Multiple Cyclic Nucleotide Phosphodiesterase Activities from Rat Tissues and Occurrence of a Calcium-Plus-Magnesium-Ion-Dependent Phosphodiesterase and its Protein Activator

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1. Supernatant fluids from rat cerebral cortex, cerebellum, kidney, heart and liver contained more phosphodiesterase activity hydrolysing cyclic GMP than that hydrolysing cyclic AMP when assayed with sub-saturating concentrations of substrate. 2. These activities were resolved into several fractions by Sephadex G-200 gel filtration; no two tissues had similar activity profiles. 3. With every tissue examined, a fraction (fraction II) with a molecular weight of about 150000 was obtained which hydrolysed cyclic GMP preferentially at sub-saturating substrate concentrations in the presence of a micromolar concentration of Ca^{2+} , millimolar concentration of Mg^{2+} and a protein activator. 4. The activity of fraction II accounted for about 60% in liver, more than 80% in heart and cerebellum, and almost 100% in cerebral cortex of the total activity for cyclic GMP hydrolysis, calculated from the activity profiles. 5. K_m values of fraction II samples from kidney, heart and liver for cyclic GMP were 1.3, 1.7 and $5\mu\text{M}$ respectively. 6. 3-Isobutyl-1-methylxanthine inhibited hydrolysis of cyclic GMP by fraction II with an I_{50} value of $3\mu\text{M}$ for heart and liver and $50\mu\text{M}$ for cerebrum. 7. The activator protein, with an estimated molecular weight of about 30000 was isolated from all the tissues listed in 1. 8. The concentrations of activator protein and of the isolated enzyme, fraction II, did not correspond exactly.

Cyclic AMP has been established as an intracellular mediator (second messenger) of the actions of a number of hormones in a variety of tissues. Current evidence now suggests that cyclic GMP may also play a biological role as a second messenger, distinct from that involving cyclic AMP (Goldberg *et al.*, 1973). The hydrolytic cleavage of cyclic AMP and cyclic GMP to their corresponding 5'-nucleotides, catalysed by cyclic nucleotide phosphodiesterase, is the only well-established mechanism for disposal of these cyclic nucleotides. There is now much evidence indicating the importance of cyclic nucleotide phosphodiesterase in the control of intracellular concentrations of both nucleotides (reviewed by Appleman *et al.*, 1973).

Earlier work in this laboratory demonstrated that a phosphodiesterase activity in the supernatant fluid of a brain homogenate was stimulated by micromolar concentrations of Ca^{2+} in the presence of an optimum concentration (3 mM) of Mg^{2+} (Kakiuchi & Yamazaki, 1970*a,b*). These observations were confirmed by Miki & Yoshida (1972). Lagarde & Colobert (1972) and Teo & Wang (1973) have shown the occurrence of this activity in human blood lymphocyte and bovine heart. The enzyme responsible was designated $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent cyclic nucleotide phosphodiesterase.

It was shown that the stimulation by Ca^{2+} of the crude enzyme was enhanced by the addition of a thermostable protein (phosphodiesterase activating factor) present in brain extract (Kakiuchi & Yamazaki, 1970*b*). This protein was separated from the enzyme by gel-filtration chromatography (Kakiuchi *et al.*, 1970, 1973). Cheung (1970, 1971) demonstrated the existence of a thermostable protein activator of brain phosphodiesterase. A similar protein was also found in heart muscle (Cheung, 1971; Goren & Rosen, 1971). Later, Teo & Wang (1973) and Liu *et al.* (1974) showed that purified samples of this protein bound Ca^{2+} , suggesting that a complex of Ca^{2+} and the protein is the true activator of phosphodiesterase. Teshima & Kakiuchi (1974) demonstrated the formation of an active enzyme-activating factor complex in the presence of Ca^{2+} ; in the absence of Ca^{2+} , it dissociated into an inactive form of enzyme and activating factor.

In the present study, phosphodiesterase activities in the supernatant fractions of cerebral cortex, cerebellum, kidney, heart and liver were resolved into several activity peaks by gel filtration. The $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent phosphodiesterase and activator protein were found in all these tissues. A method for determining the activating factor and some properties of the enzyme are also described.

