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Cyclic Nucleotide Phosphodiesterases from Rat Anterior Pituitary

Characterization of Multiple Forms and Regulation by Protein Activator and Ca^{2+}

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Phosphodiesterase activities for adenosine and guanosine 3′:5′-monophosphates (cyclic AMP and cyclic GMP) were demonstrated in particulate and soluble fractions of rat anterior pituitary gland. Both fractions contained higher activity for cyclic GMP hydrolysis than that for cyclic AMP hydrolysis when these activities were assayed at subsaturating substrate concentrations. Addition of protein activator and $CaCl_2$ to either whole homogenate, particulate or supernatant fraction stimulated both cyclic AMP and cyclic GMP phosphodiesterase activities. Almost 80% of cyclic AMP and 90% of cyclic GMP hydrolyzing activities were localized in soluble fraction. Particulate-bound cyclic nucleotide phosphodiesterase activity was completely solubilized with 1% Triton X-100. Detergent-dispersed particulate and soluble enzymes were compared with respect to Ca^{2+} and activator requirements and gel filtration profiles.

Particulate, soluble and partially purified phosphodiesterase activities were also characterized in relation to divalent cation requirements, kinetic behavior and effects of Ca^{2+}, activator and ethyleneglycol-bis-(2-aminoethyl)-*N*,*N*′-tetraacetic acid. Gel filtration of either sonicated whole homogenate or the $105000 \times g$ supernatant fraction showed a single peak of activity, which hydrolyzed both cyclic AMP and cyclic GMP and was dependent upon Ca^{2+} and activator for maximum activity. Partially purified enzyme was inhibited by 1-methyl-3-isobutylxanthine and papaverine with the concentration of inhibitor giving 50% inhibition at 0.4 μM substrate being 20 μM and 24 μM for cyclic AMP and 7 μM and 10 μM for cyclic GMP, respectively. Theophylline, caffeine and theobromine were less effective. The rat anterior pituitary also contained a protein activator which stimulated both pituitary cyclic nucleotide phosphodiesterase(s) as well as activator-deficient brain cyclic GMP and cyclic AMP phosphodiesterases. Chromatography of the sonicated pituitary extract on DEAE-cellulose column chromatography resolved the phosphodiesterase into two fractions. Both enzyme fractions hydrolyzed cyclic AMP and cyclic GMP and had comparable apparent K_m values for the two nucleotides. Hydrolysis of cyclic GMP and cyclic AMP by fraction II enzyme was stimulated 6–7-fold by both pituitary and brain activator in the presence of micromolar concentrations of Ca^{2+}.

In most biological systems intracellular levels of cyclic AMP are regulated by the relative activities of hormone-responsive adenylate cyclase and cyclic AMP phosphodiesterase. Similarly, concentration of cyclic GMP is also maintained by relative activities of guanylate cyclase and cyclic GMP phosphodiesterase. Cyclic nucleotide phosphodiesterases are present in both cytoplasmic and particulate fractions of the cell [1–5] and in many tissues exists at least in two or more molecular forms [1–15]. Recent reports have described the existence of a protein modifier for cyclic nucleotide phosphodiesterases which require Ca^{2+} as cofactor [16–18].

In rat anterior pituitary gland cyclic AMP presumably acts as a second messenger [19, 20] in gonadoliberin-induced lutropin release. This effect is also mediated by Ca^{2+} [20, 21]. In order to understand the role of Ca^{2+} and cyclic AMP in gonadoliberin-induced lutropin release, the degradation of cyclic AMP by phosphodiesterase and involvement of Ca^{2+} in this process were studied. Only in one instance high K_m cyclic AMP phosphodiesterase has been measured in rat anterior pituitary during development

Abbreviations. EGTA, ethyleneglycol-bis-(2-aminoethyl)-*N*,*N*′-tetraacetic acid; cyclic AMP, adenosine 3′:5′-monophosphate; cyclic GMP, guanosine 3′:5′-monophosphate.

Enzyme. 3′:5′-Cyclic-AMP phosphodiesterase (EC 3.1.4.17).

[22]. In view of the biologically important role of the cyclic nucleotide phosphodiesterases in determining the intracellular level of cyclic AMP [23] we have attempted to characterize the multiple forms of these enzymes in rat anterior pituitary with respect to substrate specificity, Ca^{2+}-dependence and activator requirement. Since current evidence suggests that in some systems cyclic GMP might play a critical role as a second messenger distinct from cyclic AMP [24] we also studied cyclic GMP phosphodiesterase in our system.

MATERIALS AND METHODS

Materials

[^{3}H]Adenosine 3′:5′-monophosphate (specific activity 26 Ci/mmol) and [^{3}H]guanosine 3′:5′-monophosphate (specific activity 19 Ci/mmol) were obtained from New England Nuclear. Both cyclic nucleotides were purified by Dowex-I (X-8; 200–400 mesh; Cl^- form) column chromatography as described by Kakuichi *et al.* [13]. Unlabelled cyclic GMP, cyclic AMP, papaverine, caffeine, theobromine, theophylline and snake venom *(Crotalus atrox)* were all obtained from Sigma Chemical Co. The 3-isobutyl-1-methylxanthine was supplied by Aldrich Chemical Co. Anion exchange resin (Bio-Rad AG1 X-2; 200–400 mesh) and agarose gel (Bio-Gel A-5m, 100–200 mesh) were purchased from Bio-Rad Laboratories. EGTA was a product from Sigma Chemical Co. All other reagents used were of analytical grade. All the solutions were prepared in deionized water.

