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Phosphorylation of the Platelet p47 Phosphoprotein Is Mediated by the Lipid Products of Phosphoinositide 3-Kinase*

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From the ‡Department of Medicine, Division of Signal Transduction, Beth Israel Hospital and Department of Cell Biology, Harvard Medical School, Boston Massachusetts 02115, §§Divisions of Experimental Medicine and Hematology-Oncology, Department of Medicine, Brigham and Women's Hospital, and Department of Anatomy and Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, ¶INSERM U428, Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris V, 75270 Paris, France, ||Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536, and the **University of Texas Southwestern Medical Center, Dallas, Texas 75235

Platelet stimulation by thrombin or the thrombin receptor activating peptide (TRAP) results in the activation of phosphoinositide 3-kinase and the production of the novel polyphosphoinositides phosphatidylinositol 3,4-bisphosphate (PtdIns-3,4-P₂) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P₃). We have shown previously that these lipids activate calcium-independent protein kinase C (PKC) isoforms *in vitro* (Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M. and Cantley, L. C. (1994) *J. Biol. Chem.* 269, 32358–32367). Activation of platelet PKC in response to TRAP is detected by the phosphorylation of the major PKC substrate in platelets, the p47 phosphoprotein, also known as pleckstrin. Here we provide evidence for two phases of pleckstrin phosphorylation in response to TRAP. A rapid phase of pleckstrin phosphorylation (<1 min) precedes the peak of PtdIns-3,4-P₂ production and is unaffected by concentrations of wortmannin (10–100 nM) that block production of this lipid. However prolonged phosphorylation of pleckstrin (>2 min) is inhibited by wortmannin concentrations that block PtdIns-3,4-P₂ production. Phorbol ester-mediated pleckstrin phosphorylation was not affected by wortmannin and wortmannin had no effect on purified platelet PKC activity. Phosphorylation of pleckstrin could be induced using permeabilized platelets supplied with exogenous γ -³²P[ATP] and synthetic dipalmitoyl PtdIns-3,4,5-P₃ and dipalmitoyl PtdIns-3,4-P₂ micelles, but not with dipalmitoyl phosphatidylinositol 3-phosphate or phosphatidylinositol 4,5-bisphosphate. These results suggest two modes of stimulating pleckstrin phosphorylation: a rapid activation of PKC (via diacylglycerol and calcium) followed by a slower activation of calcium-independent PKCs via PtdIns-3,4-P₂.

The activation of phosphoinositide 3-kinase (PI 3-K)¹ in agonist-stimulated cells results in the rapid formation of the two novel polyphosphoinositides PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃. A critical requirement for PI 3-K activation in a variety of cellular functions has been established. These include growth factor dependent mitogenesis, chemotaxis, receptor down-regulation, insulin-induced glucose transport, and actin-filament rearrangements leading to membrane ruffling (for a review, see Ref. 1). Despite these correlations, the direct targets for these polyphosphoinositides remain undescribed. PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ have been proposed to act as second messengers as they are not hydrolyzed by any known phospholipase type C enzymes (2, 3). The other product of PI 3-K activity, PtdIns-3-P, does not increase in agonist-stimulated cells and may be important in intracellular protein sorting mechanisms (4). Two enzymes, pp70S6 kinase (5) and the serine-threonine protein kinase Akt (6, 7) have been shown to be downstream of activated PI 3-K, but there is no evidence that these are the immediate targets of PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃. Recent data from our laboratory points to the calcium-independent isoforms of PKC (δ , ϵ , and η) as direct targets of these lipids (8), although *in vivo* evidence for this is still lacking. The diacylglycerol-insensitive isoform PKC ζ may also be a target for these phosphoinositides (9).

Thrombin activation of platelets results in the rapid activation of PI 3-K (10, 11). The major product of PI 3-K in activated platelets is PtdIns-3,4-P₂, which peaks at 2–3 min following stimulation. A small peak of PtdIns-3,4,5-P₃ at 30 s to 1 min has also been reported (11). Although the exact function of these novel phosphoinositides in platelet activation is undescribed, we recently established a critical requirement for PI 3-K activation in integrin-mediated platelet aggregation, leading to activation of the integrin GPIIb-IIIa (12). Furthermore, PI 3-K activation occurs both upstream and downstream of the

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¹ The abbreviations used are: PI 3-K, phosphoinositide 3-kinase; PKC, protein kinase C; PtdIns, phosphatidylinositol; PtdIns-3-P, phosphatidylinositol 3-phosphate; 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; DiC₈PtdIns-3,4,5-P₃, dioctanoyl phosphatidylinositol 3,4,5-trisphosphate; DiC₁₆PtdIns-3-P, dipalmitoyl phosphatidylinositol 3-phosphate; DiC₁₆PtdIns-3,4-P₂, dipalmitoyl phosphatidylinositol 3,4-bisphosphate; DiC₁₆PtdIns-3,4,5-P₃, dipalmitoyl phosphatidylinositol 3,4,5-trisphosphate; DAG, 1,2-dioctanoyl-sn-glycerol; TRAP, thrombin receptor activating peptide; BSA, bovine serum albumin; GP, glycoprotein; PH, pleckstrin homology; PGE₁, prostaglandin E₁; PMA, phorbol 12-myristate 13-acetate; vWF, von Willebrand factor.

integrin, suggesting that this enzyme contributes both to "inside-out" and "outside-in" signaling in platelets. Using the potent PI 3-K inhibitor wortmannin, we also showed that there is no critical requirement for PI 3-K in mediating actin assembly in response to thrombin-receptor activation (12), suggesting that the D4 phosphoinositides PtdIns-4-P and PtdIns-4,5-P₂ are the phospholipid mediators of this event in human platelets (13).

PKC is also rapidly activated in thrombin-stimulated platelets. PKC comprises a large family of closely-related serine/threonine protein kinases, classed according to their co-factor requirements (reviewed in Ref. 14). Conventional (α , β I, β II, and γ) family members are dependent on calcium, phospholipid, and diacylglycerol for activation, whereas non-conventional isoforms (δ , ϵ , η (L), θ , and μ) are insensitive to calcium. Atypical PKC ζ and ι/λ are insensitive to diacylglycerol and calcium. An often used measure of PKC activation in agonist-stimulated cells is the phosphorylation of defined substrate proteins. In platelets and other cells of hematopoietic origin, the major PKC substrate is the p47 phosphoprotein, pleckstrin (platelet and leukocyte C kinase substrate), which is rapidly phosphorylated in response to a variety of agonists, including thrombin, thrombin receptor activating peptide (TRAP), as well as phorbol ester (15, 16). The physiological function of pleckstrin is undefined, although recent studies reveal that interaction with polyphosphoinositides such as PtdIns-4,5-P₂ may be mediated by the pleckstrin homology (PH) domain at the N terminus of this protein (17). PH domains may also be involved in protein-protein interactions; in particular, various PKC isoforms have been reported to interact with the PH domains of the Akt protein kinase (18) and the tyrosine kinase Btk (19).

