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ADENOSINE CYCLIC 3',5'-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE FROM HUMAN PLATELETS

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Summary

A single cyclic AMP-dependent protein kinase (EC 2.7.1.37) has been isolated from human platelets by using DEAE-cellulose ion-exchange chromatography and Sephadex G-150 gel filtration. The molecular weight of the protein kinase was estimated to be 86 490. In the presence of cyclic AMP, the protein kinase could be dissociated into a catalytic subunit of molecular weight 50 000, and either one regulatory subunit of molecular weight 110 000 or two regulatory subunits of molecular weights 110 000 and 38 100, depending on the pH used. Recombination of either of the regulatory subunits with the catalytic subunit restored cyclic AMP-dependency in the catalytic subunit.

The apparent K_m for ATP in the presence of 10 μM Mg^{2+} was 4 μM (plus cyclic AMP) and 4.3 μM (minus cyclic AMP). The concentration of cyclic AMP needed for half-maximal stimulation of the protein kinase was 0.172 μM and apparent dissociation constants of 3.7 nM (absence of MgATP) and 0.18 μM (presence of MgATP) were exhibited by the "protein kinase-cyclic AMP complex". The enzyme required Mg^{2+} for maximum activity and showed a pH optimum of 6.2 with histone as substrate.

In addition to four major endogenous platelet protein acceptors of apparent molecular weights 45 000, 28 000, 18 500, and 11 100, the platelet protein kinase also phosphorylated the exogenous acceptor proteins thrombin, collagen and histone, all capable of inducing platelet aggregation. Prothrombin, a nonaggregating agent, was not phosphorylated.

Introduction

Cyclic AMP-dependent protein kinases (ATP:protein phosphotransferase, EC 2.7.1.37) have been isolated from a wide variety of sources [1] and it is

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now generally believed that these proteins are the molecular receptors for cyclic AMP and are also responsible for the various effects of this nucleotide [2,3]. These proteins are composed of a regulatory subunit and a catalytic subunit and appear to relay the effect of this nucleotide by phosphorylation of specific protein acceptors in the cell [3—10].

The ability of blood platelets to interact with each other (aggregate) in response to a wide variety of agents forms the basis of the physiological role of these cells in maintaining normal hemostasis. A major problem in studying the mechanism of platelet aggregation has been the apparent differences in mechanism of action of aggregating agents such as ADP, epinephrine, thrombin and collagen. However, compounds known to influence platelet aggregation have been shown to affect the metabolism of platelet cyclic AMP [11—15]. These data have suggested that widely different agents affecting platelet function, in fact, have a common effect on the cyclic AMP levels of the platelet. A hypothesis has emerged suggesting that agents that increase cellular cyclic AMP levels will inhibit platelet aggregation and agents that decrease cellular cyclic AMP levels will induce aggregation [11—15].

The purpose of this communication is to describe the properties of the cyclic AMP-dependent protein kinase in human platelets in order to better understand the role of cyclic AMP and protein kinase catalyzed phosphorylation of specific endogenous and exogenous protein acceptors in platelet function.

Experimental Procedure

Materials

Histone (type IIA) and cyclic nucleotides were purchased from Sigma. Crystalline bovine thrombin obtained from Dr. Dan Walz, Detroit. [γ-^{32}P] ATP (spec. act. = 30 Ci/mmol) was obtained from ICN and cyclic [^{3}H] AMP from New England Nuclear. Sephadex G-150 was obtained from Mallinckrodt Chemical Works. All other chemicals were of analytical reagent grade and were obtained from commercial sources.

Methods

Preparation of platelets. Platelets were isolated from 10—12 units of freshly drawn citrated human blood by differential centrifugation as described previously [16]. Isolated platelets were washed three times with 0.15 M NaCl.

Preparation of protein kinase from human platelets. Washed platelets (7—8 ml of packed cells) were resuspended in four times their volume of 20 mM Tris · HCl buffer, pH 7.4, containing 5 mM 2-mercaptoethanol. Cells were lysed by the addition of digitonin to a final concentration of 0.1%. After 15—30 min of extraction with shaking at 4°C, the platelet lysate was centrifuged at 48 200 × *g* (20 000 rev./min supernatant) for 30 min. The supernatant was removed and the pH adjusted to 5.5 with 1% acetic acid. The precipitate was removed by centrifugation at 48 200 × *g* for 45 min and the resulting supernatant adjusted to pH 7.0. Ammonium sulfate was added to 60% saturation and the protein precipitate collected by centrifugation at 10 000 × *g* for 30 min. The precipitated protein was resuspended in 3 ml of 5 mM 2-mercapto-

ethanol/20 mM Tris · HCl, pH 7.4, dialyzed for 14 h and chromatographed on a DEAE-cellulose column (1.5 cm × 28 cm) equilibrated with the same buffer. Each of the fractions (2.5 ml) containing cyclic AMP-binding and/or catalytic activity, were pooled separately and concentrated by ultrafiltration (1 ml; 0.5—2 mg protein). Peak II, containing both cyclic [^{3}H]AMP-binding and catalytic activity was dialyzed against 5 mM 2-mercaptoethanol/0.1 M KCl/20 mM phosphate buffer, pH 6.7 (Buffer A) for 14 h and further chromatographed on a Sephadex G-150 column (1.5 cm × 90 cm) equilibrated with Buffer A.

