Rapid stimulation of tyrosine phosphorylation signals downstream of G-protein-coupled receptors for thromboxane A₂ in human platelets

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Signals ensuing from trimeric G-protein-coupled receptors synergize to induce platelet activation. At low doses, the thromboxane A₂ analogue U46619 does not activate integrin α IIb β 3 or trigger platelet aggregation, but it induces shape changes. In the present study, we addressed whether low doses of U46619 trigger tyrosine phosphorylation independently of integrin α IIb β 3 activation and ADP secretion, and synergize with adrenaline (epinephrine) to induce aggregation in acetylsalicylic acid (aspirin)-treated platelets. Low doses of U46619 triggered tyrosine phosphorylation of different proteins, including FAK (focal adhesion kinase), Src and Syk, independently of signals ensuing from integrin α IIb β 3 or ADP receptors engaged by secreted ADP. The G_{12/13}-mediated Rho/Rho-kinase pathway was also increased by low doses of U46619; however, this pathway was not upstream of tyrosine phosphorylation, because this occurred in the presence of the

Rho-kinase inhibitor Y-27632. Although low doses of U46619 or adrenaline alone were unable to trigger platelet aggregation and integrin α IIb β 3 activation, the combination of the two stimuli effectively induced these responses. PP2, a tyrosine kinase inhibitor, and Y-27632 inhibited platelet activation induced by low doses of U46619 plus adrenaline and, when used in combination, totally suppressed this platelet response. In addition, the two inhibitors selectively blocked tyrosine kinases and the Rho/Rho-kinase pathway respectively. These findings suggest that both tyrosine phosphorylation and the Rho/Rho-kinase pathway are required to activate platelet aggregation via G_{12/13} plus G_z signalling.

Key words: G-protein, platelet, Rho, Rho kinase, thromboxane A₂ (TXA₂), tyrosine phosphorylation.

INTRODUCTION

Most platelet agonists interact with trimeric GPCRs (G-protein-coupled receptors). Signalling pathways originating from different G-proteins act in a highly integrated fashion to trigger platelet activation (reviewed in [1,2]). For example, co-stimulation of G_q-coupled P2Y₁ receptors and G_i-coupled P2Y₁₂ receptors, [3], and combined signalling via G_{12/13} and G_i or G_i and G_z [4–6] are required to trigger full platelet aggregation.

TXA₂ (thromboxane A₂) is a platelet agonist that binds to receptors linked to both G_q and G_{12/13} proteins [7,8]. At doses that stimulate full platelet activation, TXA₂ triggers G_q-mediated responses [9,10]. Besides activating the fibrinogen binding function of integrin α IIb β 3, this signalling pathway is implicated in the secretion of ADP, which amplifies the platelet response via the G_i-coupled P2Y₁₂ receptor [2]. Signal transduction by G_{12/13}-coupled TXA₂ receptors has mainly been addressed in the context of platelet shape change [7]. More recently, a G_{12/13} signalling pathway was reported to act in concert with G_i-mediated signalling to fully activate platelet aggregation and degranulation [4,5], and G₁₃-deficiency has been shown to result in a markedly reduced haemostatic response *in vivo* [11]. Signalling by G_{12/13} implicates the small GTPase Rho and its target Rho-kinase/ROCK (Rho-associated kinase) [7,12,13]. In addition, selective stimulation of G_{12/13}-coupled TXA₂ receptors by low doses of the TXA₂ analogue U46619 or the isoprostane 8-iso-PGF_{2 α}

(prostaglandin F_{2 α}) has been reported to activate cytoplasmic tyrosine kinases and the MAPK (mitogen-activated protein kinase) p38 [7,12,14,15].

Signalling by trimeric GPCRs implicate cytoplasmic tyrosine kinases in different cell types (see [16–23] and references contained therein). However, the role of tyrosine phosphorylation signals in the activation of platelet responses via distinct G_q-protein families has been addressed only peripherally [6,7,15,24,25], despite the first clear demonstration of Src kinase activation by GPCRs is in a study where rapid activation was obtained in response to thrombin in platelets from patients with Glanzmann's thrombasthenia, which do not express integrin α IIb β 3 [24].

