Characterization of the protein kinase activities of human platelet supernatant and particulate fractions

Suzette E. SALAMA and Richard J. HASLAM

Department of Pathology, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

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After human platelets were lysed by freezing and thawing in the presence of EDTA, about 35% of the total cyclic AMP-dependent protein kinase activity was specifically associated with the particulate fraction. In contrast, Ca^{2+} -activated phospholipid-dependent protein kinase was found exclusively in the soluble fraction. Photoaffinity labelling of the regulatory subunits of cyclic AMP-dependent protein kinase with 8-azido-cyclic [^{32}P]AMP indicated that platelet lysate contained a 4-fold excess of 49000-Da R_I subunits over 55000-Da R_{II} subunits. The R_I and R_{II} subunits were found almost entirely in the particulate and soluble fractions respectively. Chromatography of the soluble fraction on DEAE-cellulose demonstrated a single peak of cyclic AMP-dependent activity with the elution characteristics and regulatory subunits characteristic of the type-II enzyme. A major enzyme peak containing Ca^{2+} -activated phospholipid-dependent protein kinase was eluted before the type-II enzyme, but no type-I cyclic AMP-dependent activity was normally observed in the soluble fraction. The particulate cyclic AMP-dependent protein kinase and associated R_I subunits were solubilized by buffers containing 0.1 or 0.5% (w/v) Triton X-100, but not by extraction with 0.5M-NaCl, indicating that this enzyme is firmly membrane-bound, either as an integral membrane protein or via an anchor protein. DEAE-cellulose chromatography of the Triton X-100 extracts demonstrated the presence of both type-I cyclic AMP-dependent holoenzyme and free R_I subunits. These results show that platelets contain three main protein kinase activities detectable with histone substrates, namely a membrane-bound type-I cyclic AMP-dependent enzyme, a soluble type-II cyclic AMP-dependent enzyme and Ca^{2+} -activated phospholipid-dependent protein kinase, which was soluble in lysates containing EDTA.

The inhibition of platelet aggregation and degranulation by agents that increase platelet cyclic AMP is associated with enhanced phosphorylation of several specific platelet proteins (for review see Haslam *et al.*, 1980). Thus, in intact ^{32}P -labelled platelets, prostaglandin E_1 causes increased labelling of membrane-bound polypeptides (M_r 24000 and 22000) that may stimulate the active transport of Ca^{2+} ions (Haslam *et al.*, 1979; Fox *et al.*, 1979; Käser-Glanzmann *et al.*, 1979), as well as of various soluble polypeptides of unknown function (M_r 50000 and 36000; Haslam *et al.*, 1980). Moreover, purified platelet myosin light-chain kinase is inhibited after phosphorylation by the catalytic subunit of cyclic AMP-dependent

protein kinase (Hathaway *et al.*, 1981). These studies have emphasized the importance of cyclic AMP-dependent protein phosphorylation in the regulation of platelet function.

Although several studies using exogenous substrates have demonstrated cyclic AMP-dependent protein kinase activity in the soluble fraction of human platelets (Kaulen & Gross, 1974; Booyse *et al.*, 1976), in platelet membranes (Steiner, 1975) or in both fractions (Salzman & Weisenberger, 1972; Bishop & Rozenberg, 1975; Lyons *et al.*, 1975; Salama & Haslam, 1981a), published information on the relationship between these forms of the enzyme and their identity remains incomplete or contradictory. Two major classes of cyclic AMP-dependent protein kinase (type I and type II) can be distinguished by their order of elution from DEAE-cellulose (Corbin *et al.*, 1975; Hofmann *et al.*, 1975), which reflects differences in their regu-

Abbreviations used: SDS, sodium dodecyl sulphate; R_I and R_{II} , regulatory subunits of type-I and type-II cyclic AMP-dependent protein kinases.

latory (R_I or R_{II}) subunits (see Flockhart & Corbin, 1982). The ratio of these isoenzymes and their subcellular distribution differ according to the cell type studied (Corbin *et al.*, 1975, 1977; Dreyfuss *et al.*, 1978; Walter *et al.*, 1978). However, with human platelets, chromatography of soluble protein kinases on DEAE-cellulose has given variable results. Only one peak of cyclic AMP-dependent activity, not identified by the authors concerned, was observed in some studies (Kaulen & Gross, 1974; Booyse *et al.*, 1976; Kawahara *et al.*, 1980), whereas others found both type-I and type-II isoenzymes in the soluble fraction (Käser-Glanzmann *et al.*, 1979; Haslam *et al.*, 1980). This discrepancy may have been due to a variable release of membrane-bound enzyme. Thus much more cyclic AMP-dependent protein kinase activity was found in the platelet particulate fraction when platelets were lysed in the presence rather than in the absence of inhibitors of Ca^{2+} -dependent proteinase (Salama & Haslam, 1981a). Two studies utilizing 8-azido-cyclic [^{32}P]AMP as a photoaffinity label have shown that, when platelets were sonicated, the R_{II} regulatory subunits of platelet cyclic AMP-dependent protein kinase were found almost exclusively in the supernatant fraction, whereas at least half of the R_I subunits were found in the particulate fraction (Lyons, 1980; Salama & Haslam, 1981a). However, a third study (Chambers *et al.*, 1982) failed to detect R_{II} regulatory subunits in any subcellular fraction, though R_I subunits were found in all platelet fractions. In the present work, we have sought to clarify the subcellular distribution of the isoenzymes of cyclic AMP-dependent protein kinases in human platelets, using both DEAE-cellulose chromatography and photoaffinity labelling.