Preparation of cyclic [^{3}H]AMP-binding and catalytic units from the cyclic AMP-dependent protein kinase. The cyclic AMP-dependent protein kinase (II) from the Sephadex G-150 column was concentrated by ultrafiltration (1 ml; 0.1—0.3 mg protein). This fraction was incubated for 15 min at 4°C with 5 μM, final concentration, of cyclic [^{3}H]AMP (Spec. Act. = 50 mCi/mmol). The dissociated protein kinase fraction was rechromatographed on the same Sephadex G-150 column (described above) containing 1 μM cyclic AMP. Fractions containing cyclic [^{3}H]AMP-binding and catalytic activity were pooled separately and the fractions designated as IIa and IIc, respectively. Pooled fractions were concentrated by ultrafiltration (IIa, 1 ml, 0.1—0.15 mg protein; IIc, 1 ml, 0.05—0.1 mg protein). A second smaller cyclic [^{3}H]AMP-binding unit was prepared by dialysis of IIa against 5 mM 2-mercaptoethanol/0.1 M KCl/20 mM Tris · HCl, pH 7.4 (Buffer B) for 20 h at 4°C, followed by gel filtration on a Sephadex G-150 column, 1.5 cm × 90 cm, in Buffer B. Cyclic [^{3}H]AMP-binding fractions were pooled, concentrated by ultrafiltration (1 ml; 0.05—0.075 mg protein) and designated as IIb. Positions of the cyclic AMP-binding fractions were determined by pipetting 0.3 ml fractions into scintillation vials containing 10 ml of Aquasol scintillation medium (New England Nuclear) and counting the fractions in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3380. Catalytic activity was assayed for on 0.05—0.1 ml fractions as described in the kinase assay.

Recombination experiments were performed on these catalytic and regulatory subunits after dialysis for 10 h against Buffer B.

Gel filtration. A Sephadex G-150 column (1.5 cm × 92 cm) was equilibrated at 4°C in 1 mM 2-mercaptoethanol/0.01 M KCl/20 mM Tris · HCl, pH 7.4. Samples (0.5—2 ml; 0.1—5 ml/mg protein) were placed on the column and 0.6 ml fractions were collected. The column was calibrated with globular proteins of known molecular weight. The void volume was taken as the elution volume of blue dextran. Protein standards were detected by their absorbance at 280 nm. Apparent molecular weights of unknown proteins were estimated according to the method of Andrews [19].

Protein kinase assay. Assays were performed at pH 6.5 in a standard reaction mixture of 0.2 ml containing: 20 mM potassium phosphate; 10 mM $MgCl_2$; 2 μM cyclic AMP (where indicated); 5 μM [γ-^{32}P]ATP (Spec. Act. = 0.6 mCi/mmol); 30 μg histone and 2—50 μg of enzyme. The reaction was initiated by addition of the enzyme fraction, and incubated for 10 min at 30°C. Reactions were terminated by addition of 0.5 ml of 10% trichloroacetic acid. Bovine serum albumin (0.5 mg) was added to each tube and the tubes were left on ice for 15 min. Protein precipitates were removed by centrifugation, the

protein pellet dissolved in 0.1 ml of 1 M NaOH and precipitated again with 1 ml of 10% trichloroacetic acid. After 15 min on ice, the precipitated protein was collected by filtration on glass fiber filters (Whatman GF/A filters, 2.4 cm). Protein precipitates were washed with 20 ml of 5% trichloroacetic acid, dried and counted as described in a previous section in 10 ml of a toluene solution containing 0.4% 2,5-diphenyloxazole (PPO) and 0.03% 1,4-bis[2-(4-methyl-5-phenyloxazolol)] benzene (dimethyl POPOP).

Binding of cyclic AMP. Cyclic AMP-binding activity was assayed by a slight modification of the technique described by Gilman [20]. Binding assays were carried out in a final volume of 0.2 ml containing: 50 mM potassium phosphate buffer, pH 7.0; 2 mM 2-mercaptoethanol; 5 mM magnesium acetate; 5 nM cyclic [^{3}H] AMP (Spec. Act. = 38.15 Ci/mmol); and 2—100 μg of binding protein. Incubations were carried out at 4°C and after 1 h, 1 ml of 20 mM potassium phosphate buffer, pH 6.0 was added to each reaction mixture. Maximal binding was observed within 50 min. After 5 min, the solutions were filtered through 24 mm cellulose ester (Millipore) filters (0.45 μ). The filters were washed with 10 ml of cold potassium phosphate buffer, pH 6.0 and air-dried. Dried filters were dissolved in 1 ml of Cellosolve in scintillation vials and the radioactivity determined by liquid scintillation spectrophotometry as described by Gilman [20].

The apparent dissociation constant of the cyclic AMP-protein kinase complex was determined in Buffer A. Protein kinase (10 μg) was incubated with various concentrations (0.1—20 μM) of cyclic [^{3}H] AMP (Spec. Act. = 50 mCi/mmol) in a final volume of 0.2 ml and the cyclic [^{3}H] AMP binding determined as described above. The data from the saturation curve was replotted according to the Sips equation [21] and the number of binding sites and the dissociation constant estimated from the intercepts on the Y and X axis, respectively.

Preparation of endogenous phosphoprotein acceptors from human platelets. Freshly isolated, washed platelets (1—2 ml washed cells) were resuspended in 4 volumes of 1 mM dithiothreitol/0.6 M KCl/20 mM Tris · HCl, pH 7.4, and lysed by the addition of digitonin. The cell-lysate was stirred overnight at 4°C and centrifuged at 40 000 × *g* for 45 min. The supernatant was adjusted to 30% $(NH_4)_2SO_4$ saturation and the protein precipitate discarded. Ammonium sulfate was added further to the supernatant to 90% saturation and the protein precipitates collected by centrifugation at 10 000 × *g* for 30 min. The protein pellet was resuspended in 0.1 M KCl/1 mM dithiothreitol/20 mM potassium phosphate buffer, pH 6.5 and dialyzed for 16—18 h against the same buffer.

