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PURIFICATION AND PROPERTIES OF CYCLIC AMP PHOSPHODIESTERASE FROM RAT BRAIN

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SUMMARY

1. Cyclic AMP phosphodiesterase was partially purified from rat cerebral cortex.

2. Cerebral cyclic AMP phosphodiesterase was stimulated by imidazole but largely inhibited by ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA). Half-maximal activation was observed with $3 \cdot 10^{-7}$ M Ca^{2+} (*I* 0.05).

3. Enzymes from tissues other than cerebrum (cerebellum, heart, liver, spleen and kidney cortex) were not inhibited by EGTA.

4. Cerebral phosphodiesterase which had been partially digested with trypsin was not inhibited by EGTA, but retained nearly the same total activity and was still stimulated by imidazole (desensitization).

5. It is suggested that from rat brain a specific phosphodiesterase preparation can be obtained, which contains an inhibitory protein, the activity of which may be regulated by Ca^{2+}.

INTRODUCTION

There is much evidence that adenosine 3',5'-monophosphate (cyclic AMP) acts as an intracellular mediator of the actions of several hormones[1-4]. Cyclic AMP is synthesized by adenyl cyclase[5] and hydrolyzed by a phosphodiesterase[6]. A cyclic AMP phosphodiesterase, which is widely distributed in animal tissues, has been partially purified from heart[6,7], brain[8,9], liver[10], erythrocytes[11], *etc.*, and its properties have been investigated. This enzyme also controls level of the intracellular cyclic AMP[6,9]. However, satisfactory purification of this enzyme from various tissues has not been achieved due to its lability during the purification procedures. The present paper describes the purification of phosphodiesterase from the rat cerebral cortex and the properties of the enzyme. The regulation of cerebral phosphodiesterase activity is also discussed.

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid.

MATERIALS

Cyclic adenosine 3′,5′-monophosphate was obtained from Takeda Pharmaceutical Co. Ltd. *Crotalus atrox* venom was purchsed from Sigma Chemical Co. Ltd. Cyclic [^{3}H]AMP (4.86 Ci/mmole) was purchased from New England Nuclear Corporation. Ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) and CaEGTA were purchased from Dojindo Co. Ltd. Hydroxylapatite was prepared by a standard method[12]. Redistilled water was used throughout.

METHODS AND RESULTS

Assay of phosphodiesterase activity

Phosphodiesterase activity was assayed by a modification of the method of Butcher and Sutherland[6]. One-step or two-step assay was done as occasion required. One-step assay was done in 1.0 ml of reaction mixture containing 40 mM Tris–HCl buffer (pH 7.5), 2 mM $MgCl_2$, 0.6 mM cyclic AMP, 100 μg *Crotalus atrox* venom and phosphodiesterase sample. The reaction was started by addition of substrate and the mixture was incubated for 10 min at 30°. The reaction was stopped by adding 0.1 ml of 55% trichloroacetic acid and then released P_i was measured by the method of Takahashi[13]. The reaction mixture of two-step assay was the same as above except that it was incubated for 10 min without *Crotalus atrox* venom. The reaction was stopped by boiling the mixture for 3 min. Then the mixture was cooled to 30° and incubated with additional snake venom (100 μg) for another 10 min. The reaction was stopped with trichloroacetic acid and P_i was measured as in the one-step assay.

1 unit of phosphodiesterase activity is defined as the amount converting 1 μmole of cyclic AMP in 30 min at 30°. Specific activity was expressed in units of enzyme per mg of protein. Phosphodiesterase activity was also assayed with cyclic [^{3}H]AMP by the method of Brooker *et al.*[14]. Protein concentration was determined by the method of Lowry *et al.*[15].

Enzyme preparations at different steps of purification were dialyzed against 2 mM imidazole buffer (pH 7.5) containing 1 mM $MgSO_4$, and then their activities were assayed.

Cyclic [^{3}H]AMP binding activity

This was assayed by a modification of the method of Gill and Garren[16]. For binding, a mixture consisting of 0.1 ml of 100 mM potassium phosphate buffer (pH 5.0), 0.1 ml of cyclic [^{3}H]AMP (6500 cpm, $3 \cdot 10^{-12}$ mole) and 0.1 ml of sample (final volume 0.3 ml) was kept for 30 min at 0°. Then it was diluted with 2 ml of cold 50 mM potassium phosphate buffer (pH 5.0) and filtered through a Millipore filter (HA 0.45-μm pore size). The filter was washed with the same buffer and dissolved in 5 ml of Bray's solution in a scintillation vial and the radioactivity was counted.