In order to elucidate the role of tyrosine phosphorylation in signal transduction by TXA₂ receptors, we exploited evidence that low doses of the TXA₂ analogue U46619 trigger some platelet responses, i.e. shape change and adhesion to surface bound fibrinogen, but no detectable aggregation and secretion [5,14,26,27]. In this study, we show that U46619 at low doses triggered tyrosine phosphorylation and that this signalling pathway was not downstream of Rho/Rho-kinase activation. We also found that, whereas low doses of U46619 or adrenaline (epinephrine) alone were unable to induce platelet activation, the two stimuli used in combination were potent agonists of platelet aggregation, providing evidence that both tyrosine phosphorylation and the Rho/Rho-kinase pathway are required to activate platelet aggregation resulting from combined G_{12/13} and G_z signalling.

Abbreviations used: ASA, acetylsalicylic acid; FAK, focal-adhesion kinase; GPCR, G-protein-coupled receptor; MLC, myosin light chain; RhoGEF, Rho guanine nucleotide-exchange factor; ROCK, Rho-associated kinase; TXA₂, thromboxane A₂.

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EXPERIMENTAL

Platelet preparation and stimulation

Venous blood samples (40 ml) were collected from healthy adult human volunteers, who had not been taking any medication in the previous 3 weeks and who also provide informed consent. An acid/citrate/dextrose mixture (14 mmol/l sodium citrate, 11.8 mmol/l citric acid and 18 mmol/l dextrose; Merck) was used as an anticoagulant. Washed platelets were obtained by centrifugation at 200 *g* for 10 min, followed by 700 *g* for 15 min at room temperature in the presence of 0.4 unit/ml apyrase V (Sigma). Platelets were then suspended at 37 °C in 2–3 ml of Hepes buffer [10 mmol/l Hepes (pH 7.4), 145 mmol/l NaCl, 5 mmol/l KCl, 0.5 mmol/l Na₂HPO₄ and 6 mmol/l glucose], as described previously by Hallam et al. [28]. ASA (acetylsalicylic acid; 100 µmol/l; Sigma) was added to the platelet suspensions to inhibit platelet COX-1 (cyclo-oxygenase-1) activity. Platelet suspensions were kept at room temperature and tested within 2 h. The stable TXA₂ analogue U46619 (9,11-dideoxy-9,11-epoxymethanoprostano-5Z,13E-dien-1-oic acid; Sigma) was used at concentrations ranging between 0.05–1 µmol/l [29,30]. In some experiments, platelets were also co-stimulated with 10 µmol/l adrenaline (A. Menarini Diagnostics) and 0.05 µmol/l U46619 [5]. Platelet suspensions were pre-incubated with the following inhibitory compounds before the assays: Y-27632 [(*R*)-(+)-trans-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexane-carboxamide 2HCl; a p160^{ROCK} inhibitor; final concentration, 30 µmol/l; time of pre-incubation, 10 min at 37 °C; Calbiochem]; PP2 {4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine} or its inactive analogue PP3 (4-amino-7-phenylpyrazolo[3,4-*d*]pyrimidine; final concentration, 10 µmol/l; time of pre-incubation, 10 min at 37 °C; Calbiochem); eptifibatide (a synthetic RGD mimetic; final concentration, 10 µg/ml; time of pre-incubation, 30 min at 37 °C; Integrilin); and apyrase VII (final concentration, 10 units/ml; time of pre-incubation, 5 min at 37 °C; Sigma). To determine the contribution of ADP receptors, we used the P2Y antagonist MRS2179 (2'-deoxy-*N*⁶-methyl adenosine 3',5'-diphosphate diammonium salt; final concentration, 0.1 mmol/l; time of pre-incubation, 1 min; Sigma) and the P2Y12 antagonist AR-C69931MX (final concentration 0.5 µmol/l; time of pre-incubation, 1 min), generously given by AstraZeneca.

Platelet aggregation and cytofluorimetric assays

Washed platelets were suspended at 2×10^8 cells/ml in Hepes buffer (see above) supplemented with 1 mmol/l CaCl₂ and 1 mmol/l MgSO₄, and were maintained at 37 °C in an aggregometer cuvette under continuous stirring at 1000 rev./min. After 1 min of incubation, U46619 (0.05 or 1 µmol/l) was added to 0.5 ml of platelets, and light transmission was monitored for 3 min. Platelet aggregation was measured using a four-channel aggregometer (Aggregometer II, Daiichi) and expressed as change in percentage of transmitted light (%T). In some experiments, 10 µmol/l adrenaline and 0.05 µmol/l U46619 were added simultaneously and aggregation was monitored with platelet suspensions pre-incubated either in the presence or absence of various inhibitors.