In this report we use the phosphorylation of pleckstrin to study the contribution of PI 3-K lipid products in activating platelet PKC. We present data demonstrating that PI 3-K is essential for a late phase of TRAP-stimulated pleckstrin phosphorylation and, more specifically, that the phosphoinositides PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ mediate this effect.

EXPERIMENTAL PROCEDURES

Materials—Prostaglandin E₁ (PGE₁), wortmannin, staurosporine, bovine serum albumin (BSA), fibrinogen, phorbol 12-myristate 13-acetate (PMA), saponin, and miscellaneous chemical reagents were purchased from Sigma. TRAP (either the 14-residue or the 6-residue form) was obtained from Bachem, King of Prussia, PA. [γ -³²P]Orthophosphoric acid and [γ -³²P]ATP (3000 Ci/mmol) were from DuPont NEN. X-Omat AR5 autoradiography film was purchased from Eastman Kodak Co. Phosphatidylinositol 3-phosphate, dipalmitoyl (DiC₁₆PtdIns-3-P), and phosphatidylinositol 3,4-bisphosphate, dipalmitoyl (DiC₁₆PtdIns-3,4-P₂) were obtained from Matreya Inc, Pleasant Gap, PA. 1-O-(1, 2-Di-O-palmitoyl-sn-glycero-3-phosphoryl)-D-myo-inositol 3,4,5-trisphosphate (DiC₁₆PtdIns-3,4,5-P₃) and 1-O-(1, 2-Di-O-octanoyl-sn-glycero-3-phosphoryl)-D-myo-inositol 3,4,5-trisphosphate (DiC₈PtdIns-3,4,5-P₃) were synthesized as described previously (8, 20). PtdIns-4,5-P₂ was obtained from Upstate Biotechnology, Lake Placid, NY.

Isolation and Activation of Platelets—Human platelets were purified as described previously (12). Human blood was drawn from volunteers who had not ingested aspirin for at least 10 days into 0.1 volume of Aster-Jandl anticoagulant and centrifuged at 200 $\times$ g for 15 min. Platelet-rich plasma, to which 1 μ M PGE₁ was added, was gel-filtered over a Sepharose 2B column (Pharmacia Biotech Inc.) pre-equilibrated with platelet buffer (10 mM Hepes, 145 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 0.5 mM NaH₂PO₄, 10 mM glucose, 0.3% BSA, pH 7.4) at 37 °C. Gel-purified platelets were rested for 60 min at 37 °C before use. The platelet concentration determined on a Coulter counter (Coulter Corp., Dade County, FL) was typically 2.5–3.0 $\times$ 10⁹/ml. Platelets were activated either with 25 μ M TRAP or with 100 nM PMA. Where indicated, wortmannin and staurosporine were preincubated with platelets at 37 °C for 15 and 5 min, respectively, prior to agonist stimulation. Inhibitors were added to platelets from 10 mM stock solutions in dimethyl sulfoxide (Me₂SO). The final concentration of Me₂SO did not

exceed 0.1%.

For experiments involving platelet phospholipid labeling, platelets isolated by two cycles of centrifugation were incubated for 1 h at 37 °C with 2 mCi/ml [³²P]orthophosphoric acid, gel filtered as described above, and allowed to rest for 1 h before use. Phospholipids were extracted as described previously (12, 21). Lipids were quantitated using a Radiomatic A500 on-line radioactivity counter (Packard Instrument Co., Downers Grove, IL).

Purification of Platelet Protein Kinase C—PKC was partially purified from resting platelets by column chromatography on fast protein liquid chromatography (Pharmacia Biotech Inc.) essentially as described previously (22). Briefly, 1 $\times$ 10⁹ resting platelets isolated as described above were lysed in homogenization buffer A (20 mM Tris-HCl, pH 7.0, 2 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 0.25 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride, 4 μ M/ml pepstatin A, 4 μ M/ml leupeptin, 10 mM sodium fluoride). The lysate was cleared by ultracentrifugation at 100,000 $\times$ g for 30 min and the resulting supernatant applied to a 30 ml HiLoad Q Sepharose column (Pharmacia Biotech Inc.) and eluted with a 0–1.0 M NaCl gradient in column buffer (20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol). Fractions were assayed for PKC activity as described previously (8). Peak fractions were used for determining the effects of wortmannin on PKC activity. Activity was assayed by measuring the incorporation of ³²P from [γ -³²P]ATP into histone H1 (type III-S) or the peptide ϵ substrate (Upstate Biotechnology) derived from the pseudosubstrate sequence of PKC ϵ , with an Ala $\rightarrow$ Ser substitution (23). The reaction mixture contained 20 mM Hepes, pH 7.5, 5 mM MgCl₂, 100 μ M CaCl₂, 50 μ M [γ -³²P]ATP, 50 μ M phosphatidylserine and 10 μ M diacylglycerol vesicles, 10 μ g of histone H1 (type III-S), or 30 μ M peptide ϵ . Wortmannin was added to the reaction mixture at the indicated concentrations, and incubated for 15 min. Reactions were started by addition of PKC and incubated at 37 °C for 10 min. Reaction mixtures were spotted onto P81 phosphocellulose paper and washed four times in 500 ml of 1% phosphoric acid, or filtered onto GF/C filters, which were washed with 10% trichloroacetic acid. Incorporation of ³²P was determined by liquid scintillation counting.

Platelet Aggregation Studies—Gel-filtered platelets or ³²P-labeled platelets were added to an aggregation cuvette and the aggregation reaction was started after the addition of 500 μ g/ml fibrinogen and either 25 μ M TRAP or 100 nM PMA under constant stirring conditions, at 37 °C. The reaction was recorded on a Chrono-Log aggregometer (Chrono-Log Corp., Havertown, PA).

Analysis of Pleckstrin Phosphorylation—Platelets were labeled with [³²P]orthophosphoric acid as described above, except that 1 mCi/ml was used. ³²P-Labeled platelets were activated under aggregating conditions in the presence of 500 μ g/ml fibrinogen and the reaction stopped by the addition of SDS-polyacrylamide gel electrophoresis buffer containing β -mercaptoethanol. The samples were analyzed on a 13% SDS-polyacrylamide gel, and after drying, the gels were analyzed by autoradiography followed by densitometry, or on a Bio-Rad model GS363 molecular imager (Bio-Rad).

