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HUMAN BLOOD PLATELET 3′ : 5′-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE.

ISOLATION OF LOW-K_m AND HIGH-K_m PHOSPHODIESTERASE

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Summary

Human blood platelet contained at least three kinetically distinct forms of 3′ : 5′-cyclic nucleotide phosphodiesterase (3′ : 5′-cyclic-AMP 5′-nucleotidohydrolase, EC 3.1.4.17) (F I, F II and F III) which were clearly separated by DEAE-cellulose column chromatography. Although a few properties of the platelet phosphodiesterases such as their substrate affinities and DEAE-cellulose profile resembled somewhat those of the three 3′ : 5′-cyclic nucleotide phosphodiesterase in rat liver reported by Russell et al. [10], there were pronounced differences in some properties between the platelet and the liver enzymes: (1) the platelet enzymes hydrolyzed both cyclic nucleotides and lacked a highly specific cyclic guanosine 3′ : 5′-monophosphate (cyclic GMP) phosphodiesterase and (2) kinetic data of the platelet enzymes indicated that cyclic adenosine 3′ : 5′-monophosphate (cyclic AMP) and cyclic GMP interact with a single catalytic site on the enzyme.

F I was a cyclic nucleotide phosphodiesterase with a high K_m for cyclic AMP and a negatively cooperative low K_m for cyclic GMP. F II hydrolyzed cyclic AMP and cyclic GMP about equally with a high K_m for both substrates. F III was low K_m phosphodiesterase which hydrolyzed cyclic AMP faster than cyclic GMP. Each cyclic nucleotide acted as a competitive inhibitor of the hydrolysis of the other nucleotide by these three fractions with K_i values similar to the K_m values for each nucleotide suggesting that the hydrolysis of both cyclic AMP and cyclic GMP was catalyzed by a single catalytic site on the enzyme. However, cyclic GMP at low concentration (below 10 μM) was an activator of cyclic AMP hydrolysis by F I. Papaverine and EG 626 acted as competitive inhibitors of each fraction with virtually the same K_i value in both assays using either cyclic AMP or cyclic GMP as the substrate. The ratio of cyclic AMP hydrolysis to cyclic GMP hydrolysis by each fraction did not vary significantly after freezing/thawing or heat treatment. These facts also suggest that both

nucleotides were hydrolyzed by the same catalytic site on the enzyme. The differences in apparent K_i values for inhibitors such as cyclic nucleotides, papaverine and EG 626 would indicate that three enzymes were different from each other. Centrifugation in a continuous sucrose gradient revealed sedimentation coefficients F I and II had 8.9 S and F III 4.6 S. The molecular weight of these forms, determined by gel filtration on a Sepharose 6B column, were approx. 240 000 (F I and II) and 180 000 (F III). F III was purified extensively (70-fold) from homogenate, with a recovery of approximately 7%.

Introduction

Since the possibility that there might be more than one form of 3′ : 5′-cyclic nucleotide phosphodiesterase (3′ : 5′-cyclic-AMP 5′-nucleotidohydrolase, EC 3.1.4.17) in rat brain has been first suggested from kinetic data established by Brooker et al. [1], kinetically distinct phosphodiesterases have been separated from a number of tissues [2—9]. Russell et al. [10] have recently shown that rat liver contains at least three active cyclic nucleotide phosphodiesterase fractions which differ in their substrate affinity, specificity and subcellular distribution. The liver, of course, consists of heterogenous cells and therefore it is of interest to investigate whether multiple forms of phosphodiesterase exist also in a tissue of homogenous cell population.

Blood offers one distinct advantage in that different cells are already suspended in the plasma suitable for separation by differential centrifugation, and thus affords an excellent system for the preparation of phosphodiesterase from a homogenous cell population.

There are reports which suggest existence of at least two forms of cyclic AMP phosphodiesterase in human blood platelets [8,11—13]. However evidence that there might be more than one form of cyclic GMP phosphodiesterase in the platelets has never been presented. In order to investigate further the question of multiple forms of cyclic nucleotide phosphodiesterase in human blood platelets, chromatographic, sucrose density gradient and kinetic studies have been undertaken. This investigation shows that human blood platelets contain at least three active cyclic nucleotide phosphodiesterases which hydrolyze both nucleotides with a different substrate affinity for each other.

