Functional implications of tyrosine protein phosphorylation in platelets

Simultaneous studies with different agonists and inhibitors

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During activation of platelets by agonists, a number of proteins become phosphorylated at tyrosine residues. Using immunoblotting with a monoclonal anti-phosphotyrosine antibody, we have compared the different phosphotyrosine-protein (PTP) profiles of platelets stimulated with thrombin, collagen, ADP, arachidonic acid, phorbol myristate acetate and P256, an anti-glycoprotein-IIb-IIIa (GPIIb-IIIa) monoclonal antibody (mAb). Only a few PTPs were observed in resting platelets, of molecular masses 130, 64, 56–60 and 36 kDa. After stimulation by different agonists these proteins were more intensely phosphorylated and additional PTPs appeared with molecular masses of 170, 150, 140, 120, 105/97 (doublet), 85, 80, 75 and 45 kDa. The kinetics of phosphorylation differed from one agonist to another, but no significant differences in the overall patterns were detected, except in presence of ADP and P256-F(ab')₂, which induced only the additional tyrosine phosphorylation of the 64 kDa protein and to a lesser extent that of a 75 kDa protein. The use of various agonists and the inhibitors (staurosporine, ajoene and RGDS) permitted a better characterization of the relationship between the different steps of activation and phosphorylation on tyrosine residues. The studies suggest the following conclusions: (i) stimulation of tyrosine phosphorylation occurs after activation of protein kinase C; (ii) there is a relationship between ligand binding to GPIIb-IIIa and the tyrosine phosphorylation of the 64 kDa protein; and (iii) there is a close relationship between PTP formation and the intensity of platelet activation and aggregation.

INTRODUCTION

Human platelets circulate in the blood as quiescent cells, and can be activated by a variety of agonists to release granule constituents and to aggregate. The biochemical basis for these processes has been linked to the activation of a polyphosphoinositide-specific phospholipase C, which yields the messenger molecules inositol trisphosphate and diacylglycerol, both of which contribute to the activation of kinases (for a review see Siess, 1989). These events lead to the phosphorylation of the specific proteins myosin light chain and pleckstrin, which are the substrate for myosin light chain kinase (MLCK) and the main substrate for protein kinase C (PKC) respectively. In several studies using thrombin and other platelet agonists, specific phosphorylation of proteins on tyrosine residues occurs during platelet activation (Ferrel & Martin, 1988; Golden & Brugge, 1989; Nakamura & Yamamura, 1989). A few studies have investigated the functional role of this phosphotyrosine-protein (PTP) production and the regulatory mechanisms involved. Some have described the relationship between PTPs and intracellular signal transduction systems, such as the elevation of Ca²⁺ and PKC activation (Vostal *et al.*, 1991; Takayama *et al.*, 1991) or the elevation of cyclic AMP (Pumiglia *et al.*, 1990). Moreover, the previous observation that tyrosine kinase inhibitors can inhibit platelet aggregation and release of granule constituents suggests that tyrosine kinases may play an important role in the physiological activation of platelets (Rendu *et al.*, 1991). Other workers have studied the relationship between PTPs and the

integrin glycoprotein IIb-IIIa (GPIIb-IIIa). Thus Ferrell & Martin (1989) showed that some PTPs were dependent upon fibrinogen binding and the agonist used, whereas Golden and collaborators concluded that these same proteins required aggregation but were independent of the agonist used (Golden *et al.*, 1990). Since ligand binding to GPIIb-IIIa has previously been suggested to induce some post-occupancy events, such studies point to a role for GPIIb-IIIa not only in the aggregatory mechanism but also in signal transduction.

