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EFFECTS OF PROSTAGLANDIN E_1 ON PROTEIN KINASE ACTIVITY AND ENDOGENOUS PHOSPHORYLATION OF INTACT HUMAN PLATELETS

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Abstract—When intact human platelets were treated with prostaglandin E_1, protein kinase activity of the platelet sonicate was elevated when assayed without cyclic AMP. Total protein kinase activity (assayed with cyclic AMP) remained unchanged. Prostaglandin A_1 mimicked this effect, but other prostaglandins and anti-platelet agents had no effect on protein kinase activity. Phosphoprotein phosphatase activity was not influenced by prostaglandin E_1. Basal phosphorylation of several endogenous substrates for protein kinase was also elevated when platelets were treated with prostaglandin E_1. The results demonstrate that prostaglandin E_1 stimulates protein kinase activity and endogenous phosphorylation in intact platelets and suggest that this effect is mediated by the action of prostaglandin E_1 on cyclic AMP levels. An important role of phosphoprotein in mediating the effect of prostaglandin E_1 on platelet function is suggested.

There is ample evidence which indicates the involvement of cyclic AMP in platelet physiology [1, 2]. Prostaglandin E_1 (PGE_1), a potent inhibitor of platelet aggregation and release reaction, increases cyclic AMP in platelets [3, 4]. It has been suggested that the effects elicited by cyclic AMP are mediated by activation of protein kinase [5], and a role for cyclic AMP-dependent protein kinase has been established in some tissues [6–8]. Although a cyclic AMP-dependent protein kinase has been demonstrated in human platelets [9] and partial purification of this enzyme has been achieved [10, 11], the precise role of this enzyme in platelet function is still unknown.

In order to examine this relationship, it is necessary to determine whether protein kinase activity of intact platelets is influenced by PGE_1 or other anti-platelet agents which modulate the cyclic AMP level of platelets. The present study demonstrates that PGE_1 increases the basal activity of cyclic AMP-dependent protein kinase in intact platelets, consistent with its stimulation of the formation of cyclic AMP.

Phosphorylated protein produced by cyclic AMP-dependent protein kinase has been considered to be dephosphorylated by phosphoprotein phosphatase [12, 13]. This means that the enzyme may be involved in the switch-off mechanism of a cyclic AMP action. Therefore, it also appears important to define whether prostaglandin E_1 may have some influence on the phosphoprotein phosphatase activity of an intact platelet. This was examined and will be shown in the results.

Finally, a study of endogenous phosphorylation in platelets was undertaken in order to determine the phosphorylated intermediate proteins during the action of PGE_1 on platelet functions.

MATERIALS AND METHODS

Experimental procedure. Whole human blood collected in 5% citrate was obtained from normal adults and used within 24 hr. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood at 300 *g* for 6 min and platelets were precipitated by centrifugation of PRP at 1200 *g* for 30 min at room temperature. The platelet pellet was washed twice with EDTA (2 mM)–Tris (2 mM)–saline (pH 7.2) and resuspended in the same solution. After the addition of PGE_1 (0.05 to 5 μM), other prostaglandins (0.05 to 5 μM), adenosine (50 μM), l-epinephrine (5 μM) and thrombin (0.5 units/ml), the platelets were incubated for 20 min at room temperature. Ice-cold saline was added and the platelets were washed twice by centrifuging at 2°. Sedimented platelets were resuspended with a small volume of ice-cold saline and ultrasonicated on ice using a sonifier (Branson Sonic Power Co.). The sonicate was frozen at −60° until the protein kinase activity, endogenous phosphorylation and phosphoprotein phosphatase activities were measured.

Fractionation of the sonicate was performed by centrifuging at 27,000 *g* for 30 min using a Hitachi 65P automatic preparative ultracentrifuge. After the supernatant (cytosol) fraction was obtained, the sediment was washed with EDTA (2 mM)-Tris (2 mM)-saline (pH 7.2) and resuspended in the same solution; this was used as a particulate fraction.

