Phosphatidylinositol 3,4,5-Trisphosphate Triggers Platelet Aggregation by Activating Ca^{2+} Influx†

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ABSTRACT: Exogenous phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] stimulates the aggregation of washed rabbit platelets in a Ca^{2+}- and dose-dependent manner. This aggregation is reversible at low PtdIns(3,4,5)P_3 levels, but becomes irreversible when the concentration exceeds a threshold of about 20 μM. Other D-3 and D-4 phosphoinositides examined, including phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P_2], phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], and phosphatidylinositol 3-monophosphate [PtdIns(3)P], fail to exert appreciable platelet activation at comparable concentrations. In addition, PtdIns(3,4,5)P_3 can reverse the inhibitory effect of wortmannin on thrombin-induced platelet aggregation. Taken together with the observation that PtdIns(3,4,5)P_3 is readily incorporated into cell membranes, these findings reaffirm the second messenger role of PtdIns(3,4,5)P_3 in thrombin receptor activation. The existence of a PtdIns(3,4,5)P_3-dependent Ca^{2+} entry system on platelet membranes is supported by the partial inhibition of thrombin-induced Ca^{2+} influx by wortmannin. Evidence suggests that this system differs from receptor-operated nonselective Ca^{2+} channels. However, the mechanism by which PtdIns(3,4,5)P_3 facilitates Ca^{2+} entry remains unclear. Although PtdIns(3,4,5)P_3 has been known to stimulate phospholipase C-γ (PLC-γ), internal Ca^{2+} mobilization does not play a significant role in the cytosolic Ca^{2+} increase in response to PtdIns(3,4,5)P_3 stimulation. Collectively, these data provide a putative link between PtdIns(3,4,5)P_3 and Ca^{2+} signaling, which may, in part, account for the regulatory function of PtdIns(3,4,5)P_3 during platelet aggregation. Moreover, this study bears out the notion that individual PI 3-kinase lipid products play distinct roles in the regulation of cellular functions.

The crucial involvement of phosphoinositide 3-kinase (PI 3-kinase)[1] in platelet activation was first implicated by the accumulation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in response to diverse agonist stimulations (*1*). These external stimuli include thrombin (*2*−*5*), thrombin receptor-directed ligands (*6*, *7*), thromboxane A_2 analogue (*3*), GTPγS (*3*), ADP (*8*), lysophosphatidic acid (*9*), and von Willebrand factor (vWF) (*10*). This relationship is further supported by the finding that platelet aggregation was inhibited by a potent PI 3-kinase inhibitor, wortmannin, in a dose-dependent manner (*9*, *11*). Multiple isoforms of PI 3-kinase have been identified in platelets, including p85/p110 α and β and p110 γ (*8*). All these isozymes become more localized to the cytoskeleton after platelet activation, implying that their lipid products may act in part by affecting cytoskeletal reorganization at the membrane linkage (*12*, *13*). To date, a number of signaling molecules have been identified as putative targets for PI 3-kinase lipid products (*14*). These include Ca^{2+}-independent PKC isozymes (δ, ϵ, η, and ζ) (*15*−*17*), the integrin glycoprotein (GP) IIb-IIIa (*11*), PLC-γ (*18*), protein kinase PKB/Akt (*19*−*21*), and actin-regulating proteins such as profilin (*22*) and gelsolin (*23*). Among these, GPIIb-IIIa is especially noteworthy. GPIIb-IIIa is the receptor for fibrinogen and also a critical signal transducer both inside-out and outside-in to modulate primary signals. In essence, PI 3-kinase lipid products stimulate GPIIb-IIIa through PKC activation (*23*). The activated integrin in turn signals an increase in PtdIns(3,4)P_2, without a rise in PtdIns(3,4,5)P_3 (*23*, *24*). This integrin-activated generation of PtdIns(3,4)-P_2 depends on a PtdIns(3)P 4-kinase via PtdIns(3)P, a route distinct from that mediated by PI 3-kinase (*24*). The late accumulation of PtdIns(3,4)P_2 is thought to be crucial to the maintenance of the GPIIb-IIIa receptor in its active state as well as the stimulation of filopodial actin assembly by inhibiting gelsolin function (*23*).

Substantial evidence indicates that the syntheses of PtdIns-(3,4,5)P_3 and PtdIns(3,4)P_2 are regulated via distinct mechanisms (*24*−*28*), and that these two D-3 phosphoinositides play different functional roles. For example, PtdIns(3,4)P_2

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[1] Abbreviations: PI 3-kinase, phosphoinositide 3-kinase; PLC, phospholipase C; PKC, protein kinase C; PtdIns(3)P, phosphatidylinositol 3-monophosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; Ins(1,3,4,5)-P_4, D-*myo*-inositol 1,3,4,5-tetrakisphosphate; GroPIns(3)P, glycerophosphorylinositol 3-phosphate; GroP(3,4)P_2, glycerophosphorylinositol 3,4-bisphosphate; GroP(3,4,5)P_3, glycerophosphorylinositol 3,4,5-trisphosphate; TXA_2, thromboxane A_2; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.

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is more potent in activating Ca^{2+}-independent PKC (*16*) as well as in inhibiting gelsolin function (*23*). While PKB/Akt activation is PtdIns(3,4)P_2-specific (*19*−*21*), PLC-γ is specifically activated by PtdIns(3,4,5)P_3 (*18*). Thus, to gain insight into the role of PI 3-kinase in the regulation of platelet function, it is imperative to refine the specific functions of individual D-3 phosphoinositides. To address this issue, we have examined the regulatory effect of exogenous D-3 phosphoinositides on washed rabbit platelets. We find that PtdIns(3,4,5)P_3 at micromolar concentrations triggers platelet aggregation in a dose-dependent manner, and can reverse the inhibition of thrombin-induced platelet aggregation by wortmannin. This activation is, in part, mediated through transmembrane Ca^{2+} influx, and is not noted with other D-3 or D-4 phosphoinositides examined. This PtdIns(3,4,5)P_3-induced Ca^{2+} influx is thus of potential relevance to the functional role of PI 3-kinase during platelet activation.