Preparation of Pituitary Extract

25–26 day-old female rats (Spartan) were killed by decapitation under chloroform anaesthesia. Anterior pituitaries were immediately removed and collected in normal saline. Pituitaries (usually sixty) were homogenized unless otherwise stated in a glass homogenizer in 4 ml of buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM EGTA, 3.75 mM 2-mercaptoethanol). The homogenate was usually centrifuged at $105000 \times g$ for 60 min. The supernatant thus obtained was either subjected to gel filtration or dialyzed overnight against 2 l of the above buffer for 24 h at 0–4 °C (four changes of buffer). The sediment, where required, was also suspended in 4 ml of the above buffer and dialyzed exactly as described above. In some cases whole homogenate was sonicated under the conditions described by Thompson and Appleman [2] and centrifuged at $20000 \times g$ for 20 min. The supernatant was then subjected to gel filtration as described below.

Agarose-Gel Filtration

Gel filtration on Bio-Gel-A 5m was performed according to Thompson and Appleman [3] in columns (2.5 × 45 cm) at flow rates of 10–12 ml per h at 0–4 °C. The elution medium contained 50 mM Tris-HCl buffer, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 3.75 mM 2-mercaptoethanol and 0.1 mM EGTA. Fractions of 64 drops (≈4 ml) were collected and assayed either in the presence of 100 μM EGTA or 100 μM $CaCl_2$ and saturating concentration rat brain activator [17].

DEAE-Cellulose Column Chromatography

Chromatography on DEAE-cellulose (DE52) was carried out in columns (1.2 × 10 cm) equilibrated with 50 mM sodium acetate, pH 6.5, containing 5 mM 2-mercaptoethanol. Sonicated extract (1 ml) was applied to the column and washed with 60 ml of the column buffer. A linear gradient from 0.07 M to 1 M sodium acetate (100 ml each) was then applied and forty-drop (2.5 ml) fractions were collected. The fractions were then assayed for cyclic AMP and cyclic GMP phosphodiesterases in the presence and absence of activator.

Preparation of Activator

A heat-stable, non-dialyzable activator of phosphodiesterase from either brain or anterior pituitaries was prepared according to Cheung [16]. Briefly, tissue samples were homogenized in 3 volumes of distilled water at 0–4 °C. The homogenate was adjusted to pH 5.9 and centrifuged at $13000 \times g$ for 30 min. The sedimented material was discarded. The supernatant was next heated in boiling water bath for 5 min and after this treatment was transferred to an ice bath. After centrifugation the clear supernatant was dialyzed successively against two changes (200 volumes) of 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM $MgCl_2$ and 0.1 mM EGTA for 24 h and then against three changes (400 volumes) of 10 mM Tris-HCl, pH 7.5, for an additional 48 h. Assay for activator was performed according to Kakuichi *et al.* [17] based on the ability of the activator to enhance the activity of diluted $105000 \times g$ supernatant fraction of rat brain homogenate in the presence of 0.1 mM $CaCl_2$. The amount of the activator that doubled the enzyme activity in the standard assay system is defined as 10 units.

Assay of Phosphodiesterase

The enzyme assay was carried out according to the procedure of Thompson and Appleman [2] as modified by Boudreau and Drummond [25]. The procedure

involved the conversion of 5′-AMP (or GMP), the end product of cyclic AMP (or cyclic GMP) phosphodiesterase reaction, to adenosine (or guanosine) by 5′-nucleotidase (snake venom) and the subsequent separation of the substrate and final product by cation-exchange resin. Assay mixture in a final volume of 200 µl contained 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 3.75 mM 2-mercaptoethanol, cyclic [^{3}H]AMP (or GMP) (120 × 1000 counts/min) unlabelled cyclic AMP (or GMP) 0.2 µM (low substrate) or 200 µM (high substrate) and suitably diluted enzyme. In addition, 100 µM $CaCl_2$ and 10–30 µg of activator were also added where required. After incubation at 20 °C for usually 5 min the reaction was stopped by placing the tubes first in solid CO_2/ethanol bath and then by boiling in a water bath for 3 min. 50 µl of snake venom (1 mg/ml) were added to each tube and incubation continued at 20 °C for 10 min. The reaction was stopped by the addition of 1 ml of resin (2 parts 3 mM acetic acid, 1 part resin in the case of cyclic AMP or 3 parts 115 mM formic acid, 1 part resin in the case of cyclic GMP) and the contents spun at 2000 × *g* for 5 min and 0.5 ml aliquots counted for radioactivity in a Beckman LS230 spectrophotometer.

Protein Determinations

Protein was determined by the colorimetric procedure of Lowry *et al.* [26] using bovine serum albumin as standard.

RESULTS

Kinetic Properties of Crude Phosphodiesterase(s)

Results presented in Fig. 1 show kinetic behavior of sonicated supernatant fraction. When the apparent K_m was determined using a wider range of substrate concentration, the Lineweaver-Burk plot was not linear. Extrapolation of the linear portions of these plots showed two apparent K_m values (Table 1). The particulate free supernatant and 105000 × *g* sediment exhibited similar apparent K_m values (Table 1). Cyclic GMP phosphodiesterase activity in the sonicated fraction (Fig. 1), soluble fraction as well as particulate fraction (105000 × *g*), showed straightforward kinetic behavior with an apparent K_m of 7–20 µM (Table 1).

