$\alpha_{IIb}\beta_3$-integrin mediated adhesion of human platelets to a fibrinogen matrix triggers phospholipase C activation and phosphatidylinositol 3′,4′-bisphosphate accumulation

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Abstract This study focused on the variations in phosphoinositide metabolism depending upon $\alpha_{IIb}\beta_3$-integrin/fibrinogen interaction without previous activation of platelet agonist receptors. We found that adhesion of resting human platelets to immobilized fibrinogen stimulates phosphatidic acid production and a concomitant decrease in phosphatidylinositol 4′,5′-bisphosphate. These results, and the absence of a transphosphatidylation reaction, argue in favor of the activation of a phospholipase C. Moreover, we observed the accumulation of phosphatidylinositol 3′,4′-bisphosphate in adherent platelets as a consequence of the activation of a phosphatidylinositol 3-kinase. This effect was inhibited by ADP scavengers. Our results demonstrate that in adherent platelets, whereas phosphatidylinositol 3-kinase activation is controlled by both $\alpha_{IIb}\beta$-integrin engagement and released ADP, phospholipase C stimulation is triggered only by $\alpha_{IIb}\beta$-integrin/fibrinogen interaction.

Key words: $\alpha_{IIb}\beta_3$-Integrin; Platelet adhesion; Phospholipase C; Phosphatidylinositol 3-kinase; ADP

1. Introduction

Integrin $\alpha_{IIb}\beta_3$ is a well-known receptor for adhesive proteins responsible for platelet aggregation and adhesion to damaged vessel walls, therefore playing an important role in primary hemostasis. The main ligand of $\alpha_{IIb}\beta_3$ is fibrinogen which, in soluble form, is bound only after previous activation of $\alpha_{IIb}\beta_3$-integrin. One dodecapeptide sequence at the carboxyl terminus of the fibrinogen γ-chain is primarily involved in both platelet aggregation and adhesion [1–3]. The integrin $\alpha_{IIb}\beta_3$ does not display any intrinsic enzymatic activity (reviewed in [4]) but associates in vivo or in vitro with signaling enzymes such as $p60^{c-src}$ and $p125^{FAK}$ [5,6]. Data on the signaling pathway depending upon $\alpha_{IIb}\beta_3$ activation in platelets have greatly enhanced knowledge of integrin-mediated signal transduction. Inhibition of fibrinogen binding to $\alpha_{IIb}\beta_3$ in stimulated platelets has highlighted the role of this glycoprotein in regulating a number of signals including cytoplasmic alkalinization [7], calcium influx [8], and protein-tyrosine phosphorylation [9].

We have previously demonstrated that the inhibition of fibrinogen binding to $\alpha_{IIb}\beta_3$ inhibits the synthesis of the major phosphatidylinositol 3′,4′-bisphosphate (PtdIns(3,4)P_2) pool in thrombin-stimulated platelets [10]. *D*3-phosphorylated phosphoinositides are involved in the phosphorylation of pleckstrin [11–13] and in the activation of the receptor function of $\alpha_{IIb}\beta_3$-integrin [14] in platelets, and in mitogenesis or cell transformation in nucleated cells [15,16]. Finally, *D*3-phosphorylated phosphoinositides could regulate different events, such as activation of specific isoforms of protein kinase C, cytoskeleton organization, or intracellular trafficking [17].

In order to study signal transduction via the phosphoinositide pathway depending upon $\alpha_{IIb}\beta_3$-integrin function without previous activation of other agonist receptors, we took advantage of the property of resting $\alpha_{IIb}\beta_3$-integrin mediating cell adhesion to immobilized fibrinogen. This type of interaction has clinical relevance as it could occur during platelet-extracellular matrix, and platelet-platelet interactions in vivo. Data obtained by Haimovich et al. [18] after adhesion of platelets to immobilized fibrinogen have shown that tyrosine phosphorylation of specific proteins was triggered by $\alpha_{IIb}\beta_3$/fibrinogen interaction accompanying platelet spreading. Moreover, some of these tyrosine phosphorylation steps may be under the control of $p125^{FAK}$ [18].

