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We have shown previously that ADP released upon platelet adhesion mediated by $\alpha_{\text{IIb}}\beta_3$ integrin triggers accumulation of phosphatidylinositol 3′,4′-bisphosphate (PtdIns-3,4-P_2) (Gironcel, D., Racaud-Sultan, C., Payrastre, B., Haricot, M., Borchert, G., Kieffer, N., Breton, M., and Chap, H. (1996) *FEBS Lett.* 389, 253–256). ADP has also been involved in platelet spreading. Therefore, in order to study a possible role of phosphoinositide 3-kinase in platelet morphological changes following adhesion, human platelets were pretreated with specific phosphoinositide 3-kinase inhibitors LY294002 and wortmannin. Under conditions where PtdIns-3,4-P_2 synthesis was totally inhibited (25 μM LY294002 or 100 nM wortmannin), platelets adhered to the fibrinogen matrix, extended pseudopodia, but did not spread. Moreover, addition of ADP to the medium did not reverse the inhibitory effects of phosphoinositide 3-kinase inhibitors on platelet spreading. Although synthetic dipalmitoyl PtdIns-3,4-P_2 and dipalmitoyl phosphatidylinositol 3′,4′,5′-trisphosphate restored only partially platelet spreading, phosphatidylinositol 4′,5′-bisphosphate (PtdIns-4,5-P_2) was able to trigger full spreading of wortmannin-treated adherent platelets. Following ^{32}P labeling of intact platelets, the recovery of [^{32}P]PtdIns-4,5-P_2 in anti-talin immunoprecipitates from adherent platelets was found to be decreased upon treatment by wortmannin. These results suggest that the lipid products of phosphoinositide 3-kinase are required but not sufficient for ADP-induced spreading of adherent platelets and that PtdIns-4,5-P_2 could be a downstream messenger of this signaling pathway.

Platelets play a key role in hemostasis by their capacities to adhere and to aggregate in response to vascular injury. The most abundant platelet integrin, the $\alpha_{\text{IIb}}\beta_3$ complex, is largely responsible for platelet aggregation after binding of soluble fibrinogen. Moreover, $\alpha_{\text{IIb}}\beta_3$ integrin is required for a complete and irreversible platelet adhesion to the subendothelial matrix (1). In this case, its preferential ligand is the von Willebrand

factor, but under certain conditions fibrinogen or fibrin could also act as adhesion substrates. In its resting state, $\alpha_{\text{IIb}}\beta_3$ integrin is able to recognize immobilized fibrinogen. However, the interaction of soluble fibrinogen with $\alpha_{\text{IIb}}\beta_3$ complex requires a previous conformational change of the integrin due to an inside-out signaling pathway. When platelets adhere *in vitro* to a fibrinogen matrix, they undergo several irreversible morphological changes such as rounding and spreading. These responses are sustained by a cytoskeletal reorganization including extension of filopodia, lamellipodia, and controlled orientation of stress fibers. It has been shown that a concomitant granular secretion of ADP from adherent platelets was necessary for spreading (2), and that it controlled specific signals, *i.e.* p125[FAK] and PtdIns 3-kinase[1] activations (2, 3). Data from Haimovich *et al.* (4) show that tyrosine phosphorylation of p125[FAK] tyrosine kinase seems to be correlated with cell spreading upon platelet adhesion to a fibrinogen matrix. On the other hand, PtdIns 3-kinase activity has been involved in cytoskeletal rearrangements occurring during cell motility or platelet aggregation (5–9). Moreover, studies in whole cells have demonstrated an association of PtdIns 3-kinase with p125[FAK] (10, 11) and the small G proteins Rac and Cdc42 (12), all of them being involved in the regulation of cytoskeleton organization. Taking advantage of specific PtdIns 3-kinase inhibitors, LY294002 and wortmannin (8, 13, 14), we herein demonstrate that PtdIns 3-kinase is involved in the ADP-signaling pathway that controls platelet spreading. Nevertheless, our results suggest that PtdIns-4,5-P_2, a phospholipid tightly associated with actin-binding proteins in focal contacts and a key regulator of actin polymerization (15), could be a downstream messenger of this signaling pathway.

EXPERIMENTAL PROCEDURES

Materials—Human fibrinogen, ADP, human thrombin, phorbol 12-myristate 13-acetate (PMA), apyrase, pyruvate kinase, phosphoenolpyruvate, wortmannin, PtdIns-4-P, PtdIns-4,5-P_2, fatty acid-free bovine serum albumin, and phosphate-buffered saline (PBS) were from Sigma. Lysophosphatidic acid (LPA), the thrombospondin-1 cell binding do-

[1] The abbreviations used are: PtdIns 3-kinase, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; 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; PtdOH, phosphatidic acid; LPA, lysophosphatidic acid; PLC, phosphatidylinositol-specific phospholipase C; PKC, protein kinase C; MLCK, myosin light chain kinase; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; TXA$_2$, thromboxane A$_2$; Di-C$_{16}$, dipalmitoyl.

main peptide H-RFYVVMWK-OH, and the TXA$_2$ analog U46619 used were, respectively, from Sigma, Bachem (Voisins-le Bretonneux, France), and Calbiochem (Meudon, France). LY294002 and GF109203X were obtained, respectively, from Biomol (Plymouth Meeting, PA) and Glaxo (Les Ulis, France). Synthetic Di-C$_{16}$-PtdIns-3,4-P$_2$ (dipalmitoyl L-α-phosphatidyl-D-*myo*-inositol 3, 4-bisphosphate) and Di-C$_{16}$-PtdIns-3,4,5-P$_3$ were purchased from Matreya (Pleasant Gap, PA).