Fractionation of Anterior Pituitary Cyclic Nucleotide Phosphodiesterases by Gel Filtration

Cyclic nucleotide phosphodiesterase was partially purified by agarose A-5m column chromatography. Fig. 2 shows a typical elution profile of cyclic GMP phosphodiesterase from sonicated 20000 × *g* supernatant. The peak activity was stimulated almost 3-fold by 100 µM $CaCl_2$ and to about 6-fold in the presence of activator and $CaCl_2$. Similar results were also obtained using cyclic AMP (0.4 µM). By this procedure although separation of cyclic AMP and

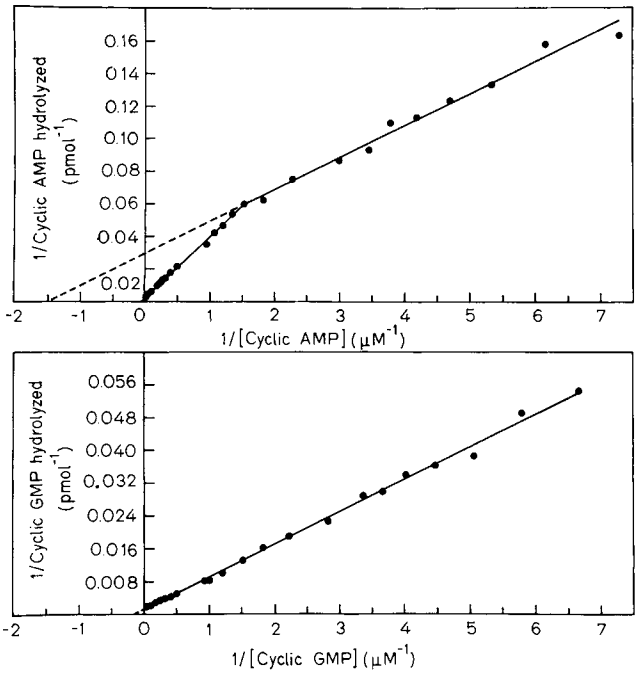

Fig. 1. *Kinetic analysis by double reciprocal plot of cyclic AMP and cyclic GMP hydrolysis by rat anterior pituitary sonicated supernatant.* Concentrations of cyclic AMP ranged from 0.14 µM to 25 µM and cyclic GMP from 0.15 µM to 25 µM. The results are mean of duplicate samples

Table 1. *Apparent* K_m *values of the particulate and soluble cyclic nucleotide phosphodiesterases*
Anterior pituitaries from thirty rats were homogenized in 7.2 ml of buffer (20 mM Tris-HCl, pH 7.4; 3.75 mM 2-mercaptoethanol) and homogenate was divided into two parts. The first part (3.6 ml) was sonicated 10 min at 0–4 °C and then centrifuged at 20000 × *g* for 20 min. The clear supernatant was used for enzyme assays. The second part was directly centrifuged at 105000 × *g* for 60 min and the clear supernatant saved. The sediment was rinsed with buffer and resuspended in 3.6 ml of buffer. The supernatant, sediment and sonicated fractions were tested for nucleotide phosphodiesterase activity. Concentrations of cyclic AMP ranged from 0.14 µM to 25 µM and cyclic GMP from 0.15 µM to 25 µM

Fraction	K_m		
	cyclic AMP		
	low	high	cyclic GMP
	µM		
Sonicated Extract	0.67	20	7
Sediment (105000 × *g*)	0.33	13	20
Supernatant (105000 × *g*)	1.80	20	10

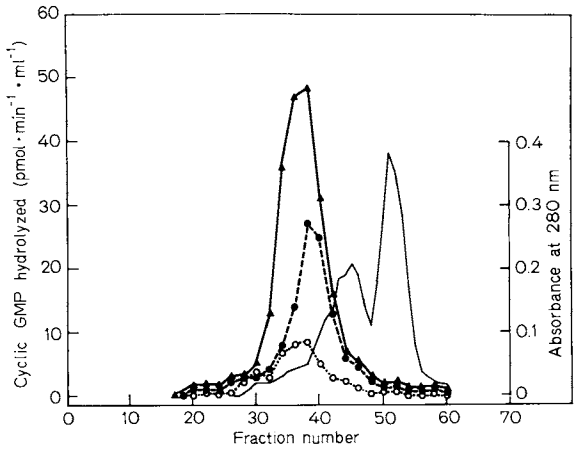

Fig. 2. *Agarose A-5m gel filtration profile of the pituitary phosphodiesterase from sonicated supernatant.* Four ml of the sonicated extract (30 pituitaries) was applied on the column (2.5 × 45 cm) and eluted with 50 mM Tris-HCl, pH 7.5, containing 10 mM NaCl, 1 mM $MgCl_2$, 3.75 mM 2-mercaptoethanol and 0.1 mM EGTA. The flow rate was 12 ml/h and 4.1-ml (64 drops) fractions were collected. Cyclic GMP (0.4 μM) was used to monitor phosphodiesterase activity. (○ ···· ○), No additions; (●---●), $CaCl_2$ 100 μM; (▲——▲), $CaCl_2$ 100 μM + activator 28 μg; (——) absorbance at 280 nm