Platelet Permeabilization Studies—Platelets were permeabilized with saponin according to the method of Authi *et al.* (24) with a few modifications. Platelets isolated by two cycles of centrifugation at 800 $\times$ g in the presence of 1 μ M PGE₁ were resuspended in permeabilization buffer (5 mM Hepes, 1 mM glucose, 1 mM MgCl₂, 0.42 mM NaH₂PO₄, 11.9 mM NaHCO₃, 140 mM KCl, 1 mM EGTA, and 0.037 mM CaCl₂, pH 7.4) at a concentration of 3 $\times$ 10⁸/ml and rested at 37 °C for 30 min before use. 200 μ l of platelets were permeabilized under non-aggregating conditions in the presence of 11 μ g/ml saponin and 100 μ Ci/ml exogenously added [γ -³²P]ATP. At time 0, phosphoinositides were added and platelets incubated at 37 °C for 2 min. The reaction was stopped by addition of SDS sample buffer and pleckstrin phosphorylation analyzed by SDS-polyacrylamide gel electrophoresis as described above.

Phosphoinositides were stored at –70 °C in methanol:chloroform:1 N HCl (2:1:0.1). For permeabilization studies, phosphoinositides were prepared by drying under a stream of nitrogen, followed by resuspension in 20 mM Hepes, 1 mM EGTA, pH 7.5, and sonicated in an ice-cold cup horn bath sonicator at 50% output for 10 min (Branson Ultrasonics, Danbury, CT). Sonicated phosphoinositides were used immediately for permeabilization studies.

RESULTS

Wortmannin Inhibits the TRAP-stimulated Sustained Phosphorylation of the p47 Phosphoprotein Pleckstrin—Under aggregating conditions, wortmannin did not affect the early phosphorylation of p47 pleckstrin in TRAP-stimulated platelets 30 s after addition of TRAP (Fig. 1A). In contrast, wortmannin

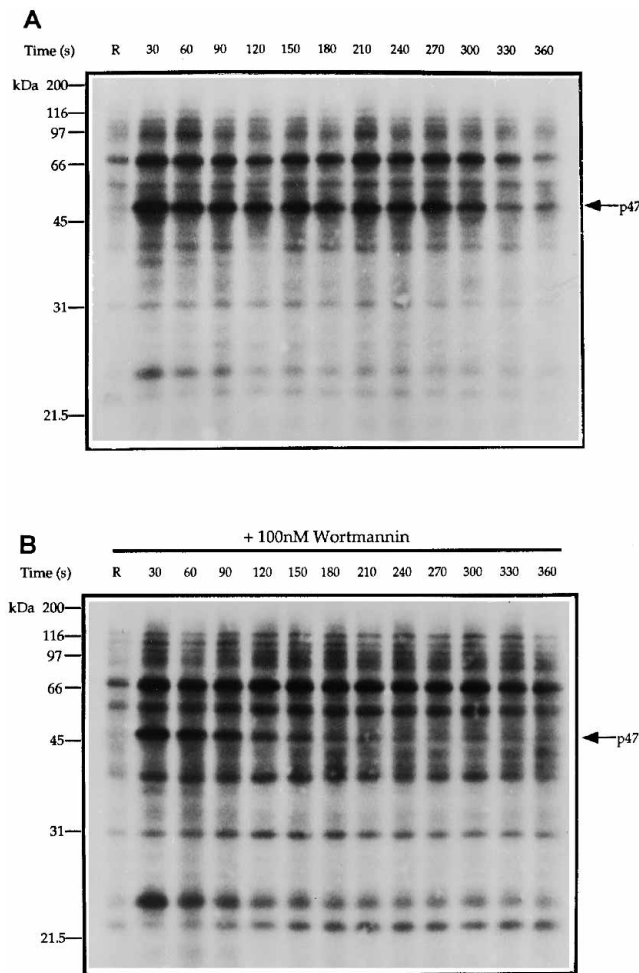


FIG. 1. Wortmannin inhibits the TRAP-stimulated phosphorylation of pleckstrin. 32 P-labeled gel-purified platelets were preincubated with the indicated doses of wortmannin (WM) for 15 min, then stimulated with 25 μ M TRAP in the presence of 500 μ g/ml fibrinogen under stirring conditions. The reaction was terminated by addition of SDS-polyacrylamide gel electrophoresis sample buffer, and heating to 100 $^{\circ}$ C for 5 min. Platelet phosphoproteins were analyzed on a 13% SDS-polyacrylamide gel followed by autoradiography. In *panel A*, platelets were stimulated with TRAP for 30-s intervals up to 6 min prior to lysis. In *panel B*, platelets were preincubated with 100 nM wortmannin, then stimulated with TRAP. The arrow points to the position of pleckstrin, with a molecular mass of 47 kDa, determined according to appropriate standards.

(100 nM) did cause significant inhibition of pleckstrin phosphorylation following 2 min or more of TRAP stimulation (Fig. 1B). A detailed analysis of the kinetics of pleckstrin phosphorylation reveals an initial burst of phosphorylation at 30 s, which is sustained up to 4 min and then begins to decline (Fig. 1A). In platelets pretreated with 100 nM wortmannin then activated with TRAP, there is an inhibition of the sustained phosphorylation of pleckstrin, but there is no effect at 30–90 s (Fig. 1B). 100 nM wortmannin only significantly affects the phosphorylation of p47 pleckstrin in the context of total platelet lysate following TRAP stimulation, leaving other phosphoproteins unaffected.

A dose response of wortmannin inhibition in platelets stimulated for 3 min reveals that doses as low as 10 nM are capable of inhibiting pleckstrin phosphorylation (17%) induced by TRAP (Fig. 2, A and B), and 100 nM wortmannin was maximally effective (61.3% inhibition). This inhibition did not increase with wortmannin concentrations above 100 nM and up to 1 μ M, indicating that other wortmannin-insensitive pathways contribute to the phosphorylation of pleckstrin. The dose-re-

