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STUDIES WITH PHOSPHODIESTERASE

III. TWO FORMS OF THE ENZYME FROM HUMAN BLOOD PLATELETS

M. SAMIR AMER AND ROBERT F. MAYOL

Department of Pharmacology, Mead Johnson Research Center, Evansville, Ind. 47721 (U.S.A.)

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SUMMARY

The existence of two forms of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) phosphodiesterase and one form of guanosine 3′,5′-cyclic monophosphate (cyclic GMP) phosphodiesterase in human blood platelets was demonstrated by kinetic analysis, DEAE-chromatography and sucrose gradient centrifugation. The two forms of cyclic AMP-phosphodiesterase differed significantly in their K_m values. The lower K_m form (phosphodiesterase-II) appears to be associated with the platelet membrane and may play the more significant role in the control of intracellular cyclic AMP levels in platelets.

INTRODUCTION

Cyclic adenosine 3′,5′-monophosphate 3′-hydrolase (cyclic AMP phosphodiesterase) exists in a number of tissues in multiple forms differing in their (a) Michaelis–Menten constants (K_m values) (refs 1–8 and Amer, M. S., unpublished); (b) stability and drug sensitivity (refs 9–11 and Amer, M. S., unpublished); (c) response to divalent cations (Amer, M. S., unpublished and refs 12–14); (d) substrate specificity[13,15–17]; (e) electrophoretic and chromatographic behavior[5,18]; and (f) subcellular localization[15,19]. However, the functions of these multiple forms of the enzyme and their relative importance in the control of intracellular cyclic AMP and/or cyclic guanosine-3′,5′-monophosphate (cyclic GMP) levels are not clear. Neither is it clear whether the different forms of the enzyme are structurally or functionally related.

Cyclic AMP appears to play a very important role in platelets, (for review see ref. 20) and compounds which affect the intracellular levels of cyclic AMP have profound effects on platelet aggregation. The intracellular levels of the cyclic nucleotide are controlled by both adenylate cyclase, the enzyme that catalyzes the formation of cyclic AMP, and phosphodiesterase which catalyzes its hydrolysis, both of which were shown to exist in platelets[20].

The present studies were designed to study in detail the multiple forms of

phosphodiesterase in human blood platelets in an effort to determine their relative importance in the control of intracellular cyclic AMP levels and hence platelet aggregation.

MATERIALS AND METHODS

Preparation of human platelets

Platelet-rich plasma was prepared by low speed centrifugation of freshly obtained citrated whole blood. Platelet-rich plasma was used as such in the studies using intact platelets. Platelet homogenates were prepared in 0.25 M sucrose from platelet pellets obtained by the centrifugation of the platelet-rich plasma using homogenizer tubes with Teflon pestles.

DEAE-chromatography

DEAE-column chromatography using gradient elution with $(NH_4)_2CO_3$ (0.05–0.3 M) was carried out as described previously for rabbit tissues[21].

Sucrose gradient fractionation

Sucrose gradient fractionation was carried out by layering* the whole homogenate onto a 5–20% sucrose gradient and centrifuging for 15 h at 27 000 rev./min and 5 °C in the SW27 rotor (Spinco). A cushion of CsCl (3 g CsCl in 3 ml 20% sucrose) was placed at the bottom of the gradient to prevent pelleting of the fast sedimenting material.

Phosphodiesterase assay

The method described by Thompson and Appleman[15] was used for the assay of phosphodiesterase. Initial reaction velocities were determined at 15 substrate concentrations, covering the range from $3 \cdot 10^{-8}$ to $8 \cdot 10^{-3}$ M. Michaelis–Menten constants (K_m values), maximal velocities (V) and the proportions of the two forms of the enzymes were calculated with the aid of computer programs as previously described[21]. In some experiments, the phosphodiesterase activity which is exposed on the platelet surface by collagen treatment[22] was assayed using the same procedure.

RESULTS

Studies with platelet whole homogenates

Preliminary experiments indicated that high speed supernatant preparations did not contain all of the phosphodiesterase activity found in the platelet whole homogenates. Studies on the kinetics of hydrolysis of cyclic AMP and cyclic GMP were therefore carried out on the 4000 × g supernate, or in some cases the whole homogenate. The results obtained with the whole platelet homogenate using 15 substrate concentrations of cyclic AMP and cyclic GMP are shown in Fig. 1. Essentially, the same results are obtained with the 4000 × g supernate.

It is clear from Fig. 1 that at least two forms of phosphodiesterase differing markedly in the K_m values for cyclic AMP are present in the platelets. These were

* The sample when layered onto the gradient, settled to a point 1 cm below the surface.