Experimental

Materials

Fresh pig brains were obtained from Nippon Ham Co. Ltd., Osaka, Japan. Cyclic [^3H]AMP (sp. radioactivity 16.3 Ci/mmol) and cyclic [^3H]GMP (sp. radioactivity 3.5 Ci/mmol) were purchased from Schwarz/Mann, Orangeburg, N.Y., U.S.A., and New England Nuclear Corp., Boston, Mass., U.S.A., respectively. Both compounds were purified before use by column chromatography on Dowex 1 (X8; 200–400 mesh: Cl^- form) (see under 'Phosphodiesterase assays'; method 2). Unlabelled cyclic AMP, cyclic GMP and Tris were obtained from Boehringer Mannheim Yamanouchi K.K., Tokyo, Japan. EGTA [ethanedioxybis(ethylamine)tetraacetic acid] was a product of Dojindo Co. Ltd., Kenguncho, Kumamoto, Japan. Snake (*Crotalus atrox*) venom was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. SQ 20009 {1-ethyl-4-(isopropylidenehydrazino)-1*H*-pyrazolo-[3,4-*b*]-pyridine-5-carboxylic acid ethyl ester hydrochloride} was a product of E. R. Squibb & Sons, Inc., Princeton, N.J., U.S.A. (Chasin *et al.*, 1972). 3-Isobutyl-1-methylxanthine was kindly given by Dr. T. Sakai, Marupi-Searle Co. Ltd., Osaka, Japan, and Dr. H. Shimizu, Nippon Roche Co., Kamakura, Japan. MgSO_4 was of analytical grade (E. Merck, Darmstadt, Germany). The collodion bags and ultrafiltration membranes (UM-10) used for concentration of preparations of enzyme and activator protein were products of Sartorius-Membranfilter GmbH, Göttingen, Germany, and Amicon Corp., Lexington, Mass., U.S.A., respectively. Instagel was a product of Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A. Deionized distilled water with a conductivity of less than $1\ \mu\text{mho}$ was used throughout the present work. Dowex 1 resins were washed six times alternately with 20 vol. each of 2*M*-HCl, 2*M*-NaOH, then with 2*M*-HCl, and finally with the deionized distilled water three times. Usually resins were also washed with acetone after the first wash with HCl. Chelex 100 (200–400 mesh) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. The resin was washed six times alternately with solutions of 1*M*-HCl and 1*M*-NaOH and finally with the deionized distilled water. Sephadex G-200 and G-15 were products of Pharmacia Fine Chemicals, Uppsala, Sweden.

Where indicated, Ca^{2+} -EGTA buffer was added to the medium to obtain the desired concentration of Ca^{2+} . The buffer was prepared as described by Weber & Winicur (1961); EGTA at a final concentration of 10 mM was mixed with an appropriate concentration of CaCl_2 to produce the desired concentration of Ca^{2+} . Usually 0.1 ml of this buffer was used per ml of medium. The concentration of Ca^{2+} in the medium was calculated by taking the apparent binding con-

stant of Ca^{2+} -EGTA as $8 \times 10^5 \text{ M}^{-1}$ at pH 6.9 (see Ogawa, 1968).

Removal of Ca^{2+} from reagents

In the experiment shown in Table 1, Ca^{2+} was removed from all reagents, enzyme preparations and protein activator samples by using Chelex 100 resin. Traces of Ca^{2+} in the deionized distilled water and 100 mM-imidazole-HCl buffer containing 0.3 mM-dithiothreitol were removed by passage through columns (2.5 cm $\times$ 22 cm) of Chelex 100; 20 mM-cyclic nucleotides were similarly passed through columns (0.9 cm $\times$ 12 cm) of Chelex 100. Columns (1.5 cm $\times$ 25 cm) of Sephadex G-15 were washed each with approx. 300 ml of the Chelex 100-treated imidazole-HCl buffer containing 0.3 mM-dithiothreitol. Enzyme preparations and protein activator samples, to which EGTA had been added to a final concentration of 0.5 mM, were separately desalted on the Sephadex G-15 columns by using the Chelex 100-treated buffer. MgSO_4 was essentially free from Ca^{2+} contamination.

Methods

Preparation of supernatant fluids. Male Sprague-Dawley rats (3–8 months old) were killed by decapitation under ether anaesthesia. Tissues were rapidly removed and freed from connective tissue. Cerebral cortices were also dissected from pig brains. The interval between death and dissection of the pig brains was approx. 2 h; during that period, the brains were kept in ice. Tissues were minced with scissors and then washed in homogenizing medium to remove blood if present (this step was omitted with cerebral cortex and cerebellum). They were then homogenized in glass homogenizers with 3 vol. of 10 mM-Tris-HCl buffer (pH 7.5) – 1 mM- MgCl_2 – 0.1 mM-EGTA – 2 mM-2-mercaptoethanol. The homogenates were centrifuged at 105 000 *g* for 1 h. The supernatants were either subjected to gel filtration on Sephadex G-200 immediately, or dialysed overnight against 50–200 vol. of the above homogenizing medium with three changes of the medium. All procedures for preparation of supernatants were carried out at 0–4°C.

Gel filtration. Gel filtration was carried out as a routine at 0–4°C on columns (2.5 cm $\times$ 86 cm) of Sephadex G-200 which had been equilibrated with the elution medium. The elution medium contained 20 mM-Tris-HCl buffer (pH 7.5), 100 mM-NaCl, 1 mM- MgCl_2 , 0.1 mM-dithiothreitol and 0.1 mM-EGTA. The supernatant from a tissue was applied to a column, and the column was then eluted with the above medium at a flow rate of about 10–15 ml/h. Fractions of volume 4.4 ml were collected. Usually elution was completed in about 48 h. The molecular weights of fractions eluted from the column were

estimated from their V_e/V_0 ratios, with horse heart cytochrome c, bovine plasma albumin, rabbit muscle aldolase, bovine γ -globulin and Blue Dextran-2000 as standards. The fractions containing the (Ca²⁺ + Mg²⁺)-dependent cyclic nucleotide phosphodiesterases (fraction II, Figs. 2 and 3) were pooled and concentrated in collodion bags or through UM-10 membranes to 1–10 mg of protein/ml (or 0.3–1 mg of protein/ml for cerebral cortex and cerebellum). The concentrated preparations were divided into small portions, frozen in a bath at -80°C and stored at this temperature; they were fairly stable for several months. Preparations were used within a day after thawing and were not refrozen and stored again.

