Tyrosine kinases and phosphoinositide metabolism in thrombin-stimulated human platelets

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In this study we have examined the implication of tyrosine kinase activities in aggregation, 5-hydroxytryptamine secretion and mainly phosphoinositide metabolism in response to human platelet stimulation by thrombin. Using the potent tyrosine kinase inhibitor tyrphostin AG-213, we have observed a significant inhibition of aggregation and 5-hydroxytryptamine release; however, this percentage inhibition was lower at high thrombin concentrations. On the other hand, tyrphostin treatment of metabolically ^{32}P -labelled platelets significantly inhibited the thrombin-dependent accumulation of $\text{PtdIns}(3,4)\text{P}_2$, which involves at least a PtdIns 3-kinase and/or a $\text{PtdIns}3\text{P}$ 4-kinase, whereas the synthesis of phosphatidic acid (PtdOH), a good reflection of the phospholipase C (PLC) activation in platelets,

was partially blocked. Inositol phosphate production was also inhibited by about 40 % when tyrphostin-treated platelets were stimulated with thrombin. In addition, we show by Western-blot analysis that $\text{PLC}\gamma 1$, as well as the regulatory subunit (p85) of the PtdIns 3-kinase, were present in the anti-phosphotyrosine immunoprecipitate isolated from thrombin-stimulated platelets. Furthermore, tyrphostin treatment clearly decreased the $\text{PLC}\gamma 1$ and p85 contents in such an anti-phosphotyrosine immunoprecipitate. Our results provide the first evidence for a direct or indirect regulation of $\text{PtdIns}(3,4)\text{P}_2$ accumulation and $\text{PLC}\gamma 1$ activity by tyrosine phosphorylation during thrombin stimulation of human platelets.

INTRODUCTION

The signal-transduction cascade known as the phosphoinositide pathway is activated by a wide variety of hormones and growth factors [1,2]. The receptors for these agonists are either coupled to G-proteins, like the thromboxane A_2 -receptor and the muscarinic receptor [3,4], or contain an intrinsic tyrosine kinase, like the epidermal growth factor (EGF) receptor and platelet-derived growth factor (PDGF) receptor [5]. It has been shown that different isoforms of phospholipase C (PLC) are implicated in each pathway; for example, $\text{PLC}\beta_1$ is regulated by Gq [6], whereas a cytosolic isoform called $\text{PLC}\gamma 1$ is phosphorylated and activated by several tyrosine-kinase-containing receptors [7]. Recently, the receptor for thrombin has been cloned; it belongs to the seven-transmembrane-domain receptor family, suggesting its coupling to G-proteins [8]. Moreover, the activation of PLC in thrombin-stimulated human platelets has been demonstrated to occur through a G-protein [9]. However, it is now well established that thrombin also induces specific tyrosine phosphorylation of several proteins in platelets [10,11]. In this respect, it is noteworthy that tyrosine phosphorylation of some of these proteins may correlate with 5-hydroxytryptamine (serotonin) secretion and aggregation [12], cyclic AMP elevation [13] or increased phosphoinositide turnover [14]. The tyrosine kinase(s) responsible for these specific phosphorylations, as well as its (their) regulation, have not been elucidated so far. One can suggest that non-receptor tyrosine kinases of the *src* family, which are abundant in platelets [15], could play this role. On the other hand, we and others have reported a relationship between receptor and non-receptor tyrosine kinases and the phosphoinositide metabolism [16,17]. Indeed, activation of

PtdIns 3-kinase or $\text{PLC}\gamma 1$ has been shown to involve tyrosine kinase activities [7,18], and some studies suggest that PtdIns 4-kinase and/or $\text{PtdIns}4\text{P}$ 5-kinase may be associated with and regulated by the EGF receptor [17,19]. Therefore, it was decided to investigate the implication of tyrosine kinase activities in the different aspects of the thrombin-dependent activation of phosphoinositide metabolism in human platelets.