In the endogenous acceptor assay, histone was substituted with 10 μg of acceptor. Incorporation of ^{32}P into protein was studied by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Frozen gels were sliced (2 mm slices) and each slice dissolved in 1 ml of 2% periodic acid at 50°C for 12 h. Solubilized gels were counted in Aquasol as described in a previous section.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was carried out on protein samples dissolved and dialyzed at room temperature for 14—16 h against solubilizing buffer containing 1% sodium dodecyl sulphate, 2.5% (v/v) 2-mercaptoethanol, 5 M urea, and 0.1 M sodium phosphate buffer, pH 7.1. Polyacrylamide gels were prepared by

a modification of the method described by Dunker and Rueckert [22]. The cross-linking agent, *N,N'*-methylene-bis-acrylamide, was substituted with *N,N'*-diallyltartardiamide to achieve solubilization of gel slices in 2% periodic acid. Electrophoresis on single 7.5% acrylamide gels, 12 cm in length, containing 0.1% sodium dodecyl sulphate, 5 M urea and 1% 2-mercaptoethanol, was carried out at room temperature for 18—20 h at 2 mA per gel. Gels were stained with 0.25% Amido black in 50% methanol for 1 h at room temperature. Gels were destained with 70% methanol containing 7% acetic acid. Radioactivity was determined on unstained, sliced gels as described in a previous section. The molecular weights of the proteins studied were determined by electrophoresis of these proteins with standard proteins of known molecular weight in the same experiment.

Protein determination. Protein was determined by a microbiuret method using bovine serum albumin as the standard [23]. Prior to each determination, samples were dialyzed against distilled water for 14 h at 4°C to eliminate interference by Tris, dithiothreitol or mercaptoethanol.

Results

Isolation of platelet cyclic AMP-dependent protein kinase. The dialyzed 0—60% $(NH_4)_2SO_4$ precipitate (2—4 ml, 6—12 mg protein) was applied onto a DEAE-cellulose column and after washing the column with 100 ml of equilibrating buffer (5 mM 2-mercaptoethanol/20 mM Tris · HCl, pH 7.4), elution was carried out with a 600 ml linear concentration gradient of 0—0.35 M KCl, in the same buffer. Both cyclic [^{3}H] AMP-binding and kinase activities were measured on each of the 2.5 ml effluent fractions as described under Methods. Three peaks of activity were eluted and designated as I, II and III (Fig. 1). Peak I had only cyclic [^{3}H] AMP-binding activity, whereas II had both cyclic [^{3}H] AMP-binding and kinase activity, and III kinase activity only. II alone was stimulated by cyclic AMP. Gel filtration of II (1 ml, 1.2 mg, see Table I),

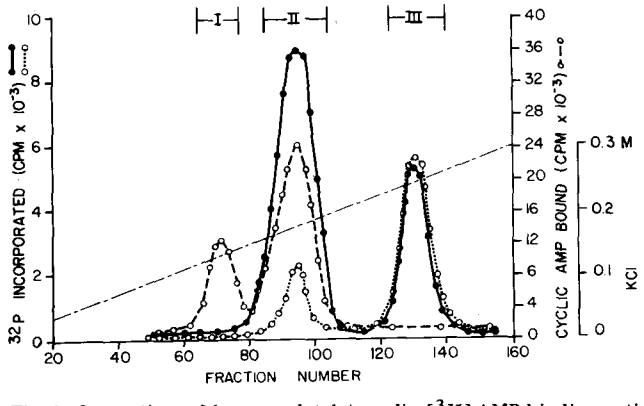

Fig. 1. Separation of human platelet cyclic [^{3}H] AMP-binding activity (I), cyclic AMP-dependent protein kinase activity (II), and cyclic AMP-independent protein kinase activity (III) by DEAE-cellulose (1.5 cm × 28 cm) chromatography; 2.5 ml fractions were collected. Detailed experimental procedure was as described in the text. Kinase activity was assayed (●———●) in the presence and absence (○- - - - -○) of cyclic AMP.

TABLE I

PURIFICATION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE FROM HUMAN PLATELETS

Freshly isolated human platelets (7—8 ml packed cells) were treated as described in the text. Total activity was determined in the presence of 5 μM cyclic AMP. One activity unit is defined as that amount of enzyme necessary to catalyze the transfer of 1 pmol of ^{32}P from [γ-^{32}P]ATP into histone per min at 30° C.

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
48200 × *g* supernatant	144.2	7943	55.08	100
Acid supernatant	30.7	6489	211.4	81.7
$(NH_4)_2SO_4$ (0—60%)	12.9	6315	489.5	79.5
DEAE-cellulose	1.2	4130	3441.7	51.8
Sephadex G-150	0.27	2478	9177	31.2

dialyzed against Buffer A, on Sephadex G-150 (see Methods) gave a single peak which contained both cyclic [^{3}H] AMP-binding and cyclic AMP-dependent protein kinase activity (Fig. 2A). This protein fraction was concentrated by ultrafiltration (1 ml, 0.2 mg protein), and used within 24 h after preparation. The cyclic AMP-dependent protein kinase (II) was purified about 167-fold with an overall recovery of about 31% of the total initial kinase activity (48 200 × *g* supernatant) (Table I). Total kinase activities were determined in the presence of 5 μM cyclic AMP. A typical stepwise isolation procedure is shown in Table I.

