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CYCLIC 3′,5′-AMP PHOSPHODIESTERASE OF RABBIT AORTA

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Summary

Cyclic AMP and cyclic GMP phosphodiesterase activities (3′ : 5′-cyclic AMP 5′-nucleotidohydrolase, EC 3.1.4.17) were demonstrated in the isolated intima, media, and adventitia of rabbit aorta. The activity for cyclic AMP hydrolysis in the intima was 2.7-fold higher than that for cyclic GMP hydrolysis. The activity for cyclic AMP hydrolysis in the media was approximately equal to that for cyclic GMP hydrolysis, but in the adventitia, cyclic GMP hydrolytic activity was 2.1-fold higher than cyclic AMP hydrolytic activity. Distribution of the activator of the phosphodiesterase was studied in the three layers. Each layer contained the activator. The activator was predominantly localized in the smooth muscle layer (the media). The effect of the activator and Ca^{2+} on the media cyclic AMP and cyclic GMP phosphodiesterase was also briefly studied. The activity of the cyclic GMP phosphodiesterase was stimulated by micromolar concentration of Ca^{2+} in the presence of the activator. However, the activity of the cyclic AMP phosphodiesterase was not significantly stimulated by Ca^{2+} up to 100 μM in the presence of the activator.

Above 90% of cyclic nucleotide phosphodiesterase activity in the whole aorta was found to be derived from the media. A major portion (60—70%) of the media enzyme was found in 105 000 × *g* supernatant. Cyclic AMP phosphodiesterase in the supernatant was partially purified through Sepharose 6B column chromatography and partially separated from cyclic GMP phosphodiesterase. Using a partially purified preparation from the 105 000 × *g* supernatant the main kinetic parameters were specified as follows:

1) The pH optimum was found to be about 9.0 using Tris—maleate buffer. The maximum stimulation of the enzyme by Mg^{2+} was achieved at 4 mM of $MgCl_2$.

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2) High concentration of cyclic GMP (0.1 mM) inhibited noncompetitively the enzyme activity, and the activity was not stimulated at any tested concentration of cyclic GMP.

3) Activity—substrate concentration relationship revealed a high affinity (K_m = 1.0 μM) and low affinity (K_m = 45 μM) for cyclic AMP. The homogenate and 105 000 × *g* supernatant of the media also showed non-linear kinetics similar to the Sepharose 6B preparation and their apparent K_m values for cyclic AMP hydrolysis were 1.2 μM and 36—40 μM and an enzyme extracted by sonication from 105 000 × *g* precipitate also exhibited non-linear kinetics (K_m = 5.1 μM and 70 μM).

4) Papaverine exhibited much stronger inhibition on the aorta cyclic AMP phosphodiesterase (50% inhibition of the intima enzyme, I_{50} at 0.62 μM, I_{50} of the media at 0.62 μM and I_{50} of the adventitia at 1.0 μM) than on the brain (I_{50} at 8.5 μM) and serum (I_{50} at 20 μM) cyclic AMP phosphodiesterase, while theophylline inhibited these enzymes similarly. However, cyclic GMP phosphodiesterases in all tissues examined were inhibited similarly, not only by theophylline but also by papaverine.

Introduction

Experimental evidence implying that there is a relationship between cyclic AMP and vasodilation, has accumulated during the last few years [1—6]. The level of cyclic AMP in any tissue is a result of a balance between its rate of synthesis by adenylate cyclase and that of hydrolysis by cyclic AMP phosphodiesterase. The activity of phosphodiesterase in a tissue is, therefore, critical in regulating its level of cyclic AMP. Amer [6] suggested that phosphodiesterase activity (especially the low Michaelis—Menten constant form) of the aorta from spontaneously hypertensive and stress hypertensive rats was significantly elevated. Volicer et al. [7] have reported that cyclic AMP system (adenylate cyclase and cyclic AMP phosphodiesterase activity) is predominantly localized in the parts of the bovine carotid artery that contain smooth muscle cells. The present studies were initiated to evaluate whether phosphodiesterases derived from three different layers and having different functions, possess characteristics correlated with each function of three layers of the aorta. It is of interest to note that cyclic AMP and cyclic GMP phosphodiesterase are distributed differently in the three layers.

Materials and Methods

Chemicals and reagents

Nucleotides and snake venom (*Crotalus atrox*) in a lyophilized form were obtained from Sigma Chemical Co. Tritium-labelled cyclic nucleotides (specific activity; cyclic AMP: 33.2 Ci/mmole, cyclic GMP: 3.46 Ci/mmole) were purchased from New England Nuclear. All reagents were of analytical grade. The final concentration of nucleotide was determined spectrophotometrically using molar extinction coefficients.

