Miller Laboratories, Allison Park, Pa.), were kept under diurnal lighting conditions, with lights on from 6:00 a.m. to 6:00 p.m. Bilateral superior cervical ganglionectomized animals (Zivic-Miller Laboratories) were used 1 week after surgery. Drugs were dissolved in 0.9% NaCl and injected intraperitoneally; doses are presented as the free base. Rats were killed by decapitation between 2:00 and 3:00 p.m.

Phosphodiesterase assay. Pineals were quickly removed and placed individually in small glass homogenizers containing 0.5 ml of cold buffer (50 mM Tris-HCl containing 4 mM $MgCl_2$, pH 8) and homogenized for 30 sec (about 15 strokes). An additional 0.5 ml of buffer was added, and the homogenization was repeated. Phosphodiesterase activity was assayed by the one-step method of Brooker *et al.* (1) as modified by Filburn and Karn (14). The reaction mixture contained 20 μl of homogenate; 3 μM [*G*-^{3}H]cAMP, 2×10^5 cpm (New England Nuclear Corporation), unless indicated otherwise; 50 mM Tris-HCl, pH 8; 4 mM $MgCl_2$; and excess 5′-nucleotidase, 0.16 unit (Sigma, grade IV), in a total volume of 0.1 ml. Samples were incubated for 12 min at 37°, and the reaction was terminated by adding 0.5 ml of ammonium acetate buffer, pH 4. The entire mixture was transferred to a 40 × 5 mm column of aluminum oxide (Woelm, neutral activity, grade 1, ICN); reaction tubes were rinsed with 0.5 ml of ammonium acetate buffer, which was added to the column. Radioactive adenosine was eluted from the column with 1.9 ml of acetate buffer into counting vials containing 8 ml of Aquasol (New England Nuclear), and radioactivity was counted in a Beckman liquid scintillation counter with automatic quench correction. All experiments were done in duplicate. Blank values were prepared with heat-inactivated enzyme. Protein was assayed by the method of Lowry *et al.* with bovine serum albumin as the standard (15).

Sources of drugs. *l*-Isoproterenol hydrochloride, *dl*-propranolol hydrochloride, and aminophylline were purchased from Sigma Chemical Company. Cycloheximide was obtained from Aldrich Chemical Company, and phentolamine hydrochloride was kindly supplied by Ciba-Geigy.

RESULTS

Kinetic analysis of Hydrolysis of cAMP by pineal phosphodieserase. A Lineweaver-Burk plot of the enzymatic hydrolysis of cAMP by pineal homogenate was biphasic (Fig. 1). Extrapolation of the plot revealed apparent Michaelis constants (K_m) of about 2 and 37 μM. Our studies deal with the low-K_m enzyme activity because of its probable importance for the inactivation of cAMP *in vivo* (16–18), although the high-K_m enzyme may be involved under certain conditions. Thus, unless indicated otherwise, phosphodiesterase was assayed with 3 μM cAMP, which would measure primarily the low-K_m enzyme activity.

Enhanced pineal phosphodiesterase activity after l-isoproterenol treatment. Treating rats with *l*-isoproterenol (5 mg/kg intraperitoneally) enhanced the phosphodiesterase activity of pineals (Fig. 2). Activity was significantly elevated at 1 hr and began to approach normal values by 5 hr. Phosphodiesterase activity increased in a dose-dependent manner after treatment with *l*-isoproterenol (Fig. 3). Activity could be increased by about 40% when assayed 2 hr after the injection of *l*-isoproterenol (5 mg/kg intraperitoneally). A kinetic analysis revealed that the gain of activity could be accounted for by an increase in V_{max} from 1.3 to 2.0 μmoles of cAMP hydrolyzed per milligram of protein per minute (Table 1). There was also a small but significant increase in K_m. The high-K_m enzyme activity measured in the presence of 90 μM cAMP showed a small, statistically insignificant increase in *l*-isoproterenol-treated rats. *l*-Isoproterenol in concentrations from 1 to 100 μM had no effect on enzyme activity when added to homogenates of pineal.