Measurement of protein kinase activity. Protein kinase activity was measured by a slight modification of the method of Miyamoto *et al.* [14]. Aliquots of sonicate or fractions were added to the medium containing 50 mM sodium glycerol phosphate buffered at pH 6.5, 500 μg/ml of histone, 10 mM $MgCl_2$, 2 mM theophylline, 10 mM NaF, 0.3 mM EGTA,* 5 μM ATP-Na_2 and [γ-^{32}P]ATP (0.1 to 0.3 μM) with or without 5 μM cyclic AMP in a final volume of 0.25 ml. Incubations

* EGTA = ethyleneglycolbis(aminoethylether)tetraacetate.

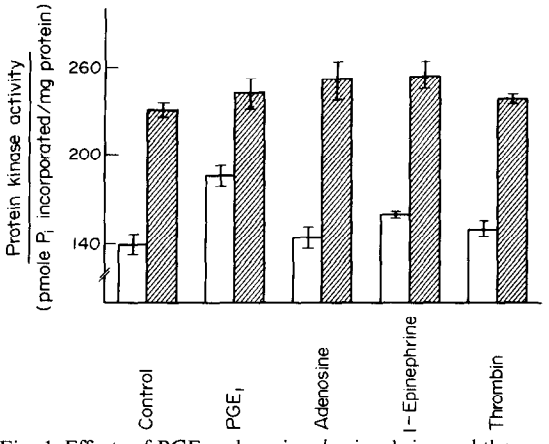

Fig. 1. Effects of PGE_1, adenosine, *l*-epinephrine and thrombin on protein kinase activity of intact human platelets. Prostaglandin E_1 (5 μM), adenosine (50 μM), *l*-epinephrine (5 μM) and thrombin (0.5 units/ml) were added to incubation medium containing intact human platelets. Protein kinase activity of platelet sonicate was assayed in the absence (□) and in the presence (■) of cyclic AMP. Bars are standard errors of duplicate determinations of triplicate tubes.

were carried out at 37° and were terminated after 5 min by the addition of 5% trichloroacetic acid (TCA) (1.0 ml); 0.2 ml of 0.63% bovine serum albumin was added to each tube as a carrier. The mixture was centrifuged, and the supernatant fraction was discarded. The precipitate was washed twice by being dissolved in 0.1 ml of 1 N NaOH and reprecipitated with 1.0 ml of 5% TCA. The resultant pellet was finally dissolved in 0.1 ml of 1 N NaOH, and 0.4 ml of distilled water was added. The whole solution in each tube was put into a vial containing Bray's solution and the radioactivity was counted in a Packard scintillation counter.

Measurement of phosphoprotein phosphatase activity. Phosphorylated histone was prepared by incubating 20 mg histone in the medium metioned above with 25 μCi [γ-^{32}P]ATP, 5 μM cyclic AMP and partially purified cyclic AMP-dependent protein kinase of dog heart muscle for 20 min at 37°. After adding trichloroacetic acid (5%) to the tube, this was centrifuged and the sediment was washed twice with water and dialyzed against 2 mM Tris–HCl (pH 7.5) for 24 hr. Phosphoprotein phosphatase activity of the platelet sonicate was determined according to the method of Meisler and Langan [12], but the last step was modified by adding an aliquot of isobutyl alcohol-benzene extract to Bray's solution and counting the radioactivity in a Packard scintillation counter.

Endogenous substrate for protein kinase in platelets was analyzed by the modified method of Suzuki and Field [15]. In brief, supernatant fractions (27,000 *g*, 30 min) of control and of sonicated PGE_1-treated platelets were phosphorylated with [γ-^{32}P]ATP either in the absence or in the presence of cyclic AMP by using histone-free protein kinase assay buffer. The reaction was terminated by additions of 2% mercaptoethanol and 0.2% Socium dodecyl sulphate (SDS) and the tubes were boiled for 2 min. A portion of each sample was applied to 0.2% SDS–5.6% polyacrylamide gel electrophoresis. The resultant gels were stained for protein by Coomassie Blue. After photography and densitography were performed, the gels were cut by equal width (2 mm) from the top. Each slice was put into a vial and was solubilyzed with hydroperoxide at about 50° incubation. The radioactivity was determined as mentioned above.

