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THE EFFECT OF ADP, CALCIUM AND SOME INHIBITORS OF PLATELET AGGREGATION ON PROTEIN PHOSPHOKINASES FROM HUMAN BLOOD PLATELETS

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Summary

A protein phosphokinase (ATP: protein phosphotransferase EC 2.7.1.37) which is stimulated by 3′,5′-cyclic adenosine monophosphate (cyclic AMP) has been partially purified from both the cytoplasmic and membrane fractions of human platelets. The kinetics of both enzyme preparations are similar in respect to cyclic AMP, ATP, ADP and AMP. $5 \cdot 10^{-7}$ M cyclic AMP stimulated both preparations by approximately 100%. Both ADP and AMP at a concentration of $5 \cdot 10^{-5}$ M inhibited protein phosphokinase activity of the soluble and membrane preparation by between 50% and 70%. The response of the two enzyme preparations to calcium differed. 10 mM Ca^{2+} inhibited soluble protein phosphokinase activity approximately 80% both in the presence and absence of $5 \cdot 10^{-7}$ M cyclic AMP whereas the same concentration of Ca^{2+} inhibited the membrane-bound enzyme by approximately 60% in the presence of $5 \cdot 10^{-7}$ M cyclic AMP and 40% in the absence of cyclic AMP. This observation may be of importance in understanding the mechanism of platelet aggregation.

Introduction

Cyclic AMP and its dibutyryl derivative have been shown to inhibit platelet aggregation [1]. In addition, substances which inhibit platelet aggregation are said to increase the level of cyclic AMP in platelets [2]. In most tissues, cyclic AMP is thought to act through cyclic AMP-dependent protein phosphokinases [3], and such an enzyme has been purified from platelets [4,5]. In an attempt to clarify the role of cyclic AMP in platelet metabolism, the kinetics of platelet protein phosphokinase were studied, and the effects of ADP, calcium and some inhibitors of platelet aggregation on phosphokinase activity were observed.

During the course of preparation of this paper a preliminary report was

published describing some of the properties of a protein phosphokinase purified from platelet membranes [6]. The conclusions inferred from the results for the kinetics of the membrane protein phosphokinase were different from those which we report in this paper using the protein phosphokinase from the soluble fraction of platelets. Consequently we prepared purified platelet membranes in an attempt to determine whether there is a significant difference between the kinetics of the protein phosphokinases from platelet soluble and membrane fractions.

Methods

Purification of protein kinase

Human platelets in plasma, prepared as platelet concentrate by the Red Cross Blood Transfusion Service from ACD blood the previous day, were centrifuged at 250 × *g* for 15 min to remove contaminating erythrocytes and leucocytes. The platelet rich plasma was spun at 2300 × *g* for 15 min to separate the platelets. The platelet buttons were washed in normal saline buffered with 10 mM Tris, pH 7.4, and disrupted by freezing and thawing 4 times. The suspension was centrifuged at 27 000 × *g* for 30 min. Platelet soluble protein phosphokinase was extracted from the supernatant according to the method of Kuo & Greengard [7] up to and including the stage of DEAE-cellulose column chromatography. Platelet membranes were purified from the 27 000 × *g* precipitate by a method similar to that of Barber & Jamieson [8]. The precipitate was resuspended in 10 vols of 20 mM Tris buffer, pH 7.4, and the suspension was layered over 27% (w:v) sucrose and centrifuged for 4 h at 62 000 × *g*. The fraction at the interface was removed, diluted with 2 vols of 20 mM Tris buffer, pH 7.4, and centrifuged at 105 000 × *g* for 1 h. The pellet was resuspended in approximately 20 vols of 20 mM Tris buffer and this suspension of membranes was used for protein phosphokinase assay. Both the soluble and membrane-bound enzymes were stored at −20° C prior to enzyme assay.