Some workers who have studied the protein kinase activities present in the supernatant fraction of platelets have observed elution of a peak of cyclic AMP-independent protamine kinase or histone kinase activity from DEAE-cellulose (Booyse *et al.*, 1976; Yamaki *et al.*, 1982) or histone-Sepharose (Lyons *et al.*, 1975), but only at a higher ionic strength than was required to elute cyclic AMP-dependent activity. In contrast, Kawahara *et al.* (1980) showed that platelet Ca^{2+} -activated phospholipid-dependent protein kinase was eluted from DEAE-cellulose before the cyclic AMP-dependent enzyme. We therefore also studied the subcellular distribution and chromatographic behaviour of the platelet cyclic AMP-independent protein kinases that could be detected with histones as substrates, with particular emphasis on the Ca^{2+} -activated phospholipid-dependent enzyme, which is considered to play an important role in platelet activation (Kawahara *et al.*, 1980; Takai *et al.*, 1982).

Some of our results have been reported in a preliminary form (Salama & Haslam, 1981b).

Experimental

Materials

Cyclic [$G\text{-}^3H$]AMP (36.7 Ci/mmol) was purchased from New England Nuclear Corp., Boston, MA, U.S.A., [$\gamma\text{-}^{32}P$]ATP (19.7 Ci/mmol) was from Amersham, Oakville, Ont., Canada, and 8-azido-cyclic [^{32}P]AMP (145 Ci/mmol) and phosphatidylserine were from ICN, Irvine, CA, U.S.A. Mixed calf thymus histone (type II-A), lysine-rich histone (type III-S), bovine serum albumin, diolein (85% 1,3-diolein, 15% 1,2-diolein), Triton X-100, cyclic AMP and ATP were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Glass-fibre filters (F2832-24) and DEAE-cellulose (DE 52) were from Whatman, Maidstone, Kent, U.K., and cellulose acetate filters (HAWP 024 12) from Millipore, Mississauga, Ont., Canada.

Methods

Preparation of platelet fractions. Platelets were prepared from freshly drawn human blood and were washed three times at 4°C in a medium containing 135 mM-NaCl, 5 mM-glucose and 13 mM-trisodium citrate that was adjusted to pH 6.5 with HCl (Haslam & Lynham, 1972). The final suspension, containing either 25 or 150 mg wet wt. of platelets/ml, was mixed with 0.02 vol. of 250 mM-EDTA (adjusted to pH 8.0 with NaOH) to give a final pH of 7.0 and an EDTA concentration of 5 mM. This suspension was rapidly frozen in solid CO_2 /acetone and then thawed in a water bath at 37°C with shaking to prevent the temperature rising above 0°C. Supernatant and particulate fractions were separated by centrifugation for 30 min at 37 500 g_{av} at 4°C. Platelet particulate fractions were resuspended in the original volume of platelet lysis medium or in half the original volume of extraction buffers. In some experiments, platelets were disrupted by sonication, and supernatant and particulate fractions were prepared as described by Salama & Haslam (1981a).

Sequential extraction of cyclic AMP-dependent protein kinase from platelet particulate fraction. This fraction was extracted first with buffer containing 10 mM- K_2HPO_4 (adjusted to pH 7.0 with H_3PO_4), 2 mM-2-mercaptoethanol and 1 mM-EDTA (Buffer A) to which 0.1 M-NaCl was added, then with Buffer A containing 0.5 M-NaCl, and finally with Buffer A containing 0.5 M-NaCl and 0.1% or 0.5% (w/v) Triton X-100. All extractions were performed with a Dounce homogenizer with a B pestle

(ten strokes), after which the homogenate was stirred for 30 min at 0°C. After each extraction, samples were centrifuged for 30 min at 37 500g_{av}. at 4°C. Some supernatants containing Triton X-100 were re-centrifuged at 100 000g_{av}. for 1 h to verify that all the enzyme activity present was soluble. Pellets were either resuspended in Buffer A containing 0.1 M-NaCl for assay or used in the next step in the extraction procedure. Supernatant fractions were dialysed overnight at 4°C against Buffer A containing 0.1 M-NaCl before assay.

