SUBCELLULAR DISTRIBUTION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY AND OF CYCLIC AMP-BINDING PROTEINS IN HUMAN PLATELETS

Modification by Ca^{2+}-dependent proteolysis

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1. Introduction

Increases in platelet cAMP inhibit platelet function [1], an effect that is associated with the phosphorylation of M_r 22 000 and 24 000 membrane polypeptides in intact platelets [2,3]. The presence of both soluble [4–7] and membrane-bound [8] cAMP-dependent protein kinases has been reported in platelets. In one study, addition of soluble protein kinase with the ion-exchange characteristics of the type I enzyme was required for the cAMP-dependent phosphorylation of the M_r 22 000 membrane polypeptide [7], but preliminary experiments in this laboratory have indicated that a membrane-bound cAMP-dependent protein kinase can phosphorylate this polypeptide [3]. We have, therefore, investigated the distribution of cAMP-dependent protein kinase activity and of photoaffinity-labelled cAMP-binding proteins between the particulate and supernatant fractions of platelets sonicated under various conditions. The results showed that provided proteolysis was prevented, the particulate fraction contained about 20% of the total platelet cAMP-dependent protein kinase activity and about 60% of the type I cAMP-binding regulatory subunits (R_I), whereas almost all the type II regulatory subunits (R_{II}) were found in the supernatant.

2. Materials and methods

Histone (Type IIA), nucleotides, EGTA, DFP, leupeptin, aprotinin and benzamidine were obtained from Sigma Chemical Co., [γ-^{32}P]ATP from Amersham Corporation and 8-azido-[^{32}P]cAMP from ICN.

Human platelets, washed as described elsewhere [9], were finally resuspended in Tyrode's solution without albumin at a concentration of 5×10^8 ml^{-1}. Samples of platelet suspension (3–5 ml) were mixed with the indicated additions, cooled to 0°C and lysed by sonication for 4 × 15 s [2]. Soluble and particulate fractions were isolated by centrifugation at 90 000 × g for 60 min at 4°C. The latter were resuspended in Tyrode's solution containing the same additions.

Rabbit skeletal and bovine heart muscle were homogenized in a Waring blender for 2 min at 0°C in 3 vol. of 4 mM EDTA, pH 7.0, containing 2 mM dithiothreitol. These homogenates were centrifuged at 25 000 × g for 30 min at 4°C to give supernatants that were stored at −50°C until photoaffinity labelled (see below).

Protein kinase assays (final vol. 0.2 ml) contained 10 mM potassium phosphate buffer, pH 7.0, 2 mM 2-mercaptoethanol, 2.5 mg of histone ml^{-1}, 10 mM $MgCl_2$, and 0.2 mM [γ-^{32}P]ATP (7.5 Ci mol^{-1}) with or without 5 μM cAMP. After addition of up to 100 μl of platelet fraction (10–100 μg of protein), the mixture was incubated for 10 min at 30°C. Incubations were terminated by addition of 0.1 ml of 12 mM ATP containing bovine serum albumin (5 mg ml^{-1}) and 0.9 ml of 10% (w/v) trichloroacetic acid containing 10 mM K_2HPO_4. The ^{32}P-labelled protein was isolated on glass fibre filters (Whatman F2832-24) and washed with 10% (w/v) trichloroacetic acid. The filters were counted for ^{32}P using Čerenkov radiation [2]. All assays were performed in triplicate. One unit of protein kinase activity was defined as that transferring 1 pmol of $^{32}PO_4^{3-}$ from [γ-^{32}P]ATP to histone per min at 30°C.

Abbreviations: cAMP, cyclic AMP; SDS, sodium dodecylsulphate; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetate; DFP, diisopropylfluorophosphate

Photoaffinity labelling of platelet fractions was carried out at 0°C, using 8-azido-[^{32}P]cAMP. Reaction mixtures (0.4 ml) contained 0.32 ml of platelet material, 1 mM 2-mercaptoethanol, 0.5 mM 3-isobutyl-1-methylxanthine and 0.09 μM or 0.5 μM 8-azido-[^{32}P]-cAMP (2–5 μCi per sample). After incubation for 20 min at 0°C, the mixtures were irradiated for 10 min using a UVS-11 Mineralight held at a distance of 5 cm. In some experiments, mixtures were preincubated at 0°C with cAMP or other agents before addition of 8-azido-[^{32}P]cAMP. After irradiation of the mixtures, protein was precipitated with 0.1 ml 30% (w/v) trichloroacetic acid, dissolved in buffer containing 3% (w/v) SDS and 5% (v/v) 2-mercaptoethanol and analysed by SDS–polyacrylamide gel electrophoresis [10], using 11% (w/v) acrylamide in the separating gel. The ^{32}P-labelled polypeptides were detected by autoradiography and the relative amounts of ^{32}P in them were quantitated by densitometry of the autoradiographs. The apparent molecular weights of labelled polypeptides were determined by parallel electrophoresis of protein markers [2]. Protein was determined according to Lowry et al. [11].