Cytofluorimetric assays

Platelet suspensions (180 µl; 8×10^4 cells/ml) were pre-incubated at 37 °C for 30 min with or without 10 µg/ml eptifibatide, and then incubated at 37 °C for 3 min in the presence of U46619 (0.05–2 µmol/l) or thrombin (0.05–1 unit/ml). FITC-labelled PAC-1 (Becton Dickinson), a ligand mimetic monoclonal antibody that specifically binds to the activated form of the integrin α IIb β 3

complex, was added to platelet suspensions before the agonists. In all of the experiments, an isotype-matched irrelevant mouse IgG₁ (FITC–IgG₁; Becton Dickinson) was included as negative control. After incubation at room temperature for 15 min, samples were analysed in duplicate by flow cytometry (FACScan; Becton Dickinson) using dual colour fluorescence. Platelets were identified on the basis of size and granularity.

Immunoblot analysis

Platelets (200 µl of washed platelets; 17.5×10^8 cells/ml) were incubated at 37 °C, under static conditions, in the presence or absence of U46619 (0.05 µmol/l) for the times indicated in the Results section. In these experiments, the ADP scavenger apyrase VII, the inhibitor of the fibrinogen receptor eptifibatide and ASA were pre-incubated with washed platelet preparations, unless indicated otherwise. After various times (see the Results section), 4 × sample buffer [100 mmol/l Tris/HCl (pH 6.8), 200 mmol/l 2-mercaptoethanol, 4 % (w/v) SDS, 20 % (v/v) glycerol and 0.4 % Bromophenol Blue] was added to the samples, which were then boiled for 3 min and, after centrifugation for 1 min at 13 000 *g*, were stored at –20 °C. To examine Syk tyrosine phosphorylation with phospho-specific antibodies (see below), ice-cold 2 × RIPA buffer [100 mmol/l Tris/HCl (pH 7.6), 300 mmol/l NaCl, 2 % (w/v) Triton X-100, 1 % sodium deoxycholate and 0.2 % SDS] supplemented with one protease inhibitor cocktail tablet (Roche Molecular Biochemicals), 2 mmol/l Na₃VO₄, 20 µmol/l phenylarsine oxide (Sigma), 10 µg/ml pepstatin (Sigma) and 2 mmol/l dithiothreitol (Sigma) was added to the samples. After incubation for 30 min on ice, lysates were clarified by centrifugation at 4 °C at 15 600 *g* for 10 min and stored at –20 °C. An aliquot of each sample (5 µl) was used to assess the protein concentration. Samples were separated by SDS/PAGE on 7.5 % or 12 % (w/v) acrylamide gels and transferred on to nitrocellulose Hybond C (Amersham Biosciences). After quenching for 1 h at room temperature in 3 % BSA (Sigma) in Tris-buffered saline [5 mmol/l Tris/HCl (pH 7.5), 170 mmol/l NaCl] containing 0.02 % Tween-20, blots were incubated overnight at 4 °C with primary antibodies, followed by incubation with secondary HRP (horseradish peroxidase)-conjugated donkey anti-(rabbit Ig) or goat anti-(mouse Ig) antibodies (Amersham Biosciences) for 1 h. Immunoreactivity was detected using ECL[®] Western Blotting detection reagent (Amersham Biosciences). After probing with anti-phosphotyrosine antibodies, the membrane was stripped in 62.5 mmol/l Tris/HCl (pH 6.7), 100 mmol/l 2-mercaptoethanol and 2 % (w/v) SDS at 50 °C for 30 min, and then re-probed with specific anti-protein antibodies. Anti-phospho Syk (Tyr³⁵²), anti-phospho Src (Tyr⁴¹⁶ of Src and equivalent tyrosine residues of other Src family members) and anti-phospho Akt (Ser⁴⁷³) antibodies were all from Cell Signalling Technology. Anti-phospho FAK (focal-adhesion kinase; Tyr³⁹⁷) was from BioSource International. Anti-phospho MLC (myosin light chain; Thr¹⁸/Ser¹⁹) was from Santa Cruz Biotechnology. Anti-phospho cortactin (Tyr⁴⁸⁶) was from Chemicon International. Anti-phosphotyrosine antibodies 4G10 and PY99 were from Upstate Biotechnology and Santa Cruz Biotechnology respectively.