MATERIALS AND METHODS

Materials. 1-*O*-(1,2-Di-*O*-palmitoyl-*sn*-glycero-3-phosphoryl)-D-*myo*-inositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], 1-*O*-(1,2-di-*O*-octanoyl-*sn*-glycero-3-phosphoryl)-D-*myo*-inositol 3,4,5-trisphosphate [di-C_8-PtdIns(3,4,5)P_3], 1-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycero-3-phosphoryl)-D-*myo*-inositol 3,4-bisphosphate [PtdIns(3,4)P_2], and 1-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycero-3-phosphoryl)-D-*myo*-inositol 3-monophosphate [PtdIns(3)P] were prepared as previously reported (*29*). [1-^{3}H]PtdIns(3,4,5)P_3 and [1-^{3}H]PtdIns(3,4)P_2 were synthesized according to modified procedures for the respective D-3 phosphoinositides, which will be published elsewhere. All synthetic phosphoinositides were characterized by ^{1}H and ^{31}P NMR and FAB mass spectrometry, in which no appreciable impurity was detected. PtdIns(4,5)P_2, PtdIns(4)P, PtdIns, Fura 2-acetoxymethyl ester (Fura 2-AM), and wortmannin were products from Calbiochem. It is worth mentioning that PtdIns(4,5)P_2 from other sources often gave inconsistent results with different batches, presumably due to the presence of impurities. Ins(1,3,4,5)P_4 was synthesized according to a previously reported procedure (*30*). [^{3}H]Ins(1,3,4,5)P_4 and $^{45}CaCl_2$ were purchased from DuPont NEN. Thrombin, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), RGDS peptide, luciferin, luciferase, and acetylsalicylic acid were purchased from Sigma.

For all studies, PtdIns(3,4,5)P_3 and other phospholipids were added to platelets from stocks in DMSO with stirring. The added phospholipid formed a homogeneous suspension, and the added DMSO did not cause cell lysis or appreciable interference to platelet function.

Preparation of Washed Rabbit Platelets. Platelets were obtained from adult New Zealand White rabbits and washed using a modified method of Baenziger and Majerus (*31*). In brief, whole blood was anticoagulated by treating 9 volumes of blood with 1 volume of anticoagulant that consisted of 3.8% (w/v) trisodium citrate and 140 mM dextrose. Platelet-rich plasma was obtained by centrifugation of whole blood at 200*g* for 15 min. The platelets were pelleted by centrifugation at 1500*g* for 10 min. The platelet pellets were resuspended in a platelet washing buffer, consisting of 4.3 mM Na_2HPO_4, 24.3 mM NaH_2PO_4, 4.3 mM K_2HPO_4, pH 6.5, 113 mM NaCl, 5.5 mM glucose, and 0.5% bovine serum albumin, washed twice, and resuspended in buffer A consisting of 20 mM Tris/HCl, pH 7.0, containing 150 mM NaCl, and 5 mM glucose. Platelet concentrations were determined by using a Coulter cell counter (Coulter Electronics, Inc., FL), and were adjusted to (1−3) × 10^8 cells/mL for all experiments. These washed platelets stood at room temperature for 1−2 h before use.

Platelet Aggregation Studies. Washed platelets were added to an aggregation cuvette, and the reaction was initiated by adding the indicated amounts of PtdIns(3,4,5)P_3 or 1 unit/mL thrombin in the presence of 1 mM Ca^{2+} with stirring at 1000 rpm. Platelet aggregation was monitored on a two-channel Chrono-Log aggregometer (Chrono-Log Corp., Havertown, PA) at 37 °C. For the inhibition study, the washed platelets were preincubated with wortmannin or various chemicals for 5 min prior to the thrombin or PtdIns(3,4,5)P_3 stimulation.

Incorporation of [^{3}H]PtdIns(3,4,5)P_3 and [^{3}H]PtdIns(3,4)P_2 into Platelets. To the washed platelets in 0.7 mL of buffer A was added 40 μM PtdIns(3,4,5)P_3 containing 0.2 μCi of [^{3}H]PtdIns(3,4,5)P_3 (specific activity, 35 mCi/mmol), dissolved in 2 μL of dimethyl sulfoxide (DMSO). One hundred microliter aliquots of the mixture were taken at the indicated times, and passed through 0.45 μm nitrocellulose filters (Millipore) presoaked with the resuspension buffer on a Millipore Model 1225 sampling manifold assembly. The membranes were washed 3 times with 3 mL of the resuspension buffer, and the retained radioactivity was quantitated by liquid scintillation spectrometry. It is worth mentioning that when the incubation mixture excluded platelets, no appreciable amounts of the radioactive phospholipid were retained by the membrane filter after washing.

In a parallel experiment, incorporation of [^{3}H]PtdIns(3,4)P_2 into washed platelets was assessed under the same conditions. The specific activity of the synthetic [^{3}H]PtdIns(3,4)P_2 was 38 mCi/mmol.

Characterization of the Membrane-Incorporated [^{3}H]PtdIns(3,4,5)P_3. In the above [^{3}H]PtdIns(3,4,5)P_3 incorporation study, the composition of ^{3}H-labeled material associated with platelets was analyzed with the 20 s exposure sample. To 100 μL of the mixture were added 5 μL of 1 M EDTA and 25 μL of 5 M HCl followed by 160 μL of chloroform−methanol (1:1; v/v). After vigorous mixing, the phases were separated by centrifugation at 6000*g* for 5 min. The organic layer was dried by a stream of N_2. Lipid deacylation was carried out by using the methylamine/methanol method as described (*32*). The deacylated product was analyzed by HPLC on a 4.6 × 200 mm Adsorbosphere Sax column (5 μm), equilibrated with H_2O (solvent A) at a flow rate of 1 mL/min. A gradient with solvent B (0.5 M $NH_4H_2PO_4$, pH 3.9) was applied as follows: 0−10% solvent B in 5 min, 10−70% solvent B in 70 min, 70−100% solvent B in 10 min, and 100−0% for 10 min. Fractions were collected every 1 min, and their radioactivity was measured by liquid scintillation.