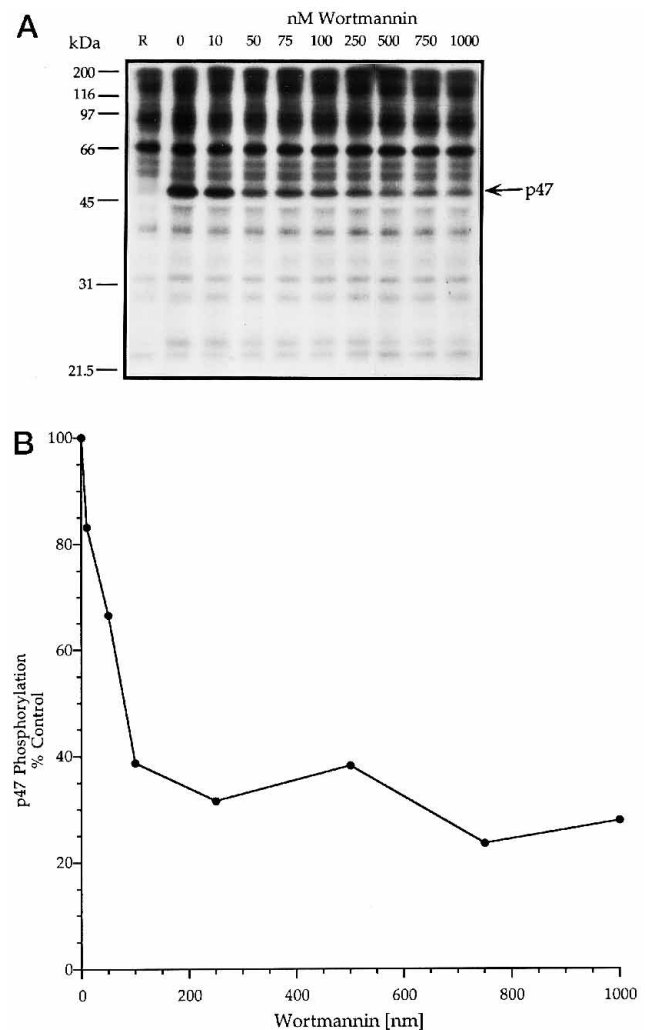


FIG. 2. Dose-response inhibition of pleckstrin phosphorylation in TRAP-stimulated platelets. *A*, platelets were preincubated with increasing doses of wortmannin up to 1000 nM, then stimulated with TRAP for 180 s. Pleckstrin phosphorylation was detected as described in the legend to Fig. 1. *B*, the 47-kDa band in *panel A* was quantified by densitometry and is plotted against increasing concentrations of wortmannin. Inhibition of pleckstrin phosphorylation is plotted as a percentage of control (no wortmannin). The results are representative of six different experiments.

sponse inhibition of pleckstrin phosphorylation by wortmannin closely correlates with that found for TRAP-stimulated PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ production (12). In contrast, concentrations as high as 1 μ M did not inhibit the initial burst of pleckstrin phosphorylation at 30 s following TRAP stimulation (data not shown).

Thrombin Receptor Activation Results in the Accumulation of D3 Phosphoinositides—Activation of PI 3-K in TRAP-stimulated platelets results in the rapid formation of D3 phosphoinositides as judged by reverse-phase high pressure liquid chromatography analysis of the deacylated lipids (Fig. 3A). The kinetics of TRAP-stimulated PI 3-K activation are similar to those previously reported for thrombin stimulation of platelets (10, 11, 25), indicating that TRAP stimulation of platelets results in the same signal transduction pathways that are activated following thrombin stimulation. Unlike other cell types, the major agonist-stimulated D3 phosphoinositide in TRAP-stimulated platelets is PtdIns-3,4-P₂, which peaks at 2–4 min following stimulation. PtdIns-3,4-P₂ accumulation is sustained up to 4 min and then begins to decline. On the other hand, PtdIns-3,4,5-P₃ synthesis is rapid and short-lived, peaking af-

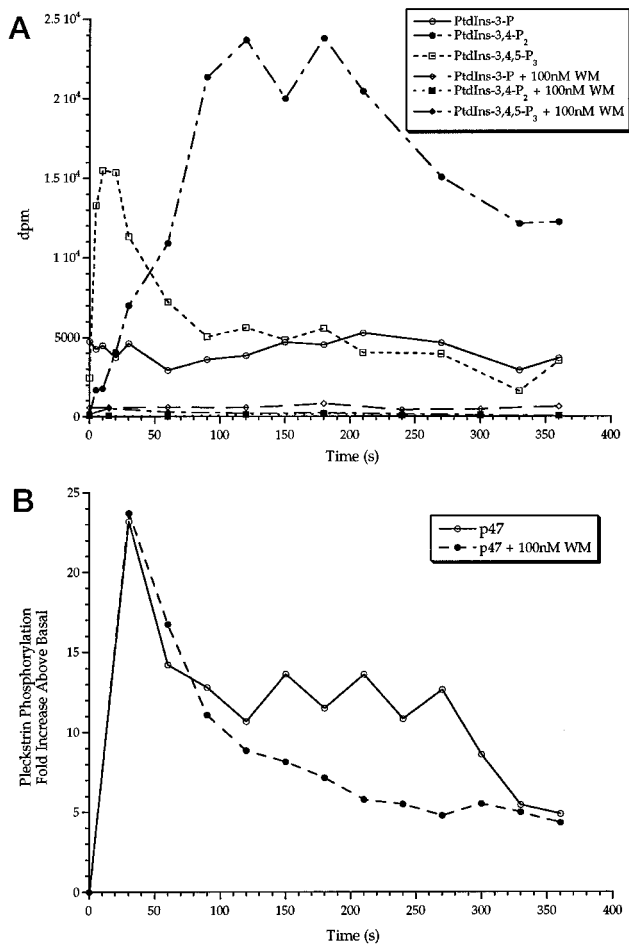


FIG. 3. TRAP stimulates production of D3 phosphoinositides in isolated human platelets. A, 32 P-labeled gel-purified platelets were stimulated with 25 μ M TRAP in the presence of 500 μ g/ml fibrinogen under stirring conditions, and in the presence or absence of 100 nM wortmannin. The reaction was stopped at the appropriate time points by the addition of the lipid extraction mixture. Lipids were extracted and analyzed as described under "Experimental Procedures." B, the 47-kDa bands in Fig. 1 (A and B) were quantified on a Molecular Imager and plotted against the time course of PtdIns-3-P, PtdIns-3,4-P₂, and PtdIns-3,4,5-P₃ production.

ter 15–30 s following stimulation and then decreasing (Fig. 3A). Kucera and Rittenhouse (11) have also reported a peak of PtdIns-3,4,5-P₃ at 30 s in thrombin-stimulated platelets. The breakdown of PtdIns-3,4,5-P₃ correlates temporally with the synthesis of PtdIns-3,4-P₂. This finding suggests that the appearance of the latter may in part be due to the action of a 5' phosphatase on PtdIns-3,4,5-P₃, as has been reported in formyl peptide-stimulated neutrophils (26), as well as thrombin-stimulated platelets (27). The levels of PtdIns-3-P in TRAP-stimulated platelets do not change dramatically following activation, consistent with results obtained in other cell types (28). The fungal metabolite wortmannin has been extensively used as a potent and specific inhibitor of PI 3-K activity in a wide variety of cell types. We have previously reported that low concentrations of wortmannin inhibit the production of PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ in TRAP-stimulated platelets, without affecting the synthesis of PtdIns-4-P or PtdIns-4,5-P₂ (12). Wortmannin inhibition of a $\beta\gamma$ -sensitive PI 3-K activity in platelets has also been reported with an IC₅₀ of 5 nM (29). In this study, wortmannin inhibited the TRAP-stimulated accumulation of D3 phosphoinositides in all time points up to 6 min (Fig. 3A).

