Functional relationship between cyclic AMP-dependent protein phosphorylation and platelet inhibition

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Exposure of human platelets to prostacyclin (PGI_2), iloprost or prostaglandin E_1 (PGE_1) elicits the cyclic AMP-dependent phosphorylation of proteins of 22, 24, 30, 39, 50, 60 and 250 kDa (P22, P24 etc.). P22 was recently identified as *rap1B*, a *ras*-like protein, and P24 was shown to be the β -chain of glycoprotein Ib. We found that cyclic AMP-dependent phosphorylation of all proteins except P22 was maximal 1 min after exposure of platelets to PGI_2 , iloprost or PGE_1 ; maximal phosphorylation of P22 occurred after 45 min of incubation. Inhibition of thrombin-induced platelet activation required only a 30 s incubation with PGI_2 or iloprost; at this time phosphorylation of P22 was only slightly increased. Although at maximal concentrations PGI_2 was more potent than PGE_1 in inhibiting thrombin-induced platelet activation, no difference in the degree and the kinetics of cyclic AMP-dependent protein phosphorylation was found. Platelets that had been preincubated and washed in the presence of PGE_1 and later resuspended in the absence of PGE_1 responded fully to activation by thrombin despite maximal phosphorylation of P22 and P24. Furthermore, addition of PGI_2 to PGE_1 -washed platelets prevented thrombin-induced platelet activation, but did not evoke further phosphorylation of P22 or P24. Phosphorylation of P39 and P50 correlated better with PGI_2 -induced inhibition of platelet activation. In experiments in which PGE_1 -induced inhibition of platelet activation was overcome by the addition of thrombin, no dephosphorylation of proteins phosphorylated by cyclic AMP-dependent kinases was observed. These experiments indicate that: (a) phosphorylation of *rap1B* and glycoprotein Ib is not related to platelet inhibition by cyclic AMP; (b) phosphorylation of other proteins such as P39 and P50 probably plays a role in mediating cyclic AMP-dependent platelet inhibition; (c) reactions other than cyclic AMP-dependent protein phosphorylation may participate in platelet inhibition by cyclic AMP.

INTRODUCTION

Prostacyclin (PGI_2) is one of the most powerful inhibitors of platelet activation [1]. Platelet shape change, secretion and aggregation are completely suppressed by pretreatment with prostacyclin. PGI_2 , prostaglandin E_1 (PGE_1) and the stable PGI_2 analogue iloprost bind to a common specific receptor on the platelet surface [2] that couples to the guanine-nucleotide-binding protein G_s [3,4]. Activation of G_s stimulates adenylate cyclase and leads to an increase in intracellular cyclic AMP and the subsequent cyclic AMP-dependent phosphorylation of specific proteins. Cyclic AMP inhibits platelet activation at several steps: one of the most important is receptor-mediated phosphoinositide hydrolysis (for references see [5]). The inhibitory effects of cyclic AMP on protein kinase C activation, Ca^{2+} mobilization, fibrinogen-receptor exposure, myosin light-chain phosphorylation, actin polymerization and cytoskeletal assembly are believed to be consequences of the inhibition of receptor-mediated phospholipase C activation (reviewed in [5]). Other studies discovered additional target sites for platelet inhibition by cyclic AMP. For example, several investigators reported that cyclic AMP inhibits secretion and aggregation induced by Ca^{2+} ionophore at steps distal to Ca^{2+} mobilization [6–9]. Also, modest increments in platelet cyclic AMP were reported to abolish platelet-activating-factor-induced aggregation and secretion, whereas they had little effect on phosphoinositide hydrolysis and elevation of cytosolic Ca^{2+} concentration induced by platelet-activating factor [10]. We recently found that cyclic AMP inhibits platelet aggregation at steps distal to activation of protein kinase C and Ca^{2+} -dependent protein kinase [11].