$^{45}Ca^{2+}$ Uptake. Washed platelets (0.5 mL) were incubated with 1 mM $CaCl_2$ containing 5 μCi of $^{45}Ca^{2+}$ with stirring at 1000 rpm at 37 °C for 5 min, and Ca^{2+} uptake was started by adding 40 μM PtdIns(3,4,5)P_3. Aliquots (100 μL) were taken at the indicated times, and passed through 0.45 μm nitrocellulose filters (Millipore) presoaked with the resus-

pension buffer on a Millipore Model 1225 sampling manifold assembly. The membranes were washed 3 times with 3 mL of the resuspension buffer, and the retained radioactivity was quantitated by liquid scintillation spectrometry. Basal Ca^{2+} uptake was measured at the indicated times in the absence of PtdIns(3,4,5)P_3. Also subtracted from each data point was the chelation of $^{45}Ca^{2+}$ by the polyphosphoinositide, in which $^{45}Ca^{2+}$ was incubated with PtdIns(3,4,5)P_3 in the absence of platelets. PtdIns(3,4,5)P_3 showed weak association with Ca^{2+}, which attributed to less than 2% of the total $^{45}Ca^{2+}$ uptake at each time point. Other phosphoinositides examined included PtdIns(3,4)P_2, PtdIns(4,5)P_2, PtdIns(3)P, and PtdIns. However, none of these showed appreciable activity in stimulating $^{45}Ca^{2+}$ uptake.

Ins(1,3,4,5)P_4 Receptor-Binding Assay. Platelet membrane preparations and radioligand competitive binding were carried out according to a modification of the methods described by Cullen et al. (*33*). Washed rabbit platelets were suspended in the aforementioned platelet washing buffer containing 1 mM DTT, 1 mM AEBSF, and 10 μg/mL leupeptin, and sonicated at 4 °C. The suspension was centrifuged at 1200*g*, and the supernatant was centrifuged at 45000*g* for 45 min to collect the platelet membrane. The pellet was suspended in 25 mM Tris/HCl, pH 7.2, containing 1 mM EDTA, 1 mM DTT, 5 mM leupeptin, and 2 mM AEBSF at a protein concentration of 4 mg/mL. The membrane preparation was immediately used for the following binding assay. The assay mixture consisted of 10 mM MES, pH 6.7, 100 mM KCl, 20 mM NaCl, 1 mM EDTA, 2 nM [^{3}H]Ins(1,3,4,5)P_4 (30 Ci/mmol), 200 μg of membrane proteins, and various concentrations of competing Ins(1,3,4,5)P_4 or di-C_8-PtdIns-(3,4,5)P_3, in a final volume of 0.3 mL. The mixture was incubated at 4 °C for 15 min, and the reaction was terminated by filtration through GF/C filters (Whatman) presoaked with the incubation buffer, followed by a wash with the same buffer (3 mL). The receptor-bound radioactivity was analyzed by liquid scintillation spectrometry. Nonspecific binding was measured in the presence of 30 μM Ins(1,3,4,5)-P_4. The modified procedure differed from the original method (*33*) in the composition of the assay buffer. In the present study, 0.1% (w/v) BSA was omitted in view of the nonspecific binding of phospholipids [PtdIns(3,4,5)P_3] to BSA.

RESULTS

PtdIns(3,4,5)P_3 Induces Platelet Activation. Washed rabbit platelets were exposed to various phosphoinositides in the presence of 1 mM Ca^{2+}, which included PtdIns(3,4,5)-P_3, PtdIns(3,4)P_2, PtdIns(4,5)P_2, PtdIns(3)P, PtdIns(4)P, and PtdIns. Among these, PtdIns(3,4,5)P_3 gave rise to cell aggregation in a dose-dependent manner (Figure 1A). At low concentrations of exogenous PtdIns(3,4,5)P_3, this platelet aggregation appeared to be reversible (10 μM; trace d) or nearly unnoticeable (5 μM; trace e). The threshold for irreversible aggregation was around 20 μM (trace c). At 40 and 80 μM, the aggregation was virtually instantaneous without an appreciable lag phase (traces b and a, respectively). This platelet aggregation required constant stirring, indicating the importance of cell−cell contacts during activation (*34*). In addition, this PtdIns(3,4,5)P_3-induced aggregation was Ca^{2+}-dependent. The platelets did not respond to PtdIns(3,4,5)P_3 in the absence of Ca^{2+}, but readily