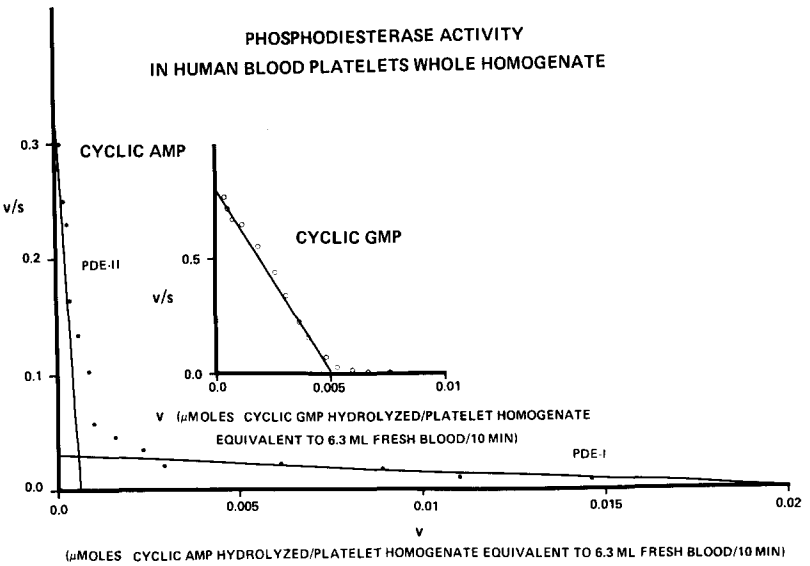

Fig. 1. Eadie plots for the hydrolysis of cyclic AMP and cyclic GMP by human blood platelet whole homogenate. A radioactive assay method[15] was used to determine initial velocities at 15 substrate concentrations covering the range from $3 \cdot 10^{-8}$ to $8 \cdot 10^{-3}$ M. In this form of Eadie plot, the slope is equal to $-1/K_m$ and thus gives direct indication of the affinity of the enzyme to its substrate. Each assay tube contained the platelet fraction equivalent to 6.3 ml of fresh blood. Incubations were carried out at 30 °C for 10 minutes. Phosphodiesterase-I (PDE-I) is the high K_m form and phosphodiesterase-II is the low K_m form of the enzyme.

tentatively identified as phosphodiesterase-I with the higher K_m value and phosphodiesterase-II with the lower K_m value. The K_m and V values for the two forms of the enzyme and the contribution of the low K_m form (phosphodiesterase-II) to the total activity were derived using the method described by Cleland[23], and the results are shown in Table I. As can be seen in the table, the low K_m form of phosphodiesterase (phosphodiesterase-II) accounts for only a small proportion of the total enzyme activity of the whole homogenate.

TABLE I

KINETIC PARAMETERS FOR THE HYDROLYSIS OF CYCLIC AMP AND CYCLIC GMP BY PLATELET WHOLE HOMOGENATES AND PURIFIED FRACTIONS FROM DEAE-COLUMNS

	Cyclic AMP			*Cyclic GMP*	*V (cyclic AMP)*
	Phosphodiesterase type	K_m	% *V-II*[a]	K_m	*V (cyclic GMP)*
Whole Homogenate	phosphodiesterase-I	$6.1 \cdot 10^{-4}$	4.2	$1.6 \cdot 10^{-5}$	2.84
	phosphodiesterase-II	$3.2 \cdot 10^{-6}$			
DEAE-Purified	phosphodiesterase-I[b]	$1.84 \cdot 10^{-3}$			
	phosphodiesterase-II[c]	$1.11 \cdot 10^{-7}$		$1.71 \cdot 10^{-6}$[d]	

[a] % of total V present as phosphodiesterase-II calculated from the kinetic data as described by Cleland[23].

[b] $V = 0.104$ μmole/mg protein per min.

[c] $V = 0.013$ μmole/mg protein per min.

[d] $V = 0.0064$ μmole/mg protein per min.