Preparation of Platelets—Human platelets were isolated from fresh platelet concentrates (Centre Régional de Transfusion Sanguine, Toulouse, France) by centrifugation as described previously (16). All washing procedures were performed at 37 °C in the presence of apyrase (1 unit/ml) as an ADP scavenger. In some experiments, platelet-rich plasma was incubated with 100 μM aspirin for 20 min to block cyclooxygenase activity. Platelets were labeled for 90 min with 0.4 mCi/ml [γ-^{32}P]phosphate (Amersham Pharmacia Biotech, Bucks, United Kingdom), as described previously (16). They were finally resuspended in modified Tyrode's buffer (pH 7.4) containing 2.5 mM CaCl$_2$.

Cell Adhesion Assays and Lipid Extract Analysis—Cell culture flasks (75 cm^2, Greiner Labortechnik, Poitiers, France) were precoated or not (control) with 100 μg/ml of fibrinogen and were then blocked with fatty acid-free bovine serum albumin (3). The cell adhesion assay was performed using 5 ml of human platelets (3 $\times$ 10^7 platelets/ml) that were added for 60 min at 37 °C to the fibrinogen-coated flasks or to the control flasks. In some experiments, the ADP scavenger pyruvate kinase plus phosphoenolpyruvate (14.3 units/ml and 1 mM, respectively; 10 min) or the protein kinase C (PKC) inhibitor GF109203X (12 μM; 60 min) or the PtdIns 3-kinase inhibitors LY294002 (0–25 μM; 10 min) or wortmannin (0–100 nM; 15 min) were added to the platelet suspension before adhesion. GF109203X, wortmannin, and LY294002 were dissolved in Me$_2$SO, which did not exceed 0.06% (v/v). Recovering of adherent cells, evaluation of the extent of cell adhesion, and lipid extract analysis by HPLC were performed as described previously (3).

Spreading Restoration Assays—After elimination of unattached platelets and two washes with PBS, adherent wortmannin-treated platelets were incubated with different agonists (20 μM ADP, 5 μM LPA, 50 μM H-RFYVVMWK-OH, 5 μM U46619, 10 nM PMA, or 1 unit/ml thrombin) or phosphoinositides (PtdIns-4-P, PtdIns-4,5-P$_2$, Di-C$_{16}$-PtdIns-3,4-P$_2$, Di-C$_{16}$-PtdIns-3,4,5-P$_3$, or a mixture of these lipids, 10–30 μM) in Tyrode's buffer for 30 min at 37 °C. Before use, phosphoinositides were dried, suspended in 10 mM Hepes (pH 7.0), and sonicated in the absence of carrier phospholipids.

Optical Microscopy—At the end of the adhesion step or the spreading restoration assay, unattached platelets were removed by washing with PBS and the buffer was replaced by 1% glutaraldehyde in 0.1 M Na$_2$HPO$_4$. Fixation was continued at room temperature for 15 min. After washing, adherent platelets were examined by interference light microscopy with a Reichert EMF4 microscope. Micrographs were taken at original magnification $\times$1250.

Immunoprecipitation of Talin—Adherent platelets (4.5 $\times$ 10^8 platelets) were scraped off at 4 °C in a lysis buffer containing 20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 10 μM pepstatin, 10 μg/ml leupeptin, and 1% (v/v) Triton X-100. Resting platelets in suspension (4.5 $\times$ 10^8 platelets) were centrifuged and resuspended in 600 μl of the lysis buffer. After sonication (20 kHz for 2 $\times$ 10 s) and centrifugation (12,000 $\times$ g for 10 min at 4 °C), the soluble fraction was collected and subsequently precleared for 30 min at 4 °C with protein G-Sepharose 4B fast flow (Sigma). Precleared suspensions were then incubated overnight at 4 °C with the polyclonal anti-talin antibody prepared as described previously at a 1:50 dilution (17). Capture of immune complex was performed by adding 50 μl of protein G-Sepharose 4B fast flow. The immunoprecipitates were then washed once with PBS without calcium and magnesium, supplemented with anti-proteases as described above and 0.1% (v/v) Triton X-100, and twice with the same buffer without Triton.

Lipid Extraction and Western Blotting on Talin Immunoprecipitates—For lipid analysis, immunoprecipitation of talin was performed after plating of ^{32}P-labeled platelets as described above. Lipids were extracted as described previously (3) and separated by TLC following the procedure established by Pignataro and Ascoli (18). Briefly, lipid extracts were applied on oxalate-EDTA-impregnated silica gel plates, which were developed twice for 120 min with CHCl$_3$, CH$_3$OH, 9.15 M NH$_4$OH (40:40:15). Individual lanes containing commercial standards PtdIns-4-P or PtdIns-4,5-P$_2$ were stained with iodine vapors. After exposition of the plates for 3–7 days, the radioactive spots were visualized and quantitated by a PhosphorImager 445 SI (Molecular Dynamics, Inc). Quantification was also performed after scraping the appropriate areas of the plate and counting in a liquid scintillation counter.