For this purpose we have used tyrphostin AG-213 as tyrosine kinase inhibitor [20], and we have shown firstly that physiological responses of platelets, such as aggregation and 5-hydroxytryptamine secretion, were markedly affected by tyrphostin treatment. Secondly, we have observed that $\text{PtdIns}(3,4)\text{P}_2$ and phosphatidic acid (PtdOH) synthesis, which is classically observed during thrombin activation of platelets, were significantly inhibited by tyrphostin treatment. Accumulation of $\text{PtdIns}(3,4)\text{P}_2$ in platelets has been reported to implicate the binding of fibrinogen to the integrin GpIIb/IIIa [21], which is also able to regulate specific tyrosine phosphorylations in platelets [22]. On the other hand, the partial inhibition of both PtdOH synthesis and inositol phosphate formation observed in the presence of tyrphostin suggested the participation of a $\text{PLC}\gamma$ isoform in thrombin-stimulated platelets. We have confirmed the relationship between PLC as well as PtdIns 3-kinase and tyrosine kinase activities by demonstrating the presence of $\text{PLC}\gamma 1$ and p85 (the regulatory subunit of PtdIns 3-kinase) in the anti-phosphotyrosine immunoprecipitate obtained from thrombin-stimulated platelets. Interestingly, tyrphostin treatment markedly decreased the $\text{PLC}\gamma 1$ and p85 contents in such an anti-phosphotyrosine immunoprecipitate.

Thus, our data indicate that tyrosine phosphorylation may play an important role in biochemical and functional platelet

Abbreviations used: PLC, phospholipase C; p85, regulatory subunit of the PtdIns 3-kinase; PtdOH , phosphatidic acid; DAG, diacylglycerol; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; ras GAP, p21^{ras} -GTPase activating protein.

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responses to thrombin, and that at least some enzymes of the phosphoinositide cycle may be partly regulated directly or indirectly by tyrosine phosphorylation in this model.

EXPERIMENTAL

Preparation and activation of platelets

Platelet concentrates were obtained from the local blood bank (Centre Régional de Transfusion Sanguine, Toulouse, France). Platelets were prepared as previously described by Ardlie et al. [23]. In some experiments platelets were labelled at room temperature with 200 μ Ci of sodium [32 P]orthophosphate (Amersham International, Amersham, Bucks., U.K.)/ml during 90 min in a Ca^{2+} -free Tyrode's buffer (pH 6.5, with 0.2 mM EGTA). 32 P-labelled platelets were then washed in the same buffer minus EGTA, 2×10^9 cells/ml were resuspended in Tyrode's buffer A (pH 7.35, 2.5 mM CaCl_2) and immediately used for activation experiments and lipid analysis.

For inositol phosphate measurements, platelets were incubated with gentle shaking in Ca^{2+} -free Tyrode's buffer (pH 7, with 0.1 mM EGTA, 1 mM MnCl_2) containing 100 μ Ci of *myo*-[^3H]inositol (Amersham International)/ml 3.5 mM glucose during 60 min and then 24 mM glucose during 120 min. Platelets were then washed with the same buffer lacking EGTA and MnCl_2 and resuspended in Tyrode's buffer A.

For activation, platelets in Tyrode's buffer A were preincubated at 37 °C for 5 min with gentle shaking, then incubated during 5 min in the presence or the absence of 100 μ M tyrphostin AG-213 (obtained from the laboratory of A. Levitzki, Hebrew University, Jerusalem, Israel) and subsequently stimulated with indicated concentrations of human thrombin (Sigma, St. Louis, MO, U.S.A.) at 37 °C in a shaking water bath, for 5 min except when indicated. The tyrphostin was stored as a 100 mM solution in dimethyl sulphoxide; the final dimethyl sulphoxide concentration in the presence of platelets did not exceed 0.1 % and was adjusted as well in all samples. For lipid or inositol phosphate analysis, activation reactions were stopped by addition of chloroform/methanol, followed immediately by an acid lipid extraction.

