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CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE PROPERTIES OF THE ENZYME OF HUMAN BLOOD PLATELETS

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SUMMARY

1. A cyclic 3',5'-nucleotide phosphodiesterase of human blood was mostly localized in the platelets and the leukocytes. The platelets accounted for the majority of the total activity. Most of the activity associated with the erythrocytes could be accounted for by the contamination of leukocytes and platelets. The plasma exhibited no phopshodiesterase activity.

2. After sonication, 80% or more of the phosphodiesterase activity in the platelets remained in a high speed supernatant fluid. The soluble enzyme hydrolyzed cyclic AMP at a rate of 2 to 5 nmoles/mg protein per min at 30°. It also hydrolyzed other cyclic 3',5'-nucleotides, and the relative rates were: cyclic AMP (100), cyclic UMP (67), cyclic TMP (58), cyclic GMP (56), cyclic IMP (38), cyclic CMP (<0.1) and dibutyryl cyclic AMP (0). The hydrolysis of cyclic AMP was inhibited by cyclic GMP, cyclic IMP, and dibutyryl cyclic AMP, but not by cyclic CMP, cyclic TMP, and cyclic UMP.

3. The platelet enzyme required divalent cations for full activity. Mn^{2+}, Mg^{2+}, and Co^{2+} were effective ions. It was inhibited by EDTA, ATP and other nucleoside triphosphates. Although the inhibition by EDTA was explainable by metal chelation, the effect of ATP appeared more complicated. The inhibition by caffeine was of a mixed type.

4. The enzyme exhibited two K_m values, one of $7 \cdot 10^{-4}$ M and another of $7 \cdot 10^{-5}$ M.

INTRODUCTION

The level of cyclic AMP in any tissue is a result of a balance between its rate of synthesis, catalyzed by adenyl cyclase, and that of hydrolysis, catalyzed by cyclic 3',5'-nucleotide phosphodiesterase[1]. The activity of phosphodiesterase in a tissue, is therefore, critical in regulating its level of cyclic AMP. Previous work from this labo-

Abbreviations: cyclic AMP, adenosine 3',5'-monophosphate: cyclic CMP, cyclic GMP, cyclic IMP, cyclic TMP, cyclic UMP: cyclic phosphates of their corresponding nucleosides.

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ratory has described the subcellular localization and regulatory properties of brain phosphodiesterase[2]. One advantage of studying the brain enzyme is that the nervous tissue offers high phosphodiesterase activity. A disadvantage of this tissue is its cellular heterogeneity, which makes the preparation of phosphodiesterase from one type of cell technically difficult. Were there more than one form of phosphodiesterase in the different tissues of the brain, it would be difficult to identify their cellular origin and properties.

The 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 homogeneous cell population. The present studies were initiated to evaluate whether phosphodiesterase derived from a homogeneous cell type, the human blood platelets, exhibits characteristics similar to an enzyme of a heterogeneous tissue, the brain. The platelet was chosen because bulk quantities of clean preparation could be obtained with simple differential centrifugation. It is of interest to note that several neurohormones found in the brain are also present in the platelets. Results presented in this paper indicate that although the platelet enzyme shares many of the properties of the brain enzyme, it exhibits some characteristics different from the enzyme of the central nervous system.

MATERIALS AND METHODS

Chemicals and reagents

Snake venom (*Crotalus atrox*) was obtained in a lyophilized form from Sigma Chemical Co. Nucleotides and nucleosides were purchased from Sigma, Schwarz BioResearch Co., Boerhinger-Manheim Corp., or CalBiochem. Myokinase and pyruvate kinase were products of Boerhinger-Mannheim Corp., and lactate dehydrogenase was from Worthington Biochemical Corp. All reagents were of analytical grade and the solutions were adjusted to pH 7 with dilute HCl or NaOH. The final concentration of nucleotides was determined spectrophotometrically using appropriate extinction coefficients from information supplied by the manufacturers.