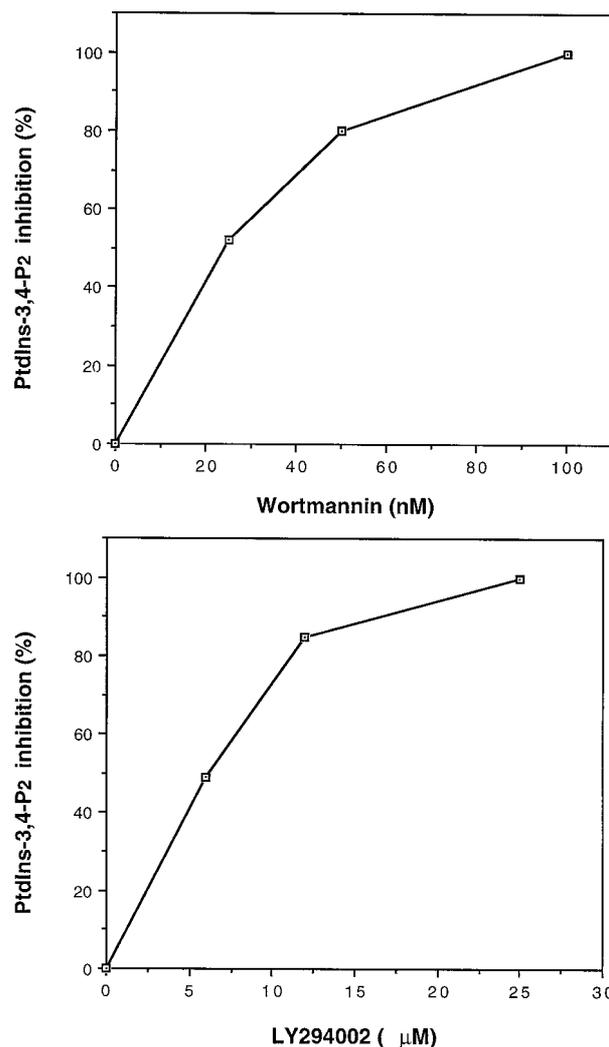For protein analysis, anti-talin immunoprecipitates were solubilized,

FIG. 1. **Dose-dependent inhibition of adhesion-induced [^{32}P]-PtdIns-3,4-P$_2$ synthesis by wortmannin and LY294002.** Washed platelets were pretreated with wortmannin (15 min) or LY294002 (10 min) at different concentrations and then were plated on the fibrinogen matrix for 60 min. After washing, adherent cells were scraped off and their lipid extract was analyzed by HPLC after deacylation. [^{32}P]PtdIns-3,4-P$_2$ was quantified as described under "Experimental Procedures."

separated on 7.5% SDS-polyacrylamide gels, and blotted onto nitrocellulose as described previously (11). Immunodetection of talin was performed with the mouse monoclonal anti-talin antibody 8d4 from Sigma. Antibody reaction was visualized using the ECL chemiluminescence system (Amersham Pharmacia Biotech). Quantification of the different bands was performed by a densitometric analysis, which determines the pixel volume in each area (Gel Doc 1000, Bio-Rad).

RESULTS

Wortmannin and LY294002 Inhibit PtdIns-3,4-P$_2$ Synthesis Triggered upon Platelet Adhesion—Wortmannin and LY294002 have been largely used in platelets as specific inhibitors of PtdIns 3-kinase, at nanomolar (10–100 nM) and micromolar (25 μM) concentrations, respectively (8, 9, 19, 20). Since we have shown previously that platelet adhesion triggers accumulation of [^{32}P]PtdIns-3,4-P$_2$ (3), we first assessed the inhibitory effects of wortmannin and LY294002 on the production of [^{32}P]PtdIns-3,4-P$_2$ as a reflection of PtdIns 3-kinase activation. ^{32}P-Labeled platelets were preincubated with increasing doses of wortmannin or LY294002, and cells were then plated for 60 min on the fibrinogen matrix before lipid extraction and analysis by HPLC. As shown in Fig. 1, LY294002 and wortmannin inhibited [^{32}P]PtdIns-3,4-P$_2$ synthesis in a dose-de-

pendent manner with 80% inhibition achieved at 12 μM and 50 nM, respectively. The production of [^{32}P]PtdIns-3,4-P$_2$ was totally abrogated at 25 μM LY294002 and 100 nM wortmannin, at which concentrations platelet adhesion was not significantly affected (Table I). At these concentrations, among other phosphoinositides (PtdIns, PtdOH, PtdIns-4-P, and PtdIns-4,5-P$_2$), only the PtdIns-4-P level was found to be somewhat decreased, but not significantly in comparison with control Me$_2$SO-treated platelets (Fig. 2).

PtdIns 3-Kinase Activation Is Necessary for ADP-dependent Platelet Spreading—As described previously (21), upon adhesion, adherent platelets undergo the following steps of morphological changes: disk to sphere shape change, extension of pseudopodia, and a much slower process, cell spreading (Fig. 3, *A* and *B*). Pretreatment of platelets with wortmannin or LY294002 inhibited platelet spreading on fibrinogen (Fig. 3, *C* and *D*). However, it should be noted that, although pretreated platelets did not fully spread, they still extended pseudopodia. The inhibitory effect of LY294002 and wortmannin on cell spreading was already detectable at 6 μM and 25 nM, respectively. Concentrations of 25 μM LY294002 and 100 nM wortmannin completely prevented platelet spreading. After removing wortmannin and LY294002 from the adhesion medium by two washes, we observed that only the inhibitory effect of LY294002 was reversible after 30 min (data not shown). Indeed, LY294002 is a competitive inhibitor at the ATP-binding site of PtdIns 3-kinase (13), whereas wortmannin induces a

TABLE I
Effects of wortmannin and LY294002 on platelet adhesion

Platelets were incubated in absence (A) or presence of Me$_2$SO (0.03%; 15 min), wortmannin (100 nM; 15 min) or LY294002 (25 μM; 10 min) and plated on the fibrinogen matrix for 60 min. Calculation of adhesion percentage was as described under "Experimental Procedures." Data are from five independent experiments.