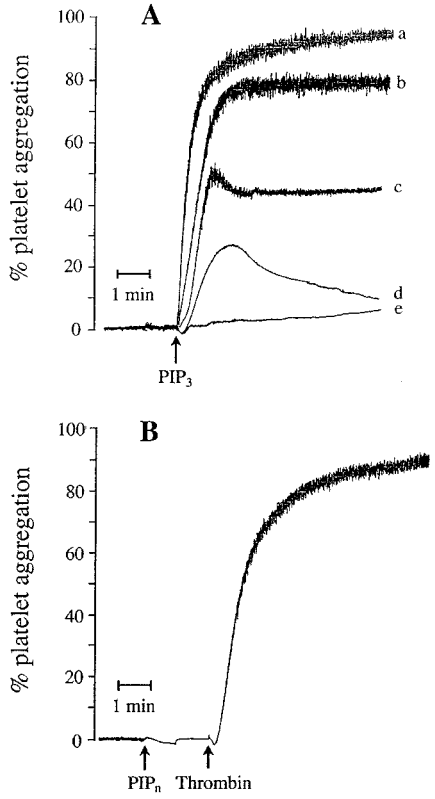

FIGURE 1: PtdIns(3,4,5)P_3-induced platelet aggregation. (A) Dose-dependency on exogenous PtdIns(3,4,5)P_3. Washed rabbit platelets were treated with 80 μM (trace a), 40 μM (trace b), 20 μM (trace c), 10 μM (trace d), or 5 μM (trace e) PtdIns(3,4,5)P_3. The arrow indicates the point of phospholipid introduction to the platelet suspension. (B) Phosphoinositide specificity. A number of other D-3 and D-4 phosphoinositides were also examined (denoted by PIP_n), which included PtdIns(3,4)P_2, PtdIns(4,5)P_2, PtdIns(3)P, PtdIns(4)P, and PtdIns. However, none of these showed appreciable platelet aggregation up to and at 100 μM. The tracings are representative of three experiments at 100 μM. All phosphoinositides were added to platelets from stocks in DMSO with stirring. The amount of DMSO added was the same for all experiments, i.e., 2 μL added to a platelet suspension of 700 μL. DMSO alone has no appreciable effect on platelets.

aggregated upon the subsequent supplement of Ca^{2+} (data not shown). Other phosphoinositides examined failed to induce appreciable aggregation even at concentrations greater than 100 μM (Figure 1B). The platelets resumed aggregation upon subsequent thrombin or PtdIns(3,4,5)P_3 activation, indicating the competency of the platelets after phosphoinositide treatment. It is worth mentioning that although DMSO (final concentration, 0.3%) was used to form a fine suspension of the phospholipid, the solvent alone did not cause appreciable interference to platelet function or cell lysis.

Moreover, exogenous PtdIns(3,4,5)P_3 could overcome the inhibitory effect of wortmannin on thrombin-induced platelet aggregation, suggesting a plausible involvement of PtdIns-(3,4,5)P_3 in the regulation of platelet function (Figure 2). In Figure 2A, trace a depicts a typical profile of thrombin-induced platelet activation without the inhibitor. In contrast, wortmannin (400 nM) pretreatment of the platelets caused a significant decrease in the amplitude of the initial aggregation phase followed by a progressive disaggregation of the cells (trace b). This finding conforms with the report on the effect of wortmannin on TRAP-induced aggregation (*11*). Sub-

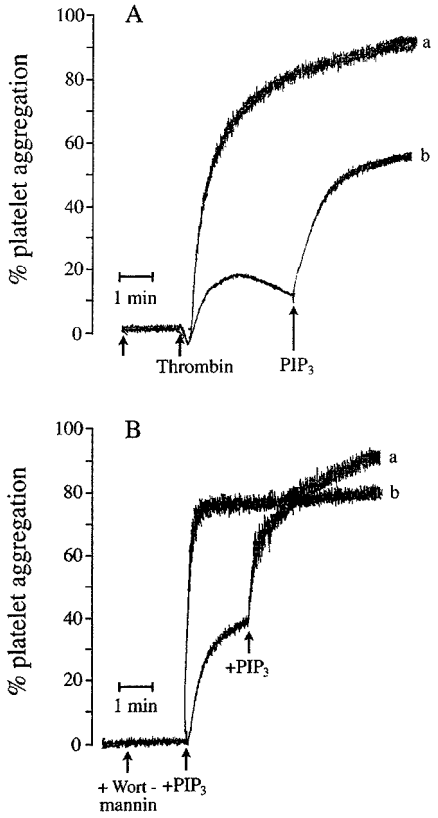

FIGURE 2: PtdIns(3,4,5)P_3 reverses the inhibition of thrombin-induced platelet aggregation by wortmannin in a dose-dependent and additive manner. (A) Washed rabbit platelets were preincubated without (trace a) or with (trace b) wortmannin (400 nM) at the point indicated by an arrow, and thrombin (1 unit/mL) was then added. Trace a showed a typical profile of thrombin-induced platelet activation without wortmannin pretreatment. In contrast, in wortmannin-pretreated cells (trace b), a significant decrease in the amplitude of the initial aggregation phase followed by a progressive disaggregation of the cells was observed. Subsequent addition of exogenous PtdIns(3,4,5)P_3 (40 μM) reversed the wortmannin inhibition. (B) Effect of PtdIns(3,4,5)P_3 on wortmannin-treated platelets. Washed rabbit platelets were treated with wortmannin (400 nM) followed by different doses of PtdIns(3,4,5)P_3. Trace a represents the stepwise addition of two equal aliquots of 40 μM; trace b indicates the addition of 80 μM PtdIns(3,4,5)P_3. The tracings are representative of three experiments.

