The platelet cytoskeleton regulates the aggregation-dependent synthesis of phosphatidylinositol 3,4-bisphosphate induced by thrombin

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Abstract Pretreatment of intact platelets with cytochalasin D prevented actin polymerization and cytoskeleton reorganization induced by thrombin, but did not affect platelet aggregation. Under these conditions, synthesis of phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) stimulated by thrombin was strongly inhibited, while production of phosphatidic acid was unaffected. The inhibitory effect of cytochalasin D was not observed when platelet aggregation was prevented by the RGDS peptide. We also found that cytochalasin D did not affect PtdIns(3,4)P_2 synthesis induced by concanavalin A (ConA), which is known to occur through an aggregation-independent mechanism. Moreover, thrombin, but not ConA, induced the translocation of phosphatidylinositol 3-kinase to the cytoskeleton. This process was equally inhibited by both the RGDS peptide and cytochalasin D. These results demonstrate that the cytoskeleton represents a functional link between thrombin-induced aggregation and synthesis of PtdIns(3,4)P_2.

Key words: Phosphatidylinositol 3-kinase; Cytoskeleton; Platelet; Cytochalasin D

1. Introduction

The 3-phosphorylated phosphoinositides, phosphatidylinositol 3-phosphate (PtdIns(3)P), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), are intracellular messengers that accumulate in stimulated cells, including human platelets treated with strong agonists like thrombin and the thromboxane A_2 analogue U46619 [1–3]. In stimulated platelets, the synthesis of 3-phosphorylated phosphoinositides occurs in two temporal waves: the totality of PtdIns(3,4,5)P_3 and a small amount of PtdIns(3,4)P_2 are synthesized rapidly after agonist addition, while the majority of PtdIns(3,4)P_2 is accumulated in a late phase of platelet stimulation [4]. This delayed but sustained production of PtdIns(3,4)P_2 has been related to platelet aggregation, because it is prevented by inhibitors of fibrinogen binding to integrin α_{IIb}-β_3 and does not occur in platelets from patients affected by Glanzmann thrombasthenia [5,6]. However, the evidence that, in these conditions, the accumulation of PtdIns(3,4)P_2 is reduced but not abolished, and the observation that some agonists, like the lectin concanavalin A (ConA) or lysophosphatidic acid, can induce the synthesis of this lipid in the absence of aggregation [7,8] suggested the existence of a dual mechanism, aggregation-independent and aggregation-dependent, for PtdIns(3,4)P_2 accumulation in stimulated platelets.

Several authors have reported the association of activated phosphatidylinositol 3-kinase (PtdIns 3-kinase) with the cytoskeleton in thrombin-stimulated platelets [9,10]. A possible correlation between the aggregation-dependent synthesis of PtdIns(3,4)P_2 and the translocation of PtdIns 3-kinase to the cytoskeleton has been hypothesized based on the evidence that the two events revealed similar kinetics and were equally prevented by the inhibition of platelet aggregation [11].

In this work, we directly investigated the role of the PtdIns 3-kinase interaction with the cytoskeleton on the regulation of the aggregation-dependent and independent synthesis of PtdIns(3,4)P_2 in intact platelets stimulated with thrombin. Platelets pretreated with cytochalasin D were used as a model to analyze the effect of thrombin-induced aggregation in the absence of actin polymerization and cytoskeleton reorganization, while platelets stimulated with the lectin ConA provided a model for PtdIns(3,4)P_2 synthesis in the absence of aggregation, but in the presence of a normal actin polymerization and cytoskeleton reorganization. The results demonstrate that intracellular cytoskeleton, probably through the interaction with PtdIns 3-kinase, selectively promotes the aggregation-dependent synthesis of PtdIns(3,4)P_2.

2. Materials and methods

2.1. Materials

Cytochalasin D, ConA, thrombin, the peptide RGDS, leupeptin and lipids were obtained from Sigma. [^{32}P]Orthophosphate (20 mCi/ml, carrier free) and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham.