Studies with intact platelets

Salzman and Weisenberger[22] demonstrated that kaolin and collagen, when added to intact platelets, exposed phosphodiesterase activity which remained platelet-bound. It was of interest to see which of the two forms of phosphodiesterase was exposed by one of these agents. The cyclic AMP-phosphodiesterase activities found in platelet whole homogenates, membrane fractions and collagen-released intact platelets were examined and the results are shown in Fig. 2. As can be seen from the

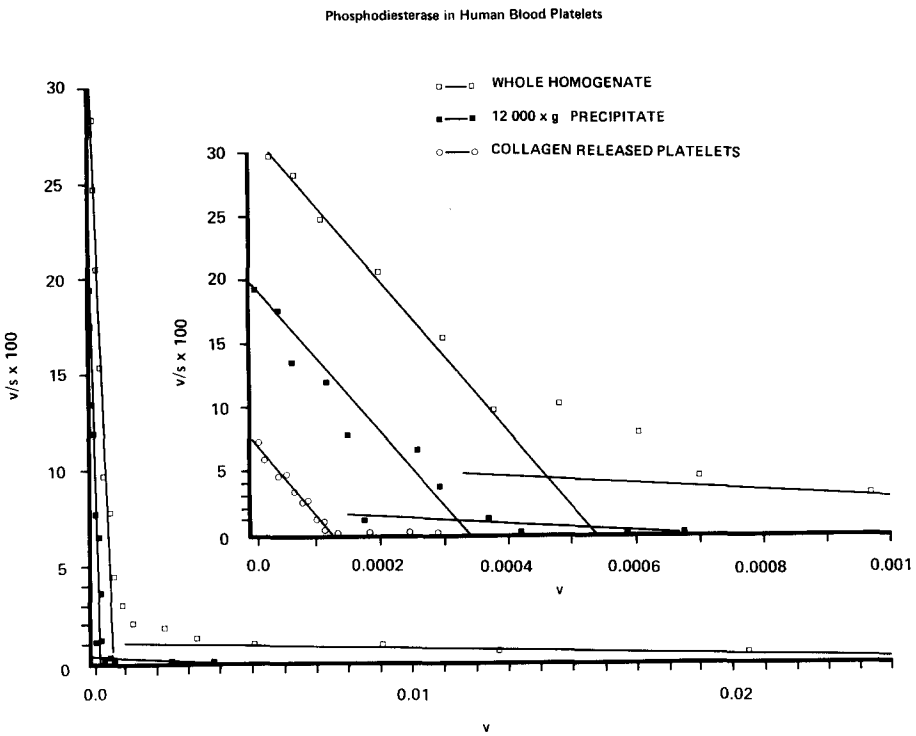

Fig. 2. Eadie plots of phosphodiesterase activity from whole homogenate, 12 000 × *g* precipitate and unhomogenized collagen-released platelets. Conditions are the same as for Fig. 1. v = initial velocity (μmoles hydrolyzed by the respective platelet fraction in 6.3 ml fresh blood/10 min), S = substrate concentration (mM).

Eadie plots, the phosphodiesterase exposed on the collagen-released platelets is of the low K_m variety, *i.e.* phosphodiesterase-II, with a K_m value of $2 \cdot 10^{-6}$ M, which is the same as that found in the 12 000 × *g* membrane fraction and the whole homogenate. A similar situation exists in rabbit and monkey brains (Amer, M. S., unblished) and supports the hypothesis that the low K_m form of phosphodiesterase is partly particulate.

DEAE-chromatography of phosphodiesterase activity

The presence of two forms of phosphodiesterase was further confirmed by DEAE chromatography of the 4000 × *g* supernate of the platelets. The results are shown in Fig. 3. Two peak areas of enzyme activity were obtained when cyclic AMP