RESULTS

Low doses of U46619 are unable to induce integrin α IIb β 3 activation and integrin α IIb β 3-dependent aggregation in platelets

The TXA₂ analogue U46619 at low doses (below 0.05 µmol/l) induces platelet shape change and adhesion to surface-bound fibrinogen, without triggering detectable platelet aggregation and

Table 1 Platelet aggregation induced by simultaneous stimulation of G_{12/13} and G₂ pathways is independent of ADP secretion and requires both tyrosine phosphorylation and Rho-kinase-mediated signals

ASA-treated platelets were stimulated in the presence or absence of 10 units/ml apyrase with the indicated doses of U46619 and adrenaline, alone or in combination. Platelet aggregation was recorded for 3 min. For inhibitory studies, ASA-treated platelets were stimulated following pre-incubation with 10 μ mol/l PP2 or PP3, 30 μ mol/l Y27632 or 10 μ mol/l PP2 + 30 μ mol/l Y27632. Values are means $\pm$ S.E.M. of five independent experiments. * P < 0.001 compared with U46619 + adrenaline without inhibitors; † P < 0.01 compared with U46619 alone.

Treatment	Aggregation (%)
U46619 (1 μ mol/l)	74.2 $\pm$ 4.8
+ apyrase	18.8 $\pm$ 6.1†
U46619 (0.05 μ mol/l)	0.4 $\pm$ 0.4
Adrenaline (10 μ mol/l)	0.0 $\pm$ 0.0
U46619 (0.05 μ mol/l) + adrenaline (10 μ mol/l)	72.5 $\pm$ 4.3
+ apyrase	67.6 $\pm$ 2.0
+ PP2 + apyrase	38.61 $\pm$ 6.5*
+ PP3 + apyrase	62.8 $\pm$ 6.8
+ Y27632 + apyrase	10.9 $\pm$ 5.8*
+ Y27632 + PP2 + apyrase	1.0 $\pm$ 1.0*

secretion [4,5,7,14,15]. In our experimental conditions, in order to exclude the activation of other platelet signalling receptors, i.e. the fibrinogen receptor integrin α IIB β 3 and receptors for ADP, we examined the effects of eptifibatide, a widely used synthetic antagonist of integrin α IIB β 3–fibrinogen interaction [31,32], and apyrase, an ADP scavenger, on platelet responses. U46619 triggered platelet aggregation at 1 μ mol/l, but not at 0.05 μ mol/l (Table 1). As eptifibatide completely inhibited the platelet response to 1 μ mol/l U46619 (results not shown), we conclude that any possible contribution of signals ensuing from integrin α IIB β 3 following its engagement by platelet-derived fibrinogen can be excluded when stimulating platelets with 0.05 μ mol/l U46619 in the presence of eptifibatide. Low doses of U46619, even in the absence of apyrase, were also unable to induce expression of the activation-dependent integrin α IIB β 3 epitope recognized by the PAC-1 antibody (results not shown). In contrast, both 1 μ mol/l U46619 (see Table 2) and thrombin (results not shown) markedly increased PAC-1 binding. The addition of apyrase to the extracellular medium inhibited U46619-induced PAC-1 binding (results not shown), suggesting that the effects of U46619 are dependent on the stimulation of ADP release and the consequent engagement of ADP receptors. We conclude that low doses of U46619 are unable to induce conformational changes in integrin α IIB β 3 detectable by an increase in PAC-1 binding and integrin α IIB β 3-dependent responses, and eptifibatide and apyrase can be used to determine any possible participation of integrin α IIB β 3 or ADP receptors in platelet signal transduction in response to low doses of U46619.

Low doses of U46619 trigger protein tyrosine phosphorylation independently of integrin α IIB β 3 outside-in or ADP receptor signalling

The capability of low doses of U46619 (0.05 μ mol/l) to trigger protein tyrosine phosphorylation was examined in the presence of eptifibatide, apyrase and ASA (see the Materials and methods section) to exclude any possible contribution of receptors for fibrinogen and ADP (see above), as well as for platelet-derived TXA₂. As shown in Figure 1(A), platelet stimulation with 0.05 μ mol/l U46619 resulted in a rapid increase in tyrosine phosphorylation of some proteins. Re-probing the same blot with specific antibodies indicated that phospho-proteins of approx. 125, 80 and 70 kDa migrated at the level of FAK, cortactin and

Syk respectively; in contrast, the FAK analogue Pyk2, whose migration in the gel was clearly distinct from that of FAK, was reproducibly found not to be phosphorylated (results not shown). To identify more clearly proteins whose tyrosine phosphorylation was increased by 0.05 μ mol/l U46619, we utilized phospho-specific antibodies (Figure 1B). We found that 0.05 μ mol/l U46619 markedly increased tyrosine phosphorylation of FAK, Src and Syk (Figures 1B and 1C), and cortactin (results not shown).