Measurement of aggregation and 5-hydroxytryptamine secretion

Aggregation was monitored by turbidometry using a Chronolog aggregometer with stirring at 600 rev./min (5×10^8 cells/ml). 5-Hydroxytryptamine secretion was performed as previously described [24]; briefly, 5-hydroxy[^{14}C]tryptamine (Amersham International)-labelled platelets (2×10^9 cells/ml) were preincubated with or without tyrphostin and stimulated with increasing concentrations of thrombin for 5 min. Incubation was stopped by addition of 10 mM EDTA, cooling on ice and centrifugation. 5-Hydroxy[^{14}C]tryptamine released from platelet dense granules was determined by liquid-scintillation counting.

Lactate dehydrogenase measurements were used to check the tyrphostin cytotoxicity and performed as described in [25].

Lipid extraction and analysis

Lipids were extracted, deacylated by methylamine treatment, identified and quantified by an h.p.l.c. technique on a Whatman Partisphere SAX column as previously described [26].

Assay of PLC activity on exogenous substrate

PLC activity present in anti-phosphotyrosine immunoprecipitates was detected with $\text{Ptd}[^3\text{H}]\text{Ins}(4,5)\text{P}_2$ (Amersham International) as exogenous substrate. The reaction was performed at 37 °C during 10 min as previously described [27].

Inositol phosphate measurement

The upper phases obtained from the lipid extraction of *myo*-[^3H]inositol-labelled platelets (2×10^9 cells/ml) were neutralized with NaOH, diluted with water to 5 ml and applied to 1 ml columns of AG1-X8 anion-exchange resin (Bio-Rad, Richmond, CA, U.S.A.). Sequential elutions were performed as described by Berridge et al. [28]; however, after elution of glycerophosphoinositol, InsP_1 , InsP_2 and InsP_3 were eluted together with 0.1 M formic acid/1.0 M ammonium formate.

Immunopurification

Reactions (6×10^8 cells/ml) were stopped by addition of 1 vol. of buffer B containing 40 mM Tris/HCl (pH 7.4), 100 mM NaCl, 100 mM NaF, 10 mM EDTA, 40 mM $\text{Na}_2\text{P}_2\text{O}_7$, 2 mM sodium orthovanadate, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 200 μ g/ml phenylmethanesulphonyl fluoride and 2 % digitonin, followed by sonication for 5 s at 20 kHz (Ultrasonic Cell Disruptor) at room temperature. After 10 min on ice, sonication (5 s, 20 kHz) and centrifugation (12000 g, 5 min at 4 °C), the soluble fraction was collected and incubated for 150 min at 4 °C with an anti-phosphotyrosine antibody coupled to agarose (Oncogene Science). After one washing step in buffer B diluted in 1 vol. of distilled water and three washing steps in 10 mM Tris/HCl (pH 7.4) containing 100 mM NaCl, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 100 μ g/ml phenylmethanesulphonyl fluoride, the phosphotyrosyl-proteins were then eluted in 10 mM Tris/HCl (pH 7.4) containing 100 mM NaCl and 10 mM phenyl phosphate as described previously [17].

Gel electrophoresis and immunoblotting

Phosphotyrosyl-proteins obtained from immunopurification were resuspended in sample buffer [100 mM Tris/HCl (pH 6.8), 15 % (v/v) glycerol, 25 mM dithiothreitol, 3 % SDS], and proteins were separated by SDS/PAGE in a 7.5 % gel. Proteins were blotted on to nitrocellulose as described previously [29]. Immunodetection was performed with a polyclonal antibody to the p85 regulatory subunit of the PtdIns 3-kinase (dilution 1/500; UBI) or a monoclonal antibody to PLC γ 1 (dilution 1/3000; Zymed Laboratories) and anti-rabbit or anti-mouse antibodies conjugated to alkaline phosphatase as secondary antibodies. 5-Bromo-4-chloro-3-indolyl phosphate was used as substrate for alkaline phosphatase.