DEAE-cellulose chromatography of platelet fractions. Platelet supernatant was first dialysed against Buffer A. The particulate fraction enzyme was solubilized with Buffer A containing 0.5% Triton X-100 (see above), and the 37 500g_{av}. supernatant was dialysed overnight against Buffer A containing 0.1% Triton X-100 and an additional 1 mM-EDTA. All column manipulations were performed at 4°C, and DEAE-cellulose columns (0.9 cm × 7 cm) were equilibrated with the buffer used for dialysis of the platelet protein fraction. The dialysed material was applied to the column, which was then washed with dialysis buffer until no further protein emerged (approx. 40 ml). Protein kinases were then eluted with a 300 ml linear gradient of 0–0.4 M-NaCl in the same buffer. Fractions (2.5 ml) were collected at a flow rate of 15 ml/h. NaCl concentrations in the column fractions were measured with a refractometer.

Protein kinase assays. For measurement of the cyclic AMP-dependent protein kinase activity, assay mixtures (final vol. 0.2 ml) contained 10 mM-potassium phosphate buffer, pH 7.0, 2 mM-2-mercaptoethanol, 2.5 mg of mixed histone/ml, 10 mM-MgCl₂ and 0.2 mM-[γ -³²P]ATP (7.5 Ci/mol) with or without 5 μ M-cyclic AMP. The reaction was started by the addition of 0.1 ml of column fraction or 0.05 ml of platelet fraction (10–50 μ g of protein), and the mixtures were incubated for 10 min at 30°C. For measurement of the Ca²⁺-activated phospholipid-dependent protein kinase activity, a modification of the method of Kishimoto *et al.* (1980) was used. The reaction mixture (final vol. 0.25 ml) contained 60 mM-Tris/HCl, pH 7.5, 5 mM-MgCl₂, 0.16 mg of lysine-rich histone/ml and 10 μ M-[γ -³²P]ATP (120 Ci/mol) with or without activators (1 mM-CaCl₂, 40 μ g of phosphatidylserine/ml and 2 μ g of diolein/ml). The reaction was started by addition of 0.1 ml of column fraction or 0.05 ml of platelet fraction and the mixture was incubated for 3 min at 30°C. In both assays, enzyme activity was terminated by addition of trichloroacetic acid, and the ³²P-labelled protein was isolated on glass-fibre filters, washed and counted for ³²P radioactivity as described previously (Salama & Haslam, 1981a). One unit of protein kinase activity is defined as that

transferring 1 pmol of [³²P]phosphate from [γ -³²P]-ATP to histone/min at 30°C. Protein phosphorylation was linear with respect to time and platelet protein in both assays.

Binding of cyclic AMP to platelet proteins. A modification of the method of Sugden & Corbin (1976) was used. Column fraction (0.2 ml) with or without 0.02 ml of 0.5 mM-cyclic AMP was added to 0.28 ml of a solution containing 50 mM-potassium phosphate buffer, pH 7.0, 1 mM-EDTA, 0.5 mg of mixed histone/ml, 2 M-NaCl, and 0.03 μ M-cyclic [³H]AMP (36.7 Ci/mmol). The mixture was incubated for 60 min at 30°C, and then 1 ml of ice-cold 10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-EDTA was added. After mixing, 1 ml of this solution was filtered through a Millipore filter (HAWP 024 12) moistened with the same buffer. The filter was rinsed with 3 × 8 ml of this buffer and dried in an oven at 150°C. The ³H retained on the filters was measured by liquid-scintillation counting in 7.5 ml of Amersham ACS counting fluid. The specific binding of cyclic [³H]-AMP to protein (i.e. that displaced by unlabelled cyclic AMP) was calculated.

Photoaffinity labelling of cyclic AMP-binding proteins. This was performed with 0.5 μ M-8-azido-cyclic [³²P]AMP, as described previously (Salama & Haslam, 1981a). After their separation by SDS/polyacrylamide-gel electrophoresis, ³²P-labelled polypeptides were detected by autoradiography and quantified by using a Joyce-Loebl microdensitometer. The areas of peaks were determined by triangulation. Rabbit skeletal and bovine heart muscle supernatants were photoaffinity labelled to provide markers for the identification of the regulatory subunits of type-I and type-II cyclic AMP-dependent protein kinases (R_I and R_{II} respectively) (Salama & Haslam, 1981a).

Other methods. Protein was determined by the method of Lowry *et al.* (1951). Lactate dehydrogenase activity in platelet fractions was determined as described by Bergmeyer *et al.* (1965).

