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STIMULATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASES FROM RAT BRAIN BY ACTIVATOR PROTEIN, PROTEOLYTIC ENZYMES AND A VITAMIN E DERIVATIVE

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Summary

DEAE-cellulose column chromatography of the 105 000 × *g* supernatant from rat brain homogenate showed the presence of three forms of cyclic AMP phosphodiesterase (3′:5′-cyclic-AMP 5′-nucleotidohydrolase, EC 3.1.4.17) : P1, P2 and P3 enzymes according to their elution order from the column. Cyclic GMP phosphodiesterase activity was found only in P2. The P2 enzyme was stimulated 2—6-fold over the basal activity by three different types of agents; Ca^{2+}-dependent activator protein, sodium α-tocopheryl phosphate (a vitamin E derivative) and proteolytic enzymes such as trypsin and chymotrypsin. Unlike the activator protein, the last two types of activators exerted their effects regardless of the presence of Ca^{2+}. In contrast to P2, P3 was unaffected by any of the above-mentioned activators, whereas the activity of the P1 was slightly enhanced in the presence of each activator. Upon activating P2 with various activators, a decrease of the K_m value for substrate (cyclic AMP or cyclic GMP) and an increase of maximum velocity were commonly observed.

Treatment of the brain supernatant with trypsin caused a substantial decrease in the molecular weight of activator-sensitive cyclic AMP phosphodiesterase; the molecular weight of the native (activator-dependent) enzyme was estimated to be approximately 150 000 by Sephadex G-200 gel filtration, while the trypsin-treated enzyme had a molecular weight of 80 000 and was insensitive to activator protein. These activator-dependent and activator-independent enzymes were separated by an affinity column, which was prepared by cyanogen bromide-activated Sepharose with purified activator protein.

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Introduction

Cyclic nucleotide phosphodiesterases were found to be present ubiquitously in mammalian tissues including liver, heart, muscle, kidney and brain [1—6]. Of these, the brain is the most abundant source of activator-dependent phosphodiesterase as well as the activator protein [6—11]. Extensive studies have recently shown that in several tissues cyclic nucleotide phosphodiesterase(s) existed in multiple forms [1—6,12—22]. In the brain multiple forms of the enzyme have been demonstrated by various methods such as gel filtration chromatography [6,23], isoelectric focusing [24], and polyacrylamide gel column [12]. We shall describe a method to prepare multiple forms of activator-dependent and activator-independent cyclic AMP phosphodiesterases from rat brains using DEAE-cellulose chromatography. These enzymes differed in the elution pattern from an affinity column of Sepharose coupled with purified activator protein.

Besides the well-known Ca^{2+}-dependent activator protein [7,8,25,26], two other types of compounds have been demonstrated to be activators of cyclic AMP phosphodiesterase both in a Ca^{2+}-independent fashion: one type is proteolytic enzymes which were first demonstrated by Cheung [7,27], and the other type a vitamin E derivative called sodium α-tocopheryl phosphate. A previous report from our laboratories [28] established that the vitamin E derivative selectively stimulated the cyclic AMP phosphodiesterase from liver; this stimulation may well be due to a detergent-like action of sodium α-tocopheryl phosphate. Indeed, the detergent-induced activation of phosphodiesterase was also confirmed recently by Wolff and Brostrom [29] who showed that both phosphatidyl inositol and lysophosphatidyl choline did effectively activate the enzyme prepared from the brain. In this communication we shall compare the activation mechanisms of cyclic nucleotide phosphodiesterase induced by activators including activator protein, proteolytic enzymes and a vitamin E derivative.

Materials and Methods

Materials. [^{3}H]adenosine 3′:5′-cyclic phosphate (cyclic [^{3}H]AMP) (39.8 Ci/mmol) and [^{3}H]guanosine 3′:5′-cyclic phosphate (cyclic [^{3}H]GMP) (2.1 Ci/mmol) were purchased from New England Nuclear Corporation, Boston, Mass. AG 1-X8 was obtained from Bio Rad Laboratories, Richmond, Calif. Unlabeled cyclic AMP, cyclic GMP and snake venom (from *Crotalus atrox*) were purchased from Sigma Chemical Co., St. Louis. Sodium α-tocopheryl phosphate was from Eisai Pharmaceutical Co. Tokyo, Japan. Trypsin, chymotrypsin, carboxypeptidase A, leucine aminopeptidase were from Worthington Biochemical Corporation, Freehold, N.J. *Bacillus subtilis* alkaline protease was a gift from Daiwa Kasei, Osaka, Japan. DEAE-cellulose (DE 52 microgranular type) was purchased from Whatman Ltd., Kent, U.K. Sepharose 2B and DEAE-Sephadex A-50 were from Pharmacia, Uppsala, Sweden. All other chemicals were of commercial analytical grade quality.

Preparation of cyclic nucleotide phosphodiesterases from rat brain. Male Wistar rats were killed by decapitation. The brain was excised, minced and

homogenized in 5 volumes (w/v) of 0.04 M Tris · HCl buffer (pH 8.0), containing 0.32 M sucrose in a glass homogenizer with 5 strokes of a Teflon pestle. The homogenate was centrifuged at 105 000 × *g* for 60 min. The resulting supernatant was used throughout the studies.