The presence of high concentrations of apyrase (10 units/ml) in our assays would safely exclude any potential contribution of secreted ADP to the tyrosine phosphorylation pathway triggered by 0.05 μ mol/l U46619. The lack of involvement of secreted ADP was also confirmed using two different experimental approaches. As shown in Figure 2(A), 0.05 μ mol/l U46619 did not cause Akt phosphorylation. In contrast, 1 μ mol/l U46619 induced Akt phosphorylation, which was abolished by high concentrations of apyrase (10 units/ml). In addition, the ADP receptor antagonists AR-C69931MX and MRS 2179, which compete for ADP binding to P2Y₁₂ and P2Y₁ receptors, did not inhibit tyrosine phosphorylation triggered by 0.05 μ mol/l U46619 either alone (results not shown) or in combination (Figure 2B). Therefore we can exclude that, upon platelet stimulation with 0.05 μ mol/l U46619 in the presence of apyrase, even trace amounts of ADP contribute to tyrosine phosphorylation.

Tyrosine phosphorylation signals triggered by low doses of U46619 are distinct from those mediated by the Rho target Rho-kinase

At low doses, the TXA₂ analogue U46619 has been shown to act primarily through G_{12/13} [7,11,12,14,15]. The G_{12/13}-dependent signalling pathway impinges on the small GTPase Rho, and Rho-kinase represents an immediate Rho target which, upon activation by GTP-loaded Rho, increases either indirectly or directly MLC phosphorylation [33]. Tyrosine phosphorylation signals in response to 0.05 μ mol/l U46619 were not inhibited by Y-27632, a specific inhibitor of Rho-kinase (Figure 3A). In contrast, phosphorylation of MLC, a specific Rho-kinase target, was markedly inhibited by Y-27632 (Figure 3B). Therefore we conclude that U46619-induced tyrosine phosphorylation is not secondary to Rho-kinase activation.

Tyrosine phosphorylation signals triggered by low doses of U46619 synergize with those ensuing from G₂-coupled receptors inducing platelet aggregation

G_{12/13} has been reported to synergize with G_i signalling pathways to activate platelets [4,5]. We therefore investigated whether engagement of the G_{12/13}-coupled TXA₂ receptors synergize with the G₂-coupled receptors for adrenaline. As shown in Figure 4 (see Table 1 for the mean results of several experiments), neither 10 μ mol/l adrenaline nor 0.05 μ mol/l U46619 alone was able to trigger platelet aggregation, but they were as effective as 1 μ mol/l U46619 when used in combination. As shown in Table 1, apyrase inhibited the platelet response to 1 μ mol/l U46619, but had no effects on the response to the combination of 0.05 μ mol/l U46619 and 10 μ mol/l adrenaline, indicating that this response is not dependent on secreted ADP.

In order to identify signals implicated in the induction of platelet aggregation in response to the combination of adrenaline and U46619, we used different inhibitory drugs (Figure 4 and Table 1). PP2, a tyrosine kinase inhibitor displaying selectivity for Src family kinases [34,35], but not its inactive analogue PP3 (Table 1), strongly reduced platelet aggregation in response to the combination of adrenaline and U46619. Interestingly, the Rho-kinase inhibitor Y-27632 also markedly inhibited platelet aggregation in response to adrenaline and U46619 and had

Figure 1 Low doses of U46619 trigger tyrosine phosphorylation signals

ASA-treated platelets were pre-incubated with 10 units/ml apyrase and 10 μ g/ml epifibatide and then stimulated with 0.05 μ mol/l U46619. At the indicated times, the reaction was stopped by the addition of 4 $\times$ sample buffer, the proteins were subjected to electrophoresis and transferred on to nitrocellulose. (A) Blots were probed with anti-phosphotyrosine antibodies. A representative experiment of three performed is shown. Molecular masses (in kDa) are indicated on the left. (B) Blots were probed with phospho-specific antibodies recognizing the indicated tyrosine residues [FAK Tyr³⁹⁷ (α FAKpY397), Src Tyr⁴¹⁶ (α SrcpY416) and Syk Tyr³⁵² (α SykpY352)], followed by stripping and re-probing with antibodies recognizing total protein. (C) Densitometric analysis of blots probed with phospho-specific antibodies. Results are means $\pm$ S.D. of three independent experiments.

an additive effect with PP2. The two inhibitors, either alone or in combination, also completely suppressed PAC-1 binding (Table 2) induced by adrenaline and U46619. We conclude that both tyrosine phosphorylation signals and Rho-kinase activity are required to induce platelet aggregation and integrin α IIB β 3 activation in response to low doses of TXA₂ and adrenaline.