Protein was determined by the method of Lowry *et al.* [16]. Partially purified cyclic AMP-dependent protein kinase of dog heart muscle was obtained using the method of Miyamoto *et al.* [14] including DEAE-cellulose chromatography (step 4). [γ-^{32}P]ATP (2.59 Ci/m-mole) was purchased from the Radiochemical Centre (Amersham). Histone (calf thymus, type 2), ATP-Na_2, cyclic AMP, adenosine and *l*-epinephrine were purchased from the Sigma Chemical Co., St. Louis, MO. Prostaglandins A_1, A_2, E_1, E_2 and $F_{2\alpha}$ were kindly provided by the Ono Pharmac. Co., and thrombin was obtained from the Mochida Pharmac. Co. Standard proteins used in the SDS-polyacylamide gel electrophoresis were: cytochrome-*c* (Sigma, type 3, mol. wt. 12,400), trypsin inhibitor (mol. wt. 21,500), rinder serum albumin (mol. wt. 68,000) and RNA-polymerase (mol. wt. α-39,000, β-155,000, β'-165,000) from the Böeringer Co.

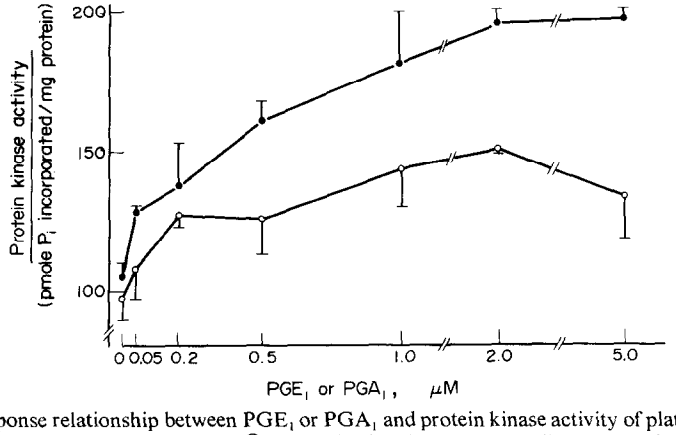

Fig. 2. Dose–response relationship between PGE_1 or PGA_1 and protein kinase activity of platelet's sonicate. Prostaglandin E_1 (●) or prostaglandin A_1 (○) was added to incubation medium containing intact human platelets. Basal protein kinase activity of platelet sonicate was assayed. Bars are standard errors of duplicate determinations of triplicate tubes.

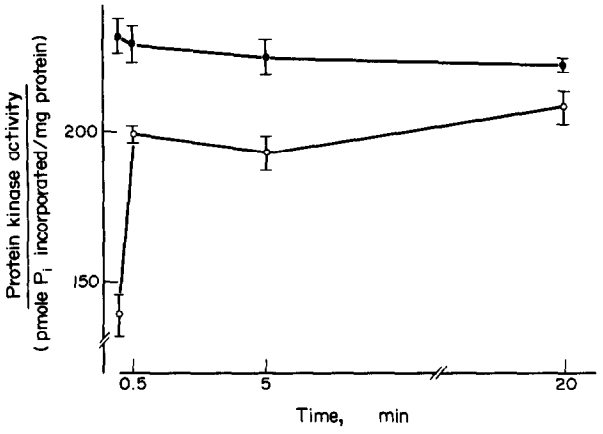

Fig. 3. Time course of protein kinase activity of human platelets treated with PGE_1. Prostaglandin E_1 (5 μM) was added to incubation medium containing intact human platelets. Protein kinase activity of platelet sonicate was assayed in the absence (○) and in the presence (●) of cyclic AMP. Bars are standard errors of duplicate determinations of triplicate tubes.