Materials and Methods

Materials. Cyclic [^{3}H]adenosine 3′ : 5′-monophosphate (specific activity 33.2 Ci per mmol) and cyclic [^{3}H]guanosine 3′ : 5′-monophosphate (3.46 Ci per mmol) were purchased from New England Nuclear. Unlabeled cyclic AMP, cyclic GMP and snake venom (*Crotalus atrox*) were purchased from Sigma Chemical Co. EG 626 is a gift from Dr. T. Shimamoto, Department of Internal Medicine, Tokyo Ikashika University, also one of the phthalazone derivatives. These are potent inhibitors of the cyclic nucleotide phosphodiesterase [14,15]. Cation exchange resin (BioRad AG 50-X4, 200—400 mesh) was extensively washed with 0.5 M NaOH, 0.5 M HCl and deionized water to a final pH of 5.0. Agarose gel (Pharmacia, Sepharose 6B) and DEAE-cellulose (Whatman, DE 52)

were equilibrated with the column buffer prior to use. All other chemicals were reagent grade or the best commercially available.

Phosphodiesterase preparation. Human platelet phosphodiesterase was prepared as previously described [14]. Careful microscopic checks showed that platelets were virtually free from erythrocyte and leukocyte contamination. Platelet-rich plasma was prepared by low-speed centrifugation of freshly obtained citrated whole blood. Platelets were isolated from the platelet-rich plasma by centrifugation. Platelet homogenates were prepared in 50 mM Tris · HCl buffer (pH 7.4) containing 1 mM $MgCl_2$ using homogenizer tubes with Teflon pestles. The homogenized solutions were sonicated (30 s per ml) and soluble phosphodiesterase preparation was obtained from the sonicated homogenates by centrifugation at 105 000 × *g* for 60 min. This preparation, referred to as the sonicated extract, was used for kinetic and chromatographic studies.

Phosphodiesterase assay. The two-step assay for enzymatic activity is similar to that previously described [16]: 5′-[^{3}H] AMP or 5′-[^{3}H] GMP formed by the phosphodiesterase is converted to [^{3}H] adenosine or [^{3}H] guanosine by the action of nucleotidase and the product which is isolated by cation exchange resin is counted in a liquid scintillation counter. An appropriate dilution of enzyme is incubated in 50 mM Tris · HCl, pH 8.0, 5 mM $MgCl_2$, 50 μg of bovine serum albumin, 0.4 μM cyclic [^{3}H] AMP or 0.4 μM cyclic [^{3}H] GMP, in a total volume of 0.5 ml. When higher concentrations of substrate are required, indicated amounts of unlabeled cyclic nucleotide are included. After 20 min at 30°C, the reaction is terminated by boiling for 5 min. Next 0.05 ml of snake venom (1 mg per ml in H_2O) is added with an additional incubation for 10 min at 30°C. Then 1.0 ml of water is added and the mixture applied to a small ion exchange resin column (AG 50-X4, 0.8 × 2 cm column). Adenosine or guanosine was eluted with 1.5 ml of 3 M NH_4OH after washing the column with 15 ml of distilled water. Recovery of adenosine or guanosine from the column is 95%.

Assay of the activator. Assay of the activator was made according to the method of Kakiuchi et al. [17] based on the ability of the activator to enhance the activity of diluted (105 000 × *g*) supernatant fluid from rat brain homogenate in the presence of 50 μM Ca^{2+}. The amount of the activator that doubled the enzyme activity in the standard system was defined as 10 units.

Identification of the product

The incubated reaction mixtures, their AG 50 column effluents or eluates were evaporated, and the residues of each preparation were each dissolved in 25 μl of distilled water. 5 μl of this solution was applied to a cellulose thin layer and separated by chromatography as described previously [16]. Three different solvent systems such as ethanol/*n*-butanol/88% formic acid/water (60 : 20 : 5 : 15, v/v/v/v), *n*-butanol/88% formic acid/water (77 : 10 : 13) and 2-propanol/ammonia/water (70 : 10 : 20) were used, with ascending procedure. Cyclic AMP, cyclic GMP and nucleoside (adenosine, inosine, guanosine and xanthosine) spots detected under an ultraviolet lamp were compared with the spots on the plate (1 cm^2 each). These were collected and transferred into counting vials with 10 ml each of scintillation fluid (120 g naphthalene, 0.4 g POPOP, 8 g PPO, 200 ml methanol, 40 ml ethylene glycol, 2 l dioxane). Blank tubes contained heat-denatured enzyme instead of native enzyme.

