Platelets express a single class of Fcγ receptor (FcγRIIA), which is involved in heparin-associated thrombocytopenia and possibly in inflammation. FcγRIIA cross-linking induces platelet secretion and aggregation, together with a number of cellular events such as tyrosine phosphorylation, activation of phospholipase C-γ2 (PLC-γ2), and calcium signaling. Here, we show that in response to FcγRIIA cross-linking, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) is rapidly produced, whereas phosphatidylinositol (3,4)-bisphosphate accumulates more slowly, demonstrating a marked activation of phosphoinositide 3-kinase (PI 3-kinase). Inhibition of PLC-γ2 was not related to its tyrosine phosphorylation state, since wortmannin actually suppressed its dephosphorylation, which requires platelet aggregation and integrin αIIbβ3 engagement. In contrast, the stable association of PLC-γ2 to the membrane/cytoskeleton interface observed at early stage of platelet activation was fully abolished upon inhibition of PI 3-kinase. In addition, PLC-γ2 was able to preferentially interact in vitro with PtdIns(3,4,5)P3. Finally, exogenous PtdIns(3,4,5)P3 restored PLC activation in permethylized platelets treated with wortmannin. We propose that PI 3-kinase and its product PtdIns(3,4,5)P3 play a key role in the activation and adequate location of PLC-γ2 induced by FcγRIIA cross-linking.

In addition to specific interactions involving their Fab domains, immunoglobulins G can interact with various membrane receptors by their Fc region. These so-called Fc receptors are coded by a heterogeneous family of at least eight genes forming a complex locus on chromosome 1. Based on both functional and structural criteria, these proteins can be differentiated into three groups (I–III) displaying various expression patterns in cells of the immune system (1, 2). Platelets possess a single class of Fcγ receptors, FcγRIIA.1 As reviewed by Anderson et al. (3), clustering of FcγRIIA induces shape change, secretion, and aggregation, which are typical platelet responses contributing to their hemostatic function. In addition, there is a close link between secretion and aggregation, since released ADP was shown recently to be very critical for platelet aggregation evoked by FcγRIIA cross-linking (4). The precise role of platelet FcγRIIA is still obscure, although it explains how these cells are activated by specific antibodies directed against various membrane antigens, by immune complexes or by aggregated IgG (3). On a pathophysiological point of view, platelet FcγRIIA might involve an hemostatic response at inflammatory sites displaying IgG deposits, and there is increasing evidence for a direct involvement of FcγRIIA in heparin-associated thrombocytopenia occurring in patients under heparin therapy (3).

FcγRIIA is a 40-kDa polypeptide bearing two IgG-like domains in its extracellular region, a single transmembrane segment, and an ITAM, also known as Reth motif, in the cytoplasmic tail (5). ITAM sequences contain two variably spaced tyrosine residues; they are present in Fcγ receptors, FcεR, various subunits of both T cell and B cell antigen receptors, and the γ-chain of Fc receptor in platelets (6); and they support the paradigm of immune cell activation (7–12). Indeed, clustering of membrane receptors present in cells of the immune system promotes the phosphorylation, presumably by Src kinases, of the two tyrosine residues of ITAM. This results in the specific anchoring to ITAM sequences of tyrosine kinases bearing two SH2 domains, i.e. either Syk or ZAP-70. These two tyrosine kinases then induce a complex set of signaling events including calcium mobilization as well as activation of PI 3-kinase and mitogen-activated protein kinases, the latter ones via an upstream cascade involving Grb2-Sos and the small GTPase Ras (7–12).

Phosphoinositide metabolism plays a key role during the stimulation of immune cell receptors and involves two types of enzymes, PLC and PI 3-kinase. Among various isoforms identified so far, PLC-γ2 is abundant in hematopoietic cells, it is activated downstream of tyrosine kinases, and it promotes both

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1 The abbreviations used are: FcγRIIA, low affinity Fcγ receptor IIA; ITAM, immunoreceptor tyrosine-based activation motif; FcεR, high affinity IgE receptor; SH2, Src homology-2; PI 3-kinase, phosphoinositide 3-kinase; PLC, phospholipase C; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PtdOH, phosphatic acid; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; TRAP, thrombin-receptor activating peptide; Pipes, 1,4-piperazinediethanesulfonic acid.