Differential blockade by adrenergic receptor antagonists of increase in phosphodiesterase activity induced by l-isoproterenol treatment. Prior treatment of rats with the *beta*-adrenergic blocking agent *dl*-pro-

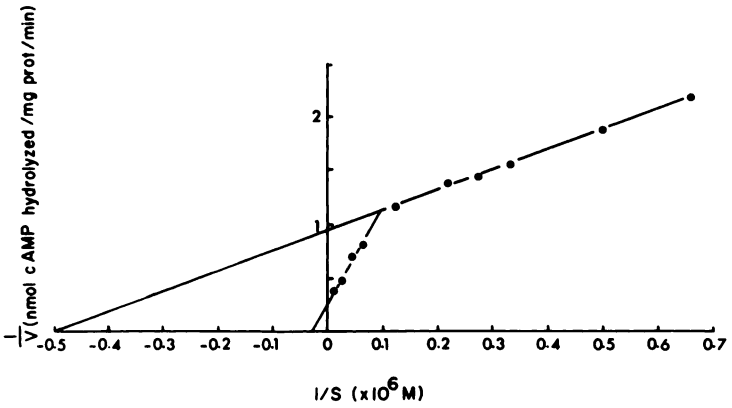

FIG. 1. *Lineweaver-Burk plot of cAMP hydrolysis by pineal phosphodiesterase.*
Assays were performed as described in MATERIALS AND METHODS. Values shown are the means of triplicate determinations on a single homogenate.

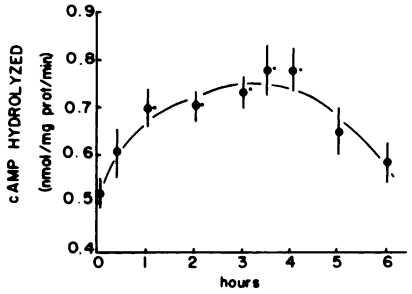

FIG. 2. *Time course of enhancement of pineal low-K_m phosphodiesterase activity after l-isoproterenol (5 mg/kg intraperitoneally) treatment*
Assays were performed as described in MATERIALS AND METHODS with 3 μM cAMP as substrate. Each value represents the mean for four to six rats ± standard error.
*$p<0.05$ when compared with control animals.

pranolol prevented the increase in phosphodiesterase activity induced by *l*-isoproterenol. In contrast, the *alpha* adrenergic blocking agent phentolamine did not prevent the increase in phosphodiesterase activity following *l*-isoproterenol treatment (Table 2).

Influence of cycloheximide or sympathectomy on phosphodiesterase activity following l-isoproterenol treatment. Acute treatment of rats with cycloheximide prevented the increase in phosphodiesterase activity seen after *l*-isoproterenol treatment (Table 3). *l*-Isoproterenol was still capable of increasing pineal phosphodiesterase activity after bilateral superior cervical ganglionectomy, a procedure which destroys the sympathetic neurons that innervate the rat pineal (19) (Table 4).

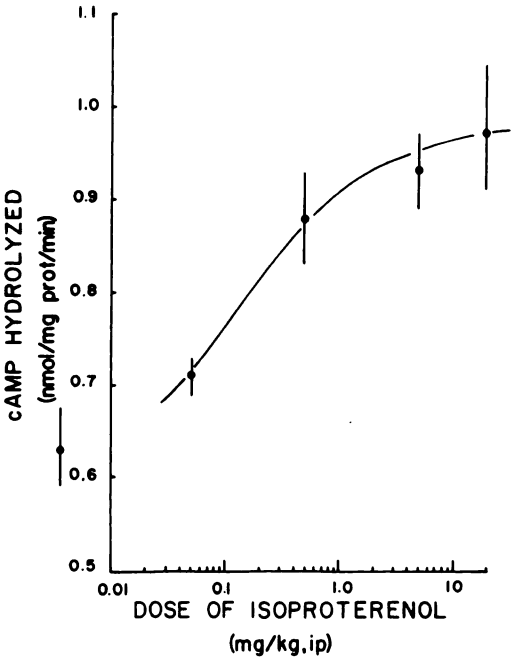

FIG. 3. *Phosphodiesterase activity as a function of dose of l-Isoproterenol*
Animals were killed 2 hr after the administration of various doses of drug or 0.9% NaCl. Assays were performed as described in MATERIALS AND METHODS with 3 μM cAMP as substrate. Phosphodiesterase activity for NaCl-treated rats is shown to the left of the ordinate. Each value represents the mean for four to six rats ± standard error.