Chromatography. Gel filtration on Sepharose 6B was performed by the method of Hidaka et al. [18] in columns (2.6 × 95 cm) at flow rates of 18 ml per h using 50 mM Tris · HCl (pH 7.5) buffer containing 1 M KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The column was calibrated with Blue Dextran 2000, catalase, aldolase and bovine serum albumin. DEAE-cellulose chromatography was performed by the method of Russell et al. [10] in columns (1.5 × 20 cm) with bed volumes of 35 ml. The buffer was 50 mM Tris/acetate, pH 6.0, containing 3.75 mM 2-mercaptoethanol. The enzyme preparation was applied to the column, followed by elution with several column volumes of buffer. The initial wash contained no detectable phosphodiesterase activity. A linear gradient from 0 to 1 M sodium acetate was then applied with a flow rate of 0.5 ml per min and a total gradient volume of 300 ml. Hydroxyapatite was prepared by the method of Levin [19] and chromatography was performed in columns (1 × 25 cm) with bed volumes of 20 ml. The enzyme preparation was applied to the column, followed by elution with several column volumes of 10 mM potassium phosphate buffer, pH 7.5. A linear gradient from 10 to 200 mM potassium phosphate buffer, pH 7.5 was applied with flow rates of 0.3 ml per min and a total gradient volume of 120 ml. Fractions were pooled and concentrated by ultrafiltration using collodion bags. The concentration by ultrafiltration was performed only when it was necessary, because a large loss (approx. 50%) of the activity was observed, probably due to inactivation during concentration by ultrafiltration. The enzyme activity was more labile when the preparation was diluted. Then, purified enzyme preparation was usually stored at 4° C in the presence of 0.1 mg/ml of bovine serum albumin. When it was stored in this condition, 50% of the activity of purified preparation (F I, F II, F III) remained after 3 weeks.

Sucrose density gradient study. Sucrose density gradient centrifugation with a continuous gradient from 5% to 20% sucrose (w/v) was performed in tubes (1.3 × 5 cm) also containing 50 mM Tris · HCl, pH 7.5, in a total volume of 5.0 ml. The appropriate enzyme, in a volume of 0.1 ml, was applied to the top of gradient solution and centrifuged at 100 000 × g for 16 h in a Hitachi 65P centrifuge with an RPS-50 rotor. After centrifugation, 0.2-ml fractions were collected from bottom of the tubes and the activity of the phosphodiesterase was determined. Bovine serum albumin and catalase were usually centrifuged as standards in each experiment.

Results

Validation of assay and identification of product

The specificity of this assay for human blood platelet cyclic nucleotide phosphodiesterase in the presence of enough nucleotidase was examined by submitting the incubated reaction mixtures, their AG 50 column effluents and eluates to cellulose thin layer chromatography. The three different solvent systems used were as described previously [16]. Two radioactive spots in the incubated reaction mixtures agreed with those for cyclic AMP and adenosine or cyclic GMP and guanosine in three different solvent systems. Moreover, these spots were not separated from those of the standard nucleotides or nucleosides, when the two were mixed together before thin layer chromatography, in any

solvent system. Only one radioactive spot in column effluent agreed with each authentic nucleotide spot (cyclic AMP or cyclic GMP) and only one radioactive spot in the eluate agreed with each authentic nucleoside (adenosine or guanosine). Under the conditions of our assay using human blood platelets, the reaction product was confirmed to be almost entirely adenosine or guanosine. Linearity of enzyme reaction was then examined. The hydrolysis of cyclic AMP or cyclic GMP by human blood platelets as a function of the time of incubation is shown in Fig. 1. The rate of the hydrolysis of cyclic AMP or cyclic GMP by the platelet enzyme was linear for at least 50 min in the presence of 50 μg of bovine serum albumin. Bovine globulin or catalase had similar effect to that of bovine serum albumin. Incubation of the enzyme without bovine serum albumin resulted in apparent reduction of the activity as a function of incubation time as shown in Fig. 1. Accordingly, the apparent stimulation of low-K_m enzyme activity by bovine serum albumin as demonstrated in Fig. 1 seems to be due to protection from inactivation of the enzyme by dilution. A similar effect of bovine serum albumin on the phosphodiesterase of other tissues has been also reported [5]. This stimulation was not observed in the assay of high-K_m enzyme because the amount of enzyme protein used for high-K_m phosphodiesterase assay was 10 times more than the amount employed for low-K_m enzyme assay (1—10 μg protein).