Enzyme purification

Step 1 (homogenate and 11 500 × g supernatant). Wistar strain rats were decapitated and their cerebral cortices were removed. These were homogenized in 10 vol. of chilled 0.32 M sucrose (pH 7.4) in a Teflon homogenizer. The homogenate was

centrifuged for 30 min at 11 500 × *g* and the supernatant (11 500 × *g* supernatant) was frozen at −20° for several days.

Step 2 (105 000 × g supernatant). The 11 500 × *g* supernatant was thawed and centrifuged for 30 min at 105 000 × *g*. A clear yellow supernatant (105 000 × *g* supernatant) was obtained.

Step 3 (freeze–thawing). The 105 000 × *g* supernatant was frozen in dry ice–acetone and thawed in water bath at 37°. This procedure was repeated. The heavy flocculent material formed was removed by centrifugation for 30 min at 105 000 × *g* and a clear supernatant (freeze–thawing fraction) was obtained. More than two cycles of freeze–thawing caused much loss of activity. The clear supernatant could be kept at −20° for 1 month without loss of activity.

Step 4 (hydroxylapatite column). The freeze–thawing fraction was adsorbed on a hydroxylapatite column. The gradient elution was obtained by putting 100 ml of 1 mM sodium phosphate buffer (pH 7.5) in the mixing chamber and 100 ml of 200 mM sodium phosphate buffer (pH 7.5) in the reservoir. The flow rate was 0.3–0.4 ml/min. Protein was eluted as a large initial peak followed by a shoulder (Fig. 1). The enzyme activities in Fractions I, II and III in Fig. 1 are shown in Table I. Only Fraction II had appreciable activity. The enzyme was extremely labile on freezing at this stage, so the next procedure was carried out immediately.

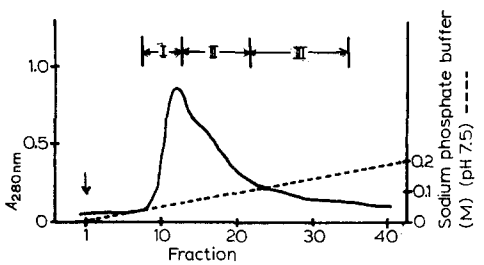

Fig. 1. Hydroxylapatite column. A hydroxylapatite column (2 cm × 12 cm) was equilibrated with 2 mM imidazole buffer (pH 7.5) containing 1 mM $MgSO_4$. Undialyzed preparation was applied to the column at a load of 3 mg protein per ml column bed volume. The column was washed with 2 mM imidazole buffer containing 1 mM $MgSO_4$, and then protein fractions were eluted by gradient elution. Fractions of 5 ml were collected and their absorbance at 280 nm was measured. The eluates in Tubes 8–12 (Fraction I), Tubes 13–22 (Fraction II) and Tubes 23–34 (Fraction III), respectively, were combined. Fraction II had activity.

TABLE I

ACTIVITIES OF FRACTIONS FROM A HYDROXYLAPATITE COLUMN

The protein in each fraction in Fig. 1 was precipitated with 60% saturation of $(NH_4)_2SO_4$. The precipitates were dialyzed against 2 mM imidazole buffer (pH 7.5) containing 1 mM $MgSO_4$ and assayed in 40 mM Tris–HCl buffer (pH 7.5).

Fraction	*Specific activity (units/mg protein per 30 min)*	*Yield (%)*
I	0	—
II	22.4	40
III	9.65	8

Step 5 (60% $(NH_4)_2SO_4$). Fraction II was adjusted to 60% saturation with $(NH_4)_2SO_4$ by adding solid $(NH_4)_2SO_4$ without neutralization. After 45 min standing, the precipitate was collected by centrifugation. It was dissolved in 1/3 vol. of 2 mM imidazole buffer (pH 7.5) containing 1 mM $MgSO_4$ and dialyzed against three changes of the same buffer for 18 h. This enzyme preparation was labile and lost half its activity within 2 weeks on storage at −20°. All procedures were carried out at 4°.

Table II shows the purification and yield of phosphodiesterase.

TABLE II

SUMMARY OF PURIFICATION AND YIELD OF CYCLIC AMP PHOSPHODIESTERASE

Each preparation was dialyzed against 2 mM imidazole buffer (pH 7.5) containing 1 mM $MgSO_4$ and assayed in 40 mM Tris–HCl buffer (pH 7.5).