Preparation of phosphodiesterase from various components of human blood

Fresh bank blood collected in acid–citrate–dextrose bag was fractionated by differential centrifugation into plasma, erythrocytes, and platelets according to WOLFE AND SCHULMAN[3]. Leukocytes were isolated with a Dextran solution according to FALLON *et al.*[4]. The platelet preparation contained 0–10 leukocytes or erythrocytes per $1 \cdot 10^5$ platelets. The number of platelets was adjusted to $1 \cdot 10^9$ per ml, and the suspension was sonicated for 10 sec using a Sonifier Cell Disrupter, Model W140D (Ultrasonics, Inc., Long Island, N.Y.) at a setting of 40 W. The treatment was repeated twice with a 3-min interval between each sonication. The sonicated solution was dialyzed exhaustively against 20 mM Tris–HCl, pH 7.5. The dialyzed solution was centrifuged at $100\,000 \times g$ for 60 min and the supernatant fluid was used. The erythrocytes and leukocytes were sonicated and dialyzed similarly as the platelets, and the whole sonicated preparations were used. The plasma was dialyzed and used without further handling. All procedures were performed at 0–4° unless otherwise stated.

Assay of phosphodiesterase

Three procedures were used, depending on the experimental conditions. Assay I measured P_i, which was released from AMP, the product of the reaction, by a nucleotidase of snake venom[5]. Briefly, the reaction mixture of 0.5 ml containing 40 mM Tris–HCl (pH 8.0), 1 mM $MnCl_2$, 2 mM cyclic AMP and an appropriate amount of protein was incubated for various times at 30°. The incubation time varied from 45 to 210 min, depending on the experimental conditions. The reaction was stopped in a boiling water bath. After thermal equilibrium to 30°, 0.1 mg of snake venom was added and the incubation was continued for another 20 min to convert AMP, the product of phosphodiesterase, into adenosine and P_i. The reaction was stopped by 55% trichloroacetic acid or 0.4 M ammonium acetate, pH 4. Denatured protein was removed by centrifugation and the supernatant fluid was assayed for P_i according to FISKE AND SUBBAROW[6], or LOWRY AND LOPEZ[7]. It was found that when a reaction mixture contained 100 μg or more protein, the sedimented protein removed some P_i color which was proportional to the amount of protein in the incubation system. Therefore, a standard curve for the determination of P_i was made in the presence of a known amount of the enzyme protein to correct for this loss of color.

Assay II determined AMP using the coupled reaction of myokinase, pyruvate kinase, and lactate dehydrogenase[8]. The reaction was terminated by the addition of 0.05 ml of 3 M $HClO_4$, and the incubation mixture was then neutralized with solid $KHCO_3$. Insoluble $KClO_4$ and denatured proteins were removed by centrifugation at 4° and an aliquot of the supernatant fluid was assayed for AMP.

Assay III used cyclic [8-^{14}C]AMP (specific activity 0.1–0.5 mC/mmole). The reaction mixture was reduced to 0.2 ml, and the conditions were otherwise identical to that of Assay II. At the end of the reaction, carrier AMP and cyclic AMP were added to the solution and an aliquot of the supernatant fluid was put on Whatman No. 1 paper for descending chromatography[9]. AMP spots were located by ultraviolet illumination. They were removed and counted in a liquid scintillation counter. The scintillation fluid contained 3 g PPO and 100 mg POPOP per 1 l toluene.

All assays were done in duplicate, under conditions such that the rate was linear with the time of incubation and the concentration of protein. The data had been corrected for a control containing no enzyme or a heat inactivated enzyme. Protein was measured according to GORNALL *et al.*[10]. using bovine serum albumin as a standard.

RESULTS

Distribution of phosphodiesterase in various components of human blood

Phosphodiesterase in the human blood was mainly localized in the platelets and leukocytes. Under the conditions described in Table I, $1 \cdot 10^7$ platelets hydrolyzed 0.12 nmoles of cyclic AMP per min (or 1.2 nmoles per $1 \cdot 10^8$ platelets per min) whereas $1 \cdot 10^7$ leukocytes contaminated with $1.0 \cdot 10^7$ platelets hydrolyzed 0.5 nmoles. This gave a rate of hydrolysis equivalent to 0.38 nmoles per $1 \cdot 10^7$ white cells per min, after correcting the activity attributable to the platelets. About 70% of the enzyme activity in the erythrocyte fraction or whole blood could be accounted for by the presence of platelets and leukocytes. Since the platelets far outnumber the leukocytes and the

TABLE I

DISTRIBUTION OF PHOSPHODIESTERASE IN THE VARIOUS CELLULAR COMPONENTS OF HUMAN BLOOD

Various components of blood were prepared as described in the text. 0.1 ml of each fraction was determined for phosphodiesterase activity according to Assay III.