	A	Me$_2$SO	LY294002	Wortmannin
Adhesion ± S.E. (%)	25 ± 5	29 ± 11	20 ± 5	19 ± 5

covalent modification of the catalytic site of the enzyme (14). In agreement with Haimovich *et al.* (2, 4), pretreatment of platelets with the ADP scavenger pyruvate kinase plus phosphoenolpyruvate just before the adhesion assay induced the same effects as treatment with the PtdIns 3-kinase inhibitors, *i.e.* absence of spreading but persistence of pseudopodal extension (Fig. 3*E*). After washing, ADP (20 μM) was added to adherent platelets to overcome the ADP scavenging system. Addition of ADP restored full spreading of all adherent platelets as shown in Fig. 3*F*. On the other hand, addition of 20 μM ADP to adherent platelets pretreated with wortmannin did not reverse inhibition of platelet spreading (Fig. 3*G*). These data demonstrate that PtdIns 3-kinase signaling pathway is required for ADP-induced spreading of adherent platelets.

PtdIns-4,5-P$_2$ but Not Lipid Products of PtdIns 3-Kinase Is Sufficient for a Full Platelet Spreading—Our previous measurements of PtdIns 3-kinase activation in adherent platelets (3) have shown that, although the PtdIns-3,4,5-P$_3$ level was not significantly modified between 5 and 30 min of adhesion, PtdIns-3,4-P$_2$ accumulated as a function of the adhesion time. Time course of PtdIns-3,4-P$_2$ production closely paralleled platelet spreading upon adhesion (data not shown). In order to determine whether products of PtdIns 3-kinase could be involved in platelet spreading, we used Di-C$_{16}$-PtdIns-3,4-P$_2$ and Di-C$_{16}$-PtdIns-3,4,5-P$_3$, which were reported to trigger biologic responses when added to whole cells (7, 22). As shown in Fig. 4*A*, addition of Di-C$_{16}$-PtdIns-3,4-P$_2$ (20 μM) on adherent platelets pretreated with wortmannin only partially restored platelet spreading. After 30 min of incubation with Di-C$_{16}$-PtdIns-3,4-P$_2$, some adherent platelets have lost their round shape and have undergone pseudopodal and hyalomere extension. Nevertheless, these modifications concerned only a small proportion of adherent platelets (5%), as compared with 35% of spread platelets obtained with non-pretreated control cells (Fig. 4*B*). Using amounts of Di-C$_{16}$-PtdIns-3,4-P$_2$ between 10 and 30 μM, we obtained a dose-dependent increase in the response rate (detected as early as 15 min of adhesion) and in the proportion
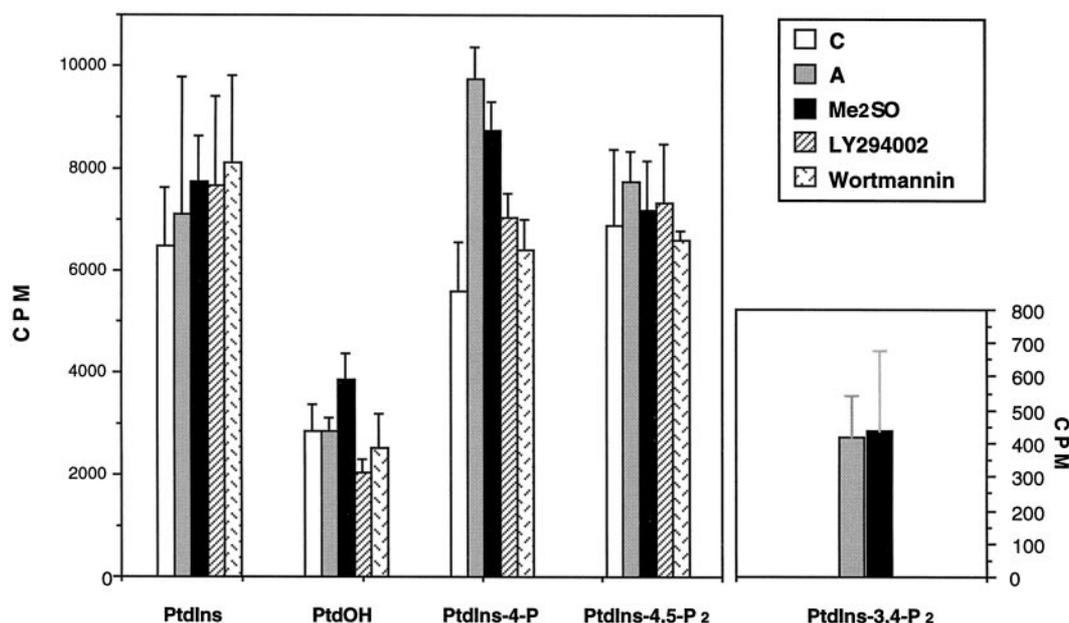