The sustained production of PtdIns-3,4-P₂ therefore correlates temporally with sustained pleckstrin phosphorylation in

response to TRAP. This correlation is further examined in Fig. 3B. Between 2 and 4 min, activation of the thrombin receptor with TRAP under aggregating conditions results in the sustained accumulation of PtdIns-3,4-P₂ and the sustained phosphorylation of p47. Both of these events are inhibited in platelets pretreated with 100 nM wortmannin. The decline in PtdIns-3,4-P₂ production at 4–6 min also correlates with a decline of p47 phosphorylation. The initial burst of pleckstrin phosphorylation also correlates with the appearance of PtdIns-3,4,5-P₃, but at this early time point (30 s), p47 phosphorylation is not affected by wortmannin pretreatment. Activation of PKC from DAG synthesis has been reported at 30–60 s (30), and this may account for pleckstrin phosphorylation at these early times, although it is likely that PtdIns-3,4,5-P₃ may also contribute.

Wortmannin Has No Effect on Platelet PKC or on Phorbol Ester-stimulated Pleckstrin Phosphorylation—As p47 pleckstrin is the major PKC substrate in platelets, it was important to determine that wortmannin inhibition of pleckstrin phosphorylation is not due to a nonspecific inhibition of PKC activity in TRAP-stimulated platelets. This was achieved by two separate approaches. First, p47 phosphorylation was analyzed in platelets stimulated with phorbol ester, which directly activates PKC independent of second messenger production. Under aggregating conditions, there was no effect of wortmannin on pleckstrin phosphorylation in platelets stimulated with PMA at 1 or 4 min following stimulation (Fig. 4A). In contrast, the potent protein kinase inhibitor staurosporine showed a significant reduction in the PMA-stimulated phosphorylation of pleckstrin. This result suggests that wortmannin inhibition of pleckstrin phosphorylation in agonist-stimulated platelets is not due to a direct inhibition of PKC activity.

Second, PKC was partially purified from resting platelets by column chromatography and assayed in the presence of increasing concentrations of wortmannin. Using concentrations as high as 10 μ M, there was no effect of wortmannin on the ability of platelet PKC to phosphorylate either histone III-S or the peptide ϵ substrate (Fig. 4B). Histone III-S was used to assay for conventional calcium-dependent PKCs (α , β , and γ). However, histone III-S is a poor substrate for non-conventional and atypical PKCs (δ , ϵ , η , θ , ζ , and λ) and therefore peptide ϵ , based on the PKC ϵ pseudosubstrate sequence was used (23). These results are in agreement with previous observations where wortmannin failed to significantly affect PKC activity (22, 31, 32).

Synthetic PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ Stimulate Pleckstrin Phosphorylation in Permeabilized Platelets—Thus, the critical question is whether the lipid products of PI 3-K can circumvent the wortmannin inhibition of pleckstrin phosphorylation. We investigated whether addition of PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ to permeabilized platelets could induce pleckstrin phosphorylation. Platelets were permeabilized with saponin and provided with exogenous [γ - 32 P]ATP to monitor only those cells that had been permeabilized. Addition of two different synthetic phosphoinositides, a long-chain dipalmitoyl PtdIns-3,4,5-P₃ (DiC₁₆PtdIns-3,4,5-P₃) and a water-soluble, short-chain dioctanoyl PtdIns-3,4,5-P₃ (DiC₈PtdIns-3,4,5-P₃) induced pleckstrin phosphorylation (Fig. 5A). Pleckstrin phosphorylation could also be induced by TRAP and PMA stimulation, indicating that agonist-mediated signaling from the thrombin receptor to pleckstrin phosphorylation was intact in these permeabilized cells. Importantly, 100 nM wortmannin also caused inhibition of pleckstrin phosphorylation following TRAP stimulation in this permeabilized platelet assay. In contrast, PtdIns-3,4,5-P₃-mediated pleckstrin phosphorylation was insensitive to wortmannin, indicating that the phosphoinositides are acting directly to induce pleckstrin phosphoryla-

FIG. 4. Phorbol ester-stimulated pleckstrin phosphorylation is not inhibited by wortmannin. A, platelets were preincubated with the indicated doses of wortmannin for 15 min, or staurosporine for 5 min. After the addition of 500 μ g/ml fibrinogen, platelets were activated with 100 nM PMA for the times indicated. The reaction was stopped, and platelet phosphoproteins were detected as described in the legend to Fig. 2. The arrow points to the position of pleckstrin. B, PKC purified from resting platelets was preincubated with the indicated doses of wortmannin for 5 min, then assayed for the ability to transfer 32 P from [γ - 32 P]ATP into histone III-S or peptide ϵ as described under "Experimental Procedures." section. The results are plotted as percentage of stimulation above basal, in the absence of phosphatidylserine/DAG.

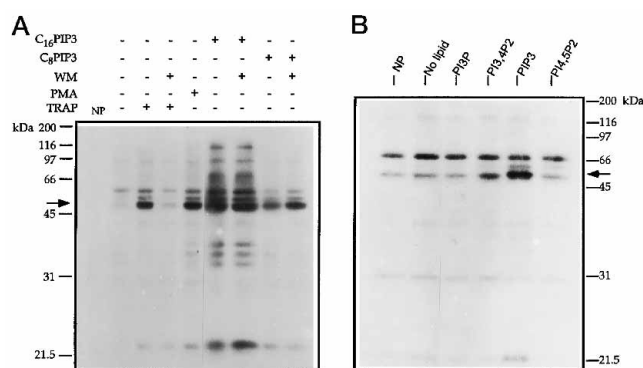
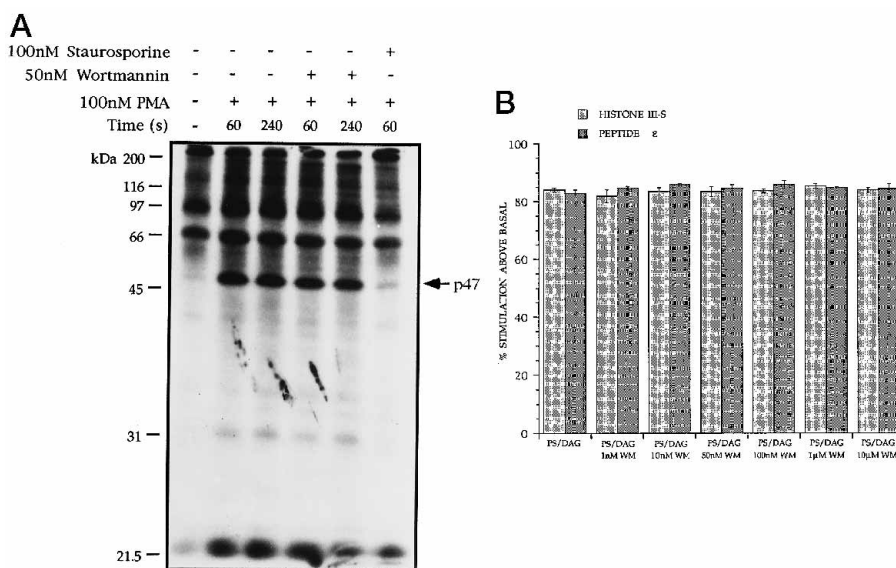


