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3′:5′-CYCLIC-NUCLEOTIDE PHOSPHODIESTERASES OF MAMMALIAN SERA

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Summary

Cyclic AMP and cyclic GMP phosphodiesterases (3′:5′-cyclic-nucleotide 5′-nucleotidohydrolase EC 3.1.4.17) were found in the sera of human, dog, rabbit and rat. The formed elements of blood were not present in sera and thus not the source of the phosphodiesterase. More rapid hydrolysis of cyclic GMP than cyclic AMP is observed in the sera of these four species when 0.4 μM of cyclic AMP or cyclic GMP is used as the substrate. Protein activator of the phosphodiesterase is not detectable in the sera of these four species. Serum cyclic AMP and cyclic GMP phosphodiesterase activities are not stimulated by protein activator prepared from bovine brain. The serum phosphodiesterases of these four species are purified through Sepharose 6B column chromatography. Cyclic AMP phosphodiesterase are found in a broad area corresponding to molecular weights ranging from approximately 150 000 to 340 000 with 2 to 3 peaks in all animals tested. Cyclic GMP phosphodiesterase is found in a single area corresponding to molecular weights of 230 000 (rabbit and rat) and 270 000 (human and dog).

Serum cyclic AMP and cyclic GMP phosphodiesterase activities of these four species have pH optimum of 7.5—8.5. Optimal concentration of Mg^{2+} is about 5 mM for cyclic GMP phosphodiesterase activities of these four species as well as for cyclic AMP phosphodiesterase activities except rabbit. Rabbit serum cyclic AMP phosphodiesterase requires higher concentration of Mg^{2+} (50 mM) for its optimal activity. The double reciprocal plots are non-linear for cyclic AMP phosphodiesterases of all animals and cyclic GMP phosphodiesterases of human, dog and rat. Rabbit cyclic GMP phosphodiesterase exhibits a linear reciprocal plot. Cyclic GMP is inhibitor of serum cyclic AMP phosphodiesterase. Rabbit enzyme was most effectively inhibited by cyclic GMP.

Introduction

Cyclic AMP and cyclic GMP appear to play a very important role in many tissues and compounds which affect the intracellular levels of these nucleotides which have profound effects on the function of a tissue. The intracellular levels of these nucleotides are controlled by both adenyl or guanyl cyclase and cyclic AMP or cyclic GMP phosphodiesterase. Serum normally contains many enzymes derived from not only the formed elements of blood but also from many other tissues. Although cyclic nucleotide phosphodiesterase was found in rat plasma [1,2] and differed from erythrocytes enzyme [2], the source of the phosphodiesterase is unknown. We have recently demonstrated cyclic AMP and cyclic GMP phosphodiesterases in human and rabbit serum as well as rat serum [3]. This report summarizes the properties of serum cyclic AMP and cyclic GMP phosphodiesterases of several mammals.

Materials and Methods

Chemicals and reagents

Cyclic nucleotides and snake venom (*Crotalus atrox*) were obtained from Sigma Chemical Co. ^{3}H-labeled cyclic nucleotides (spec. act.: cyclic AMP 33.2 Ci/mmole, cyclic GMP 3.46 Ci/mmole) were purchased from New England Nuclear. All other chemicals were of reagent grade or commercially available.

Preparation of serum and plasma

Fresh blood was standing for 2 h at room temperature and then the serum was isolated by centrifugation at 3000 rev./min for 15 min. Plasma was obtained from fresh citrated blood by centrifugation. Blood was collected in 3.8% sodium citrate (1v/4v blood). All the materials used for collection of blood were siliconized. Both serum and plasma were used after dialysed against 100 volumes of 50 mM Tris buffer (pH 7.5) containing 1 mM $MgCl_2$, twice changed, for 6 h. Enzymic activity of both serum and plasma was not different before and after dialysis. These preparations were stored at $-20°$C before use.