Results

Subcellular distribution of protein kinase activities in platelets

On separation of platelets lysed by freezing and thawing into supernatant and particulate fractions, 45 ± 2% (mean ± S.E.M., 22 expts.) of platelet protein, but only 17 ± 1% (13 expts.) of platelet lactate dehydrogenase, was found in the particulate material. This indicates that freezing and thawing released enzymes present in the platelet cytosol fairly effectively, though not quantitatively. Two sets of assay conditions were applied to these fractions, one appropriate for measurement of cyclic AMP-dependent activity and one appropriate for

Table 1. *Distribution of histone kinase activities in platelet supernatant and particulate fractions; effects of cyclic AMP and of Ca^{2+} + phosphatidylserine + diolein on enzyme activities*

Assays were all performed with material from the same platelet suspension (25 mg wet wt./ml). After separation of platelet lysate (0.9 mg of protein/ml) into supernatant and particulate fractions, the latter (48% of the total protein) was resuspended in the lysis medium (see the Experimental section). Assays were performed as described in the Experimental section, under conditions appropriate for measurement of cyclic AMP-dependent protein kinase activity (A) and of Ca^{2+} -activated phospholipid-dependent protein kinase activity (B). Results are means $\pm$ S.E.M. from triplicate assays. The percentages of the enzyme activities in the particulate fractions were calculated with respect to the sum of the activities found in both particulate and supernatant fractions.

Assay conditions	Substrate	Activators	Protein kinase activity (units/mg of protein)		Percentage of activity in particulate fraction
			Supernatant fraction	Particulate fraction	
A	Mixed histone	None	500 $\pm$ 29	680 $\pm$ 25	55.7
		Cyclic AMP	1172 $\pm$ 29	960 $\pm$ 34	43.2
		Ca^{2+} + phosphatidylserine + diolein	493 $\pm$ 21	610 $\pm$ 42	53.5
B	Lysine-rich histone	None	68 $\pm$ 16	91 $\pm$ 8	55.4
		Cyclic AMP	89 $\pm$ 10	148 $\pm$ 14	60.8
		Ca^{2+} + phosphatidylserine + diolein	856 $\pm$ 19	156 $\pm$ 14	14.5

Table 2. *Effects of different methods of preparing platelet particulate fractions on their content of cyclic AMP-dependent protein kinase*

In each experiment, platelets from a single donor were suspended at 25 mg wet wt./ml, half in the medium described in the Experimental section of the present paper (A) and half in Tyrode's solution containing 5 mM-EDTA (B), as described by Salama & Haslam (1981a). Samples of both platelet suspensions were frozen and thawed, and particulate material was isolated by centrifugation at 37500 g_{av} for 30 min (see the Experimental section). Other samples of the same suspensions were sonicated for 4 $\times$ 15 s and the particulate fractions were isolated by centrifugation at 100000 g_{av} for 60 min (Salama & Haslam, 1981a). Each particulate fraction was resuspended in the same medium as the platelets from which it was derived. The protein content, lactate dehydrogenase activities and cyclic AMP-dependent protein kinase activities of both the particulate and supernatant fractions were determined and the percentages of protein and of enzyme activity in the particulate fractions calculated. Results are means $\pm$ S.E.M. from three experiments.

Procedure for preparing platelet particulate fraction		Percentage of protein or enzyme activity in particulate fraction		
Lysis medium	Method of lysis	Protein	Lactate dehydrogenase	Cyclic AMP-dependent protein kinase
A	Freeze-thaw	49.3 $\pm$ 1.8	16.8 $\pm$ 2.1	55.4 $\pm$ 3.6
	Sonication	41.0 $\pm$ 1.1	3.5 $\pm$ 0.1	25.8 $\pm$ 1.8
B	Freeze-thaw	39.2 $\pm$ 1.2	11.5 $\pm$ 2.3	47.4 $\pm$ 4.4
	Sonication	34.0 $\pm$ 4.5	4.1 $\pm$ 0.8	19.2 $\pm$ 0.2

measurement of the Ca^{2+} -activated phospholipid-dependent enzyme (see the Experimental section). Thus, in the presence of mixed histone and 0.2 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at pH 7.0, cyclic AMP stimulated the protein kinase activity of platelet supernatant by about 2-fold [activity ratio (ratio of protein kinase activity in the absence of cyclic AMP to that in its presence) 0.49 ± 0.04 , 20 expts.] and that of the particulate fraction by about 1.4-fold (activity ratio 0.73 ± 0.02 , 20 expts.), but no Ca^{2+} -activated phospholipid-dependent activity was observed in either fraction (Table 1, A). In contrast, when lysine-rich histone, 10 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and pH 7.5 were used,

addition of Ca^{2+} , phosphatidylserine and diolein together increased the protein kinase activity of platelet supernatant by about 11-fold (10.7 ± 0.8 -fold, 5 expts.), but little cyclic AMP-dependent activity was detected (Table 1, B). In a series of experiments similar to that in Table 1, $52 \pm 3\%$ (mean $\pm$ S.E.M., 20 expts.) of cyclic AMP-dependent protein kinase activity, but only $17 \pm 3\%$ (5 expts.) of the Ca^{2+} -activated phospholipid-dependent protein kinase, was found in the particulate fraction. The latter activity had the same subcellular distribution as lactate dehydrogenase and was therefore soluble, at least under the condi-

tions of lysis used. The results suggested that about 35% of the platelet cyclic AMP-dependent protein kinase activity is genuinely particle-bound.