FIG. 5. Synthetic PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ induce pleckstrin phosphorylation in permeabilized platelets. Isolated human platelets were permeabilized with saponin as described under "Experimental Procedures," in the presence of exogenously-added [γ - 32 P]ATP. Under non-aggregating conditions, 25 μ M TRAP in the presence or absence of 100 nM wortmannin (WM) or 100 nM PMA were added to detect pleckstrin phosphorylation. A, synthetic short-chain DiC₈PtdIns-3,4,5-P₃ (C₈PIP₃, 5 μ M) was presented to the permeabilized platelets as a free monomer in solution, in untreated or wortmannin (WM, 100 nM) pretreated platelets. Similarly, long-chain DiC₁₆PtdIns-3,4,5-P₃ (C₁₆PIP₃, 5 μ M) was sonicated in the absence of carrier phospholipids and presented as micelles in untreated or wortmannin pretreated platelets (NP, non-permeabilized platelets). B, the specificity of phosphoinositide-mediated pleckstrin phosphorylation was assayed using synthetic DiC₁₆PtdIns-3-P (PI3P), DiC₁₆PtdIns-3,4-P₂ (PI3,4P₂), DiC₁₆PtdIns-3,4,5-P₃ (PIP₃), or PtdIns-4,5-P₂ (PI4,5-P₂) micelles (5 μ M) in the absence of carrier phospholipids, as described above. The arrow points to the position of pleckstrin. The results are representative of four separate experiments.

tion, rather than by acting on a cell surface receptor to activate PI 3-K. In addition, pleckstrin phosphorylation was only induced by the D3 phosphoinositides PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃, not by PtdIns-3-P or PtdIns-4,5-P₂ (Fig. 5B), showing specificity for D3 phosphoinositides that are synthesized only after agonist stimulation of cells. The likely targets of the D3 phosphoinositides are the calcium-independent PKCs as synthetic D3 phosphoinositides activate the calcium-independent PKC family members δ , ϵ , and η in *in vitro* assays (8).²

Phorbol Ester Can Rescue Wortmannin Inhibition of Irreversible Platelet Aggregation—Finally, we further investigated the role of PI 3-K and PKC in the platelet aggregation response to TRAP. PI 3-K plays a central role in platelet aggregation

through the glycoprotein GPIIb-IIIa and appears to be activated both upstream and downstream of this integrin (12). As we previously reported, preincubation of platelets with wortmannin followed by TRAP stimulation leads to an initial aggregation, followed by disaggregation of the platelets (Ref. 12 and Fig. 6A). Aggregation in the presence of wortmannin could be rescued to control levels by the addition of the phorbol ester PMA, showing that direct activation of PKC to bypass wortmannin inhibition of PI 3-K leads to GPIIb-IIIa activation and fibrinogen binding (Fig. 6A). In similar fashion, wortmannin had no effect on aggregation when platelets were activated using both TRAP and PMA (Fig. 6A). Phorbol ester alone can induce platelet aggregation in the presence of fibrinogen, although the rapid initial wave of aggregation induced by thrombin or TRAP is not observed. Staurosporine pretreatment completely abolished PMA-induced aggregation, but wortmannin was without effect (Fig. 6B), consistent with the inability of wortmannin to directly inhibit PKC.

DISCUSSION

The results presented in this paper show that activation of the thrombin receptor in isolated platelets results in the sustained phosphorylation of the PKC substrate p47 pleckstrin. This event closely correlates with the sustained accumulation of the PI 3-K lipid product, PtdIns-3,4-P₂ (Fig. 3). Pretreatment of platelets with the potent PI 3-K inhibitor wortmannin leads to a loss of the sustained phosphorylation of pleckstrin and inhibition of the sustained synthesis of PtdIns-3,4-P₂. An initial burst of pleckstrin phosphorylation is also observed at 30 s to 1 min, but this is not affected by wortmannin concentrations as high as 1 μ M, suggesting that PKC activation by diacylglycerol and/or other PI 3-K-insensitive pathways are responsible for mediating this early event. Both DAG and PtdIns-3,4-P₂ may contribute at later times as wortmannin only inhibits 61% of TRAP-stimulated pleckstrin phosphorylation.

Wortmannin inhibition of pleckstrin phosphorylation has previously been reported, but has provided conflicting results. Yatomi *et al.* (22) have reported inhibition of platelet pleckstrin phosphorylation in response to suboptimal doses of thrombin and phorbol ester stimulation in wortmannin-treated platelets. Hashimoto *et al.* (33), however, failed to reproduce the wortmannin inhibition of pleckstrin phosphorylation in response to phorbol ester. In this report, we have found no evidence for a direct inhibition of wortmannin on PKC. Phorbol ester-induced pleckstrin phosphorylation was not inhibited in platelets pre-

² A. Toker, unpublished observations.