DEAE-cellulose chromatography. DEAE-cellulose column (bed volume, 20 ml) was equilibrated at 4° C with 70 mM acetate buffer (pH 6.5), containing 0.1 mM EGTA and 5 mM 2-mercaptoethanol. 2—4 ml of the brain supernatant were applied to the column and 7-g fractions were collected. The column was eluted with a linear 200 ml gradient from 70 mM to 1 M acetate buffer (pH 6.5), containing 0.1 mM EGTA and 5 mM 2-mercaptoethanol. This chromatography system was a modification of the method reported by Russell et al. [4] for rat liver.

Preparation of activator protein. Phosphodiesterase activator protein [7,8, 25,26] was prepared from bovine brain by the procedure of Kato et al. [30]. Briefly, 2 kg of bovine brain were homogenized in 8 l of 0.1 M potassium phosphate buffer (pH 7.1), containing 1 mM EDTA and 55%-saturated ammonium sulfate. The homogenate was centrifuged at 10 000 × *g* and the supernatant was kept. To the supernatant solid ammonium sulfate was added to 85% saturation and the pH of the supernatant was adjusted to 4.6 by the addition of phosphoric acid. The precipitate was collected by centrifugation and dissolved in a minimum amount of 0.1 M potassium phosphate buffer (pH 7.1), containing 1 mM EDTA and 0.05 M sodium chloride (Buffer A). After dialysis against the Buffer A, the dialyzed solution was applied to a DEAE-Sephadex A-50 column (100 ml) which had been equilibrated with Buffer A. The column was eluted by successive application of 300-ml portions of Buffer A containing increasing amounts of sodium chloride (0.22 M and 0.3 M). The activator protein was eluted by the buffer containing 0.3 M sodium chloride. The homogeneity and physical properties of the purified activator protein were described elsewhere [30].

Immobilization of activator protein to Sepharose 2B. Phosphodiesterase activator protein purified from bovine brain [30] was immobilized to Sepharose 2B by the cyanogen bromide method [34] with some modifications [35]. To 5 ml of Sepharose 2B suspension containing 3.5 ml of packed Sepharose gel were added 2.5 ml of 2% BrCN solution. The activation reaction was allowed to proceed for 9 min by the dropwise addition of 3 M NaOH, keeping the pH of reaction mixture to 11. BrCN-activated Sepharose 2B was immediately washed on a glass filter with 60 ml of 0.1 M borate/HCl buffer (pH 8.15), and then mixed with 5 ml of the same buffer containing 9.0 mg of activator protein and 10 mM $CaCl_2$. The coupling reaction was performed for 24 h at 25° C. Subsequently, the Sepharose was washed with 60 ml of 0.05 M borate/HCl buffer (pH 8.15), containing 1 M NaCl and 1 mM EDTA. The concentration of activator protein coupled to the Sepharose was 1.5 mg/ml of packed gel.

Phosphodiesterase assay. The modified procedure [31] of the radioisotopic method of Thompson and Appleman [23] was employed for the assay of cyclic nucleotide phosphodiesterase activities. The volume of the final assay mixture was 0.2 ml which contained 10 mM $MgCl_2$, 40 mM Tris · HCl (pH 8.0), 1 μM cyclic nucleotide (90 000 cpm) and 25—50 μl of enzyme preparation, unless

otherwise indicated. The reaction was initiated by the addition of substrates, and was terminated by boiling for 45 s. After incubation for 5 min at 37°C, enzyme activities were determined in such a manner that no more than 15% of the substrate was hydrolyzed during the reaction. Experiments were conducted in duplicate.

Assay of phosphodiesterase activator activity. The ability of the activator protein to stimulate the activity of rat brain phosphodiesterase was assayed using the same assay procedure of phosphodiesterase as described above. The P2 enzyme which was prepared as described in Results from rat brain was used as activator protein-deficient phosphodiesterase. The reaction mixture (0.2 ml) contained 40 mM Tris · HCl (pH 8.0), 10 mM $MgCl_2$, 1 μM cyclic AMP, 1 mM $CaCl_2$ and an appropriate amount (0—1 μg/tube) of the activator protein. The amount of activator protein was estimated from the standard activation curve using the purified bovine brain activator protein (prepared as described in the previous section).

Gel filtration. The Sephadex G-200 column was 2 × 30 cm with a gel bed volume of 100 ml. The gel was equilibrated in 70 mM acetate buffer (pH 6.5), containing 5 mM 2-mercaptoethanol and 0.1 mM EGTA and was charged with 1—2 ml of 105 000 × *g* brain supernatant. 1.8 g of the fractions were collected. The equilibration and elution buffers were identical. The molecular weights of the fractions eluted from the column were estimated from their V_e/V_o ratios, with cytochrome *c*, bovine serum albumin, catalase, human myeloma γ-globulin and Blue Dextran 2000 as standards.

Results

DEAE-cellulose column chromatography of brain supernatant

2 ml of rat brain 105 000 × *g* supernatant (see Methods) were applied to a column of DEAE-cellulose and the column was eluted with a linear gradient of 70 mM acetate buffer (pH 6.5). Fig. 1 shows a typical elution profile of cyclic nucleotide phosphodiesterase. The peak of cyclic GMP phosphodiesterase activity was single and nearly symmetrical, whereas the activity of cyclic AMP phosphodiesterase (assayed at 1 μM substrate concentration) gave three peaks designated as P1, P2 and P3 as shown in Fig. 1. The P2 fraction was aligned with the peak of cyclic GMP phosphodiesterase activity. A single peak of the endogenous activator protein was observed around Fraction 18 (Fig. 1), to which none of the phosphodiesterase peaks corresponded. The fractions of P1 (Fraction Nos. 5, 6 and 7 in Fig. 1), P2 (Fraction Nos. 9, 10 and 11) and P3 (Fraction Nos. 15, 16 and 17) were pooled separately and were used to conduct further characterization.