Inhibition of platelet activation by cyclic AMP is associated

with the phosphorylation of proteins with molecular masses of 22, 24, 36, 50, 130 and 250 kDa [12–16]. The 24 kDa protein has been identified as the β -chain of glycoprotein Ib [17], and the 250 kDa protein is actin-binding protein [18]. Glycoprotein Ib binds thrombin and is a receptor for von Willebrand factor (for references see [5]). In resting platelets, actin-binding protein links glycoproteins Ia and Ib to actin filaments [5]. The microsomal 22 kDa protein (P22) has been named thrombolamban and is a *ras*-related protein [19]. Earlier studies demonstrated that cyclic AMP-dependent phosphorylation of P22 stimulates Ca^{2+} uptake into platelet membrane vesicles [20]. P22, a major protein of human platelets, is present in membranes and cytosol and binds GTP [21,22]. We recently identified this protein as *rap1B*, a *ras*-like protein that belongs to the family of small GTP-binding proteins [22a]. *Rap1B* is 95 % identical with *rap1A* [23], which is also known as *Krev-1* [24]. *Krev-1* has been shown to reverse the transformed phenotype of *ras*-transformed cells [24].

Although several proteins that are phosphorylated by cyclic AMP-dependent protein kinase have been identified in intact platelets, it is not known whether and how these proteins are involved in cyclic AMP-dependent inhibition of platelet activation. The present study explored the functional relationship between cyclic AMP-dependent protein phosphorylation and inhibition of platelet activation.

EXPERIMENTAL

Materials

PGI_2 , PGE_1 , human thrombin, leupeptin, apyrase and saponin were purchased from Sigma (St. Louis, MO, U.S.A.). Iloprost

Abbreviations used: PGI_2 , prostacyclin; PGE_1 , prostaglandin E_1 .

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was obtained from Schering (Berlin, Germany). All other materials were obtained as described previously [11].

Isolation and ^{32}P -labelling of human platelets

Human blood (120 ml) was drawn from healthy volunteers (0.38 % trisodium citrate as anticoagulant), and was centrifuged at 180 *g* for 20 min to yield platelet-rich plasma. Platelet-rich plasma was incubated with 1 mM-acetylsalicylic acid for 15 min at 37 °C. Citric acid (9 mM) and EDTA (5 mM) were added, and platelets were pelleted by centrifugation at 800 *g* for 15 min. They were resuspended in 2 ml of pre-warmed resuspension buffer (pH 7.4; 20 mM-Hepes, 138 mM-NaCl, 2.9 mM-KCl, 1 mM-MgCl₂ and 1 mM-EGTA) containing apyrase (3 units of ADPase/ml) and 10 % (v/v) autologous platelet-poor plasma. The last was obtained by centrifugation of 0.5 ml of platelet-rich plasma in a Microfuge. The platelet suspension was incubated with 10 mCi of $\text{H}_3^{32}\text{PO}_4$ (10 Ci/mmol; ICN) for 90 min at 37 °C, diluted with 20 ml of prewarmed resuspension buffer (pH 6.2) containing 0.36 mM- NaH_2PO_4 , and pelleted by centrifugation at 800 *g* for 10 min. Platelets [(10–12) $\times 10^9$] were resuspended in 8 ml of resuspension buffer without apyrase and platelet-poor plasma.

Samples (0.5 ml) of the platelet suspension were transferred into aggregometer cuvettes and incubated at 37 °C with stirring. PGI_2 , iloprost, PGE_1 (stock solutions in ethanol) or ethanol (< 0.1 %) were added. In some experiments, thrombin was added subsequently. Aggregation was measured in a Chronolog aggregometer (Havertown, PA, U.S.A.). Portions (0.05 ml) were transferred to an equal volume of sample buffer [11] containing 3 % SDS, 15 mg of dithiothreitol/ml and 1 % glycerol for measurement of protein phosphorylation.

In some experiments samples (0.1 ml) were transferred into Eppendorf tubes, and 2 mM-EGTA, 1 mM-leupeptin and 50 μg of saponin/ml were added. Platelets were left for 15 min at room temperature. Membranes were pelleted by centrifugation for 5 min in a Microfuge. The pellet was immediately resuspended in resuspension buffer containing 5 mM-EGTA. An equal volume of sample buffer was added to the supernatant and pellet, and the samples were boiled for 2 min.