RESULTS

Inhibition of thrombin-induced platelet aggregation and 5-hydroxytryptamine release by tyrphostin treatment

When platelets were stimulated with 0.3 or 0.7 i.u. of thrombin/ml, typical aggregation profiles were observed (Figures 1a and 1b respectively). Treatment with tyrphostin fully inhibited the aggregation obtained at 0.3 i.u./ml (Figure 1a), whereas a partial inhibition was observed at 0.7 i.u. of thrombin/ml (Figure 1b).

As indicated in Figure 2, 5-hydroxytryptamine release from dense granules was also fully inhibited by tyrphostin at 0.1 i.u. of thrombin/ml, and partially inhibited at higher thrombin concentration. Furthermore, we have checked that tyrphostin treatment had no lytic effect. Indeed, lactate dehydrogenase activity in the supernatant of tyrphostin-treated platelets represented 2.5 ± 0.3 % ($n = 2$) of the total activity measured in platelet homogenate and was similar to that measured in the supernatant of control cells.

These inhibitory effects of tyrphostin on the physiological

Figure 1 Effect of tyrphostin treatment on thrombin-induced platelet aggregation

Aggregation profiles were obtained as indicated in the Experimental section and are representative of 3–4 different experiments. Profiles (a) and (b) represent the aggregation obtained with 0.3 and 0.7 i.u./ml of thrombin respectively, in the absence or presence of 100 μ M tyrphostin.

Figure 2 Effect of tyrphostin treatment on thrombin-induced 5-hydroxytryptamine secretion

5-Hydroxy[14 C]tryptamine released from dense granules by increasing concentrations of thrombin (5 min of stimulation) was measured as indicated in the Experimental section. Data are from a typical experiment representative of two separate experiments with very similar results: $\circ$, secretion measured with control platelets; $\bullet$, secretion obtained with 100 μ M tyrphostin-treated platelets. Maximal 5-hydroxytryptamine release measured in normal platelets was obtained at 0.7 i.u. of thrombin/ml and represents 60% of the total 5-hydroxy[14 C]tryptamine incorporated into platelets.

activation of platelets, previously reported [30], suggest that tyrosine kinases, via specific phosphorylations, may play an important role in other biochemical events measured during thrombin activation of human platelets.

Effect of tyrphostin on PLC activation and $\text{PtdIns}(3,4)\text{P}_2$ production on thrombin activation of platelets

We have investigated whether both PLC activation and $\text{PtdIns}(3,4)\text{P}_2$ accumulation, classically observed during thrombin activation of platelets and potentially regulated via tyrosine phosphorylation, may be affected by tyrphostin treatment.

As shown in Figure 3, when metabolically ^{32}P -labelled platelets were stimulated for 5 min by increasing concentrations of thrombin, we observed a dose-dependent appearance of [^{32}P]PtdOH. It is admitted that in platelets the synthesis of PtdOH results mainly from the activation of a PtdIns -specific PLC producing diacylglycerol (DAG), which is subsequently phosphorylated to PtdOH by a DAG kinase. As shown in Figure 3, PtdOH synthesis was significantly decreased in the presence of tyrphostin. However, inhibition of PtdOH synthesis (23, 44 and 38% at 0.1,

Figure 3 Effect of tyrphostin treatment on [^{32}P]PtdOH formation

^{32}P -labelled platelets treated ($\bullet$) or not ($\circ$) with 100 μ M tyrphostin were stimulated by increasing concentrations of thrombin for 5 min. Results are expressed as arbitrary units (d.p.m. of PtdOH produced/d.p.m. of PtdOH produced in control) and are means $\pm$ S.E.M. of 6 independent experiments. Probability of significance by Student's t test: * $P < 0.02$, $n = 6$; ** $P < 0.006$, $n = 6$.