Cell fraction	*Cell counts per 0.1 ml*		*Activity (nmoles/0.1 ml per min)*
Whole blood	Platelets:	$2.7 \cdot 10^7$	0.45
	Leukocytes:	$6.0 \cdot 10^5$	
	Erythrocytes:	$5.0 \cdot 10^8$	
Erythrocytes	Platelets	$6.0 \cdot 10^6$	0.14
	Leukocytes:	$2.0 \cdot 10^5$	
	Erythrocytes:	$8.5 \cdot 10^8$	
Leukocytes	Platelets:	$1.0 \cdot 10^7$	0.5
Erythrocytes	Platelets:	$6.0 \cdot 10^6$	0.14
	Erythrocytes:	0	
Platelets	Platelets:	$1.0 \cdot 10^8$	1.2
	Leukocytes:	0	
	Erythrocytes:	0	
Plasma		0	0

specific activity of phosphodiesterase in the platelets is 4–5 times that in the leukocytes, the platelets accounted for the majority of the activity in the blood. The plasma exhibited no phosphodiesterase activity.

Sonication of the platelets rendered most of the activity soluble. As shown in Table II, the 100 000 × *g* supernatant fluid contained 80% or more of the total activity. Neither further sonication nor treatment with Triton X-100 (0.005–0.5%) revealed more activity in the whole sonicated fraction or the 100 000 × *g* precipitate, indicating that sonication exposed all the enzyme activity. When rat brain was homogenized with the usual technique, most of the phosphodiesterase activity was associated with the microsomes and more than half of the microsomal activity appeared latent, which was unmasked by exposure to Triton X-100 (ref. 11).

Effect of pH

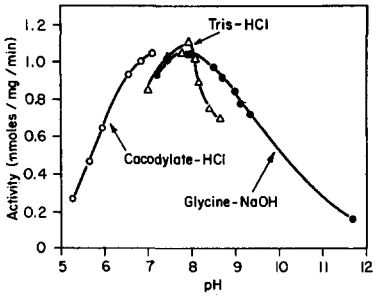
Fig. 1 illustrates the activity of phosphodiesterase as a function of pH. The maximal activity was observed at pH 8, and was comparable to that of the enzyme of rat brain[9], bovine brain[2], and frog erythrocytes[12]. However the enzyme from dog heart[13] and fish brain[14] exhibited optimum pH between 8.5 and 9.2.

TABLE II

DISTRIBUTION OF PHOSPHODIESTERASE IN SOLUBLE AND PARTICULATE FRACTIONS OF HUMAN BLOOD PLATELETS

Platelet was prepared from one pint of fresh bank blood. Phosphodiesterase was determined according to Assay I.

Fraction	*Protein (mg)*	*Specific activity (nmoles/mg per min)*	*Total activity (nmoles/1 pint blood per min)*
Whole sonicated fraction	194	1.6	314
100 000 × *g* supernatant fluid	130	2.0	260
100 000 × *g* precipitate	62	0.8	50



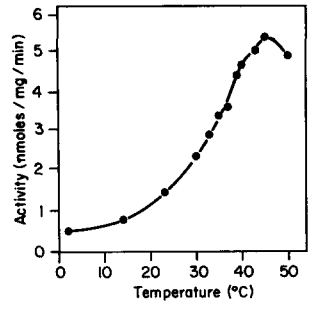


Fig. 1. Effect of pH on phosphodiesterase activity. The standard reaction mixture containing 0.8 mg protein, 1 mM Mn^{2+} and 40 mM buffer. Phosphodiesterase was determined by Assay I.

Fig. 2. Effect of temperature on phosphodiesterase activity. The standard reaction mixture containing 0.8 mg protein was incubated for 60 min at various temperatures. Assay I was performed.

Temperature dependency

The effect of incubation temperature on the activity of phosphodiesterase was examined in Fig. 2. The activity was maximal at 45° and declined thereafter. The activity at 2° and at 30° was about 10% and 50% of the activity at 45°, respectively.

Stability of the enzyme

The thermal stability of phosphodiesterase was studied by exposing the enzyme to different temperatures before assay. When the platelet enzyme was exposed to 55° for 5 min in the absence of cyclic AMP and then assayed at 30°, it retained about 50% of the original activity. In the case of rat brain enzyme[9], exposure to 55° for 5 min resulted in complete loss of activity.

Storage at −20° for various times affected the phosphodiesterase activity.