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Phosphatidylinositol 3,4,5-Trisphosphate-dependent Stimulation of Phospholipase C-γ2 Is an Early Key Event in FcγRIIA-mediated Activation of Human Platelets*

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calcium mobilization and activation of protein kinase C via the two second messengers produced upon hydrolysis of PtdIns(4,5)P2 (13, 14). On the other hand, the heterodimeric IA class PI 3-kinase is also regulated by tyrosine kinases and promotes the accumulation of D3-phosphoinositides, which are considered as potential second messengers (15–17). Two recent studies have shown specific interactions between PLC-γ1 and PtdIns(3,4,5)P3, but depending on the authors this might involve either the N-terminal PH domain or the SH2 domains of the protein (18, 19). However, no direct link between PLC-γ2, which is inactive toward the products of PI 3-kinase, and PI 3-kinase itself has been demonstrated so far in cells activated via FcγRIIA cross-linking.

Besides the fact that FcγRIIA might play a key role in the hemostatic and inflammatory function of platelets, these cells are interesting to consider in so far they contain a single class of Fcγ receptor (FcγRIIA), in contrast to neutrophils or monocytes, for instance (1, 3). Clustering of platelet FcγRIIA promotes phosphorylation of the two tyrosine residues of the ITAM motif, which is followed by the classical set of signaling events occurring under similar conditions, i.e., various tyrosine phosphorylations and calcium mobilization, through activation of PLC-γ2 (3, 20–22). Moreover, Chacko et al. (23) provided evidence that PI 3-kinase associates transiently with FcγRIIA upon platelet receptor clustering, probably via Syk. The present study was thus undertaken in order to determine possible changes of phosphoinositide metabolism occurring upon clustering of platelet FcγRIIA. In addition, taking advantage of the use of two specific and unrelated inhibitors (wortmannin and LY294002), we have focused our interest on signaling events occurring downstream of PI 3-kinase. Our present data unravel a causal relationship between PI 3-kinase and PLC-γ2, which might also function in the signaling cascade evoked by other membrane receptors involved in the immune response.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-FcγRIIA monoclonal antibody IV.3 and mouse anti-phosphotyrosine 4G10 antibody were purchased from Upstate Biotechnology, rabbit polyclonal anti-PLC-γ1 antibodies were from Immunoresearch Laboratories, rabbit polyclonal anti-PLC-γ2 antibody was from Santa Cruz Biotechnology Inc. [γ-32P]ATP (3,000 Ci/mmol) was from NEN Life Science Products. DiC16-PtdIns(3,4,5)P3 and diC16-PtdIns(3,4)P2 were from Matreya Inc. PtdIns, PtdIns(4)P, PtdIns(4,5)P2, crude brain phosphoinositides, phosphatidyserine, thrombin, wortmannin, and RGDS were purchased from Sigma, and TLC plates from Merck. All other reagents were obtained from Sigma unless otherwise indicated.

**Preparation and Activation of Platelets**—Platelets were isolated from concentrates obtained from the local blood bank (Etablissement de Transfusion Sanguine, Toulouse, France) essentially as described previously (24). They were washed in washing buffer (pH 6.5) containing 140 mM NaCl, 5 mM KCl, 5 mM KH2PO4, 1 mM MgSO4, 10 mM Hepes, 5 mM glucose, 0.35% (w/v) bovine serum albumin. The same buffer plus 1 mM CaCl2 was added to the final suspension, and pH was adjusted to 7.4. In experiments dealing with inositol lipid analysis, platelets were labeled with 0.5 mM [32P]orthophosphate during 60 min in a phospho-free washing buffer (pH 6.5) at 37 °C. [32P]Labeled platelets were then washed once in the same buffer and finally suspended at a final concentration of 1 × 10^9 cells/ml (pH 7.4). Cross-linking of FcγRIIA was achieved by preincubation of platelets for 1 min with the monoclonal antibody IV.3 (2 μg/ml) followed by addition of anti-mouse IgG F(ab)’2 (30 μg/ml) at 37 °C under gentle shaking as described previously (25).

**Lipid Extraction and Analysis**—Reactions were stopped by addition of chilled methanol (1/1, v/v), then incubated at 4 °C for 1 h, and lipids were immediately extracted following the modified procedure of Bligh and Dyer (26, 27).