3. Results

Various chelating agents and protease inhibitors were added to samples of platelet suspension prior to their sonication and the protein kinase activities of the platelet lysates and of supernatant and particulate fractions prepared from them were then determined (table 1). The percentages of both the cAMP-dependent and independent activities recovered in the particulate fraction were increased 2 to 3-fold when EGTA or EDTA was used. This reflected statistically significant increases in the specific activities of these fractions. Leupeptin (0.2 mM) had similar though slightly weaker effects. At the same time, these agents tended to decrease the supernatant protein kinase activities (table 1). In contrast, neither aprotinin (1 trypsin inhibitory unit per ml) nor benzamidine (10 mM) had any effect on the subcellular distribution of protein kinase activities.

Photoaffinity labelling of platelet lysates (prepared in the presence of 10 mM EDTA and 1 mM DFP) with 0.5 μM 8-azido-[^{32}P]cAMP, followed by electrophoresis of the platelet protein, always revealed two ^{32}P-labelled polypeptides with apparent M_r values of 55 000 and 49 000 (fig.1). Less consistently, labelled polypeptides with apparent M_r values of 38 000 and 52 000 were also observed. The 49 000 and 55 000 M_r polypeptides had the same electrophoretic mobilities as polypeptides that could be photoaffinity labelled in rabbit skeletal and bovine heart muscle supernatants (fig.1) and their molecular weights corresponded to those of the R_I and R_{II} subunits of

Table 1
Effects of different inhibitors of proteolysis on the subcellular distribution of protein kinase activity in platelets

Addition to platelet suspension	No. of expts	Specific activity of whole lysate (units/mg protein)		Specific activity of supernatant fraction (units/mg protein)		Specific activity of particulate fraction (units/mg protein)		Percentage of total activity in particulate fraction	
		−cAMP	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP
None	9	322	579	411	860	131	216	11.3	9.2
EGTA (5 mM)	7	236	620	224[a]	791	338[a]	582[a]	37.3[b]	23.6[b]
EDTA (5 mM)	7	288	631	320[a]	776	316[a]	558[a]	28.4[b]	22.6[b]
Leupeptin (0.2 mM)	5	241	484	272	614	268	365[a]	26.2[a]	17.3[a]
Aprotinin (1 unit ml^{-1})	4	288	550	359	756	119	175	13.1	8.4
Benzamidine (10 mM)	5	268	650	357	853	122	207	11.3	8.7

Mean values from the no. of expts indicated are given; values significantly different from the controls with no additions are shown: [a] $P < 0.05$, [b] $P < 0.01$ (paired t test)

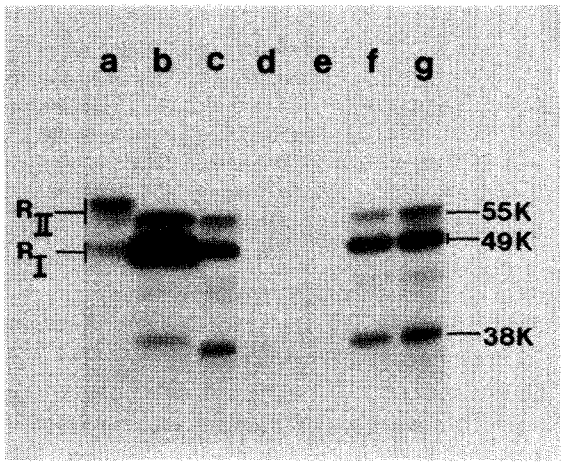

Fig.1. Photoaffinity-labelling of platelet lysate and of bovine heart and rabbit skeletal muscle supernatants by 8-azido-[^{32}P]cAMP (0.5 μM). EDTA (10 mM) and DFP (1 mM) were added to platelet suspension before sonication. After photoaffinity labelling, protein samples were electrophoresed as follows: (a) bovine heart supernatant, (b) rabbit skeletal muscle supernatant and (c–g) platelet lysate. Before the addition of 8-azido-[^{32}P]cAMP, samples of platelet lysate were incubated for 20 min with (c) no addition, (d) 1 μM cAMP, (e) 10 μM cAMP, (f) 10 μM cyclic GMP and (g) 10 μM AMP. An autoradiograph is shown.