Preparation of the intima, media, and adventitia from rabbit aorta

The rabbit was sacrificed by decapitation. The aorta was removed quickly and opened by a longitudinal dissection. The intima (endothelial cells) was obtained by short contact of the intima surface to a solid CO_2-treated glass plate. With this technique, only endothelial cells remained on the glass plate and were collected by washing the plate with physiological saline containing 1 mM $MgCl_2$. The intima prepared by this technique was confirmed to be homogeneous endothelial cells under microscope (Fig. 1). The arterial wall separated from the intima was placed on an ice-cold glass plate with the adventitia side up. The adventitia was scratched off with small knife from the smooth muscle layer (media layer). The adventitia and media so isolated are also shown in Fig. 1. The media preparation so treated contained only smooth muscle layer, but the adventitia preparation had some contamination of the media and connective tissue outside of the aorta. Distribution of the enzyme or the activator in the three layers was studied by determining their activities using the samples isolated from 1 g (wet weight) of the aorta. Forty rabbits were needed for collecting endothelial cells of the aorta for this experiment, whereas the aorta of one rabbit was enough to obtain the preparation of the media for purification.

Phosphodiesterase preparation

The isolated media and adventitia of rabbit aorta were homogenized with 9 volumes of 50 mM Tris—HCl buffer (pH 7.5), containing 1 mM $MgCl_2$ using a glass tissue homogenizer. The intima isolated was very little, below 1 mg out of 1 g (wet weight) of the aorta and could not be weighed accurately. Endothelial cells on the glass plate were collected by washing with 1 ml of the saline per sample isolated from 1 g (wet weight) of the aorta. The intima suspended with physiological saline containing 1 mM $MgCl_2$ was homogenized with glass tissue homogenizer. All steps were carried out at 0—4° C. The homogenized solution of the media was centrifuged at 105 000 × *g* for 90 min. The 105 000 × *g* precipitate was washed with the same buffer, sonicated (30 s per ml) and centrifuged at 105 000 × *g* again for 90 min. With this treatment, a part (20—30%) of the enzyme activity in the precipitate could be solubilized. This preparation referred to as the sonicated extract, was used for gel filtration studies.

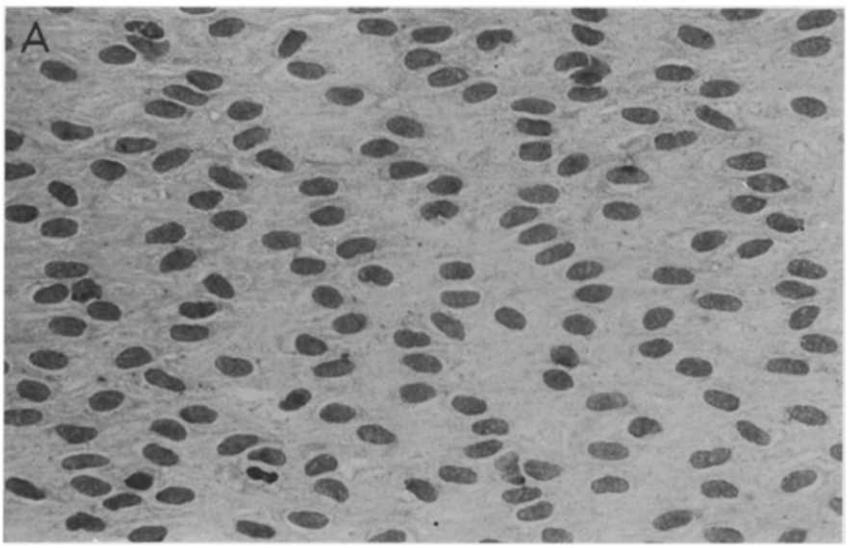
Assay of phosphodiesterase

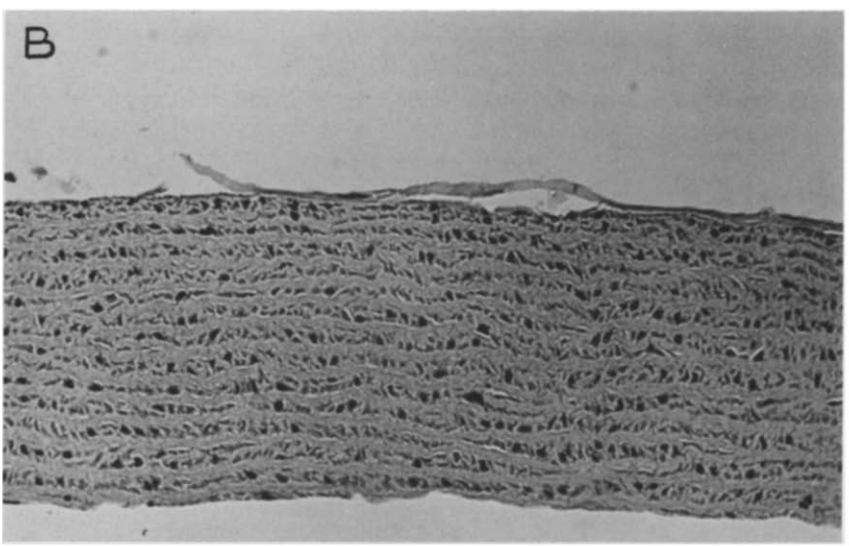
The method is essentially that of Butcher and Sutherland [8], employing snake venom as a source of 5′-nucleotidase. The [^{3}H]adenosine or [^{3}H]guanosine was isolated by cation exchange resin [9]. The assay for phosphodiesterase activity consists of a two step procedure. In the first stage of the incubation, the reaction mixture of 0.5 ml contained 32 mM Tris—HCl (pH 8.0), 4 mM $MgCl_2$, 0.4 μM cyclic [^{3}H]AMP or 0.4 μM cyclic [^{3}H]GMP (100 000 cpm) and an appropriate concentration of enzyme. The reaction was initiated by the addition of cyclic AMP or cyclic GMP. At the end of 15 min at 30° C, the tube containing the reaction mixture was transferred to a boiling water bath for 5 min to terminate the reaction. After thermal equilibration to 30° C, 0.05 ml of snake venom (1 mg per ml) was added for a second 10 min incubation. The