For PtdIns(4)P and PtdIns(4,5)P2, lipids were immediately decylated by 20% methanolic and analyzed by HPLC on a Whatman Partisphere 5 SAX column (Whatman International Ltd., UK) as described previously (27).

For PtdIns(3,4)P2 and PtdIns(3,4,5)P3, quantification, lipids were first resolved by thin-layer chromatography (TLC) using chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14, v/v). The spots corresponding to PtdIns(3,4)P2 and PtdIns(3,4,5)P3 were then scraped off, deacylated, and analyzed by HPLC as described above. For quantification of PtdOH, lipids were resolved by TLC using chloroform/methanol/10 N HCl (87:12:1, v/v) as described previously (28). The spots corresponding to PtdOH were directly quantified by PhosphorImager analysis.

**Measurement of Aggregation and 5-Hydroxytryptamine Secretion**—Aggregation was monitored using a Chrono-log dual channel aggregometer with stirring at 900 rev/min at 37 °C. Aggregation was stopped by addition of 3% formaldehyde and 0.1 M EDTA, cooling on ice, and centrifugation. The radioactivity of 5-hydroxy[14C]tryptamine released from platelet dense granules was determined by liquid-scintillation counting.

**Gel Electrophoresis and Immunoblotting**—Proteins were resuspended in electrophoresis sample buffer containing 100 mM Tris-HCl, pH 6.8, 15% (v/v) glycerol, 25 mM dithiothreitol, and 3% SDS, boiled for 5 min, separated on 7.5% SDS-polyacylamide gel electrophoresis, transferred to nitrocellulose membranes (Gelman Science Products). The nitrocellulose was blocked for 60 min at room temperature with 1% (w/v) milk powder, 1% (w/v) bovine serum albumin in a buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% (v/v) Tween 20. Immunodetection was achieved using the relevant antibody, peroxidase-conjugated secondary antibody, and ECL system. The various bands were quantified by densitometric analysis measuring the pixel volume in each area (Gel Doc 1000, Bio-Rad).

**Immunoprecipitation**—Reactions were stopped by addition of twice-concentrated ice-cold lysis buffer containing 80 mM Tris-HCl, pH 7.4, 200 mM NaCl, 200 mM NaF, 20 mM EDTA, 80 mM Na2VO4, 4 mM Na3VO4, 2% (v/v) Nonidet P-40, and 10 μg/ml each of aprotinin and leupeptin. After gentle shaking during 20 min at 4 °C and centrifugation (12,000 × g for 10 min at 4 °C), the soluble fraction was collected and preclarified for 30 min at 4 °C with protein A-Sepharose CL4B. The preclarified suspensions were then incubated overnight at 4 °C with the adequate antibody, and immune complexes were then precipitated by addition of 10% (w/v) protein A-Sepharose CL4B for 1 h at 4 °C and centrifugation (6,000 × g for 5 min at 4 °C). The immunoprecipitates were washed twice in washing buffer and twice in washing buffer containing 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 100 μM Na2VO4, 1 μg/ml each of aprotinin and leupeptin. Immunoprecipitated proteins were resolved by 7.5% SDS-polyacylamide gel electrophoresis and analyzed by Western blotting.

**Isolation of Cytoskeleton**—Reaction was stopped and the cytoskeleton immediately extracted by adding one volume of ice-cold twice-concentrated cytoskeleton buffer containing 100 mM Tris-HCl, pH 7.4, 200 mM EGTA, 2 mM Na2VO4, 4 μg/ml each of aprotinin and leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 2% (v/v) Triton X-100 as described previously (30, 31). After 10 min at 4 °C, the cytoskeleton was pelleted by centrifugation (12,000 × g for 10 min at 4 °C), washed once in cytoskeleton buffer with 0.5% Triton X-100, and once with the same buffer without Triton X-100. Cytoskeleton was then immediately suspended in electrophoresis sample buffer (32).

**Cytosol Depletion**—After stimulation, platelets were centrifuged (3,000 × g for 30 s) and suspended in 20 mM Pipes buffer (pH 6.8) containing 150 mM KCl, 2 mM EDTA, and 30 μg/ml misonidazole. After 3 min at room temperature under shaking, supernatant and pellet fractions were separated by centrifugation (12,000 × g for 40 s). The pellet was twice washed in Laemmli’s sample buffer, and PLC-γ2 was then precipitated by Western blotting using a specific antibody as described above.