In this paper, we have examined the tyrosine phosphorylation induced by a variety of receptor-dependent and receptor-independent agonists upon platelet activation. Notable among the former was a monoclonal antibody (mAb) specific for GPIIb-IIIa, which was shown to activate platelets (Bachelot *et al.*, 1990; Stuttle *et al.*, 1991). Whereas the antibody induced full platelet activation, the F(ab')₂ fragments induced only weak aggregation, without activation of phospholipase C or granule release (Bachelot *et al.*, 1991). Therefore this mAb represented a good tool to the specific study of 'post-occupancy events' which occur after ligand binding to GPIIb-IIIa (Ginsberg *et al.*, 1990; Phillips *et al.*, 1991). Our data indicate that: (i) the pattern of PTPs is different depending on the potency of the agonist to induce either a strong and irreversible aggregation or only a weak one; (ii) with strong agonists, PTP production occurs downstream of protein kinase C activation; and (iii) the PTP doublet of 105/97 kDa seemed to be correlated with strong aggregation, but GPIIb-IIIa activation alone (no aggregation) was sufficient to induce the phosphorylation of a 64 kDa tyrosine protein.

Abbreviations used: GPIIb-IIIa, glycoprotein IIb-IIIa; mAb, monoclonal antibody; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTP, phosphotyrosine-protein; RGDS, Arg-Gly-Asp-Ser.

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Fig. 1. Protein-tyrosine phosphorylation in isolated human platelets stimulated by thrombin or collagen

The platelets were stirred in the cuvette of an aggregometer at 37 °C. At the end of the stimulation period, proteins were solubilized and separated on a 10 % polyacrylamide gel, transferred and immunoblotted with the anti-phosphotyrosine antibody as described in the Materials and methods section. (a) Time course in the presence of thrombin (0.035 unit/ml) or collagen (7.5 µg/ml). (b) Dose responses after 120 s of stimulation with various concentrations of thrombin (unit/ml: b, 0.025; c, 0.035; d, 0.05; e, 0.075; f, 0.1; g, 0.2; h, 0.5 or various concentrations of collagen (µg/ml: i, 1.25; j, 2.5; k, 3.5; l, 5; m, 7.5; n, 12.5). 5-Hydroxytryptamine release (Rel.) and/or aggregation intensity (Agg.) are given below each lane. Immunoblots are representative of four experiments.

MATERIALS AND METHODS

Reagents

Phorbol 12-myristate 13-acetate (PMA), staurosporine and Arg-Gly-Asp-Ser (RGDS) were purchased from Sigma. Bovine thrombin and collagen were obtained from Hoffmann-La Roche (Basel, Switzerland) and Stago (Asnières, France) respectively. Metrizamide was from Nycomed AS (Oslo, Norway). Electrophoresis chemicals and nitrocellulose were supplied by Bio-Rad.

Antibodies

P256, an mAb of isotype IgG₁ specific for GPIIb-IIIa, was that used by Bai *et al.* (1984). The anti-phosphotyrosine mAb was produced by Druker *et al.* (1989). The specificity of this antibody was confirmed by competition with phenyl phosphate (40 mM), phosphotyrosine (1 mM), phosphoserine (1 mM) and phosphothreonine (1 mM). In presence of phenyl phosphate or phosphotyrosine the immunoblotted bands were completely abolished, whereas phosphoserine and phosphothreonine had no significant effect (results not shown).

Platelet preparation and stimulation

Human platelets were isolated as previously described (Rendu *et al.*, 1983). Briefly, blood was anticoagulated with 0.1 vol. of 12 mM-trisodium citrate, 13 mM-citric acid and 11 mM-glucose. Platelet isolation was carried out from platelet-rich plasma at

room temperature using a metrizamide gradient and a concentration of $(3-4) \times 10^8$ platelets/ml was obtained in 10 mM-Hepes, 140 mM-NaCl, 3 mM-KCl, 0.5 mM-MgCl₂, 5 mM-NaHCO₃ and 10 mM-glucose, pH 7.4.

Aggregation was assessed using 0.4 ml aliquots at 37 °C under constant stirring (1200 rev./min) in a Payton dual-beam aggregometer, after addition of the agonist. The amplitude of the change in absorbance was expressed as a percentage of the absorbance of a blank sample (buffer without platelets). In few experiments the samples were fixed and observed under a phase-contrast microscope in order to quantify the number of small aggregates. Some of the samples were analysed with a multisizer counter (Coultronics). This allowed us to classify large aggregates [diameter of the particle (d) > 50 µm], medium-sized aggregates (20 µm < d < 50 µm) and small aggregates (maximum of four platelets attached together; d < 20 µm), which were registered by the aggregometer as intensity over 35 %, between 5 % and 35 %, and under 5 % respectively. In some experiments, fibrinogen, RGDS, ajoene [a gift from Dr. R. Apitz-Castro (IVIC, Caracas, Venezuela)] or staurosporine was added 1 min before activation.