Previous work (Salama & Haslam, 1981a) with platelets suspended in Tyrode's solution containing EDTA and lysed by sonication indicated that the particulate fraction contained only 20–25% of the total cyclic AMP-dependent protein kinase activity. The present procedure was therefore compared with that followed in that previous study, by using platelets from the same donors (Table 2). The results indicated that the method of platelet lysis rather than the suspending medium was the main factor affecting the distribution of cyclic AMP-dependent protein kinase. Particulate fraction from sonicated platelets contained less than half the cyclic AMP-dependent protein kinase activity found in a similar fraction from frozen and thawed platelets. Parallel measurements of lactate dehydrogenase indicated that only about one-third of this difference could be accounted for by the presence of incompletely lysed platelets in the latter preparation (Table 2). These findings indicate the existence of a substantial fraction of platelet cyclic AMP-dependent protein kinase (15–20% of the total activity) that is normally associated with the particulate fraction, but is released into the supernatant when platelets are sonicated.

Photoaffinity labelling of cyclic AMP-binding proteins in platelet supernatant and particulate fractions

Photoaffinity labelling of fractions from frozen and thawed platelets by 8-azido-cyclic [32 P]AMP (see, e.g., Fig. 1, lanes *a*, *b* and *f*) showed that the supernatant contained $88 \pm 4\%$ (mean $\pm$ S.E.M., 6

expts.) of the platelet content of a labelled polypeptide of 55000Da, suggesting that this polypeptide was entirely soluble, whereas the particulate fraction contained $85 \pm 4\%$ (mean $\pm$ S.E.M., 6 expts.) of a more highly labelled polypeptide of 49000Da. Comparison of these polypeptides with those observed on photoaffinity labelling of supernatants from bovine heart (lane *l*) and rabbit skeletal muscle (lane *m*) indicated, as previously reported (Salama & Haslam, 1981a), that the first represents non-phosphorylated R_{II} subunits of type-II cyclic AMP-dependent protein kinase and the second represents R_I subunits of the type-I enzyme. Densitometry of the autoradiographs showed that the ratio of R_I to R_{II} subunits in platelet lysate was $4.2 \pm 0.6:1$ (6 expts.). In addition, photoaffinity labelling of supernatant fraction revealed a minor 52000-Da labelled polypeptide (lane *f*), and the particulate fraction contained labelled polypeptides (e.g. one of 38000Da) that are probably proteolytic breakdown products of R_I subunits (lane *b*).

Extraction of cyclic AMP-dependent protein kinase from platelet particulate fraction

The nature of the binding of cyclic AMP-dependent protein kinase by platelet particulate fraction was investigated by sequential extraction of this fraction with various media. Both cyclic AMP-dependent enzyme activity and the photoaffinity labelling of polypeptides by 8-azido-cyclic [32 P]AMP were determined in the extracts and corresponding pellets. Homogenization of particulate fraction with Buffer A containing 0.1 M-NaCl

Fig. 1. Extraction of cyclic AMP-binding proteins from platelet particulate fraction; analysis by photoaffinity-labelling with 8-azido-cyclic [32 P]AMP

Platelet particulate fraction was prepared from suspension containing 25mg wet wt. of platelets/ml and was extracted sequentially with various media, as described in the Experimental section. The particulate fractions and corresponding extracts were photoaffinity-labelled with 8-azido-cyclic [32 P]AMP and electrophoresed on SDS/polyacrylamide slab gels. An autoradiograph is shown: *a*, platelet lysate; *b*, untreated platelet particulate fraction; *c*, residual particulate fraction after extraction with Buffer A + 0.1 M-NaCl; *d*, residual particulate fraction after extraction with Buffer A + 0.5 M-NaCl; *e*, residual particulate fraction after extraction with Buffer A + 0.5 M-NaCl + 0.5% Triton X-100; *f*, dialysed platelet supernatant fraction; *g*, extract obtained with Buffer A + 0.1 M-NaCl; *h*, extract obtained with Buffer A + 0.5 M-NaCl; *i*, dialysed extract as in *h*; *j*, extract obtained with Buffer A + 0.5 M-NaCl + 0.5% Triton X-100; *k*, dialysed extract as in *j*; *l*, bovine heart supernatant; *m*, rabbit skeletal muscle supernatant.