reaction was stopped by boiling for 5 min. Then, 1 ml of water was added, and denatured proteins were removed by centrifugation. 1 ml of the clear supernatant fluid was applied to a small ion exchange column (AG 50W-X4, 200—400 mesh, H^+ form). The product, [^{3}H]adenosine or [^{3}H]guanosine, was eluted with 1.5 ml of 3 M ammonium hydroxide after washing the column with 10 ml of water. The amounts of product were determined in a liquid scintillation spectrometer.

Gel filtration

Gel filtration was performed on a Sepharose 6B column (2.6 × 95 cm) with a bed volume of 504 ml at flow rates of 18 ml per h using 50 mM Tris—HCl buffer (pH 7.5), containing 1 M KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$.





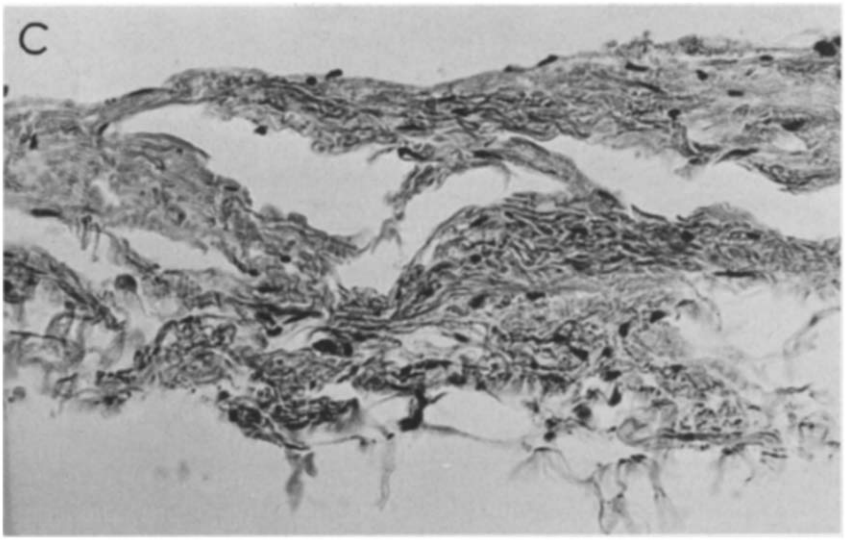


Fig. 1. A. Endothelial cells of rabbit aorta stained by Hematoxylin—Eosine (×400) B. Media of rabbit aorta stained by Hematoxylin—Eosine (×200) C. Adventitia of rabbit aorta stained by Hematoxylin—Eosine (×200)

Preparation and assay of the activator

The activator of rabbit aorta was prepared according to Cheung [10]. Fresh rabbit aorta was homogenized with 3 vols of glass distilled water chilled to 0° C. The homogenate was adjusted to pH 5.9 and spun at 13 000 × *g* for 30 min. The sediment was discarded. The supernatant was heated in a boiling water bath. After heating for 5 min, the beaker was transferred to an ice-bath for quick cooling. Denatured proteins were removed by centrifugation. The boiled supernatant (pH 5.9) was dialyzed twice against 300 vols of 10 mM Tris—HCl (pH 7.5) buffer containing 1 mM $MgCl_2$ and 0.1 mM EGTA (ethyleneglycol-bis-(β-aminoethyl ether)*N'*,*N'*-tetraacetic acid) for 24 h and used for the experiment. Assay of the activator was made according to Kakiuchi et al. [11] based on the ability of the activator to enhance the activity of diluted 105 000 × *g* supernatant fluid of the rat brain homogenate in the presence of 100 μM Ca^{2+}. The amount of the activator that doubled the enzyme activity in the standard system was defined as 10 units. The 105 000 × *g* supernatant of the media was dialyzed twice against 300 vols of 10 mM Tris—HCl buffer containing 1 mM $MgCl_2$ and 0.1 mM EGTA. This preparation, referred to as Ca^{2+}-free phosphodiesterase, was used for the studies on the Ca^{2+} effect. Effect of Ca^{2+} on the Ca^{2+}-free phosphodiesterase was studied by using Ca^{2+}-EGTA buffer according to Kakiuchi et al. [11].