Phosphodiesterase assays. Phosphodiesterase activity was determined by three methods, 1, 2 and 3, which were modifications of the methods of Butcher & Sutherland (1962), Beavo *et al.* (1970) and Thompson & Appleman (1971a) respectively. In method 1, the standard reaction mixture contained, in a final volume of 0.75 ml, 80 mM-imidazole-HCl buffer (pH 6.9), 3 mM-MgSO₄, 0.3 mM-dithiothreitol, 1.2 mM-cyclic nucleotide as substrate, enzyme protein and other additions as indicated. Unless otherwise stated, the partially purified pig brain activator protein (usually more than 1 unit/tube, which gives nearly maximum stimulation of the enzyme activity in the presence of Ca²⁺) was added to the reaction mixtures. Incubation at 30°C was started by addition of substrate, and stopped after 30 min by immersing the tubes in boiling water for 3 min (first-stage incubation). Then 6 μmol of MnCl₂ and 0.1 mg of *C. atrox* venom (5'-nucleotidase source) were added and the mixtures were reincubated for 30 min at 30°C (second-stage incubation). The reaction was stopped by adding HClO₄ to a final concentration of 0.3 M and the mixture was centrifuged. P_i in the supernatant was then measured by the method of Ames (1966). Control values, obtained with heat-inactivated enzyme, were subtracted from those obtained with native enzyme. It was confirmed in the present work that 0.4 mg of the *C. atrox* venom used did not release any detectable P_i from cyclic nucleotides.

Assay methods 2 and 3 were essentially the same as method 1 except that tritiated cyclic nucleotide substrate (4×10^5 d.p.m./assay tube) was used and the radioactive nucleoside formed was determined. The first-stage incubation was terminated by heating for 1 min instead of 3 min. The second-stage incubation was terminated in method 2 by applying the reaction mixture to a column (0.5 cm diam.; bed volume 0.75 ml) loaded with Dowex 1 (X8; 200–400 mesh) which had been equilibrated with 2 mM-HCl, or in method 3 by adding 1.0 ml of a slurry of Dowex 1 (X2; 200–400 mesh), containing 0.3 ml of packed resin, to the reaction tube. HClO₄ was not used in either case. In method 2, the column was then eluted successively with 20 ml of 2 mM-HCl, 20 ml of 2 mM-

HCl, and finally 30 ml of 0.1 M-HCl. Tritiated adenosine or guanosine was recovered quantitatively in the first 20 ml fraction and remaining substrate in the third 30 ml fraction. Samples (5 ml) of the first and third fractions were mixed with 10 ml of Instagel and their radioactivities were determined in a liquid-scintillation spectrometer. In method 3, the reaction mixture, containing resin, was shaken vigorously for 5 min and then centrifuged. A 1 ml sample of the supernatant was mixed with 10 ml of Bray's solution (Bray, 1960) and its radioactivity was determined. Assay methods 2 and 3 were used mainly for the determination of enzyme activity with non-saturating substrate concentration. Therefore the enzyme concentration and/or incubation time (10–30 min) was adjusted so that less than 30% of the initial amount of substrate was hydrolysed. Under these conditions, the amount of substrate hydrolysed was proportional to the concentration of enzyme or the incubation time. This was confirmed with enzymes from cerebellum and heart with 0.4 μM -cyclic GMP as substrate.

A unit of phosphodiesterase is defined as the amount which hydrolyses 1 μmol of substrate/min at 30°C in the presence of a saturating amount of activator protein and Ca²⁺. All the analytical procedures, including assays of enzyme, activator factor and protein were carried out at least in duplicate and results are given as average values.

Preparation of phosphodiesterase activating factor. This was isolated from the supernatants from a variety of rat tissues by gel filtration (see Figs. 2 and 3). Fractions containing activator protein were concentrated and then stored at -80°C . Activator protein was also prepared and purified in bulk from supernatant from fresh pig brains by the procedure of Teo *et al.* (1973) except that EGTA was included in the medium; these preparations gave a single protein band on disc-gel electrophoresis, and were used for most of the experiments.

Assay of activator protein. The assay was based on the ability of activator protein to stimulate the activity of activator-deficient (Ca²⁺ + Mg²⁺)-dependent cyclic nucleotide phosphodiesterase of brain in the presence of Ca²⁺. Two methods were used. In method A, previously described by Kakiuchi *et al.* (1973), the assay was performed with 20 μg of protein of dialysed supernatant from the pig brain homogenate and 2–2.5 μM -free Ca²⁺ (Ca²⁺-EGTA buffer). Other incubation conditions were as for enzyme assay by method 1. Under these conditions, the enzyme was relatively deficient in activator protein because of dilution, and enzyme activity increased as the concentration of activator factor added to the reaction mixture increased. With the above system the amount of activator protein that doubled the enzyme activity was defined as 10 units.

