Biochemical Pharmacology, Vol. 44, No. 5, pp. 881–888, 1992.
Printed in Great Britain.

0006–2952/92 $5.00 + 0.00

INHIBITION OF PLATELET ACTIVATION BY TYROSINE KINASE INHIBITORS

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(*Received* 20 *August* 1991; *accepted* 5 *June* 1992)

Abstract—Protein tyrosine kinase (PTK) blockers (tyrphostins) inhibit in a dose-dependent fashion thrombin-induced aggregation and serotonin release with IC_{50} values in the 10–35 μM concentration range. The inhibition of thrombin-induced aggregation correlates with their potency in inhibiting phosphorylation of proteins on tyrosine residues. Using metabolically ^{32}P-labelled human platelets, it was found that the tyrphostins have no effect on the decrease in [^{32}P]phosphatidylinositol bisphosphate but prevent the replenishment of [^{32}P]polyphosphoinositide. Tyrphostins decreased [^{32}P]phosphatidic acid production induced by thrombin, although never by more than 50%, and only delayed the peak of diacylglycerol, suggesting that phospholipase C was still activated. Tyrphostins inhibited the thrombin-elicited early phosphorylation of p43 and p20, substrates for protein kinase C (PKC) and myosin light chain kinase, respectively, at short times of activation. This inhibition, however, was overcome after 1 min of stimulation with thrombin. Tyrphostin AG213 also inhibited platelet aggregation and tyrosine protein phosphorylation induced by phorbol myristate acetate (PMA), but did not inhibit pleckstrin phosphorylation. These results suggest that thrombin induces the phosphorylation of proteins on tyrosine residues which most probably results in the activation of phosphoinositide kinases. The ability of tyrphostins to inhibit phosphorylation of p43 and p20 when induced by thrombin but not when induced by PMA confirms that PTKs may be involved subsequent to PKC activation.

Platelets are widely used for studying the mechanism of signal transduction and protein phosphorylation. *In vivo* labelling of platelets with ^{32}P results in the phosphorylation of a number of proteins. Upon activation, two proteins which are not phosphorylated in the resting state become heavily phosphorylated: myosin light chain (p20) and pleckstrin (p43), the latter being the main substrate for protein kinase C (PKC¶) [1, 2]. These phosphorylations depend on the generation of second messenger molecules, namely inositol trisphosphate and diacylglycerol (DG), from phosphoinositide breakdown by a phospholipase C activity. Inositol trisphosphate participates in calcium mobilization from intracellular stores which together with DG promotes the activation of PKC. In addition, following platelet stimulation protein phosphorylation is also increased on tyrosine residues [3–8], the function of which is still unclear. Interest in tyrosine-specific phosphorylation has greatly increased in recent years, since it was shown to be involved in numerous pathways including regulation of the cell cycle, cell growth and proliferation, and many metabolic processes [9]. In several cell types tyrosine phosphorylation regulates phosphatidylinositol kinase (for review see Ref. 10). In order to elucidate further the role of the phosphorylation of proteins on tyrosine residues, we examined the effect of protein tyrosine kinases (PTKs), inhibitors of the tyrphostin family [11–14], on platelet aggregation and release, and the correlation, if any, between these biochemical occurrences.

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¶ Abbreviations: MGP, metrizamide gradient platelets; PMA, phorbol myristate acetate; PTK, protein tyrosine kinase; PA, phosphatidic acid; PIP_2, phosphatidylinositol (4, 5) bisphosphate; DG, diacylglycerol; PKC, protein kinase C; TBS, Tris-buffered saline; E/PDGF, epidermal/p'latelet derived growth factor.

MATERIALS AND METHODS

Materials. [^{14}C]5-Hydroxytryptamine and [^{32}P]-PO_4 were obtained from Oris (CEA, France). Metrizamide was from Nyegaard Norway; bovine thrombin was from Roche (Basel) and monoclonal anti-phosphotyrosine antibody was a gift from B. Druker [15].