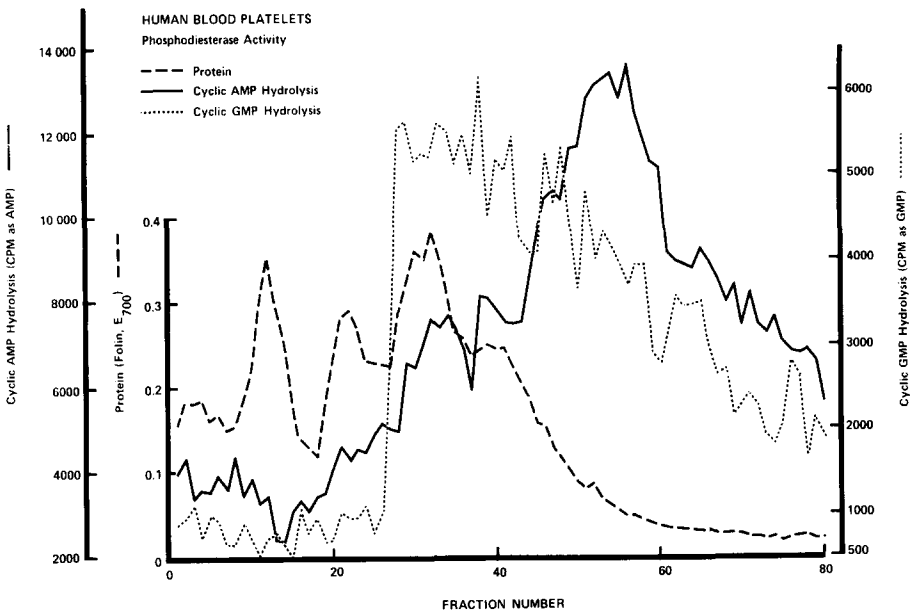

Fig. 3. DEAE-chromatogram of human blood platelets 4000 × *g* supernate. 10 ml platelet 4000 × *g* supernate (320 mg wet platelets/ml) were added to each column prior to chromatography (equivalent to 325 ml of whole blood). 6-ml fractions were collected every 6 min. The cyclic AMP and cyclic GMP hydrolysis were determined as described by Thompson and Appleman[15] in 0.2-ml aliquots of the eluate fractions. The assays were carried out at substrate concentrations of $2 \cdot 10^{-6}$ M which results in an apparent exaggeration of the low K_m activity. Phosphodiesterase-I is the high K_m form and phosphodiesterase-II is the low K_m form of the enzyme.

was used as substrate. Only one peak, although not sharp, was apparent when cyclic GMP was used as substrate. The kinetic properties of the peak tubes are shown in Fig. 4 and the kinetic parameters included in Table I. As can be seen from Table I, the K_m values in the separated peaks differed significantly from the crude supernatant preparations. This may reflect the presence of activators or inhibitors in the crude homogenates that could be lost on purification. The presence of activators and inhibitors of phosphodiesterase in other tissues has been described[24–26].

It should be noted that in Fig. 4 there may be an indication of a second form of phosphodiesterase with a high K_m value when cyclic GMP was used as a substrate. However, if this is true, it would be present in extremely low concentration.

Sucrose gradient fractionation of phosphodiesterase activity

Sucrose gradient studies also indicated that the fast sedimenting material contained a higher proportion of the low K_m enzyme than the more slowly sedimenting fraction. These results are shown graphically in Fig. 5. The lighter fractions contained the majority of the total phosphodiesterase activity and still contained a smaller proportion of phosphodiesterase-II.

Effects of additions on platelet phosphodiesterase activity

The effects of EDTA, EGTA, Ca^{2+} and Mg^{2+} on phosphodiesterase activity in the 4000 × *g* supernatant fraction of human blood platelets were also studied using

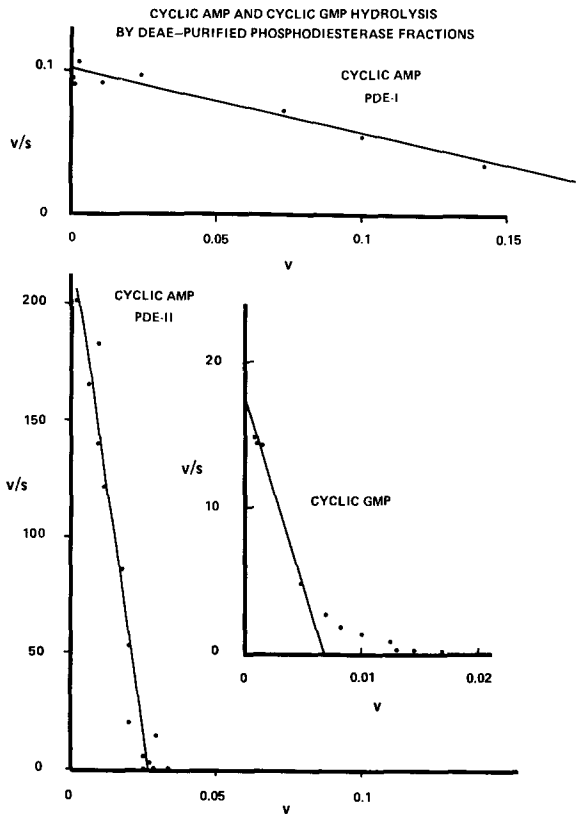

Fig. 4. Kinetics of hydrolysis of cyclic AMP and GMP by DEAE-purified fractions. Enzyme assays were carried out on the peak tubes (Tubes 34 and 55) corresponding to the two activity peaks as shown in Fig. 3. v = initial velocity (μmoles hydrolyzed/2 mg protein per min), s = substrate concentration (mM). PDE, phosphodiesterase.

conditions similar to those described for Fig. 1. In general, the results obtained were similar to those already reported[3]. EDTA completely inhibited the total enzyme activity. EGTA, on the other hand, had almost no effect on the basal total activity of the enzyme. Ca^{2+} appeared to be the ion more important for the activity of the enzyme although Mg^{2+} was capable of replacing Ca^{2+} to a significant extent.