Results

Distribution of cyclic AMP and cyclic GMP phosphodiesterase and its activator in various layers of rabbit aorta

Cyclic nucleotide phosphodiesterase activity in rabbit aorta was investi-

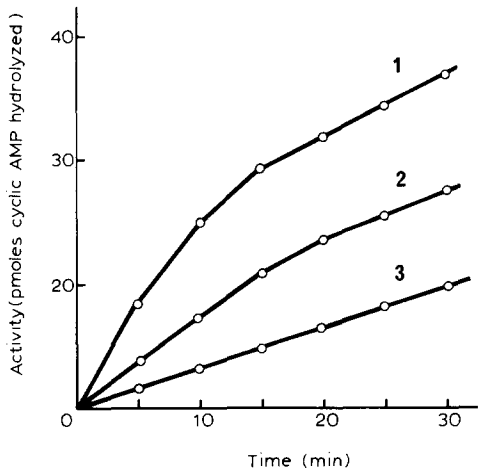


Fig. 2. Cyclic AMP hydrolysis by the 105 000 × *g* supernatant solution (1.4 mg protein/ml) of the rabbit aorta as a function of the time of incubation at 30°C and as a function of the amount of the enzyme preparation. The assay was performed as described in the text. 1: 40 μl (56 μg protein) of the supernatant, 2: 20 μl and 3: 10 μl was added.

gated using a low substrate concentration (0.4 μM). The linearity of the enzyme reaction with time and enzyme concentration was shown in Fig. 2. 1 g (wet weight) of fresh aorta obtained from a rabbit was divided into three layers as described in Materials and Methods. Cyclic nucleotide phosphodiesterases were mainly localized in the media of the aorta (Table I). Although the intima and adventitia had significant activities of cyclic AMP and cyclic GMP phosphodiesterase, their activities were low. Table I shows that cyclic AMP phos-

TABLE I

DISTRIBUTION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE AND ITS ACTIVATOR IN VARIOUS LAYERS OF THE RABBIT AORTA

1 g (wet weight) of rabbit aorta was divided into three layers. The amount of the enzyme or the activator was determined in each layer as described in the text. The enzyme or the activator preparation was a homogenate fraction or its heated fraction of each layer. Duplicate determinations were made for each experiment. Results obtained from four experiments were averaged. Values indicate the mean ± S.E.

Preparation from 1g of aorta	Protein (mg)	Activity (pmoles hydrolyzed per min)					Activator (units**)	
		Cyclic AMP		Cyclic GMP		A/G***	Net units**	Units** per mg protein
		Net activity	Units* per mg protein	Net activity	Units* per mg protein			
Intima	0.122 ± 0.021	12.8 ± 1.4	99.1	4.74 ± 1.0	36.7	2.7	1.77 ± 0.33	14.5
Media	44.1 ± 8.4	4460 ± 580	101	4860 ± 490	110	0.92	3580 ± 330	78.3
Adventitia	9.93 ± 3.4	189 ± 71	19.0	400 ± 78	40.3	0.47	178 ± 40	18.4

* pmoles per min.

** 10 units: the amount of the activator doubled the enzyme activity (see text).

*** Cyclic AMP hydrolytic activity/cyclic GMP hydrolytic activity.

phodiesterase activity in the intima was 2.7-fold higher than cyclic GMP phosphodiesterase activity. Table I also shows that cyclic AMP phosphodiesterase activity in the media is approximately equal to cyclic GMP phosphodiesterase activity, but cyclic GMP phosphodiesterase activity of the adventitia is much higher (2.1-fold) than its cyclic AMP phosphodiesterase activity. The distribution of the activator of the phosphodiesterase in the three layers was investigated. The homogenates of the three layers were boiled for 3 min. The amount of the activator in these supernatants was determined as described in Materials and Methods. The results were summarized in Table I. Each layer contained the activator. The activator was predominantly localized in the smooth muscle layer.

Purification of cyclic nucleotide phosphodiesterase of the media

Cyclic AMP phosphodiesterase was purified through a Sepharose 6B column. Fig. 3A shows the typical elution profile of cyclic nucleotide phos-