Fraction	*Protein (mg)*	*Activity (units)*	*Specific activity (units/mg protein per 30 min)*	*Yield (%)*
Homogenate	1193	1490	1.25	100
11 500 × *g* supernatant	265	895	3.37	60
105 000 × *g* supernatant	230	1010	4.35	68
Freeze–thawing	112	824	7.35	54
Hydroxylapatite column and $(NH_4)_2SO_4$	9.75	222	22.4	22

Phosphodiesterase activity and cyclic [^{3}H]AMP binding activity

The binding activity increased with the phosphodiesterase activity until Step 5 of purification (Table III). However, sucrose density gradient centrifugation separated phosphodiesterase activity from binding activity (Fig. 2, Table III). Phosphodiesterase activity was assayed with cyclic [^{3}H]AMP (ref. 14) to see if there was any interaction between enzyme activity and binding activity, but activity of phosphodiesterase (K_m or V) was not affected by binding protein. The K_m of the enzyme after sucrose gradient centrifugation was $0.6 \cdot 10^{-5}$–$1.0 \cdot 10^{-5}$ M.

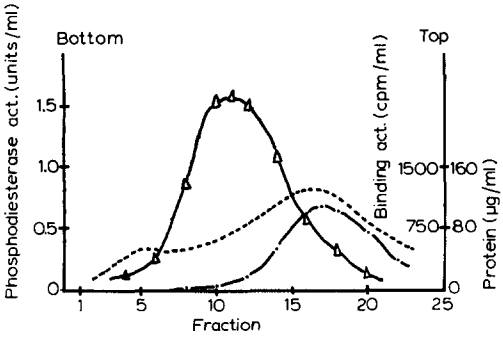

Fig. 2. Sucrose density gradient centrifugation of phosphodiesterase and binding protein. A 0.3-ml aliquot of phosphodiesterase preparation (spec. act., 13.0; protein, 2.6 mg/ml) was layered over a 5-ml, 5–20% sucrose gradient in 10 mM Tris–HCl buffer (pH 7.5). It was centrifuged at 36 000 rev./min for 16.5 h at 4° in a SW 39 rotor. Then 0.25-ml fractions were collected from the bottom of the tube. △—△, phosphodiesterase activity; — — —, protein concentration; — · — · —, binding activity.

TABLE III

PHOSPHODIESTERASE AND CYCLIC[^{3}H]AMP-BINDING ACTIVITIES IN VARIOUS PREPARATIONS

Fraction	*Specific activity (units/mg protein per 30 min)*	*Cyclic[^{3}H]AMP-binding activity (cpm/mg protein)*
11 500 × *g* supernatant*	1.73	5 300
105 000 × *g* supernatant	3.0	8 100
Freeze–thawing	4.75	7 900
Hydroxylapatite column and 60% $(NH_4)_2SO_4$	13.0	14 000
Sucrose density	18.0	235

* The 11 500 × *g* supernatant used in this experiment had been frozen at −20° for several months with some loss of activity.

Phosphodiesterase activity in various fractions of rat cerebral cortex

The specific activities of the enzyme in various fractions of rat cerebral cortex are shown in Table IV. The supernatant fraction contained about 60% of the total activity (Table II) and its specific activity was the highest. Among the particulate fractions, the synaptic membrane fraction showed high specific activity.

TABLE IV

PHOSPHODIESTERASE ACTIVITIES IN VARIOUS FRACTIONS OF RAT CEREBRAL CORTEX

Fractionation was carried out by the method of Whittaker *et al.*[17]. Enzyme activities were assayed in 40 mM Tris–HCl buffer (pH 7.5).

Fraction	*Specific activity (units/mg protein per 30 min)*
Homogenate	1.25
Mitochondria	1.13
Synaptic membranes	1.79
Synaptic vesicles	1.23
Microsomes	1.47
Supernatant	3.0

Stimulation by imidazole and inhibition by EGTA

Enzyme activity was about 1.5-fold higher in imidazole buffer (40 mM, pH 7.5) than in Tris–HCl buffer (Table V). Moreover, addition of 1 mM EGTA completely abolished the stimulatory effect of imidazole.

TABLE V

IMIDAZOLE STIMULATION AND EGTA INHIBITION IN TRIS BUFFER AND IMIDAZOLE BUFFER

The phosphodiesterase activity was 16.1 units per mg protein. Incubation was carried out for 10 min. Activity is expressed as the absorbance (at 750 nm) given by P_i released.

