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

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters.

Citation
Toker, Alex, Christilla Bachelot, Ching-Shih Chen, J. R. Falck, John H. Hartwig, Lewis C. Cantley, and Tibor J. Kovacsovics. 1995. “Phosphorylation of the Platelet P47 Phosphoprotein Is Mediated by the Lipid Products of Phosphoinositide 3-Kinase.” Journal of Biological Chemistry 270 (49): 29525–31. doi:10.1074/jbc.270.49.29525.

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:41543151

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Phosphorylation of the Platelet p47 Phosphoprotein Is Mediated by the Lipid Products of Phosphoinositide 3-Kinase*

(Received for publication, August 30, 1995)

Alex Toker§§, Christilla Bachelot†, Ching-Shih Chen‡, J. R. Falck**††, John H. Hartwig§§, Lewis C. Cantley‡, and Tibor J. Kovacsics§§††

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 Pharmacaultics, 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 (the Yan, 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 3,4-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-3,4,5-P3.

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, pp7056 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 isoenzymes of PKC (δ, ε, and ζ) as direct targets of these lipids (8), although in vivo evidence for this is still lacking. The diacylglycerol-insensitive isoform PKCζ 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-3,4,5-P3, 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

1 The abbreviations used are: PI 3-K, phosphoinositide 3-kinase; PKC, protein kinase C; PtdIns, phosphatidylinositol; PtdIns-3-P, phosphatidylinositol 3-phosphate; PtdIns-4,5-P2, phosphatidylinositol 4,5-bisphosphate; PtdIns-3,4-P2, phosphatidylinositol 3,4-bisphosphate; PtdIns-3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; DiC2PtdIns-3,4,5-P3, dioctanoyl phosphatidylinositol 3,4,5-trisphosphate; DiC2PtdIns-3,4-P2, dioctanoyl phosphatidylinositol 3,4-bisphosphate; DiC2PtdIns-3,4,5-P3, dioctanoyl phosphatidylinositol 3,4,5-trisphosphate; DAG, 1,2-diacylglycerol; 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.

* This work was supported in part by grants from the Fondation Henri Dunois-Ferrière-Dinu-Lipatti (to T. J. K.) and from the Edwin S. Webster Foundation (to J. H. H.) and by United States Public Health Service Grants GM 41890 (to L. C. C.) and HL47874 (to J. H. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by the Medical Foundation Inc. (Boston, MA). To whom correspondence should be addressed. 200 Longwood Avenue, Boston, MA 02115. Tel.: 617-278-3051; Fax: 617-278-3313; E-mail: atoker@mercury.bih.harvard.edu.

†† Present address: Division of Hematology, Centre Hospitalo-Universitaire Vaudois, Lausanne, Switzerland.

29525
Phosphoinositide 3-Kinase Mediates Pleckstrin Phosphorylation

pi

PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ mediate this effect.

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, β₁, 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 η 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), and platelet PKC. We present data demonstrating that PI 3-K is essential for a late phase of TRAP-stimulated pleckstrin phosphorylation.

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 μCi/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 × 10⁸ resting platelets were isolated and lysed in homogenization buffer A (20 mM Tris-HCl, pH 7.0, 2 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 0.25 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride, 4 μg/mL pepstatin A, 4 μg/mL leupeptin, 10 mM sodium fluoride). The lysate was cleared by ultracentrifugation at 100,000 × 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 [³²P]ATP into histone H1 (type III-S) or the peptide e substrate (Upstate Biotechnology, Inc.) essentially as described previously (23). The reaction mixture contained 20 μL Hepes, pH 7.5, 5 mM MgCl₂, 100 μM CaCl₂, 50 μM [³²P]ATP, 50 μM phosphatidylserine and 10 μM diacylglycerol vesicles, 10 μg of histone H1 (type III-S), or 30 μM peptide e. 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 μg/mL fibrinogen and either 25 μM 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 Platelet Phosphorylation—Platelets were labeled with [³²P]orthophosphoric acid as described above, except that 1 μCi/mL was used. The reaction was started after the addition of 500 μg/mL fibrinogen and either 25 μM 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).

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 × g in the presence of 1 μM 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 KC₁, 1 mM EGTA, and 0.037 mM CaCl₂) at pH 7.4 at a concentration of 3 × 10⁹/mL and rested at 37°C for 30 min before use. 200 μL of platelets were permeabilized under non-aggregating conditions in the presence of 500 μg/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 Laboratories, Hercules, CA).

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 × g in the presence of 1 μM 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 KC₁, 1 mM EGTA, and 0.037 mM CaCl₂) at pH 7.4 at a concentration of 3 × 10⁹/mL and rested at 37°C for 30 min before use. 200 μL of platelets were permeabilized under non-aggregating conditions in the presence of 11 μg/mL saponin and 100 μCi/mL exogenously added [³²P]ATP. At time 0, phosphoryositides 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.