Table 1 Effect of tyrphostin treatment on inositol phosphate production upon thrombin activation of human platelets

myo-[^3H]inositol-labelled platelets were stimulated for 5 min as indicated in the Experimental section, and inositol phosphates produced were isolated by Dowex chromatography and quantified. Results are expressed in d.p.m. of inositol phosphates ($\text{InsP}_1 + \text{InsP}_2 + \text{InsP}_3$) produced and are means $\pm$ S.E.M. of three different experiments: ^aprobability of significance, by Student's t test (thrombin 1 i.u./ml versus control), $P < 0.05$, $n = 3$; ^bprobability of significance, by Student's t test (thrombin 1 i.u./ml + tyrphostin versus thrombin 1 i.u./ml), $P < 0.05$, $n = 3$.

	[^3H]inositol phosphates (d.p.m.)
Control	1322 $\pm$ 170
Thrombin (1 i.u./ml)	3756 $\pm$ 395 ^a
Thrombin (1 i.u./ml) + tyrphostin	2125 $\pm$ 375 ^b

Figure 4 Effect of tyrphostin treatment on [³²P]PtdIns(3,4)P₂ accumulation

³²P-labelled platelets treated (●) or not (○) with 100 μM tyrphostin were stimulated by increasing concentrations of thrombin for 5 min. Results are expressed as d.p.m. of PtdIns(3,4)P₂ produced and are means ± S.E.M. of 4–5 different experiments. Probability of significance by Student's *t* test: **P* < 0.02; *n* = 5; ***P* < 0.01, *n* = 4.

0.3 and 0.7 i.u. of thrombin/ml, respectively) did not strictly parallel inhibition of aggregation or secretion, which decreased when thrombin concentrations were raised (Figures 1 and 2). For instance, secretion was inhibited by 93 %, 46 % and 22 % at 0.1, 0.3 and 0.7 i.u. of thrombin/ml respectively. Inhibition of PtdOH accumulation could be due to a partial inhibition of PLC and/or DAG kinase. To discriminate between these possibilities, we have measured the production of inositol phosphate in *myo*-[³H]inositol-prelabelled platelets (Table 1). The same percentage inhibition by tyrphostin has been measured for inositol phosphate production induced by thrombin. This indicates that tyrphostin treatment partially affected PLC activation by thrombin, but we cannot exclude formally a possible effect on DAG kinase.

On the other hand, as we previously reported [26], Figure 4 shows a dose-dependent production of PtdIns(3,4)P₂ by human platelets upon thrombin stimulation. Using thrombin at 0.1 i.u./ml, this production was almost fully abolished in tyrphostin-treated cells, while at 0.3 i.u./ml the inhibition was 61 ± 10 %. Interestingly, one should note that, compared with PtdOH labelling there was a better parallelism between inhibition of PtdIns(3,4)P₂ accumulation and platelet aggregation in the presence of tyrphostin (Figures 1 and 4; C. Guinebault, unpublished work).

Under our experimental conditions, we detected no significant modification of the labelling of the other classes of phospholipids upon tyrphostin treatment of control or thrombin-stimulated platelets (results not shown). This is particularly important to consider in the case of PtdIns(4,5)P₂ and PtdIns4P, suggesting that, in contrast with another lipophilic substance, chlorpromazine [31], tyrphostin was without effect on the level of metabolic ATP [32]. Therefore our results suggest regulation of a PLC as well as PtdIns 3-kinase and/or PtdIns3P 4-kinase [33] by tyrosine kinases in human platelets.

Presence of PLCγ1 and p85 in anti-phosphotyrosine immunoprecipitate

In order to confirm a relationship between tyrosine phosphorylation and PLC or PtdIns 3-kinase, we have performed immunopurification of phosphotyrosyl-proteins from a digitonin-soluble fraction of platelets and probed them with anti-PLCγ1 and anti-p85 antibodies.

Figure 5 Immunodetection of PLCγ1 in the anti-phosphotyrosine immunoprecipitate from a digitonin-soluble fraction of platelets

Lane 1, resting platelets; lane 2, thrombin-stimulated platelets (5 min, 1 i.u./ml); lane 3, thrombin stimulation (5 min, 1 i.u./ml) of 100 μM-tyrphostin-treated platelets.