Electron microscopy of platelet membrane preparation

The 105 000 × *g* pellet was fixed in glutaraldehyde and osmium tetroxide, stained in uranyl acetate, dehydrated and embedded in Durcupan ACM (Fluka). Thin sections were cut and mounted on collodion and carbon coated grids and stained in Reynolds lead citrate stain. Sections were observed in a Phillips EM 300 electron microscope. Electron micrographs were taken at magnifications ranging from 3000 to 30 000 times.

Protein phosphokinase assay

The activity of cyclic AMP-dependent protein phosphokinase was assayed by the method of Kuo and Greengard [9]. The incubation mixture for the standard assay contained, in a final volume of 0.2 ml: sodium acetate buffer, pH 6.0, 10 μmol; histone protein 400 μg; [γ-^{32}P]ATP, 0.53 nmol containing about 0.5 μCi and magnesium acetate, 2 μmol. The reaction was initiated by addition of platelet enzyme extract (15—28 μg of protein for the soluble enzyme and 60—130 μg of protein for the membrane enzyme). Incubation was

carried out for 4 min for the soluble enzyme and 8 min for the membrane-bound enzyme. The reaction was terminated by adding 4 ml of 5% trichloroacetic acid containing 0.25% sodium tungstate, pH 2.0, followed by 0.2 ml of 0.63% serum albumin. After standing for 5 min at 0°C, the mixture was centrifuged for 10 min at 2300 × *g* and the supernatant decanted. The precipitate was dissolved in 0.1 ml of 1 M NaOH and 2.0 ml of 5% trichloroacetic acid 0.25% tungstate solution was added. The protein was reprecipitated by addition of 0.1 ml of 0.6 M H_2SO_4 and the mixture was centrifuged. The washing process was repeated and the protein was finally dissolved in 0.1 ml of 1 M NaOH and counted in 10 ml of distilled water in a Packard Tri-Carb liquid scintillation spectrometer.

Cyclic AMP, cyclic GMP, AMP, ADP, ATP, adenine, adenosine and histone were obtained from Sigma and bovine serum albumin from Calbiochem. [γ-^{32}P]ATP (PB. 132) was obtained from the Radiochemical Centre, Amersham, RA233 (2,6-bis (diethanalamino) 4-piperidino-pyrimido (5,4-d) pyrimidine) was obtained from Boehringer Ingelheim.

Protein was determined by Miller's modification of the method of Lowry [10].

Results

Purification

The distribution of protein phosphokinase activity in the protein peaks of a DEAE-cellulose column is shown in Fig. 1. The main activity is located in Peak 2, with slight activity in Peaks 3 and 4 (which showed the same cyclic AMP dependence as the enzyme from Peak 2). Dialysis of the Peak 2 enzyme against 5 mM phosphate buffer, pH 7.0, decreased basal enzyme activity by an average of 50% but increased cyclic AMP dependence by 80%. After storage for 6 months at −20°C the dialysed enzyme lost all activity whereas the undialysed

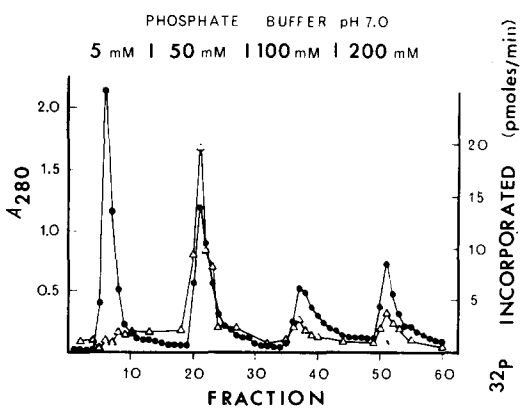

Fig. 1. DEAE-cellulose column chromatography of extract of platelet soluble fraction. Approximately 100 mg of protein from the dialyzed 55% $(NH_4)_2SO_4$ precipitate step was applied to a DEAE-cellulose column 2.0 × 14 cm and eluted stepwise with 5, 50, 100 and 200 mM phosphate buffers, pH 7.0, (all buffers used in the enzyme preparation contained 2 mM EDTA). The effluent was collected in 5 ml fractions at a rate of 1 ml per min. The absorbance at 280 nm, ●———●, and protein phosphokinase activity, △———△, (in the presence of $5 \cdot 10^{-7}$ M cyclic AMP) of the fractions were measured.