DEAE-cellulose and Sepharose 6B chromatographic separation

90% of the cyclic AMP hydrolytic activity and 80% of the cyclic GMP hydrolytic activity of human blood platelets was found in the (105 000 × *g*)-supernatant fraction after sonication. Sonicated extracts of human blood platelet when chromatographed on DEAE-cellulose yielded three active cyclic nucleotide phosphodiesterase fractions designated as F I, F II and F III in Fig. 2. The protein activator of the phosphodiesterase was also eluted in the fractions

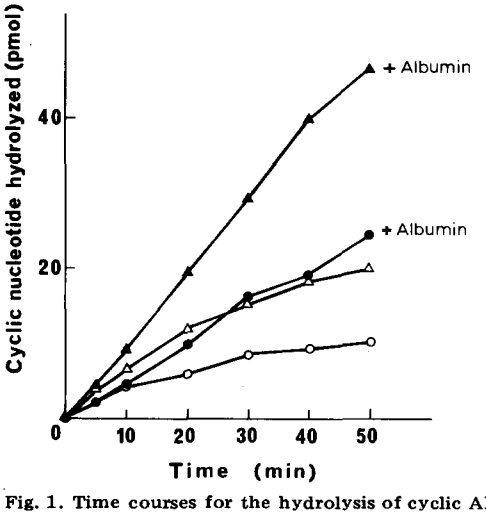

Fig. 1. Time courses for the hydrolysis of cyclic AMP or cyclic GMP by human blood platelets (sonicated extract) with or without 50 μg of bovine serum albumin. ○,●: cyclic AMP hydrolysis, △,▲: cyclic GMP hydrolysis. Substrate concentration was 0.4 μM (200 pmol per assay). Conversion of labeled substrate was below 20%.

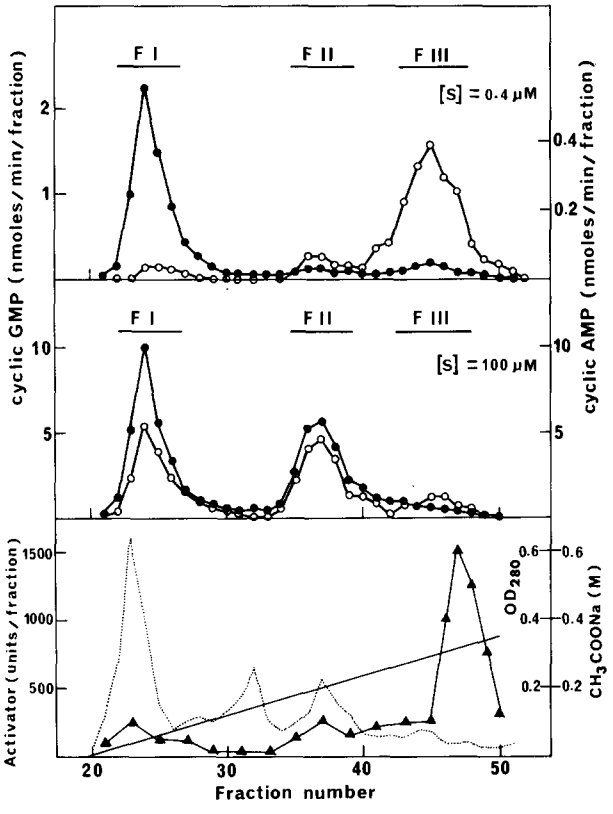

Fig. 2. DEAE-cellulose profile of platelet phosphodiesterase from a sonicated extract. The gradient was from fraction 20 to 75. Aliquots, 0.2 ml for high substrate (100 μM) or 0.01 ml for low substrate level (0.4 μM), were assayed directly. ○———○, cyclic AMP hydrolysis; ●———●, cyclic GMP hydrolysis. Aliquots, 0.05 ml, were used after boiling for 5 min for the estimation of the activator. ▲———▲, activator; - - - - - -, absorbance at 280 nm; ———, sodium acetate gradient.