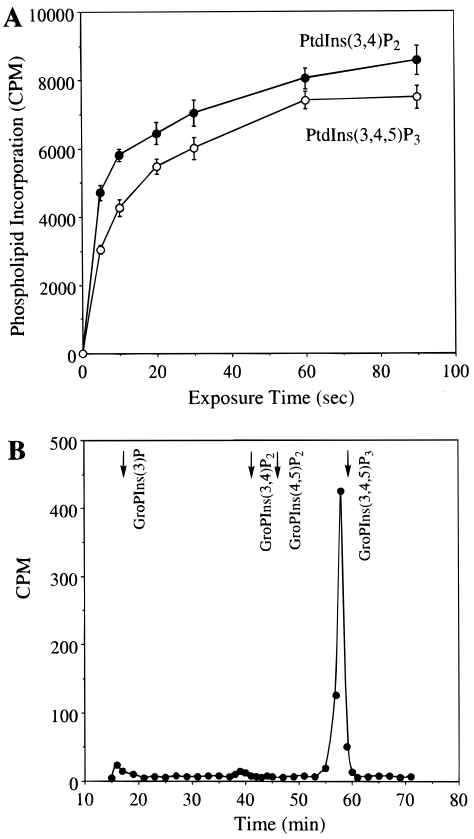

FIGURE 3: (A) Time course of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 incorporation into washed rabbit platelets. Washed rabbit platelets were exposed to 40 μM PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 containing 0.2 μCi of the corresponding tritium-labeled analogue (sp act., 35−38 mCi/mmol). The radioligand incorporation was analyzed by a membrane filtration method described under Materials and Methods. When platelets were omitted from the incubation, the retained radioactivity on the membrane filter was negligible. Each data point represents the mean of three determinations. (B) HPLC profile of the deacylated product of ^{3}H-labeled phospholipids associated with platelets after exposure to [^{3}H]PtdIns(3,4,5)P_3 for 20 s. Lipid extraction and HPLC analysis are described under Materials and Methods. ^{3}H-Labeled standards of glycerophosphorylinositol 3-phosphate [GroPIns(3)P], GroPIns(3,4)P_2, GroPIns(4,5)P_2, and GroPIns(3,4,5)P_3 were prepared from the respective ^{3}H-labeled phosphoinositides by the same procedure.

sequent addition of exogenous PtdIns(3,4,5)P_3 (40 μM) reversed the wortmannin inhibition (Figure 2A, trace b). By the same token, in wortmannin-pretreated cells, PtdIns(3,4,5)P_3 triggered platelet activation in a dose-dependent and additive manner (Figure 2B). Traces a and b represent the responses from the stepwise addition of two equal aliquots of 40 μM PtdIns(3,4,5)P_3 and from a single addition of 80 μM PtdIns(3,4,5)P_3, respectively, to wortmannin-pretreated platelets.

Phosphoinositide Uptake by Platelets. A crucial issue that warranted clarification was whether exogenous PtdIns(3,4,5)P_3 or related phosphoinositides could be efficiently incorporated into platelets. This question was of relevance to the underlying mechanism(s) for PtdIns(3,4,5)P_3-triggered platelet aggregation. Thus, we examined the uptake of [^{3}H]-PtdIns(3,4,5)P_3 and [^{3}H]PtdIns(3,4)P_2 into washed rabbit platelets by using a membrane filtration method. Figure 3A indicates that the D-3 phosphatidylinositol polyphosphates readily permeated the plasma membrane in a time-dependent fashion. Within 10 s, substantial quantities of the phospholipids were incorporated into the platelets. According to the cell-associated radioactivity at saturation, there were approximately 2.5 pmol of PtdIns(3,4,5)P_3 incorporated per 10^6 platelets.

To examine whether PtdIns(3,4,5)P_3 underwent metabolism immediately after being incorporated, the platelets that had been exposed to [^{3}H]PtdIns(3,4,5)P_3 for 20 s were treated with HCl and EDTA followed by solvent extraction. The recovered phospholipids were subjected to deacylation, and the identity of the tritium-labeled deacylated product was verified by HPLC in reference to glycerolphosphorylinositol phosphate standards. As shown in Figure 3B, the incorporated [^{3}H]PtdIns(3,4,5)P_3 remained largely intact with negligible formation of [^{3}H]PtdIns(3,4)P_2 and [^{3}H]PtdIns(3)P, supporting the specificity of PtdIns(3,4,5)P_3 in inducing platelet activation.

Although this study could not distinguish between specific uptake versus nonspecific adsorption of the phosphoinositide to the platelet surface, the aggregation data suggest that at least a fraction of the incorporated PtdIns(3,4,5)P_3 could

rapidly reach the cytosolic leaflet of plasma membranes to act on its target. The exact mechanism of phosphoinositide transport across membranes, however, remains unclear.

Effect of PtdIns(3,4,5)P_3 on Cytosolic Ca^{2+} Levels. Several lines of evidence indicate that PtdIns(3,4,5)P_3-induced platelet aggregation was Ca^{2+}-dependent. First, platelets did not respond to PtdIns(3,4,5)P_3 stimulation in the absence of Ca^{2+}, but readily aggregated upon the subsequent supplement of Ca^{2+}. Second, the PtdIns(3,4,5)-P_3-induced aggregation could be abolished by EDTA or EGTA to remove Ca^{2+} from the melieu (data not shown). These data prompted us to examine the effect of PtdIns-(3,4,5)P_3 on the uptake of external Ca^{2+}. The D-3 phosphoinositide was added to a platelet suspension containing $^{45}Ca^{2+}$. As shown in Figure 4A, PtdIns(3,4,5)P_3 stimulated a sharp increase in intracellular $^{45}Ca^{2+}$, followed by a gradual decline. The decrease might arise from the action of Ca^{2+}-ATPase in an effort to regulate Ca^{2+} homeostasis (*35*).

It is worth mentioning that the rate of spontaneous Ca^{2+} uptake (without agonist) accounted for less than 2% of the PtdIns(3,4,5)P_3-induced influx. Again, the stimulation of Ca^{2+} inflow was not observed with any other phosphoinositide examined, including PtdIns(3,4)P_2, PtdIns(4,5)P_2, and PtdIns(3)P (data not shown).