2.2. Platelet preparation and labeling

Human washed platelets were prepared from platelet concentrates as previously described [3]. Platelets were labeled with [^{32}P]orthophosphate (0.3 mCi/ml) at the concentration of 10^9 cells/ml in 135 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.16 mM NaH_2PO_4, 2 mM $MgCl_2$, 0.2 mM EGTA, 5.5 mM glucose, 0.34% bovine serum albumin, pH 6.5 (buffer A), for 90 min at room temperature. Platelets were then washed with buffer A without glucose and EGTA (buffer B) and finally resuspended at the concentration of 10^9 cells/ml in buffer B, pH 7.4, containing 1 mM $MgCl_2$ and 2.5 mM $CaCl_2$.

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Abbreviations: PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns 3-kinase, phosphatidylinositol 3-kinase; ConA, concanavalin A

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2.3. Platelet incubation and lipid extraction

Platelet samples (1 ml, 10^9 cells/ml) were equilibrated at 37°C. Cytochalasin D (10 μM final concentration), RGDS (1 mM final concentration) or buffer were added 2 min before stimulation with 1 U/ml thrombin or 100 μg/ml ConA. As cytochalasin D was stored as a 1 mM stock solution in dimethylsulfoxide, an equal volume of dimethylsulfoxide was added to control samples. Stimulation with thrombin was performed for 5 min at 37°C with shaking, to induce full platelet aggregation, while stimulation with ConA was performed for the same time, but without shaking to prevent platelet aggregation. In some experiments, performed to evaluate platelet aggregation, cell activation was performed in siliconized cuvettes placed in an aggregometer with continuous stirring at 37°C. In these cases, platelet aggregation was monitored continuously. Reactions were stopped by addition of chloroform/methanol (1:2, v/v) and lipids were extracted, deacylated by methylamine treatment, separated and identified by high performance liquid chromatography (HPLC) on a Whatman Partisphere strong ion exchange column, as previously described [3].

2.4. Cytoskeleton preparation

Platelet samples were lysed by addition of an equal volume of ice-cold cytoskeletal buffer (100 mM Tris–HCl, pH 7.4, 20 mM EGTA, 4 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 mM Na_3VO_4, 2% Triton X-100). Samples were mixed and placed on ice for 10 min. Triton X-100-insoluble material was recovered by centrifugation at 13 000×*g* for 5 min at 4°C, washed twice with cytoskeletal buffer without Triton X-100 diluted 1:1, and resuspended in the PtdIns 3-kinase assay buffer as described below. For electrophoresis analysis, samples were solubilized with 2% sodium dodecyl sulfate (SDS), 0.5% dithiothreitol, 10% glycerol, 0.05% bromophenol blue and boiled for 5 min. Proteins were separated on a 5–15% acrylamide gel and stained with Coomassie blue.

2.5. PtdIns 3-kinase assay

Cytoskeletal samples were mechanically resuspended with 50 μl of ice-cold 50 mM Tris–HCl, pH 7.4, containing 0.1 mM Na_3VO_4. Then, 50 μl of PtdIns 3-kinase assay buffer (50 mM Tris–HCl, pH 7.4, 3 mM dithiothreitol, 200 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 10 μM ATP) was added. Reaction was started by addition of 30 μl of exogenous lipid vesicles (100 μM phosphatidylinositol plus 200 μM phosphatidylserine obtained by sonication in 50 mM Tris–HCl, pH 7.4) and 1.5 μCi of [γ-^{32}P]ATP. Samples were incubated at 37°C for 20 min with shaking. Reactions were stopped by addition of 800 μl of chloroform/methanol (1:2, v/v) and lipids were extracted, deacylated and analyzed by HPLC as described [3].

3. Results

Pretreatment of washed platelets with 10 μM cytochalasin D for 2 min did not affect the aggregation induced by 1 U/ml thrombin (Fig. 1A). However, the analysis of the composition of the cytoskeleton from resting and thrombin-stimulated platelets revealed that, under the same conditions, the agonist-induced actin polymerization was almost totally prevented (Fig. 1B, 45 kDa band), as well as the interaction of cytoskeletal proteins, including actin binding protein (235 kDa band), myosin (200 kDa band) and α-actinin (99 kDa band), with the Triton X-100-insoluble material.