as shown in Fig. 2. This DEAE-cellulose profile of human platelet phosphodiesterase from sonicated extract was similar to that of rat liver phosphodiesterase from sonicated extract [10]. All fractions hydrolyzed both cyclic AMP and cyclic GMP but there was no highly specific cyclic GMP phosphodiesterase as in rat liver [10]. F I was high-K_m (500 μM) cyclic AMP phosphodiesterase and low-K_m (0.5 μM) negatively cooperative cyclic GMP phosphodiesterase. F II was high-K_m phosphodiesterase and was comparable to the rat liver phosphodiesterase designated as D II [10]. The apparent K_m for both nucleotides was the same (40 μM), and was similar to the K_m value of D II. The elution characteristics of these major fractions on DEAE-cellulose columns and their substrate affinities were highly reproducible. When F I, F II or F III was rechromatographed individually after isolation, it was eluted in exactly the same fraction as the original. The sonicated extracts apparently retained the sum of the kinetic properties of the individual enzymes. When each fraction was submitted for gel filtration on Sepharose 6B individually, F I and II were eluted in the fraction corresponding to a molecular weight of 240 000, and F III was eluted in the later fraction corresponding to a molecular weight of 180 000.

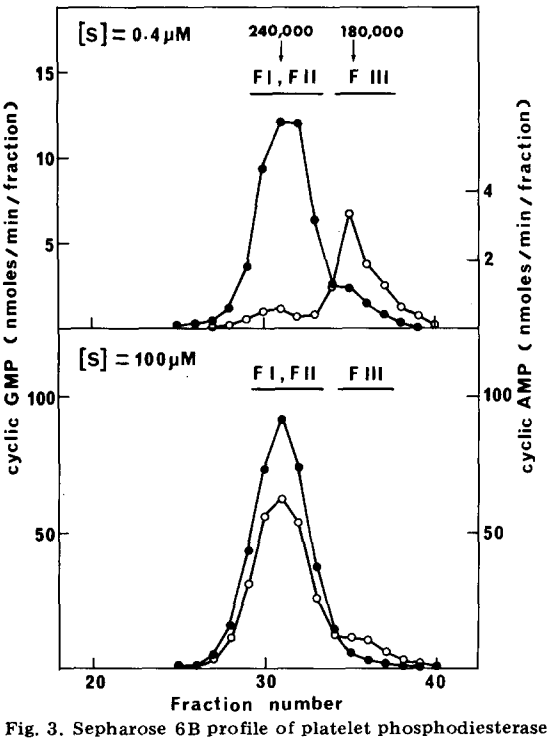

Fig. 3. Sepharose 6B profile of platelet phosphodiesterase from a sonicated extract. Gel filtration was performed on a column (2.6 X 95 cm) at a flow rate of 18 ml per hour using 50 mM Tris · HCl buffer pH 7.5 containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 M KCl. 10 ml fractions were collected. When F I, F II and F III were applied to the same column separately, cyclic nucleotide phosphodiesterase in F I, II and F III was eluted in the fraction indicated in the figure. The phosphodiesterase activity was determined at low (0.4 μM) and high (100 μM) substrate concentrations. o———o, cyclic AMP hydrolysis; •———•, cyclic GMP hydrolysis.

When sonicated extracts were applied to the same Sepharose 6B column, the elution profile was virtually identical to the sum of the profiles of the individual enzymes (Fig. 3). The extracts when centrifuged in a sucrose density yielded two active cyclic nucleotide phosphodiesterase, of sedimentation coefficient values 8.9 and 4.6 S respectively (Fig. 4). F I and II were found in the heavier fraction (8.9 S) and F III in the lighter fraction (4.6 S) when the individual enzyme was centrifuged separately under the same conditions.

Properties of F I

This enzyme eluted from DEAE-cellulose at about 0.08 M sodium acetate and contained about 35% cyclic GMP phosphodiesterase activity at low substrate level (0.4 μM) in the homogenate, about 25% cyclic GMP hydrolytic activity at the higher substrate level (100 μM) and about 20% cyclic AMP hydrolytic activity at the higher substrate concentration (100 μM) in the homogenate. Cyclic AMP hydrolytic activity in F I displayed normal Michaelis-Menten kinetics with a K_m of 500 μM but the hydrolysis of cyclic GMP by F I displayed abnormal Michaelis-Menten kinetics (negatively cooperative), with a K_m of 0.5 μM. Cyclic competitively inhibited the hydrolysis of cyclic GMP by