Phosphodiesterase assay

Activity of phosphodiesterase was determined by the method of Hidaka and Shibuya [3]. The linearity of the enzyme reaction with time and enzyme concentration has been described previously [3]. The reaction mixture (final volume, 0.5 ml) contained 50 mM Tris · HCl, pH 8.0, 5 mM $MgCl_2$, 0.4 μM cyclic [^{3}H]AMP or cyclic [^{3}H]GMP (100 000 cpm) and an appropriate amount of the enzyme, and was incubated at 30°C for 15—60 min. Fifty mM of $MgCl_2$ was used for the assay of rabbit serum cyclic AMP phosphodiesterase. The reaction was terminated by boiling for 5 min, and then 50 μg of snake venom was added and the mixture incubated for another 10 min. Then, 1 ml of water was added, and denatured protein was removed by centrifugation. The clear supernatant fluid was applied to a small cation exchange column (AG 50W-X4, 200-400 mesh, 0.7 × 1.5 cm). The product, [^{3}H]adenosine or [^{3}H]-guanosine, was eluted with 1.5 ml of 3N ammonium hydroxide after washing the column with 10 ml of water. The amount of product was determined in a

liquid scintillation spectrometer. Over 95% of adenosine or guanosine is recovered from the column using authentic compounds by this method. About 10% of added cyclic nucleotide is generally hydrolyzed during the incubation.

Preparation and assay of the activator

The activator of the bovine brain was prepared according to Cheung [4]. Fresh bovine brain was homogenized with 3 vol of glass-distilled water chilled to 0° C. The sediment was discarded. The supernatant was heated for 5 min in a boiling bath. Denatured proteins were removed by centrifugation. The boiled supernatant was used for the experiments. Assay of the activator was made according to Kakiuchi et al. [5] based on the ability of the activator to enhance the activity of diluted 105 000 × *g* supernatant fluid of the rat brain homogenate.

Sepharose 6B column chromatography

Gel filtration was carried out on Sepharose 6B column (2.6 × 95 cm). The column was eluted with 50 mM Tris · HCl buffer, pH 7.5, containing 1 M KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ at a flow rate of 18 ml per h. Rat, rabbit and dog sera (4—7 ml) were applied to the column. Human serum or plasma contained such a low activity of cyclic nucleotide phosphodiesterase that ammonium sulfate fractionation was carried out in order to obtain the preparation containing the highest activity as follow; 24.5 ml of $(NH_4)_2SO_4$ saturated solution was added to 30 ml of human serum or plasma (45% saturation), stirring for 30 min at 0° C, and centrifuged. The pecipitate was dissolved with 7 ml of 50 mM Tris · HCl (pH 7.5) containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Above 90% of original activity was recovered in the precipitate. The solution was applied to Sepharose 6B column. The column were calibrated with Blue Dextran 2000, catalase, aldolase and bovine serum albumin.

Results

Phosphodiesterase activities in the sera of the various animals

Cyclic nucleotide phosphodiesterase activities in human, dog, rabbit and rat sera were investigated using a low substrate concentration (0.4 μM). The

TABLE I

CYCLIC AMP AND CYCLIC GMP HYDROLYSIS BY HUMAN, DOG, RABBIT AND RAT SERA

The activity was measured using 0.4 μM cyclic AMP or cyclic GMP as the substrate in the presence of 5 mM $MgCl_2$ at pH 8.0. Values are mean ± S.E. of four experiments.

Species	Activity (pmol/min/ml)		
	Cyclic AMP	Cyclic GMP	G/A*
Human	1.80 ± 0.20	3.78 ± 0.40	2.1
Dog	22.1 ± 5.0	40.0 ± 7.6	1.8
Rabbit	52.3 ± 5.3	242.6 ± 30.4	4.6
Rat	208.6 ± 47.4	2465.3 ± 858.9	11.8

* Cyclic GMP hydrolytic activity/cyclic AMP hydrolytic activity.

highest phosphodiesterase activity was found in rat serum and the lowest in human serum (Table I). All preparations of phosphodiesterase from sera were stored at −20° C and the effect of storage in −20° C freezer on cyclic AMP and cyclic GMP hydrolytic activity in sera was examined. When sera were stored at −20° C without freeze-thawing, appreciable loss of both cyclic AMP and cyclic GMP hydrolytic activity was not observed at least within 2 weeks. Moreover, identical elution profiles of Sepharose 6B column chromatography of the sera from four species are obtained from both each fresh and stored serum (−20° C, 2 weeks). The activity in rat serum was several hundred-fold higher than that in human serum. More rapid hydrolysis of cyclic GMP than cyclic AMP was observed in all sera tested. Rat serum cyclic GMP phosphodiesterase was 12-fold higher than its cyclic AMP phosphodiesterase (Table I).