**Synthesis of Radiolabeled D3-phosphorylated Inositol Lipids**—[32P]PtdIns(3)P, [32P]PtdIns(3,4)P2, and [32P]PtdIns(3,4,5)P3 were prepared by incubating crude brain phosphoinositides (60 μg) mixed with phosphatidyserine (120 μg) in 50 mM Tris-HCl, pH 7.4 (1 μg of phosphoinositides/μl), vortexed, and sonicated (20 kHz for 5 min). Lipid suspension was then added to platelet PI 3-kinase in the presence of 30 μCi [γ-32P]ATP (3,000 Ci/mmol), 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1.5 mM dithiothreitol, 0.5 mM EDTA, 5 mM MgCl2, and 100 μM ATP (33). After 30 min at 37 °C, reaction was stopped by addition of acidified chloroform/methanol and the lipids were immediately extracted as described above.

**Phosphoinositide Binding Assay**—Radiolabeled phosphoinositides
were dried under a nitrogen stream and suspended in HNE buffer containing 30 mM Heps, pH 7.0, 100 mM NaCl, and 1 mM EDTA. After sonication, HNE buffer supplemented with 0.5% (v/v) Nonidet P-40 was added to obtain a final concentration of 0.02% (v/v) Nonidet P-40 (34). Thirty μl of this suspension were added to immunoprecipitated PLC-γ2 resuspended in 30 μl of HNE buffer supplemented with 0.02% (v/v) Nonidet P-40. After 45 min of incubation at room temperature under gentle shaking, samples were washed twice with 1 ml of HNE buffer supplemented with 0.2% (v/v) Nonidet P-40 (34). Lipids that remained associated with the immune complex were extracted as described above and resolved by TLC using chloroform/acetone/methanol/acetate acid/water (20/30/26/24/14, v/v) (35).

Studies with Permeabilized Platelets—Platelet suspensions were adjusted to 5 × 10^9 cells/ml in 132 mM NaCl, 2.8 mM KCl, 0.86 mM MgCl2, 8.9 mM NaHCO3, 2 mM Heps, 5.6 mM glucose, 12.2 mM Na3 citrate, and 10 mM Tris, pH 7.1 as described (36). Then 100 μl of platelet suspension was mixed with 400 μl of buffer containing 120 mM KCl, 4 mM MgCl2, 25 mM NaCl, 1 mM NaHPO4, 1 mM EGTA, 0.269 mM CaCl2 and 15 mM Hepes, pH 7.1. Platelets were incubated with 10 nM wortmannin or MeSO for 15 min at 37 °C and then permeabilized during 3 min at 37 °C with 20 μg/ml saponin in the presence of 40 μM [γ-32P]ATP (100 μM) with or without 15 μM diC16-PtdIns(3,4,5)P3 or diC16-PtdIns(3,4)P2. The F(ab′)2 fragments were added 1 min after monoclonal antibody IV.3. The incubation was stopped after 1 min by addition of chloroform/methanol (v/v), and lipids were extracted and resolved by TLC for PtdOH quantification as described above.

RESULTS

PI 3-Kinase and PLC Are Rapidly Activated upon FcγRIIA Cross-linking—Cross-linking of platelet FcγRIIA has been shown to induce a transient association of PI 3-kinase to the ITAM sequences present in the cytoplasmic tail of the receptor; however, its consequences on a possible in vivo activation of the enzyme were not emphasized (23). Therefore, using an HPLC technique, we have first measured the time course of D3-phosphoinositide synthesis during FcγRIIA-mediated activation of 32P-labeled platelets. We found that PtdIns(3,4,5)P3 was rapidly produced, whereas PtdIns(3,4)P2 accumulated with a slower time course (Fig. 1A).

In addition, we observed a rapid drop in the substrate of PLC, PtdIns(4,5)P2, followed by its resynthesis (Fig. 1B). This sharp decrease in PtdIns(4,5)P2 was concomitant with the production of PtdOH (Fig. 1C), an event considered to reflect PLC activation in platelets. In these cells, the main part of diacylglycerol produced by PLC is converted into PtdOH by a diacylglycerol kinase, the contribution of phospholipase D being relatively minor (37, 38). In agreement with this, the production of PtdOH evoked by FcγRIIA cross-linking was abolished by the PLC-specific inhibitor U73122 (data not shown). Finally, the radioactivity of phosphatidylinositol 4-phosphate (PtdIns(4)P) did not change significantly over the whole period of platelet stimulation by FcγRIIA cross-linking (Fig. 1D).