cAMP-dependent protein kinases from these tissues [13]. However, in bovine heart the main photoaffinity-labelled polypeptide had an apparent M_r 57 000 (the phosphorylated form of the R_{II} subunit [14]) and did not correspond to any labelled platelet polypeptide. The 49 000 M_r ^{32}P-labelled polypeptide often appeared as a closely spaced doublet, with equally labelled components. Photoaffinity labelling of both the 55 000 and the 49 000 M_r polypeptides was suppressed by preincubation of the platelet lysate with 1 μM or 10 μM cAMP, but not by 10 μM cyclic GMP or 10 μM AMP, indicating that specific cAMP-binding sites were labelled (fig.1). Labelling of the 38 000 M_r polypeptide, which is probably a proteolytic degradation product, and of the 52 000 M_r polypeptide were also suppressed by preincubation with cAMP, but labelling of a few minor polypeptides in the 80 000–85 000 M_r range was not affected.

Photoaffinity labelling of the supernatant and particulate fractions of lysates prepared in the presence and absence of various chelating agents and protease inhibitors showed that EGTA, EDTA and leupeptin markedly increased the amount of ^{32}P-labelled polypeptide with M_r 49 000 found in the particulate fraction (fig.2 and 3). In four experiments in which EDTA was used, 61 ± 4% (mean ± S.E.M.) of this labelled polypeptide was particle-bound. These agents also increased the labelling of the 55 000 M_r polypeptide, which was found almost exclusively in the supernatant fraction, and largely prevented the appearance in the supernatant of several smaller labelled polypeptides (M_r 45 000, 41 000, 38 000 and 36 000), presumably proteolytic breakdown products of the 49 000 and 55 000 M_r species. Benzamidine and aprotinin, on the other hand, did not prevent loss of the 49 000 and 55 000 M_r polypeptides. DFP (1 mM, not shown) had variable effects, depending on the batch used, which were independent of its ability to inhibit trypsin. When photoaffinity labelling was carried out with a lower concentration of 8-azido-[^{32}P]cAMP (0.09 μM), label-

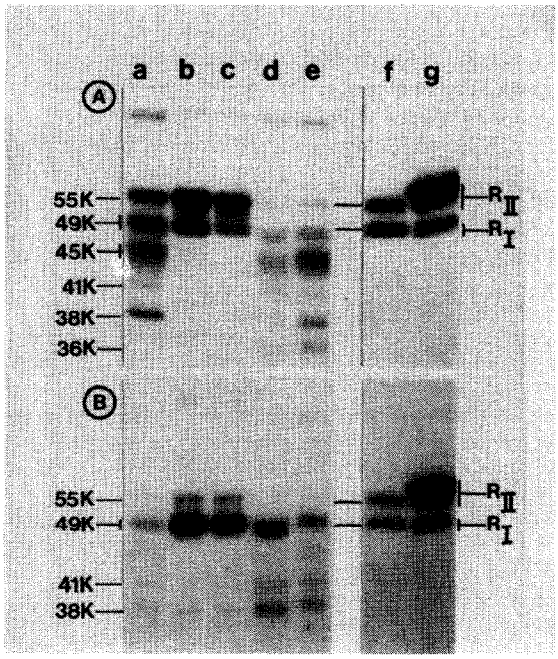

Fig.2. Effects of inhibitors of proteolysis on the photoaffinity labelling of (A) supernatant and (B) particulate fractions of platelet lysate by 0.5 μM 8-azido-[^{32}P]cAMP. Prior to their sonication, samples of platelet suspension were mixed with the following additions (final concentrations): (a) none, (b) 5 mM EGTA, (c) 5 mM EDTA, (d) 10 mM benzamidine and (e) 1 trypsin inhibitory unit of aprotinin per ml. The amount of supernatant fraction protein electrophoresed was derived from half as many platelets as the amount of particulate fraction used. As markers for the R_I and R_{II} regulatory subunits of cAMP-dependent protein kinases, rabbit skeletal muscle (f) and bovine heart muscle (g) supernatants were photoaffinity labelled and electrophoresed in parallel with the platelet samples. An autoradiograph is shown.