In this study, we investigated phosphoinositide metabolism in human platelets adherent on a matrix of fibrinogen. We show: (1) the stimulation of a phospholipase C during the early stages of the adhesion process; and (2) slower synthesis of PtdIns(3,4)P_2 in adherent platelets involving ADP released upon adhesion. These results outline similarities in changes of phosphoinositide metabolism triggered by integrins and agonist receptors.

2. Materials and methods

2.1. Reagents

Human fibrinogen, ADP, apyrase, pyruvate kinase, phosphoenolpyruvate, fatty acid free-BSA, and PBS were from Sigma (Sigma Chemical Co., St. Louis, MO).

2.2. Preparation of platelets

Human platelets were isolated from fresh platelet concentrates (Centre Régional de Transfusion Sanguine, Toulouse, France) by centrifugation as already described [19]. In some experiments, platelet-rich plasma was incubated with 100 μM aspirin for 20 min to block cyclooxygenase activity. All washing procedures were performed at 37°C in the presence of apyrase (1 U/ml) as ADP scavenger. Label-

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Abbreviations: PLC, phospholipase C; PLD, phospholipase D; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3′-phosphate; PtdIns(4)P, phosphatidylinositol 4′-phosphate; PtdIns(3,4)P_2, phosphatidylinositol 3′,4′-bisphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4′,5′-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3′,4′,5′-trisphosphate; PtdIns 3-kinase, phosphatidylinositol 3-kinase; PtdOH, phosphatidic acid; TXA_2, thromboxane A_2

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ing of platelets was performed for 90 min with 0.4 mCi/ml [γ-^{32}P]phosphate (Amersham, Ltd. Bucks, UK) as previously described [19]. Platelets were finally resuspended in modified Tyrode's buffer (pH 7.4) containing 2.5 mM $CaCl_2$.

2.3. Cell adhesion assay

Cell culture flasks (75 cm^2, CEB, Angers, France) were precoated for 16 h at 4°C with 100 μg/ml of fibrinogen within 20 ml of Tyrode's buffer without BSA. Control flasks were treated according to the same protocol but without adhesive protein. After three washes with Tyrode's buffer without BSA, fibrinogen-coated and control flasks were blocked for 3 h at 4°C with fatty acid free-BSA (10 mg/ml; 20 ml) in order to decrease non-specific platelet adhesion to polystyrene. Prior to the adhesion assay, flasks were washed three times with Tyrode's buffer without BSA.

The cell adhesion assay was performed using 10 ml of human platelets (3×10^7 platelets/ml) that were added for 5–30 min at 37°C to the fibrinogen-coated or control flasks. 6–24 flasks were used to recover sufficient numbers of platelets allowing measurement of the *D*3-phosphorylated phosphoinositides by HPLC. In some experiments, the ADP scavenger pyruvate kinase plus phosphoenolpyruvate (14.3 U/ml and 1 mM, respectively) was added to the platelet suspension for 10 min before adhesion. At the end of the experiment, non-adherent platelets were removed by aspiration. Flasks were then washed twice with PBS. The extent of cell adhesion was evaluated both by counting the remaining non-adherent platelets and by comparison of the total radioactivity incorporated into the lipid fraction from non-adherent and adherent platelets.

2.4. Lipid extraction and analysis

Adherent platelets were rapidly scraped off in the presence of CH_3OH/EDTA (8 mM) and immediately transferred into $CHCl_3$. Lipids from adherent and non-adherent cells were extracted as previously described [19]. Lipids were then deacylated by methylamine treatment, and separated by HPLC on a Partisphere SAX column (Whatman International Ltd., UK) using an elution gradient of 0–1 M ammonium phosphate as previously described [19]. Radioactivity was detected on line with a Berthold LB506C radioactivity monitor.