FIG. 2. **Effects of wortmannin and LY294002 on ^{32}P labeling of various phospholipids from resting and adherent platelets.** Washed platelets were pretreated in absence (*A*) or presence of wortmannin (100 nM; 15 min), LY294002 (25 μM; 10 min), or Me$_2$SO (vehicle; 0.03%; 15 min) and were plated on the fibrinogen matrix for 60 min. Control resting platelets (*C*) were added to a flask without fibrinogen. Control and adherent platelets were then recovered, and their lipid extracts were analyzed by HPLC after deacylation as described under "Experimental Procedures." ^{32}P radioactivity incorporated into various phosphoinositides is expressed in counts/min from 3×10^8 platelets, and data are means ± S.E. from three independent experiments. Radioactivity of [^{32}P]PtdIns-3,4-P$_2$ was undetectable in samples of non-adherent platelets or platelets treated with PtdIns 3-kinase inhibitors and is not represented.
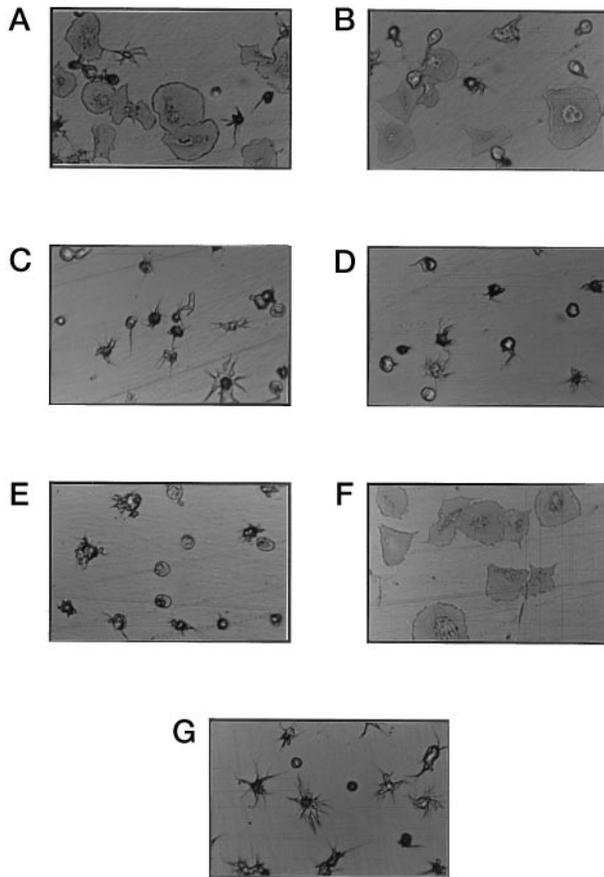
FIG. 3. **Effects of wortmannin, LY294002, and ADP on platelet spreading.** Platelets were incubated in absence (*A*) or presence of Me$_2$SO (0.03%; *B*), wortmannin (100 nM; *C* and *G*), LY294002 (25 μM; *D*) as described previously in the legend to Fig. 2. In some experiments, the ADP scavenger pyruvate kinase plus phosphoenolpyruvate was added to the platelet suspension before adhesion (*E* and *F*) as described under "Experimental Procedures." Adhesion was performed for 60 min, and after two washes, ADP (20 μM) was added to the adherent platelets for 30 min (*F* and *G*). After fixation, adherent cells were observed by interference light microscopy.

FIG. 4. **Effects of different phosphoinositides on spreading of platelets pretreated with wortmannin.** *A*, platelets were incubated in presence of 0.03% Me$_2$SO (*A*) or 100 nM wortmannin (*W*, PtdIns-3,4-P$_2$, PtdIns-4,5-P$_2$) as described previously in the legend to Fig. 2. Adhesion was performed for 60 min and after two washes, Di-C$_{16}$-PtdIns-3,4-P$_2$ or PtdIns-4,5-P$_2$ (20 μM) was added to the adherent platelets for 30 min as described under "Experimental Procedures." After fixation, adherent cells were observed by interference light microscopy. Platelets in a fully spread stage are indicated by *large arrowheads*, whereas those in a partial spread stage are indicated by *thin arrowheads*. *B*, different lipids were prepared and added to adherent platelets pretreated with wortmannin (*W*) as described under "Experimental Procedures." Adherent platelets pretreated with 0.03% Me$_2$SO (*A*) were taken as a control. The histogram was used to express the percentages of platelets in a partial spread stage (*black box*) or in a full spread stage (*hatched box*). One hundred randomly chosen platelets were counted from each experiment ($n = 2$–6), and each experiment has a paired control.

of responsive cells (Fig. 4*B*). In no case did we observe a full spreading of platelets, even after 60 min of incubation. Addition of Di-C$_{16}$-PtdIns-3,4,5-P$_3$ or PtdIns-4-P or both (data not shown) together with Di-C$_{16}$-PtdIns-3,4-P$_2$ was not more efficient in restoring full spreading (Fig. 4*B*). Surprisingly, addition of PtdIns-4,5-P$_2$ to adherent platelets pretreated with wortmannin triggered full spreading (Fig. 4, *A* and *B*). Moreover, the number of fully spread platelets was increased by addition of both Di-C$_{16}$-PtdIns-3,4-P$_2$ and PtdIns-4,5-P$_2$ (Fig. 4*B*). Nevertheless, under these conditions, the amount of fully spread platelets was far below that observed in the control situation.

PtdIns-4,5-P$_2$ effects on cell spreading are unlikely to be dependent on induction of platelet release reaction, as ADP (20 μM), LPA (5 μM), the thrombospondin-1 cell binding domain peptide (H-RFYVVMWK-OH; 50 μM), could not restore spreading of platelets treated with 100 nM wortmannin (Table II). By contrast, the TXA$_2$ analog U46619 (5 μM) triggered full spreading of adherent platelets pretreated with 100 nM wortmannin. However, addition of PtdIns-4,5-P$_2$ (20 μM) to adherent platelets pretreated with aspirin (100 μM) and wortmannin (100 nM) was still able to restore full platelet spreading (Table II).