In other experiments, a part of the platelet suspension was incubated with 1 μM - PGE_1 for 20 min at 37 °C, centrifuged at 800 *g* for 10 min, and platelets were resuspended in an equal volume of resuspension buffer without PGE_1 . The platelet suspension was left at room temperature for 30–40 min before starting the experiment.

Measurement of protein phosphorylation

^{32}P -labelled proteins were separated by SDS/polyacrylamide (7.5, 10 or 12.5 %)-gel electrophoresis on 1.5 mm-thick and 20 cm-long gels (Bio-Rad, Rockville Center, NY, U.S.A.). Proteins were then stained with Coomassie Blue. The gels were dried with BioGelWrap (Bioscience Inc., Carmel, NY, U.S.A.) and were then exposed for 1–2 days to Kodak BB5 films. Specific zones of the dried gels localized by autoradiography were cut out and measured for ^{32}P radioactivity by liquid-scintillation counting. The experimental results shown are representative of three to four other experiments.

RESULTS

Exposure of human platelets to PGI_2 , PGE_1 or the PGI_2 analogue iloprost induced the phosphorylation of proteins with molecular masses of 22, 24, 30, 39, 50, 60, and 250 kDa (P22, P24 etc.) similarly to previous observations [12–16]. P24, P30, P60 and P250 were present mainly in the platelet particulate fraction, whereas P50 was predominantly a soluble protein. P22 was

Fig. 1. Time course of protein phosphorylation induced by iloprost

^{32}P -labelled platelets were incubated with iloprost (1 μM). Phosphoproteins: ●, P22; ▲, P24; □, P30; ○, P50.

present in both the cytosolic and the membrane fractions (results not shown). Time-course studies showed that phosphorylation of P24, P30, P39 and P50 was maximal 1 min after exposure of platelets to iloprost, whereas maximal phosphorylation of P22 (*rap1B*) was only reached after an incubation period of 30–60 min (Fig. 1). A similar time course was observed after exposure of platelets to PGI_2 and PGE_1 (results not shown). The finding of slower P22 phosphorylation and more rapid P24 and P50 phosphorylation confirms earlier observations [13].

We found that PGI_2 was more potent than PGE_1 in inhibiting thrombin-induced platelet aggregation; therefore we compared the extent of protein phosphorylation after platelet exposure to maximal concentrations of PGI_2 and PGE_1 . To our surprise, there were no significant differences between PGI_2 and PGE_1 in the degree and kinetics of cyclic AMP-dependent phosphorylation of P22, P24, P39 and P50 (Fig. 2). PGI_2 completely suppressed thrombin-induced platelet aggregation and phosphorylation of myosin light chain and P47 for at least 2 min, whereas after platelet incubation with PGE_1 , thrombin-induced protein phosphorylation and platelet aggregation were only delayed and slightly suppressed (Table 1).

For PGI_2 , an incubation period of 30 s was needed to inhibit thrombin-induced platelet activation. After 30 s, cyclic AMP-dependent phosphorylation of P22 only slightly increased,

Fig. 2. Comparison of protein phosphorylation induced by PGI_2 (200 nM) and PGE_1 (1 μM)

Phosphoproteins: ●, P22; ▲, P24; △, P39; ○, P50.

Table 1. Effect of PGI₂ and PGE₁ on aggregation and protein phosphorylation induced by thrombin

³²P-labelled platelets were preincubated for the times indicated with either PGI₂ (200 nM) or PGE₁ (1 μM) before addition of thrombin (10 nM). Protein phosphorylation was measured before (control, PGI₂, PGE₁) and 10 s (P20) and 30 s (P47) after addition of thrombin. Values are means ± s.d. of triplicate determinations.