Properties of platelet cyclic AMP-dependent protein kinase

Dissociation of cyclic AMP-dependent protein kinase. Freshly isolated cyclic AMP-dependent protein kinase (II from Fig. 2A, 1 ml, 0.2 mg protein) was dissociated with cyclic [^{3}H] AMP and rechromatographed on Sephadex G-150 in Buffer A containing cyclic AMP (1 μM) as described under Methods.

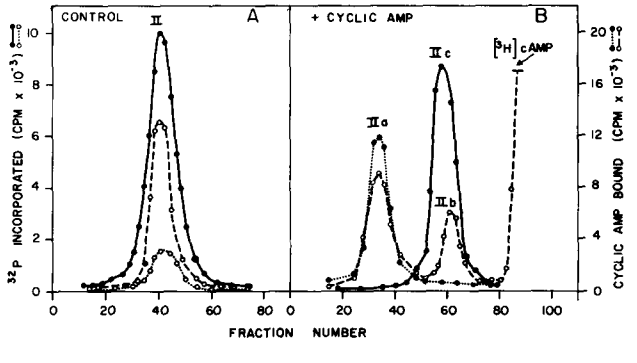

Fig. 2. Gel filtration of cyclic AMP-dependent protein kinase on Sephadex G-150 (1.5 cm × 90 cm). Preparation and application of the enzyme onto column and assay methods for protein kinase and cyclic [^{3}H] AMP-binding are as described in the text; 1 ml fractions were collected. Kinase activity was assayed in the presence (●———●) and absence (○- - - - -○) of cyclic AMP. A, undissociated protein kinase II in Buffer A; B, rechromatography of protein kinase II (from Fig. 2A) after dissociation with 5 μM cyclic AMP (Spec. Act. = 50 mCi/mmol). ●- - - - -●, in Buffer A; ○— — —○ in Buffer B.

Under these conditions (pH 6.7), the cyclic [^{3}H] AMP-binding and kinase activities were completely separated as shown in Fig. 2B. A single cyclic [^{3}H] AMP-binding peak (IIa) and a single kinase peak (IIc) were eluted. Peak IIc kinase activity was not affected by cyclic AMP. Peaks IIa and IIc were pooled separately, concentrated by ultrafiltration (IIa, 1 ml, 0.1—0.15 mg; IIc, 1 ml, 0.05—0.1 mg protein, Spec. Act. = 900—1000 units/mg) and used in subsequent recombination experiments.

However, if the cyclic AMP-dependent protein kinase (II from Fig. 2A) was first dialyzed against Buffer B (pH 7.4), dissociated with cyclic AMP and rechromatographed on Sephadex G-150 (see Methods), equilibrated with Buffer B, two cyclic [^{3}H] AMP-binding peaks, IIa and IIb and a single kinase peak IIc, were eluted (Fig. 2B). The larger of the two cyclic [^{3}H] AMP-binding peaks (fractions 30—35) eluted in the same position as the single cyclic [^{3}H] AMP-binding peak IIa obtained after dissociation in Buffer A. A second smaller cyclic [^{3}H] AMP-binding peak IIb was eluted as part of the kinase activity peak IIc (Fig. 2B). Fractions 55—65, containing both cyclic [^{3}H] AMP-binding and kinase activity were not affected by cyclic AMP and eluted in the same position as the dissociated kinase activity IIc obtained in Buffer A. Further attempted dissociation and rechromatography of this peak (fractions 55—65) in the presence of 1 μM cyclic AMP, did not alter the elution volumes of either the cyclic [^{3}H] AMP-binding or kinase activities. The appearance of a second smaller cyclic [^{3}H] AMP-binding activity (IIb) did suggest the possible conversion of the larger cyclic [^{3}H] AMP-binding protein IIa to a lower molecular weight form.

Effect of pH on cyclic [^{3}H] AMP-binding subunits. Cyclic [^{3}H] AMP-binding subunit IIa (Buffer A) was concentrated by ultrafiltration (1 ml, 0.1—0.15 mg protein; $1-2 \cdot 10^5$ cpm) and applied onto Sephadex G-150, equilibrated with Buffer A (see Methods). A single cyclic [^{3}H] AMP-binding peak was eluted without any apparent change in molecular weight or significant loss of bound cyclic [^{3}H] AMP (Fig. 3A).

In a separate experiment, cyclic [^{3}H] AMP-binding peak IIa was concentrated by ultrafiltration (1 ml, 0.1—0.15 mg protein), dialyzed against Buffer B for 20 h, and rechromatographed on the same Sephadex G-150 colum described above (in Buffer B). Two cyclic [^{3}H] AMP-binding peaks were eluted (Fig. 3B). The first peak (fraction number 75) eluted in the same position as the large cyclic [^{3}H] AMP-binding subunit IIa. The second smaller cyclic [^{3}H]-AMP-binding peak eluted in the same position (fraction number 102) as the cyclic [^{3}H] AMP-binding subunit IIb produced from II by dialysis against Buffer B (see Figs 2B and 3B).

These data suggested that the low molecular weight subunit IIb, produced by dissociation of II in Buffer B, was similar to the low molecular weight subunit produced from subunit IIa by dialysis against Buffer B.

Dialysis of IIa against Buffer B for less than 12—14 h resulted in very little or no detectable conversion of IIa to the lower molecular weight form IIb.