A new method that is more sensitive than method A was also devised for determination of activator

protein (see Fig. 1). In this (method B) the assay mixture contained, in a final volume of 0.75 ml, 80 mM-imidazole-HCl buffer (pH 6.9), 3 mM-MgSO₄, 0.3 mM-dithiothreitol, 100 mM-NaCl (or KCl), 50 μ M-CaCl₂, 1.2 mM-cyclic AMP, 9–10 units of fraction II enzyme of either rat or pig cerebral cortex, and the test sample or a standard sample of activator protein. The assay was performed as in enzyme assay method 1. Method B gave slightly variable results depending on the preparation of enzyme, and was less reproducible than method A. Therefore standard preparations of activator protein which had been calibrated by method A served as controls in method B. The amount of activator protein that gave 50% of the full stimulation of the enzyme activity in method B corresponded to about 0.13–0.15 unit.

Protein assay. Protein was determined by the method of Lowry *et al.* (1951). Solutions of crystalline bovine plasma albumin were used as standard.

Results

Dependence of the activity of fraction II from brain on Ca²⁺, Mg²⁺ and activator protein

The activity of fraction II (see Fig. 2) was dependent on the simultaneous presence of Ca²⁺, Mg²⁺ and activator protein in the reaction mixture (Table 1). The results are consistent with those obtained previously with Ca²⁺-EGTA buffer (Kakiuchi *et al.*, 1973). In the presence of activator protein the hydrolysis of cyclic GMP was stimulated by lower concentrations of Ca²⁺ than of cyclic AMP; 3 μ M-Ca²⁺ and 12 μ M-Ca²⁺ caused nearly maximum stimulation of the hydrolysis of cyclic GMP and cyclic AMP respectively.

Dose response of activator protein

In the presence of Ca²⁺, the activity of fraction II increased sigmoidally with increase in the concentra-

tion of activator protein (Fig. 1). Hill plots of the data gave a straight line, from which the Hill coefficient was calculated as approx. 2. The results are consistent with the interpretation previously described by Teshima & Kakiuchi (1974) that more than one molecule of activator protein interacts with a molecule of enzyme. The reaction mixture contained 100 mM-KCl because, as shown in Table 2, inclusion of NaCl or KCl in the reaction medium enhanced the effect of activator protein by lowering the control activity. (NH₄)₂SO₄ was also effective, and the lack of specificity indicates that the salt effect may be due to ionic strength. The maximum effect was obtained with 100 mM-NaCl or 100 mM-KCl. Higher concentrations were less effective, owing to inhibition of the enzyme activity in the presence of activator protein. Therefore either 100 mM-KCl or 100 mM-NaCl was included in the reaction mixture in assay of activator protein.

Fractionation of the supernatants by gel filtration

Fresh supernatants from a variety of rat tissues were subjected to gel filtration. The presence of EGTA in the medium used for gel filtration was necessary to obtain enzyme free from activator protein since activator and the enzyme reversibly associate together in the presence of Ca²⁺. When EGTA was not present, a trace of Ca²⁺ in the medium caused incomplete separation of activator protein and enzyme. The elution profiles of enzyme activity and activator protein shown in Figs. 2 and 3 are consistent in repeated runs (three times for cerebral cortex, liver and kidney, two times for cerebellum and heart) of gel filtration with different samples of the tissues.

(i) *Cerebral cortex.* When enzyme activity was assayed with 1.2 mM substrate, three fractions (Ia, Ib, II) were found with estimated molecular weights of 400 000, 250 000 and 150 000 (Figs. 2a and 2b). Fraction II required Ca²⁺, Mg²⁺ and activator protein for

Table 1. *Dependence of the activity of fraction II from cerebral cortex on Ca²⁺, Mg²⁺ and activator protein*

Fraction II, from rat cerebral cortex, the partially purified pig brain activator protein, and all reagents used were purified from Ca²⁺ by using Chelex 100 resin, as described in the Experimental section. Ca²⁺-EGTA buffer was not used in this experiment. Enzyme activity was measured at pH 7.2 (imidazole-HCl buffer) by assay method 1, by using 1.2 mM-cyclic AMP or 1.2 mM-cyclic GMP as substrate. When indicated in the Table, 0.43 unit of activator protein was added.

Cations added	Activator protein	Enzyme activity (nmol/min per ml of enzyme)	
		With cyclic AMP	With cyclic GMP
None	+	5.1	10.7
Mg ²⁺ (2 mM)	+	13.1	18.7
Mg ²⁺ (2 mM) + Ca ²⁺ (3 μ M)	+	20.1	58.3
Mg ²⁺ (2 mM) + Ca ²⁺ (12 μ M)	+	56.9	60.7
Mg ²⁺ (2 mM) + Ca ²⁺ (20 μ M)	+	68.6	57.4
Mg ²⁺ (2 mM) + Ca ²⁺ (20 μ M)	—	10.7	15.4
Mg ²⁺ (2 mM) + EGTA (50 μ M)	+	9.3	12.6

activity and thus represents (Ca²⁺ + Mg²⁺)-dependent cyclic nucleotide phosphodiesterase. Fraction Ia and Ib did not require Ca²⁺ for activity. When assay was carried out with 0.4 μ M substrate, activity was only detected in fraction II (Figs. 2c and 2d), indicating that the enzymes in fractions Ia and Ib have high K_m values. With 0.4 μ M substrate, fraction II hydrolysed cyclic GMP much faster than cyclic AMP, whereas with 1.2 mM substrate, activity was greater with cyclic AMP than with cyclic GMP. Fractions Ia and Ib hydrolysed the two substrates at almost equal rates. In the following experiments 0.4 μ M substrate was used unless otherwise stated.