Platelet preparation. Platelets were prepared as described previously [7]. Briefly, blood was obtained from healthy volunteers who had not ingested any drug for at least 1 week and centrifuged for 10 min at 100 *g* to obtain platelet-rich plasma. When necessary, platelets were labelled at this step by incubation without stirring with either [^{14}C]5-hydroxytryptamine (0.8 μM, sp. radioact. 48 mCi/mol, 30 min at 37°) or [^{32}P]phosphoric acid (1 mCi/10 mL platelet-rich plasma, 90 min at 37°). Platelets were isolated from plasma on a metrizamide gradient. The "metrizamide gradient platelets" (MGP) thus obtained were freeed of metrizamide by a final wash and resuspended in a buffer of 140 mM NaCl–

5 mM $NaHCO_3$–0.5 mM $MgCl_2$–3 mM KCl–10 mM glucose–10 mM Hepes, pH 7.4 and adjusted to a concentration of 4–5 × 10^8 cells/mL.

The tyrphostins were dissolved in dimethyl sulphoxide to generate a 100 mM stock solution. The tyrphostins were further diluted in double distilled water and the proper buffers such that the final dimethyl sulphoxide concentration in the presence of platelets did not exceed 0.1%. Platelet aliquots (0.4 mL) were prewarmed to 37° in the presence of the dissolution medium or tyrphostin for 1 min and activation was initated by the addition of agonists under constant stirring (1100 rpm) in an aggregometer cuvette (Chronolog, U.S.A.). With longer times of incubation of up to 5 min different results were not obtained.

The release reaction was measured on the ^{14}C-labelled samples and the reaction stopped after 2 min by transfer in 1/5 vol. of EDTA (0.1 M) followed by immediate centrifugation as described previously [2]. The results were expressed as per cent release of total [^{14}C]5-hydroxytryptamine in the platelets.

Metabolic phosphorylation labelling. Phosphoproteins, phosphoinositide and phosphatidic acid (PA) were measured on the same sample of [^{32}P]-MGP. Briefly, activation by thrombin was stopped by the addition of 3.75 vol. of chloroform–methanol–HCl 12 M–EDTA 0.1 M (20:40:1:2, by vol.) to each ^{32}P-labelled sample. Chloroform and water (1.25 vol. of each) were added and the mixture was shaken vigorously. The aqueous phase was discarded. Proteins, concentrated at the interphase, were solubilized in a mixture containing 2% (w/v) SDS, 0.0625 M Tris, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue and analysed by SDS–PAGE under reducing conditions with 2-mercaptoethanol (5%, v/v) using a 10% resolving gel and 3% stacking gel. After staining with Coomassie blue, dried gels were exposed to β Max films (Amersham). After autoradiography ^{32}P-labelled proteins were detected by scanning densitometry (LKB Ultroscan). The lower organic phase was evaporated, washed and resuspended in chloroform–methanol–water (75:25:2, by vol.) [16]. Phosphoinositide and PA were separated by TLC on silica gel using chloroform–acetone–methanol–acetic acid–water (40:15:13:12:7, by vol.) as the migration solvent. Individual ^{32}P-labelled phospholipids were visualized by autoradiography and recovered by scraping for liquid scintillation counting.

DG production from unlabelled platelets stimulated by thrombin in the presence or absence of tyrphostin was measured using the assay reagents system from Amersham (code RPN 2009).

Immunoblotting. Platelet activation was stopped by addition of a mixture of SDS 2% (w/v) and EDTA 1 mM. After solubilization and reduction in the presence of 2-mercaptoethanol (5%, v/v) for 30 min at 60°, phosphoproteins were subjected to electrophoresis using a 7% resolving gel and 3% stacking gel. The gel was electroblotted onto a nitrocellulose filter by semi-dry transfer (Hoeffer Scientific Instruments, U.S.A.). After staining with Red Ponceau (0.2%, w/v in 0.3% trichloroacetic acid), the filter was soaked overnight with Tris-buffered saline (TBS: 10 mM Tris, pH 7.4, NaCl 170 mM) containing 0.05% azide, 0.05% Tween 20 and 5% (w/v) low fat powder milk. The filter was then incubated with the monoclonal anti-phosphotyrosine antibody [15] for 2 hr at room temperature, rinsed five times with TBS and finally incubated with 0.4 μCi [^{125}I]rabbit anti-mouse immunoglobulin. After five rinses in TBS, the filter was dried and autoradiographed using β Max films (Amersham). The specificity of the antibody was ascertained by incubation of the blot with 50 mM of phenylphosphate for 30 min at room temperature prior to immunostaining with [^{125}I]-rabbit anti-mouse immunoglobulin.