3. Results

3.1. Stimulation of PLC and PtdIns 3-kinase activities upon platelet adhesion to a fibrinogen matrix

As previously reported [1,20], we found that unstimulated platelets adhered to, and spread on a fibrinogen-coated polystyrene surface. In our experiments, platelets adhered primarily as single cells without forming aggregates. No platelet adhesion to control flasks was observed. Adhesion of platelets to fibrinogen-coated flasks was time-dependent, increasing from 0.5×10^7 to 2×10^7 and 3×10^7 platelets/flask after 5, 15, and 30 min of incubation, respectively. After 15 min of incubation, an increase in [^{32}P]phosphatidic acid (PtdOH) (288 ± 71%), and a decrease in [^{32}P]PtdIns(4,5)P_2 (39 ± 5%) in adherent platelets were observed (Fig. 1). As shown in Fig. 2A, maximal increase in [^{32}P]PtdOH production was observed as early as 5 min of adhesion. At the same time, maximal decrease in

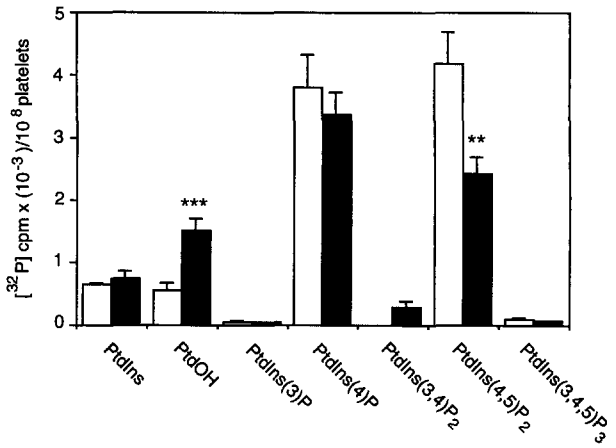

Fig. 1. Effect of platelet adhesion to immobilized fibrinogen on ^{32}P labeling of various phosphoinositides. Platelets were incubated for 15 min in flasks coated with fibrinogen or albumin (control). Adherent platelets from fibrinogen-coated flasks (solid bars) and non-adherent ones from control flasks (open bars) were recovered and their lipids extracted as described in Section 2. ^{32}P radioactivity incorporated into various phosphoinositides is expressed in cpm from 10^8 platelets and data are means ± S.E.M. from 9 independent experiments. Radioactivity of [^{32}P]PtdIns(3,4)P_2 was undetectable in samples of non-adherent platelets and is not represented. Significance of adhesion-induced variations of platelet PtdOH and PtdIns(4,5)P_2 was calculated with the Student's *t*-test: ***$p < 0.001$; **$p < 0.01$.

[^{32}P]PtdIns(4,5)P_2 was measured (Fig. 2A). Thereafter, these two metabolites returned gradually to a new plateau. Thus, our results suggest that a phospholipase active on PtdIns(4,5)P_2 is rapidly and transiently stimulated in adherent platelets. PtdOH could come either from a PLC/diglyceride kinase or a PLD. Transphosphatidylation is an exclusive property of PLD that produces phosphatidylethanol (PEt) instead of PtdOH in the presence of ethanol. We assessed the formation of [^{32}P]PEt within labeled platelets added to the fibrinogen-coated flask in the presence of 0.5% ethanol. After lipid extraction, separation by TLC and autoradiography, [^{32}P]PEt was not detected in adherent platelets (data not shown). Thus, our results indicate the activation of a PLC promoted by platelet adhesion to immobilized fibrinogen. We also observed that [^{32}P]PtdIns(3,4)P_2, which was undetectable in control platelets, was synthesized in adherent platelets (Fig. 1). Synthesis of this phosphoinositide 3-kinase (PtdIns 3-kinase) product was time-dependent (Fig. 2B) and represented an average of 10% of total the [^{32}P]PtdInsP_2 level (PtdIns(3,4)P_2+PtdIns(4,5)P_2). Beside the variations in PtdOH and PtdIns(3,4)P_2, no significant change of ^{32}P incorporation into PtdIns, PtdIns(3)P, PtdIns(4)P and PtdIns(3,4,5)P_3 was observed in adherent platelets (Fig. 1).