Secretion was studied using part of the platelet-rich plasma, which was preincubated with 0.6 µM-[¹⁴C]5-hydroxytryptamine (Amersham) for 30 min at room temperature before platelet isolation. The reaction was stopped by transfer into 0.2 vol. of ice-cold 0.1 M-EDTA and the mixture was immediately centrifuged (15000 g, 1 min). The [¹⁴C]5-hydroxytryptamine was

measured in the supernatant by liquid scintillation counting. Release is expressed as a percentage of the total platelet content.

Studies of PTPs were performed using unlabelled platelets. Stimulation was stopped by addition of 0.25 vol. of 10% (v/v) SDS and 5 mM-EDTA.

Immunoblot procedure

Samples were heated for 1 h at 60 °C in the presence of 5% 2-mercaptoethanol. Proteins were separated using either 10% or 7% polyacrylamide gel electrophoresis and transferred to nitrocellulose by semi-dry transfer (2 h at 200 mA), between 7 and 15 V.

Non-specific binding was blocked with 5% low-fat powder in a buffer of Tris (10 mM), NaCl (0.17 M), NaN_3 (0.05%) and Tween 20 (0.05%) before probing with the anti-phosphotyrosine mAb. Blots were washed five times with the above buffer prior to detection of bound antibody with ^{125}I -labelled rabbit anti-mouse antibody. After five further washings, the membranes were exposed to Hyperfilm β -max (Amersham).

RESULTS

Kinetics and dose-response induced by thrombin and collagen

In resting platelets only a few proteins were significantly phosphorylated on tyrosine, including proteins with molecular masses of 130 kDa, 64 kDa, 56–60 kDa (identified as the src kinase; Rendu *et al.*, 1991) and 36 kDa (Fig. 1). However, some variation was observed between different experiments. Although this could be explained by a very low level of activation of platelets inherent in the preparation of isolated platelets, the most important differences we observed in non-stimulated platelets were due to stirring. Thus control platelets (Fig. 1b, lane a) which were stirred for 2 min in the aggregometer had more intense bands than those which were not stirred (Fig. 1a, thrombin 0 s and collagen 0 s). After stimulation with thrombin or collagen, the intensity of phosphorylation of these proteins increased and additional PTPs appeared, with molecular masses of 170, 150, 140, 120, 105/97 (doublet), 85, 80, 75 and 45 kDa (Fig. 1a). The differences between the kinetics of phosphorylation stimulated by the two agonists correlated with the prolonged lag observed with collagen-induced platelet activation. However, no differences in the overall patterns were detectable. Some of these PTPs appeared rapidly (64, 75 and 130 kDa), preceding the formation of platelet aggregates over 20 μm in diameter, whereas some were phosphorylated when medium-sized aggregates were formed (140, 150 and 170 kDa) and others only at later times when large aggregates were formed (105/97 kDa doublet), i.e. when aggregation was nearly complete. Fig. 1(b) shows the dose-dependence of PTP formation for the two agonists after 120 s of stimulation. Proteins were maximally tyrosine-phosphorylated with 0.075 unit of thrombin/ml (lane e) or 7.5 μg of collagen/ml (lane m). The 64, 75 and 130 kDa proteins were phosphorylated in the presence of low doses of agonists, i.e. during weak activation (Fig. 1b, lanes b, i and j) when aggregation and release were lower than 20% and 15% respectively. These PTPs corresponded to those shown in Fig. 1(a) to appear shortly after activation, i.e. when only small aggregates had formed.