Phosphate acceptor proteins. Human platelet cyclic AMP-dependent protein kinase, in the presence of cyclic AMP, phosphorylated several exogenous proteins that induced platelet aggregation. Fig. 4 shows the relative effectiveness of substrate proteins histone, thrombin, collagen and prothrombin. It

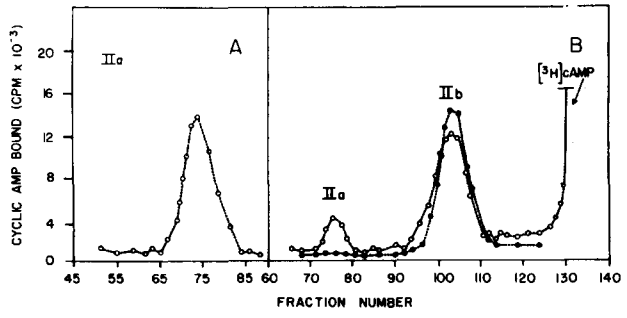

Fig. 3. Gel filtration of large cyclic [^{3}H] AMP-binding subunit IIa on Sephadex G-150 (1.5 cm × 90 cm). Subunit IIa was obtained by dissociation of the protein kinase in Buffer A as described in Fig. 2B. Experimental details are as described in text; 1 ml fractions were collected. A, ○- - - - -○, IIa in Buffer A; B, ○- - - - -○, rechromatography of IIa (from experiment in Fig. 3A) after dialysis against Buffer B for 20 h. ●- - - -●, small cyclic [^{3}H] AMP-binding subunit IIb obtained by dissociation of the protein kinase in Buffer B (see Fig. 2B).

should be noted that high concentrations of histone (>30 μg) are inhibitory and that histone is a better substrate for kinase catalyzed phosphorylation than either thrombin or collagen (Fig. 4). Thrombin and collagen showed no inhibitory effects over the concentration range studied. Prothrombin, a non-aggregating agent, was not phosphorylated by the cyclic AMP-dependent protein kinase. Bovine serum albumin, bovine casein and protamine sulfate were inactive as substrates, as reported previously [24].

The cyclic AMP-dependent protein kinase also phosphorylated four major

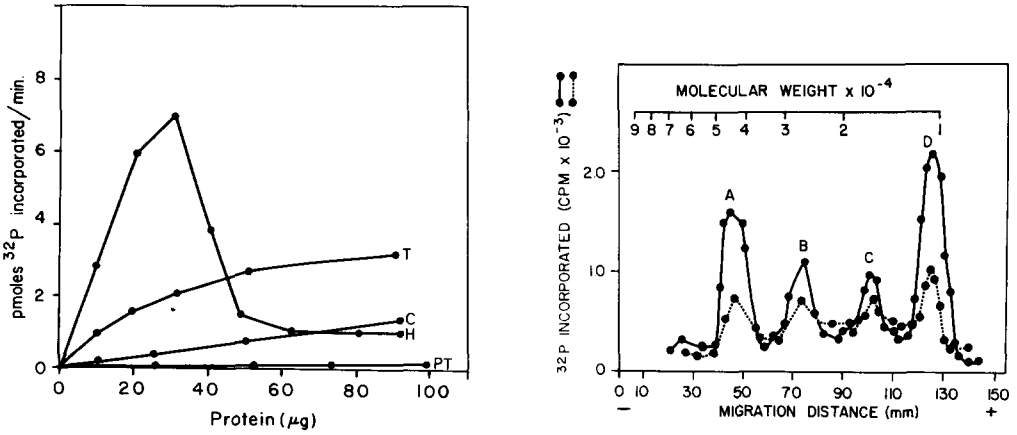

Fig. 4. Platelet protein kinase catalyzed phosphorylation of exogenous acceptor proteins capable of inducing platelet aggregation. T, thrombin; C, collagen; H, histone; and PT, prothrombin control (non-aggregating agent). Conditions for assay of protein kinase were as described in text. Reactions were carried out in the presence of 2 μM cyclic AMP. All values have been corrected for ^{32}P incorporation in the absence of added enzyme.

Fig. 5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of platelet protein kinase catalyzed phosphorylation of endogenous platelet acceptor proteins A, B, C, and D. Presence (●———●) and absence (●- - - - -●) of cyclic AMP. Conditions were as described in text.

endogenous human platelet acceptors. As shown in Fig. 5, the cyclic AMP-dependent protein kinase, in the presence of cyclic AMP, caused a marked increase in the phosphorylation of the platelet substrate proteins A, B, C and D with apparent subunit molecular weights of 45 000, 28 000, 18 500 and 11 100 respectively.

Effect of enzyme concentration, incubation time and pH. Incorporation of ^{32}P from [γ-^{32}P]ATP into histone with increasing enzyme concentration was linear up to 30 min at 30°C. Using histone as substrate, a pH optimum of 6.2 was found.

Effect of cyclic nucleotides. Cyclic AMP was far more effective than any other cyclic nucleotide in stimulating kinase activity and inhibiting subsequent cyclic [^3H]AMP-binding in the 10^{-6} M concentration range. Cyclic cytosine 3′,5′-monophosphate was about 30% as active as cyclic AMP in stimulating kinase activity.

A double reciprocal plot of cyclic AMP concentration against histone phosphorylation is shown in Fig. 6. The concentration of cyclic AMP necessary for half-maximal stimulation of kinase activity was 0.172 μM.

Cyclic AMP-binding. Binding of cyclic AMP to the cyclic AMP-dependent protein kinase from human platelets in the presence of MgATP, is shown in Fig. 7. A double reciprocal plot of the data yielded an apparent dissociation constant of 0.18 μM for the protein-cyclic AMP complex. A dissociation constant of 3.7 nM was obtained in the absence of MgATP. At saturating nucleotide concentrations, the protein kinase exhibited a maximum binding capacity of 16.6 pmol of cyclic AMP per μg of protein (Fig. 7).