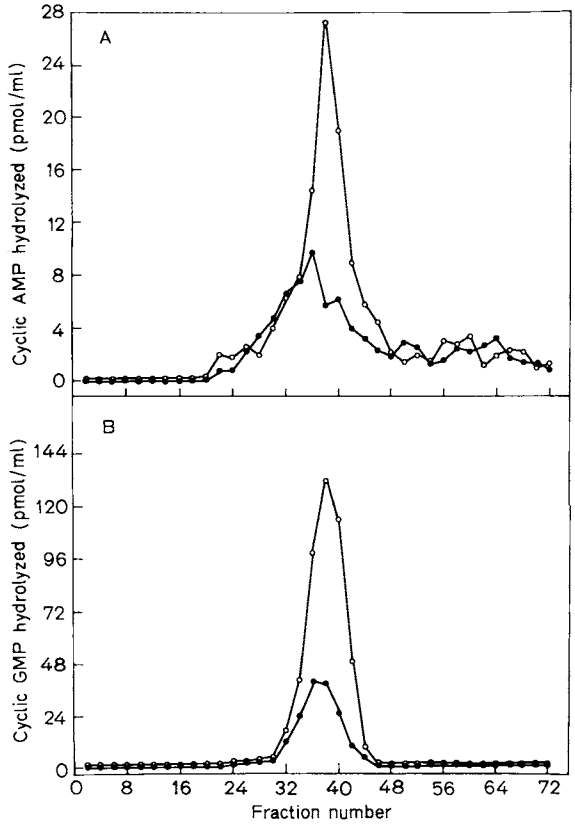

Fig. 3. *Agarose A-5m gel filtration of the particulate free pituitary supernatant.* Experimental conditions were identical to those described under Fig. 2. Each fraction was assayed for cyclic AMP(A) and cyclic GMP (B) phosphodiesterase activities in the presence and absence of activator. The concentration of substrates used was 0.4 μM. (○——○), Activator 28 μg + $CaCl_2$ 100 μM; (●——●), EGTA 0.1 mM

cyclic GMP phosphodiesterases could not be achieved, it did purify both activities between 10 – 15-fold with 30 – 40% recovery. The concentrated preparation or column eluate containing 1 mg/ml albumin were stable for a few weeks at −20 °C. Results presented in Fig. 3 show the elution profile of particulate free supernatant on agarose columns. Both the activities were eluted in the same position as in the case of sonicated extract. Measurement of activities in the presence of 100 μM EGTA or 100 μM $CaCl_2$ and activator did not affect the elution profile. Similar results were also obtained using higher concentrations (200 μM) of either cyclic AMP or cyclic GMP.

PROPERTIES OF PARTIALLY PURIFIED ENZYME

K_m *for Cyclic GMP and Cyclic AMP*

Lineweaver-Burk plots of the rates of hydrolysis of cyclic GMP by partially purified enzyme either in the presence or absence of activator and 100 μM $CaCl_2$ gave a straight line with an apparent K_m of 8 μM. The presence of activator did not change the K_m, but it increased V. When K_m determinations were carried out in the presence of 100 μM EGTA the Lineweaver-Burk plots showed two K_m values of 1 μM and 20 μM. Identical results were obtained when cyclic GMP was used as substrate (Table 2).

Table 2. *Effect of activator on EGTA on the* K_m *of partially purified cyclic AMP and cyclic GMP phosphodiesterases*
Partially purified enzyme was obtained by agarose A-5m gel chromatography as described in the text. The active fractions were pooled and dialyzed for 24 h against four changes of buffer (20 mM Tris-HCl, pH 7.4; 3.75 mM 2-mercaptoethanol). After dialysis the samples were assayed for cyclic AMP and cyclic GMP phosphodiesterases in the presence of activator (28 μg protein) and EGTA (0.1 mM). Other details were the same as described under Table 1

Treatment	K_m	
	cyclic AMP	cyclic GMP
	μM	
None	5.7	8
EGTA (0.1 mM)	0.5, 3.6	1, 20
Activator (28 μg) + $CaCl_2$ (0.1 mM)	5.7	8

Effect of Activator, $CaCl_2$ *and EGTA*

The effect of EGTA on pituitary cyclic nucleotide phosphodiesterases is shown in Table 3. In the presence of excess Mg^{2+}, EGTA (100 μM) inactivated the activator deficient cyclic AMP and cyclic GMP phosphodiesterases to about 67 – 72%, respec-

Table 3. *Effect of EGTA, activator and $CaCl_2$ on partially purified cyclic nucleotide phosphodiesterase*
Partially purified enzyme was obtained as described under Table 1. The active fractions were dialyzed against four changes of buffer (20 mM Tris-HCl, pH 7.4; 3.75 mM 2-mercaptoethanol). After dialysis samples were assayed for cyclic AMP and cyclic GMP phosphodiesterases as described in the text. The concentration of substrate used was 0.4 μM

Additions	Cyclic AMP hydrolyzed	Cyclic GMP hydrolyzed
	pmol × min^{-1} × mg $protein^{-1}$	
None	18.3	125
$CaCl_2$ 100 μM	20.4	141
Activator (28 μg)	43.9	179
EGTA 100 μM	5.9	35
$CaCl_2$ 100 μM + activator (28 μg)	59.3	212
$CaCl_2$ 100 μM + EGTA 100 μM	21.5	145
Activator (28 μg) + EGTA 100 μM	4.3	26
Activator (28 μg) + EGTA 100 μM + $CaCl_2$ 100 μM	38.6	148

tively, whereas in the presence of saturating concentrations of protein activator, the enzyme activities were stimulated five fold and eight-fold. Addition of 100 μM $CaCl_2$ along with activator further increased the enzyme activity that was observed without added divalent metal ion. The activation of cyclic nucleotide phosphodiesterase by protein activator was completely abolished by low concentrations of EGTA in the presence of excess Mg^{2+}. In the absence of activator and chelating agent 100 μM Ca^{2+} alone did not activate enzyme activity more than 5–10%. These results indicate that the activation of cyclic AMP and cyclic GMP phosphodiesterases in the pituitary requires the presence of both protein activator and Ca^{2+}.