Effects of various phosphodiesterase stimulators on DEAE-cellulose fractions

Several agents are currently known to activate cyclic AMP phosphodiesterase from a variety of tissues. These agents are classified into three groups: (1) activator protein or Ca^{2+}-dependent regulatory protein [8,25,26]; (2) certain phosphate containing detergents such as sodium α-tocopheryl phosphate (TPNa) [28] and lysophosphatidyl choline [29]; and (3) proteolytic enzymes [7,27, 33]. A comparison of the phosphodiesterase activities of P1, P2 and P3 frac-

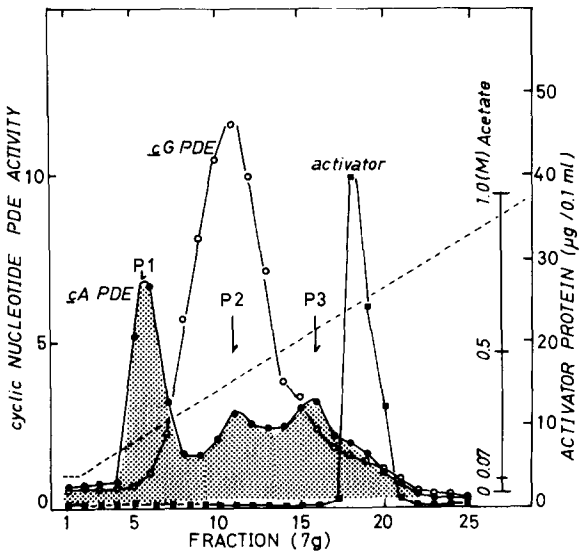

Fig. 1. DEAE-cellulose profile of cyclic nucleotide phosphodiesterase and endogenous activator protein from rat brain. Rat brain 105 000 X *g* supernatant fraction was obtained as described in Methods. Two ml of the supernatant (10 mg protein) was applied to the DEAE-cellulose column equilibrated with 70 mM acetate buffer (pH 6.5) containing 0.1 mM EGTA and 5 mM 2-mercaptoethanol. The column was developed with a linear 200 ml gradient from 0.07 M to 1.0 M sodium acetate buffer (pH 6.5) containing 0.1 mM EGTA and 5 mM 2-mercaptoethanol. Fractions (7 g) were collected. Aliquots of 25 μl were used for phosphodiesterase assay. Activity was measured using 1 μM cyclic AMP (●———●, shaded), or 1 μM cyclic GMP (○———○) and was expressed as pmol of cyclic nucleotide hydrolyzed/min per tube. The major peaks of cyclic AMP phosphodiesterase activity were designated to P1, P2 and P3 in order of elution. To measure endogenous activator protein in each fraction, aliquots of fractions were first boiled for 60 s to inactivate the phosphodiesterases in the fraction and the boiled aliquots were used for the assay of activator protein as described in Methods. The activator protein was expressed as μg protein/0.1 ml of fraction (■———■).

tions revealed the following facts with regard to the extent of stimulation by these agents (Table I). Firstly, all three types of the activators preferentially stimulated the P2 phosphodiesterase. The P1 fraction was also stimulated both by activator protein and by tryptic treatment, although the extent of activation was much smaller than that of the P2 fraction. On the other hand, the P3 enzyme was affected by none of the stimulators. Secondly, cyclic GMP phosphodiesterase of the P2 fraction, too, was stimulated by the same three stimulators; however, only 4-fold increase in cyclic GMP phosphodiesterase activity was observed at a substrate concentration of 1 μM, as compared to 8-fold augment of the P2 cyclic AMP phosphodiesterase. As with cyclic AMP phosphodiesterase, the P3 cyclic GMP phosphodiesterase was not activated in the presence of activators. Likewise, the cyclic GMP phosphodiesterase of the P1 fraction was insensitive to activators. Thirdly, once the P2 cyclic AMP phosphodiesterase was activated by one of the effectors, there was no combined stimulatory effect by the further addition of any of the other agent. Moreover, the addition of sodium α-tocopheryl phosphate to the activated P2 enzyme constantly decreased cyclic AMP phosphodiesterase activity.

TABLE I

EFFECTS OF VARIOUS ACTIVATORS ON THE CYCLIC NUCLEOTIDE PHOSPHODIESTERASES FRACTIONATED BY DEAE-CELLULOSE CHROMATOGRAPHY

Rat brain 105 000 × *g* supernatant fraction was obtained and was chromatographed on DEAE-cellulose as described under Methods. The P1, P2 and P3 enzymes were assayed for cyclic AMP as well as cyclic GMP phosphodiesterase activities with or without various activators. Activators were: activator protein (0.1 μg/tube) plus 1 mM $CaCl_2$, sodium α-tocopheryl phosphate (0.5 mM) and trypsin (0.5 μg/tube). Enzyme activities were expressed as pmoles of cyclic nucleotide hydrolyzed/min per tube. In parenthesis, given the relative activity to each basal activity. Values are the mean of duplicate experiments.