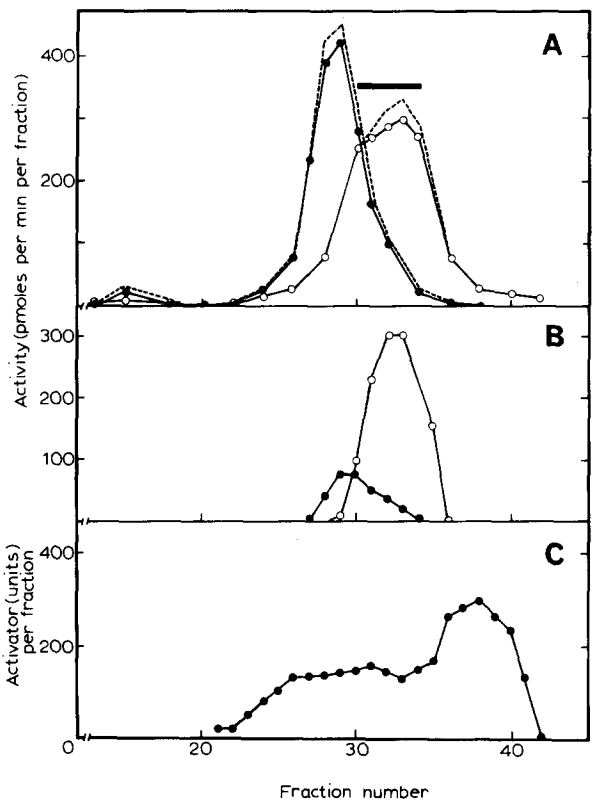


Fig. 3. Sepharose 6B profile of the media phosphodiesterase and its activator from 105 000 × *g* supernatant. 7.5 ml of the media supernatant (1.4 mg protein/ml) was placed on the column (2.6 × 95 cm) and eluted with 0.05 M Tris—HCl (pH 7.5), containing 1 M KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The flow rate was 18 ml/h and 10.3 ml per fraction was collected. The solid bar indicates the fractions pooled. A: ●———●, cyclic GMP hydrolytic activity; ○———○, cyclic AMP hydrolytic activity; - - - - - -, the activity when assay was performed in the presence of the activator. B: Rechromatography of cyclic AMP phosphodiesterase. The fractions pooled were concentrated with a collodion bag and applied to the same Sepharose 6B column. ●———●, cyclic GMP hydrolytic activity; ○———○, cyclic AMP hydrolytic activity. C: Elution pattern of the activator. 0.3 ml aliquots of the eluate fraction were heated for 3 min in boiling water bath and its activity was assayed in the standard system, as described in the text.

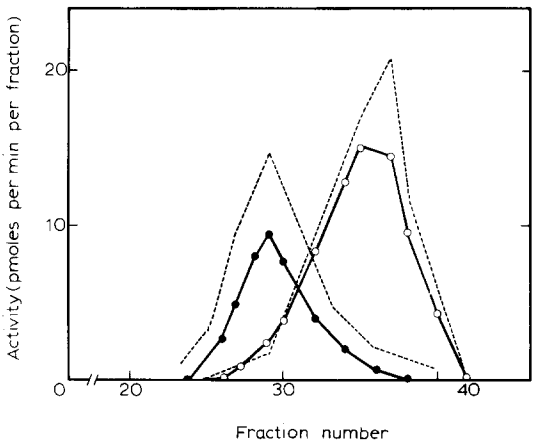


Fig. 4. Sepharose 6B profile of the media phosphodiesterase from the sonicated extract of the 105 000 × *g* precipitate. 6 ml of the sonicated extract (100 mg wet smooth muscle/ml) was placed on the column (2.6 × 95 cm) and eluted with 0.05 M Tris—HCl (pH 7.5), containing 1 M KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The flow rate was 18 ml/h and 10.3 ml fractions were collected. ●———●, cyclic GMP hydrolytic activity; ○———○, cyclic AMP hydrolytic activity; - - - - - -, the activity when assay was performed in the presence of the activator.

phodiesterase in the 105 000 × *g* supernatant of the media. The fractions containing cyclic AMP phosphodiesterase were pooled, concentrated in collodion bags at reduced pressure at 4°C and applied to the same Sepharose 6B column. The cyclic AMP hydrolytic activity was more clearly separated by this rechromatography (Fig. 3B). This separation of cyclic AMP and cyclic GMP phosphodiesterase through Sepharose 6B column was also observed with human platelet preparations [12]. Purification of cyclic AMP and cyclic GMP phosphodiesterase was 20- and 62-fold with 27% recovery of total homogenate activity after Sepharose 6B column chromatography. These partially purified preparations, when concentrated, were stable for a few weeks at −20°C. Fig. 4 shows the typical elution pattern of the sonicated extract of the 105 000 × *g* precipitate of the media. Cyclic AMP phosphodiesterase was eluted in fractions later than in the eluate fractions of 105 000 × *g* supernatant cyclic AMP phosphodiesterase. The cyclic GMP hydrolytic activity in the sonicated extract was lower than its cyclic AMP hydrolytic activity.

Effect of the activator and Ca^{2+} on the cyclic nucleotide phosphodiesterase of the media

Addition of the activator to each fraction from gel filtration on Sepharose 6B column barely altered its elution profile which was obtained by determining the activity without the addition of the activator (Fig. 3A). This result indicates the possibility that the activator was also eluted in the same fractions containing the enzyme activity. Assay of the activator in the eluate fractions was performed in 0.3 ml aliquots of the fractions which were heated for 3 min in boiling water bath as described in Materials and Methods. Fig. 3C shows that the activator was eluted widely in both fractions containing cyclic AMP and cyclic GMP phosphodiesterase. The effect of the activator on the cyclic nucleotide phosphodiesterase of the 105 000 × *g* supernatant of the media was inves-