RESULTS

Aggregation and release

A series of tyrphostins [14] were examined as blockers of thrombin-induced platelet aggregation and serotonin release. Table 1 shows that a number of tyrphostins are quite effective in inhibiting these processes. All compounds were tested at the same concentration of 25 μM (Table 1). As a representative of the active tyrphostins for further studies we chose AG213 (RG 50864) which we found earlier to be an effective blocker of epidermal growth factor (EGF) receptor kinase [17] but a less potent inhibitor of platelet derived growth factor (PDGF) receptor kinase-mediated events [18] and of bcr/abl kinases [19]. We also examined AG370, which is a poor EGF receptor kinase blocker but a potent blocker of PDGF receptor kinase-mediated phosphorylation in intact cells [18].

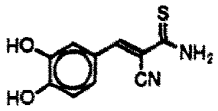
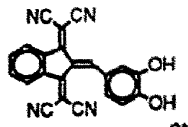
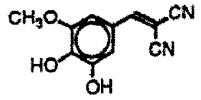
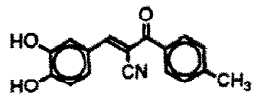
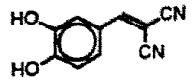
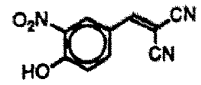
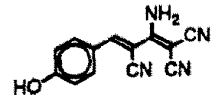
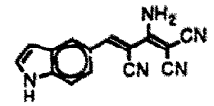
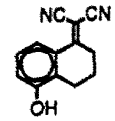
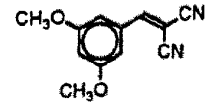
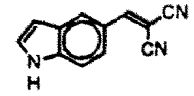
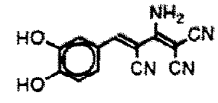
Figure 1 shows that AG213 inhibited in a dose-dependent manner both thrombin-induced platelet aggregation (IC_{50} = 35 μM) and [^{14}C]5-hydroxytryptamine release (IC_{50} = 20 μM). AG370 (Table 1, Fig. 1) had no effect at concentrations of up to 200 μM. AG 213 was also a potent inhibitor of platelet aggregation induced by ADP, collagen and phorbol myristate acetate (PMA) (not shown). The effect of AG213 on platelets does not result from lysis since no lactate dehydrogenase was detectable in the supernatant of platelets incubated with 100 μM AG 213 (data not shown).

Anti-phosphotyrosine immunoblot assays

Thrombin-induced platelet activation results in tyrosine phosphorylation of a number of proteins with M_r values of 170,000, 150,000, 140,000, 120,000, a doublet of 105,000–97,000, 85,000, a doublet of 80,000–75,000, 55,000, 36,000, 28,000, and intensification of the tyrosine phosphorylation of proteins with M_r values of 130,000, 64,000 and 56,000–60,000 (Fig. 2). A similar profile was observed following PMA-induced activation. The band of 56,000–60,000 M_r is most likely $pp60^{c\text{-}src}$ as demonstrated by western blotting using anti-src antibody [12].

Tyrphostin AG213 inhibited tyrosine phosphorylation induced by either thrombin or PMA (Fig. 2) whereas AG370 had no effect (data not shown). The inhibitory dose response towards AG213 was similar for thrombin-induced aggregation, serotonin release and tyrosine phosphorylation (Figs 1 and 2, and Ref. 12), but somewhat variable

Table 1. Inhibition of thrombin-induced platelet activation by tyrphostins

AG No.	Structure	Inhibitory effect (%)		
		Aggregation	Serotonin release	N
213		49 (95.6)	83.1 (86)	7
372		56	82.6	3
34		45.4	86.5	4
364		40.8	65.4	1
18		38.2 (85.3)	58.6 (83)	3
127		4	0	3
112		4	31.7	3
370		2.6 (9)	1.7 (1.2)	2
307		2.4	1.4	1
81		0.7	4.8	3
410		0.7	13.8	2
114		−7	2	1

Platelets (4×10^8 cells/mL) were preincubated with tyrphostins 25 μM (or 50 μM; values in parentheses) at 37° in the absence of stirring. Stirring started with the addition of thrombin (0.05 U/mL), and aggregation intensity and release of [^{14}C]5-hydroxytryptamine were measured 120 sec after thrombin addition.

N, number of determinations.