Figure 6 Immunodetection of the regulatory subunit (p85) of the PtdIns 3-kinase in the anti-phosphotyrosine immunoprecipitate from a digitonin-soluble fraction of platelets

Lane 1, resting platelets; lane 2, thrombin-stimulated platelets (5 min, 1 i.u./ml); lane 3, thrombin stimulation (5 min, 1 i.u./ml) of 100 μM-tyrphostin-treated platelets.

As indicated in Figure 5, when anti-phosphotyrosine immunoprecipitate from resting platelets (lane 1) was probed with anti-PLCγ1 antibody, we could detect a single protein with an apparent molecular mass of 145 kDa, corresponding to the expected size of PLCγ1. An increase in PLCγ1 content was observed in the anti-phosphotyrosine immunoprecipitate isolated from thrombin-activated platelets (Figure 5, lane 2). Finally, PLCγ1 was hardly detectable when platelets were pretreated with tyrphostin (lane 3). In parallel, PLC activity was measured by an assay *in vitro* in the anti-phosphotyrosine immunoprecipitate from resting or thrombin-activated platelets. A

weak PLC activity could be detected in control (100 ± 30 fmol of $[^3\text{H}]\text{InsP}_3/\text{min}$ produced; mean $\pm$ S.E.M., $n = 3$), whereas an increase (270 ± 100 fmol/min of $[^3\text{H}]\text{InsP}_3$ produced; mean $\pm$ S.E.M., $n = 3$) was observed upon thrombin stimulation.

In the anti-phosphotyrosine immunoprecipitate from resting platelets (Figure 6, lane 1) we also detected by Western blotting, using an anti-p85 antibody, a single protein band of 85 kDa corresponding to the described mass of the regulatory subunit (p85) of PtdIns 3-kinase. A clear increase in the p85 content was observed in the immunoprecipitate from thrombin-activated platelets (Figure 6, lane 2), and this increase was significantly decreased by tyrphostin treatment (Figure 6, lane 3).

DISCUSSION

Our present data argue in favour of a role of tyrosine phosphorylation in several aspects of platelet activation by thrombin. We demonstrate that thrombin-induced platelet aggregation as well as 5-hydroxytryptamine secretion are strongly inhibited by tyrphostin treatment. However, although significant, this inhibition is clearly decreased at high thrombin concentration, suggesting that several signals may converge to regulate these processes. These observations already reported [30] indicate that, besides specific tyrosine phosphorylations, downstream biochemical reactions classically observed during thrombin activation of platelets may be affected by tyrosine kinase inhibitors. However, the precise regulatory role of these tyrosine phosphorylations is still unclear. In other models, PLC γ 1 and PtdIns 3-kinase regulatory subunit, two proteins participating in phosphoinositide metabolism, are tyrosine-phosphorylated and activated by several tyrosine-kinase-containing receptors [7,18].

Although it is well established that PLC activation in thrombin-stimulated platelets is largely dependent on G-proteins [9], we have demonstrated here that tyrphostin partially blocks the PtdOH synthesis and the inositol phosphate production induced by thrombin stimulation. These observations suggest a contribution of tyrosine phosphorylation in PLC activation upon thrombin stimulation. The discrepancy observed between our present results and those previously published [30] may be due to the fact that we have used for the present study higher concentrations of thrombin for a longer stimulation time, and may emphasize the existence of a G-protein-dependent and a G-protein-independent pathway by which thrombin may activate PLC isoforms in platelets. This could also explain the lack of parallelism between the inhibition of PLC and of aggregation/secretion. On the other hand, we have shown that PtdIns(3,4) P_2 accumulation is significantly inhibited by tyrphostin in thrombin-stimulated platelets. This inhibition seems to be parallel to the inhibition of the thrombin-dependent aggregation. Our data suggest that PtdIns 3-kinase and/or PtdIns3P 4-kinase may be directly or indirectly regulated via tyrosine phosphorylation in platelets. In this respect, it is noteworthy that PtdIns(3,4) P_2 synthesis and tyrosine phosphorylation of a pool of proteins have been shown to be dependent on fibrinogen binding to GpIIb/IIIa, since they are inhibited by the fibrinogen-binding-site analogue RGDS [21,22].