DISCUSSION

It is clear from the studies already described that human blood platelets possess at least two forms of cyclic AMP-phosphodiesterase differing mainly in their K_m values. This was established by kinetic analysis and DEAE chromatography. Furthermore, it seems that the low K_m form of the enzyme (phosphodiesterase-II) is associated with the platelet membranes while the other form (phosphodiesterase-I) is soluble. This is similar to what we observed in other tissues particularly the brain of rabbits and monkeys (Amer, M. S., unpublished).

It is interesting to note that only one form of cyclic GMP-phosphodiesterase

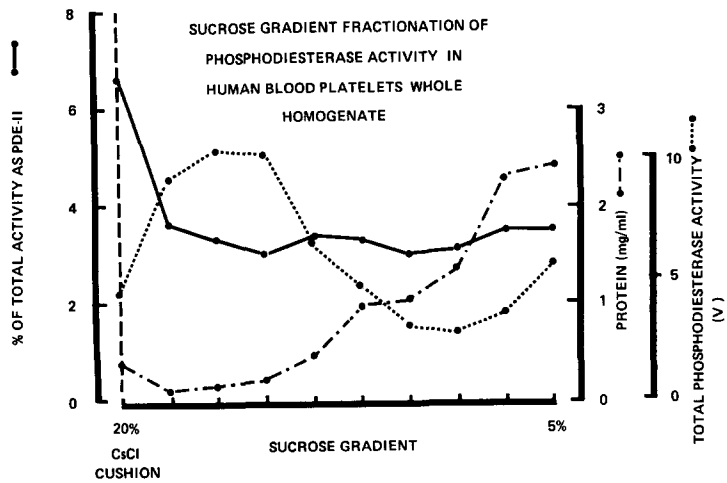

Fig. 5. Sucrose gradient fractionation of platelet whole homogenate. The fractions were collected by puncturing the bottom of the tube and were dialyzed against 0.05 M potassium phosphate buffer, pH 7.5 and analyzed for protein and for phosphodiesterase activity at 15 substrate concentrations covering the range from $3 \cdot 10^{-8}$ to $8 \cdot 10^{-3}$ M to allow for the calculation of the relative proportion of activity attributable to either form of the enzyme. The percentage of the total activity present as phosphodiesterase-II was calculated after the method of Cleland[23]. V is expressed in μmoles hydrolyzed/0.5 ml per 10 min at 30 °C.

seems to exist. This may re-emphasize the separate identity of cyclic GMP-phosphodiesterase. It seems, therefore, that the hydrolysis of cyclic GMP may be catalyzed by an enzyme system distinct from that responsible for the hydrolysis of cyclic AMP. This is not unexpected since both nucleotides are formed through the intervention of separate cyclases.

The relative importance of the two forms of phosphodiesterase in the control of intracellular cyclic AMP levels is becoming clearer. Since the concentration of cyclic AMP in platelets is about 10^{-6} M or less[20], the form of the enzyme that can act on these small levels is likely to be the particulate phosphodiesterase-II since it possesses appreciably higher affinity for the substrate cyclic AMP. Phosphodiesterase-I has a K_m value at least two orders of magnitude higher than the substrate concentration available *in vivo* and thus appears of doubtful importance in the control of cyclic AMP levels in the platelets. The importance of phosphodiesterase-II in the control of cyclic AMP levels in platelets is further strengthened by its apparent sensitivity to inhibition by adenosine and modulation by agents strongly influencing platelet aggregation, such as epinephrine, aspirin, and prostaglandin E_1 (ref. 27). Although these agents did not significantly alter the total platelet phosphodiesterase activity, they changed the proportion of the total activity present in the phosphodiesterase-II form when added to platelet homogenates *in vitro*[27].

The fact that phosphodiesterase-II is probably located on the membrane and its activity appears on the surface of the platelets immediately prior to the collagen-induced release reaction[22] indicates that it may play a significant role in that reaction. This is particularly true since platelet aggregation involves the interaction of membranes of different platelets adhering in some way to each other. Inhibition or induction of this interaction might be expected to occur at or near the cell surface.

It is tempting to speculate that a pool of cyclic AMP may be membrane-associated and normally inhibits the release reaction. Interaction of this membrane pool of cyclic AMP with the membrane-bound phosphodiesterase-II may result in the hydrolysis of that pool concomitant with the initiation of the release reaction. This hypothesis agrees with the observed inhibition of aggregation by cyclic AMP and compounds that elevate its intracellular concentration.

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