Using ^{32}P-labeled platelets, we analyzed the effect of cytochalasin D on thrombin-induced activation of PtdIns 3-kinase and phospholipase C. We found that treatment of intact platelets with 10 μM cytochalasin D caused a strong and specific inhibition of the synthesis of PtdIns(3,4)P_2 induced by thrombin (Fig. 2). By contrast, accumulation of phosphatidic acid was not affected by this metabolite (Fig. 2).

Previous studies using RGD-containing peptides or platelets from thrombasthenic patients revealed that in the absence of aggregation, the thrombin-induced synthesis of PtdIns(3,4)P_2 was reduced by about 60% [5,6]. This inhibition was quantitatively very similar to that observed in cytochalasin D-treated platelets (Fig. 2), although, in this case, platelets underwent aggregation normally. Therefore, we compared the effects of cytochalasin D and RGDS peptide on PtdIns(3,4)P_2 synthesis in thrombin-stimulated platelets. As shown in Fig. 3,

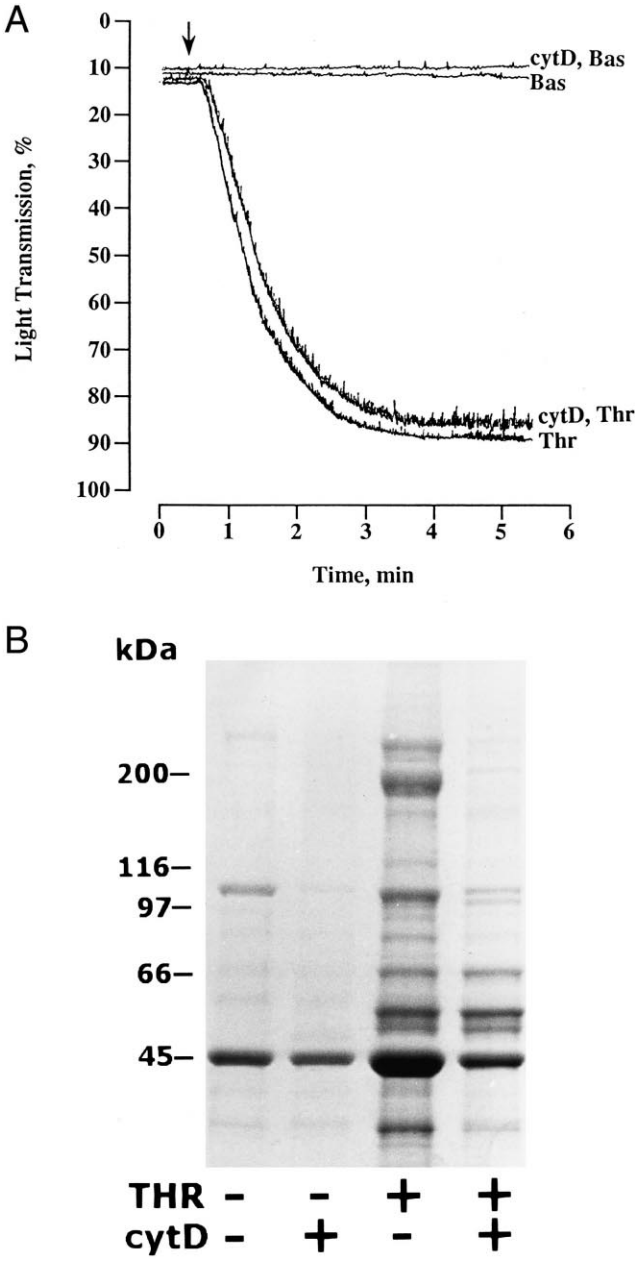

Fig. 1. Effect of cytochalasin D on platelet aggregation and cytoskeleton reorganization. Washed platelets were incubated at 37°C in a lumiaggregometer under constant stirring and treated with 10 μM cytochalasin D (cytD) or with an equal volume of dimethylsulfoxide for 2 min. Samples were then stimulated with buffer (Bas) or 1 U/ml thrombin (Thr) for 5 min and aggregation was monitored. After cell lysis with Triton X-100, cytoskeleton was prepared and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). A: Optical pattern of platelet aggregation induced by thrombin in dimethylsulfoxide- or cytochalasin D-treated cells. The arrow indicates the addition of the agonist. B: Analysis of the cytoskeletal proteins by SDS–PAGE on a 5–15% polyacrylamide gradient gel and Coomassie blue staining. The positions of the molecular weight markers are indicated on the left. The main cytoskeletal proteins are identified as actin binding protein (235 kDa), myosin (200 kDa), α-actinin (99 kDa) and actin (45 kDa).