Apparent K_m for ATP. A double reciprocal plot of ATP concentration

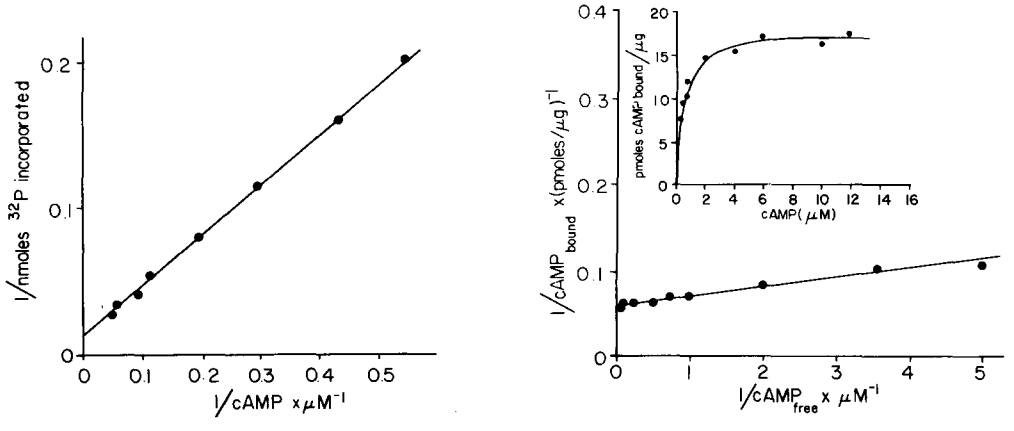

Fig. 6. Double reciprocal plot illustrating the effect of cyclic AMP concentration on the rate of histone phosphorylation by platelet protein kinase. Each assay contained 4.7 μg of enzyme and the assay conditions were as described in the text. The basal activity of the protein kinase was subtracted from the activity observed in the presence of cyclic AMP.

Fig. 7. Binding of cyclic AMP to platelet protein kinase in the presence of MgATP. Binding assays were carried out as described in text. The concentration of cyclic [^3H]AMP was varied over the range of 0.1—20 μM using 10 μg of protein kinase per assay. The data from the saturation curve (inset) were replotted according to Sips [21]. An estimate of the number of binding sites and dissociation constant is provided by the intercepts on the Y and X axes, respectively.

TABLE II

RECOMBINATION OF CYCLIC AMP BINDING SUBUNITS IIa AND IIb WITH CATALYTIC SUBUNIT IIc

Dialyzed subunits were recombined and incubated for 90 min at 4°C. Activities were determined under standard assay conditions as described in text. Each assay contained 10 μg of catalytic subunit IIc.

Fractions	Enzyme activity (units)	
	—Cyclic AMP	+5 μM Cyclic AMP
IIc	8.9	8.4
IIc + IIa*	5.7	8.8
IIc + IIb*	6.9	9.6
IIc + IIa**	0.9	8.2
IIc + IIb**	1.1	7.9

*Weight ratio (μg:μg) of IIa and IIb to IIc is 0.33:1.
**Weight ratio of IIa and IIb to IIc is 1.67:1.

against histone phosphorylation in the presence of 10 mM $MgCl_2$, is shown in Fig. 8. An apparent K_m for ATP of 4 μM and 4.2 μM was obtained in the presence and absence of 1 μM cyclic AMP, respectively. The presence or absence of cyclic AMP had little effect on the apparent K_m value, but increased the maximal velocity of the phosphorylation reaction 3 to 6-fold.

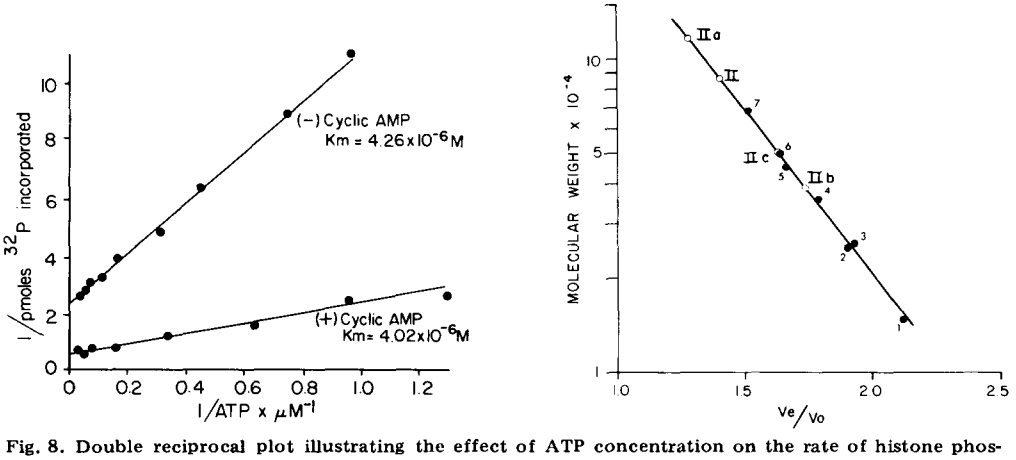

Fig. 8. Double reciprocal plot illustrating the effect of ATP concentration on the rate of histone phosphorylation by platelet kinase in the presence and absence of cyclic AMP (2 μM final concentration). Assays were performed in the presence of 10 mM Mg^{2+}. Assays conditions were as described in text. Each assay contained 5 μg of enzyme. Basal activities were not subtracted from activities observed in the presence of cyclic AMP.