In order to elucidate whether PP2 and Y-27632 display selectivity in the inhibition of tyrosine phosphorylation or the Rho/Rho-kinase pathway, we examined activation of these pathways by adrenaline and U46619. As shown in Figure 5, 0.05 μ mol/l U46619 alone triggered both tyrosine phosphorylation and MLC phosphorylation, whereas 10 μ mol/l adrenaline failed to elicit either of these responses. In addition, a combination of the two stimuli did not consistently enhance either protein tyrosine phosphorylation or MLC phosphorylation. PP2 suppressed tyrosine phosphorylation triggered by 0.05 μ mol/l U46619 either alone or in combination with 10 μ mol/l adrenaline, but only partially inhibited MLC phosphorylation. In contrast, Y-27632 suppressed MLC phosphorylation, but had a negligible effect on protein tyrosine phosphorylation induced by U46619 and adrenaline. Despite PP2 having some inhibitory effect on MLC phosphory-

lation (see the Discussion section) and its use in combination with Y-27632 having an even stronger inhibitory effect on tyrosine phosphorylation (compare the last three lanes of Figure 5), these findings suggest that protein tyrosine phosphorylation and MLC phosphorylation are two substantially distinct pathways triggered by G_{12/13}-coupled TXA₂ receptors and are both required to trigger platelet aggregation in the presence of additional signals ensuing from G_z-coupled receptors.

DISCUSSION

Our major findings can be summarized as follows. Low doses of the TXA₂ analogue U46619 triggered tyrosine phosphorylation of FAK, Syk and Src, which was independent of fibrinogen binding to α IIB β 3 integrin or the interaction of secreted ADP receptors with its platelet receptors P2Y₁ and P2Y₁₂. Moreover, this signalling pathway was independent of the Rho target Rho-kinase. Confirming previous findings [5,14,26,27], this signalling pathway was insufficient to trigger full platelet activation, but it can synergize with the G_z-linked adrenaline receptor to trigger platelet aggregation.

Figure 2 Low doses of U46619 are unable to trigger Akt phosphorylation, and their capability to trigger protein tyrosine phosphorylation is not inhibited by ADP receptor antagonists

(A) ASA-treated platelets were stimulated with the indicated concentrations of U46619 in the presence (+) or absence (–) of apyrase (10 units/ml). After 5 min of incubation, the reaction was stopped by the addition of 4 × sample buffer, and equal amounts of protein (15 µg) were analysed by Western blot analysis with a phospho-specific anti-Akt antibody (α AktpSer 473), followed by stripping and re-probing with an antibody recognizing total Akt (α Akt). A representative experiment of three performed is shown. (B) ASA-treated platelets, pre-incubated with 10 units/ml apyrase and 10 µg/ml eptifibatide, were incubated with 0.5 µmol/l AR-C66931MX and 0.1 mmol/l MRS2179 prior to stimulation with 0.05 µmol/l U46619. At the times indicated, the reaction was stopped by the addition of 4 × sample buffer, proteins were subjected to electrophoresis and transferred on to nitrocellulose. Blots were probed with an anti-phosphotyrosine antibody. A representative experiment of two performed is shown. Molecular masses (in kDa) are indicated on the left.

Responses triggered by low doses of TXA₂, or other arachidonate-derived products such as F₂-isoprostanes [5,7,14,26,27, 29], have been shown to depend on stimulation of G_{12/13}-coupled receptors [7,11]. Thus it is likely that the tyrosine-kinase-based signalling pathway that we describe in the present study depends on the stimulation of the G_{12/13}-coupled TXA₂ receptor; consistent with this hypothesis is the observation that its stimulation resulted in activation of the Rho/Rho-kinase pathway (Figures 3 and 5), a classical G_{12/13}-activated pathway [7,36,37]. As studies in non-haemopoietic cells have shown that G_{q/11} can also impinge on Rho, via activation of the RhoGEFs (Rho guanine nucleotide-exchange factors), LARG (leukaemia-associated RhoGEF) or p53RhoGEF [38,39], we cannot formally exclude that G_q-coupled TXA₂ receptors are also implicated in the platelet response to low doses of U46619. However, it is important to note that other G_q-mediated signalling pathways, such as increases in intracellular free calcium [5,26] or Akt phosphorylation [32,40] (Figure 2) are not activated by low doses of U46619.