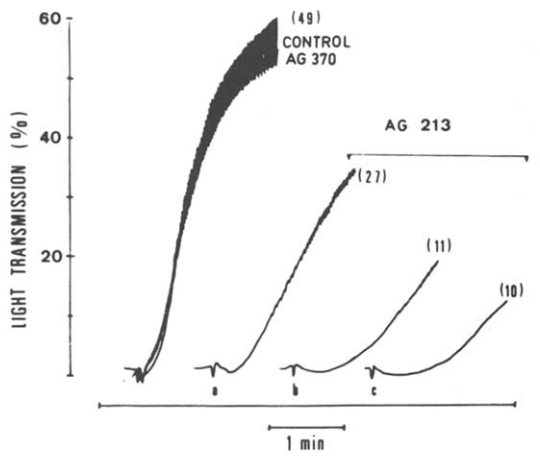


Fig. 1. Tracings of thrombin-induced platelet aggregation in the presence of tyrphostins. MGP (4×10^8 cells/mL) were incubated with tyrphostins AG213 or AG370 before addition of thrombin (0.05 U/mL). (a) 25 μM; (b) 50 μM; (c) 100 μM AG213. Per cent of release of [^{14}C]5-hydroxytryptamine is indicated in brackets. Aggregation was expressed as described previously [2].

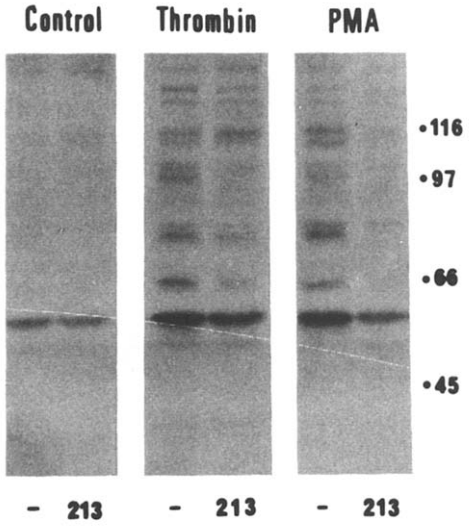


Fig. 2. Tyrphostin inhibition of thrombin- and PMA-induced tyrosine phosphorylation. Tryphostin AG213 (50 μM) was incubated without stirring for 1 min at 37° with MGP (4×10^8 cells/mL). At the end of the incubation, the mixture was stirred in the cuvette of an aggregometer either in the presence of saline (−) or 0.05 U/mL thrombin or 200 nM PMA, for 2 min. Immunoblotting was performed with monoclonal antibody anti-phosphotyrosine protein. Data are representative of 3–5 experiments.

between platelet preparations depending on the donor. However, there was a consistent parallel between the inhibition of aggregation and inhibition of tyrosine protein phosphorylation.

Protein phosphorylation

An autoradiogram obtained from the direct electrophoretic pattern of metabolically ^{32}P-labelled platelet proteins is shown in Fig. 3. Under these conditions the three kinds of phosphorylated proteins are visualized, i.e. tyrosine, serine and threonine protein phosphorylations.

Tyrphostin AG213 inhibited thrombin-induced phosphorylation of p20 and p43 within platelets at short times of activation (up to 1 min) (Fig. 3A). These two protein bands represent myosin light chain [20] and pleckstrin, respectively [21]. However, as reported previously [12], this inhibition was overcome when platelets were stimulated with thrombin for 2 min.

In order to determine whether tyrphostin directly inhibits PKC or acts at a step regulating PKC activation, the effect of tyrphostin was studied on PMA-induced platelet aggregation. Tyrphostin AG213 (50 μM) almost completely inhibited aggregation induced by 200 nM PMA. In contrast PMA-induced phosphorylation of p20 and p43 was unaffected by this tyrphostin (Fig. 3B) whereas tyrosine phosphorylation induced by PMA was abolished by 50 μM AG213 (Fig. 2). Furthermore, AG213 had no effect on the PMA-evoked phosphorylation of proteins other than tyrosine proteins, nor any effect on the phosphorylated proteins present in resting platelets. These results taken together demonstrate the selectivity of the inhibitor towards PTKs.

Metabolism of phosphoinositide

In order to examine in more detail the signalling pathway(s) intercepted by tyrphostins we examined the effect of AG213 on the changes in ^{32}P associated with polyphosphoinositides and on the rate of [^{32}P]-PA production subsequent to thrombin challenge.