(ii) *Cerebellum*. One major fraction (fraction II) was separated (Fig. 3a). The activity of fraction II, which was greater with cyclic GMP than with cyclic AMP, was dependent on Ca²⁺, Mg²⁺ and activator protein. Its molecular weight was about 150000. The activity profile of cerebellum differed from that of cerebral cortex in that there was a shoulder peak of activity (with cyclic GMP) at the higher molecular weight side of fraction II, as well as small peak(s) of activity for cyclic AMP hydrolysis with a lower molecular weight(s) than fraction II.

(iii) *Kidney*. One major peak (fraction II) was separated with preferential activity for cyclic GMP (Fig. 3b). Its molecular weight was estimated to be 150000 and it required Ca²⁺, Mg²⁺ and activator protein for activity. Fraction III, and possibly IV, was distinguished from fraction II. These two fractions showed activity with cyclic AMP, which were slightly stimulated by Ca²⁺. The molecular weight of fraction IV was estimated to be 50000, which is close to the value obtained by Jard & Bernard (1970) for a fraction, PDE II of rat kidney.

(iv) *Heart*. Two distinct peaks were obtained (Fig. 3c). Fraction II, with an estimated molecular weight of 150000, required Ca²⁺, Mg²⁺ and activator protein for activity and, in the presence of these, it hydrolysed cyclic GMP much faster than cyclic AMP. Fraction I with an estimated molecular weight of about 250000 hydrolysed the two nucleotides equally well, and its activity was not affected by Ca²⁺.

Fig. 1. Effect of protein activator on the activity of rat brain fraction II (assay method B)

Experimental conditions were as described in the text. Enzyme activity is expressed as μ mol/min per ml of enzyme. Hill plots of the same data are shown in the inset. v and V_{max} are expressed in terms of activity and the maximum activity, respectively, above that of the control containing no protein activator.

Table 2. Effects of salts on enzyme activity with and without activator protein

The activity of fraction II from rat cerebral cortex was determined by assay method 1 by using 1.2 mM-cyclic AMP as substrate with 50 μ M-CaCl₂, the concentrations of salts indicated in the Table and 3.75 units of the partially purified pig brain activator protein where indicated.

Addition (concentration in mM)	Enzyme activity (μ mol/min per ml)		Activity ratio With activator protein Without activator protein	Ionic strength (mol/l)
	Without activator protein	With activator protein		
Expt. 1				
None	0.190	0.539	2.8	0.092
NaCl (100)	0.112	0.494	4.4	0.192
KCl (100)	0.091	0.466	5.1	0.192
Expt. 2				
None	0.210	0.665	3.2	0.092
KCl (50)	0.178	0.638	3.6	0.142
KCl (100)	0.120	0.590	4.9	0.192
KCl (200)	0.144	0.594	4.1	0.292
KCl (600)	0.124	0.244	2.0	0.692
(NH ₄) ₂ SO ₄ (100)	0.129	0.500	3.9	0.392

Fig. 2. Gel-filtration (Sephadex G-200) profile of the activity of the supernatant from rat cerebral cortex

Supernatant containing 15.2 mg of protein (corresponding to about 0.67 g of tissue) was subjected to gel filtration as described in the text. Phosphodiesterase activities in the fractions were assayed in the presence of $0.06 \mu\text{M}$ - Ca^{2+} (○) (Ca^{2+} -EGTA buffer), or $12 \mu\text{M}$ - Ca^{2+} (●) at substrate concentrations of 1.2 mM (a and b) and $0.4 \mu\text{M}$ (c and d) by using assay methods 1 and 3 respectively. The activities with cyclic GMP (a and c) and cyclic AMP (b and d) are illustrated separately. The partially purified pig brain protein activator (2.4 units) was added to all enzyme assay tubes. Protein activator in the fractions was assayed by method B on samples that had been boiled for 3 min. Δ , Protein activator; $\cdots$, E_{280} .

(v) *Liver*. At least five fractions with phosphodiesterase activity were separated (Fig. 3d). Three of these, fractions Ia, Ib and II, with estimated molecular weights of 400000, 300000 and 150000 respectively, were active with both cyclic GMP and cyclic AMP, the activities with cyclic GMP being higher than those with cyclic AMP. Fraction IV, with an estimated molecular weight of 50000, and fraction III were distinguished from fraction II. These two fractions were only active with cyclic AMP. The activity of fraction II was dependent on Ca^{2+} , Mg^{2+} and activator protein and, in their presence, its activity with cyclic GMP was the highest of the activity peaks. The activities of fractions III and IV were also slightly stimulated by Ca^{2+} .

Distribution of activator protein in rat tissues

Activator protein was also isolated from these tissues, i.e. cerebral cortex, cerebellum, kidney, liver and heart (Table 3). Samples of activator protein from all these tissues had the same molecular weight, approx. 30000, which is close to the value reported for samples

of activator protein from heart (Teo *et al.*, 1973) and brain (Teshima & Kakiuchi, 1974), estimated by gel filtration.