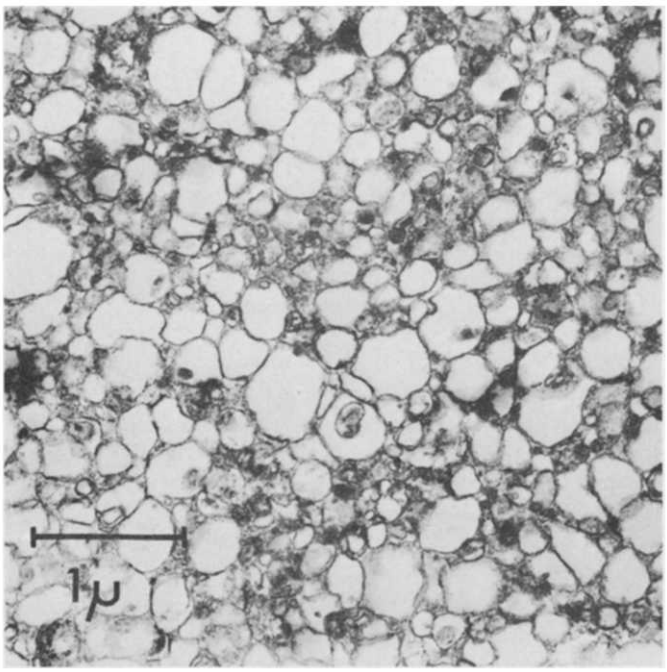

Fig. 2. Electron micrograph of human platelet membrane fraction.

enzyme could be kept indefinitely at that temperature. The results reported here were obtained using an undialysed enzyme preparation.

An electron micrograph of the membrane pellet is shown in Fig. 2. The fraction contains mainly membrane-like structures. The supernatant from the 105 000 × *g* membrane pellet showed only slight protein phosphokinase activity and so it was assumed that virtually all enzyme activity in the preparation was membrane-bound.

Properties

The reaction velocity of the platelet protein phosphokinase was measured and found to be linear during the incubation time. The specific activity of the soluble preparation ranged from 190 to 407 pmol ^{32}P incorporated/mg protein per min while the membrane preparation ranged from 12.3 to 20.9 pmol ^{32}P/mg per min. Total protein phosphokinase activity recovered in the soluble fraction was about 20 times greater than that recovered in the membrane preparation. $5 \cdot 10^{-7}$ M cyclic AMP activated the soluble enzyme by an average of 110% and the membrane enzyme by an average of 105%. Fig. 3 shows the inverse plots of percentage stimulation of protein phosphokinase activity as a function of cyclic AMP concentration. The K_m for cyclic AMP ranged from $4.0 \cdot 10^{-8}$ M to $1.7 \cdot 10^{-7}$ M for the soluble enzyme and from $9.3 \cdot 10^{-8}$ M to $1.1 \cdot 10^{-7}$ M for the membrane-bound enzyme. Cyclic GMP, the only other cyclic nucleotide tested, had no effect on any of the protein peaks eluted from the DEAE-cellulose column at a concentration of $5 \cdot 10^{-7}$ M although at $5 \cdot 10^{-6}$ M it activated the Peak 2 enzyme approximately 45%. The Lineweaver-Burk plots