At a concentration of AG213 which yielded the maximum inhibitory effect on thrombin-induced aggregation and release, the rapid decrease in [^{32}P]-phosphatidylinositol (4,5) bisphosphate (PIP_2), measured during the first 20 sec after thrombin addition, was unaffected by the drug (Fig. 4). In contrast, the replenishment of the labelled [^{32}P]PIP_2 was consistently lower in the presence of AG213 than in control platelets.

Under similar conditions, tyrphostin AG213 retarded thrombin-stimulated DG production (Fig. 5). The basal value of 112 pmol/10^9 platelets was increased two to three times by stimulation with 0.05 U/mL thrombin. In the presence of the inhibitor, the peak of DG appeared 20 sec after thrombin addition whereas it appeared at 10 sec in the absence of tyrphostin and was reduced by 15–20%.

[^{32}P]PA production, after 60 sec of thrombin stimulation, was inhibited by over 50% in the presence of tyrphostin AG213 (Fig. 6) and maximal inhibition was already achieved at 20 μM AG213. After prolonged thrombin stimulation (120 sec), [^{32}P]PA production measured in the presence of tyrphostin was decreased by around 25% (Table in Fig. 6).

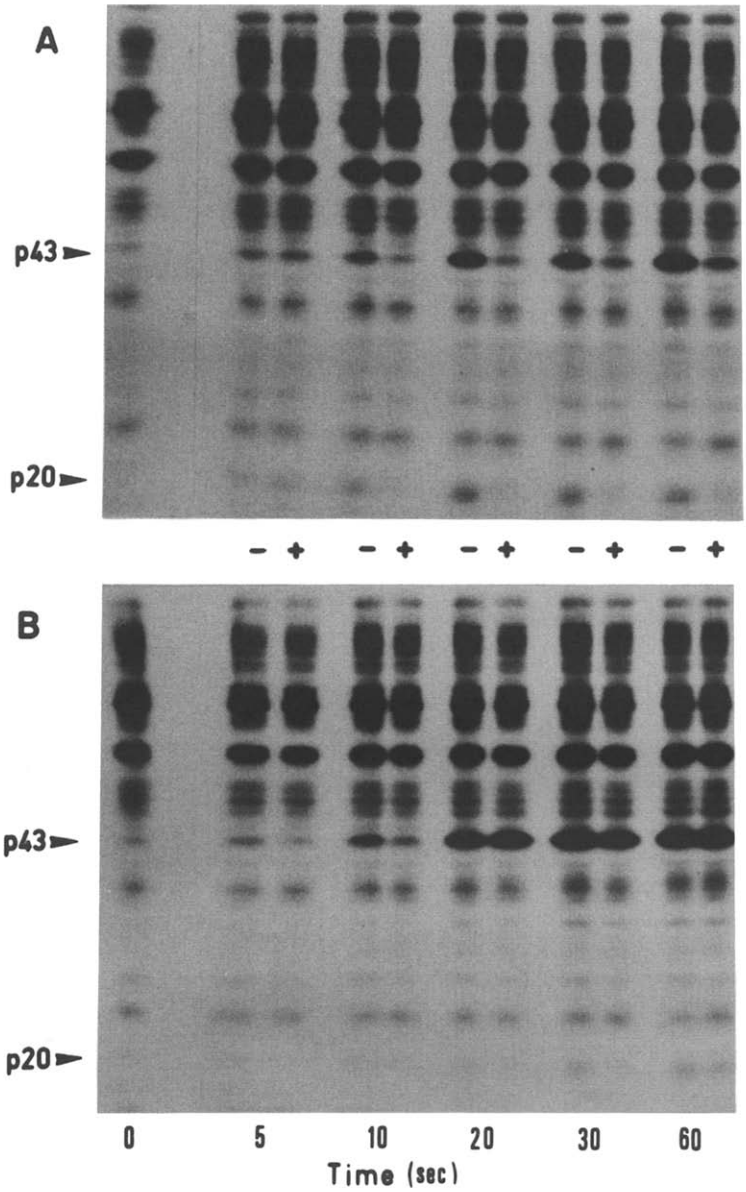


Fig. 3. Autoradiograms of p20 and pleckstrin phosphorylation during thrombin and PMA-induced aggregation in the presence or absence of tyrphostin. [^{32}P]MGP were incubated without stirring with tyrphostin AG213 (50 μM) before addition of (A) thrombin (0.05 U/mL) or (B) PMA (200 nM) for indicated times. Proteins were separated on a 10% acrylamide gel. Data shown are representative of four experiments.