PI 3-Kinase Is Upstream of the FcγRIIA-dependent Activation of PLC—To determine whether PI 3-kinase was required for FcγRIIA-mediated physiological responses, wortmannin and LY294002, two unrelated PI 3-kinase inhibitors, were used in platelet secretion and aggregation assays. As reported previously (23), platelet aggregation induced by FcγRIIA cross-linking was fully inhibited by low doses of wortmannin, whereas, in similar conditions, platelet aggregation induced by TRAP became reversible and thrombin-induced aggregation was not significantly affected (Fig. 2A). Similar data were obtained with LY294002, which was able to fully inhibit FcγRIIA-mediated aggregation (95 ± 5% inhibition at 25 μM). Interestingly, platelet secretion evoked by FcγRIIA cross-linking was also strongly inhibited by wortmannin or LY294002 in a dose-dependent manner (Fig. 2B). These results clearly indicated a critical role for PI 3-kinase in the early steps of FcγRIIA-dependent platelet aggregation.

Under these conditions (10 nM wortmannin), the synthesis of both PtdIns(3,4,5)P3 and PtdIns(3,4)P2 was totally suppressed, while no change was observed for the other phosphoinositides (data not shown). However, interestingly, PtdOH production was almost abolished in platelets challenged with FcγRIIA cross-linking, whereas thrombin-induced accumulation of PtdOH remained insensitive to wortmannin (Fig. 3, A and B). Inhibition of PtdOH and D3-phosphoinositide production displayed very similar dose-response curves using the two inhibitors of PI 3-kinase, with IC50 of 4 nM and 2 μM for wortmannin and LY294002, respectively (Fig. 3C). These values are comparable to those determined for inhibition of serotonin secretion (6 nM and 5 μM, see Fig. 2B). Finally, myrecithine, a natural flavonoid with inhibitory activity toward PI 3-kinase (39), also blocked PtdOH formation. 2

Specific inhibition of PI 3-kinase thus appeared to secondar-

2 M.-P. Gratacap, B. Payrastre, C. Viala, G. MaucO, M. Plantavid, and H. Chap, unpublished observations.
ily block PLC activation evoked by FcγRIIA cross-linking, at a variance with the signaling pathway evoked by thrombin. As a main difference between thrombin and FcγRIIA cross-linking, the former activates PLC-β2 and -β3, which are regulated by heterotrimeric G-proteins and are present in significant amounts in platelets (40, 41). In contrast, PLC-γ2 appears as an essential intermediate in the signaling pathway evoked downstream of FcγRIIA, which involves its tyrosine phosphorylation presumably by Syk (25, 42). Indeed, inhibition of Syk by a specific inhibitor, piceatannol, was found to inhibit secretion, aggregation, and PtdOH production induced by FcγRIIA cross-linking, the same effects being reproduced with the specific PLC inhibitor U73122 (data not shown). This led us to explore the effect of wortmannin on the tyrosine phosphorylation of PLC-γ2.

PI 3-Kinase Regulates PLC-γ2 by a Mechanism Independent of Its Tyrosine Phosphorylation—We first investigated the whole pattern of phosphotyrosyl proteins in platelets stimulated by FcγRIIA cross-linking. As shown in Fig. 4A, this was not affected even at a high concentration of wortmannin (100 nM). In contrast to thrombin, FcγRIIA cross-linking promoted its own transient tyrosine phosphorylation, as reported by other
PI 3-Kinase Triggers the Stable Interaction of Cytosolic PLC-γ2 to the Platelet Membrane/Cytoskeleton—In a first series of experiments, the cytoskeleton from platelets stimulated by FcγRIIA cross-linking was isolated by precipitation in Triton X-100 according to established procedures (30, 31). To focus on the initial signaling events occurring under these conditions and to avoid major cytoskeletal changes occurring downstream of αIIIβ3 engagement, aggregation was inhibited either by the lack of shaking or upon addition of RGDS. Immunoblotting of cytoskeletal pellet allowed us to observe the appearance of PLC-γ2 upon FcγRIIA cross-linking, thus reflecting the translocation of the enzyme from the cytosol to the cytoskeleton (Fig. 6A). However, this was abolished in the presence of wortmannin (Fig. 6A).