Comparison of PTPs induced by different platelet activators

In order to determine whether tyrosine phosphorylations depended on the agonist, we used several inducers [thrombin, collagen, ADP, arachidonic acid (a precursor of thromboxane) and PMA] in order to study the effect of direct activation of PKC, and mAb P256, specific for GPIIb-IIIa, previously described to induce platelet activation (Bachelot *et al.*, 1990). As shown in Fig. 2, all strong agonists, which could induce both

aggregation beyond 35–40% (large aggregates) and release (i.e. thrombin, collagen, arachidonic acid, PMA and P256-IgG) induced phosphorylation of a common set of proteins on tyrosine. The band at 75 kDa corresponded to at least two bands (75 kDa and a slightly lower molecular mass) which were not always separated, and was more easily observed when only medium-sized aggregates were formed.

By contrast, weaker agonists, which induced less than 35% aggregation (medium-sized aggregates) without release, induced

Fig. 2. Comparison of PTPs induced by various agonists

Platelets were stimulated for 120 s in the presence of Ca^{2+} (1 mM) with thrombin (0.05 unit/ml; lane b), collagen (10 $\mu\text{g}/\text{ml}$; lane c), ADP (20 μM) alone (lane d) or in the presence of fibrinogen (100 $\mu\text{g}/\text{ml}$; lane e), arachidonic acid (1 μM ; lane f), P256 (4 $\mu\text{g}/\text{ml}$; lane g), P256-F(ab')₂ (8 $\mu\text{g}/\text{ml}$; lane h) or PMA (200 nM; lane i). Proteins were separated on a 7% polyacrylamide gel, transferred and immunoblotted with the anti-phosphotyrosine mAb as described in the Materials and methods section. The arrows designate successively PTPs of 130 kDa, the 105/97 kDa doublet, 75 kDa, 64 kDa and the 56–60 kDa (src). Aggregation intensities (Agg.) are given below each lane. Immunoblots are representative of at least four experiments.

Fig. 3. Effects of ajoene on thrombin- or collagen-induced PTPs

Isolated platelets were stimulated for 120 s with thrombin (0.05 unit/ml; lanes a and b) or collagen (10 $\mu\text{g}/\text{ml}$; lanes c and d), with (b, d) or without (a, c) ajoene (50 μM), an inhibitor which induces membrane structural modifications and inhibits aggregation and release (Rendu *et al.*, 1989). Proteins were separated on a 7% polyacrylamide gel, transferred and immunoblotted with the anti-phosphotyrosine mAb as described in the Materials and methods section. The arrow indicates the lower band of the 75 kDa proteins. Aggregation intensities (Agg.) are given below each lane. Addition of ajoene to resting platelets was without effect. Immunoblots are representative of four experiments.

Fig. 4. Effects of RGDS on PTPs

Isolated platelets were stimulated for 120 s with thrombin (0.05 unit/ml; lanes c and d), P256-IgG (4 μ g/ml; e and f) or P256-F(ab')₂ (8 μ g/ml; g and h), with (+) or without (–) RGDS (200 μ M). Lanes a and b are controls. Proteins were separated on a 7% polyacrylamide gel, transferred and immunoblotted with the anti-phosphotyrosine mAb as described in the Materials and methods section. Immunoblots are representative of four experiments.

only certain PTPs. F(ab')₂ induced only the phosphorylation of the 64 kDa and (to a lesser extent) the 75 kDa proteins. As previously described, the F(ab')₂ fragments induced weak aggregation which varied from 10 to 20% depending on the donor. In some experiments, when fibrinogen was added together with F(ab')₂, the aggregation reached around 30% intensity without the formation of large aggregates; nevertheless, the pattern of PTPs remained identical to that shown in Fig. 2. Thus only the phosphorylation of the 64 kDa and the 75 kDa PTPs seemed to be related to F(ab')₂-induced activation. During stimulation by ADP in the presence of added fibrinogen, aggregation reached at most 35% intensity (no large aggregates) and the doublet of 105/97 kDa was not observed. Noteworthy were the PTPs obtained in the presence of ADP and in the absence of fibrinogen. Under such conditions, where only small aggregates were formed, PTPs were similar to those in the presence of both fibrinogen and ADP, but less phosphorylated.