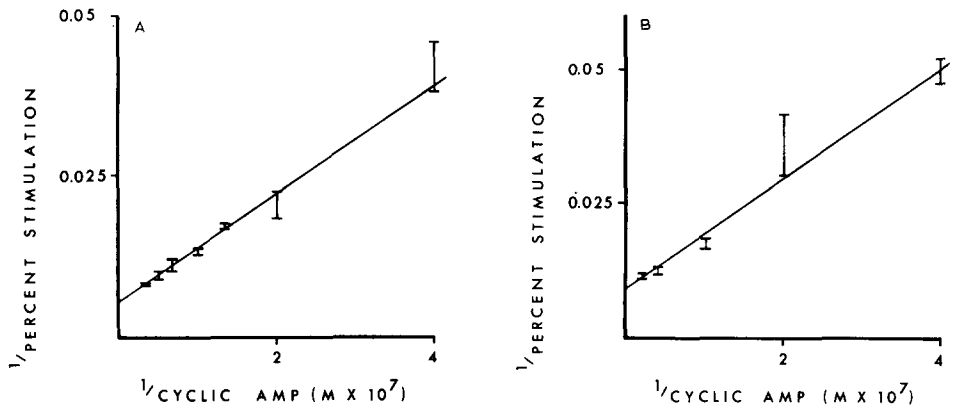

Fig. 3. Inverse plot of percentage stimulation of protein phosphokinase activity as a function of cyclic AMP concentration (error bars show range of duplicates). (A) soluble enzyme preparation, (B) membrane-bound preparation.

for ATP are shown in Fig. 4. The K_m for ATP ranged from $6.6 \cdot 10^{-6}$ M to $9.0 \cdot 10^{-6}$ M for the soluble enzyme and from $1.4 \cdot 10^{-5}$ M to $1.6 \cdot 10^{-5}$ M for the membrane-bound enzyme.

The effect of ADP, AMP, adenosine, adenine and RA233 is shown in Table I. $5 \cdot 10^{-5}$ M ADP inhibited the soluble enzyme approximately 70% both in the presence and absence of cyclic AMP. The activity of the membrane-bound enzyme was similarly inhibited by ADP by approximately 60%. $5 \cdot 10^{-6}$ M ADP inhibited the soluble enzyme about 20% but had no significant effect on the activity of the membrane protein phosphokinase. $5 \cdot 10^{-5}$ M AMP inhibited the membrane protein phosphokinase approximately 60% and the soluble enzyme by 50%. $5 \cdot 10^{-6}$ M AMP inhibited the soluble enzyme approximately 20% and the membrane enzyme to a slightly lesser degree. $5 \cdot 10^{-6}$ M adenosine had a slight inhibitory effect on the soluble protein phosphokinase preparation. $5 \cdot 10^{-6}$ M adenine and $5 \cdot 10^{-6}$ M RA233 had no significant effect on the soluble enzyme.

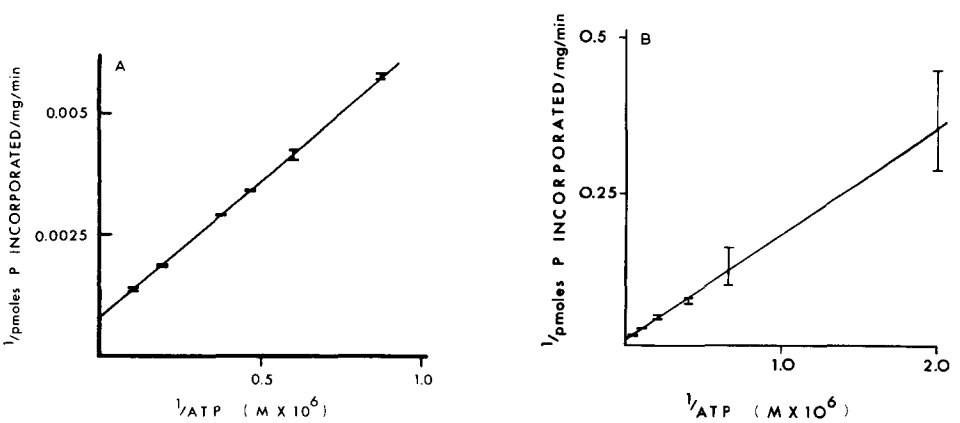

Fig. 4. Lineweaver-Burk plot of protein phosphokinase activity as a function of ATP concentration (error bars show range of duplicates). (A) soluble enzyme preparation, (B) membrane-bound preparation.