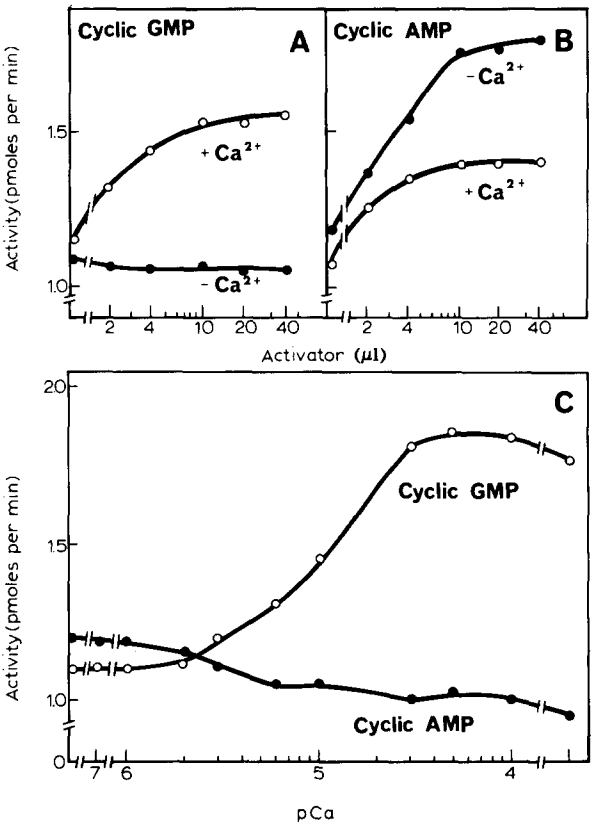


Fig. 5.A: Effect of the activator of rabbit aorta on cyclic GMP phosphodiesterase (10 μg protein, 105 000 × *g* supernatant of the media) in the presence of 0.1 mM $CaCl_2$ (○———○) and in the presence of 0.1 mM EGTA instead of $CaCl_2$ (●———●). Enzyme and activator preparations were dialyzed twice against Tris—HCl (pH 7.5), containing 1 mM $MgCl_2$ and 0.1 mM EGTA for 24 h. Assay of enzyme was performed as described in the text. B: Effect of the activator on cyclic AMP phosphodiesterase (10 μg protein, 105 000 × *g* supernatant of the media) in the presence of 0.1 mM $CaCl_2$ (○———○) and in the presence of 0.1 mM EGTA instead of $CaCl_2$ (●———●). C: Effect of Ca^{2+} on the hydrolysis of 0.4 μM cyclic AMP (●———●) and cyclic GMP (○———○) by a Ca^{2+}-free preparation of the media phosphodiesterase. Assay was performed in the presence of the saturated amounts of the activator. Ca^{2+}—EGTA buffer was used according to Kakiuchi et al. [11].

tigated in the presence of 0.1 mM $CaCl_2$ and in the presence of 0.1 mM EGTA instead of $CaCl_2$. The activator stimulated cyclic GMP hydrolysis in the presence of $CaCl_2$ but no stimulation occurred in the absence of $CaCl_2$ (Fig. 5A). However, the activator stimulated the activity for cyclic AMP hydrolysis both in the presence and in the absence of $CaCl_2$ (Fig. 5B). The rate of hydrolysis of 0.4 μM cyclic AMP or cyclic GMP by Ca^{2+}-free preparation of the cyclic nucleotide phosphodiesterase of the media was titrated with Ca^{2+} in the presence of the activator by the method of Kakiuchi et al. [11]. Ca^{2+}-stimulated cyclic GMP phosphodiesterase activity above 3 μM, but did not stimulate the activity of cyclic AMP phosphodiesterase (Fig. 5C). The effect of the activator on the rat brain phosphodiesterase was seen when the concentration of Ca^{2+} was above 2 μM and stimulation of cyclic GMP phosphodiesterase activity was more marked than that of cyclic AMP phosphodiesterase activity [11].

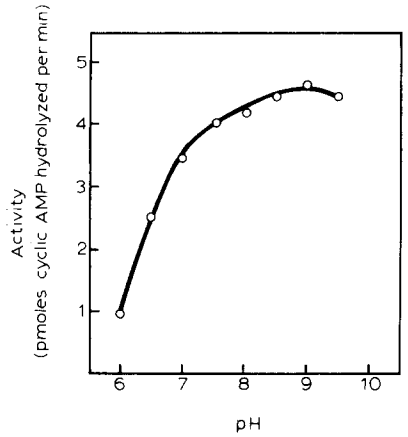


Fig. 6. Effect of pH on rabbit media cyclic AMP phosphodiesterase. Enzyme purified through Sepharose 6B was employed in the assay, and the buffer was 32 mM Tris—maleate. The pH was adjusted to 7.5 for the nucleotidase reaction. Assay conditions and procedures were described under Materials and Methods.

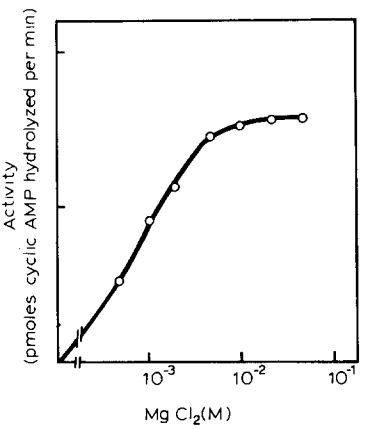


Fig. 7. Effect of Mg^{2+} on rabbit media cyclic AMP phosphodiesterase. Enzyme purified through Sepharose 6B was employed in the assay. Assay conditions and procedures were described under Materials and Methods.