Conditions for optimal activity

Both cyclic AMP and cyclic GMP phosphodiesterase activities in the sera of human, dog, rabbit and rat were dependent upon Mg^{2+}. The dependence of those enzymes on Mg^{2+}, when Tris · HCl (pH 8.0) was employed as buffer, is shown in Fig. 1. Optimal concentration of Mg^{2+} was about 5 mM for human, dog and rat serum phosphodiesterases and rabbit cyclic GMP phosphodiesterase, but it was about 50 mM for rabbit serum cyclic AMP phosphodiesterase. Therefore 5 mM Mg^{2+} was employed in usual assay except in the assay of rabbit serum cyclic AMP phosphodiesterase. Rabbit serum cyclic AMP phosphodiesterase was assayed in the presence of 50 mM Mg^{2+}.

pH Optima of both cyclic AMP and cyclic GMP phosphodiesterase were similar from various species when Tris-maleate and Tris · HCl buffer were employed. Since pH optima of all serum phosphodiesterases tested were found to range from pH 7.5 to pH 8.5, the assay for serum phosphodiesterase was performed at pH 8.0.

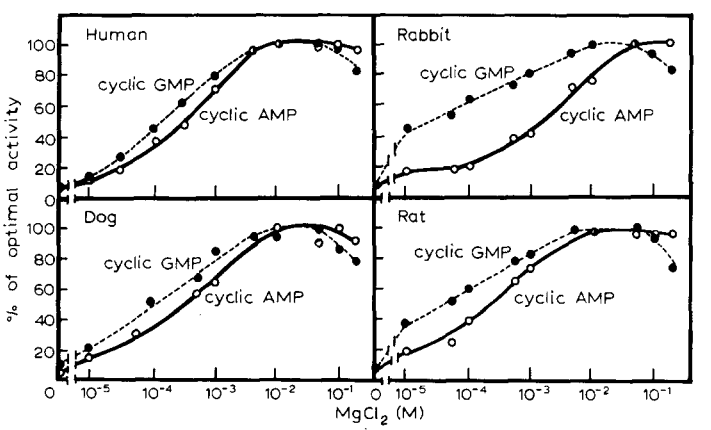

Fig. 1. Effect of $MgCl_2$ on serum cyclic AMP and cyclic GMP phosphodiesterases. The sera were dialyzed against 50 mM Tris · HCl buffer (pH 7.5) before assay. Various amounts of $MgCl_2$ indicated in the figure were added to the reaction mixtures at the first stage of assay. At the second stage of assay $MgCl_2$ was added to a final concentration of 10^{-2} M for the nucleotidase reaction. 0.4 μM Cyclic AMP or cyclic GMP was used as substrate. Assay conditions and procedures were described under Materials and Methods.

TABLE II

CYCLIC AMP AND CYCLIC GMP HYDROLYSIS BY SERUM AND PLASMA

Serum and plasma phosphodiesterase activities of human and rat were determined using 0.4 μM of cyclic AMP or cyclic GMP as substrate. Plasma was obtained from citrated fresh blood by centrifugation. Blood was collected in 3.8% sodium citrate (1v/4v blood). Real plasma volume was obtained by calculation. Both serum and plasma were dialyzed against 50 mM Tris · HCl buffer, pH 7.5, containing 1 mM $MgCl_2$ before use. Assay conditions and precedures were described in Materials and Methods.

Species		pmol/min/ml	
		Cyclic AMP hydrolysis	Cyclic GMP hydrolysis
Human	serum	1.80 ± 0.20	3.78 ± 0.40
	plasma	1.63 ± 0.32	2.80 ± 0.42
Rat	serum	208.6 ± 47.4	2465.0 ± 859
	plasma	7.91 ± 1.06	18.4 ± 8.7

Phosphodiesterases in serum and plasma

Cyclic AMP and cyclic GMP phosphodiesterase activities were compared in serum and plasma of human and rat after dialysis of each preparation (Table II). Human serum phosphodiesterase activity was slightly higher than its plasma activity. However, rat serum cyclic AMP and cyclic GMP phosphodiesterase activities were 26- and 134-fold higher than the plasma activities, respectively. Higher serum phosphodiesterase activity was thought to be due to the release of the enzyme from the formed elements of blood during coagulation. This hypothesis was supported by the fact that a different elution profile of serum