RESULTS

In the preliminary investigations, the ultrasonicate of human platelets showed protein kinase activity augmented by cyclic AMP. A freezing–thawing method produced a platelet lysate of comparable protein kinase activity in the absence of cyclic AMP, but this was enhanced less markedly by cyclic AMP than was the enzyme obtained by an ultrasonication method.

Although no direct effect was observed when PGE_1 (5 μM) was added to the assay medium for protein kinase activity, it increased the basal activity of the protein kinase by 34 per cent in incubated human platelets (Fig. 1). Total protein kinase activity (measured in the presence of cyclic AMP) remained unchanged. Thus, the ratio of the activity in the absence, to the activity in the presence, of cyclic AMP rose from 0.60 to 0.77. On the other hand, the anti-platelet agents, adenosine (50 μM), *l*-epinephrine (5 μM) and thrombin (0.5 units/ml), had no effect on protein kinase activity assayed in the absence or in the presence of cyclic AMP.

The dose–response relationship revealed that 0.05 μM PGE_1 caused a significant increase and 2–5 μM produced maximal increase in the basal protein kinase activity (Fig. 2). This increase was very rapid as almost maximal increase was observed at 0.5 min of incubation (Fig. 3). The activity remained elevated during a 20 min incubation. The effect was equally apparent in the presence and in the absence of theophylline (10^{-2} M) in the incubating medium (data not shown).

The increase in the basal protein kinase in the sonicate caused by PGE_1 was seen in both the supernatant (cytosol) and the particulate fractions (Table 1). The protein kinase activity, which was expressed in terms of protein concentration, was higher in the cytosol than in the particulate fraction, but a greater per cent increase occurred in the latter (125 per cent) as compared to the former fraction (50 per cent).

PGA_1 also increased the basal protein kinase activity and the activity ratio (−cyclic AMP/+cyclic AMP) (Table 2). The dose–response relationship showed that the maximal activity observed in PGA_1 treatment was smaller than that in PGE_1 treatment (Fig. 2). Prostaglandin $F_{2\alpha}$ tended to increase the activity, but PGE_2 and PGA_2 did not affect appreciably the enzyme activity or the activity ratio.

Table 1. Protein kinase activity of subcellular fractions of control and PGE_1-treated human platelets*

	Protein kinase activity (pmoles Pi incorporated/mg protein)					
	−cAMP		+cAMP		−cAMP/+cAMP	
	Control	PGE_1	Control	PGE_1	Control	PGE_1
Sonicate	44.3 ± 3.2	71.7 ± 0.3†	107.3 ± 8.3	93.6 ± 2.4	0.41 ± 0.03	0.77 ± 0.02†
Supernatant fraction‡	37.9 ± 2.4	58.1 ± 0.9§	60.4 ± 5.1	77.9 ± 4.4	0.63 ± 0.08	0.75 ± 0.06
Particulate fraction‖	18.9 ± 1.6	43.4 ± 4.2§	48.1 ± 1.1	54.6 ± 2.0	0.39 ± 0.02	0.79 ± 0.03†

* Fractionation of platelet sonicate was performed at 2° as described in Materials and Methods. Data are the means ± S.E. of triplicate determinations.
†$P < 0.01$, compared to control values.
‡Centrifuge (27,000 *g*, 30 min).
§ $P < 0.02$, compared to control values.
‖Saline-washed pellet by centrifuge (27,000 *g*, 30 min).