TABLE III

RELATIVE EFFECT OF DIVALENT CATIONS ON ENZYME ACTIVITY

The reaction mixture contained the standard components, with the divalent cation varied as indicated in the table. Assay I was used with the following modification. At the end of 90 min incubation, the reaction was terminated by boiling. After the tubes were cooled, 1 mM of EDTA was added before the second stage incubation was started with snake venom. EDTA was added to release the inhibition by some of the divalent cations on the 5′-nucleotidase of the venom. EDTA has no effect on 5′-nucleotidase activity or on P_i assay. Activity in the presence of 1 mM Mn^{2+} was taken as 100%.

Additions (1 mM)	*Relative activity*
$MnCl_2$	100
$MgSO_4$	100
$CoCl_2$	80
$BaCl_2$	32
$CaCl_2$	8
$ZnSO_4$	7
$NiCl_2$	5
$CuCl_2$	3
None	30
EDTA	0

TABLE IV

ENZYMIC HYDROLYSIS OF VARIOUS CYCLIC 3',5'-NUCLEOTIDES BY PLATELET PHOSPHODIESTERASE
The standard reaction mixture was incubated for 90 min. Assay I was used.

Nucleotides	*Specific activity (nmoles/mg per min)*	*Relative rate*	*Total activity (nmoles/ 1 pint blood per min)*
Purine base			
Cyclic AMP	2.4	100	180
Cyclic GMP	1.4	56	100
Cyclic IMP	0.9	38	69
Dibutyryl cyclic AMP	0	0	0
Pyrimidine base			
Cyclic UMP	1.6	67	119
Cyclic TMP	1.4	58	104
Cyclic CMP	0	0	0

After 2 and 5 months, the enzyme lost 20% and 50% of its original activity, respectively.

Effect of divalent cations

Phosphodiesterase from various tissues requires a divalent cation to express full activity[2]. Table III demonstrates that for the platelet enzyme 1 mM Mn^{2+}, Mg^{2+}, and Co^{2+} were effective, while Ba^{2+}, Ca^{2+}, Zn^{2+}, Ni^{2+}, and Cu^{2+} were inhibitory. In the absence of added divalent cations, the enzyme retained about 15–30% of its activity observed at 1 mM Mn^{2+}. The residual activity was abolished when assayed in the presence of 0.25 mM EDTA.

The effect of the concentrations of Mn^{2+} on phosphodiesterase activity was examined (see Fig. 6). One millimolar Mn^{2+} gave full activity, and the K_m was found to be 0.05 mM.

TABLE V

EFFECT OF CYCLIC NUCLEOTIDES ON THE HYDROLYSIS OF CYCLIC AMP BY PHOSPHODIESTERASE
The reaction mixture of 0.2 ml contained 40 mM Tris–HCl (pH 8.0), 1 mM Mn^{2+}, 0.5 mg protein (spec.act. 2.5 nmoles/mg per min), 2 mM cyclic [8-^{14}C]AMP and various concentrations of cyclic nucleotides as indicated. Phosphodiesterase was determined by Assay III.

Other addition (mM)	*Relative activity*
None	100
Cyclic GMP (0.4)	33
Cyclic GMP (2)	18
Cyclic IMP (0.4)	34
Cyclic IMP (2)	20
Dibutyryl cyclic AMP (0.4)	69
Dibutyryl cyclic AMP (2)	49
Cyclic CMP (0.4)	100
Cyclic CMP (2)	100
Cyclic TMP (0.4)	100
Cyclic TMP (2)	90
Cyclic UMP (0.4)	100
Cyclic UMP (2)	96

Substrate specificity of phosphodiesterase

The soluble enzyme of sonicated platelets hydrolyzed cyclic 3',5'-nucleotides with the following relative rates: cyclic AMP (100), cyclic GMP (56), cyclic IMP (38), dibutyryl cyclic AMP (0), cyclic UMP (67), cyclic TMP (58), and cyclic CMP (<0.1), Table IV. Note that the platelet enzyme hydrolyzed cyclic TMP and cyclic UMP as effectively as cyclic IMP and cyclic GMP. The rate of hydrolysis of cyclic TMP relative to that of cyclic AMP is high compared to phosphodiesterase of other tissues[2]. Occasionally, platelet preparations hydrolyzed cyclic UMP 20–50% faster than other cyclic nucleotides. In the case of bovine brain[2], the following relative rates were observed: cyclic AMP (100), dibutyryl cyclic AMP (0), cyclic GMP (20), cyclic IMP (30), cyclic UMP (2), cyclic CMP (0), and cyclic TMP (0).*