These data suggest a plausible involvement of PtdIns-(3,4,5)P_3 in platelet signaling by effecting Ca^{2+} influx. However, one might raise a question concerning the impact of PtdIns(3,4,5)P_3 on internal Ca^{2+} mobilization since the inositol lipid has been reported to activate PLC-γ isozymes (*18*, *22*). To answer this question, the effect of PtdIns(3,4,5)-P_3 on cytosolic [Ca^{2+}] was examined in Fura 2-preloaded platelets in the presence of 1 mM free Ca^{2+} versus EGTA (Figure 4B; traces a and b, respectively). In the presence of Ca^{2+}, the addition of PtdIns(3,4,5)P_3 resulted in a sharp rise in cytosolic [Ca^{2+}], which reached a plateau 16 s after the lipid addition and was maintained at the same level for up to 2 min (trace a). This result was consistent with that from the above $^{45}Ca^{2+}$ uptake. On the other hand, when the external Ca^{2+} was depleted by EGTA, PtdIns(3,4,5)P_3 stimulation only caused a small increase in cytosolic [Ca^{2+}] with a lag phase of approximately 20 s, followed by a slow decline to the basal level (trace b). The maximum could only account for less than 5% of that in the presence of external Ca^{2+}. This small increase was likely attributable to internal Ca^{2+} release by Ins(1,4,5)P_3 generated by the PtdIns(3,4,5)P_3-activated PLC-γ.

Mechanism of PtdIns(3,4,5)P_3-Induced Ca^{2+} Entry. Taken together, these findings provided evidence that PtdIns(3,4,5)-P_3 triggered platelet activation in part by activating transmembrane Ca^{2+} influx. The existence of a PtdIns(3,4,5)P_3-dependent Ca^{2+} entry mechanism on platelet membranes was supported by the inhibitory effect of wortmannin on thrombin-induced Ca^{2+} influx (Figure 5A). As shown, 100 nM wortmannin lowered the rate of thrombin-stimulated Ca^{2+} uptake by 50%, implicating the involvement of PI 3-kinase in the Ca^{2+} influx. To further delineate the mechanism(s) by which thrombin induced Ca^{2+} entry, three plausible Ca^{2+} transport systems were assessed. First, in the literature, agonist-stimulated Ca^{2+} entry into platelets has been associated with receptor-operated Ca^{2+} channels (*34*, *36*). These agonists include platelet-activating factor, thrombin, vasopressin, ADP, and thromboxane A_2 analogues. These nonselective Ca^{2+} channels are permeable to various monovalent and divalent cations, and have been reported to be inhibited by Cd^{2+} and Ni^{2+} at submillimolar concentrations (*37*−*41*). Second, the GPIIb-IIIa complex has been suggested to function as a Ca^{2+} channel, which could be inhibited by RGDS peptide (*42*−*44*). Third, an Ins(1,3,4,5)-P_4 receptor-mediated Ca^{2+} transport system on platelet plasma membranes has been proposed (*33*, *45*−*47*). In view of the largely shared structural motifs between PtdIns(3,4,5)-P_3 and Ins(1,3,4,5)P_4, it is plausible that PtdIns(3,4,5)P_3 activates the Ins(1,3,4,5)P_4 receptor by mimicking the essential structural features (*33*).

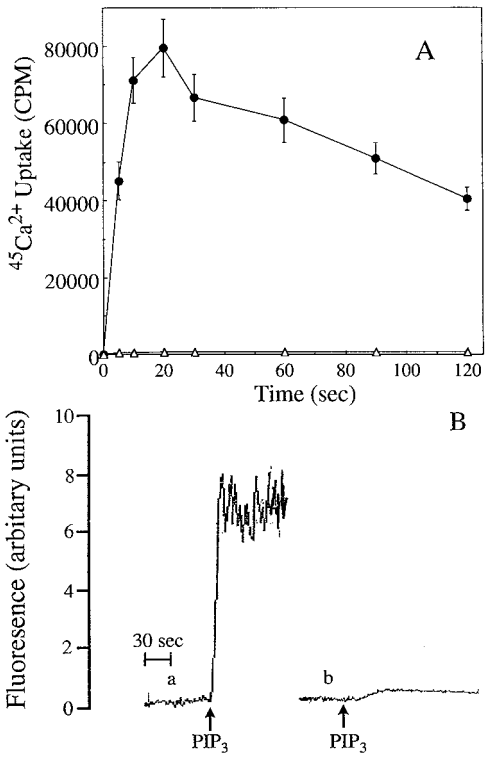

FIGURE 4: PtdIns(3,4,5)P_3-induced Ca^{2+} influx. (A) Time course of $^{45}Ca^{2+}$ uptake by washed rabbit platelets. Washed platelets were incubated with 1 mM $CaCl_2$ containing 5 μCi of $^{45}Ca^{2+}$ with stirring at 1000 rpm at 37 °C for 5 min, and Ca^{2+} uptake was initiated by adding 40 μM PtdIns(3,4,5)P_3. $^{45}Ca^{2+}$ uptake was measured by a membrane filtration method described under Materials and Methods. Spontaneous Ca^{2+} influx (open triangles) accounted for less than 2% of the PtdIns(3,4,5)P_3-induced uptake. Each data point represents the mean of data from three independent experiments. (B) Fluorescence traces of Fura 2-loaded platelets in response to PtdIns-(3,4,5)P_3 stimulation in a buffer containing 1 mM Ca^{2+} (trace a) or in an EGTA-treated buffer (trace b). Washed platelets were suspended in a buffer consisting of 4.3 mM Na_2HPO_4, 24.3 mM NaH_2PO_4, 4.3 mM K_2HPO_4, pH 6.8, 113 mM NaCl, 5 mM D-glucose, 1 mM $CaCl_2$, and 0.5% BSA at a concentration of 3×10^8 cells/mL. The suspension was incubated with 10 μM Fura 2-AM at 37 °C for 1 h, pelleted by centrifugation at 1000*g* for 10 min, washed with the same buffer but without Ca^{2+} twice, and resuspended at approximately 5×10^7 cells/mL in the same buffer. The effect of PtdIns(3,4,5)P_3 on the cytosolic [Ca^{2+}] was examined by Fura 2 fluorescence in a Hitachi F-2000 spectrofluorometer at 37 °C with excitation and emission wavelengths at 340 and 510 nm, respectively. To the mixture was supplemented 1 mM Ca^{2+}, and the reaction was initiated by adding 40 μM PtdIns(3,4,5)P_3. To avoid platelet aggregation, stirring of the solution was stopped 2 s after PtdIns(3,4,5)P_3 addition. In the Ca^{2+}-free experiment, 1 mM EGTA was added to deplete residual Ca^{2+} in the suspension, followed by 40 μM PtdIns(3,4,5)P_3.