Some properties of the enzyme

(i) K_m for cyclic GMP. Lineweaver-Burk plots of the rates of hydrolysis of cyclic GMP by fraction II enzymes from rat kidney, liver, and heart gave the straight lines, from which K_m values of 1.3, 5 and $1.7 \mu\text{M}$ respectively, were calculated. As reported, brain fraction II enzyme also has a similar K_m value of $2 \mu\text{M}$ (Kakiuchi *et al.*, 1973). The assays were carried out in the presence of Ca^{2+} and activator protein by using substrate concentrations of 0.4 – $50 \mu\text{M}$ for kidney, 0.25 – $50 \mu\text{M}$ for liver, and 0.4 – $100 \mu\text{M}$ for heart.

(ii) *Effects of inhibitors*. Table 4 summarizes effects of inhibitors on hydrolysis of cyclic GMP by fraction II. The activity of the brain enzyme was one order of magnitude less susceptible to inhibition by these inhibitors than the activities of the enzymes from liver and heart.

Fig. 3. Sephadex G-200 gel-filtration profiles of the activities of supernatants from rat cerebellum, kidney, heart and liver

Supernatants from cerebellum (a), kidney (b), heart (c) and liver (d), containing 12.2mg of protein (corresponding to 0.46g of tissue), 48.4mg of protein (corresponding to 0.88g of tissue), 126mg of protein (corresponding to 4.1g of tissue), 197mg of protein (corresponding to 3.3g of tissue) respectively, were subjected to gel filtration. Phosphodiesterase activity was determined in the presence of 0.4 μ M substrate. The other conditions and symbols are as for Fig. 2.

(iii) *Effects of Mg²⁺*. Mg²⁺ is essential for the activity of fraction II since the addition of Ca²⁺ alone revealed none of the activity. Mg²⁺ has another effect on the enzyme. Addition of Mg²⁺ to the enzyme preparation was found to stabilize the activity of the enzyme to some extent. Aging of a sample of dia-

lysed supernatant from rat brain, containing 50 μ g of protein/ml and 10mM-Tris-HCl buffer (pH 7.5), for 20h at room temperature (approx. 20°C) caused a decrease in its activity with 12 μ M-Ca²⁺ from 100m-units/mg of protein to 43munits/mg of protein. When 1mM-MgCl₂ was present in the sample, activity after

the aging was 90 munits/mg of protein. The activity with EGTA, 30 munits/mg of protein, was not influenced by the aging or the inclusion of MgCl_2 .

(iv) *Effect of dithiothreitol.* The inclusion of dithiothreitol (0.05–0.1 mM) partially prevented the inactivation of fraction II during aging for several days at 4°C. Enzyme activity of fraction Ia (brain) was also stabilized to some extent by addition of dithiothreitol (Fig. 4). Dithiothreitol could be replaced by 2-mercaptoethanol.

(v) *Enzyme stability.* On purification, the enzyme was unstable even when dithiothreitol and Mg^{2+} were present in the medium. The activity of fraction II measured with EGTA was much more stable than the activity in the presence of Ca^{2+} and activator protein. Therefore purification or aging frequently caused the decrease in the responsiveness of the enzyme to stimulation by Ca^{2+} and activator protein. Brain enzyme was the least stable and lost more than a half of the activity on gel filtration or DEAE-cellulose column chromatography. The activity could not be recovered by adding an excess of activator protein to the assay mixture. Liver and heart enzymes were more stable than brain enzyme on gel filtration. Fraction II was also unstable on dilution, although concentrated solutions of the enzyme in the elution medium could be stored at -80°C for several months, as described in the Experimental section.

Discussion

By using reagents and preparations of enzyme and activator protein relatively free from contaminating Ca^{2+} , we have shown conclusively that a cyclic nucleotide phosphodiesterase (fraction II) from the supernatant from cerebral cortex requires the simultaneous presence of Ca^{2+} , Mg^{2+} and activator protein in the reaction mixture for the full activity. Results are consistent with those obtained by Teo & Wang (1973) with bovine heart enzyme, and support the previous results in which Ca^{2+} –EGTA buffer was used (Kakiuchi & Yamazaki, 1970*a,b*; Kakiuchi *et al.*, 1971; Miki & Yoshida, 1972; Kakiuchi *et al.*, 1973).

The $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent cyclic nucleotide phosphodiesterase (fraction II) was isolated together with activator protein from all the tissues examined, including cerebral cortex, cerebellum, kidney, heart and liver. The concentrations of the isolated enzyme (fraction II) and activator protein did not correspond exactly (Table 3). Smoake *et al.* (1974) found that the concentration of activator protein and the total phosphodiesterase activity in homogenates of various rat tissues were not parallel. The enzyme from cerebral cortex slightly differed from those from other tissues

Table 3. *Distribution of enzyme (fraction II) and protein activator in rat tissues*

$(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent cyclic nucleotide phosphodiesterase (fraction II) and protein activator were isolated from supernatants of homogenates of the indicated tissues by gel filtration as shown in Figs. 2 and 3. Protein activator was assayed by assay method B. The activity of fraction II with $0.4 \mu\text{M}$ -cyclic GMP was calculated from the elution profiles. The precipitate was not washed when it was separated from the supernatant fluid by centrifugation, so some supernatant fluid was lost in the precipitate fraction. To correct for this, results were first expressed as enzyme activity/mg of protein of the supernatant, or amount of protein activator/mg of protein of the supernatant and then converted into values per g of tissue by multiplying by mg of total soluble protein/g of tissue. For this the conversion factors, 26.4, 22.8, 55.3, 59.7 and 30.5 mg of protein/g of cerebellum, cerebral cortex, kidney, liver and heart respectively, were obtained in a separate experiment, where the soluble proteins were recovered quantitatively in the combined supernatant fluids obtained by repeated homogenization and centrifugation of the homogenates.