The fact that strong platelet agonists, such as thrombin (1 unit/ml), the TXA$_2$ analog U46619, and the PKC activator PMA (10 nM), were able to restore spreading of platelets pretreated
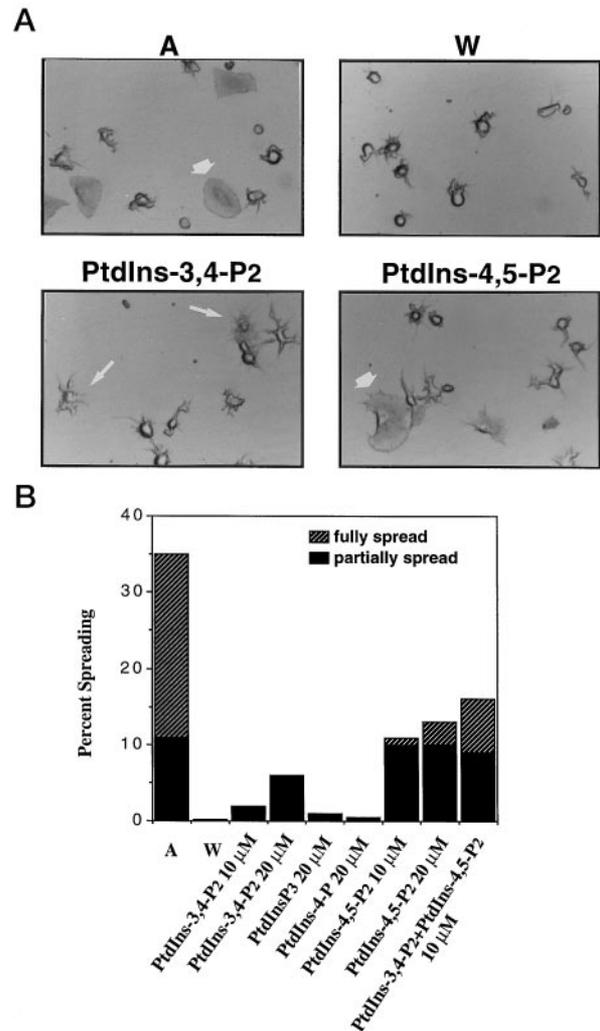
with 100 nM wortmannin (Table II) might suggest a PtdIns 3-kinase-independent pathway of platelet spreading. In order to determine a possible activation of PKC isoforms by high concentrations of PtdIns-4,5-P$_2$ (23), we used the specific PKC inhibitor GF109203X (24). Preincubation of platelets with GF109203X (12 μM) induced inhibition of platelet spreading as described previously by Haimovich and co-workers (4). When platelets had been pretreated by both GF109203X (12 μM) and wortmannin (100 nM) together, exogenous PtdIns-4,5-P$_2$ was still able to restore full spreading of adherent platelets with a similar efficiency as in the case of a pretreatment with wortmannin alone (Table II). These experiments argue in favor of a specific PtdIns-4,5-P$_2$ effect on the restoration of cell spreading that is independent on PKC activation.

TABLE II
Spreading restoration of wortmannin-treated platelets by different agonists

Platelets were incubated with wortmannin (100 nM; 15 min) and/or GF 109203X (12 μM; 60 min) and then plated on the fibrinogen matrix for 60 min. In some experiments, platelet-rich plasma was incubated with 100 μM aspirin. Spreading restoration assays were performed by addition of 20 μM ADP, 5 μM LPA, 50 μM H-RFYVVMWK-OH, 1 unit/ml thrombin (THR), 5 μM U46619, 10 nM PMA, or 20 μM PtdIns-4,5-P$_2$ as described under "Experimental Procedures." After 30 min, efficiency in full spreading restoration was observed by microscopy and was expressed as follows: percentage of adherent platelets in a full spread state (100%) (++++), 50% (++), 10% (+), or 0% (−).

Treatment	Agonist	Spreading restoration
Wortmannin	ADP	−
Wortmannin	LPA	−
Wortmannin	H-RFYVVMWK-OH	−
Wortmannin	THR	++++
Wortmannin	U46619	++
Wortmannin	PMA	++
Wortmannin	PtdIns-4,5-P$_2$	+
Wortmannin + aspirin	PtdIns-4,5-P$_2$	+
Wortmannin + GF109203X	PtdIns-4,5-P$_2$	+

The partial rescue of platelet spreading with lipids suggests that there may be additional components to the PtdIns 3-kinase-dependent pathway. We addressed the possibility that the myosin light chain kinase (MLCK) could be involved in platelet spreading. Indeed, inhibitors of MLCK have been shown to disassemble focal adhesions and to reduce their phosphotyrosine staining (25), and wortmannin is a MLCK inhibitor, but at micromolar concentrations (26). Preincubation of platelets with 10 μM ML-7 (specific inhibitor of MLCK from Biomol; Ref. 27) during the 30 min before adhesion did not modify platelet spreading (data not shown). We concluded that MLCK does not seem to be involved in platelet spreading.

In Vivo Association of PtdIns-4,5-P$_2$ with Talin Is Decreased upon Wortmannin Treatment—Since the level of [^{32}P]PtdIns-4,5-P$_2$ was not significantly modified *in vivo* upon treatment of platelets with wortmannin, we looked for possible changes of the lipid content of focal adhesion sites. Talin is a prominent actin-binding protein that links integrins and the cytoskeleton and has been shown to be required for cell spreading (28, 29). Although some variations could be observed in the amount of talin immunoprecipitated from platelets (Fig. 5*A*), mean values obtained from two experiments were essentially the same (Fig. 5*B*). Under these conditions, only traces of PtdIns-4-P were recovered in anti-talin immunoprecipitates from resting platelets, whereas, after adhesion, the amount of PtdIns-4-P increased, PtdIns-4,5-P$_2$ and traces of PtdIns-3,4-P$_2$ becoming detectable (Fig. 5*C*). For instance, the amount of talin isolated from activated platelets was 1.4- and 0.9-fold (two experiments) the amount obtained from control platelets, whereas associated phospholipids were increased by 7.5- and 11-fold, respectively. This indicated that adhesion promoted specific association of phosphoinositides with talin. Moreover, by comparison with the relative amounts of polyphosphoinositides found in total platelets, there was an enrichment in PtdIns-4-P and PtdIns-3,4-P$_2$ in the anti-talin immunoprecipitates (compare Figs. 2 and 5*D*). Treatment of platelets with wortmannin induced a strong decrease (60–100%) of these three lipids associated with talin (Fig. 5, *C* and *D*). Thus, we conclude that PtdIns 3-kinase inhibitors may modify the association of PtdIns-4,5-P$_2$ with talin and therefore influence its possible function in platelet spreading.