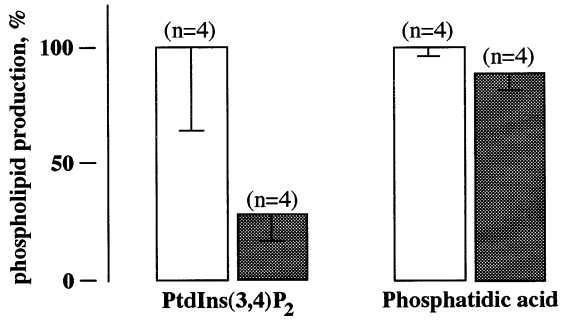

Fig. 2. Effect of cytochalasin D on the production of PtdIns(3,4)P_2 and phosphatidic acid induced by thrombin. ^{32}P-Labeled platelets were treated with 10 μM cytochalasin D or an equal volume of dimethylsulfoxide for 2 min at 37°C, and then stimulated with 1 U/ml thrombin for 5 min with shaking. Lipids were extracted, and the production of PtdIns(3,4)P_2 and phosphatidic acid was determinated by HPLC analysis of the deacylated products. Synthesis of PtdIns(3,4)P_2 or phosphatidic acid in dimethylsulfoxide-treated, thrombin-stimulated platelets is reported as 100% of production, and corresponds to 3510 ± 702 cpm and 13 749 ± 4424 cpm, respectively. The results are the mean ± S.D. of four separated experiments.

pretreatment of platelets with RGDS or cytochalasin D caused a similar inhibition of PtdIns(3,4)P_2 production. Moreover, preincubation of platelets with RGDS before treatment with cytochalasin D did not result in an enhanced inhibition of the synthesis of PtdIns(3,4)P_2 induced by thrombin. These results suggested that, when the aggregation-dependent pathway of PtdIns 3-kinase activation was inhibited by RGDS peptide, cytochalasin D had no more effects. To confirm the correlation between the aggregation-dependent and the cytochalasin D-sensitive synthesis of PtdIns(3,4)P_2, we analyzed ^{32}P-labeled platelets stimulated with the lectin ConA. We have previously demonstrated that ConA induces the accumulation of PtdIns(3,4)P_2 exclusively through a mechanism independent of integrin α_{IIb}-β_3 and aggregation [7]. As shown in Fig. 3, preincubation of platelets with cytochalasin D did not significantly affect the ability of ConA to stimulate the synthesis of PtdIns(3,4)P_2. Under the same conditions, ConA-induced actin polymerization and cytoskeleton reorganization was completely prevented by cytochalasin D (data not shown).

It is known that, in aggregated platelets, the PtdIns 3-kinase associates with the cytoskeleton [9,10]. Fig. 4 shows that the PtdIns 3-kinase activity in the cytoskeleton strongly increased upon platelet aggregation induced by 1 U/ml thrombin. This effect was clearly aggregation-dependent, as it was almost totally prevented by the RGDS peptide. Moreover, treatment of intact platelets with cytochalasin D almost totally abolished the ability of thrombin to promote the association of PtdIns 3-kinase activity with the cytoskeleton. In addition, the lectin ConA, which induced an aggregation-independent and cytochalasin D-insensitive accumulation of PtdIns(3,4)P_2 in intact platelets, was not able to stimulate the association of PtdIns 3-kinase activity with the cytoskeleton (Fig. 4). Similar results were obtained by analyzing the presence of the regulatory subunit of PtdIns 3-kinase, p85, in the Triton X-100-insoluble material, using a specific antiserum (data not shown).