Tissue	Activity of fraction II (nmol/min per g of tissue)	Protein activator (units/g of tissue)
Cerebral cortex	24.4	449
Kidney	10.4	98
Cerebellum	7.14	659
Heart	1.21	36
Liver	1.06	85

Table 4. *Effects of inhibitors on the rates of hydrolysis of cyclic GMP by fraction II isolated from rat tissues*

Hydrolysis of $0.4 \mu\text{M}$ -cyclic GMP in the presence of $50 \mu\text{M}$ - CaCl_2 , 1 unit of the partially purified pig brain protein activator, and the test compound as indicated in the Table was determined by assay method 3.

Enzyme source	Concentration of compound that causes 50% inhibition of hydrolysis of cyclic GMP (μM)			
	3-Isobutyl-1-methylxanthine	SQ 20009	Papaverine	Theophylline
Cerebral cortex	50	200	100	1200
Heart muscle	3	15	15	100
Liver	3	15	15	150

Fig. 4. Effects of dithiothreitol and aging on the activity profile of the dialysed supernatant from a homogenate of rat cerebral cortex

Equal volumes of the supernatant were subjected to gel filtration on the day after killing the animals (*a* and *b*), or after aging the supernatant for 12 days at 4°C (*c* and *d*). The medium used for preparation of the enzyme consisted of 20 mM-Tris-HCl (pH 7.5), 1 mM-MgCl₂ and 0.1 mM-EGTA with (*b* and *d*), or without (*a* and *c*) dithiothreitol. The medium used for gel filtration consisted of 40 mM-Tris-HCl (pH 7.5), 1 mM-MgCl₂, 100 mM-NaCl and 0.1 mM-EGTA with (*b* and *d*), or without (*a* and *c*) dithiothreitol. In (*b*) and (*d*) dithiothreitol, at a final concentration of 0.05 mM, was added when the homogenate was made and this concentration of dithiothreitol was maintained in the media in all procedures including preparation of enzyme and subsequent gel filtration. Enzyme was assayed by method 1 (with 1.2 mM-cyclic AMP) in the presence of either 1 mM-EGTA (○) or 0.1 mM-CaCl₂ + protein activator (●).

in being more labile after purification and less susceptible to inhibition by methylxanthine derivatives and other compounds.

Miki & Yoshida (1972) could not detect any effect of Ca²⁺ on phosphodiesterase activities of supernatants from a variety of tissues, other than cerebral cortex. They used a saturating concentration of cyclic AMP as substrate in their assay. The present

results show that the magnitude of the stimulatory effect of Ca²⁺ depends on the substrate concentration. As shown in Table 5, the effect of Ca²⁺ can be observed consistently with fresh supernatants from a variety of tissues when incubations were carried out with a sub-saturating concentration of cyclic nucleotides, preferably cyclic GMP. But with a saturating concentration of cyclic nucleotides, the effect of

Table 5. *Effect of Ca^{2+} on hydrolysis of 1 μM -cyclic AMP and 1 μM -cyclic GMP in supernatants from rat tissues*

The phosphodiesterase activities of dialysed supernatant fractions of homogenates of tissues were assayed by method 2. Values are means $\pm$ S.E.M. of four determinations.

Tissue	Hydrolysis of cyclic AMP (nmol/30min per mg of protein)		Hydrolysis of cyclic GMP (nmol/30min per mg of protein)	
	With 0.06 μM - Ca^{2+}	With 12 μM - Ca^{2+}	With 0.06 μM - Ca^{2+}	With 12 μM - Ca^{2+}
Cerebral cortex	12.9 $\pm$ 0.3	56.6 $\pm$ 0.5*	23.6 $\pm$ 0.7	297.5 $\pm$ 2.8*
Heart muscle	2.85 $\pm$ 0.07	3.30 $\pm$ 0.13†	1.61 $\pm$ 0.06	4.61 $\pm$ 0.02*
Kidney	4.86 $\pm$ 0.18	8.43 $\pm$ 0.47*	7.28 $\pm$ 0.22	15.0 $\pm$ 0.3*
Liver	1.27 $\pm$ 0.03	1.72 $\pm$ 0.09*	1.47 $\pm$ 0.03	2.42 $\pm$ 0.07*
Skeletal muscle	1.21 $\pm$ 0.01	1.40 $\pm$ 0.02*	0.32 $\pm$ 0.00	0.54 $\pm$ 0.01*

* The activity with 12 μM - Ca^{2+} was significantly higher than that with 0.06 μM - Ca^{2+} (Ca^{2+} -EGTA buffer), $P < 0.001$.

† The activity with 12 μM - Ca^{2+} was significantly higher than that with 0.06 μM - Ca^{2+} , $P < 0.05$.

Table 6. *Effect of Ca^{2+} on hydrolysis of 1.2 mM-cyclic AMP and 1.2 mM-cyclic GMP in supernatants from rat tissues*

Experimental conditions were as for Table 5 except that 1.2 mM substrate was used and activities were assayed by method 1.