Effect of cyclic nucleotides on the hydrolysis of cyclic AMP

To determine whether the same substrate site was responsible for the hydrolysis of cyclic AMP and the other cyclic nucleotides, we studied the effect of these compounds on the hydrolysis of cyclic AMP. Table V showed that the purine nucleotides were inhibitory, whereas the pyrimidine nucleotides were without effect. Dibutyryl cyclic AMP inhibited the activity of the enzyme even though it was not hydrolyzed. It has been shown that the 2'-*O*-butyryl side group of dibutyryl cyclic AMP may be hydrolyzed chemically under mild conditions[17,18]. The possibility existed that the monobutyryl derivative may be the inhibitory compound in our experiment. The fact that the platelet phosphodiesterase hydrolyzed cyclic AMP as well as cyclic TMP and cyclic UMP and the fact that these pyrimidine nucleotides did not affect the hydrolysis of cyclic AMP suggested that there was more than one substrate site. To examine whether these sites are on one or different proteins, we performed heat inactivation experiments. The enzyme was preincubated at 58° for various times before the usual assay. The time to inactivate the enzymic hydrolysis of these nucleotides varied from 15 min for cyclic IMP to 35 min or longer for cyclic AMP. This suggested different sensitivity of the substrate sites to heat inactivation. However, physical separation of the substrate sites using conventional fractionation techniques such as ammonium sulfate, ethanol and calcium phosphate gel have not been successful. The question of one enzyme or multiple enzymes remains to be established.

MARQUIS *et al.*[19] noted that cyclic AMP and dibutyryl cyclic AMP inhibited ADP-induced platelet aggregation and that dibutyryl cyclic AMP was much more effective. The fact that the platelet enzyme hydrolyzed both the purine and pyrimidine cyclic nucleotides suggested the possibility that these nucleotides might affect platelet aggregation. Using the procedure of MUSTARD *et al.*[20] with a Payton Aggregation Module, we found that cyclic GMP and cyclic IMP were about 50% as effective as cyclic AMP, and cyclic nucleotides with a pyrimidine base were only slightly effective. We also found that dibutyryl cyclic AMP was more effective than cyclic AMP in counteracting the ADP-induced aggregation. Dibutyryl cyclic AMP might inhibit the aggregation process directly, or indirectly through its inhibition on the hydrolysis of endogenous cyclic AMP. The effectiveness of dibutyryl cyclic AMP may be due to its resistance to hydrolysis by phosphodiesterase and to its more

* After the completion of this work, two reports came to our attention showing broad substrate specificity of a phosphodiesterase from a bacteria[15] and from the adipose cells[16].

TABLE VI

EFFECT OF PURINE, PYRIMIDINE AND THEIR NUCLEOSIDES OR NUCLEOTIDES ON PHOSPHODIESTERASE ACTIVITY

Reaction mixture of 0.5 ml contained 40 mM Tris–HCl (pH 8.0), 1.4 mg protein (spec. act. 3 nmoles/mg per min), 2 mM cyclic AMP, and 1 mM purine, pyrimidine and their derivatives. Phosphodiesterase was determined by Assay II or Assay III. The result of both assays were correlated well with each other.

Purine	*Relative activity***	*Pyrimidine*	*Relative activity***
ATP	9	UTP	22
GTP	22	TTP	16
ITP	26	CTP	18
ADP*	64		
5′-AMP*	100	UMP*	100
5′-GMP*	100	5′-GMP*	100
5′-IMP*	100	Thymidine*	84
Adenosine*	56	Uridine*	100
Guanosine	68	Cytidine	100
Inosine	66	Uracil	100
Adenine	73	Thymine	100
Guanine	77	Cytosine*	95
Hypoxanthine	60		

* Assay III was used.
** The activity without inhibitor is taken as 100.

lipophilic nature. Cyclic GMP and cyclic IMP may affect the aggregation by inhibiting the hydrolysis of endogenous cyclic AMP.