DISCUSSION

We have shown previously that synthesis of PtdIns-3,4-P$_2$ in adherent platelets is under the control of the released ADP, since addition of ADP reversed the inhibitory effects of an ADP
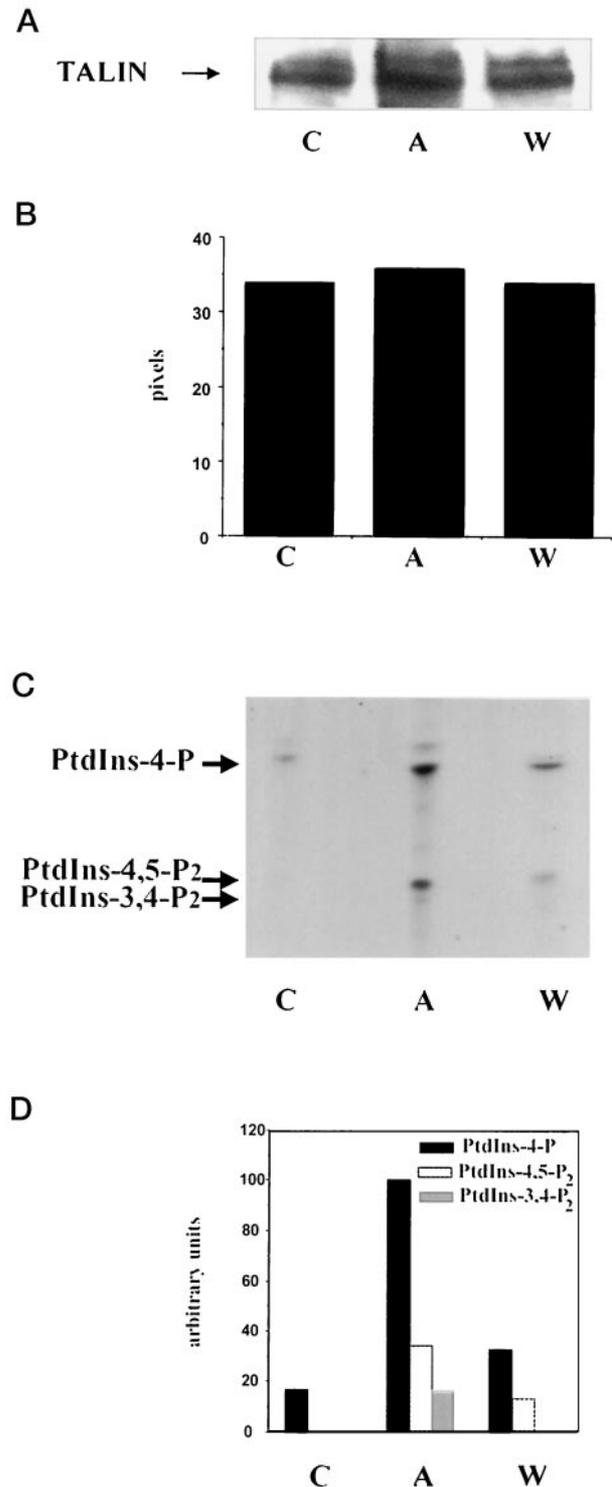

FIG. 5. **Effects of wortmannin treatment on association of polyphosphoinositides with talin *in vivo*.** Platelets were labeled with [^{32}P]orthophosphate (*C* and *D*) or not (*A* and *B*) and plated on the fibrinogen matrix (*A*, *W*) or kept in suspension (*C*) for 60 min as described in the experimental procedure. In some cases, platelets were pretreated with 100 nM wortmannin before plating (*W*). There was no detectable difference in the amount of lipid-incorporated ^{32}P between the adherent and suspended samples. Immunoprecipitation of talin from 4.5×10^8 platelets was performed with a polyclonal anti-talin antibody followed by Western blotting (*A*), and lipids were extracted and separated by TLC (*C*) as described under "Experimental Procedures." No lipid was precipitated by protein G-Sepharose alone. Quantification of the amounts of talin immunoprecipitated was performed by a densitometric analysis (*B*), and the lipid content (*D*) is expressed taking the amount of PtdIns-4-P in assay *A* as 100% (74 cpm). *B* and *D* are means from two independent experiments.

scavenger on PtdIns-3,4-P_2 synthesis (3). Our present results, in agreement with those of Haimovich *et al.* (2, 4), demonstrate that ADP release occurring upon platelet adhesion is required for full platelet spreading. Thus, ADP released by adherent platelets controls both PtdIns-3,4-P_2 synthesis and cell spreading. In conditions where PtdIns-3,4-P_2 synthesis was totally abolished by inhibitors of PtdIns 3-kinase, we observed an inhibition of platelet spreading while pseudopodal extension was maintained. We demonstrate here that the PtdIns 3-kinase signaling pathway is required for ADP-induced spreading of adherent platelets.

In order to determine which lipid is responsible for platelet spreading, we have added synthetic PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 to adherent wortmannin-treated platelets. PtdIns-3,4-P_2 appears to be the most efficient in restoring partial spreading, *i.e.* pseudopodal and hyalomere extension. However, among all phosphoinositides tested, PtdIns-4,5-P_2 alone was able to trigger the full spreading of wortmannin-treated platelets. Although all phospholipid solutions were prepared under similar conditions, their packing into micelles or vesicles was not characterized. Moreover, differences in the acyl chains of PtdIns-3,4-P_2 (palmitate) and PtdIns-4,5-P_2 (stearate and arachidonate) may have influenced their activity. Nevertheless, our results suggest a role for both PtdIns-4,5-P_2 and lipid products of PtdIns 3-kinase in the signal transduction pathway leading to platelet spreading.