In the presence of ajoene (Rendu *et al.*, 1989), which by inducing membrane structural modifications prevents 5-

hydroxytryptamine release and inhibits aggregation by around 70%, the lower band at 75 kDa was easily detectable (Fig. 3, arrow). In the presence of this inhibitor, however, the 105/97 kDa doublet was absent.

Effects of RGDS on PTP formation

RGDS is a specific inhibitor of platelet aggregation (Plow *et al.*, 1987) which has no effect on release. It inhibits the binding of fibrinogen and other ligands, such as fibronectin and von Willebrand factor, which bind to activated GPIIb-IIIa and allow aggregation. As previously reported (Ferrell & Martin, 1989; Golden *et al.*, 1990), RGDS did not change the PTP profile in resting platelets, whereas it completely inhibited the phosphorylation of the 105/97 kDa doublet induced by thrombin, P256-IgG (Fig. 4) and other agonists (results not shown). These results confirm that the 105/97 kDa PTP was dependent on fibrinogen binding to GPIIb-IIIa. In contrast, the phosphorylation of the 64 kDa PTP was not dependent on fibrinogen binding to GPIIb-IIIa since, in the presence of RGDS, the 64 kDa band was still phosphorylated during thrombin- or IgG-induced activation, as well as during F(ab')₂-induced activation.

Inhibition of fibrinogen binding by RGDS caused a slight enhancement of the phosphorylation of the 170 and 130 kDa proteins following thrombin- and P256-IgG-induced platelet activation (Fig. 4).

Effects of a PKC inhibitor on PTP formation

Staurosporine is a potent inhibitor of protein kinase C. It inhibited PMA-induced aggregation (by 95%) and phosphorylation of pleckstrin, the main substrate of PKC (results not shown) at a concentration compatible with those used to inhibit platelet PKC (Watson *et al.*, 1988). It almost totally suppressed all PTPs induced by PMA (Fig. 5). Staurosporine strongly, although not completely, inhibited the aggregation induced by collagen, whereas aggregation induced by thrombin or IgG was inhibited by almost 50% and that induced by F(ab')₂ was not significantly modified. It also caused an inhibition of the phosphorylation of the majority of tyrosine proteins induced by thrombin and collagen (Fig. 5). The 105/97 kDa doublet appeared to be totally absent in the presence of staurosporine, whereas phosphorylation of the 64 kDa protein with thrombin was only partially inhibited and that with P256 was unaffected. Phosphorylation of the 75 kDa proteins was also partially inhibited. Staurosporine did not affect F(ab')₂-induced PTP formation. Thus the profiles of

Fig. 5. Effects of staurosporine on PTPs

Isolated platelets were stimulated for 120 s with thrombin (0.05 unit/ml; lanes c and d), collagen (10 μ g/ml; e and f), P256-IgG (4 μ g/ml; g and h), P256-F(ab')₂ (8 μ g/ml; i and j) or PMA (200 nM; k and l), with (+) or without (–) staurosporine (3 μ M Stau.). Lanes a and b are controls. Proteins were separated on a 7% polyacrylamide gel, transferred and immunoblotted with the anti-phosphotyrosine mAb as described in the Materials and methods section. Aggregation intensities (Agg.) are given below each lane. Immunoblots are representative of three experiments.

PTP induced by thrombin in the presence of staurosporine were similar to those induced by $F(ab')_2$. Similar results were obtained in the presence of 100 μM -H-7, another PKC inhibitor (results not shown).

DISCUSSION

Blood platelets contain high levels of protein kinase activity, and it is now well established that physiological activators cause tyrosine phosphorylation of several platelet proteins. However, the role of this phosphorylation is unclear. The platelet signal transduction cascade (generation of inositol phosphate and diacylglycerol) that leads to an increase in cytosolic Ca^{2+} and PKC activation has been extensively described. Nevertheless, the relationship between these systems and the activation of tyrosine kinases remains unclear. The present study attempts to further define the roles of PKC and the integrin GPIIb-IIIa in the phosphorylation on tyrosine residues during platelet activation. Moreover, we have attempted to specify the relationship between PTPs and the different steps of aggregation (reflecting different states of activation).