Properties of partially purified cyclic AMP phosphodiesterase of the media

Fig. 6 illustrates the activity of cyclic AMP phosphodiesterase as a function of pH using 32 mM Tris—maleate buffer containing 4 mM $MgCl_2$. The maximal activity was observed at pH 9.0 and is comparable to that of the enzyme of dog heart [13]. However the enzymes of rat brain [14], bovine brain [15], human platelet [16], human serum [9], and frog erythrocytes [17] exhibited the optimum pH at 8.0.

The effect of Mg^{2+} on cyclic AMP phosphodiesterase is shown in Fig. 7. Maximal activity was observed at 4 mM and is comparable to the enzyme of human serum [9].

Fig. 8 illustrates the effect of cyclic GMP on cyclic AMP phosphodi-

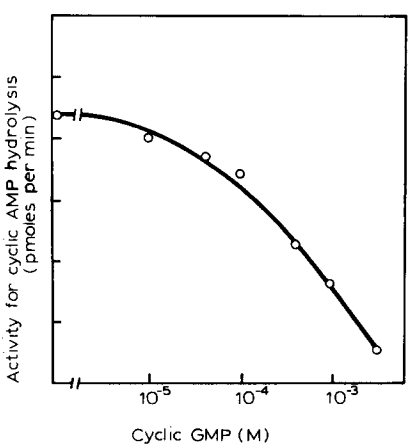


Fig. 8. Effect of cyclic GMP on rabbit media cyclic AMP phosphodiesterase. Enzyme purified through Sepharose 6B was employed in the assay. Assay conditions and procedures were described under Materials and Methods.

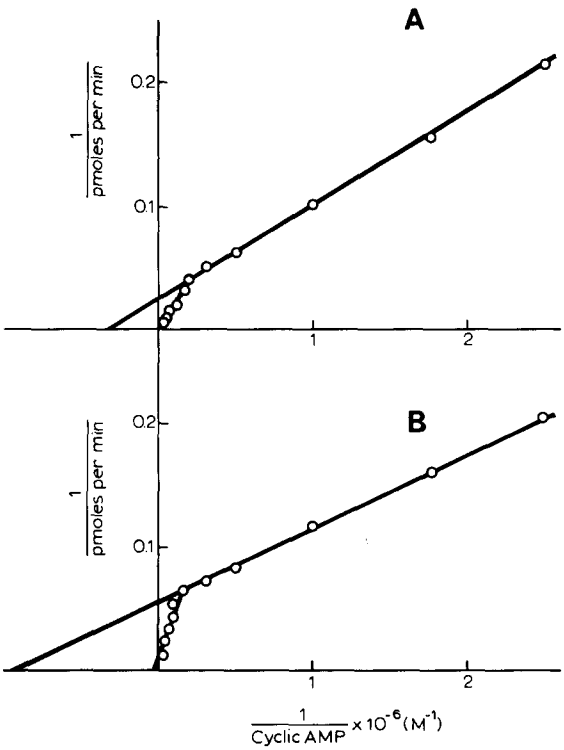


Fig. 9. Kinetic analysis of cyclic AMP hydrolysis by partially purified enzyme through Sepharose 6B, obtained from 105 000 × *g* supernatant and its precipitate (sonicated extract) of isolated media. Concentrations of cyclic AMP ranged from 0.2 μM to 1 mM. Velocities were expressed as pmoles per min at 30°C. A: 105 000 × *g* precipitate (sonicated extract); B: 105 000 × *g* supernatant. Assay was performed as described in the text.

esterase. Significant inhibition by cyclic GMP was observed above $2 \cdot 10^{-4}$ M. However the inhibition of cyclic AMP phosphodiesterase of human platelet of cyclic GMP was observed at a far lower cyclic GMP concentration ($1 \cdot 10^{-7}$ M) [12]. Cyclic GMP inhibited cyclic AMP phosphodiesterase noncompetitively and K_i was $3.0 \cdot 10^{-4}$ M.

Fig. 9 illustrates the kinetic analysis of partially purified cyclic AMP phosphodiesterase of the media through Sepharose 6B column chromatography.

TABLE II

APPARENT K_m VALUES OF SEVERAL PREPARATIONS OF THE MEDIA CYCLIC AMP PHOSPHODIESTERASE

Preparation of the media	Apparent K_m (μM)	
	Low	High
Homogenate	1.2	40
105000 × *g* supernatant	1.1	36
Sephatose 6B (supernatant)	1.0	45
105000 × *g* precipitate	3.2	59
Sepharose 6B (precipitate)	5.1	70

TABLE III

EFFECT OF PAPAVERINE AND THEOPHYLLINE ON CYCLIC AMP AND CYCLIC GMP PHOSPHODIESTERASE OF RABBIT TISSUES

Concentration of inhibitors was decreased over the range of 500 μM to 0.5 μM until the inhibition fell below 50%. The inhibitor concentration producing 50% inhibition (I_{50}) was determined graphically. The 105000 × *g* supernatants of tissues and serum were added to reaction mixture. Assay was performed as described in the text.