TABLE I

EFFECTS OF INDUCERS AND INHIBITORS OF PLATELET AGGREGATION ON PROTEIN PHOSPHOKINASE ACTIVITY OF PLATELET MEMBRANE AND SOLUBLE FRACTIONS

The results are calculated from at least 6 observations using at least 2 different enzyme preparations.

	Soluble fraction				Membrane fraction			
	−cyclic AMP		$5 \cdot 10^{-7}$M cyclic AMP		−cyclic AMP		$5 \cdot 10^{-7}$M cyclic AMP	
	% Change in activity	S.E. (%)	% Change in activity	S.E. (%)	% Change in activity	S.E. (%)	% Change in activity	S.E. (%)
$5 \cdot 10^{-5}$M ADP	−67.2*	4.6	−68.9*	1.2	−60.0*	3.2	−56.0*	1.6
$5 \cdot 10^{-6}$M ADP	−24.4*	4.6	−17.6*	4.1	−4.5	2.6	−2.2	3.7
$5 \cdot 10^{-5}$M AMP	−51.4*	4.5	−52.8*	4.1	−60.5*	5.7	−64.6*	3.8
$5 \cdot 10^{-6}$M AMP	−16.0*	1.6	−26.2*	1.7	−12.4	2.3	−19.2*	1.4
$5 \cdot 10^{-6}$M Adenosine	− 8.7*	0.8	− 8.1*	1.0				
$5 \cdot 10^{-6}$M Adenine	− 4.7	2.4	− 8.1	2.3				
$5 \cdot 10^{-6}$M RA233	+ 3.0	3.4	+ 4.3	3.8				

* Denotes a value significantly different from the control at a 1% level of confidence as calculated by a one tailed paired "t" test.

The effect of calcium on protein phosphokinase activity in the presence of Mg^{2+} is shown in Fig. 5. 10 mM Ca^{2+} inhibited soluble protein phosphokinase activity approximately 79% in the absence of cyclic AMP and approximately 82% in the presence of $5 \cdot 10^{-7}$ M cyclic AMP. The inhibition of the membrane-bound enzyme by Ca^{2+} was significantly different from that of the soluble enzyme. 10 mM Ca^{2+} inhibited the enzyme only 38% in the absence of cyclic AMP and 62% with $5 \cdot 10^{-7}$ M cyclic AMP.