Besides the PTPs observed in resting platelets, activation by different inducers [physiological (i.e. thrombin, collagen, arachidonic acid) or non-physiological (i.e. PMA, P256-IgG)] elicited similar tyrosine-phosphorylation profiles: proteins of 170, 150, 140, 130, 120, 105/97 (doublet), 85, 80, 75, 64 and 45 kDa became tyrosine-phosphorylated for the first time or became more heavily tyrosine-phosphorylated. The fact that similar PTP profiles were observed with non-receptor-dependent inducers suggests that tyrosine phosphorylation is a late step in platelet activation. The inhibitory effect of staurosporine suggests that tyrosine phosphorylation is consecutive to PKC activation. The

specificity of this inhibitor has been recently questioned, however, since it may also inhibit tyrosine kinases (Fallon, 1990). Nevertheless, the PTP profile observed in the presence of PMA, a direct PKC activator, lends support to the idea that tyrosine phosphorylation occurs after PKC activation. Moreover, similar PMA-induced PTP profiles were obtained in both untreated and aspirin-treated platelets (results not shown). This excluded the possibility of the PMA action being due to arachidonate oxygenation products. Post-PKC tyrosine phosphorylation has also been proposed in sea urchin egg cortex (Jiang *et al.*, 1990). The fact that all of these inducers caused similar modifications contrasts with previous reports (Ferrell & Martin, 1988; Nakamura & Yamamura, 1989; Lerea *et al.*, 1989). The discrepancies may be due to differences in the platelet isolation procedure, since it is difficult to prepare isolated unactivated platelets. Platelets partially activated during the isolation steps would then not react after stimulation by weak agonists. This has previously been discussed (Lerea *et al.*, 1989; Golden *et al.*, 1990); however, these groups only focused on the 50 kDa protein, and the 85, 95 and 97 kDa proteins, respectively. The different anti-PTP mAbs used could also explain the discrepancies between the different reports. In our study, low-molecular-mass PTPs could not always be detected (Rendu *et al.*, 1991).

Indeed, the phosphorylation of the 97/95 kDa PTP described by Golden *et al.* (1990), which we assume to be identical to our doublet of 105/97 kDa (given the identical distributions obtained by both groups), was related to platelet aggregation. It occurred late during thrombin- or collagen-induced platelet activation, and in agreement with both Ferrell and Golden's groups it was effectively inhibited by RGDS (Ferrell & Martin, 1989; Golden *et al.*, 1990). These PTPs appeared only after activation with strong agonists which caused more than 35–40% aggregation, i.e. the formation of large aggregates. With weak agonists, e.g. ADP or $F(ab')_2$, these 105/97 kDa proteins were never phosphorylated. This could be explained either by the weak aggregation induced by these agonists (no large aggregates are formed) or by the fact that the 105/97 kDa PTP is dependent upon both aggregation and an additional mechanism not induced by ADP and $F(ab')_2$. The phosphorylations, which require both the activation of the GPIIb-IIIa complex and the fibrinogen binding responsible for aggregation, could be one of the post-occupancy events (Ginsberg *et al.*, 1990; Phillips *et al.*, 1991). Indeed, there is emerging evidence that the binding of adhesive proteins to GPIIb-IIIa might mediate receptor functions such as enhanced affinity for ligand binding or signal transduction events (Frelinger *et al.*, 1990; Du *et al.*, 1991). GPIIb-IIIa activation was also suggested to be achieved directly with certain mAbs (O'Toole *et al.*, 1990). Recently Huang *et al.* (1991) found some tyrosine kinases associated with GPIV, the thrombospondin receptor (Asch *et al.*, 1987; McGregor *et al.*, 1989). This glycoprotein was proposed to be involved in irreversible aggregation through a macromolecular complex (Nachman *et al.*, 1987). This macromolecular complex may also be responsible for the 105/97 kDa protein phosphorylation.