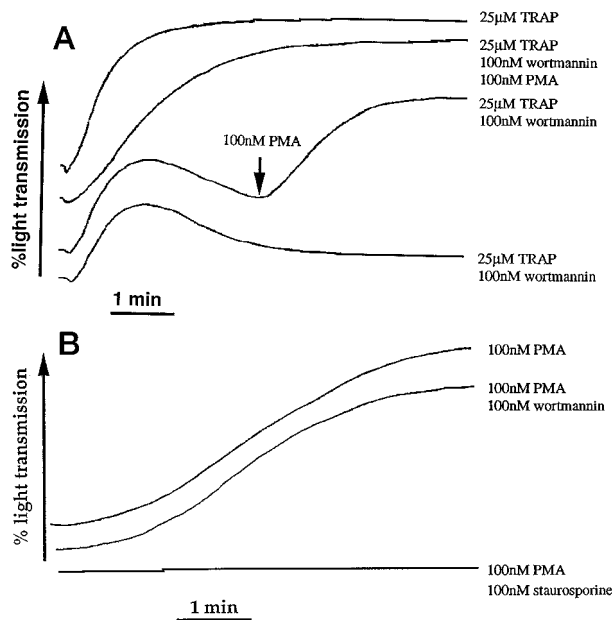


FIG. 6. Phorbol ester can overcome wortmannin inhibition of irreversible platelet aggregation. Gel-filtered platelets were preincubated with 100 nM wortmannin (WM) for 15 min or with 100 nM staurosporine for 15 min prior to stimulation where indicated. After the addition of 500 μg/ml fibrinogen, aggregation was started by the addition of 25 μM TRAP (A) or 100 nM PMA (B) under constant stirring. At the indicated time point, 100 nM PMA was added to the aggregation cuvette (A). The tracings are representative of three experiments.

incubated with wortmannin, although a complete inhibition was observed with the protein kinase inhibitor staurosporine (Fig. 4). Similarly, purified platelet PKC was not inhibited with wortmannin concentrations as high as 10 μM in *in vitro* assays. These results are in agreement with results from other laboratories (31–33).

The specificity of wortmannin as a PI 3-K inhibitor is of particular relevance to these studies, as a recent report described a hormone-stimulated PI 4-kinase activity that is sensitive to 100 nM wortmannin (34). We have shown that treatment of platelets with 100 nM wortmannin does not affect the synthesis of PtdIns-4-P and PtdIns-4,5-P₂ induced by TRAP (12). Synthesis of the D3 phosphoinositides, however, was completely abolished by 100 nM wortmannin. Wortmannin-sensitive PI 4-K activity is therefore either absent in platelets or not stimulated by activation of the thrombin receptor.

Based on the observations that sustained PtdIns-3,4-P₂ production correlates with pleckstrin phosphorylation and our previous results showing activation of calcium-independent PKCs δ, ε, and η (8), we devised a permeabilization scheme to introduce phosphoinositides into platelets and measure pleckstrin phosphorylation. In this assay, both TRAP and PMA were able to stimulate pleckstrin phosphorylation in platelets provided with exogenous [γ -³²P]ATP. Both PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ were also able to stimulate pleckstrin phosphorylation above control levels (Fig. 5). Neither PtdIns-3-P nor PtdIns-4,5-P₂ was able to stimulate pleckstrin phosphorylation, suggesting that this event is limited to those phosphoinositides which accumulate in TRAP-stimulated platelets. These data provide additional evidence that the lipid products of PI 3-K activate one or more PKC family members, which in turn phosphorylate pleckstrin. This model is further supported by the finding that wortmannin inhibition of irreversible platelet aggregation can be rescued by phorbol ester treatment (Fig. 6), once again implicating PKC as a downstream target of PI 3-K.

The results presented here argue that activation of PKC

family members by TRAP-stimulated PI turnover, by PtdIns-3,4-P₂ synthesis, or by phorbol ester addition can result in similar cell responses. Protein kinase C activation has previously been presumed to result from the agonist-mediated activation of phospholipase activity leading to DAG generation and calcium release from intracellular stores. However, we have observed the lipid products of PI 3-K, PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ to be capable of activating novel, calcium-independent PKCs *in vitro* (8). The atypical PKC family member, PKC ζ has also been shown to be activated by PtdIns-3,4,5-P₃ (9). Both calcium-dependent (α, βI, and βII) and calcium-independent (δ, η, θ, and ζ) PKC family members have been detected in platelets (30, 35–38). PKC ε may also be present in platelets, although only minor amounts were detected by Western immunoblotting (38). A number of groups have investigated the mechanism of PKC activation in stimulated platelets and have provided conflicting results. Baldassare *et al.* (30) showed thrombin activation of human platelets to result in a rapid biphasic increase in DAG mass and correlated this mass increase with translocation of PKC α, β, and ζ to the membrane fraction. However, a subsequent report showed bryostatin stimulation of platelets failed to affect a translocation of PKC α, β, δ, or ζ (36). Bryostatin is a macrocyclic lactone that binds to and activates PKC and induces the phosphorylation of PKC substrate proteins, including pleckstrin. A recent study indicated that DAG levels in resting, unstimulated platelets can fluctuate to levels comparable to that seen with thrombin stimulation but that these fluctuations do not correlate with the phosphorylation of pleckstrin (39). This finding indicates that signaling pathways other than those that lead to DAG production also may promote PKC phosphorylation of pleckstrin. Consistent with this idea, platelets subject to pathological stress and activated in response to von Willebrand factor (vWF) have been shown to induce pleckstrin phosphorylation, without stimulating PtdIns-4,5-P₂ hydrolysis and DAG generation (40). vWF stimulation of platelets has also been shown to lead to PI 3-K activation and translocation to the cytoskeletal fraction (41), suggesting that pleckstrin phosphorylation in response to vWF may be mediated by PI 3-K.

Little is known concerning the function of pleckstrin in agonist-stimulated cells. Although the phosphorylation of pleckstrin correlates with platelet aggregation, there is no direct evidence that it is involved in the activation of the platelet integrin GPIIb-IIIa. The observation that PH domains found in pleckstrin and other signaling proteins bind specifically to phosphoinositides such as PtdIns-4,5-P₂ indicates that pleckstrin may under some conditions be recruited to the plasma membrane (17). It is conceivable that phosphorylation of pleckstrin may affect this interaction or promote its activity. The N-terminal PH domain of pleckstrin was recently shown to inhibit both phospholipase Cβ- and Cγ-mediated phosphoinositide hydrolysis (42). PH domains have also been shown to mediate protein-protein interactions, and several PKC isoforms have now been shown to interact with the PH domains of pleckstrin and of the Btk and Akt protein kinases (18, 19). PH domains may also tether proteins to membranes by interacting with the βγ subunits of heterotrimeric G-proteins. The PH domain of the β-adrenergic receptor kinase (BARK) has been shown to interact with both PtdIns-4,5-P₂ and Gβγ, and both of these ligands appear to be necessary for the full catalytic activity of this kinase (43, 44).

The data presented here show that activation of PI 3-K in response to TRAP correlates with a slow phase of PKC activation leading to the phosphorylation of pleckstrin. Addition of PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ to permeabilized platelets stimulates phosphorylation of pleckstrin. More over, D3 phos-

phoinositides and DAG may act synergistically to modulate GPIIb-IIIa. The initial wave of pleckstrin phosphorylation may result from a DAG burst activating PKC, but DAG mass is then rapidly lost by 60 s (30). Sustained PKC activation and pleckstrin phosphorylation may require PI 3-K activation and synthesis of D3 phosphoinositides, particularly PtdIns-3,4-P₂, whose product correlates with sustained platelet aggregation. These processes could sustain the activation of PKC.