Very similar data were obtained using another experimental procedure. In this case, platelets were permeabilized with saponin under conditions allowing the release of over 92% of the cytoplasmic enzyme lactate dehydrogenase. It was then possible to quantify the amounts of PLC-γ2 remaining associated with the cells, presumably by interacting with the membrane and/or the cytoskeleton. As shown in Fig. 6B, FcγRIIA cross-linking promoted platelet retention of PLC-γ2 only when PI 3-kinase was fully active. These various data suggested that PI 3-kinase products might be implicated in the behavior of PLC-γ2 in platelets stimulated by FcγRIIA cross-linking. The possibility that PLC-γ2 might directly interact with D3-phosphoinositides was thus examined.

**Fig. 4. Effects of PI 3-kinase inhibitors on protein tyrosine phosphorylation evoked upon FcγRIIA-mediated activation of human platelets.** Platelets were preincubated in the absence or in the presence of wortmannin (10 nM) or LY294002 (10 μM), and stimulated by FcγRIIA cross-linking by thrombin (1 IU/ml). A, immunoblotting of platelet whole proteins with anti-phosphotyrosine antibody 4G10. B, PLC-γ2 was immunoprecipitated and submitted to immunoblotting with 4G10 antibody (a–c) or with anti-PLC-γ2 antibody (d). Incubation conditions were as follows: a and b, no wortmannin; b, wortmannin; c, LY294002. C, the intensity of the spots shown in a and b was quantified by densitometric analysis. DMSO, Me2SO. Data (percentages of maximal phosphorylation of PLC-γ2 occurring at 1 min) are means ± standard errors (three independent experiments).
lated from resting or activated platelets. To see if this observation was of significant relevance, we then tested whether PtdIns(3,4,5)P$_3$ was able to relieve the inhibition of PLC-γ2 observed in wortmannin-treated platelets.

In a last series of experiments, $^{32}$P-labeled platelets were permeabilized with saponin and challenged by FcγRIIA cross-linking. Although their responses were reduced compared with intact platelets, these conditions allowed us to detect a stimulation-dependent production of PtdOH. Apparently, permeabilization induced some spontaneous activation of PLC in resting platelets, resulting in a lower increase of PtdOH radioactivity in intact platelets, these conditions allowed us to detect a stimulation-dependent production of PtdOH.

**DISCUSSION**

Combined to other data previously reported in the literature, the results obtained in the present study allow us to describe a sequence of signaling events occurring during FcγRIIA-mediated platelet activation and illustrated in Fig. 8. Cross-linking of FcγRIIA leads immediately to tyrosine phosphorylation of its ITAM sequences, which allows recruitment and activation of the tyrosine kinase Syk (23, 25). There is some evidence that Syk then interacts with PI 3-kinase (23), which is recruited to the membrane and activated, leading to the rapid accumulation of PtdIns(3,4,5)P$_3$. Another downstream effector of Syk has been identified as PLC-γ2, which is converted into an active form upon tyrosine phosphorylation, although this might involve additional protein-tyrosine kinases such as Bruton’s tyrosine kinase (Btk), as shown for the B cell receptor (49–51).

In the following steps, PLC-γ2 catalyzes the hydrolysis of PtdIns(4,5)P$_2$ into diacylglycerol and inositol 1,4,5-trisphosphate, which then separately activate a number of enzymes involved in the generation of 32P-labeled platelets were used and the radioactivity of PtdOH was quantified by densitometric analysis. Data are means ± standard errors (three experiments).

**FIG. 5.** Inhibition of aggregation impairs tyrosine dephosphorylation without affecting PLC activity in platelets stimulated by FcγRIIA cross-linking. Platelets were incubated in the absence or in the presence of RGDS (200 μg/ml) and stimulated by FcγRIIA cross-linking with or without shaking, which is necessary for aggregation to occur. A, PLC-γ2 was immunoprecipitated and checked by immunoblotting with the anti-phosphotyrosine 4G10 antibody as in Fig. 4. B, $^{32}$P-labeled platelets were used and the radioactivity of PtdOH was determined as in Fig. 3. Data (±fold increase compared with nonstimulated platelets) are means of two independent experiments.