solubilized about one-quarter of the particle-bound enzyme ($13 \pm 2\%$ of total platelet activity; mean $\pm$ S.E.M., 9 expts.). As much of the residual lactate dehydrogenase ($9 \pm 1\%$ of total platelet activity, 4 expts.) was extracted at the same time, this protein kinase activity largely represents release of soluble enzyme from incompletely lysed platelets. However, photoaffinity labelling showed that homogenization with 0.1M-NaCl also removed some particle-bound R_I subunits (Fig. 1, lane g). The cyclic AMP-dependent protein kinase activity remaining in the particulate fraction after this step amounted to $38 \pm 2\%$ of the total platelet enzyme activity (mean $\pm$ S.E.M., 9 expts.). Very little of this protein kinase activity (on average only 2% of the

total platelet enzyme), or of the R_I subunits remaining (Fig. 1, lanes h and i), were solubilized by further extraction with Buffer A containing 0.5M-NaCl. However, subsequent extraction with Buffer A containing 0.5M-NaCl and 0.1% or 0.5% Triton X-100 solubilized most of the remaining particulate enzyme activity and R_I subunits (Fig. 1, lanes j and k). Thus, when 0.1% Triton X-100 was used, $84 \pm 3\%$ (mean $\pm$ S.E.M., 4 expts.) of this particulate enzyme activity was solubilized. Traces of R_{II} subunits found in the particulate fraction were also extracted with Triton X-100 (Fig. 1, lanes j and k). These results suggest that about 35% of the total platelet cyclic AMP-dependent protein kinase activity is firmly membrane-bound.

Fig. 2. DEAE-cellulose chromatography of the protein kinases in platelet supernatant fraction. Platelets suspended in an iso-osmotic medium containing 5mM-EDTA were lysed by freezing and thawing. After centrifugation of these lysates, the supernatants were dialysed against Buffer A and chromatographed on DEAE-cellulose (see the Experimental section). (a) and (b) show elution profiles from different supernatants that were chromatographed in an identical manner. (a) Column fractions were assayed for protein kinase activity in the presence ($\blacktriangle$) and in the absence ($\triangle$) of cyclic AMP, with mixed histone as the substrate. Binding of cyclic [3 H]AMP to protein was also measured ($\bullet$). The concentrations of NaCl in the fractions are shown ($\blacklozenge$). Arrows (a–d) indicate fractions subjected to photoaffinity labelling with 8-azido-cyclic [32 P]AMP and then electrophoresed on a SDS/polyacrylamide slab gel. An autoradiograph of the dried gel is shown in the insert. R_I and R_{II} indicate the positions of marker photoaffinity-labelled regulatory subunits that were electrophoresed in parallel (see Fig. 1). (b) Column fractions were assayed for protein kinase activity in the presence ($\blacktriangledown$) and in the absence ($\triangledown$) of Ca^{2+} + phosphatidylserine + diolein, with lysine-rich histone as the substrate. Concentrations of protein ($\blacksquare$) and NaCl ($\blacklozenge$) in the fractions are also shown.

Fig. 3. DEAE-cellulose chromatography of cyclic AMP-dependent protein kinase in a Triton X-100 extract of platelet particulate fraction

Platelet suspension containing 25mg wet wt. of platelets/ml was used. The particulate fraction was isolated and extracted with Triton X-100 as described in the Experimental section, and the dialysed extract (25mg of protein) was chromatographed. Fractions were assayed for protein kinase activity in the presence (●) and in the absence (○) of cyclic AMP, with mixed histone as the substrate, and for binding of cyclic [^3H]AMP by protein (■). The NaCl concentration is shown (▲). The insert shows the autoradiograph obtained after the column fractions indicated (a, b) were photoaffinity-labelled with 8-azido-cyclic [^{32}P]AMP and analysed by SDS/polyacrylamide-gel electrophoresis. R_I and R_{II} indicate the positions of marker photoaffinity-labelled regulatory subunits that were electrophoresed in parallel (see Fig. 1).

DEAE-cellulose chromatography of platelet protein kinases

Enzymes in the supernatant fraction. Chromatography of supernatant prepared from platelets frozen and thawed in the presence of EDTA revealed a minor peak of cyclic AMP-independent enzyme activity eluted by 0.03–0.10M-NaCl and a major peak of cyclic AMP-dependent enzyme eluted by 0.11–0.18M-NaCl (Fig. 2a). The second peak alone contained protein that could bind cyclic AMP, as determined by using cyclic [^3H]AMP or by photoaffinity labelling with 8-azido-cyclic [^{32}P]AMP. The latter technique demonstrated the presence of both the 55000-Da R_{II} subunit and the 52000-Da polypeptide, but little or no 49000-Da polypeptide (R_I subunit), indicating that this peak contains type-II cyclic AMP-dependent enzyme activity (Fig. 2a). Assay of Ca^{2+} -activated phospholipid-dependent protein kinase after DEAE-cellulose chromatography of platelet supernatant demonstrated high activity only in the fractions eluted by 0.03–0.13M-NaCl (Fig. 2b).