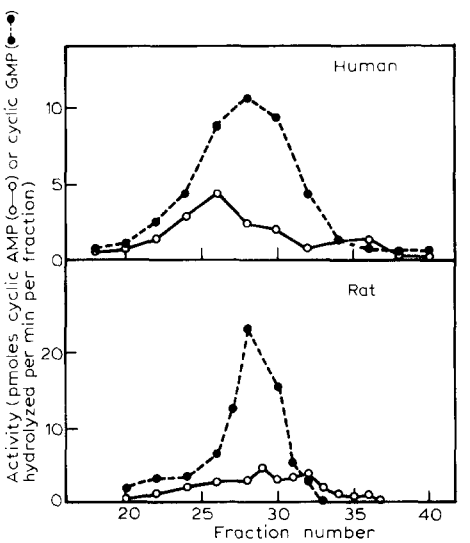

Fig. 2. Gel filtration of human and rat plasma on Sepharose 6B. Rat plasma or the preparation of human plasma obtained from ammonium sulfate fractionation were placed on the column (2.6 × 95 cm) and eluted with 50 mM Tris · HCl (pH 7.5) containing 1M KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The flow rate was 18 ml/h and 10.3 ml/fraction was collected. 0.4 μM Cyclic AMP or cyclic GMP was used as substrate. Assay conditions and procedures were described under Materials and Methods.

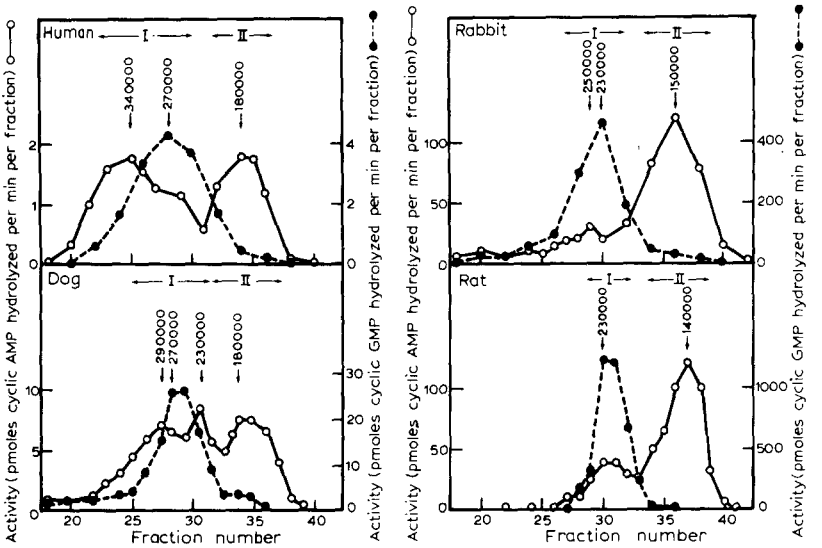

Fig. 3. Gel filtration of the sera of various animals on Sepharose 6B. The sera or the preparation of human serum obtained from ammonium sulfate fractionation were placed on the column (2.6 × 95 cm) and eluted with 50 mM Tris · HCl (pH 7.5) containing 1 M KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The flow rate was 18 ml/h and 10.3 ml/fraction was collected. 0.4 μM cyclic AMP or cyclic GMP was used as substrate. Assay conditions and procedures were described under Materials and Methods. The numbers in the squares indicate the molecular weight of each peak.

phosphodiesterase from that of plasma enzyme was observed (Fig. 2). As shown in Fig 2, human and rat plasma did not contain appreciable amounts of cyclic AMP phosphodiesterase corresponding to Fraction II shown in Fig. 3. Therefore, Fraction II at least of human and rat serum seems to come from the formed elements of blood during coagulation. Most activity of rat serum cyclic GMP phosphodiesterase probably originated from the formed elements of blood, because cyclic GMP hydrolytic activity in rat plasma was very low compared to the activity in the serum. Both rat serum and plasma did not contain any inhibitory or stimulatory substances because when the serum was mixed with the plasma, the phosphodiesterase activity was equal to the sum of individual activities. It is not clear which formed elements are responsible for this increased phosphodiesterase activity of rat serum.