Finally, we confirm the relationship between tyrosine phosphorylations and phosphoinositide metabolism by demonstrating the presence of both PLC γ 1 and the regulatory subunit of PtdIns 3-kinase (p85) in the anti-phosphotyrosine immunoprecipitate obtained from thrombin-activated platelets. The immunodetection of PLC γ 1 and p85 in such an immunoprecipitate does not obligatorily mean a direct phosphorylation on tyrosine residues. Actually, using an anti-phosphotyrosine antibody as probe in Western blotting, we have not so far

detected a direct tyrosine phosphorylation of PLC γ 1 or p85 in our samples (results not shown). However, with respect to the existence of SH2 and SH3 domains in these molecules [34], one can expect the formation of signalling complexes which may include both tyrosine-phosphorylated and non-tyrosine-phosphorylated proteins. Since we have solubilized the platelets with the mild detergent digitonin, such associations of proteins may be relatively well conserved and may explain the presence of non-tyrosine-phosphorylated proteins in the anti-phosphotyrosine immunoprecipitate. In this respect, it is noteworthy that an association between PLC γ 1, p21^{ras}-GTPase activating protein (ras GAP) and rap1B in thrombin-activated human platelets was recently reported [35]. The latter authors suggested that these associations may allow the interaction of PLC γ 1 with its substrate located in the membrane. They further showed that, upon thrombin stimulation, PLC γ 1 was indeed phosphorylated, but not on a tyrosine residue, by contrast with ras GAP, which was tyrosine-phosphorylated. Furthermore, an association of ras GAP with three *src*-related tyrosine kinases (*fyn*, *lyn* and *yes*) has been reported in thrombin-activated human platelets [36]. With respect to the presence of SH2 domains in most of these proteins, one can expect that the formation of signalling complexes may be under control of tyrosine phosphorylations.

Altogether these results suggest that non-receptor tyrosine kinases, as well as the formation of signalling complexes including PLC γ 1, ras GAP, rap1B and possibly *src*-related proteins, may play an important role in signalling via the thrombin receptor. The partial inhibition by tyrphostin of PLC activation that we have observed may be due to the lack of formation of such a complex; this is consistent with the fact that PLC γ 1 is weakly present in the anti-phosphotyrosine immunoprecipitate from thrombin-stimulated tyrphostin-treated platelets.

On the other hand, p85 may also participate in this complex, since an association of PtdIns 3-kinase with p21^{ras} has been shown in Ha-ras-transformed epithelial cells [37]. It is also noteworthy that Torti et al. [38] have recently shown an association of a small G-protein with GpIIb/IIIa. Furthermore, the inhibition of thrombin-dependent PtdIns(3,4) P_2 accumulation by RGDS that we have previously reported [21] and the inhibition of its synthesis by tyrphostin reported here correlated with the inhibition of aggregation. However, recent reports from King et al. [39] and Sorisky et al. [40] demonstrated the role of both protein kinase C and Ca^{2+} in thrombin-dependent PtdIns(3,4) P_2 accumulation, suggesting that this synthesis involves different intracellular signals to be total.

In conclusion, our data strongly argue for the existence of a tyrosine-phosphorylation-dependent pathway to activate PLC in human platelets, probably by involving a PLC γ 1 isoform and the formation of multienzymic complexes. Our results also suggest a relationship between the synthesis of a 3-phosphorylated inositol lipid [PtdIns(3,4) P_2] and tyrosine kinase activities in thrombin-stimulated platelets, possibly via GpIIb/IIIa. Further investigations concerning the direct or indirect role of tyrosine kinases in the regulation of PLC and PtdIns 3-kinase in human platelets are now in progress.

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