If EDTA was omitted from the platelet lysis medium, a completely different pattern of protein kinase activities was observed (results not shown).

Two peaks of cyclic AMP-dependent histone kinase and of cyclic [^3H]AMP-binding were obtained, and photoaffinity labelling of these showed that the first contained the 49000-Da R_I subunits normally confined to the particulate fraction and the second contained smaller labelled polypeptides, apparently proteolytic breakdown products of R_I and R_{II} subunits. The peak of Ca^{2+} -activated phospholipid-dependent activity was markedly attenuated when EDTA was omitted from the platelet lysis medium and was largely replaced by a peak of Ca^{2+} - and phospholipid-insensitive activity, detectable with mixed or lysine-rich histone, that was eluted by 0.17–0.22M-NaCl.

Enzyme in the particulate fraction. DEAE-cellulose chromatography of a 0.5% Triton X-100 extract of platelet particulate fraction showed one major peak of protein kinase activity that was eluted by 0.04–0.10M-NaCl and that was stimulated 6-fold by cyclic AMP (Fig. 3). Measurement of cyclic [^3H]AMP binding showed two peaks, one of which corresponded to the enzyme activity and the second of which was eluted by 0.10–0.15M-NaCl. Photoaffinity labelling of fractions with 8-azido-cyclic [^{32}P]AMP showed that both peaks of

cyclic AMP-binding protein contained 49000-Da R_1 subunits of cyclic AMP-dependent protein kinase. Type-II activity was either absent or present in very small amounts.

Discussion

We have used a combination of DEAE-cellulose chromatography and photoaffinity labelling with 8-azido-cyclic [32 P]AMP to identify the cyclic AMP-dependent protein kinases present in the supernatant and particulate fractions of platelets. The results explain many of the discrepancies in the literature and show that platelets contain a soluble type-II isoenzyme and a particulate type-I isoenzyme, part of which can be released into the supernatant fraction by sonication of the platelets or endogenous Ca^{2+} -activated proteolysis. When platelets were lysed by freezing and thawing in the presence of EDTA, a mild procedure that releases most of the soluble enzymes as monitored by lactate dehydrogenase activity, a complete separation of the two forms of cyclic AMP-dependent protein kinase was achieved.

The platelet supernatant fraction contained cyclic AMP-dependent protein kinase activity that was eluted from DEAE-cellulose at a salt concentration typical of the type-II enzyme. This enzyme peak contained the approx. 55000-Da platelet polypeptide previously shown to be photoaffinity labelled by 8-azido-cyclic [32 P]AMP (Lyons, 1980; Salama & Haslam, 1981a). This polypeptide co-electrophoreses with both the non-phosphorylated form of the bovine heart R_{II} subunit and the R_{II} subunit from rabbit skeletal muscle (Salama & Haslam, 1981a). In addition, the platelet soluble fraction contained a minor photoaffinity-labelled polypeptide of 52000 Da (Lyons, 1980; Salama & Haslam, 1981a). This was also eluted with the type-II enzyme and could represent a variant or proteolytic fragment of the R_{II} subunit. A similar peptide has been observed in brain (Lohmann *et al.*, 1980). The failure of Chambers *et al.* (1982) to detect R_{II} subunits in human platelets probably stems from the low concentration of 8-azido-cyclic [32 P]AMP that they used (0.01–0.1 μ M). Lyons (1980) has shown that, for unknown reasons, complete photoaffinity labelling of the platelet R_{II} subunit requires at least 0.4 μ M reagent.

After correction for trapped soluble enzyme, about 35% of the total cyclic AMP-dependent protein kinase activity of frozen and thawed platelets was calculated to be particle-bound. A similar value was obtained after extraction of trapped enzymes by gentle homogenization. As these steps were performed in buffers containing at least 0.1 M NaCl, it is unlikely that any of this activity reflects

non-specific binding of catalytic subunits to the platelet membranes (Keely *et al.*, 1975). Moreover, 0.5 M NaCl, which dissociates the subunits of type-I enzyme (Corbin *et al.*, 1975), extracted neither catalytic activity nor regulatory subunits from the particulate fraction, whereas solutions containing 0.1–0.5% Triton X-100 extracted both. Thus the platelet particle-bound enzyme could be an integral membrane protein, perhaps inserted as a result of post-translational modification. Alternatively, it could be firmly bound via an anchor protein. Triton X-100 has previously been shown to extract holoenzyme from the membranes of several other tissues and cell types, including red cells (Uno *et al.*, 1976; Corbin *et al.*, 1977; Dreyfuss *et al.*, 1978; Rubin, 1979). Similarly to the red-cell enzyme, the solubilized platelet protein kinase was eluted from DEAE-cellulose at a salt strength typical of the type-I isoenzyme and was shown to contain R_1 subunits.