Effect of Divalent Metal Ions

Effect of divalent cations on cyclic GMP and cyclic AMP phosphodiesterase activities present in the particulate free and sediment fractions are given under Fig. 4. Cyclic AMP and cyclic GMP phosphodiesterase activities in dialyzed particulate, supernatant and partially purified (by agarose column chromatography) fractions were detectable without added metal ions and these activities were stimulated by the activator. Both Mg^{2+} and Mn^{2+} stimulated the activities with increasing concentrations of divalent metal ions both in the presence and absence of added activator. The overall stimulation in the case of dialyzed particulate or soluble fraction was higher when Mg^{2+} was used as the metal ion compared to Mn^{2+} (Fig. 4A–D). Ca^{2+}, on the other hand, either slightly stimulated enzyme activities or had no effect on the concentrations tried both in the presence or absence of activator.

Partially purified cyclic nucleotide phosphodiesterase in the presence or absence of added activator hydrolyzed both cyclic AMP and cyclic GMP in the absence of added metal ion. Addition of activator itself increased the hydrolytic activities about eight-fold and six-fold respectively, when enzyme activities were tested against cyclic AMP and cyclic GMP (Fig. 4E, F). The effect of Mn^{2+} on partially purified enzyme was different with the cyclic AMP and cyclic GMP as substrates. Cyclic AMP hydrolysis in the presence of added activator and Mn^{2+} was much higher than that in the presence of Mg^{2+} at low concentrations, but in the case of cyclic GMP both metal ions were almost equally effective. Ca^{2+}, as in the case of particulate or soluble enzyme also did not significantly stimulate partially purified activity.

Effect of Inhibitors

The effect of phosphodiesterase inhibitors on partially purified enzyme is shown in Fig. 5 and Table 4. Papaverine and 1-methyl-3-isobutylxanthine were more effective than caffeine and theophylline. Theophylline was more potent compared to caffeine and theobromine (Fig. 5). The concentration of 1-methyl-3-isobutylxanthine and papaverine required to produce 50 percent inhibition was 21 and 24 μM for cyclic AMP and 7 and 10 μM for cyclic GMP, respectively (Table 4). Other inhibitors required higher concentrations to produce similar inhibition.

Separation of Cyclic Nucleotide Phosphodiesterase by DEAE-Cellulose Column Chromatography

The elution profile of sonicated pituitary extracts on DEAE-cellulose column is shown in Fig. 6. Following chromatography, phosphodiesterases were resolved into one minor (I) and one major (II) peaks and hydrolyzed both cyclic AMP and cyclic GMP. When higher cyclic AMP concentrations (100 μM) were utilized, only one peak of activity was observed (Fig. 6C) which coincided with peak II activity (Fig. 6A, B). In all the cases peak II was greatly stimulated by protein activator (5–7-fold).