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REFERENCES

- Kapeller, R., and Cantley, L. C. (1994) *BioEssays* **16**, 565–576
- Serunian, L. A., Haber, M. T., Fukui, T., Kim, J. W., Rhee, S. G., Lowenstein, J. M., and Cantley, L. C. (1989) *J. Biol. Chem.* **264**, 17809–17815
- Lips, D. L., Majerus, P. W., Gorga, F. R., Young, A. T., and Benjamin, T. L. (1989) *J. Biol. Chem.* **264**, 8759–8763
- Liscovitch, M., and Cantley, L. C. (1995) *Cell* **81**, 659–662
- Chung, J., Grammer, T. C., Lemon, K. P., Kazlauskas, A., and Blenis, J. (1994) *Nature* **370**, 71–75
- Franke, T. F., Yang, S.-I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) *Cell* **81**, 1–20
- Burgering, B. M., and Coffey, P. J. (1995) *Nature* **376**, 599–602
- Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C. (1994) *J. Biol. Chem.* **269**, 32358–32367
- Nakanishi, H., Brewer, K. A., and Exton, J. H. (1993) *J. Biol. Chem.* **268**, 13–16
- Sultan, C., Breton, M., Mauco, G., Grondin, P., Plantavid, M., and Chap, H. (1990) *Biochem. J.* **269**, 831–834
- Kucera, G. L., and Rittenhouse, S. E. (1990) *J. Biol. Chem.* **265**, 5345–5348
- Kovacsos, T. J., Bachelot, C., Toker, A., Vlahos, C. J., Duckworth, B., Cantley, L. C., and Hartwig, J. H. (1995) *J. Biol. Chem.* **270**, 11358–11366
- Hartwig, J. H., Bokoch, G., Carpenter, C., Janmey, P., Taylor, L., Toker, A., and Stossel, T. P. (1995) *Cell* **82**, 643–653
- Nishizuka, Y. (1995) *FASEB J.* **9**, 485–496
- Gailani, D., Fisher, T. C., Mills, D. C., and Macfarlane, D. E. (1990) *Br. J. Haematol.* **74**, 192–202
- Tyers, M., Haslam, R. J., Rachubinski, R. A., and Harley, C. B. (1989) *J. Cell. Biochem.* **40**, 133–145
- Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) *Nature* **371**, 168–170
- Konishi, H., Kuroda, S., and Kikkawa, U. (1994) *Biochem. Biophys. Res. Commun.* **205**, 1770–1775
- Yao, L., Kawakami, Y., and Kawakami, T. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9175–9179
- Gou, D.-M., and Chen, C.-S. (1994) *J. Chem. Soc. Chem. Commun.* 2125–2126
- Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P., and Cantley, L. C. (1989) *Cell* **57**, 167–175
- Yatomi, Y., Hazeki, O., Kume, S., and Ui, M. (1992) *Biochem. J.* **285**, 745–751
- Koide, H., Ogita, K., Kikkawa, U., and Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 1149–1153
- Authi, K. S., Evenden, B. J., and Crawford, N. (1986) *Biochem. J.* **233**, 707–718
- Nolan, R. D., and Lapetina, E. G. (1990) *J. Biol. Chem.* **265**, 2441–2445
- Stephens, L. R., Hughes, K. T., and Irvine, R. F. (1991) *Nature* **351**, 33–39
- Carter, A. N., Huang, R., Sorisky, A., Downes, C. P., and Rittenhouse, S. E. (1994) *Biochem. J.* **301**, 415–420
- Arcaro, A., and Wymann, M. P. (1993) *Biochem. J.* **296**, 297–301
- Thomason, P. A., James, S. R., Casey, P. J., and Downes, C. P. (1994) *J. Biol. Chem.* **269**, 16525–16528
- Baldassare, J. J., Henderson, P. A., Burns, D., Loomis, C., and Fisher, G. J. (1992) *J. Biol. Chem.* **267**, 15585–15590
- Nakanishi, S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H., and Matsuda, Y. (1992) *J. Biol. Chem.* **267**, 2157–2163
- Powis, G., Bonjouklian, R., Berggren, M. M., Gallegos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W. F., Dodge, J., Grindey, G., and Vlahos, C. J. (1994) *Cancer Res.* **54**, 2419–2423
- Hashimoto, Y., Togo, M., Tsukamoto, K., Horie, Y., Watanabe, T., and Kurokawa, K. (1994) *Biochim. Biophys. Acta* **1222**, 56–62
- Nakanishi, S., Catt, J. K., and Balla, T. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5317–5321
- Grabarek, J., Raychowdhury, M., Ravid, K., Kent, K. C., Newman, P. J., and Ware, J. A. (1992) *J. Biol. Chem.* **267**, 10011–10017
- Grabarek, J., and Ware, J. A. (1993) *J. Biol. Chem.* **268**, 5543–5549
- Pelech, S. L., Samiei, M., Charest, D. L., Howard, S. L., and Salari, H. (1991) *J. Biol. Chem.* **266**, 8696–8705
- Khan, W. A., Blobel, G., Halpern, A., Taylor, W., Wetsel, W. C., Burns, D., Loomis, C., and Hannun, Y. A. (1993) *J. Biol. Chem.* **268**, 5063–5068
- Fukami, M. H., and Holmsen, H. (1995) *Eur. J. Biochem.* **228**, 579–586
- Kroll, M. H., Hellums, J. D., Guo, Z., Durante, W., Razdan, K., Hrbolich, J. K., and Schafer, A. I. (1993) *J. Biol. Chem.* **268**, 3520–3524
- Jackson, S. P., Schoenwaelder, S. M., Yuan, Y., Rabinowitz, I., Salem, H. H., and Mitchell, C. A. (1994) *J. Biol. Chem.* **269**, 27093–27099
- Abrams, C. S., Wu, H., Zhao, W., Belmonte, E., White, D., and Brass, L. F. (1995) *J. Biol. Chem.* **270**, 14485–14492
- Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G., and Lefkowitz, R. J. (1994) *J. Biol. Chem.* **269**, 10217–10220
- Pitcher, J. A., Touhara, K., Payne, E. S., and Lefkowitz, R. J. (1995) *J. Biol. Chem.* **270**, 11707–11710

Phosphorylation of the Platelet p47 Phosphoprotein Is Mediated by the Lipid Products of Phosphoinositide 3-Kinase

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