Effect of the activator on serum phosphodiesterase

Sera obtained from various animals were incubated with the activator obtained from bovine brain in the presence and absence of Ca^{2+}. In Table III, the effect of the activator in the presence of 100 μM Ca^{2+} on serum cyclic GMP and cyclic AMP phosphodiesterases was summarized. The activator stimulated rat brain phosphodiesterase but did not significantly stimulate serum phosphodiesterase activity in the presence or absence of Ca^{2+}. We have been unable to obtain activation of rat brain phosphodiesterase activity using material present in the dialyzed sera from four species. These sera did not contain detectable amounts of the protein activator found in other tissues [4—9]. These results agree with the report of Smoake et al. [7].

TABLE III

EFFECT OF THE ACTIVATOR ON SERUM PHOSPHODIESTERASES

Phosphodiesterase activity was determined in the presence or absence of the activator (284 μg protein). Enzyme preparations were dialyzed against 50 mM Tris buffer (pH 7.5) containing 1 mM $MgCl_2$ before use. Assay was carried out in the presence of 100 μM $CaCl_2$ and procedures were described in Materials and Methods. 0.4 μM cyclic AMP or cyclic GMP was used as substrate.

	Cyclic GMP hydrolysis (pmoles/min/ml)		Cyclic AMP hydrolysis (pmoles/min/ml)	
	Without activator	With activator	Without activator	With activator
Rat brain	3036*	6571*	646.7*	1012*
Human serum	4.05	3.94	1.60	1.27
Dog serum	34.0	31.9	20.1	19.9
Rabbit serum	212.3	193.5	49.8	44.8
Rat serum	1985	1959	171.6	163.0

* pmoles/min/mg protein.

Gel filtration of sera of various mammals on Sepharose 6B

The elution profiles of Sepharose 6B column chromatography of the sera from several animals are presented in Fig. 3. The recovery of cyclic AMP and cyclic GMP phosphodiesterase activities after gel filtration was as follows: cyclic AMP phosphodiesterase (rat, 90%; dog, 97%; rabbit, 105%; human, 82%) and cyclic GMP phosphodiesterase (rat, 67%; dog, 75%; rabbit, 61%; human, 58%). The results indicate that all cyclic GMP phosphodiesterases of the four species tested were of one molecular form and all cyclic AMP phosphodiesterases of the four species were of multiple form. Molecular weights of these serum phosphodiesterases are given in Fig. 3. Major activities of rabbit and rat

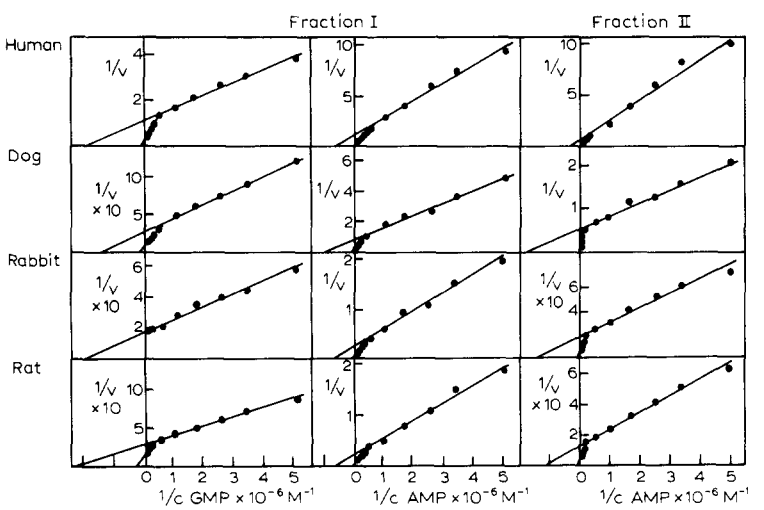

Fig. 4. Lineweaver-Burk plots of cyclic AMP and cyclic GMP hydrolysis by serum preparations prepared chromatographically. Fraction I and II were the preparations indicated in Fig. 3. Substrate concentration used to measure K_m ranged from 0.2 μM to 1.0 mM. Assay procedures are as given in Materials and Methods. Velocities were expressed as pmoles cyclic nucleotide hydrolyzed per minutes at 30° C. Determined values of the Michaelis constants are given in Table IV.