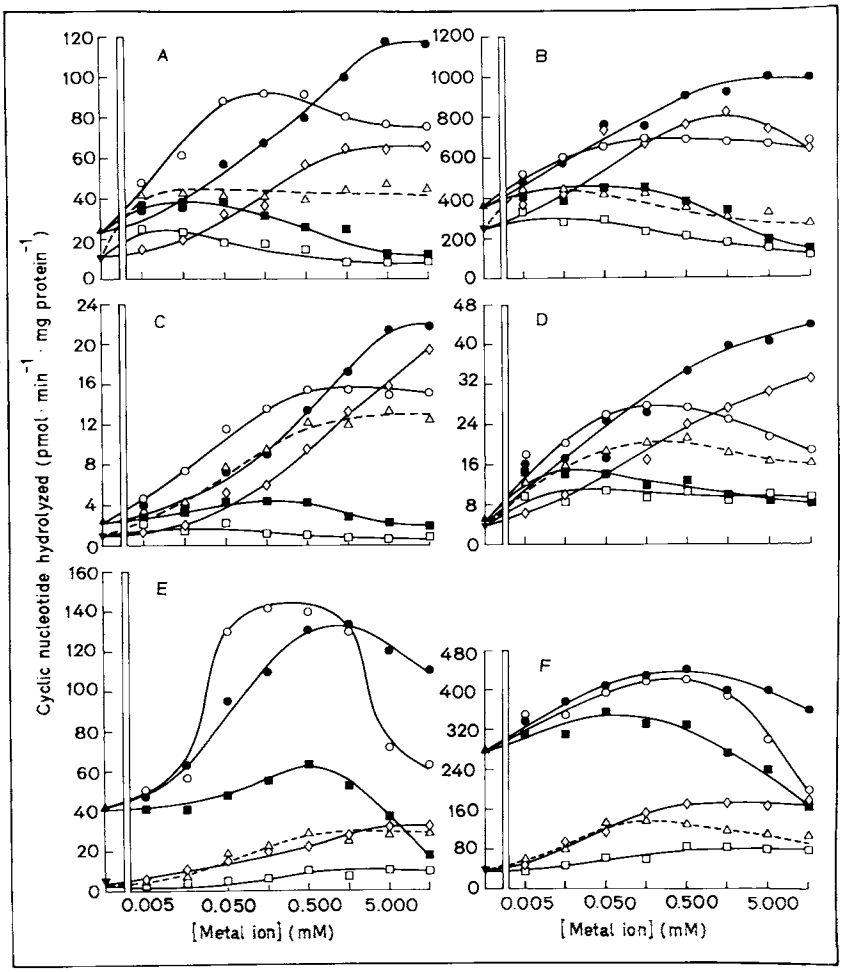

Fig. 4. *Effect of divalent metal ions on particulate, soluble and partially purified phosphodiesterases.* Fifty pituitaries were homogenized in 4 ml of buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM EGTA, 3.75 mM 2-mercaptoethanol) and homogenate was centrifuged at 105000 × *g* for 60 min to isolate particulate-free supernatant and sediment fractions. Both fractions were dialyzed against buffer for 24 h as described under Materials and Methods. Partially purified enzyme was obtained by gel filtration as described under Table 2 and was dialyzed. All the fractions were then tested for cyclic GMP and cyclic AMP hydrolysis in the presence and absence of activator and various divalent metal ions. All solutions of divalent metal ions were prepared in deionized water as suggested by Lin *et al.* [27]. (▲▼) No metal ion; (○—○, △---△) Mn^{2+}; (●—●, ◇—◇) Mg^{2+}; (■—■, □—□) Ca^{2+}; (●—●, ○—○, □—□, ▲) with activator; (△---△, ◇—◇, □—□, ▼) without activator; (A,B) particulate free supernatant; (C,D) sediment (105000 × *g*); (E,F) partially purified enzyme; (A,C,E) cyclic AMP hydrolysis; (B,D,F) cyclic GMP hydrolysis

Peak I with cyclic AMP gave two K_m values of 1.5 μM and 13 μM while with cyclic GMP it gave essentially straight kinetic plots with apparent K_m of 10 μM in the presence of saturating concentrations of activator. Peak II showed straight kinetic plots with both cyclic AMP and cyclic GMP with apparent K_m values of 5 μM and 10 μM, respectively.

Effect of Different Protein Activators on the Peak II Phosphodiesterase Activity

Using both low (0.4 μM) and high (100 μM) cyclic AMP peak II phosphodiesterase activity was stimulated about 5-fold in the presence of brain activator and about 6-fold in the presence of pituitary activator (Table 5). Both activators almost equally stimulated cyclic GMP hydrolysis.

Apparent Distribution of Cyclic Nucleotide Phosphodiesterase in Soluble and Particulate Fractions

In addition to characterization of soluble enzyme attempts were also made to follow the distribution of phosphodiesterase in particulate and soluble fractions and solubilization of the particulate enzyme. Results presented in Table 6 show the distribution of cyclic AMP and cyclic GMP phosphodiesterases between 105000 × *g* sediment and particulate fractions. Almost 80% of cyclic AMP hydrolyzing activity was confined to the supernatant fraction. In contrast,

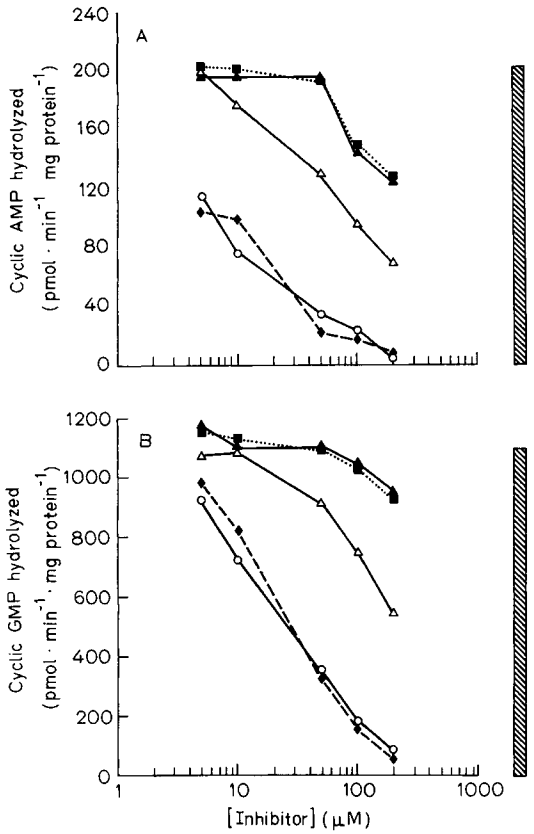

Fig. 5. *Effect of inhibitors on partially purified phosphodiesterases.* Partially purified enzyme was isolated as described under Fig. 1. Results are the mean of duplicate determinations. The hatched bars represent enzyme activity without activator, ○——○, 1-methyl-3-isobutylxanthine; ◆---◆, papaverine; △——△, theophylline; ▲——▲, caffein; ■···■, theobromine

Table 4. *Inhibition of partially purified phosphodiesterase by 1-methyl-3-isobutylxanthine, papaverine, theophylline and caffeine*
Experimental conditions were the same as described in Fig. 5. I_{50} value is the concentration of inhibitor required to produce 50% inhibition of 0.4 μM substrate

Inhibitor	I_{50}	
	cyclic AMP	cyclic GMP
	μM	
1-Methyl-3-isobutylxanthine	21	7
Papaverine	24	10
Theophylline	> 200	40
Caffeine	> 200	> 200

more than 90% of cyclic GMP phosphodiesterase activity was localized in the soluble fraction. However, when Triton X-100 (1%) was included in the reaction mixtures, both activities in soluble fractions were decreased considerably while detergent showed almost no effect on particulate associated enzymes. When high-speed sediment fraction was resuspended in the