Table 1
Consequences of platelet treatment with ADP scavenger or cyclooxygenase inhibitor on [^{32}P]PtdOH and [^{32}P]PtdIns(3,4)P_2 production upon adhesion

	[^{32}P]PtdOH (cpm)		[^{32}P]PtdIns (3,4)P_2 (cpm)	
	Control	Adherent cells	Control	Adherent cells
No treatment ($n=5$)	844 ± 273	2048 ± 641	N.D.	333 ± 141
PK-PEP ($n=3$)	1409 ± 69	3364 ± 624	N.D.	traces
Aspirin ($n=2$)	700 ± 51	2450 ± 362	N.D.	358 ± 30

Treatment of platelets and measurement of [^{32}P]PtdOH and [^{32}P]PtdIns(3,4)P_2 were performed as described in Section 2. cpm of [^{32}P]PtdOH and [^{32}P]PtdIns(3,4)P_2 are from 10^8 platelets (mean ± S.E.M.) after 30 min of adhesion to a fibrinogen matrix. In the control platelets, [^{32}P]PtdIns(3,4)P_2 was not detectable (N.D.) under our experimental conditions. The number of experiments (n) is indicated on the table.

3.2. ADP is involved in adhesion-dependent PtdIns 3-kinase activation

ADP present in dense granules and cyclooxygenase products (TXA_2) released after platelet activation are platelet agonists. Since Haimovich et al. [18] have suggested that small amounts of ADP could be released by adherent platelets, we performed a platelet adhesion assay in the presence of pyruvate kinase plus phosphoenolpyruvate as an ADP scavenger system. The number of adherent platelets recovered on the fibrinogen matrix was not significantly changed by this treatment. Whilst the [^{32}P]PtdOH increase triggered by adhesion was unchanged, the production of [^{32}P]PtdIns(3,4)P_2 was hardly measurable in adherent platelets that had been treated with ADP scavenger (Table 1). Moreover, the addition of ADP (20 μM) to adherent platelets after removing the ADP scavenger resulted in the recovery of [^{32}P]PtdIns(3,4)P_2 production: in two independent experiments the [^{32}P]PtdIns(3,4)P_2 level increased from a trace amount to 144 and 740 cpm, respectively, for 10^8 adherent platelets after 30 min of treatment by ADP. As a control, adherent platelets that were not incubated with ADP did not produce PtdIns(3,4)P_2. On the other hand, treatment of platelets with aspirin in order to inhibit cyclooxygenase activity and TXA2 production modified neither PtdOH nor PtdIns(3,4)P_2 production upon platelet adhesion (Table 1). These results suggest that ADP but not TXA_2 is involved in adhesion-dependent PtdIns(3,4)P_2 synthesis.

4. Discussion

The aim of this study was to determine modifications of phosphoinositide metabolism promoted by direct $\alpha_{IIb}\beta_3$-integrin/fibrinogen interaction without previous activation of platelets by other agonists. Since $\alpha_{IIb}\beta_3$-integrin in resting platelets is able to recognize immobilized fibrinogen, we used a platelet adhesion assay on fibrinogen-coated flask to measure ^{32}P incorporation into phosphoinositides.

We showed for the first time that a PLC could be activated secondary to platelet adhesion to a fibrinogen matrix. Indeed, we measured the synthesis of PtdOH concomitantly with the decrease in PtdIns(4,5)P_2 in platelets adherent on a fibrinogen matrix. Moreover, we did not detect PEt as a consequence of PLD activation in adherent platelets. These results, together with the facts that PtdOH is the most sensitive indicator of PLC activity [21,22] and that PLD-dependent PtdOH formation is negligible in fully activated platelets [23], are in favor of PLC activation dependent on $\alpha_{IIb}\beta_3$-integrin engagement. Other integrins have been shown to regulate PLC activity: PLC-γ1 and G-protein coupled PLC in LFA-1 and $\alpha_v\beta_3$-integrin-dependent signaling, respectively [24,25]. We are currently performing experiments to identify the PLC isoform involved in our model.

The second signal evoked upon platelet adhesion to a fibrinogen matrix is PtdIns 3-kinase activation. Indeed, we have measured in adherent platelets an increase in PtdIns(3,4)P_2. Even though PtdIns(3,4)P_2 synthesis in platelets could be under the possible control of three enzymes (PtdIns(4)P 3-kinase, PtdIns(3)P 4-kinase, $PtdInsP_3$ 5-phosphatase), in all cases a PtdIns 3-kinase must be stimulated. This result is of importance as we have demonstrated that the physiological integrin/ligand interaction is able to stimulate PtdIns 3-kinase without previous cell activation by other agonists. In agreement with our results, Kovacsovics et al. [14], using anti-$\alpha_{IIb}\beta_3$ monoclonal antibodies, have recently shown that PtdIns 3-kinase activation is involved in the signal transduction pathway from activated $\alpha_{IIb}\beta_3$-integrins.