	I_{50} (μM)				
	Intima	Media	Adventitia	Brain	Serum
Cyclic AMP as substrate					
Papaverine	0.62	0.62	1.0	8.5	20
Theophylline	127	113	172	235	167
Cyclic GMP as substrate					
Papaverine	4.5	4.9	7.4	22	6.9
Theophylline	110	133	156	347	312

The substrate concentration used to measure the K_m in Fig. 9 ranged from 0.2 μM to 1 mM. When the apparent K_m was determined using a wider range of substrate concentrations, we found that the double reciprocal plot is not linear.

Extrapolation of this curve indicates that the enzyme owns two apparent K_m values. The homogenate, 105 000 × *g* supernatant and Sepharose 6B preparations exhibited similar apparent K_m values (Table II). Slightly different apparent K_m values were obtained by 105 000 × *g* precipitate or its sonicated extract after Sepharose 6B column (Table II).

Inhibition of cyclic AMP phosphodiesterase by papaverine and theophylline

To determine why papaverine has an extensive dilatory or relaxant effect on blood vessels, we studied effects of papaverine and theophylline on cyclic AMP and cyclic GMP phosphodiesterase in various tissues of the rabbit. Table III shows that papaverine inhibited the enzyme of the aorta more markedly than that of brain or serum, whereas theophylline did not inhibit selectively aorta enzymes. However there was no obvious difference in the inhibition of cyclic GMP phosphodiesterase in the tissues tested by papaverine (Table III).

Discussion

Cyclic nucleotide phosphodiesterase and its activator were predominantly localized in those parts of the rabbit aorta that contain smooth muscle cells. A major portion (60—70%) of cyclic AMP phosphodiesterase in smooth muscle layers was found in 105 000 × *g* supernatant fraction. Cyclic AMP phosphodiesterase in the 105 000 × *g* precipitate seems to be different from the enzyme in the 105 000 × *g* supernatant, judging from its apparent K_m values and the elution pattern from Sepharose 6B column. The properties of the 105 000 × *g* precipitate enzyme were not studied further, because the solubilization of the enzyme from the precipitate was difficult and in the sonication as described in the text, only 20—30% of the total activity in the precipitate was solubilized. The drawing of any conclusion concerning the difference between these en-

zymes should be withheld until a better soluble preparation of the precipitate can be obtained. The aorta contained the activator of cyclic nucleotide phosphodiesterase which was predominantly localized in smooth muscle layer. As shown in Fig. 5, the extent of the stimulation of the phosphodiesterase by the activator was not so great as reported by Cheung [10] and Kakiuchi et al. [11]. This is simply attributed to the fact that the enzyme preparation used in our experiment is crude and still contains a large amount of the activator. The stimulation of cyclic GMP phosphodiesterase of the 105 000 × *g* supernatant of the media by the activator was dependent upon Ca^{2+}, but that of cyclic AMP phosphodiesterase was not dependent on Ca^{2+}. The existence of relatively Ca^{2+}-independent cyclic AMP phosphodiesterase was investigated only in the 105 000 × *g* supernatant fraction of the smooth muscle layer and these data do not even preclude the possibility that the arterial wall, as well as the media, contains Ca^{2+}-dependent cyclic AMP phosphodiesterase. Moreover, there is a possibility that the activator preparations used for our experiments still contained a small amount of firmly bound Ca^{2+} even after its dialyzation against the buffer containing 0.1 mM EGTA. Our results might suggest only the relative difference of the sensitivity of cyclic GMP and cyclic AMP phosphodiesterase of the media, to Ca^{2+}-dependent stimulation by the activator. Papaverine inhibited the aorta phosphodiesterase at one tenth of the concentration necessary for inhibition of the brain enzyme. Perhaps, this property is responsible for the vasodilatory activity of this drug. Kukovetz et al. [4] have demonstrated that papaverine and some other smooth muscle relaxants are very potent inhibitors of phosphodiesterase. Papaverine also strongly inhibits the activity of human platelet cyclic AMP phosphodiesterase and the effect of this drug upon platelet phosphodiesterase is consistent with the inhibitory effect of papaverine on platelet aggregation [18]. Similarly theophylline inhibited the phosphodiesterase of the aorta as well as brain and serum enzymes. This property of theophylline would suggest that this drug acts not only on blood vessels but also on other organs, such as the brain simultaneously. Separation of cyclic AMP and cyclic GMP phosphodiesterase of the media (smooth muscle layer) was observed by Sepharose 6B column chromatography. This separation would indicate that cyclic AMP phosphodiesterase of the media is different from cyclic GMP phosphodiesterase. This is true in the case of liver cyclic nucleotide phosphodiesterase [19].

Many investigators [16,20—22] demonstrated the existence of two K_m values for phosphodiesterase of various tissues. The present paper shows that the media enzyme also exhibits two K_m values.

Previous work from this laboratory indicated that both human serum and plasma contain cyclic AMP and cyclic GMP phosphodiesterase [9]. The origin of the serum phosphodiesterase is not yet known at the present time, but there is a possibility that a part of the serum enzyme is derived from blood vessels. These problems should be studied in future.

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