Buffer	*Absorbance/tube*	
	Control	*1 mM EGTA*
40 mM Tris (pH 7.5)	0.110	0.060
40 mM Imidazole (pH 7.5)	0.157	0.058

Determination of the effective Ca^{2+} concentration

The relation between the free Ca^{2+} concentration and phosphodiesterase activity was investigated using CaEGTA buffer. The free Ca^{2+} concentration was calculated by the method of Weber *et al.*[18]. The experiment was carried out at pH 6.9 and I 0.05. 2 mM CaEGTA had no effect on phosphodiesterase activity. Fig. 3 shows that $3 \cdot 10^{-7}$ M free Ca^{2+} causes half-maximal stimulation. The results suggest that cerebral phosphodiesterase may be regulated by Ca^{2+} *in vivo*, as suggested by Kakiuchi *et al.*[19].

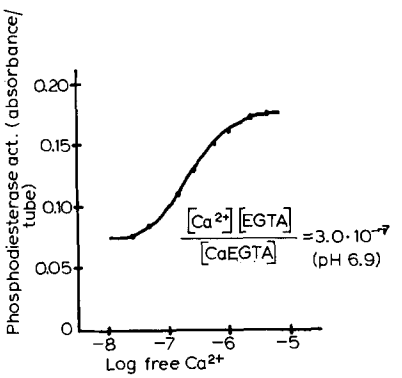

Fig. 3. Activation of phosphodiesterase by Ca^{2+} and determination of the effective free Ca^{2+} concentration. Enzyme with a specific activity of 9.05 units per mg protein was adjusted to the activity shown in 40 mM imidazole buffer (pH 6.9) and incubated for 30 min. The free Ca^{2+} concentration was varied by changing the EGTA:CaEGTA ratio.

Trypsin digestion and EGTA inhibition

Table VI shows that the inhibitory effect of EGTA was lost after treatment of phosphodiesterase with trypsin (desensitization). These results suggest the presence in the enzyme preparation of an inhibitory protein, the activity of which is regulated by Ca^{2+}. This effect of trypsin was not due to the binding of trypsin protein with inhibitory protein, because the effect of trypsin increased with time.

TABLE VI

THE EFFECT OF TRYPSIN DIGESTION ON PHOSPHODIESTERASE ACTIVITY

A suitably diluted sample of phosphodiesterase (spec. act. 9.05) was preincubated for the period shown in 0.5 ml of a mixture consisting of 80 mM imidazole buffer (pH 7.5), 4 mM $MgSO_4$ and trypsin (50 μg), and then trypsin inhibitor was added. *Crotalus atrox* venom (100 μg) and cyclic AMP (0.6 μmole) were added. The reaction mixture was made up to 1.0 ml and incubated with or without EGTA for 10 min. Activity is expressed as the absorbance given by P_i released. To obtain the zero time value, trypsin was added after trypsin inhibitor.

Trypsin treatment	*Absorbance/tube*	
time (min)	*Control*	*1 mM EGTA*
0	0.145	0.030
1	0.140	0.084
5	0.145	0.135
10	0.095	0.105
15	0.093	0.098

Phosphodiesterases from various tissues

Table VII shows that rat liver, heart, cerebellar, spleen and kidney cortical phosphodiesterases were stimulated by imidazole, but were not inhibited by EGTA. The same results were obtained using the two-step assay method, so this effect was

TABLE VII

PHOSPHODIESTERASE ACTIVITIES IN VARIOUS TISSUES AND THEIR INHIBITION BY EGTA

Samples of various tissues were homogenized with 0.32 M sucrose, and the homogenates were centrifuged for 30 min at 11 500 × *g*. The supernatants were dialyzed against 2 mM imidazole buffer, 1 mM $MgSO_4$ for 18 h. Activities were assayed with suitably diluted samples giving values for activity which were nearly equal to 0.1 absorbance unit in 40 mM imidazole buffer (pH 7.5). In estimating specific activity, activities were assayed in 40 mM Tris–HCl buffer (pH 7.5). Cerebral phosphodiesterase was incubated for 10 min, the other preparations for 30 min. The same results were also obtained with the samples dialyzed for 3 h.