The particulate fraction from platelets sonicated in the presence of EDTA contained only about half the cyclic AMP-dependent protein kinase activity found in the same fraction from platelets frozen and thawed in the presence of EDTA. Even less activity was present when sonication was performed in the absence of inhibitors of Ca^{2+} -dependent proteinase (Salama & Haslam, 1981a). The latter result indicates that Ca^{2+} -dependent proteolysis can release the type-I isoenzyme into the supernatant fraction. It is less clear how sonication can solubilize some of this enzyme in the presence of EDTA, though it is possible that sonication liberates a Ca^{2+} -insensitive proteinase that attacks membrane-bound proteins. However, Käser-Glanzmann *et al.* (1979), who observed type-I activity in the supernatant from sonicated platelets, found that this enzyme fraction was more effective in phosphorylating a 22000-Da membrane polypeptide than the type-II enzyme. This suggests that the enzyme may be released in a form that can still associate with platelet membranes under appropriate conditions. Very similar observations to ours have been made with human leucocytes by Juhl & Esmann (1979), who found that type-I enzyme activity did not appear in the supernatant from gently disrupted cells, but was present after vigorous sonication.

Although the total particulate type-I cyclic AMP-dependent protein kinase activity present in platelets appeared less than the soluble type-II activity, the ratio of R_1 to R_{II} subunits, as measured by photoaffinity labelling, was about 4:1 in the present study and 2.4:1 in a previous study in which the 52000-Da polypeptide was included with that of 55000 Da (Lyons, 1980). This suggests that R_1 subunits may be present in excess of the available catalytic subunits. In support of this

view, we observed free R_1 subunits, as well as holoenzyme, on DEAE-cellulose chromatography of the solubilized particulate material. Cyclic AMP-binding activity free of catalytic activity has also been observed on chromatography of platelet supernatants prepared under conditions in which some endogenous proteolysis is likely to have occurred (Booyse *et al.*, 1976; Haslam *et al.*, 1980). In the present study, in which 5 mM-EDTA was used, only trace amounts of R_1 subunit were encountered in the first supernatant, though homogenization of the particulate fraction in 0.1 M-NaCl released some R_1 subunits. The functional significance of excess R_1 subunits in the platelet is uncertain, but it is possible that they buffer the effects of changes in platelet cyclic AMP.

Our results also show that Ca^{2+} -activated phospholipid-dependent protein kinase is found exclusively in the soluble fraction of platelets lysed in the presence of EDTA, as was previously noted by Kikkawa *et al.* (1982). As this enzyme is soluble under conditions in which the type-I cyclic AMP-dependent protein kinase is membrane-bound, DEAE-cellulose chromatography of platelet supernatant permits rapid preparation of Ca^{2+} -activated phospholipid-dependent protein kinase free of cyclic AMP-dependent activity. In brain, the Ca^{2+} -activated enzyme binds to synaptic membranes in the presence of Ca^{2+} ions (Takai *et al.*, 1979). We have similarly found that 70–80% of the platelet enzyme remains in the particulate fraction when platelets are frozen and thawed in the presence of 1 mM- $CaCl_2$, provided that leupeptin is added to block Ca^{2+} -dependent proteolysis (S. E. Salama & R. J. Haslam, unpublished work). However, when neither EDTA nor leupeptin was present in the platelet lysis medium, DEAE-cellulose chromatography of the supernatant fraction showed that most of the Ca^{2+} -activated phospholipid-dependent protein kinase was replaced by a Ca^{2+} - and phospholipid-insensitive enzyme eluted at a higher ionic strength. This phenomenon may explain the results of others (Booyse *et al.*, 1976; Yamaki *et al.*, 1982), who found a cyclic AMP-independent protein kinase activity in platelets that was eluted from DEAE-cellulose after the type-II cyclic AMP-dependent enzyme, as those workers also lysed or froze their platelets in media that did not contain EDTA. Nishizuka and colleagues have shown that Ca^{2+} -activated phospholipid-dependent protein kinase from brain is converted by Ca^{2+} -dependent proteolysis into a Ca^{2+} -independent enzyme that is eluted from DEAE-cellulose at a higher ionic strength (Takai *et al.*, 1977; Inoue *et al.*, 1977; Kishimoto *et al.*, 1983). Our results suggest that the same process readily occurs in platelet material in the absence of EDTA.

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