**FIG. 6.** Wortmannin inhibits translocation of PLC-γ2 to the cytoskeleton and (or) the cytosol-depleted FcγRIIA-activated platelets. A, Platelets were stimulated for indicated times by FcγRIIA cross-linking under nonshaking conditions (NS, ●), in the presence of RGDS (200 μg/ml) (■) or of wortmannin (10 nm) (▲). At the end of the incubation, cytoskeletons were precipitated by addition of Triton X-100 and centrifugation as described under “Experimental Procedures.” Cytoskeletal proteins were then probed by immunoblotting and collected by centrifugation as described under “Experimental Procedures.” The cell pellet was then probed by immunoblotting with anti-PLC-γ2 antibody, and the intensity of the spots was quantified by densitometric analysis. Data are means ± standard errors (three independent experiments). B, platelets preincubated or not with 10 nm wortmannin were stimulated by FcγRIIA cross-linking. Incubations were blocked by addition of saponin (30 μg/ml), followed by centrifugation as described under “Experimental Procedures.” The cell pellet was then probed by immunoblotting with anti-PLC-γ2 antibody, and the intensity of the spots was quantified as in A. DMSO, Me$_2$SO. Data are means ± standard errors (three experiments).
Fig. 7. PtdIns(3,4,5)P$_3$ interacts with PLC-γ and overcomes the inhibitory effect of wortmannin on PLC activity. A, PLC-γ was immunoprecipitated from resting (lane 2) or activated (lane 3) platelets and incubated with a mixture of $^{32}$P-labeled PI 3-kinase substrates, unlabeled phosphoinositides and phosphatidylyserine as indicated under "Experimental Procedures." The phosphoinositides that remained associated with immunoprecipitated PLC-γ after several washing steps were extracted, separated by TLC, and detected by autoradiography. Shown is a representative result from three different experiments with very similar data. Lane 1, total $^{32}$P-labeled phosphoinositides incubated with the different immune complex; lane 4, control with nonimmune complex. B, $^{32}$P-labeled platelets were incubated with or without wortmannin (10 nM, 15 min, 37 °C), then permeabilized with saponin (20 μg/ml) and activated by FcγRIIA cross-linking in the absence or in the presence of diC16-PtdIns(3,4,5)P$_3$ or diC16-PtdIns(3,4)P$_2$ (15 μM) as described under "Experimental Procedures." After 1 min of stimulation, lipids were extracted and the radioactivity of PtdOH was determined as in Fig. 3. Data (percentage of PtdOH increase, over control) are means ± standard errors of three independent experiments. α, significant difference (p < 0.002) with platelets stimulated in the absence of wortmannin; b, significant difference (p < 0.041) with platelets stimulated in the presence of wortmannin (Student's t test).

To ascertain our pharmacological evidence that PI 3-kinase plays a crucial role in the activation of PLC-γ2 evoked by FcγRIIA cross-linking, it is important to notice that wortmannin and LY294002, which act on PI 3-kinase by different mechanisms, were added at concentrations previously shown to be specific for PI 3-kinase and used to demonstrate a role of this enzyme in various cell responses including histamine secretion as secretion and aggregation induced by FcγRIIA cross-linking; 2) accumulation of PtdIns(3,4,5)P$_3$ precedes the formation of PtdOH; 3) in the absence of PI 3-kinase inhibitors, phosphorylation of PLC-γ2 coincides with the time where the enzyme is fully active, as detected by the accumulation of PtdOH; 4) whatever the step involved (PI 3-kinase or integrin αIIb/β3), inhibition of aggregation abolishes the secondary dephosphorylation of PLC-γ2; 5) the inhibition of PI 3-kinase can be overcome by introduction, into permeabilized platelets, of PtdIns(3,4,5)P$_3$, which was found to bind specifically to PLC-γ2.

As secretion and aggregation induced by FcγRIIA cross-linking; 2) accumulation of PtdIns(3,4,5)P$_3$ precedes the formation of PtdOH; 3) in the absence of PI 3-kinase inhibitors, phosphorylation of PLC-γ2 coincides with the time where the enzyme is fully active, as detected by the accumulation of PtdOH; 4) whatever the step involved (PI 3-kinase or integrin αIIb/β3), inhibition of aggregation abolishes the secondary dephosphorylation of PLC-γ2; 5) the inhibition of PI 3-kinase can be overcome by introduction, into permeabilized platelets, of PtdIns(3,4,5)P$_3$, which was found to bind specifically to PLC-γ2.