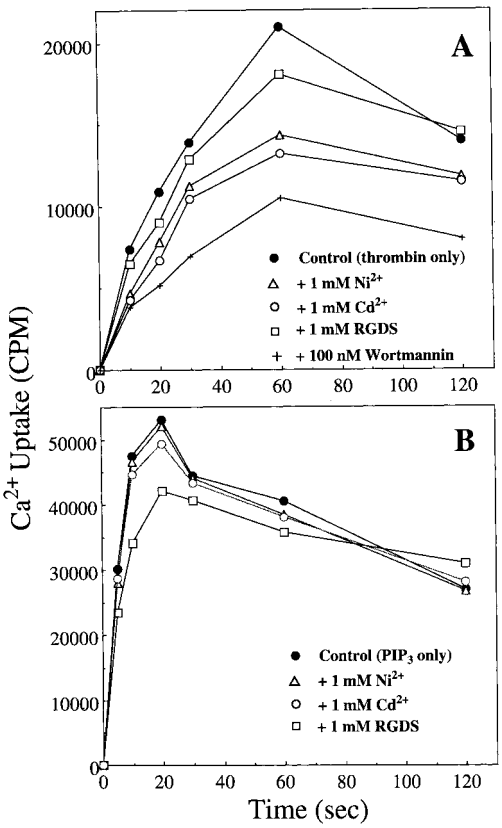

FIGURE 5: (A) Effect of wortmannin, Ni^{2+}, Cd^{2+}, and RGDS peptide on thrombin-induced Ca^{2+} influx. (B) Effect of Ni^{2+}, Cd^{2+}, and RGDS peptide on PtdIns(3,4,5)P_3-induced Ca^{2+} influx. Ca^{2+} uptake was examined using 10^8 platelets as described under Materials and Methods with thrombin (100 milliunits) or PtdIns-(3,4,5)P_3 (40 μM) in the absence and presence of individual inhibitors. Inhibitor concentrations were 1 mM for Ni^{2+}, Cd^{2+}, and RGDS peptide, and 100 nM for wortmannin.

Figure 5A shows that RGDS, Ni^{2+}, or Cd^{2+} at 1 mM could also partially inhibit thrombin-induced Ca^{2+} entry, however, with a potency lower than that of wortmannin. The fact that none of these inhibitors exerted a complete inhibition suggests the involvement of multiple Ca^{2+} transport mechanisms in thrombin-stimulated Ca^{2+} influx.

To further discern these Ca^{2+} entry pathways, we examined the inhibitory activity of Ni^{2+}, Cd^{2+}, and RGDS peptide on PtdIns(3,4,5)P_3-induced Ca^{2+} influx. However, none of these compounds significantly blocked the $^{45}Ca^{2+}$ inflow at millimolar concentrations (Figure 5B). This finding suggests that the PtdIns(3,4,5)P_3-induced Ca^{2+} entry was independent of the nonselective receptor-operated Ca^{2+} channel and GPIIb-IIIa.

We thus turned our attention to the Ins(1,3,4,5)P_4 receptor by examining the displacement of [^{3}H]Ins(1,3,4,5)P_4 binding to platelet membranes by di-C_8-PtdIns(3,4,5)P_3 vis-à-vis Ins-(1,3,4,5)P_4 (Figure 6). The C_8 analogue of the phospholipid, a water soluble derivative, was used in place of micellar di-C_{16}-PtdIns(3,4,5)P_3 because the latter would be readily incorporated into membranes.

The displacement of [^{3}H]Ins(1,3,4,5)P_4 by its cold counterpart (closed circles) displayed a profile characteristic of two ligand-binding sites of vastly different affinities, which closely resembled that described in the literature for the Ins-(1,3,4,5)P_4 receptor in pig platelet membranes (*33*). The potency of di-C_8-PtdIns(3,4,5)P_3 in displacing [^{3}H]Ins-

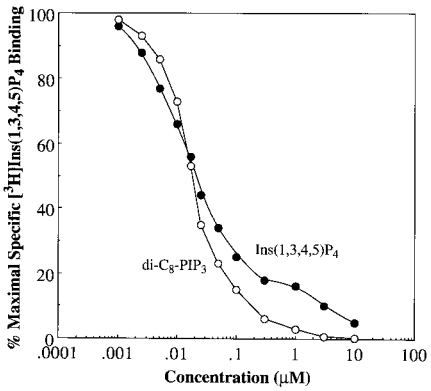

FIGURE 6: Inhibition of specific [^{3}H]Ins(1,3,4,5)P_4 binding to platelet membranes by increasing concentrations of Ins(1,3,4,5)P_4 (closed circles) or PtdIns(3,4,5)P_3 (open circles). The displacement assay was carried out as described under Materials and Methods. Nonspecific binding was measured in the presence of 30 μM Ins-(1,3,4,5)P_4. Each data point represents the mean of three determinations.

(1,3,4,5)P_4 binding (open circles) was of the same order of magnitude as that of Ins(1,3,4,5)P_4, though the phospholipid showed a higher potency in displacing [^{3}H]Ins(1,3,4,5)P_4 than Ins(1,3,4,5)P_4 at concentrations greater than 0.03 μM. The IC_{50} values were estimated to be 21 nM for di-C_8-PtdIns-(3,4,5)P_3, and 25 nM and 2 μM for the high and low binding sites, respectively, for Ins(1,3,4,5)P_4.