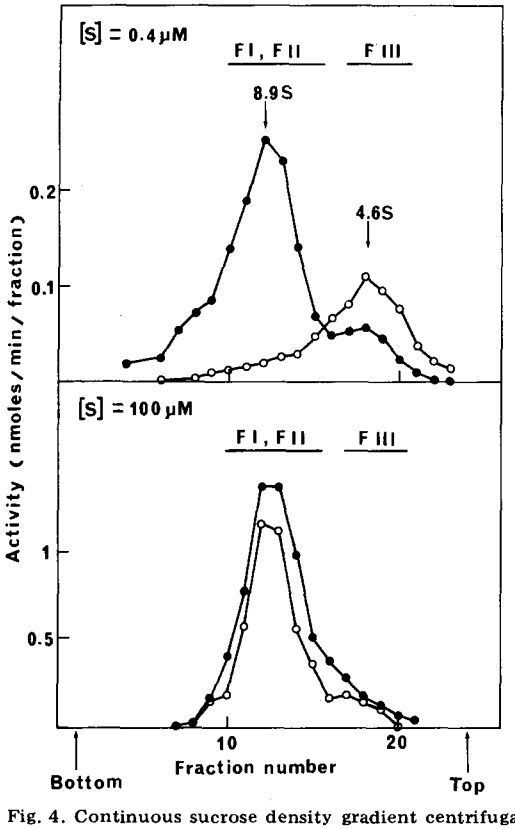

Fig. 4. Continuous sucrose density gradient centrifugation studies of a sonicated extract of human blood platelets. A linear gradient from 5% to 20% sucrose containing 50 mM Tris · HCl, pH 7.5, was used and centrifugation at 100 000 × *g* was performed for 16 h. Aliquots, 10 μl for low substrate (0.4 μM) and 100 μl for high substrate (100 μM), were assayed directly. The activities of F I, F II and F III for both nucleotides eluted at the fractions indicated in the figure when individual fractions were centrifuged separately under the same conditions. ○———○, cyclic AMP hydrolysis; ●———●, cyclic GMP hydrolysis.

this enzyme. For an inhibitor used as an alternative substrate by the same enzyme, one would predict that the apparent K_m would equal the apparent K_i. The apparent K_i for cyclic AMP was found to be 490 μM, virtually the same as the apparent K_m for cyclic AMP hydrolytic activity of this enzyme. Apparent K_i for papaverine or EG 626 was found to be virtually the same in both assays, employing either cyclic AMP or cyclic GMP as the substrate (Table I). Therefore cyclic AMP and cyclic GMP would be hydrolyzed by the same catalytic site on the enzyme in F I. However, cyclic GMP activated the hydrolysis of cyclic AMP by this enzyme at low concentration (below 10 μM) and inhibited cyclic AMP hydrolysis at higher concentration (above 50 μM) (Fig. 5).

Properties of F II

The active fraction which eluted from DEAE-cellulose at about 0.2 M sodium acetate hydrolyzed both nucleotides, exhibited normal kinetics for both substrates, and contained about 20% of both cyclic AMP and cyclic GMP phosphodiesterase activity at high substrate level (100 μM) in the homogenate. The

TABLE I

PROPERTIES OF HUMAN BLOOD PLATELET PHOSPHODIESTERASE

Inhibitor		F I		F II		F III	
		Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
	K_m	500 μM	0.5 μM	40 μM	40 μM	0.37 μM	0.15 μM
Cyclic AMP	K_i	—	490	—	21	—	0.25
Cyclic GMP	K_i	—	—	40	—	0.16	—
Papaverine	K_i	0.72	0.86	2.1	1.6	0.27	0.16
EG 626	K_i	43	37	9.5	6.3	1.0	1.3

activity in this fraction had the same affinity for cyclic AMP and cyclic GMP (Table I). A higher concentration (above 10 μM) of each of these nucleotides competitively inhibited the hydrolysis of the other, with K_i values similar to the K_m values for each nucleotide (Table I). The apparent K_i for papaverine as well as EG 626 was virtually the same using either cyclic AMP or cyclic GMP as the substrate (Table I). These results would suggest that the same enzyme in F II hydrolyzed both nucleotides.