	Cyclic AMP phosphodiesterase activity		
	P1	P2	P3
Basal	5.19 (100)	2.04 (100)	3.14 (100)
Activator protein	7.42 (143)	17.1 (840)	3.12 (99.4)
Sodium α-tocopheryl phosphate (TPNa)	7.11	11.3 (552)	3.61 (115)
Trypsin	7.00 (135)	13.3 (653)	3.29 (105)
Activator protein + TPNa	—	4.37 (214)	—
Activator protein + Trypsin	—	9.85 (483)	—
TPNa + Trypsin	—	3.10 (152)	—

	Cyclic GMP phosphodiesterase activity		
	P1	P2	P3
Basal	1.02 (100)	8.09 (100)	2.43 (100)
Activator protein	1.04 (102)	33.6 (415)	2.38 (98.0)
Sodium α-tocopheryl phosphate	0.84 (82.3)	30.8 (381)	2.48 (102)
Trypsin	0.92 (90.5)	32.8 (405)	2.53 (104)

Sodium α-tocopheryl phosphate effects on cyclic AMP phosphodiesterase of P2 fraction

Fig. 2A illustrates that the activity of cyclic AMP phosphodiesterase rose with increasing sodium α-tocopheryl phosphate concentration. The activity velocity plots show that the half-maximal stimulation took place at 0.1 mM (Fig. 2B). Based on this experiment, sodium α-tocopheryl phosphate at the concentration of 0.5 mM was routinely used to examine the maximum stimulation of phosphodiesterase. The addition of Ca^{2+} at the concentrations from 1 μM to 1 mM did not modify sodium α-tocopheryl phosphate stimulation (data not shown) indicating that Ca^{2+} was not required in the activation process by sodium α-tocopheryl phosphate unlike phosphodiesterase stimulation by activator protein [8,25,26].

Activation of P2 enzyme by proteolytic digestion

It has been shown [7,27,33] that cyclic nucleotide phosphodiesterases are stimulated by proteolytic digestion. We compared the proteolytic activation with other activators. Fig. 3 depicts the time course of the trypsin activation of cyclic AMP phosphodiesterase in the P2 fraction. The phosphodiesterase stimulation by tryptic digestion was time- and temperature-dependent. The rate of activation was linear for at least 1 min of incubation, and thereafter the rate gradually decreased (Fig. 3, curves B and C) and inactivation was observed in

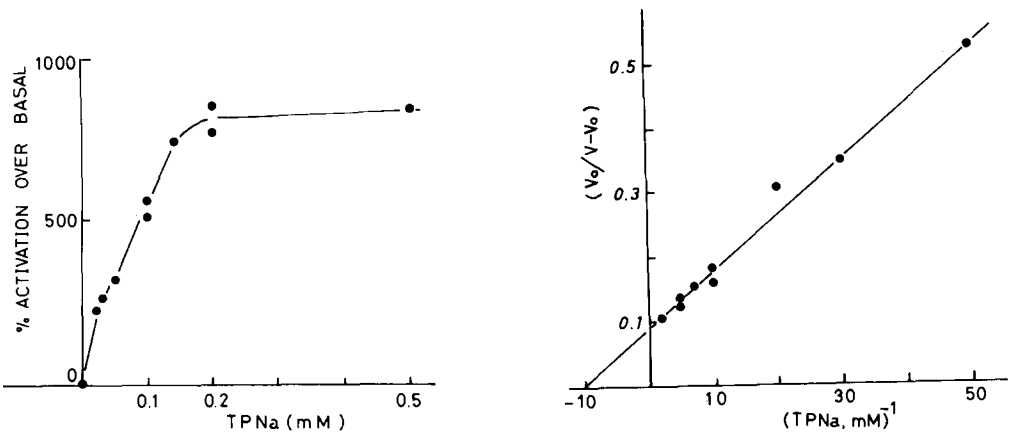

Fig. 2A (left): Effect of sodium α-tocopheryl phosphate concentration on cyclic AMP phosphodiesterase activity from the P2 fraction. The P2 enzyme (see Fig. 1) was preincubated with varying concentration of sodium α-tocopheryl phosphate at 0°C for 10 min. Sodium α-tocopheryl phosphate concentration were those which were present in final assay tubes. Cyclic AMP phosphodiesterase activity was assayed in the substrate concentration of 1 μM and was expressed as per cent over the basal (control) activity of 1.34 pmoles cyclic AMP hydrolyzed/min per tube. B (right): Double reciprocal representation of cyclic AMP phosphodiesterase activation by sodium α-tocopheryl phosphate. The data of Fig. 2A were plotted in double reciprocal manner. V_0 is the basal cyclic AMP phosphodiesterase activity measured in the absence of sodium α-tocopheryl phosphate and V is the enzyme activity at a given concentration of sodium α-tocopheryl phosphate. The half maximum effect of sodium α-tocopheryl phosphate was 0.1 mM, which was calculated from the x-axis intersection.

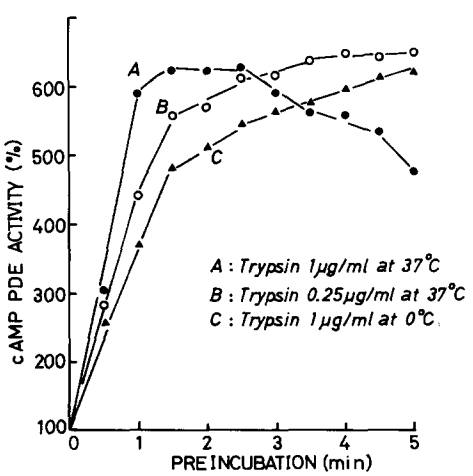

Fig. 3. Time course of the activation of cyclic AMP phosphodiesterase by trypsin treatment. 1-ml aliquots (50 μg protein) of the P2 fraction (see Fig. 1) was preincubated at 37°C (A and B) or at 0°C (C) with trypsin at the concentration of 1 μg/ml (A and C) or 0.25 μg/ml (B) for 0 to 5 min as indicated. Trypsin concentrations were those which were present in the preincubation tubes. At designated time, 50 μl aliquots were transferred to the tubes which contained Trasylol (500 KIE) to terminate the trypsin digestion. Cyclic AMP phosphodiesterase was assayed at a substrate concentration of 1 μM. The results are expressed as per cent of each zero-time control.