TABLE 1

Change of apparent kinetic constants for low-K_m phosphodiesterase of rat pineal gland following l-isoproterenol treatment

NaCl (0.9%) or *l*-isoproterenol (5 mg/kg intraperitoneally) was administered 2 hr before the animals were killed. Values shown are averages for three separate studies. Concentrations of cAMP ranged between 1.5 and 7.5 μM; kinetic constants were determined from Lineweaver-Burk plots.

Treatment	Apparent K_m	V_{max}
	μM ± SEM	*nmole cAMP/mg protein/min ± SEM*
NaCl	2.3 ± 0.1	1.3 ± 0.1
Isoproterenol	3.5 ±0.3[a]	2.0 ± 0.2[a]

[a] $p < 0.02$.

TABLE 2

Effect of adrenergic receptor antagonists on l-isoproterenol-induced increase of pineal gland phosphodiesterase activity

NaCl (0.9%), *dl*-propranolol (20 mg/kg intraperitoneally), phentolamine (10 mg/kg intraperitoneally), or *l*-isoproterenol was administered in the order indicated. Propranolol was injected 20 min whereas phentolamine was injected 75 min before the second injection. Rats were killed 2 hr after the second injection. Each group consisted of four to six rats.

Treatment	Phosphodiesterase activity
	nmole cAMP/mg protein/min ± SEM
NaCl + NaCl	0.46 ± 0.05
NaCl + isoproterenol	0.74 ± 0.06[a]
Propranolol + NaCl	0.45 ± 0.02
Propranolol + isoproterenol	0.55 ± 0.05
NaCl + NaCl	0.46 ± 0.04
NaCl + isoproterenol	0.71 ± 0.06[a]
Phentolamine + NaCl	0.56 ± 0.02
Phentolamine + isoproterenol	0.67 ± 0.03[a]

[a] $p < 0.01$ when compared with NaCl + NaCl group.

Enhancement of responsiveness of pineal phosphodiesterase to low doses of *l*-isoproterenol by aminophylline. Aminophylline treatment by itself significantly enhanced pineal phosphodiesterase activity. Moreover, prior treatment with aminophylline resulted in a further increase of enzyme activity when combined with a dose of *l*-isoproterenol that was ineffective by itself (Table 5).

Pineal phosphodiesterase activity in mixed homogenates of control and l-isoproterenol-treated rats. To test for the presence of an activator Z20) in samples from *l*-isoproterenol-treated rats, homogenates from these animals were mixed with samples from control animals. The mixed samples were no more active than would be predicted from the sum of their individual activities (Table 6).

DISCUSSION

Two forms of phosphodiesterase have been identified by kinetic analysis in

TABLE 3

Prevention of l-isoproterenol-induced increase of rat pineal phosphodiesterase activity by cycloheximide treatment

Cycloheximide (20 mg/kg intraperitoneally) or 0.9% NaCl was injected 30 min before *l*-isoproterenol or NaCl, and the animals were killed 3.5 hr after the second injection. Each group contained five or six rats.

Treatment	Phosphodiesterase activity
	nmole cAMP/mg protein/min ± SEM
NaCl + NaCl	0.67 ± 0.04
NaCl + isoproterenol	0.93 ± 0.04[a]
Cycloheximide + isoproterenol	0.59 ± 0.04
Cycloheximide + NaCl	0.54 ± 0.02

[a] $p < 0.01$ when compared with other groups.

TABLE 4

l-Isoproterenol-induced increase of rat pineal gland phosphodiesterase activity after bilateral superior cervical ganglionectomy

NaCl (0.9%) or *l*-isoproterenol (5 mg/kg intraperitoneally) was injected 2 hr before the animals were killed. Bilateral superior cervical ganglionectomy was performed 1 week before treatment. Each group contained five or six rats.

Treatment	Phosphodiesterase activity
	nmole cAMP/mg protein/min ± SEM
NaCl	0.45 ± 0.04
Isoproterenol	0.64 ± 0.03[a]

[a] $p < 0.01$ when compared with NaCl treatment.

TABLE 5

Enhancement by aminophylline of effect of l-isoproterenol on rat pineal phosphodiesterase activity

Drugs were injected intraperitoneally in the order shown. NaCl (0.9%) or aminophylline (80 mg/kg) was given 20 min before the second injection, and rats were killed 2 hr later. Each group contained four to six rats.