In a previous report, we have shown that the binding of P256 $F(ab')_2$ alone could induce an activation of GPIIb-IIIa, allowing weak aggregation (Bachelot *et al.*, 1991). However, only the cross-talk between the two pathways evoked by $F(ab')_2$ and Fc receptors respectively allowed the activation of all platelet activation systems (Bachelot *et al.*, 1991; the present work, Fig. 6). The conformational change in GPIIb-IIIa induced by $F(ab')_2$ can allow the binding of fibrinogen, but to a lesser extent than that induced by IgG. Thus the different conformational states of GPIIb-IIIa could be associated with distinct post-occupancy events (Fig. 6). The use of the mAb P256 $F(ab')_2$ directed against GPIIb-IIIa allows us to demonstrate that phosphorylation of

Fig. 6. Occurrence of tyrosine-protein phosphorylation during platelet activation

The figure summarizes our knowledge of the relationship between PTPs and GPIIb-IIIa in platelet activation, based upon data reported in the present paper and in the literature. On the left is shown signal transduction induced by strong agonists; on the right are shown the different conformational states of GPIIb-IIIa (resting state, weakly activated and fully activated). Three groups of PTPs are defined, namely: PTP1 (64 kDa and 75 kDa), PTP3 (97/105 kDa doublet) and PTP2 (all other newly phosphorylated proteins) (see the Discussion). The arrow from the Fc receptor represents the cross-talk between the two pathways evoked by the Fc receptor and the IIB-IIIa epitope, as previously discussed (Bachelot *et al.*, 1991). Abbreviations: DG, diacylglycerol; IP_3 , inositol trisphosphate; L, ligand; MLCK, myosin light chain kinase; PKC and *PKC, primed and activated states of PKC respectively (Bell, 1985); PPI, polyphosphoinositides susceptible to phospholipase C; TX, thromboxane; FcγRII, receptor of the Fc part of IgG on platelet membrane.

the 64 kDa band is related to GPIIb-IIIa activation. However, tyrosine phosphorylation of this protein was not inhibited by RGDS, which is known to inhibit fibrinogen binding to GPIIb-IIIa. RGDS can permit the conformational change, i.e. GPIIb-IIIa activation, induced by the agonists. Such a modification of GPIIb-IIIa could be sufficient to allow the tyrosine-phosphorylation of the 64 kDa protein. On the other hand, we cannot exclude the possibility that tyrosine-phosphorylation of the 64 kDa protein could be induced by two separate mechanisms, one dependent on GPIIb-IIIa activation and one dependent on the platelet signal transduction cascade induced by the platelet agonists (Fig. 6). From several conflicting reports it is clear that ADP does not allow full platelet activation (Suzuki *et al.*, 1988; Siess, 1989), but addition of fibrinogen together with ADP could induce a modification of GPIIb-IIIa allowing the tyrosine-phosphorylation of the 64 kDa protein.

Functional platelet responses (i.e. aggregation and granule release) and the state of phosphorylation of tyrosine proteins seem to be closely related. Indeed, it was demonstrated that inhibitors of tyrosine kinases inhibit platelet aggregation and release (Rendu *et al.*, 1991). Moreover, in the present study we show that inhibition of aggregation (by RGDS) and/or release (by ajoene) resulted in either inhibition or enhancement of phosphorylation of tyrosine proteins induced by strong agonists. Thus under these conditions some of the PTPs were absent (105/97 kDa), whereas other were more phosphorylated or not dephosphorylated (170 and 130 kDa proteins; lower band of the 75 kDa proteins). These results suggest that phosphorylation or dephosphorylation of regulatory proteins may be important in platelet activation. A role for both tyrosine kinase and tyrosine phosphatase activities has indeed been previously proposed (Lerea *et al.*, 1989).

In conclusion, our results are consistent with the presence of three groups of PTPs whose phosphorylation is dependent on the mechanism involved in platelet activation and occurs after PKC activation (Fig. 6). Of these, one group was dependent on GPIIb-IIIa activation [PTP1 (64, 75 kDa)] and one was dependent on strong aggregation [PTP3 (105/97 kDa)].

This work was supported by Dret grant 89/247 (to F.R.). We are grateful to Dr. Rafael Apitz-Castro for providing ajoene.

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Received 30 December 1991; accepted 17 January 1992