TABLE II

EFFECTS OF PROTEASES ON CYCLIC AMP PHOSPHODIESTERASE FROM THE P2 FRACTIONS

The P2 enzyme was separated through a DEAE-cellulose column according to the procedure as described in Fig. 1. Phosphodiesterase assay tubes contained 50 μl of the P2 fraction (3 μg protein). Proteases (0.5 or 5 μg), were applicable, were directly added to assay tubes. After preincubation for 15 s on ice, the assay was started by the addition of substrate. Tubes (c) and (d) received soy bean trypsin inhibitor and Trasylol, respectively, prior to the addition of trypsin. Activities are expressed as a percentage of the control. The specific activity of the control experiment was 0.58 pmol cyclic AMP hydrolyzed/min per μg protein. Substrate concentration was 1 μM. Values are the mean of duplicate experiments.

	Cyclic AMP phosphodiesterase activity (% of control)
(a) Control	100
(b) Trypsin, 0.5 μg/tube	594
(c) Trypsin, 0.5 μg/tube + soy bean trypsin inhibitor, 3 μg/tube	107
(d) Trypsin, 0.5 μg/tube + Trasylol, 170 KIE/tube	102
(e) Chymotrypsin, 0.5 μg/tube	426
(f) *B. subtilis* alkaline protease, 0.5 μg/tube	586
(g) Carboxypeptidase A, 0.5 μg/tube	94.4
(h) Carboxypeptidase A, 5 μg/tube	87.5
(i) Leucine amino peptidase, 0.5 μg/tube	97.1
(j) Leucine amino peptidase, 5 μg/tube	93.9

the presence of a large amount of the proteinase (Fig. 3, curve A).

The specificity of the proteolytic enzymes in activation of cyclic AMP phosphodiesterase was tested as shown in Table II. Because in this assay system proteases were directly added to the phosphodiesterase assay tubes, proteolytic modifications of phosphodiesterase occured simulutaneously with cyclic AMP hydrolysis. Of the five proteolytic enzymes tested (trypsin, chymotrypsin, alkaline protease, carboxypeptidase A and leucine aminopeptidase), endopeptidase (trypsin, chymotrypsin and alkaline protease) only had a stimulatory effect while remaining two exopeptidases were essentially ineffective. As the case of sodium α-tocopheryl phosphate, Ca^{2+} was not required for proteolytic activation (data not shown). The trypsin activation of the phosphodiesterase was almost completely blocked by the addition of such inhibitors as soy bean trypsin inhibitor or Trasylol (Table II).

Trypsin treatment of crude brain supernatant

Experiments were undertaken to see whether the molecular forms of cyclic nucleotide phosphodiesterases were altered upon trypsin activation of the 105 000 × *g* supernatant. Fig. 4A shows a typical profile of cyclic AMP phosphodiesterase from the untreated supernatant after Sephadex G-200 gel filtration. Two activity peaks were observed when assayed with 1 μM cyclic AMP. The V_e/V_0 ratios of these activity peaks were 1.06 and 1.50 with estimated molecular weights of 400 000 and 150 000, respectively. In the presence of activator protein and calcium ions, there was a 6-fold activation of the second peak cyclic AMP phosphodiesterase, while the first peak phosphodiesterase was activated to a much smaller extent (Fig. 4A). There was a broad peak (V_e/V_0 value 1.50) of cyclic GMP phosphodiesterase with a leading shoulder when