Tissue	Hydrolysis of cyclic AMP (μmol /30min per mg of protein)		Hydrolysis of cyclic GMP (μmol /30min per mg of protein)	
	With 0.06 μM - Ca^{2+}	With 12 μM - Ca^{2+}	With 0.06 μM - Ca^{2+}	With 12 μM - Ca^{2+}
Cerebral cortex	0.96	3.33	1.10	2.19
Heart muscle	0.09	0.11	0.09	0.09
Kidney	0.20	0.28	0.10	0.14
Liver	0.19	0.21	0.12	0.14
Skeletal muscle	0.05	0.06	0.03	0.03

Ca^{2+} can scarcely be detected in supernatants of tissues other than cerebral cortex (Table 6). Beavo *et al.* (1970) demonstrated the importance of measuring the activity of cyclic nucleotide phosphodiesterase at a sub-saturating substrate concentration. Loten & Sneyd (1970) and Manganiello & Vaughan (1973) showed an effect of insulin on adipose tissue cyclic nucleotide phosphodiesterase with low concentrations of substrate.

The present results show that, when assayed with sub-saturating concentrations of substrate, supernatant fractions from a variety of tissues except skeletal muscle contain much more activity for hydrolysis of cyclic GMP than for that of cyclic AMP (see Table 5; Figs. 2 and 3). Similar results were obtained by Beavo *et al.* (1970) with rat brain, liver and heart. These phosphodiesterase activities for cyclic GMP are now shown to be mainly due to the activity of fraction II. Indeed, the activity of fraction II accounted for about 60% in liver, more than 80% in heart and cerebellum and almost 100% in cerebral cortex of the total activity for cyclic GMP hydrolysis, calculated from the enzyme activity profiles shown in Figs. 2 and 3.

Fraction II from all the tissues examined hydro-

lysed cyclic GMP preferentially at sub-saturating substrate concentrations. Results are compatible with our previous conclusion from studies on the brain enzyme, that the function of the (Ca^{2+} + Mg^{2+})-dependent cyclic nucleotide phosphodiesterase is probably to hydrolyse cyclic GMP (Kakiuchi *et al.*, 1973). It must be noted, however, that fraction II also hydrolysed cyclic AMP to some extent at a substrate concentration of 0.4 μM . The activity of brain fraction II with cyclic AMP increased progressively with increasing substrate concentration and, at a saturating concentration, it hydrolysed cyclic AMP faster than cyclic GMP (Table 6). Concentrations of cyclic AMP in various tissues are usually at least one order of magnitude higher than those of cyclic GMP (Kuo *et al.*, 1972; Steiner *et al.*, 1972). Therefore the possibility that cyclic AMP may also serve as substrate of this enzyme cannot be excluded.

It is now well established that cyclic nucleotide phosphodiesterase exists as several different molecular entities (Brooker *et al.*, 1968; Cheung, 1969; Jard & Bernard, 1970; Rosen, 1970; Monn & Christiansen, 1971; Kakiuchi *et al.*, 1971; Thompson & Appleman, 1971a,b; Uzunov & Weiss, 1972; Campbell & Oliver, 1972; Hrapchak & Rasmussen, 1972;

Russell *et al.*, 1973; Pichard *et al.*, 1973; Schröder & Rickenberg, 1973). Of particular interest are observations described by Thompson & Appleman (1971*a,b*) that cyclic nucleotide phosphodiesterase from rat brain, heart, skeletal muscle, kidney and adipose tissue yielded two enzyme species on gel filtration. One had a high molecular weight (about 400000) and preferential affinity for cyclic GMP and the other had a low molecular weight (about 200000) and preferential affinity for cyclic AMP. From liver, they obtained only one peak of activity with a molecular weight of 400000, which hydrolysed both nucleotides. In the present work no two tissues had similar activity profiles. For instance, we found only one peak with activity for both nucleotides from cerebral cortex when assayed with 0.4 μ M substrate and at least five distinct peaks of activity from liver, two with activity for cyclic AMP and three with activity for both nucleotides with molecular weights ranging from 50000 to 400000. Moreover, the molecular weight of the (Ca²⁺ + Mg²⁺)-dependent cyclic nucleotide phosphodiesterase hydrolysing cyclic GMP was about 150000 (in the absence of activator protein) compared with 400000 for the cyclic GMP enzyme of Thompson & Appleman (1971*b*). These discrepancies may reflect differences of experimental conditions, including the enzyme preparations, gel-filtration techniques and assay conditions. The effects of Ca²⁺ and activator protein have not been examined under the experimental conditions used by Appleman's group. Russell *et al.* (1973) separated three forms of liver enzyme with different substrate specificities by DEAE-cellulose chromatography. We have obtained results supporting their work (S. Kakiuchi & Y. Teshima, unpublished work).

At present the physiological significance of the (Ca²⁺ + Mg²⁺)-dependent cyclic nucleotide phosphodiesterase is unknown. It seems likely that this enzyme actually functions *in vivo* in regulation of the tissue concentration of cyclic GMP for the following reasons. (a) This enzyme accounted for most of the cyclic GMP-hydrolysing activity found in the supernatants of all the tissues examined. (b) The concentration of Ca²⁺ that regulates the activity of this enzyme seems to be within the physiological concentration range. (c) The effect of Ca²⁺ is greatest at substrate concentrations close to physiological concentrations.

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