Table 2. Protein kinase activity of human platelets treated with various prostaglandins*

	Protein kinase activity (pmoles Pi incorporated/mg protein)		
	−cAMP	+cAMP	−cAMP/ +cAMP
Control	37.3 ± 2.0	75.0 ± 6.5	0.49 ± 0.05
PGE_1	67.5 ± 6.9†	82.0 ± 12.0	0.82 ± 0.08‡
PGE_2	40.5 ± 2.8	73.5 ± 8.5	0.55 ± 0.10
PGA_1	49.6 ± 2.5†	61.6 ± 4.4	0.80 ± 0.02†
PGA_2	43.0 ± 4.1	60.8 ± 3.1	0.71 ± 0.05
PGF_2	53.0 ± 4.9‡	76.1 ± 4.3	0.69 ± 0.04

* Prostaglandins were added to incubation medium of intact human platelets at a 5 μM concentration. Data are the means + S.E. of triplicate determinations.

† $P < 0.02$.

‡ $P < 0.05$.

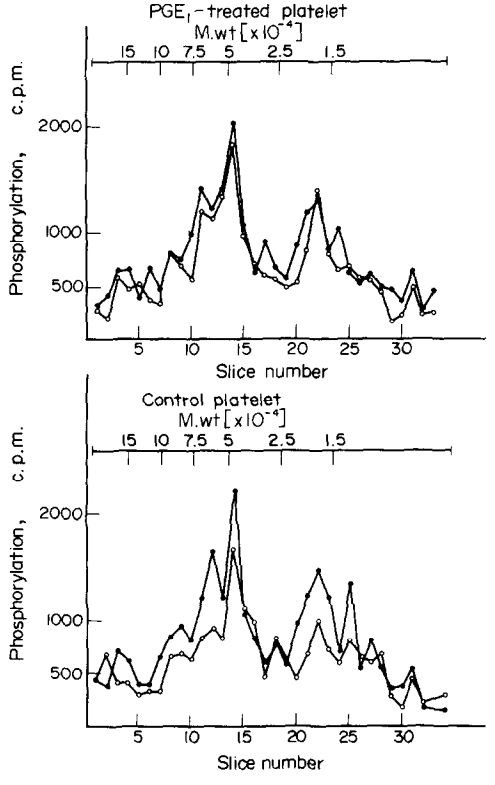

Fig. 4. Endogenous phosphorylation in control and PGE_1-treated platelets. Supernatant fractions of control (lower) and PGE_1-treated (upper) platelet sonicates were phosphorylated using [γ-^{32}P]ATP as phosphoryl donor. Fifty-eight μg of protein of each sample was applied to SDS-polyacrylamide gel electrophoresis and the resultant gels were sliced, solubilized and processed for counting the radioactivity as described in the text. Open circle (○) denotes phosphorylation in the absence of cyclic AMP, and closed circles (●) in the presence of cyclic AMP. Molecular weight (M.W.) represented by inserted bars was determined from standard proteins (described in Materials and Methods) electrophoresed in parallel. Each point was determined by duplicate gels. This figure represents one of four experiments.

Phosphoprotein phosphatase activity was demonstrated in the sonicate of human platelets. Both NaF (5 mM) and $CaCl_2$ (5 mM) inhibited about 50 per cent of the control activity, and increasing concentrations of these compounds exhibited greater inhibitions. $ZnCl_2$ was also found to have an inhibitory effect. The activity of phosphoprotein phosphatase in the sonicates prepared from platelets which had been treated with PGE_1 (5 μM), adenosine (50 μM), *l*-epinephrine (5 μM) and thrombin (0.5 units/ml) for 20 min or 120 min at room temperature remained unchanged when compared to the controls (platelets incubated without test substances).

A study of endogenous phosphorylation disclosed that the control platelets contained multiple proteins as endogenous substrates for protein kinase, and some of these (mol. wt: 62,000, 48,000, 17,000, and 12,000) showed increased phosphorylation in the presence of cyclic AMP (Fig. 4). In the PGE_1-treated platelet, although the densitogram of protein staining was precisely similar to the control (data not shown), basal (in the absence of cyclic AMP) phosphorylation in regions of some proteins (mol. wt: 70,000–48,000 and 17,000) increased close to the level observed in the presence of cyclic AMP. The pattern of the phosphorylation in the presence of cyclic AMP was similar in the PGE_1-treated platelets and in the control.