DISCUSSION

Platelets are unique in that they possess high levels of the tyrosine kinases src [7, 22, 23] and fyn [22, 24, 25] as well as other proteins phosphorylated on tyrosine residues during platelet activation [3–5, 8]. Typically, receptor and receptor-associated tyrosine kinase activities are associated with signals involved in cell growth and proliferation, and cell metabolism. In platelets, the role of tyrosine proteins phosphorylated subsequent to thrombin-induced stimulation has not yet been clarified. In this study we have demonstrated that thrombin-induced activation of human platelets is inhibited by tyrosine kinase blockers of the tyrphostin family (Table 1). Specifically, inhibition of thrombin-induced platelet aggregation and 5-hydroxytryptamine release by the inhibitor tyrphostin AG213 (also known as RG50864) correlates with its inhibitory action on thrombin-induced tyrosine phosphorylation of a number of proteins, including those which are known to possess tyrosine kinase activity in the 60 kD range, i.e. src [7] and fyn [22, 24], yes and lyn [25]. Since AG213 is an extremely poor inhibitor of serine/threonine kinases (Refs 13, 17 and 26 and Table 2), as confirmed by our experiments using PMA, it is most likely that the inhibitory effect of AG213 on human platelets is due to its PTK-blocking activity.

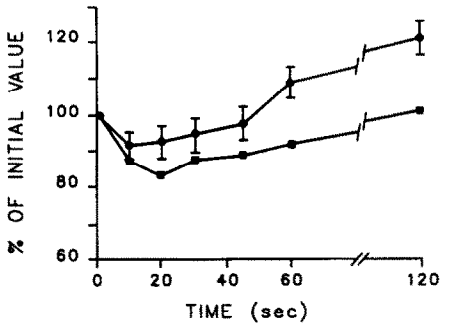


Fig. 4. Tyrphostin effect on thrombin-induced changes in ^{32}P associated with PIP_2. [^{32}P]MGP were incubated for 1 min at 37° with tyrphostin (50 μM) (■) or with buffer (●), before addition of thrombin (0.05 U/mL). Identical results were obtained in the presence of 75 μM tyrphostin. Results are given as the per cent of initial values in unstimulated platelets. Mean + SD (N = 3).

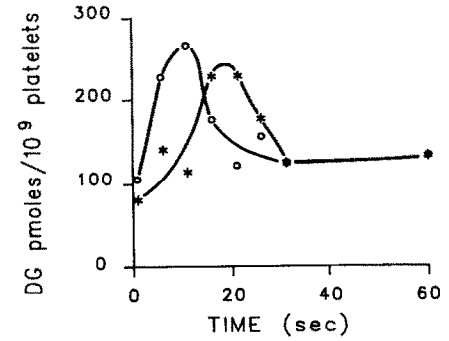


Fig. 5. Effect of tyrphostin on production of DG during thrombin-induced aggregation. Unlabelled MGP were incubated without stirring with buffer (○) or with tyrphostin AG213 (50 μM) (*) before addition of 0.05 U/mL thrombin for indicated times. Results are given in picomoles of DG produced by 10^9 platelets and are the means of two different experiments.

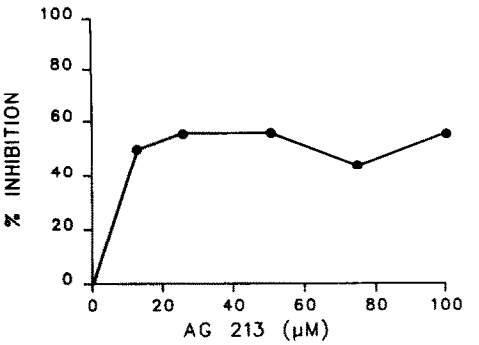


Fig. 6. Tyrphostin inhibitory effect on thrombin-induced [^{32}P]PA production. [^{32}P]MGP were incubated for 1 min at 37° with tyrphostin before addition of thrombin (0.05 U/mL). [^{32}P]PA was measured after 1 min of activation.

[^{32}P]PA production during thrombin-induced aggregation

Time (sec)	0	10	20	30	45	60	120
Control	1	1.15	1.55	2.1	2.4	3.25	2.6
AG213 (50 μM)	1	1.2	1.2	1.4	1.6	1.5	2.2

Results are given as the ratio of the number of cpm measured at each time point to that at time zero.