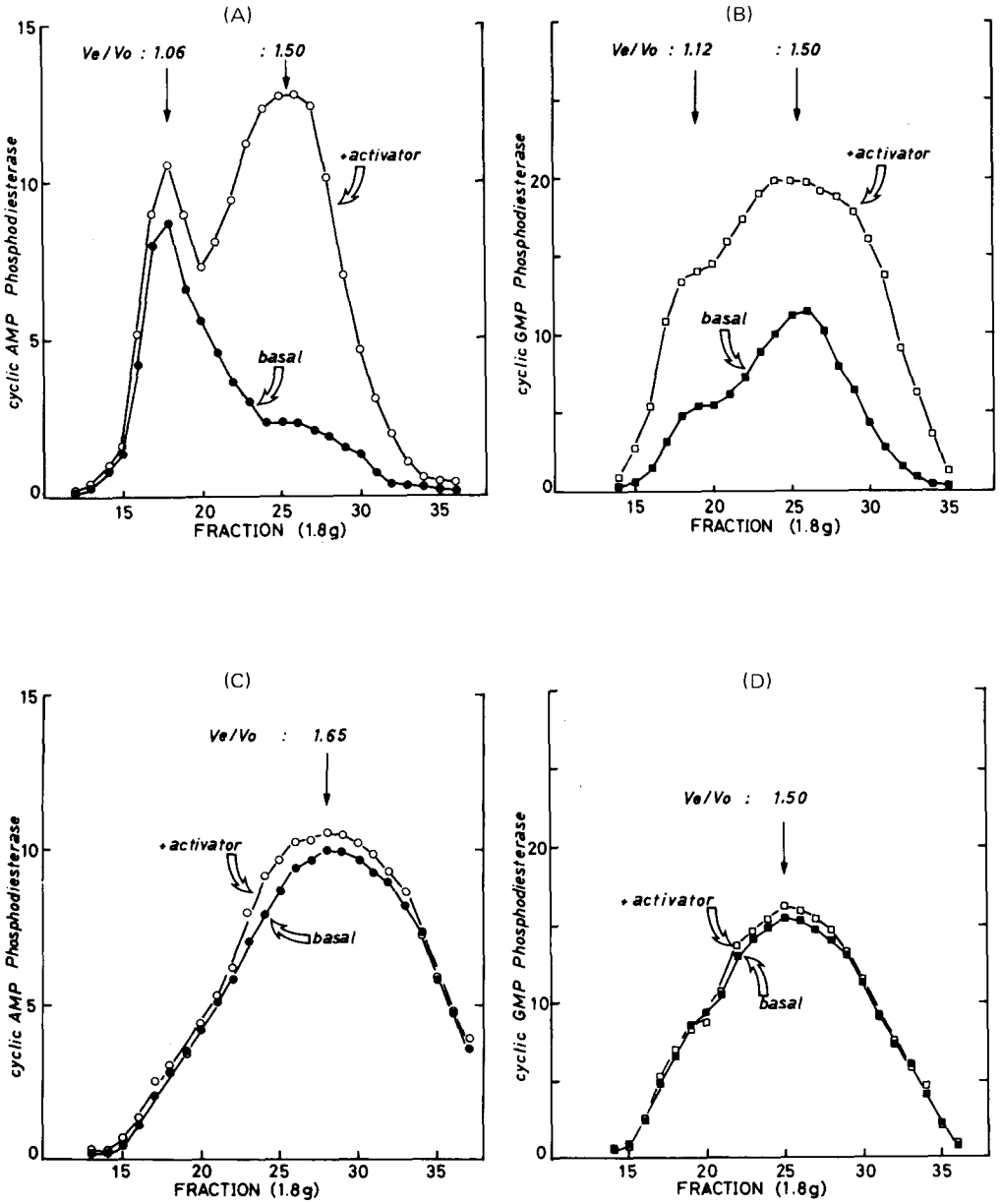

Fig. 4. Sephadex G-200 gel filtration of the brain supernatant fraction. The supernatant fraction from rat brain was prepared as described under Methods. One ml of the supernatant was applied to a Sephadex G-200 column equilibrated with 70 mM acetate buffer (pH 6.5) containing 0.1 mM EGTA and 5 mM 2-mercaptoethanol. Panel (A), Cyclic AMP phosphodiesterase activity from the native supernatant. Panel (B), cyclic GMP phosphodiesterase activity from the native supernatant. Panel (C), cyclic AMP phosphodiesterase activity from the trypsin-treated supernatant. Panel (D), cyclic GMP phosphodiesterase activity from the trypsin-treated supernatant. To prepare the trypsin-treated supernatant, one ml of the brain supernatant (5 mg protein) was preincubated with trypsin (0.2 mg) at 0°C for 5 min, and at the end of the preincubation soy bean trypsin inhibitor (0.4 mg) was added. 50 μl aliquots were used for enzyme assay. Substrate (cyclic AMP or cyclic GMP) concentration was 1 μM. 0.1 μg of purified activator protein in the presence of 1 mM $CaCl_2$ was added, where indicated, to each assay tube. Cyclic nucleotide phosphodiesterase activity was expressed as pmoles of substrate hydrolyzed/min per assay tube.

enzyme activity was assayed with 1 μM cyclic GMP as substrate (Fig. 4B). The phosphodiesterase activity of the peak and shoulder was equally stimulated by activator protein, although the degree of activation was merely twice the basal activity. In the next experiments, the supernatant was applied to the Sephadex G-200 column after a treatment with trypsin. As illustrated in Fig. 4C, a broad single peak of cyclic AMP phosphodiesterase activity appeared with V_e/V_0 ratio of 1.65. The estimated molecular weight of this peak was approximately 80 000. A wide activity peak of cyclic GMP phosphodiesterase was also found with V_e/V_0 ratio of 1.50 which was identical with that of the second peak of the untreated enzyme (Fig. 4D). It should be noted that both cyclic AMP and cyclic GMP phosphodiesterases from the trypsin-treated supernatant were insensitive to activator protein (Fig. 4C and 4D), and that the basal activities of cyclic AMP as well as cyclic GMP phosphodiesterases from the trypsin-treated supernatant were higher than those from the untreated extract.

Chromatography of cyclic AMP phosphodiesterase on activator protein-Sepharose column

The P2 fraction of DEAE-cellulose chromatography was sensitive to activator protein, while the P3 fraction was insensitive to the activator as seen in Table I, and the trypsin-treated enzyme lost the sensitivity to the activator protein (Fig. 4). Therefore, the P2 enzyme was chosen as an activator-dependent phosphodiesterase, and, conversely, the P3 and the trypsin-treated enzymes were employed as activator-independent enzymes. The both activator-dependent and independent enzymes were examined by means of affinity chromatography in order to see their difference in the binding characteristics to the activator protein. The affinity column was made of an activator protein that had been purified from bovine brain and had been bound to Sepharose 2B particles. As seen in Table III, the activator-dependent phosphodiesterase was retained in the column, from which the enzyme was not eluted unless a high concentration of EGTA and NaCl were present. The recoveries of "basal" as well as "activated" activities of native P2 enzyme were more than 100%, suggesting that a spontaneous activation of the enzyme might occur. It was of great interest that after trypsin treatment the activator-dependent enzyme not only was converted into activator-independent form (Fig. 4C) but no longer kept the affinity to the activator-Sepharose column. Since trypsin-treated P2 enzyme was unstable (data not shown), the recoveries of the enzyme from the affinity column was less than 100%. The activator-independent enzyme of P3 was only loosely adsorbed to the affinity column; i.e. most of the enzyme activity was recovered in the elution buffer containing 1 mM EGTA alone. Regardless of the enzyme types used in this experiment, the enzymatic property with regard to the sensitivity to activator protein was not modified upon passing through the affinity column.