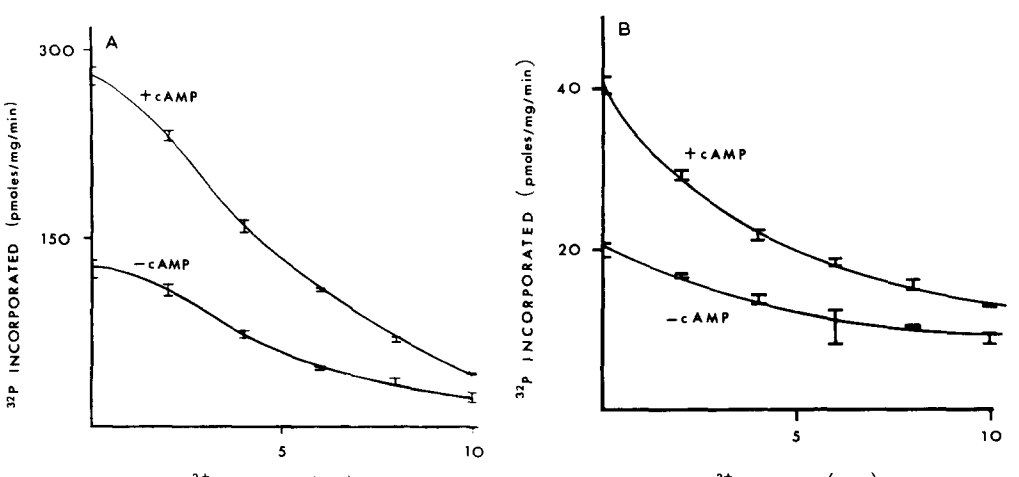

Fig. 5. The effect of Ca^{2+} on protein phosphokinase activity in the presence and absence of $5 \cdot 10^{-7}$ M cyclic AMP (error bars show range of duplicates). (A) soluble enzyme preparation, (B) membrane-bound preparation.

Discussion

Cyclic AMP has been implicated in platelet aggregation and one possible mode of action is through cyclic AMP-dependent protein phosphokinase. This paper reports results for protein phosphokinases purified from both the soluble and membrane fractions of platelets. Both enzyme preparations exhibit similar kinetics, being partially dependent on cyclic AMP. ADP and AMP have an inhibitory effect on the enzyme activity of both fractions. The K_m for cyclic AMP and for ATP are similar for both the soluble and membrane-bound enzymes and correspond to the preliminary results of Salzman [5]. However, there is a significant difference between the membrane-bound and soluble enzymes in their reaction to Ca^{2+}. 10 mM Ca^{2+} inhibits soluble protein phosphokinase approximately 80% both in the presence and absence of $5 \cdot 10^{-7}$ M cyclic AMP whereas the same concentration of Ca^{2+} inhibits membrane-bound protein phosphokinase activity approximately 60% in the presence of $5 \cdot 10^{-7}$ M cyclic AMP and only 40% in the absence of cyclic AMP. The difference between the soluble and membrane-bound enzymes in their reaction to Ca^{2+} may be due to either the presence of modifier proteins in the platelet membrane or soluble preparation or a mixture of protein phosphokinases (probably in the platelet membrane fraction) which display different kinetics. ADP, which is a potent initiator of platelet aggregation inhibited both the soluble and membrane-bound protein phosphokinases approximately 50—60% at a concentration of $5 \cdot 10^{-5}$ M. The significance of this result to in vitro or in vivo aggregation is questionable because ADP exerts its physiological effects (in vitro) at a concentration range between $5 \cdot 10^{-7}$ M and $5 \cdot 10^{-6}$ M [11]. At these concentrations ADP causes little or no inhibition of protein phosphokinase activity. Booyse et al., in a preliminary report [6], have demonstrated a 70%—95% inhibition of protein phosphokinase activity by ADP although they do not quote the concentration of ADP which was used. (The enzyme was purified by DEAE-cellulose column chromatography of an $(NH_4)_2SO_4$ fraction of platelet membranes). They conclude that inhibition of membrane-bound protein phosphokinase by ADP is associated with initiation of aggregation. Our results do not support this hypothesis because (a) the concentration of ADP needed to cause a 50% inhibition of our membrane-bound enzyme is two orders of magnitude greater than the concentration which induces platelet adhesiveness, and (b) AMP, which does not induce platelet aggregation [12] has an effect similar to ADP on protein phosphokinase activity. Of possible interest is the relationship between Ca^{2+} and cyclic AMP in their effects on protein phosphokinase activity. Feinman and Detwiler have shown that disturbance of the intracellular platelet calcium flux by divalent cation ionophores induces the platelet release reaction [13] and propose a tentative model in which the increased concentration of available intraplatelet calcium is the common pathway of induction of the platelet release reaction. The platelet release reaction is a necessary precursor to irreversible platelet aggregation [14,15]. In contrast, increasing the concentration of intraplatelet cyclic AMP appears to be the common pathway of inhibition of platelet aggregation [2].

The opposite effects of cyclic AMP and Ca^{2+} on platelet aggregation are reflected in their opposite effects on platelet protein phosphokinase activity.

This may indicate a central role for this enzyme in the regulation of platelet aggregation.

Acknowledgements

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