Properties of F III

The cyclic nucleotide phosphodiesterase which contained 55% cyclic AMP hydrolytic activity at low substrate level in the homogenate had a low K_m value for both cyclic AMP and cyclic GMP (Table I). F III exhibited normal kinetic behavior for both substrates. F III was purified by column chromatography on Sepharose 6B, and then an hydroxyapatite and DEAE-cellulose. The specific activity was 10 432 pmol/min per mg, a 70-fold purification for cyclic AMP hydrolytic activity from the homogenate. Purification of F III is summarized in Table II. Purified preparation of F III was not stable in diluted solution, but retained full activity for a week stored at 4°C when 100 μg per ml of

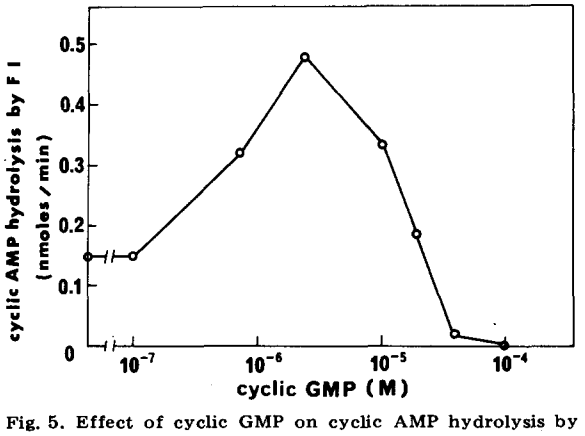

Fig. 5. Effect of cyclic GMP on cyclic AMP hydrolysis by F I. The concentration of substrate (cyclic AMP) was 100 μM.

TABLE II

PURIFICATION OF F III FROM HUMAN BLOOD PLATELET

The activity of phosphodiesterase was determined at low substrate concentration (0.4 μM).

	Total activity pmol/min		Specific activity pmol/min/mg protein		A/G *
	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP	
Homogenate	5 490	31 600	149	859	0.17
Sonicated extract	4 944	25 214	191	974	0.20
Sepharose 6B	2 076	945	365	166	2.2
Hydroxyapatite	1 025	446	1 661	723	2.3
DEAE-cellulose	386	161	10 432	4 351	2.4

* The ratio of cyclic AMP to cyclic GMP hydrolysis.

bovine serum albumin was added. A low concentration of each of these nucleotides competitively inhibited the hydrolysis of the other with K_i values similar to the K_m values for each nucleotide (Table I). F III was inhibited the most effectively by papaverine or EG 626 among these three phosphodiesterases. The apparent K_i value for papaverine or EG 626 did not vary in either assay when either cyclic AMP or cyclic GMP was employed as the substrate (Table I). This would suggest that the same enzyme hydrolyzed both nucleotides.

The ratio of the hydrolysis of cyclic AMP to that of cyclic GMP by each fraction did not vary after heat or freezing/thawing treatment (Table III). These results also indicated that the hydrolysis of both nucleotides was catalyzed by the same catalytic site on the enzyme in each fraction. The loss of enzyme activities by these treatments was markedly different among F I, F II and F III. The differential stability of the various isolated phosphodiesterase peaks of rat brain has also been reported [20,21]. F I activity was removed easily by freezing/thawing treatment and most of the activity of F III was removed by heat treatment (Table III). F II activity was relatively resistant to these treatments. These facts would suggest that F I, F II and F III were different enzymes from each other.

TABLE III

EFFECT OF FREEZING/THAWING AND HEAT TREATMENT ON HUMAN PLATELET PHOSPHODIESTERASE

The substrate concentrations for determination of cyclic AMP hydrolysis by F I and F II and cyclic GMP hydrolysis by F II were 100 μM and of cyclic GMP hydrolysis by F I and F III and cyclic AMP hydrolysis by F III were 0.4 μM.