Kinetic properties of the activator-dependent phosphodiesterase

Lineweaver-Burk plots [36] of the basal cyclic AMP phosphodiesterases of the P2 fraction showed only a single kinetic form of enzyme activity with a K_m of 31 μM for cyclic AMP and the maximum velocity of 25 nmol of cyclic AMP hydrolyzed/min per mg protein (Table IV). Activation of the phospho-

TABLE III

AFFINITY CHROMATOGRAPHY OF CYCLIC AMP PHOSPHODIESTERASE

The cyanogen bromide-activated Sepharose 2B was coupled with activator protein as described under Methods. The affinity column (2.5 ml bed volume) was equilibrated with 70 mM acetate buffer (pH 6.5) containing 1 mM $CaCl_2$ (buffer I). (A) 5 ml of the P2 fraction (300 μg protein) in the presence of 1 mM $CaCl_2$ was applied to the affinity column. (B) 5 ml of the P2 enzyme was first activated with trypsin (50 μg at 0°C for 5 min and then soy bean trypsin inhibitor (100 μg) was added. The trypsin-treated P2 enzyme in the presence of 1 mM $CaCl_2$ was applied to the column. (C) 5 ml of the P3 fraction (200 μg protein) in the presence of 1 mM $CaCl_2$ was applied to the column. The column was washed stepwise, first with 5 ml of buffer I, subsequently with 10 ml of the acetate buffer (pH 6.5) containing 1 mM EGTA (buffer II) and finally with 10 ml of the acetate buffer (pH 6.5) containing 1 mM EGTA and 0.1 M NaCl (buffer III). 5-g fractions were collected and 50-μl aliquots of each fraction were used for cyclic AMP phosphodiesterase activity at a substrate concentration of 1 μM. Org. shows the original enzyme activity applied to the column. 0.7 μg/tube of activator protein in the presence of 1 mM $CaCl_2$ was added where indicated. Enzyme activity was expressed as pmol of cyclic AMP hydrolyzed/min per tube. Values are the mean of duplicate experiments.

Fractions applied to the affinity column	Cyclic AMP phosphodiesterase activity							
		Org.	Fraction number					
			1	2	3	4	5	6
(A) P2 (native)	Basal	2.32	0	0	0	0.28	2.42	1.37
	+Activator protein	14.60	0	0	0	1.32	11.10	4.30
(B) P2 (trypsinized)	Basal	11.30	0.72	3.00	2.87	0	0	0
	+Activator protein	11.10	0.55	3.33	2.95	0	0	0
(C) P3 (native)	Basal	1.31	0	0.02	1.03	0.27	0.16	0.10
	+Activator protein	1.39	0	0.07	1.28	0.38	0.21	0.10
			I		II		III	
Composition of elution buffer			70 mM Acetate (pH 6.5) + 1 mM $CaCl_2$		70 mM Acetate (pH 6.5) + 1 mM EGTA		70 mM Acetate (pH 6.5) + 1 mM EGTA + 0.1 M NaCl	

diesterase by activator protein, by trypsin and by sodium α-tocopheryl phosphate was equally accompanied by a decrease of the K_m for cyclic AMP (17 μM). At the same time, the maximum velocity of the activated enzyme increased more than 6-fold after incubation with various stimulators. Cyclic GMP phosphodiesterase of the P2 fraction also showed a change in kinetic behavior when the enzyme was activated (Table IV). The double reciprocal plot of the P2 cyclic GMP phosphodiesterase was also linear and the apparent K_m value for cyclic GMP shifted from 13 μM (for basal state enzyme) to 6 μM (for activated state enzyme). Likewise, activation of the P2 enzyme with stimulators increased the maximum velocity of cyclic GMP phosphodiesterase 2—4-fold (Table IV). Thus, upon incubating the activator-sensitive P2 phosphodiesterase with various activators, a decrease of the K_m value for substrate (cyclic AMP or cyclic GMP) and an increase of maximum velocity were commonly observed.

TABLE IV

SUMMARY OF KINETIC PARAMETERS OF P2 PHOSPHODIESTERASE

	A Basal *	B Activator protein **	C Trypsin ***	D Sodium α-tocophery phosphate †
Cyclic AMP phosphodiesterase ††				
K_m (M) × 10^5	3.1	1.7	1.7	1.7
V ‡	25.0	167	181	153
Cyclic GMP phosphodiesterase †††				
K_m (M) × 10^5	1.3	0.62	0.63	0.63
V ‡	22.2	82.6	86.2	43.5

* Basal cyclic nucleotide phosphodiesterase activities of the P2.

** The P2 enzyme stimulated by activator protein (0.5 μg/tube) in the presence of 1 mM $CaCl_2$.

*** The P2 fraction (3 μg/tube) were preincubated with trypsin (0.5 μg) at 0°C for 5 min and then soy bean trypsin inhibitor (1 μg) was added.