	Protein phosphorylation (c.p.m. of ³² P)		Aggregation (mm)
	P20	P47	
Control	520 ± 27	677 ± 72	0
+ Thrombin	1882 ± 112	3793 ± 112	31
PGI ₂ , 15 s	521 ± 3	686 ± 48	0
+ Thrombin	663 ± 30	1155 ± 80	Start after 30 s
PGI ₂ , 30 s	548 ± 37	724 ± 39	0
+ Thrombin	529 ± 35	1061 ± 60	0
PGI ₂ , 2 min	359 ± 13	632 ± 65	0
+ Thrombin	469 ± 5	972 ± 5	0
PGE ₁ , 2 min	363 ± 24	705 ± 54	0
+ Thrombin	874 ± 105	2157 ± 182	Start after 30 s
After PGE ₁ wash			
Control	364 ± 23	657 ± 52	0
+ Thrombin	1234 ± 148	3377 ± 184	18
PGI ₂ , 30 s	291 ± 10	555 ± 5	0
+ Thrombin	424 ± 14	812 ± 77	9

whereas phosphorylation of P24 and P50 was half-maximal and that of P39 was almost maximal (Table 1, Fig. 2).

These experiments indicate that (a) inhibition of platelet activation by PGI₂ occurs without significant phosphorylation of *rap1B* and (b) the degree of phosphorylation of P24, P39 and P50 does not correlate with the differing degrees of platelet inhibition induced by PGI₂ and PGE₁.

A second strategy was employed to explore the functional relationship between cyclic AMP-dependent protein phosphorylation and inhibition of platelet function. Platelets were incubated and centrifuged in the presence of PGE₁, and then were resuspended in the absence of PGE₁. Thrombin induced aggregation with phosphorylation of P20 and P47, despite maximal cyclic AMP-dependent phosphorylation of P22 and P24 observed in these platelets (Fig. 3). Apparently, P22 and P24 (in contrast with P39 and P50) were not dephosphorylated after resuspension of platelets in the absence of PGE₁. Moreover, addition of PGI₂ to PGE₁-washed platelets prevented thrombin-induced aggregation and phosphorylation of P20 and P47 without a further increase in cyclic AMP-dependent phosphorylation of P22 and P24. In contrast, PGI₂ induced a further increase in P39 and P50 phosphorylation in PGE₁-washed platelets. These results indicate that phosphorylation of P22 and P24 is not correlated with the cyclic AMP-dependent inhibition of platelet activation; it is more likely that phosphorylation of P39 and P50 is functionally related to platelet inhibition by PGI₂.

The difference in the inhibitory action of PGE₁ and PGI₂ could reflect the ability of thrombin to decrease cyclic AMP levels below the concentration required for maximal activation of protein kinase A after platelet incubation with PGE₁, but not with PGI₂. Experiments were therefore carried out to determine whether dephosphorylation of cyclic AMP-phosphorylated proteins correlates with the reversal of platelet inhibition. As shown in Fig. 3, there seemed to be some indication of thrombin-induced dephosphorylation of P50, especially after platelet preincubation with PGE₁. This point is important and was

Fig. 3. Effects of PGI₂ and PGE₁ on cyclic AMP-dependent protein phosphorylation (a) and thrombin-induced protein phosphorylation (b): effect of PGE₁ wash

PGI₂ (200 nM) and PGE₁ (1 μM) were added 2 min before thrombin (10 nM). Protein phosphorylation was measured 15 s before, and 10 and 30 s after, addition (zero time) of thrombin (Thr). For PGE₁ wash, platelets were incubated with PGE₁ (1 μM) for 20 min at 37 °C, pelleted by centrifugation, and resuspended in the absence of PGE₁. Platelets were left at room temperature for 30 min before activation with thrombin. Platelet aggregation 30 s after addition of thrombin was for control platelets 27 (saline), 0 (PGI₂) and 3 mm (PGE₁), and for PGE₁-washed platelets 19 (saline) and 0 mm (PGI₂). Control values are means ± s.d. of triplicates. Phosphoproteins in (a): ●, P22; ▲, P24; △, P39; ○, P50.

therefore addressed in further experiments. As shown in Table 2, there was, however, no significant dephosphorylation of P50 at 10 and 30 s after addition of thrombin. Since results shown in Table 1 and Fig. 1 had indicated that 60% of maximal P50 phosphorylation might be sufficient to inhibit thrombin-induced platelet aggregation and protein phosphorylation, it is concluded that dephosphorylation of P50 is not responsible for the reversal of platelet inhibition observed, particularly after PGE₁. Also none of the other proteins (P22, P24, P39 and P60) were significantly dephosphorylated after addition of thrombin (Fig. 4, Table 2). This information suggests that cyclic AMP-dependent phosphorylation of these proteins is not solely responsible for platelet inhibition, and that the phosphorylated state of these proteins is not a simple 'off' switch for platelets.