Figure 3 Tyrosine phosphorylation signals triggered by low doses of U46619 are independent of the Rho/Rho-kinase pathway

ASA-treated platelets, pre-incubated with 10 units/ml apyrase and 10 µg/ml eptifibatide, were incubated with 30 µmol/l Y-27632 prior to stimulation with 0.05 µmol/l U46619. At the times indicated, the reaction was stopped by the addition of 4 × sample buffer, proteins were subjected to electrophoresis and transferred on to nitrocellulose. (A) Blots were probed with an anti-phosphotyrosine antibody. Molecular masses (in kDa) are indicated on the left. (B) Blots were probed with phospho-specific antibodies recognizing the indicated threonine/serine residues of MLC [MLC Thr¹⁸/Ser¹⁹ (α MLCpThr18/Ser19)], followed by stripping and re-probing with an antibody recognizing total MLC (α MLC). A representative experiment of three performed is shown.

Analogous with studies in different cell types showing that GPCRs impinge on Src, as well as other families of tyrosine kinase, agonists of trimeric GPCRs have been reported to activate tyrosine phosphorylation signals independently of integrin α IIB β 3 in platelets [6,7,15,24]. In the present study, we show that, in these cells, a putative G_{12/13}-mediated signalling pathway enhances phosphorylation of members of three distinct cytoplasmic tyrosine kinase families, i.e. Src, Syk and FAK. This tyrosine-kinase-based pathway was detected as early as 15 s after platelet stimulation and in conditions (ASA treatment, the presence of the ADP scavenger apyrase and ADP receptor antagonists, and the presence of the α IIB β 3 integrin antagonist eptifibatide) allowing us to exclude a contribution of signalling pathways triggered by platelet-derived TXA₂ and ADP, or by the binding of fibrinogen to the α IIB β 3 integrin. Our findings strengthen the notion [7,15,24,25] that cytoplasmic tyrosine kinases are implicated in two different steps of platelet responses. G_{12/13}-coupled, as well as G_{i/z}-coupled [6], platelet receptors trigger an early wave of protein tyrosine phosphorylation. Following

Figure 4 Platelet aggregation induced by simultaneous stimulation of $G_{12/13}$ and G_i pathways requires both tyrosine phosphorylation and Rho-kinase-mediated signals

(A–C) ASA-treated platelets were stimulated in the presence or absence of 10 units/ml apyrase with the indicated doses of U46619 and adrenaline alone or in combination. (D) ASA-treated platelets were stimulated with the indicated doses of U46619 and adrenaline in combination following pre-incubation with 10 $\mu\text{mol/l}$ PP2, 30 $\mu\text{mol/l}$ Y-27632 or 10 $\mu\text{mol/l}$ PP2 + 30 $\mu\text{mol/l}$ Y-27632.

activation of the integrin $\alpha\text{IIb}\beta 3$ binding to fibrinogen, a second sustained wave of tyrosine phosphorylation ensues [41,42].

In comparison with Src and Syk [7,15], tyrosine phosphorylation of FAK in response to $G_{12/13}$ -coupled TXA_2 receptors in platelets has not been reported previously. However, thrombin has been reported to induce phosphorylation of FAK in both platelets [43] and human embryonic kidney cells [16]. As opposed to studies with HeLa cells expressing $G\alpha_{13}$ or $G\alpha_q$ [17,20], we did not find any evidence implicating Pyk2 in platelets stimulated with low doses of U46619.

Mechanisms of activation of cytoplasmic tyrosine kinases by GPCRs have been elucidated only in part. In the case of Src-family kinases, several mechanisms have been described (for a review, see [23]). Among these, some are direct and involve binding of the kinase to the GPCR [44], the $G\alpha$ subunits [45] or β -arrestins bound to the receptor itself [46,47]. Among indirect mechanisms of tyrosine kinase activation by GPCRs, the best characterized is certainly represented by the integrin-mediated assembly of cytoskeletal and signalling proteins in focal adhesion sites [16,19,23,42]. A typical feature of this pathway is that it is blocked by inactivation of Rho with the *Clostridium botulinum* C3 exoenzyme, the Rho-kinase inhibitor Y-27632 and disruption of the actin cytoskeleton with cytochalasin D [16,19]. Notably, at doses that blocked MLC phosphorylation, the Rho-kinase inhibitor Y-27632 had a negligible effect on the tyrosine-kinase-based signalling pathway induced by low doses of U46619 (Figure 3) or by U46619 and adrenaline in combination (Figure 5).