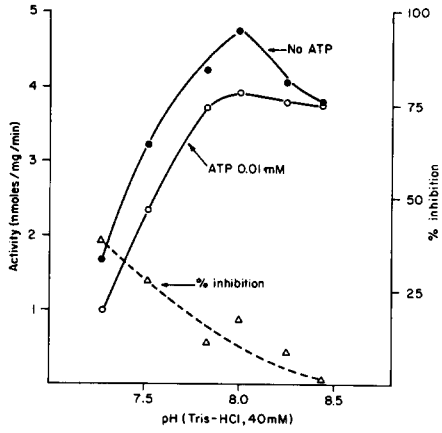
Inhibition by metabolites

As seen in Table VI, all the nucleoside triphosphates were potent inhibitors of phosphodiesterase in the absence of added divalent cations. Purine nucleosides and bases inhibited the enzyme moderately, while pyrimidine derivatives were ineffective. Nucleoside monophosphates were without effect.

Other metabolites at 1 mM concentration were tested for their effect on phosphodiesterase activity. PP_i caused 86% inhibition; citrate 70%, pyruvate, 48%; glucose 6-phosphate, 15%; glucose 1-phosphate, 10%. Fructose 6-phosphate and fructose 1,6-diphosphate were ineffective.

Inhibition of phosphodiesterase by ATP was shown with the rat brain[9]. As with the brain enzyme, the inhibition of the platelet enzyme by ATP was pH dependent (Fig. 3). The inhibition was more pronounced below pH 7.5 than above pH 8. The inhibition by 0.01 mM ATP was about 40% at pH 7, and was negligible at pH 8.4. The degree of inhibition by ATP also varied with the concentration of Mn^{2+} in the incubation mixture. In the presence of 1 mM Mn^{2+} half maximal inhibition was observed at 0.5 mM ATP, and in the absence of added Mn^{2+}, it was noted at 0.03 mM ATP.

Although 5′-GMP did not affect phosphodiesterase activity, it protected partially the enzyme from ATP inhibition. Fig. 4 shows that 0.2 mM GMP decreased the inhibition by ATP 20–35% throughout the ATP range of 0.005 to 0.2 mM. The protective effect of GMP on ATP inhibition has been observed previously[9].



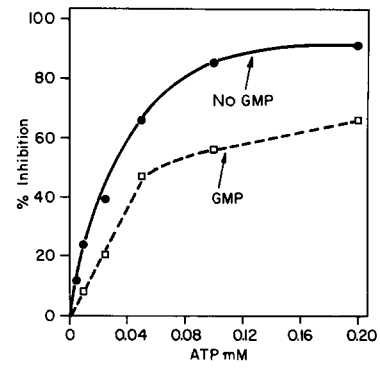
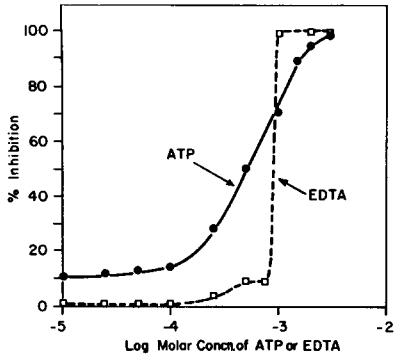


Fig. 3. Effect of pH on the inhibition of phosphodiesterase by ATP. Each tube contained in a final volume of 0.5 ml: 0.5 mg protein, 40 mM Tris–HCl and 2 mM cyclic AMP with or without 0.01 mM ATP. Assay II was used. The inhibition by 0.01 mM ATP at each pH was expressed as per cent of the corresponding control.

Fig. 4. Partial relief of ATP inhibition by GMP. The reaction mixture of 0.5 ml contained 1.2 mg protein, 40 mM Tris–HCl (pH 8.0), 0.2 mM GMP and various concentrations of ATP as indicated. The control tubes did not have 5′-GMP. Assay II was used.

Comparison of the inhibition of phosphodiesterase by ATP to that by EDTA

The effect of ATP and EDTA on the activity of the platelet enzyme as a function of inhibitor concentration is compared in Fig. 5. The inhibition of ATP appeared gradually in the presence of 1 mM Mn^{2+}; it was 10% at 0.01 mM ATP, 70% at 1 mM and 100% at 3 mM. In contrast, the onset of the inhibition by EDTA was abrupt; no inhibition was observed until EDTA reached a critical concentra-



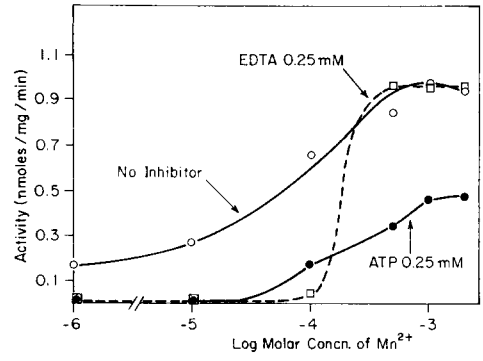


Fig. 5. Inhibition by ATP and EDTA on phosphodiesterase activity as a function of inhibitor concentration. The reaction mixture of 0.5 ml contained 40 mM Tris–HCl (pH 8.0), 1 mM Mn^{2+}, 0.7 mg of protein and the indicated amount of ATP or EDTA. Assay II was used. Control tubes did not have ATP or EDTA and were taken as 0% inhibition.