Two recent studies have shown specific interactions between PLC-γ1 and PtdIns(3,4,5)P$_3$, but depending on the authors this might involve either the N-terminal PH domain or the SH2 domains of the protein (18, 19). The present investigation was not aimed at identifying which region of PLC-γ2 participates in binding to PtdIns(3,4,5)P$_3$ but one may suppose that, actually, both domains might be involved. Whatever the precise mechanism would be, PtdIns(3,4,5)P$_3$ synthesis was found to be required for the association of PLC-γ2 with either Triton X-100-insoluble cytoskeleton or the whole particulate fraction remaining after cell permeabilization. A critical role of cytoskeleton in the signaling events described in the present study is strongly suggested by the recent observation that the small GTPase Rho, which stabilizes focal adhesion plaques and promotes the generation of stress fibers (56), tightly regulates calcium signaling and phagocytosis evoked by macrophage Fcγ receptors (57). It is difficult at this stage to conclude whether PLC-γ2 interacts with PtdIns(3,4,5)P$_3$ bound to cytoskeletal proteins, as repeatedly observed for this and other phosphoinositides (58), or whether some membrane domains remained associated with the platelet cytoskeleton, thus suggesting that

![Diagram of FcγRIIA clustering](image)
an appropriate localization of the enzyme might be at specific contact points between membrane and cytoskeleton. Identification of the fine structure of membrane/cytoskeleton microdomains targeting PLC to its substrate via PtdIns(3,4,5)P₃ will require additional investigations.

In studies mentioned above, interaction of PLC-γ1 with PtdIns(3,4,5)P₃ was used to explain how PI 3-kinase regulates the activity of PLC-γ1 in cells stimulated with platelet-derived growth factor, which involves a membrane receptor displaying intrinsic protein-tyrosine kinase activity (18, 19). In the present work, we obtained the first evidence that PI 3-kinase is absolutely required for the activation of PLC-γ2 by FcγRIIA cross-linking, the tyrosine phosphorylation of PLC-γ2 being necessary but not sufficient for its activation. It will thus be important to see whether this can be extended to different receptors acting by a similar mechanism in various immune cells, for instance other FcγR, FcεR, T cell antigen receptors, and B cell antigen receptors. A recent report indicates that this is the case for the B cell surface glycoprotein CD19 induced PLCγ isozyme activation (59).

Another example giving indirect evidence of a similar mechanism, i.e. human neutrophils where calcium mobilization triggered by FcγR clustering also requires PI 3-kinase activity (60). However, upon antigen stimulation of RBL-2H3 mast cells, PI 3-kinase is required for activation, translocation and also tyrosine phosphorylation of PLCγ1 whereas it has no effect on PLCγ2 activation (61). As we have observed for PLCγ2 in platelets stimulated by FcγRIIA cross-linking, upon platelet-derived growth factor treatment of transfected COS-1 cells, PI 3-kinase does not regulate the activity of PLCγ1 at the level of tyrosine phosphorylation but rather allow a stable interaction of PLCγ1 to the membrane probably leading to efficient substrate hydrolysis (18). Bae et al. (19) have observed that in vitro PtdIns(3,4,5)P₃ is sufficient, at elevated concentration, to activate PLCγ1 and PLCγ2; they also noticed that production of PtdIns(3,4,5)P₃ within the cell does not always result in PLCγ activation. Altogether, these results suggest that, according to the model and the agonist used, PLCγ isozymes may be stimulated by different events including tyrosine phosphorylation, PtdIns(3,4,5)P₃ production and subtle compartmentalization mechanisms that seems to be critical for the modulation of their regulation.

In conclusion, we took advantage of the use of a simple cellular model, human platelets, which express a single class of FcγR, to elucidate a very crucial point of cell signaling involving tight regulation of PLCγ2 by PtdIns(3,4,5)P₃ generation upon activation of PI 3-kinase. This mechanism is an obligatory step in FcγRIIA cross-linking-dependent platelet activation and might also function in the signaling cascade evoked by other membrane receptors containing ITAM.

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