TABLE IV

APPARENT K_m VALUES OF SERUM CYCLIC NUCLEOTIDE PHOSPHODIESTERASES

Species	K_m for cyclic GMP hydrolysis	K_m for cyclic AMP hydrolysis	
	Fraction I	Fraction I	Fraction II
Human	$5.35 \cdot 10^{-7}$ M	$1.61 \cdot 10^{-6}$ M	$3.03 \cdot 10^{-6}$ M
	$9.17 \cdot 10^{-6}$	$5.88 \cdot 10^{-5}$	$3.13 \cdot 10^{-5}$
Dog	$6.13 \cdot 10^{-7}$	$8.13 \cdot 10^{-7}$	$5.68 \cdot 10^{-7}$
	$4.59 \cdot 10^{-6}$	$4.44 \cdot 10^{-5}$	$1.76 \cdot 10^{-4}$
Rabbit	$5.00 \cdot 10^{-7}$	$1.20 \cdot 10^{-6}$	$6.10 \cdot 10^{-7}$
		$7.58 \cdot 10^{-5}$	$7.58 \cdot 10^{-5}$
Rat	$4.15 \cdot 10^{-7}$	$1.85 \cdot 10^{-6}$	$8.93 \cdot 10^{-7}$
	$4.48 \cdot 10^{-6}$	$4.31 \cdot 10^{-5}$	$1.05 \cdot 10^{-4}$

serum cyclic AMP phosphodiesterases eluted under the fraction of lower molecular weights. The fractions that contained both cyclic GMP and cyclic AMP phosphodiesterase activities were referred to as Fraction I and the fractions that contained lower molecular weight cyclic AMP phosphodiesterase were referred to as Fraction II. Fraction I of human and dog serum contained at least two cyclic AMP phosphodiesterases with different molecular weights. Each fraction was pooled, concentrated in collodion bags at reduced pressure at 4° C and used for the experiments.

Michaelis constants

Kinetic studies of cyclic AMP and cyclic GMP hydrolysis by serum phosphodiesterase preparations prepared chromatographically are presented in Fig. 4. The substrate concentration used to measure the K_m in Fig. 4 ranged from 0.2 μM to 1 mM. The apparent K_m values were summarized in Table IV. The apparent K_m values of human and dog serum cyclic AMP phosphodiesterases in Fraction I have limited meaning because these fractions contained at

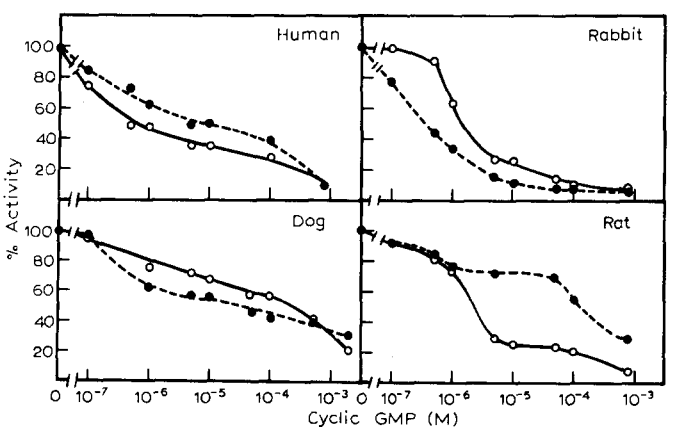

Fig. 5. Effect of cyclic GMP on cyclic AMP hydrolysis by serum preparations of various animals prepared chromatographically. Serum cyclic AMP phosphodiesterase preparations used in this study were Fraction I (○———○) and II (●- - - - -●) of sera of various animals shown in Fig. 3. 0.4 μM of cyclic AMP was used as substrate. Assay conditions and procedures were described under Materials and Methods.