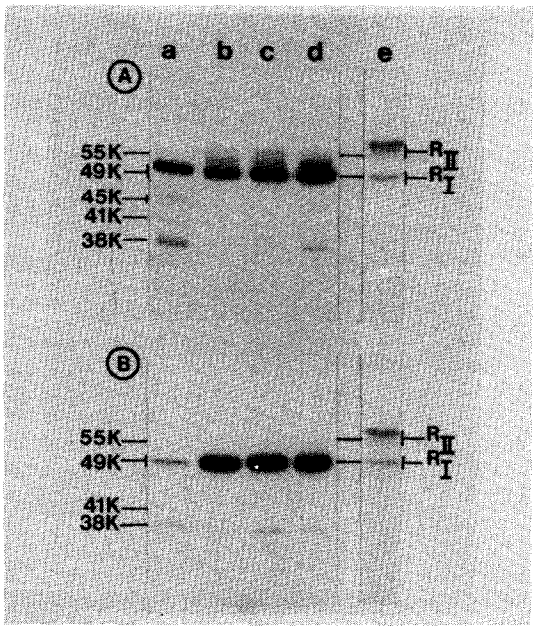

Fig.3. Effects of inhibitors of proteolysis on the photoaffinity labelling of (A) supernatant and (B) particulate fractions of platelet lysate by 0.09 μM 8-azido-[^{32}P]cAMP. Prior to their sonication, samples of platelet suspension were mixed with the following additions (final concentrations): (a) none, (b) 5 mM EDTA, (c) 5 mM EGTA and (d) 0.2 mM leupeptin. The amounts of supernatant and particulate fraction protein electrophoresed were obtained from the same number of platelets. Bovine heart muscle supernatant was photoaffinity labelled and electrophoresed in (e).

ling of the 55 000 M_r polypeptide was decreased relative to that of the 49 000 M_r polypeptide and, as a result, the 52 000 M_r species was more readily observed and shown to be soluble (fig.3).

4. Discussion

Our results indicate that EGTA, EDTA and leupeptin markedly increase both the cAMP-dependent protein kinase activity and the amount of photoaffinity polypeptide with M_r of 49 000 found in the particulate fraction of platelet lysates. At the same time, these agents increased the amount of a 55 000 M_r photoaffinity-labelled polypeptide detected in the supernatant fraction; very little of the latter polypeptide was found in the particulate fraction. As the photoaffinity labelling of both of these polypeptides was specifically inhibited by cAMP, they are likely to be the regulatory subunits of type I and type II cAMP-dependent protein kinases, which have corresponding molecular weights [13] and show similar patterns of photoaffinity labelling in other tissues [14–18]. We infer that under conditions in which endogenous proteolysis is prevented, over half of the type I platelet enzyme is particle bound, whereas almost all of the type II enzyme is soluble. These results are consistent with those of Lyons [19]. Membrane-bound type I cAMP-dependent protein kinase has also been observed in synaptic membranes [16] and in red cells [20].

Although only one form of the photoaffinity-labelled R_I subunit has previously been resolved by SDS–polyacrylamide gel electrophoresis [15,16,18, 19], two or more forms, which may be related to the doublet we often observed, can be separated by isoelectric focussing [19,21]. In bovine heart and porcine skeletal muscle, both phosphorylated and non-phosphorylated R_{II} subunits are present and can be separated by SDS–polyacrylamide gel electrophoresis [14,18]. However, electrophoresis of photolabelled polypeptides from platelets in parallel with those from bovine heart suggests that platelets do not, as claimed by Lyons [19], normally contain phosphorylated R_{II} subunits. Incubation of intact platelets with agents that increase cAMP leads to the endogenous phosphorylation of a 50 000 M_r polypeptide but this has an electrophoretic mobility significantly greater than that of the platelet R_{II} subunit [3]. The 52 000 M_r polypeptide sometimes observed after photoaffinity labelling of platelets lysate or supernatant may be related to that recently detected in bovine brain [22] and could be either a proteolytic fragment or minor variant of the R_{II} subunit.

Supernatant fractions from platelets sonicated in the absence of additions showed consistent patterns of photoaffinity-labelled fragments of the cAMP-binding proteins. Endogenous proteolysis of the R_I and R_{II} subunits has also been observed in other tissues [17,23]. The appearance of these fragments under conditions in which loss of particulate protein kinase activity was observed, suggests that the enzyme was released by a proteolytic mechanism. This could be due to proteolysis of R_I subunits, if they bind the catalytic subunits to the particulate fraction, or to the additional proteolysis of an anchor protein. The observation that EGTA prevented the release of cAMP-dependent protein kinase activity suggests that the Ca^{2+}-activated protease, that is known to be pres-

ent in platelets [24], was responsible for this effect. In support of this view, leupeptin which unlike aprotinin is a potent inhibitor of this enzyme [25], also reduced enzyme release. Our results demonstrate the importance of preventing endogenous proteolysis in studies on the phosphorylation of platelet membrane proteins and suggest that membrane-bound type I enzyme may normally mediate the cAMP-dependent phosphorylation of the membrane polypeptides thought to be important in the inhibition of platelet function by cAMP [2,3,7].

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