The interaction between the Ins(1,3,4,5)P_4 receptor and PtdIns(3,4,5)P_3 provided a putative link between PI 3-kinase and Ca^{2+} signaling during platelet activation. However, the present study could not exclude the possibility that PtdIns-(3,4,5)P_3 facilitated Ca^{2+} entry by affecting membrane permeability, which is currently under investigation.

PtdIns(3,4,5)P_3-Induced Platelet Aggregation Is Independent of TXA_2. Several physiological mediators other than Ca^{2+} might also be involved in the induction of shape change and aggregation. These include TXA_2 generated by the oxidation of arachidonate (*34*). To shed light into its potential involvement in PtdIns(3,4,5)P_3-induced platelet aggregation, we assessed the effect of aspirin on the aggregation. However, no inhibition of PtdIns(3,4,5)P_3-induced platelet activation was noted with aspirin pretreatment.

DISCUSSION

This study represents the first evidence that PtdIns(3,4,5)-P_3 induces platelet aggregation in a dose-dependent manner by activating Ca^{2+} influx. Several lines of evidence indicate that this stimulatory effect is PtdIns(3,4,5)P_3-specific. First, a series of D-3 and D-4 phosphoinositides examined were not able to induce appreciable Ca^{2+} influx or platelet aggregation even at concentrations substantially higher than that of PtdIns(3,4,5)P_3. Second, the rates of phospholipid uptake for [^{3}H]PtdIns(3,4,5)P_3 and [^{3}H]PtdIns(3,4)P_2 were comparable, excluding the possibility of differential phosphoinositide uptake by platelets. Third, no significant metabolism of [^{3}H]PtdIns(3,4,5)P_3 was noted after being incorporated into plasma membranes for 20 s. These observations confirm the notion that individual PI 3-kinase lipid products play different roles in the regulation of cell function.

The dose dependency data in Figure 1 suggest a subtle relationship between the in vitro PtdIns(3,4,5)P_3 concentration and platelet function. With exogenous PtdIns(3,4,5)P_3, the induced aggregation was reversible at low concentrations, but became irreversible with a shortened lag phase when the concentration exceeded a threshold ($\geq 20\ \mu$M). It is conceivable that the in vivo threshold, though unknown, would be substantially lower than its exogenous counterpart. The present finding may also provide a basis to account for the inhibitory effect of wortmannin on agonist-induced platelet aggregation (*11*). Presumably, the wortmannin treatment attenuates PtdIns(3,4,5)P_3 production in vivo, which results in incomplete platelet activation in a manner similar to that observed with low levels of external PtdIns(3,4,5)P_3.

As aforementioned, the immediate consequence of PtdIns(3,4,5)P_3 activation is the elevation of intracellular [Ca^{2+}], which represents a driving force for platelet aggregation. Evidence suggests that the increase was attributable to transmembrane Ca^{2+} influx, and that internal Ca^{2+} mobilization via the PLC-γ pathway did not have a significant contribution (Figure 4B). The fact that wortmannin partially inhibited thrombin-induced Ca^{2+} uptake suggests the presence of a PtdIns(3,4,5)P_3-dependent Ca^{2+} entry mechanism (Figure 5A) that differs from receptor-operated nonselective Ca^{2+} channels or the putative GPIIb-IIIa pathway. However, the mechanism by which PtdIns(3,4,5)P_3 induced Ca^{2+} influx remains unclear. Preliminary binding data shown in Figure 6 imply the potential involvement of the putative Ins(1,3,4,5)P_4 receptor in PtdIns(3,4,5)P_3-induced Ca^{2+} influx. On the other hand, the present data cannot rule out the possibility that PtdIns(3,4,5)P_3 translocates Ca^{2+} by affecting membrane permeability.

Irvine and co-workers first suggested that Ins(1,3,4,5)P_4-binding sites on platelet plasma membranes might in fact represent PtdIns(3,4,5)P_3 receptors since PtdIns(3,4,5)P_3 contained Ins(1,3,4,5)P_4 as its headgroup (*33*). This proposition, however, was dismissed due to the weak binding of GroPIns(3,4,5)P_3, a deacylated product of PtdIns(3,4,5)P_3, to the receptor (*33*). However, it was corroborated in this study by the competitive binding experiment shown in Figure 6. Di-C_8-PtdIns(3,4,5)P_3 effectively displaced [^{3}H]Ins(1,3,4,5)P_4 in receptor binding with an efficacy virtually identical to that of unlabeled Ins(1,3,4,5)P_4. Previously, Ins(1,3,4,5)P_4-binding proteins on platelet plasma membranes have been identified by two groups. Irvine and co-workers reported the characterization of this binding protein as a GTPase-activating protein whose involvement in Ca^{2+} entry remains unclear (*33*, *46*). Data reported by O'Rourke and co-workers suggest that the Ins(1,3,4,5)P_4-binding protein mediates Ca^{2+} influx in platelets (*47*). These results add to the evidence for a multi-tiered system for Ca^{2+} regulation in platelets. As suggested by the inhibition data of thrombin-induced Ca^{2+} entry (Figure 5A), the receptor-coupled Ca^{2+} channel and the PtdIns(3,4,5)P_3-mediated Ca^{2+} transport may represent the major contributors to cytosolic Ca^{2+} increase during the early phase of agonist stimulation.

In summary, the present study provides a putative link between PI 3-kinase activation and Ca^{2+} signaling in platelets. It remains unclear how PtdIns(3,4,5)P_3 facilitates Ca^{2+} transport across plasma membranes. Nevertheless, the influx of Ca^{2+} represents the initial step of a series of biochemical cascades leading to platelet aggregation. Delineation of the molecular basis by which PtdIns(3,4,5)P_3 activates Ca^{2+} transport will give useful insights concerning the control of in vivo platelet activation.

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