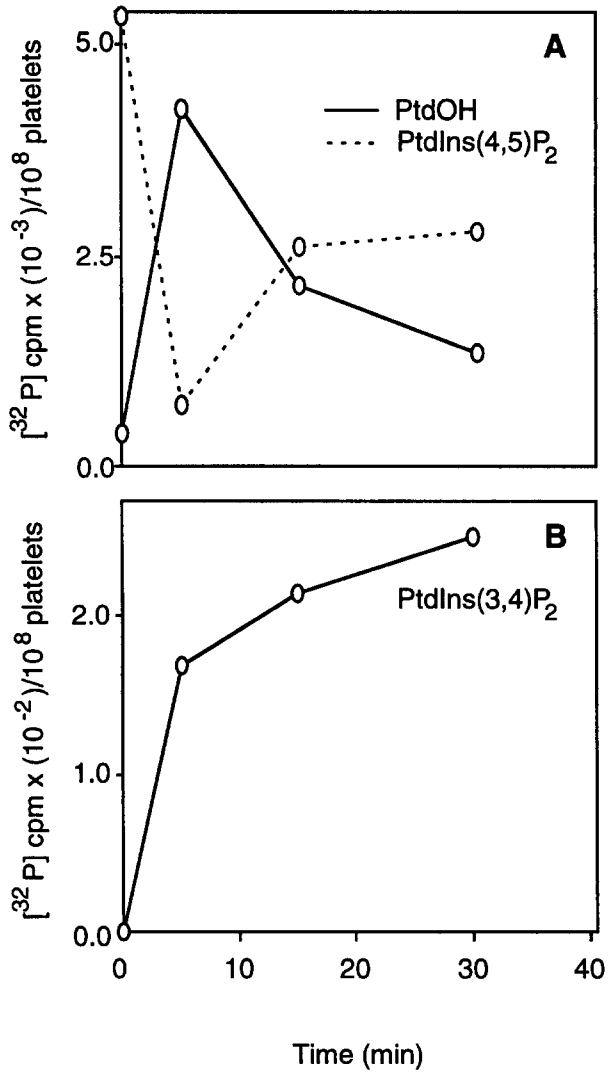

Fig. 2. Time course of ^{32}P-labeled PtdOH, PtdIns(4,5)P_2 (A) and PtdIns(3,4)P_2 (B) upon platelet adhesion to a fibrinogen matrix. Platelets were allowed to adhere to a fibrinogen matrix for the indicated times and then lipids were extracted as described in Section 2. ^{32}P radioactivity of phospholipids is expressed in cpm from 10^8 platelets. Data are from one experiment representative of three that gave similar results.

We have demonstrated here that ADP is involved in the major part of the PtdIns 3-kinase activation in adherent platelets to a fibrinogen matrix. Haimovich et al. [18,26] have shown that ADP, and a protein kinase C activated downstream, are involved in p125^{FAK} tyrosine phosphorylation upon platelet adhesion to a fibrinogen matrix. p125^{FAK}, a protein-tyrosine kinase found in focal adhesion sites and activated after $\alpha_{IIb}\beta_3$/fibrinogen binding [27], coimmunoprecipitates with PtdIns 3-kinase in thrombin-stimulated platelets and binds in vitro to the SH3 domain of the regulatory subunit p85α of the PtdIns 3-kinase [28]. This association is sufficient to stimulate PtdIns 3-kinase activity [28]. Thus, upon platelet adhesion to a fibrinogen matrix, the ADP-dependent

PtdIns 3-kinase activity could be regulated by $p125^{FAK}$, or, the PtdIns 3-kinase products could be necessary for the activation of the PKC involved in $p125^{FAK}$ stimulation.

The results of this study delineate an activation pathway after adhesion of unstimulated platelets which could be of relevance in physiological hemostasis as well as cardiovascular diseases, and offers an excellent model to study the relationship between phosphoinositide metabolism and platelet morphological changes occurring in these processes.

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