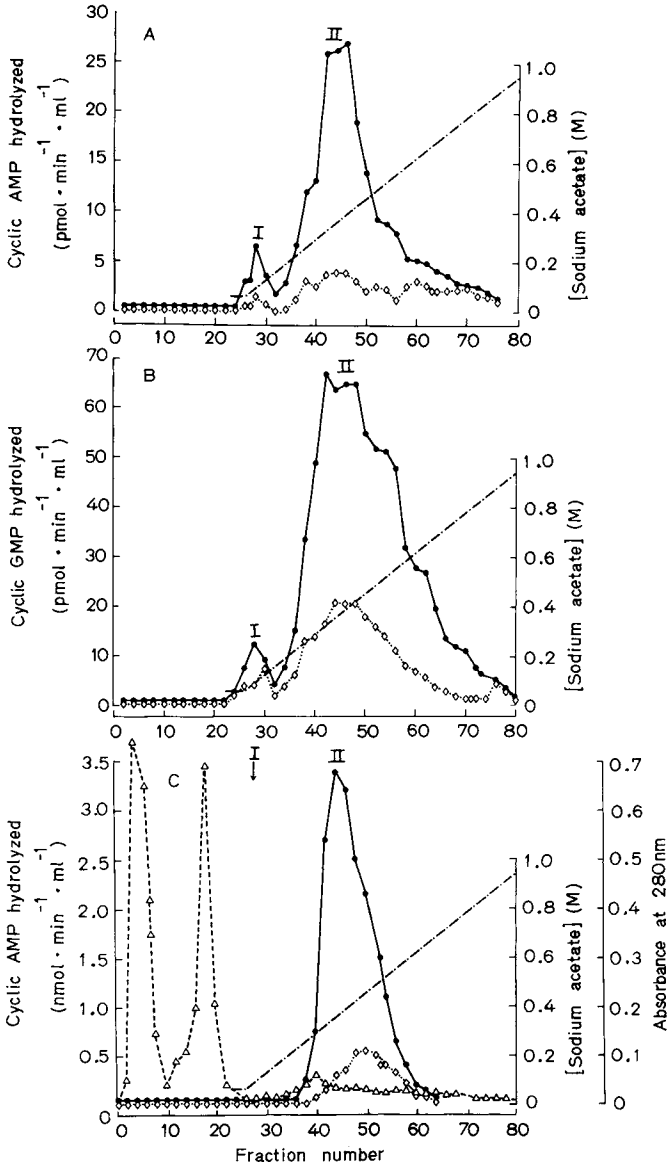

Fig. 6. *DEAE-cellulose column chromatography of pituitary phosphodiesterases.* Experimental details are given in Materials and Methods. (◇···◇) Without activator; (●——●) with activator (28 μg) + $CaCl_2$ (100 μM). (A) Cyclic AMP (0.4 μM); (B) cyclic GMP (0.4 μM); and (C) cyclic AMP (100 μM). (–·–·–) Sodium acetate gradient; (△----△) absorbance at 280 nm

presence of the detergent and then centrifuged at 105000 × *g* (60 min) almost all of the activity seen in the original sediment remained in the soluble fraction. Addition of protein activator and $CaCl_2$ in the incubation medium increased the enzyme activity in almost all fractions particularly when cyclic AMP was the substrate (Table 6).

Gel Filtrations

Further comparative characterization of soluble and particulate associated enzymes was carried out

Table 5. *Effect of different activators on the peak II phosphodiesterase activity*
Peak I and peak II phosphodiesterase activities were separated by DEAE-cellulose colum chromatography as described in the text. Active fractions from peak II were used in the present studies. The activities were determined either in the absence or presence of brain activator (28 μg protein) or pituitary activator (15 μg protein). Results are mean of duplicate experiments

Substrate	Concn	Activity with		
		no activator	brain Activator	pituitary activator
	μM	pmol hydrolysed/min		
Cyclic AMP	0.4	100	530	625
Cyclic GMP	0.4	525	1520	1625
Cyclic AMP	100	14000	71000	87000

Table 6. *Effect of Triton X-100 on activity and solubilization of cyclic nucleotide phosphodiesterase from rat anterior pituitary*
Forty pituitaries were homogenized in 5 ml of buffer A (20 mM Tris-HCl, pH 7.4; 10 mM KCl; 10 mM NaCl; 1 mM $MgCl_2$; 3.75 mM 2-mercaptoethanol) and centrifuged at 105000 × *g* for 60 min. The supernatant a fluid was removed and 105000 × *g* sediment was washed and either resuspended in buffer (sediment a) or resuspended in buffer A containing 1% Triton X-100, centrifuged at 105000 × *g* for 1 h, the supernatant b removed, the sediment washed with buffer A and finally resuspended in buffer A (sediment b). 20–40-μl aliquots of each fraction were assayed for cyclic AMP and cyclic GMP phosphodiesterase activities in the presence or absence of Triton X-100 (1%). Activator + $CaCl_2$ as indicated. Other details were the same as described under the Methods. Values are the mean of duplicate determinations

Material	Fraction used for enzyme assay	Cyclic nucleotide hydrolyzed with			
		no additions	Triton X-100 (1%)	activator + $CaCl_2$	activator + $CaCl_2$ + Triton X-100 (1%)
		pmol/min			
A. *Cyclic AMP*					
1. Tissue	whole homogenate	500	321	755	601
	supernatant a	416	226	553	432
	sediment a	67	62	82	59
2. Sediment a	supernatant b	52	58	91	102
	sediment b	19	14	31	17
B. *Cyclic GMP*					
1. Tissue	whole homogenate	1137	1086	1400	1490
	supernatant a	1120	994	1200	1260
	sediment a	114	136	234	310
2. Sediment a	supernatant b	98	102	262	282
	sediment b	16	7	50	38

by gel filtration. When whole 40000 × *g* supernatant prepared in the presence of 1% Triton X-100 was applied to a Bio-Gel A-5m column (equilibrated with 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 3.75 mM 2-mercaptoethanol and 0.1 mM EGTA) two peaks of activities were detected with cyclic AMP and cyclic GMP as substrates. Filtration of particulate enzyme showed a single peak which coincided with the first peak of the whole homogenate. Similarly particulate free supernatant prepared in the absence of Triton X-100 showed a single peak of activity following gel filtration. Addition of Triton X-100 to the elution buffer did not influence the elution profile of this enzyme. This peak eluted in the same position as was peak II of the whole homogenate. These results demonstrate that the particulate and soluble enzymes have different physical characteristics.