Fig. 9. Calibration of a Sephadex G-150 column (1.5 cm × 92 cm) with globular proteins of known molecular weight (closed circles). 1, lysozyme; 2, light chain γ-globulin; 3, trypsin inhibitor; 4, pepsin; 5, ovalbumin; 6, heavy chain γ-globulin; 7, bovine serum albumin. Molecular weights of the platelet protein kinase and its subunits (open circles) were estimated according to the method of Andrews [19]; 0.6 ml fractions were collected. II, protein kinase; IIa and IIb, cyclic [^{3}H] AMP-binding subunits; IIc, catalytic subunits. Experimental conditions were as described in text.

Effect of divalent cations. The cyclic AMP-dependent protein kinase required Mg^{2+} for maximal activity. In the presence of 1 μM cyclic AMP, a 4 to 5-fold stimulation of protein kinase activity was observed with 10 mM Mg^{2+}, whereas no stimulation was seen with Zn^{2+}, Mn^{2+}, Fe^{2+} or Co^{2+}. In the presence of Ca^{2+}, the lowest basal kinase- and cyclic AMP-stimulated activities were found. When Co^{2+} was used, the highest basal kinase activity was obtained.

Molecular weight estimates. Molecular weights of the cyclic AMP-dependent protein kinase and its subunits were estimated by gel filtration on Sephadex G-150 as described in Methods. The Sephadex G-150 column was calibrated with seven standard proteins (Fig. 9). The undissolved protein kinase had an estimated molecular weight of 86 490. In the presence of cyclic AMP in Buffer A, the catalytic subunit IIc and the single cyclic [^{3}H] AMP-binding subunit IIa had estimated molecular weights of 50 000 and 110 000, respectively. In the presence of cyclic AMP in Buffer B, IIc eluted in the same position as above and the cyclic [^{3}H] AMP-binding subunits IIa and IIb eluted at apparent molecular weights of 110 000 and 38 100.

Recombination of regulatory and catalytic subunits. Catalytic subunit IIc was recombined with two different amounts of each of the cyclic [^{3}H] AMP-binding subunits, IIa and IIb. As shown in Table II, both cyclic [^{3}H] AMP-binding subunits inhibited the catalytic activity when the experiments were carried out in the absence of added cyclic AMP. No inhibition was present when cyclic AMP was added.

Discussion

A single major cyclic AMP-dependent protein kinase has been isolated from human platelets. This enzyme exhibits the same general properties as those described for protein kinases from skeletal muscle [25—27], heart muscle [28], erythrocytes [3,29,30], corpus luteum [31] and epididymal spermatozoa [32]. The cyclic AMP-dependent protein kinase from human platelets has a molecular weight of 86 490 and can be dissociated into a single catalytic (50 000) and either one (110 000) or two (110 000 and 38 100) regulatory subunits, depending on the conditions used. From the data on the molecular weight relationships and interconversion of the two species of cyclic [^{3}H]-AMP-binding subunits, we have concluded that the platelet cyclic AMP-dependent protein kinase consisted of one catalytic subunit and one monomer regulatory subunit.

The human platelet enzyme showed a requirement for Mg^{2+} and had an apparent K_m for ATP of 4 μM. This value is in agreement with the values of 0.52 μM [33] and 1 μM [34] reported previously for the crude platelet enzyme. Values ranging from 0.15 μM to 10 μM have been reported for protein kinases from various sources [3,25,29,32]. Half-maximal stimulation of platelet kinase activity was achieved at a concentration of 0.172 μM cyclic AMP. This value was essentially the same as the dissociation constant for cyclic AMP (0.18 μM), determined in the presence of MgATP. Recently, Beavo et al. [27] reported that the dissociation constant for cyclic AMP (0.2—0.3 μM) for the rabbit skeletal muscle enzyme in the presence of MgATP, was essentially the

same as the concentration of cyclic AMP needed (0.2—0.3 μM) to produce half-maximal activity.

Kaulen and Gross recently reported on the partial purification and properties of a cyclic AMP-dependent protein kinase from human platelets [35]. These authors obtained an apparent molecular weight of 200 000 by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. However, we have also been unable to detect the presence of a second larger platelet kinase under the conditions described in this paper. Whether these two isolated proteins, in fact, represent different protein kinases in the platelet or merely different forms of the same enzyme remains to be further established.

A general hypothesis has been proposed, suggesting that the diverse effects of cyclic AMP in various tissues and species are mediated through regulation of the activity of protein kinases [1,36]. Increased or decreased phosphorylation of a specific protein kinase acceptor(s) would result in an altered functional state of the protein acceptor, thereby producing the specific physiological effects of cyclic AMP.

Protein kinase-catalyzed phosphorylation of a variety of enzymes [4,5], membrane proteins [6—8] and contractile proteins [9,37,38] have been described. The function of the protein kinase in human platelets is not known. However, this enzyme phosphorylates at least four major endogenous protein substrates (acceptor proteins A, B, C, and D, see Fig. 5). Preliminary data has been published on the nature, function and localization of endogenous acceptor protein D [39,40,41]. This membrane-associated acceptor can also be phosphorylated in intact platelets in the presence of [γ-^{32}P] ATP and exhibits high Ca-binding in the phosphorylated state. In addition, the platelet kinase shows a remarkable specificity for several exogenous protein acceptors that have the common action of inducing platelet aggregation.