† The P2 fraction was preincubated with sodium α-tocopheryl phosphate at 0°C for 15 min and the enzyme assay was started. Final concentration of sodium α-tocopheryl phosphate was 0.5 mM.

†† The concentration of cyclic AMP varied from 1 μM to 50 μM.

††† The concentration of cyclic GMP varied from 1 μM to 50 μM.

‡ Velocities were expressed as nmoles cyclic nucleotide hydrolyzed/min per mg protein.

Discussion

In the high speed supernatant of rat brain we found three physically distinct forms of cyclic AMP phosphodiesterase separated by DEAE-cellulose column chromatography. The second phosphodiesterase corresponding to the P2 in the chromatography was the activator-sensitive enzyme. The activator-dependent form of the phosphodiesterase displayed some activity even in the absence of activators whichever separation technique was employed, ion-exchange chromatography or gel filtration chromatography (Figs. 1 and 4). There are a few possible explanations for this fact. (a) The activator-dependent phosphodiesterase may exhibit by itself no activity in the absence of activators. Therefore the observed "basal" activity may be a consequence of a conversion of the inactive enzyme into a partially active form in the course of preparation. This possibility is not totally deniable, since a neutral proteinase has been reported to be present in the soluble fraction of rat brain [37], and since the activator-dependent enzyme was shown to be converted in to the independent form by endopeptidases (Table II). (b) Although the activator-dependent enzyme may not possess any "basal" activity, the dependent enzyme may be contaminated with the independent form due to a poor preparative procedure. (c) The dependent phosphodiesterase fraction may contain some amount of endogenous activator protein. This is very unlikely, however, because the endogenous activator protein was completely removed from any forms of the phosphodiesterase by means of DEAE-cellulose chromatography (Fig. 1). (d) The dependent enzyme may have some intrinsic basal activity and activators may simply further stimulate the enzyme. It is our opinion that the last explanation is the most probable one, although the first and second could not be completely ruled out. The affinity chromatography using the activator protein coupled to Sepharose proved to be successful to distinguish the activator-independent form from the

dependent form of cyclic AMP phosphodiesterase. The trypsinized phosphodiesterase (independent form) passed through the affinity column, whereas the activator-dependent enzyme was retained by the column, from which it was eluted only after the addition of a buffer containing EGTA and NaCl at high concentrations. The activator-dependent phosphodiesterase which was purified through the affinity chromatography did still contain the "basal" enzyme activity and, in addition, the activity ratio of dependent to independent enzyme was comparable between original and purified preparation. These lines of evidence suggest that observed "basal" activity in the activator-dependent phosphodiesterase is attributed to its intrinsic properties.

Although we made observations which were similar to those of Cheung [7] who first demonstrated the proteolytic activation of phosphodiesterase, we were able to obtain some additional results to give a deeper insight. It should be stressed that, among the multiple forms of phosphodiesterase, the enzyme which was stimulated by the Ca^{2+}-dependent activator protein was also stimulated by proteolytic enzymes. It is also noteworthy that kinetic changes induced by two different types of activators were common and no additive effect was observed in activating the enzyme. Nevertheless, these facts may support the idea that a common mechanism is underlying in the activation process. A substantial reduction of molecular weight was accompanied by the proteolytic modification of the cyclic AMP phosphodiesterase. From our results it is difficult to discern how phosphodiesterase was activated by proteolytic modification, but one of attractive explanations is that the enzyme was dissociated into active units upon proteolytic digestion. An alternative explanation is that the proteolytic enzyme exerted its effect by destroying a phosphodiesterase inhibitor, the presence of which was proposed by Miki and Yoshida [38] and recently by Wang and Desai [39]. The possible explanation for the trypsin activation of cyclic GMP phosphodiesterase, which was not accompanied by the change of molecular weight, is the existence of less active forms of the enzyme consisting of enzyme-inhibitor complexes which were converted into more active forms as a result of destruction and removal of the inhibitor moieties.

The mechanism by which sodium α-tocopheryl phosphate (TPNa) activates cyclic nucleotide phosphodiesterase was not established by our studies. Since we demonstrated here that there was a stimulatory effect of sodium α-tocopheryl phosphate on a soluble enzyme that was obtained after centrifugation at 105 000 $\times g$, its action to stimulate the phosphodiesterase(s) was not attributable merely to the solubilizing effect. The contention is substantiated by our unpublished data that the sodium α-tocopheryl phosphate-induced activation of phosphodiesterase was only noted in rat liver supernatant, not in the particulate fraction. However, it could not be ascertained in our current experiments whether the dissociation of enzyme occurred upon exposure to the phosphate-containing detergent, since the sodium α-tocopheryl phosphate-treated enzyme was not sufficiently stable to endure the separation process by gel-filtration to confirm the alteration of molecular weight. In agreement with the results of Wolff and Brostrom [29] who have shown a partially purified cyclic nucleotide phosphodiesterase from brain was activated by phosphatidyl inositol and lysophosphatidyl choline, we observed the detergent-induced activation was cal-

cium-independent, and the sodium α-tocopheryl phosphate shared the enzyme stimulation with activator protein. Our studies indicate, in addition, that among the multiple types of phosphodiesterases, only the P2 enzyme was preferentially activated by both sodium α-tocopheryl phosphate and activator protein.

The activator sensitive enzyme, P2, has a specific affinity to the Sepharose-activator protein column, which will enable us to purify large quantities of this enzyme. We shall then be able to evaluate the regulatory mechanisms of the cyclic nucleotide phosphodiesterase in more detail, which is now in progress in our laboratories.

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