Tissue	*Absorbance/tube*			*Specific activity (units/mg protein per 30 min)*
	Imidazole buffer	*Imidazole buffer + 1 mM EGTA*	*Tris buffer*	
Cerebrum	0.120	0.042	0.084	3.9
Cerebellum	0.112	0.112	0.072	0.41
Heart	0.145	0.145	0.105	0.20
Liver	0.110	0.110	0.078	0.21
Spleen	0.067	0.077	0.040	0.14
Kidney cortex	0.117	0.119	0.079	0.34

not due to the proteolytic action[8] of snake venom. The specific activities of phosphodiesterase preparations from liver, heart, cerebellum, spleen, kidney cortex were approx. 1/10–1/20 of that of cerebrum, so much more protein was required for assay of enzyme activity. Therefore, proteolytic enzymes contaminating the phosphodiesterase preparations of low specific activity may destroy part of the phosphodiesterase molecule, resulting in loss of its sensitivity to EGTA. Accordingly, this possibility was examined. As shown in Table VIII, the activities of the mixture were equal

TABLE VIII

EFFECT OF COMBINING THE LIVER AND CEREBRAL ENZYMES, AND THE CEREBELLAR AND CEREBRAL ENZYMES

Incubation was carried out in 40 mM imidazole buffer (pH 7.5) for 15 min. Activity is expressed as the absorbance given by P_i released.

Expt No.	*Enzyme*	*Absorbance/tube*	
		Imidazole buffer	*Imidazole buffer + 1 mM EGTA*
A	Cerebrum	0.130	0.070
	Liver	0.056	0.052
	Cerebrum + liver	0.190	0.130
B	Cerebrum*	0.063	0.020
	Cerebellum**	0.117	0.112
	Cerebrum + cerebellum	0.185	0.138

* Protein concentration was 30 μg.
** Protein concentration was 300 μg.

to the sum of the two separate activities, and preparations of enzymes from rat liver and cerebrum and from rat cerebellum and cerebrum, had no influence on each other. So the fact that phosphodiesterase preparations other than cerebrum were not inhibited by EGTA was not due to the action of proteolytic enzymes in the assay system.

DISCUSSION

The purification of phosphodiesterase from various tissues has been reported, but it is difficult to compare the purities of the preparations obtained as the assay conditions were not the same. It was reported that the specific activity of enzyme from beef heart[6] was 25.6 units/mg protein, that from dog heart[7] was 28 units/mg protein, that of rabbit brain[9] was 1.2 units/mg protein and that of rat brain[8] was 10 units/mg protein. But no satisfactory purification of any of these enzymes, and especially the brain enzymes, has been achieved because the enzyme was labile during purification. We purified phosphodiesterase from rat cerebrum to a specific activity of 22 units/mg protein, but the enzyme was still not pure. When hydroxylapatite column chromatography was carried out after $(NH_4)_2SO_4$ fractionation, no purification could be achieved. Use of DEAE-cellulose chromatography during purification resulted in great loss of activity. Cheung[20] reported that this was due to dissociation of an activator from enzyme.

Imidazole is known to stimulate phosphodiesterase[6]. We found that a portion of activity which was stimulated by imidazole was completely suppressed by EGTA. A preparation of phosphodiesterase which had been digested with trypsin and was no longer inhibited by EGTA was still stimulated by imidazole. This suggests that the site of action of imidazole is nearer to the active center than the site of action of EGTA (inhibitory protein). Roberts *et al.*[21] found that imidazole-4-acetic acid caused "uncompetitive" activation of the enzyme, probably interacting with it in a manner so as to increase the rate of dissociation from the enzyme of the product of the reaction, 5'-AMP. Kakiuchi *et al.*[22] previously reported the inhibition of phosphodiesterase by EGTA, and the existence of a Ca^{2+} + Mg^{2+}-dependent phosphodiesterase and a Ca^{2+}-independent + Mg^{2+}-dependent phosphodiesterase, suggesting the possibility of regulation of phosphodiesterase activity by Ca^{2+}. We confirmed that EGTA was inhibitory using our partially purified enzyme. When cerebral enzyme had been treated with trypsin it was no longer inhibited by EGTA, total activity remaining at nearly the same level as before treatment. From these results, it seems likely that cerebral phosphodiesterase contains an inhibitory protein which is selectively destroyed with trypsin and the activity of this protein may be regulated by Ca^{2+}. We have not yet separated a protein with an inhibitory effect. It is interesting that this inhibitory protein is similar to a troponin[23] from skeletal muscle, in its sensitivity to trypsin and regulation by a low concentration of Ca^{2+}.

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