4. Discussion

During the past years, it was reported that accumulation of PtdIns(3,4)P_2 in thrombin-stimulated platelets was maximal only when aggregation occurred and was reduced by about 60% by antagonists of fibrinogen binding to integrin α_{IIb}-β_3 [5,6]. On the other hand, it was found that, in thrombin-stimulated platelets, the PtdIns 3-kinase associated with the reorganized cytoskeleton through a mechanism strictly dependent on fibrinogen binding and platelet aggregation [9–11]. Therefore, since both synthesis of PtdIns(3,4)P_2 and interaction of PtdIns 3-kinase with the cytoskeleton were similarly regulated and showed similar kinetics during aggregation, a possible correlation has been suggested [11]. However, it could not be ruled out that the two events were independently promoted by aggregation and represented concomitant, but functionally unrelated, effects of cell-cell adhesion.

In this work, we tried to establish a functional correlation among platelet aggregation, interaction of PtdIns 3-kinase with the cytoskeleton and synthesis of PtdIns(3,4)P_2, and we demonstrated that interaction of PtdIns 3-kinase with the cytoskeleton is strictly required for the aggregation-dependent

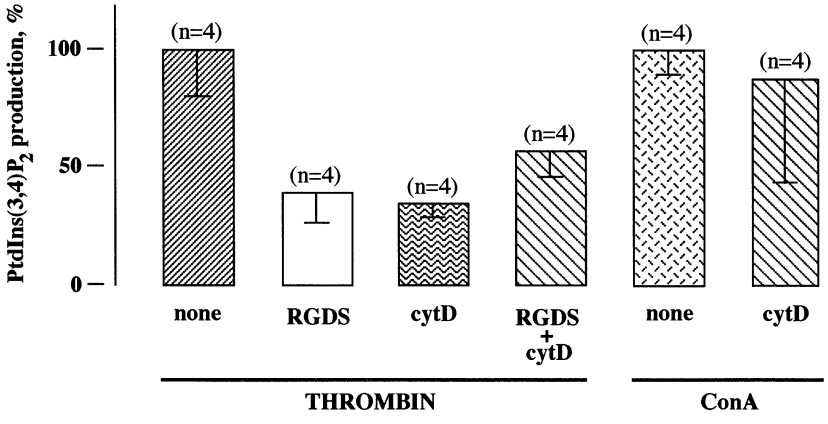

Fig. 3. Inhibition of PtdIns(3,4)P_2 production by RGDS and cytochalasin D. ^{32}P-Labeled platelets were incubated at 37°C and treated for 2 min with buffer (none), 1 mM RGDS (RGDS), 10 μM cytochalasin D (cytD) or both compounds (RGDS+cytD). Samples were then stimulated with 1 U/ml thrombin for 5 min. Other samples were treated with buffer (none) or 10 μM cytochalasin D (cytD), and then stimulated with 100 μg/ml ConA (ConA) for 5 min in the absence of shaking. The synthesis of PtdIns(3,4)P_2 in thrombin- or ConA-stimulated platelets with no added inhibitors is reported as 100% of production, and corresponds to 3510 ± 702 cpm and 5512 ± 596 cpm, respectively. The results are the mean ± S.D. of four separated experiments.