Table 2. Effect of thrombin on the dephosphorylation of P50 and P39 phosphorylated by preincubation of platelets with PGE₁ or PGI₂

³²P-labelled platelets were exposed for 2 min at 37 °C to PGE₁ (1 µM) or PGI₂ (200 nM). Then saline or thrombin (10 nM) was added for 10 or 30 s. ³²P radioactivity of P50 and P47 in control samples ranged from 500 to 1000 c.p.m., and that of P39 from 450 to 700 c.p.m. Values (% of control) are means ± S.D. of three experiments.

		PGI ₂				PGE ₁			
		+ saline		+ thrombin		+ saline		+ thrombin	
	Control	10 s	30 s	10 s	30 s	10 s	30 s	10 s	30 s
P50	100	191 ± 13	195 ± 10	201 ± 8	197 ± 32	180 ± 15	195 ± 8	195 ± 27	193 ± 17
P39	100	148 ± 6	148 ± 9	149 ± 19	148 ± 14	148 ± 11	145 ± 19	161 ± 9	152 ± 9
P47	100	90 ± 10	78 ± 5	188 ± 34	212 ± 52	81 ± 15	76 ± 6	243 ± 60	378 ± 91

Fig. 4. Effect of thrombin on PGE₁-induced protein phosphorylation

PGE₁ (1 µM) was added at 10 s, and thrombin (10 nM) was added at 120 s. Aggregation started at 150 s. Phosphoproteins in (a): ●, P22; ▲, P24; □, P30; ○, P50; ▼, P60.

DISCUSSION

In mammalian cells, the effect of cyclic AMP is believed to be mediated by the activation of cyclic AMP-dependent protein kinase and the phosphorylation of specific target proteins [25]. It has been known for some time that prostaglandins such as PGI₂ and PGE₁ inhibit platelet function by increasing cyclic AMP levels that lead to the cyclic AMP-dependent phosphorylation of several platelet proteins [12–16]. However, the functional relationship of protein phosphorylation and platelet inhibition has not been fully explored. In the present study, PGI₂, iloprost and PGE₁ induced the phosphorylation of the platelet proteins P22, P24, P39, P50, P60 and P250 similarly to previous findings [12–16]. P22 was recently identified as *rap1B*, a GTP-binding *ras*-like protein [22a]. Its function is not known. *rap1B* is 95% identical with *rap1A* [23], also called *Krev-1* [24], which has been

shown to reverse the phenotype of *K-ras* transformed cells [24]. In the present study we found that phosphorylation of P22 was very slow as compared with the cyclic AMP-induced phosphorylation of all other proteins, a result that confirms earlier observations [13]. Phosphorylated P22 was present in both membranes and cytosol. We recently showed [22a] that phosphorylation of *rap1B* in the particulate fraction occurs at a C-terminal serine residue in close vicinity to cysteine-181. In *ras* this amino acid residue is C-terminal, carboxymethylated and farnesylated; the latter modification is essential for the membrane attachment of *ras* [26]. Phosphorylation of *rap1B* does not seem to affect the kinetics and extent of GTP binding *in vitro* [27]. *rap1B* is also identical with thrombolamban [19]. Earlier studies indicated that cyclic AMP-dependent phosphorylation of P22 stimulates Ca²⁺ uptake into platelet membrane vesicles [20]. That finding, however, was questioned in a recent study [28].

The present results clearly indicate that cyclic AMP-dependent phosphorylation of *rap1B* is not involved in the inhibition of thrombin-induced platelet activation. Platelet activation can be completely prevented by PGI₂ without significant phosphorylation of P22 (Table 1). Conversely, platelets can be fully activated by thrombin despite maximal phosphorylation of P22 (Fig. 3).