Fig. 6. Effect of various concentrations of Mn^{2+} on the reversal of the inhibition of phosphodiesterase by ATP or EDTA. The reaction mixture of 0.5 ml contained 40 mM Tris–HCl (pH 8.0), 2 mM cyclic AMP, 0.4 mg protein, various concentrations of Mn^{2+} and where indicated, 0.25 mM ATP or EDTA. Assay II was used.

tion, whereby the effect was fully manifested. The critical concentration appeared to be equivalent to the concentration of Mn^{2+} (1 mM) present in the incubation mixture. Note that when the concentration of inhibitors was below 1 mM, the inhibition by ATP was more severe than that by EDTA; and when the concentration was higher than 1 mM, the effect of EDTA exceeded that of ATP.

Fig. 6 depicts a reversed experiment in which the effect of an increasing concentration of Mn^{2+} on the release of inhibition by ATP or EDTA on phosphodiesterase activity is examined. Below 0.05 mM Mn^{2+}, the inhibition by 0.25 mM ATP or by 0.25 mM EDTA was complete. At 1 mM Mn^{2+} completely reversed the inhibition by EDTA. However, the same concentration of Mn^{2+} released only 50% of the

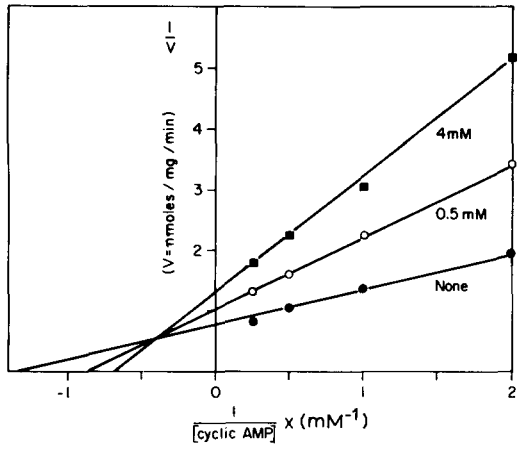


Fig. 7. Inhibition of phosphodiesterase by caffeine. The reaction mixture of 0.2 ml contained 40 mM Tris–HCl (pH 8.0), 1 mM Mn^{2+}, 0.5 mg protein, 0.5 to 4 mM cyclic [8-^{14}C]AMP and where indicated, different concentrations of caffeine. Assay III was performed.

inhibition by ATP and a higher concentration was equally ineffective. These experiments showed that the effect of EDTA on the activity of phosphodiesterase could be explained by metal chelation but that of ATP appeared more complicated.

Inhibition by caffeine

Like other mammalian enzymes, phosphodiesterase of human blood platelet was inhibited by caffeine. The mode of inhibition, however, appeared different. Fig. 7 shows a double-reciprocal plot of the effect of caffeine. Inhibition appeared to be of a mixed type. The K_i for caffeine was calculated to be 1.5 mM. With rat brain[9], the inhibition by caffeine was competitive and the K_i was 3 mM. In the case of dog heart[13], it was non-competitive and the K_i was 50 mM.

Determination of high and low K_m

The substrate concentration used to measure the K_m in Fig. 7 ranged from 0.5 to 4 mM. When the K_m was determined using a wider range of substrate concentrations, we found that the double reciprocal plot was not linear, and a discontinuity was observed between 0.25 and 0.4 mM (Fig. 8). Extrapolation of this curve indicated that the enzyme exhibited two K_m values, one of $7 \cdot 10^{-4}$ M and another of $7 \cdot 10^{-5}$ M.