Although the full significance of this specificity is not understood at this time, we have obtained data indicating that addition of an aggregating agent (e.g., thrombin) to intact platelets, in the presence of [γ-^{32}P] ATP, decreased phosphorylation of the Ca-binding protein with simultaneous phosphorylation of the added agent [39—41]. On the basis of these data, we have suggested that this Ca-binding protein is required to maintain a low level of free Ca^{2+} within the platelet and that aggregating agents decreased the phosphorylated state and hence Ca-binding function of the membrane associated protein. This in turn may result in the net influx of Ca^{2+} into, and/or mobilization of membrane-bound Ca^{2+} within the platelet [39—41]. Presumably, this available Ca^{2+} within the cell triggers the sequence of events leading to platelet aggregation. Cyclic AMP-dependent protein kinase phosphorylation, subsequent Ca-binding (immobilization) and non-aggregating platelets are consistent with the hypothesis that high platelet cyclic AMP levels do not favor aggregation [11—15].

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References

1 Kuo, J.F. and Greengard, P. (1969) Proc. Natl. Acad. Sci. U.S. 64, 1349—1355
2 Corbin, J.D., Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1970) J. Biol. Chem. 245, 4849—4851
3 Rubin, C.S., Erlichman, J. and Rosen, O.M. (1973) J. Biol. Chem. 247, 6135—6139
4 Walsh, D.A., Perkins, J.P. and Krebs, E.G. (1968) J. Biol. Chem. 243, 3763—3765
5 Friedman, D.L. and Larner, J. (1965) Biochemistry 4, 2261—2264
6 Weller, M. and Rodright, R. (1970) Nature 225, 187—188
7 Rose, A.D. and Appel, S.H. (1973) J. Biol. Chem. 248, 1408—1411
8 Delorenzo, R.J., Walton, K.G., Curran, P.F. and Greengard, P. (1973) Proc. Natl. Acad. Sci. U.S. 70, 880—884
9 Perrie, W.T., Smillie, L.B. and Perry, S.V. (1972) Biochem. J. 128, 105P—106P
10 Traugh, J.A., Mumby, M. and Traut, R.R. (1973) Proc. Natl. Acad. Sci. U.S. 70, 373—376
11 Marquis, N.R., Vigdahl, R.P. and Tauormina, P.A. (1969) Biochem. Biophys. Res. Commun. 36, 965—972
12 Salzman, E.W. and Neri, L.L. (1969) Nature 224, 609—610
13 Salzman, E.W. and Levine, L. (1971) J. Clin. Invest. 50, 131—141
14 Salzman, E.W., Rubino, E.B. and Sims, R.V. (1971) Ser. Haematol. III (No. 4), 197—208
15 Wolfe, S.M. and Shulman, N.R. (1970) Biochem. Biophys. Res. Commun. 41, 128—134
16 Booyse, F.M., Hoveke, T.P. and Rafelson, M.E., Jr. (1968) Biochim. Biophys. Acta 157, 660—663
17 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
18 Schachman, H.K. and Edilstein, S.J. (1966) Biochemistry 5, 2681—2705
19 Andrews, P. (1964) Biochem. J. 91, 222—233
20 Gilman, A. (1970) Proc. Natl. Acad. Sci. U.S. 67, 305—312
21 Eisen, H.N. (1964) Methods Med. Res. 10. 106—111
22 Dunker, A.K. and Rueckert, R.R. (1969) J. Biol. Chem. 244, 5074—5080
23 Itzhaki, R.F. and Gill, D.M. (1964) Anal. Biochem. 9, 401—410
24 Booyse, F.M., Guiliani, D., Marr, J. and Rafelson, M.E., Jr. (1973) Ser. Haematol. VI (No. 3), 351—366
25 Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1970) J. Biol. Chem. 246, 1986—1995
26 Corbin, J.D., Brostrom, C.O., King, C.A. and Krebs, E.G. (1972) J. Biol. Chem. 247, 7790—7798
27 Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3580—3583
28 Rubin, C.S., Erlichman, J. and Rosen, O.M. (1972) J. Biol. Chem. 247, 36—44
29 Tao, M. and Hackett, P. (1973) J. Biol. Chem. 248, 5324—5332
30 Traugh, J.A. and Traut, R.R. (1974) J. Biol. Chem. 249, 1207—1212
31 Menon, K.M.J. (1973) J. Biol. Chem. 248, 494—501
32 Garbers, D.L., First, N.L. and Lardy, H.A. (1973) J. Biol. Chem. 248, 875—879
33 Salzman, E.W. and Weisenberger, H. (1972) Adv. Cyclic Nucl. Res. 1, 231—247
34 Marquis, N.R., Vigdahl, R.L. and Tauormina,P.A. (1971) Fed. Proc. 30, 423
35 Kaulen, H.D and Gross, R. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 471—480
36 Kuo, J.F. and Greengard, P. (1969) J. Biol. Chem. 244, 3417—3419
37 Stull, J.T. Brostrom, C.O. and Krebs, E.G. (1972) J. Biol. Chem. 247, 5272—5274
38 Adelstein, R.S. and Conti, M.A. (1973) Fed. Proc. 32, 569
39 Booyse, F.M., Marr, J., Guiliani, D. and Rafelson, Jr, M.E. (1974) Circulation 50 (Suppl. 3), 289
40 Booyse, F.M., Marr, J., Yang, D., Guiliani, D. and Rafelson, Jr, M.E. (1975) Excepta Medica in press
41 Rafelson, Jr, M.E. and Booyse, F.M. (1975) A Dynamic Phosphorylation-Dephosphorylation Model for Platelet Aggregation and its Control: Frontiers in Biology. (S. Karger, ed.) AG, Basel, Switzerland.