Treatments		F I		F II		F III	
		Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
Control		100	100	100	100	100	100
Heat at 30°C	10 min	78	80	90	95	54	50
	30 min	63	71	68	75	9	11
Freeze thaw	Once	35	34	72	74	88	84
	3 times	2	8	52	58	65	62

Discussion

Studies on platelet phosphodiesterase would have the distinct advantage that the enzyme obtained is derived from virtually homogenous cell. Studies using partially purified preparation from human blood platelets indicate that at least three active cyclic nucleotide phosphodiesterases (F I, F II and F III) are present which are kinetically distinct from each other. There is a pronounced difference in the affinities for substrates and inhibitors between F I, F II and F III. This is most easily explained by considering that three different cyclic nucleotide phosphodiesterases are present in the sonicated (105 000 × *g*)-supernatant of human blood platelets. It is possible that these differences result from the modification of the phosphodiesterase during preparation. Approximately 60—70% of 3′ : 5′-cyclic nucleotide phosphodiesterase activity of human blood platelets was found in (105 000 × *g*)-supernatant fraction without sonication. This non-sonicated extract of human blood platelets when chromatographed on DEAE-cellulose yields the three active cyclic nucleotide phosphodiesterase fractions which are exactly like those in sonicated extracts.

The allosteric activation of cyclic AMP hydrolysis by micromolar amounts of cyclic GMP has been reported by other workers [6,10,22]. Cyclic AMP hydrolysis by F I is also activated by micromolar amounts of cyclic GMP and this activation of cyclic AMP hydrolysis by cyclic GMP is not observed with F II or F III. The same phenomenon has been reported in D II fraction of the rat liver phosphodiesterase [10]. F I is also a unique enzyme compared to F II and F III because it is the only low-K_m negatively cooperative enzyme in human blood platelets and accounts most of the low-K_m cyclic GMP phosphodiesterase activity found in the homogenate. F III accounts for most of the low-K_m cyclic AMP phosphodiesterase activity found in the homogenate. The three fractions of the platelet phosphodiesterase hydrolyzed both cyclic AMP and cyclic GMP. In particular, the following facts described in this report do suggest that both nucleotides are hydrolyzed by a single catalytic site on the enzyme in each fraction: (1) the apparent competitive inhibitory effect of each nucleotide on the hydrolysis of the other is observed to have a K_i value similar to the K_m value for each nucleotide; (2) virtually identical K_i values for inhibitors are obtained with each fraction when either cyclic AMP or cyclic GMP is employed as the substrate; (3) the ratio of cyclic AMP hydrolysis to cyclic GMP hydrolysis in each fraction does not vary after treatments such as heat and freezing/thawing; (4) cyclic AMP and cyclic GMP hydrolytic activities in F III are purified at the same ratio in each purification step after Sepharose 6B.

The platelets do not seem to contain specific cyclic GMP phosphodiesterase separable on DEAE-cellulose as reported in rat liver [10]. F I was a specific cyclic GMP phosphodiesterase at low substrate concentration but hydrolyzed both cyclic GMP and cyclic AMP at higher substrate level. The high K_m cyclic AMP phosphodiesterase activity of F I was not due to the contamination of F II fraction, because the apparent K_m value of F I for cyclic AMP (500 μM) was markedly different from that of F II (40 μM). However, some enzymatic characteristics of the human blood platelet preparation presented in this communication do agree to those of rat liver preparation [10]. F I, F II and F III fractions of the platelet phosphodiesterase are comparable to the D I, D II and

D III enzymes of rat liver reported [10] in the following points: (1) F I has a higher affinity for cyclic GMP than for cyclic AMP and hydrolyzed mainly cyclic GMP at low substrate levels; (2) F II exhibits low affinity similarly for both cyclic nucleotides; (3) F III hydrolyzes cyclic AMP faster than cyclic GMP with high affinity. The hypothesis as offered by Russell et al. [10] that there are three 3′ : 5′-cyclic nucleotide phosphodiesterases which are a cyclic GMP-, cyclic nucleotide- and cyclic AMP-hydrolyzing enzyme would be applicable, in principle, to human platelet phosphodiesterase if the definition were made properly. However, of course, it would be noticed that there are several differences in properties between the platelet and the liver enzymes.

Compounds which affect the intracellular levels of cyclic AMP have profound effects on platelet aggregation [23,24]. Recently several laboratories have suggested a relation of cyclic AMP to platelet function and have studied the metabolism of this nucleotide in platelets [23,24]. Salzman and Levine [25] offered the hypothesis that platelet aggregation is accompanied by a reduction of the platelet cyclic AMP level and inhibition of aggregation by an increase of platelet cyclic AMP level.

It is interesting and significant from a physiological standpoint that each of these nucleotides acts as a competitive inhibitor of the hydrolysis of the other, and that cyclic GMP, in addition to whatever independent role it may have in cell regulation, is intricately involved in the regulation of cyclic AMP degradation in platelets.

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