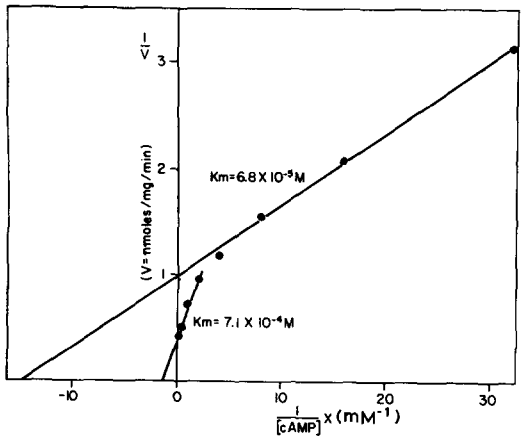


Fig. 8. Determination of low and high K_m values. The reaction mixture of 0.2 ml contained the standard components except varying concentrations of cyclic [8-^{14}C]AMP (cAMP). Assay III was used.

DISCUSSION

Phosphodiesterase of human blood platelets is in many respects similar to the brain enzyme. It exhibits optimal activity at pH 8. A divalent cation is required for full activity. It is inhibited by ATP and protected partially from ATP inhibition by 5′-GMP. Other nucleoside triphosphates and caffeine were also inhibitory. However, unlike phosphodiesterase of most other tissues, inhibition by caffeine appears to be a mixed type.

Perhaps the one property which distinguishes the platelet phosphodiesterase from the brain enzyme is its effective hydrolysis towards cyclic UMP and cyclic TMP. Since nucleotides with a pyrimidine base do not affect the hydrolysis of cyclic AMP, the substrate site for cyclic nucleotides with a purine base appears distinct from those with a pyrimidine base. Present evidence does not indicate whether the substrate sites represent different enzymes or one enzyme with multiple sites.

BROOKER *et al.*[21] demonstrated the existence of two K_m values for phosphodiesterase of rat brain. Since then, similar observations have been made on the enzyme from bovine brain[2], bovine heart[22], and frog bladder epithelial cells[23]. The present paper shows that the platelet enzyme also exhibited two K_m values.

The fact that phosphodiesterase exhibits two affinities for cyclic AMP may be of physiological significance. It is conceivable that the low K_m site may be responsible for maintaining cyclic AMP level under steady state conditions of the cell and that the high K_m site becomes critical when the cell is confronted with an upsurge of cyclic AMP as a result of perturbation. A pronounced transient accumulation of cyclic AMP has been observed in many tissues in response to hormonal stimulation of adenyl cyclase of these tissues[1]. Since cyclic AMP regulates acutely many cellular reactions and processes, it is important that its hydrolysis should be under effective control at all times. A prolonged accumulation of cyclic AMP after its specific influence has been exerted would be disadvantageous to the cell. A phos-

phodiesterase with a high K_m would minimize the time to hydrolyze this critical cellular regulator.

Previous work from this laboratory indicated that phosphodiesterase of bovine brain was a metalloenzyme[24]. It is known that EDTA and ATP are metal chelators. The logarithms of the stability constant of the Mn–EDTA and Mn–ATP are high in the order of 12 and 5, respectively[25]; and one would not expect that in the presence of an excess Mn^{2+} there would be any significant quantities of unsequestered EDTA nor ATP available to chelate the metal in the enzyme. Thus, the inhibition of phosphodiesterase by EDTA may be explained by metal chelation, but that by ATP appears more than a simple chelation of the metal on the enzyme. This conclusion is supported by our previous kinetic analysis which indicates that the inhibition of phosphodiesterase is of a mixed type[8].

HOLMSEN *et al.*[26] have shown that there are three pools of adenine nucleotides in the platelets, two metabolically active, and one inert. The nucleotides in the inert pool are extruded into the surrounding medium during the release reaction, while the ATP in one of the active pools is consumed concommittably. Since ATP inhibits the activity of phosphodiesterase, it is important to elucidate how the tissue level of ATP affects the concentration of cyclic AMP which in turn may affect the aggregation of platelet. The protection of the enzyme by GMP from ATP inhibition may also be significant in this regard.

A specific protein activator of phosphodiesterase from bovine brain has been demonstrated[27]. A similar activator was noted in various bovine and rabbit tissues and in the human brain[28]. Preliminary experiments indicated that the activator is present also in the human blood. Since the cellular components of the blood are already suspended in the plasma, they can be isolated with minimal damage to their ultrastructure. Thus, the blood promises to provide a model system to examine the cellular and subcellular localization of the protein activator relative to phosphodiesterase. Such work is now in progress.

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