DISCUSSION

In the present communication we have attempted to characterize rat anterior pituitary phosphodiesterase which exist in multiple forms. More than 80 percent of cyclic AMP and cyclic GMP hydrolyzing activities were localized in particulate free fractions. The particulate enzyme seems to be different from soluble enzyme as judged by differences in K_m values, metal ion requirements, activator dependence and elution pattern from agarose columns. Under our experimental conditions we could not solubilize particulate enzyme more than 15–20 percent by sonication but were able to solubilize it completely by detergent treatment. Gel filtration studies showed that the particulate and soluble enzymes differ in physical properties suggesting that the apparent subcellular distribution

seen in Table 6 was not due to adsorption of soluble enzyme to particulate material during the isolation process.

The anterior pituitary was also rich in cyclic nucleotide phosphodiesterase activator protein. As shown in Tables 3 and 5, the extent of stimulation of cyclic nucleotide phosphodiesterases by the activator was comparable to that reported by Cheung [16] and Kakuichi *et al.* [17] when the activities were assayed in the presence of Ca-EGTA buffer [17]. However, when the enzyme assays were carried out in the absence of EGTA, the extent of stimulation by the activator was not as pronounced as that seen in the presence of EGTA. This phenomenon can simply be explained on the basis that the enzyme preparations used after gel filtration may still contain enough endogenous activator. This possibility was further strengthened by the observation that DEAE-cellulose-purified peak II enzyme was stimulated five–six-fold even in the absence of EGTA which was devoid of most of the endogenous activator. The activation of enzyme seems to be dependent on the presence of divalent metal ions. In other systems, Ca^{2+} has been shown to be required for the interaction of activator with phosphodiesterases [18, 27–29]. In the present system, Ca^{2+} failed to exhibit any stimulatory effect either by itself or to potentiate the stimulatory effect of activator. However, Ca^{2+} did reverse the inhibitory action of EGTA when added along with EGTA or after the EGTA treatment of the enzyme suggesting that Ca^{2+} is involved in the regulation of phosphodiesterases. Somewhat similar observations have been made for phosphodiesterase activities from pig coronary arteries [30]. These data also do not preclude the possibility that the anterior pituitary contains both Ca^{2+}-dependent and independent forms of cyclic AMP phosphodiesterase(s). Further, there is a possibility that the activator preparations employed in our studies still contained a small amount of firmly bound Ca^{2+} even after its dialysis against buffer containing EGTA [31]. In addition, although deionized water or in some cases Ca^{2+}-EGTA buffer was used to follow Ca^{2+} effect, we cannot rule out the possibility that some of the solutions employed had enough Ca^{2+} to activate phosphodiesterase activity.

The partially purified (gel filtration) pituitary cyclic nucleotide phosphodiesterase showed more activity with cyclic GMP compared to cyclic AMP when activities were assayed in the presence of sub-saturating concentrations of substrate. These results are comparable to those reported for heart, brain and liver enzymes [1, 13]. In the pituitary, like heart and cerebellum [13], cyclic GMP hydrolyzing enzyme accounted for more than 80% total cyclic nucleotide hydrolyzing activity. Following gel filtration, the peak activity fraction hydrolyzed cyclic GMP to a greater extent compared to cyclic AMP but activity increased with increasing concentrations of cyclic AMP and at saturating concentration, it hydrolyzed cyclic AMP at a faster rate than cyclic GMP probably because the tissue concentrations of cyclic AMP are far greater than cyclic GMP [32, 33].

It is now believed that cyclic nucleotide phosphodiesterase exists in multi-molecular forms [2, 3, 6, 10, 34–43]. Cyclic nucleotide phosphodiesterases from rat brain, heart, skeletal muscle, kidney and adipose tissue have been separated into two forms by gel filtration [2, 3, 10, 11]. However, in the present studies only one peak of activity was observed with both substrates. These differences may be due to the experimental conditions including enzyme isolation, gel filtration and assay conditions. Further, in most of the above instances effects of Ca^{2+} and activator have not been studied. On the contrary, pituitary phosphodiesterase could be separated by DEAE-cellulose column chromatography into two species with different substrate specificities and activator dependency. But none of the enzymes seem to be specific for cyclic GMP as has been reported in rat liver [10, 11]. However, some enzymatic characteristics of pituitary enzymes do agree with those of rat liver preparations, at least, partially.

The physiological role of the regulation of these phosphodiesterase(s) is currently unknown. It seems likely that these enzymes may regulate cyclic AMP concentrations *in vivo*. Since in the pituitary, Ca^{2+} is known to modulate the accumulation of cyclic AMP [20] and the present finding that Ca^{2+} modulates phosphodiesterase activity, it is likely that the concentration of Ca^{2+} and the capacity of activator to bind Ca^{2+} may be one of the determining factors in regulating cyclic-AMP-mediated [19] and gonadotropin-induced lutropin release from anterior pituitary.

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