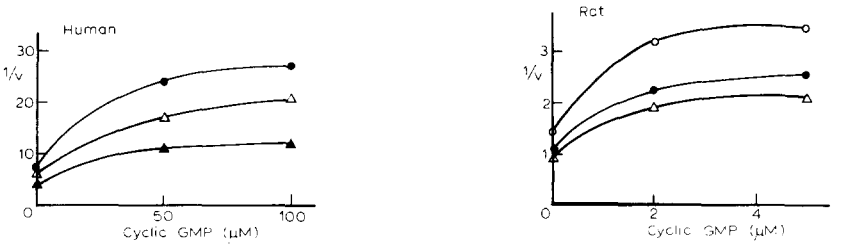

Fig. 6. Dixon plot of cyclic GMP inhibition of cyclic AMP hydrolysis for Fraction I of human and rat sera respectively. Substrate concentrations were 0.2 μM (○), 0.3 μM (●), 0.4 μM (△), 0.6 μM (▲) cyclic AMP.

least two enzymes with different molecular weight as shown in Fig. 3. As in other tissue studies, double reciprocal plots are non-linear for cyclic AMP hydrolysis for all preparations and linear for cyclic GMP hydrolysis by rabbit serum. Non-linearity was also observed in a double reciprocal plot obtained from cyclic GMP phosphodiesterase preparations of human, dog and rat sera.

Inhibition of cyclic AMP phosphodiesterase by cyclic GMP

The effect of cyclic GMP on cyclic AMP hydrolysis by serum phosphodiesterase preparations prepared chromatographically is shown in Fig. 5. Cyclic GMP mostly inhibited cyclic AMP hydrolysis by these preparations when 0.4 μM of cyclic AMP was used as the substrate. As shown in Fig. 5, cyclic AMP phosphodiesterase of rabbit was inhibited most effectively by low concentration of cyclic GMP. Cyclic GMP inhibited these cyclic AMP phosphodiesterases in a hyperbolic fashion as typified in Fig. 6.

Discussion

Serum cyclic nucleotide phosphodiesterase activities vary largely from animal to animal. Rat serum phosphodiesterase activity is the highest among animals examined and several hundred-fold higher than human serum activity. These differences of activity do not seem to be due to the difference of the stability of each serum activity. All serum phosphodiesterase activities of the four species tested are dependent on Mg^{2+} and have same range of pH optimum (7.5—8.5) and same order of apparent K_m values. These phosphodiesterases are not stimulated by protein activator prepared from bovine brain. All sera examined do not contain detectable amounts of protein activator. These sera do not contain any inhibitory substances because when the serum of one animal and the serum of another animal are mixed, the phosphodiesterase activity was equal to the sum of individual activity (unpublished observation). Consequently the marked difference of activity among species is not due to a difference of the amount of activator or inhibitors, but probably due to a difference in the amounts of enzyme contained. Rat serum had markedly higher phosphodiesterase activity than its plasma, but phosphodiesterase activity in human serum was not markedly different from that of the plasma. It is likely that the marked difference of phosphodiesterase activity among sera of several animals was due to the difference of the amounts of enzyme released from the formed elements of blood during coagulation. Cyclic GMP phosphodiesterase was released more

effectively from the formed elements of blood into serum than cyclic AMP phosphodiesterase, because serum cyclic GMP phosphodiesterase activity was higher than its cyclic AMP phosphodiesterase, whereas plasma cyclic GMP hydrolytic activity was similar to its cyclic AMP hydrolytic activity.

The presence of protein activator of phosphodiesterase in various mammalian tissues was reported from several different laboratories [4—9]. As shown in the present study the activator was not detected in the sera up to 150 μl. This agrees with the results reported by Smoake et al. [7]. However, there are still possibility that very small amount of activator is present in these sera.

Elution profiles of gel filtration indicate that all cyclic GMP phosphodiesterases of the four species are one molecular form and all cyclic AMP phosphodiesterases are multiple forms. Cyclic GMP inhibits sera cyclic AMP phosphodiesterases in a hyperbolic fashion and this is in agreement with the result obtained from rat liver enzyme [10].

The source of these serum cyclic nucleotide phosphodiesterases is yet unknown, but several tissues are supposed to be the source of these enzymes as follows. The formed elements of blood such as platelets [11] or erythrocytes and blood vessels [12] could be possible sources. Patterson et al. [1] reported that some plasma phosphodiesterase activity is due to destruction of blood elements. The elution profile of rat serum phosphodiesterases through a Sepharose 6B column is comparable to those of brain and kidney phosphodiesterase demonstrated by Thompson and Appleman [13]. Although the serum cyclic nucleotide phosphodiesterase might come from the several tissues discussed above, most of the activity was thought to be derived from the formed elements of blood.

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