We have observed a trend toward a decrease of [^{32}P]PtdIns-4-P in adherent platelets pretreated with PtdIns 3-kinase inhibitors. This result suggests that a wortmannin-sensitive PtdIns 4-kinase might exist in platelets, as has been shown in other models (30, 31). Nevertheless, in a cell system, wortmannin was reported to inhibit PtdIns 4-kinase at μM concentration, and LY294002 has not been described as an inhibitor of known PtdIns 4-kinases (13). Thus, PtdIns 3-kinase activity could be upstream of a PtdIns 4-kinase and/or a PtdIns-4-P phosphatase. Finally, even though we have not measured a significant decrease of [^{32}P]PtdIns-4,5-P_2 in whole platelets pretreated with PtdIns 3-kinase inhibitors, a decrease of PtdIns-4-P level could impair the synthesis of a particular pool of PtdIns-4,5-P_2 required for platelet spreading.

Recent studies from the Schlessinger and Rhee laboratories (32, 33) demonstrate that γ isoforms of phospholipase C (PLC) could be activated by PtdIns-3,4,5-P_3, either by targeting to cell membrane through their PH domain or by direct activation through their SH2 domain. One could thus expect an increase of PtdIns-4,5-P_2 level when adherent platelets have been pretreated with PtdIns 3-kinase inhibitors. Nevertheless, at least two reasons could explain why in our experiments this variation is not observed. First, in our previous paper (3), we have shown that upon platelet adhesion on a fibrinogen matrix a PLC active on PtdIns-4,5-P_2 was rapidly and transiently stimulated. Maximal increase of PtdOH production and PtdIns-4,5-P_2 decrease was observed as early as 5 min of adhesion. Thereafter, these two metabolites returned gradually to their basal level, and that corroborates the absence of PtdOH and PtdIns-4,5-P_2 variations after 60 min of adhesion, as shown in Fig. 2 of our present article. We thus believe that in the late steps of platelet adhesion PLC activity is not involved. However, it should be of importance to check PLC activity during the early steps of adhesion of platelets treated with PtdIns 3-kinase inhibitors. Second, since our present data show a decrease of PtdIns-4-P level upon platelet treatment with PtdIns 3-kinase inhibitors, an eventual increase of the PtdIns-4,5-P_2 level might be impaired.

PtdIns-4,5-P_2 regulates several actin-binding proteins as profilin, gelsolin, α-actinin, and vinculin (34). One of the major proteins of focal adhesions, talin, has been shown to be involved in cell spreading (28, 29). Its interaction with lipids has been documented *in vitro* and could be of importance for talin nucleated actin polymerization (34). Here, we show that PtdIns-4,5-P_2 as well as PtdIns-4-P and PtdIns-3,4-P_2 become associated with talin upon platelet adhesion. Moreover, treatment of platelets by wortmannin strongly reduces the amounts of polyphosphoinositides recovered in the anti-talin immunoprecipitate. Even though it remains to be determined whether this association is direct or not, our results support the notion of a possible regulation by PtdIns 3-kinase of a pool of PtdIns-4,5-P_2 potentially involved in cell spreading.

Hartwig *et al.* (35) have reported that D3 and D4 polyphosphoinositides uncap F-actin in resting permeabilized platelets. At low concentrations (10 μM), PtdIns-4,5-P_2 and PtdIns-3,4-P_2 are more effective than PtdIns-3,4,5-P_3. Synthesis of PtdIns-4-P and PtdIns-4,5-P_2, which are correlated with the exposure of barbed filament ends, seem to be under the control of the small G protein Rac (35). This small G protein has been shown to regulate extension of peripheral lamellipodia (36), to associate *in vivo* with both PtdIns 3-kinase and PtdIns-4-P 5-kinase (12), and it was suggested that PtdIns 3-kinase functions upstream of Rac (37, 38). Moreover, PtdIns-4,5-P_2 and PtdIns-3,4-P_2 both regulate, *in vitro*, the severing and capping of the protein gelsolin (9), whose genetic defect is responsible for the absence of lamellae although the filopod formation is maintained, upon platelet activation (39). Thus, in our model, PtdIns 3-kinase could regulate actin remodeling directly through PtdIns-3,4-P_2 synthesis and/or indirectly through PtdIns-4,5-P_2 synthesis.

Recent results from King *et al.* (40), showing that spreading of COS 7 cells attached to fibronectin is delayed after treatment by wortmannin and LY294002, support the view that the PtdIns 3-kinase signaling pathway is required for cell spreading, as controlled by integrins and/or by tyrosine kinase receptors (41). It has been suggested that ADP released from adherent platelets supports some specific signals such as Vav phosphorylation via an indirect mechanism involving activation of $\alpha_{IIb}\beta_3$ (42). Furthermore, an integrin-associated protein agonist peptide triggers activation of $\alpha_{IIb}\beta_3$ integrin resulting in platelet spreading on immobilized fibrinogen (43). Thus, upon platelet adhesion to immobilized fibrinogen, it remains to be clarified whether platelet spreading is secondary to the $\alpha_{IIb}\beta_3$ integrin engagement.

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Lipid Products of Phosphoinositide 3-Kinase and Phosphatidylinositol 4′,5′ -Bisphosphate Are Both Required for ADP-dependent Platelet Spreading

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