Phosphorylation of P24 also does not seem to play a role in cyclic AMP-mediated inhibition of thrombin-induced platelet activation (Fig. 3). The degree of phosphorylation of P24 was found to be unrelated to the inhibition of thrombin-induced protein phosphorylation and platelet aggregation. P24 is the β-chain of glycoprotein Ib. The α-chain of glycoprotein Ib is considered to be a binding site for thrombin and a receptor for von Willebrand factor on the platelet surface [5]. In a study of Bernard-Soulier platelets that are deficient of glycoprotein Ib and in which, consequently, cyclic AMP-dependent phosphorylation of the β-chain of glycoprotein Ib could not occur, collagen-induced polymerization of actin could not be inhibited by preincubating the platelets with PGE₁ [15]. However, collagen-induced platelet aggregation and P20 and P47 phosphorylation was inhibited by PGE₁ in these platelets. The effect of thrombin was not studied. It is possible that phosphorylation of glycoprotein Ib affects polymerization after exposure to thrombin, an hypothesis that was not addressed in the present study.

It is noteworthy that in a previous study [13] sodium nitroprusside, which increases cyclic GMP, and PGE₁, which increases cyclic AMP, induced equally well the phosphorylation of P50, but sodium nitroprusside was much less effective than PGE₁ in inducing the phosphorylation of P22 and P24. Both substances were potent inhibitors of collagen-induced protein phosphorylation. These results support our conclusions that cyclic AMP-dependent phosphorylation of *rap1B* and glycoprotein Ib is not involved in platelet inhibition by cyclic AMP.

Maximal concentrations of PGI₂ more potently inhibited thrombin-induced platelet activation than did maximal concentrations of PGE₁. Similar observations have been made by other investigators [29,30]. The degree and time course of cyclic AMP formation were reported to differ for platelets exposed to PGI₂ or PGE₁ [30]. PGI₂ induced a higher increase of cyclic AMP levels that was more sustained, whereas PGE₁ elicited a more rapid and smaller rise in cyclic AMP levels, followed by a rapid fall. In our experiments we found no difference in cyclic AMP-dependent protein phosphorylation after exposure of platelets to maximal concentrations of PGI₂ and PGE₁, indicating that maximal protein phosphorylation is reached by non-maximal increases of intracellular cyclic AMP levels (such as those elicited by PGE₁). It has been proposed that the observed rapid fall of cyclic AMP levels, especially after exposure to PGE₁, is caused by interaction of PGE₁ with inhibitory receptors that lead, via G_i activation, to an inhibition of adenylate cyclase [30]. Such a mechanism could co-operate with thrombin, which activates G_i in platelets [5], and might explain the smaller inhibition of platelets by PGE₁ than by PGI₂. G_i-protein activation has been recognized as an important intracellular pathway for platelet activation that mediates platelet aggregation independently of the inhibition of adenylate cyclase [5].

Proteins P39 and P50 are more likely to be involved in platelet inhibition by cyclic AMP. In our study, phosphorylation of these proteins correlated best with the inhibition of platelet activation by PGI₂. One protein, P50, has recently been purified from platelet membranes [31]. This protein is phosphorylated *in vitro* by cyclic AMP and cyclic GMP [14]. In agreement with our results, phosphorylation of P50 was recently reported to be reversible [32]. The further identification of P50 and P39 may help to identify the pathways involved in inhibition of platelet activation. However, phosphorylation of these proteins may not be the only factor in inhibition of platelet activation, since reversal of platelet inhibition after addition of thrombin was not preceded by dephosphorylation of P39 or P50. As described previously for cyclic AMP-induced exocytosis in parotid [33], one might speculate that cyclic AMP has effects in platelets that are unrelated to cyclic AMP-dependent protein phosphorylation. These effects could involve cyclic AMP receptors or cyclic AMP-binding proteins, which are well known in non-mammalian cells such as *Dictyostelium* and *Escherichia coli* [34,35].

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