DISCUSSION

The results described in this investigation demonstrated enhanced protein kinase activity in intact human platelets when the enzyme is measured in sonicates in the absence of cyclic AMP. This effect almost certainly reflects the increased intracellular cyclic AMP induced by PGE_1. It is well-established that cyclic AMP dissociates the holoenzyme of cyclic AMP-dependent protein kinase into regulatory and catalytic subunits [17]. It was reported previously that PGE_1 was a potent stimulator of platelet adenyl cyclase [3] and cyclic AMP accumulation [4]. The rapidity with which PGE_1 enhanced protein kinase activity in our study is consistent with the rate at which it stimulates cyclic AMP accumulation [4]. Furthermore, the concentrations of PGE_1 which affected protein kinase activity agree well with those that increase cyclic AMP [4]. Finally, PGA_1 mimicked the effects of PGE_1 on activation of protein kinase and has been reported to augment cyclic AMP concentrations in human platelets [4]. The absence of any increase in total protein kinase activity is consistent with dissociation of the regulatory and catalytic subunits rather than with activation of another kind of protein kinase by PGE_1. Therefore, it is reasonable to assume that PGA_1 and PGE_1 have a common mechanism of activating cyclic AMP-dependent protein kinase through intracellular accumulation of cyclic AMP. Such an activation of cyclic AMP-dependent protein kinase in intact tissue by PGE_1 was reported previously [18].

The other anti-platelet agents which were tested had no effect on protein kinase activity. Of these agents tested, adenosine inhibits platelet aggregation induced by ADP [19], and has been reported to affect the cyclic AMP-system of platelets in a complex and dual fashion [20]. *l*-Epinephrine and thrombin stimulate platelet

aggregation and release reaction [1, 2]. Although the concentrations of these agents used in the present study were high enough to cause these effects, they, by themselves, neither increased nor decreased protein kinase activity of intact human platelets. This finding, as well as the previous conflicting results concerning these agents and basal cyclic AMP levels of platelets [2], suggests that the effects of these agents on platelet physiology are not mediated solely through the cyclic AMP-protein kinase system, but through a more complicated mechanism.

Phosphoprotein phosphatase was demonstrated in platelet sonicate, and its activity was inhibited by NaF, $CaCl_2$ and $ZnCl_2$ consistent with previous results in other tissues [12, 13]. Altered activity of this enzyme by hormonal stimulations or cyclic AMP was reported in other tissues [21, 22]. In platelet, Booyse *et al.* [11] showed that thrombin caused the rapid release of labeled phosphopeptides from platelet membrane prelabeled with [γ-^{32}p]ATP. Our results have shown unchanged activity when treating intact platelets with PGE_1 and other anti-platelet agents.

Increased protein kinase activity and unchanged phosphoprotein phosphatase activity suggested that PGE_1 might produce phosphorylated intermediate protein(s) during the action of PGE_1 on platelets. Actually, increased basal phosphorylation of the segments of the gel, where endogenous substrates for cyclic AMP-dependent protein kinase exist, was found in the PGE_1-treated platelets (Fig. 4). Haslam *et al.*[23] used [^{32}P] inorganic phosphate and observed that PGE_1 caused 2-fold increases in the phosphorylation of polypeptides (mol. wt 24,000 and 22,000), as well as less significant increases in some other segments of the gels. Contrary to this, Lyons *et al.* [24] observed that PGE_1 inhibited thrombin-induced phosphorylations of peak 7 and peak 9 proteins in intact human platelets. It is difficult to explain these findings relating to the present study on the basis of several differences in the methods. The physiological role of such proteins remained to be clarified. Nevertheless, the phosphorylated proteins appear to be of considerable importance in the intermediary metabolism of a PGE_1-induced alteration in platelet functions.

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