Table 2 Activation of the integrin $\alpha\text{IIb}\beta 3$ fibrinogen receptor by simultaneous stimulation of the $G_{12/13}$ and G_i pathways requires both tyrosine phosphorylation and Rho-kinase-mediated signals

ASA-treated platelets were stimulated in the presence of 10 units/ml apyrase with the indicated doses of U46619 and adrenaline, alone or in combination. Values are expressed as a percentage of platelets binding the PAC-1 monoclonal antibody (see Materials and methods section), and are means $\pm$ S.E.M. of three independent experiments. Treatment with PP2 and Y27632 was as described in Table 1. * $P < 0.001$ compared with U46619 + adrenaline without inhibitors.

Treatment	Active fibrinogen receptor (%)
Resting	0.2 ± 0.1
U46619 (1 $\mu\text{mol/l}$)	50.5 ± 22.6
U46619 (0.05 $\mu\text{mol/l}$) + adrenaline (10 $\mu\text{mol/l}$)	25.9 ± 2.7
+ Y27632	$2.57 \pm 2.7^*$
+ PP2	$2.5 \pm 0.5^*$
+ Y27632 + PP2	$2.0 \pm 0.2^*$

Figure 5 PP2 inhibits tyrosine phosphorylation signals triggered by the combination of U46619 and adrenaline

ASA-treated platelets were stimulated in the presence of 10 units/ml apyrase with 0.05 $\mu\text{mol/l}$ U46619 and 10 $\mu\text{mol/l}$ adrenaline, alone or in combination, following pre-incubation with 10 $\mu\text{mol/l}$ PP2, 30 $\mu\text{mol/l}$ Y-27632 or 10 $\mu\text{mol/l}$ PP2 + 30 $\mu\text{mol/l}$ Y-27632. After 40 s, the reaction was stopped by the addition of 4 $\times$ sample buffer, then proteins were subjected to electrophoresis and were transferred on to nitrocellulose. (A) Blots were probed with anti-phosphotyrosine antibodies. Molecular masses (in kDa) are indicated on the left. (B) Blots were probed with phospho-specific antibodies recognizing the indicated threonine/serine residues of MLC [MLC Thr¹⁸/Ser¹⁹ ($\alpha\text{MLCpThr18/Ser19}$)], followed by stripping and reprobing with an antibody recognizing total MLC (αMLC). A representative experiment of three performed is shown.

Hence it is tempting to speculate that tyrosine kinase activation by a putative $G_{12/13}$ -mediated pathway is direct and due to kinase interaction with the receptor or the G-protein itself (see above). Certainly, evidence that $G\alpha_{13}$ binds to Pyk2, a FAK analogue, in HeLa cells overexpressing the two proteins [17] should prompt studies to challenge the possibility that $G\alpha_{13}$ and FAK interact in platelets.

The biological significance of the tyrosine-kinase-based signalling pathway in response to low doses of U46619 described in the present study is highlighted by the observation that this pathway synergizes with a G_i -mediated pathway to induce platelet aggregation (Figure 4 and Table 1). Previous findings have demonstrated that combined signalling via $G_{12/13}$ and G_i is required for full platelet activation [4,5]. Interestingly, both tyrosine-kinase-dependent and Rho-kinase-dependent signalling are required for the combined stimulation with low doses of U46619 and adrenaline. In fact, both PP2 and Y-27632 significantly inhibited platelet

aggregation induced by the combination of the two stimuli and, together, totally suppressed this response. Hence G_{12/13}-dependent Rho-kinase and tyrosine-kinase-dependent signals converge in the activation of integrin α IIb β 3 provided additional signals ensuing from the engagement of G_z-coupled receptors are generated. These two pathways are substantially independent (see, for example, Figure 3). However, we found that PP2, besides suppressing the tyrosine phosphorylation pathway, also partially inhibited MLC phosphorylation (Figure 5). These findings are consistent with data obtained with smooth muscle and endothelial cells which established a link between Src-family kinase activity and the pathway leading to MLC phosphorylation [48–50].

Apart from their heuristic content, our findings may be of clinical relevance. Local release of TXA₂ (or other bioactive eicosanoids acting through the thromboxane receptor) from activated platelet and monocytes/macrophages [51] may activate the tyrosine-kinase-based signalling pathway. Full platelet activation may then be induced in the presence of a weak platelet agonists or subthreshold concentrations of stronger agonists. Therefore our findings suggest that tyrosine kinase activation may play an important role in platelet thrombus formation.

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