Table 2. Effect of AG213 on different protein kinases

	IC_{50} (μM)	Ref.
EGF receptor	7.5	13
$pp60^{c\text{-}src}$	8	26
PKC	60	26
Protein kinase A	>100	26
	300	36

We have shown here that PIP_2 hydrolysis by phospholipase C was not inhibited by tyrphostin. This is supported by the fact that DG production was only retarded by tyrphostin AG213. By contrast, the replenishment of PIP_2 was inhibited by the PTK blocker AG213. It was proposed that thrombin promotes the interaction between $pp60^{c\text{-}src}$ kinase and phosphatidylinositol 3-kinase [24], an enzyme described as physically associating with growth factor receptors and which has been implicated in mitogenic responses [27]. In the experimental procedure we used [16], the two PIP_2 isomers, phosphorylated on positions 3 and 4, and on positions 4 and 5 on the inositol ring, cannot be separated. By inhibiting thrombin-induced activation of a PTK, tyrphostin would subsequently inhibit a phosphatidylinositol kinase. This possibility is supported by the observations that (i) tyrphostin inhibited the tyrosine kinases as shown on the western blots and (ii) the ^{32}P-labelling of the polyphosphoinositide was inhibited by tyrphostin (data not shown).

Thrombin-induced PA production was inhibited only partially by tyrphostin AG213. The bulk of PA production originates from phospholipase C activation on polyphosphoinositide (PIP_2 but also phosphatidylinositol phosphate and phosphatidyinositol) and other phospholipids (such as phosphatidylcholine) followed by the action of DG kinase on newly generated DG [28]. However, AG213 hardly affected DG production. So, the major part of the decrease of PA production cannot be due to the inhibition of this pathway. PA production can also originate from phosphatidylcholine via hydrolysis by a phospholipase D [29–31]. However, with the experimental ^{32}P-labelling we used, PA formed via phospholipase D would be barely detected. Alternatively, tyrphostin could inhibit the DG kinase although this is unlikely since such inhibition results in an increased PKC activity [32], contrary to our findings. Finally the partial inhibition of PA could also be due to the inhibition of the amplification phenomenon.

The phosphorylation of two other proteins, known to be phosphorylated on serine and threonine

residues, namely myosin light chain and pleckstrin [20, 21], was also inhibited by the PTK blocker tyrphostin AG213 at short times of activation by thrombin. This could result from the inhibition of the release of granule constituents necessary for the amplification of platelet activation. Pleckstrin (p43) and myosin light chain (p20) are phosphorylated by the activation of PKC and the calcium/calmodulin-dependent myosin light chain kinase, respectively. PKC also phosphorylates myosin light chain [33, 34], as indicated by the phosphorylation of myosin light chain induced by PMA, a direct PKC activator. The PTK blocker, tyrphostin AG213, did not modify these phosphorylations when induced by PMA. This suggests that tyrosine kinase activation could be down stream of that of PKC, a proposal supported by the identical profile of tyrosine protein phosphorylation observed under PMA and thrombin stimulation. That tyrosine phosphorylation occurs subsequent to PKC activation was also suggested in our previous experiments using staurosporin [8] as well as in PKC-deficient cells [35]. In contrast, the decrease in pleckstrin and myosin light chain phosphorylation induced by thrombin and observed at short times of activation in the presence of tyrphostin most probably results from an inhibition, by the tyrosine kinase inhibitor, of the release of granule constituents which contribute to a positive feed back mechanism.

In conclusion, the results in this study describe the inhibition of platelet aggregation and release as a consequence of the inhibition of protein kinases. The results suggest that tyrosine kinase activity, distal to that of PKC, may in turn activate phosphatidylinositol kinases, whose activities are transformed into signals which result in platelet aggregation and the release of serotonin. We are currently exploring the possibility of developing tyrphostins as therapeutic agents aimed at blocking platelet aggregation and to use them further as molecular tools to characterize signalling events in platelets.

Acknowledgements—This study was supported partially by Rhone Poulenc Rorer Pharmaceuticals, King of Prussia, PA, U.S.A. (A.L.), and DRET no 89-247 (F.R.).

mAb anti P-Tyr was a generous gift by T. Roberts and B. Drucker, in the Harvard Medical School in Boston. We thank Dr S. Fischer for helpful discussions, and S. Saleun for helpful technical assistance.

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