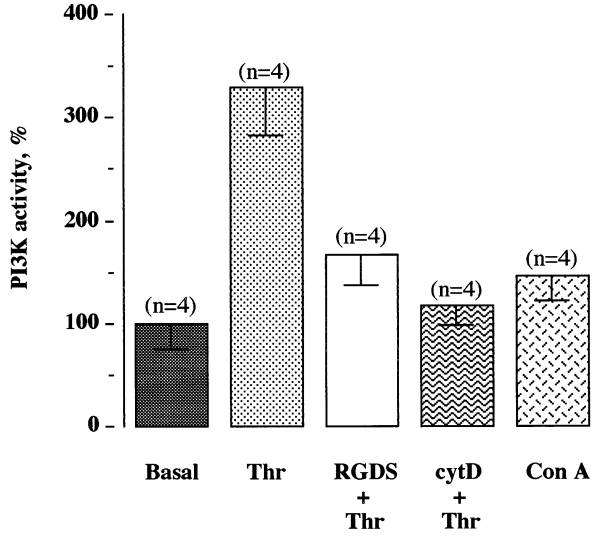

Fig. 4. Analysis of the association of PtdIns 3-kinase with the cytoskeleton. Washed platelets were treated for 5 min with buffer (basal), 1 U/ml thrombin (Thr) under constant shaking, or 100 μg/ml ConA (ConA) without shaking. In some cases, stimulation with thrombin followed the incubation of platelets with 1 mM RGDS peptide (RGDS) or 10 μM cytochalasin D (cytD) for 2 min. Samples were then lysed with Triton X-100 and the cytoskeleton was prepared. PtdIns 3-kinase activity was measured as described using phosphatidylinositol as substrate. The amount of PtdIns(3)P produced by the cytoskeleton from resting platelets is reported as 100% of activity, and corresponds to 1709 ± 645 cpm.

accumulation of PtdIns(3,4)P_2 in thrombin-stimulated platelets.

We used cytochalasin D to specifically inhibit agonist-induced actin polymerization. In agreement with previously reported results [12], we found that treatment of platelets with cytochalasin D did not affect thrombin-stimulated aggregation, but prevented actin polymerization and cytoskeleton reorganization. Under these conditions, the thrombin-induced translocation of PtdIns 3-kinase to the cytoskeleton was almost totally prevented. A similar inhibition was also observed in platelets incubated with the RGDS peptide, an inhibitor of fibrinogen binding to integrin α_{IIb}-β_3. However, while in this case both aggregation and PtdIns 3-kinase interaction with the cytoskeleton were prevented, the cytochalasin D-treated platelets represented a more informative model in which binding of PtdIns 3-kinase with the actin filaments was abolished, but aggregation occurred normally. We found that, despite platelet aggregation, the thrombin-stimulated synthesis of PtdIns(3,4)P_2 was strongly reduced by cytochalasin D treatment. This effect was specific, as no changes on the production of phosphatidic acid, resulting from the combined action of phospholipase C and diacylglycerol kinase, were observed. Moreover, the inhibition of PtdIns(3,4)P_2 synthesis induced by cytochalasin D was quantitatively very similar to that promoted by inhibitors of fibrinogen binding like the RGDS peptide. By treatment of the same platelet sample with both RGDS peptide and cytochalasin D, we found that the inhibitory effects of the two compounds on thrombin-induced accumulation of PtdIns(3,4)P_2 were neither synergistic nor additive. These results, thus, demonstrate that the aggregation-dependent synthesis of PtdIns(3,4)P_2 can occur as long as a normal cytoskeleton reorganization and interaction of PtdIns 3-kinase with the actin filaments is allowed. Thus, we can propose a model in which binding of PtdIns 3-kinase with the cytoskeleton lies downstream platelet aggregation and upstream the synthesis of PtdIns(3,4)P_2 and provides a link for the aggregation-dependent accumulation of this 3-phosphorylated phosphoinositide.

This model is also supported by our results on the effect of cytochalasin D on PtdIns(3,4)P_2 production induced by the lectin ConA. It is known that this lectin is a potent stimulator of actin polymerization and cytoskeleton reorganization, and promotes the synthesis of PtdIns(3,4)P_2 through a mechanism totally independent of platelet aggregation [7]. Thus, while two mechanisms for PtdIns(3,4)P_2 production are operating in thrombin-stimulated platelets, aggregation-independent and aggregation-dependent, only the former one takes place in ConA-treated platelets. We, therefore, checked the effect of cytochalasin D on the aggregation-independent synthesis of PtdIns(3,4)P_2 induced by the lectin ConA, and we found that this compound had no significant effects. Moreover, we found that, unlike thrombin, ConA was unable to promote the translocation of PtdIns 3-kinase to the cytoskeleton. Thus, according to our model, the aggregation-independent synthesis of PtdIns(3,4)P_2 induced by ConA was insensitive to cytochalasin D and was not paralleled by the translocation of PtdIns 3-kinase to the cytoskeleton.

In conclusion, our results demonstrate that the aggregation-dependent interaction of PtdIns 3-kinase with the cytoskeleton and the synthesis of PtdIns(3,4)P_2 are two steps of the same signaling pathway and we propose that platelet aggregation promotes the binding of PtdIns 3-kinase with the actin filaments which, in turn, results in the maximal activation of the enzyme responsible for the synthesis of PtdIns(3,4)P_2.

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