Treatment	Phosphodiesterase activity
	nmole cAMP/mg protein/min ± SEM
NaCl + NaCl	0.50 ± 0.05
NaCl + isoproterenol (0.05 mg/kg)	0.50 ± 0.05
NaCl + isoproterenol (5.0 mg/kg)	0.83 ± 0.02[a]
Aminophylline + NaCl	0.62 ± 0.02[a]
Aminophylline + isoproterenol (0.05 mg/kg)	0.75 ± 0.04[a,b]

[a] $p < 0.05$ when compared with NaCl + NaCl group.

[b] $p < 0.05$ when compared with aminophylline + NaCl group.

TABLE 6

Pineal phosphodiesterase activity in mixed homogenates from control and l-isoproterenol-treated rats

Five NaCl (0.9%)-treated and five *l*-isoproterenol (5 mg/kg intraperitoneally)-treated rats were killed 2 hr after treatment, and individual pineals were assayed for phosphodiesterase activity as described under MATERIALS AND METHODS, using 20 μl of homogenate from each sample. For analysis of combined homogenates, groups I and II were randomly paired, and 10 μl from each individual sample were combined and assayed for activity. Therefore, if enzyme activity were additive, the predicted activity should be one-half the sum of the individual activities.

Treatment	Phosphodiesterase activity
	nmole cAMP/mg protein/min ± SEM
I. NaCl	0.48 ± 0.04
II. Isoproterenol	0.71 ± 0.04[a]
Combined homogenates	0.61 ± 0.03
Predicted activity [(I + II)/2]	0.60

[a] $p < 0.01$ when compared with NaCl treatment.

many tissues, including the rat pineal (1–3). Studies *in vitro* have shown that a variety of pharmacological and physiological manipulations can change the activity of one or more forms of phosphodiesterase. Conditions which increase the level of cAMP in several cell lines have been correlated with an increase in one or both kinetic forms of the enzyme (4–10).

We found that *l*-isoproterenol, in doses that increase pineal cAMP concentrations *in vivo* (11), stimulated the activity of the low-K_m phosphodiesterase. A dose-response study for *l*-isoproterenol showed that administration of 5 mg/kg intraperitoneally produced maximal enzyme activity. A time course study using this dose revealed that activity increased by about 40% within 1 hr and returned toward normal by 5 hr. Our time course for phosphodiesterase activation is similar to that for *N*-acetyltransferase, another pineal enzyme whose activity may be modulated through changes of cAMP concentrations (11, 21, 22).

We selected the rat pineal for our study because of its unique innervation. The sympathetic neurons which innervate the pineal are readily accessible for surgical destruction (19), thus allowing differentiation of the sites of action of pharmacological mediators. Bilateral superior cervical ganglionectomy did not influence the response of pineal phosphodiesterase to *l*-isoproterenol, suggesting mediation through a postsynaptic receptor. The increase in phosphodiesterase activity induced by *l*-isoproterenol is probably mediated through a *beta* adrenergic receptor, as *beta* but not *alpha* adrenergic blocking agents were able to prevent the response to *l*-isoproterenol.

Cycloheximide prevented the response to *l*-isoproterenol, indicating that protein synthesis was required for the increase in phosphodiesterase activity. In addition, kinetic analysis revealed a significant increase in V_{max} for the low-K_m enzyme. These data support the hypothesis that *l*-isoproterenol stimulated the synthesis of enzyme protein, although other interpretations are possible. The small but significant increase in K_m seen may represent some contribution from other forms of the enzyme. We added a pineal homogenate from *l*-isoproterenol-treated animals to a

pineal homogenate from control animals to test for the presence of a phosphodiesterase activator (20, 23). No change beyond simple summation of activities was seen in mixed homogenates.

Aminophylline, a weak phosphodiesterase inhibitor which is capable of potentiating cAMP accumulation in a number of systems (21, 22, 24–26), had an intermediate effect in increasing enzyme activity. However, when combined with a low dose of *l*-isoproterenol which was ineffective alone, it induced an additional increase in phosphodiesterase activity. These studies suggest that there may be a direct relationship between cAMP concentrations and phosphodiesterase activity.

Tolerance develops to repeated doses of *beta* adrenergic agonists concomitantly with a decreased ability to produce transient increases of cAMP concentration (10, 25). Our data suggest that cAMP may influence its own destruction *in vivo* by modulating low-K_m phosphodiesterase activity. This may provide a working hypothesis to explain the tolerance that develops to repeated administration of *beta* adrenergic agonists.

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