Phosphoryositides were stored at −70°C in methanol:chloroform:1 N HCl (2:1:0.1). For permeabilization studies, phosphoryositides were prepared by drying by a steamer of nitrogen, followed by resuspension in 20 μM 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 phosphoryositides 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...
(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 μM, indicating that other wortmannin-insensitive pathways contribute to the phosphorylation of pleckstrin. The dose-response 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 μM 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-3,4,5-P3 synthesis is rapid and short-lived, peaking af-
Phosphoinositide 3-Kinase Mediates Pleckstrin Phosphorylation

The sustained production of PtdIns-3,4,5-P₃ 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-3,4,5-P₃ and the sustained phosphorylation of p47. Both of these events are inhibited in platelets pretreated with 100 nM wortmannin. The decline in PtdIns-3,4,5-P₃ 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 μM, there was no effect of wortmannin on the ability of platelet PKC to phosphorylate either histone III-S or the peptide e 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, ζ, ξ, and λ) and therefore peptide e, based on the PKC e 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⁻³²P]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 (DiC₁₀PtdIns-3,4,5-P₃) and a water-soluble, short-chain diocanoyl PtdIns-3,4,5-P₃ (DiC₂PtdIns-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 phosphorylation.
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. Stauroporine 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 products, PtdIns-3,4,5-P$_3$ (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-3,4,5-P$_3$. 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 $\mu$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-3,4,5-P$_3$ 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

---

2 A. Toker, unpublished observations.
family members by TRAP-stimulated PI turnover, by PtdIns-
3,4-P$_2$ synthesis, or by phorbol ester addition can result in
similar cell responses. Protein kinase C activation has previ-
ously been presumed to result from the agonist-mediated acti-
vation 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-P$_2$ and PtdIns-
3,4,5-P$_3$ to be capable of activating novel, calcium-independent
PKCs in vitro (8). The atypical PKC family member, PKC $\zeta$
has also been shown to be activated by PtdIns-3,4,5-P$_3$ (9). Both
calcium-dependent ($\alpha$, $\beta$, and $\lambda$) and calcium-independent ($\delta$
$\eta$, $\theta$, and $\zeta$) PKC family members have been detected in plate-
lets (30, 35–38). PKC $\epsilon$ may also be present in platelets, al-
though only minor amounts were detected by Western immu-
noblotting (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 in-
crease with translocation of PKC $\alpha$, $\beta$, $\delta$, or $\zeta$ to the mem-
brane fraction. However, a subsequent report showed bryostatin
stimulation of platelets failed to affect a translocation of PKC
$\alpha$, $\beta$, $\delta$, or $\zeta$ (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 indi-
cated that DAG levels in resting, unstimulated platelets can
fluctuate to levels comparable to that seen with thrombin stim-
ulation 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 produc-
tion 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 stimu-
lating PtdIns-3,4,5-P$_3$ 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 ago-
nist-stimulated cells. Although the phosphorylation of pleck-
strin 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-P$_2$ indicates that pleck-
strin may under some conditions be recruited to the plasma
membrane (17). It is conceivable that phosphorylation of pleck-
strin may affect this interaction or promote its activity. The
N-terminal PH domain of pleckstrin was recently shown to
inhibit both phospholipase C$\beta$- and C$\gamma$-mediated phos-
phoinositide hydrolysis (42). PH domains have also been shown
to mediate protein-protein interactions, and several PKC iso-
forms 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 interact-
ing with the $\beta$ subunits of heterotrimeric G-proteins. The
PH domain of the $\beta$-adrenergic receptor kinase ($\beta$ARK) has been
shown to interact with both PtdIns-4,5-P$_2$ and G$\gamma_{1}$, 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 activa-
tion leading to the phosphorylation of pleckstrin. Addition of
PtdIns-3,4-P$_2$ and PtdIns-3,4,5-P$_3$ to permeabilized platelets
stimulates phosphorylation of pleckstrin. More over, D3 phos-

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 $\mu$m in in vitro as-
says. 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 de-
scribed a hormone-stimulated PI 4-kinase activity that is sen-
sitive to 100 $nm$ wortmannin (34). We have shown that treat-
ment of platelets with 100 $nm$ wortmannin does not affect the
synthesis of PtdIns-4-P and PtdIns-4,5-P$_2$ induced by TRAP
(12). Synthesis of the D3 phosphoinositides, however, was com-
pletely abolished by 100 $nm$ wortmannin. Wortmannin-sensi-
tive 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-P$_2$ pro-
duction correlates with pleckstrin phosphorylation and our pre-
vious results showing activation of calcium-independent PKCs
$\delta$, $\epsilon$, and $\eta$ (8), we devised a permeabilization scheme to intro-
duce 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 [$\gamma$-$32$P]ATP. Both PtdIns-3,4-P$_2$ and PtdIns-
3,4,5-P$_3$ were also able to stimulate pleckstrin phosphorylation
above control levels (Fig. 5). Neither PtdIns-3-P nor PtdIns-
4,5-P$_3$ was able to stimulate pleckstrin phosphorylation, sug-
uggesting 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

![Image](https://www.jbc.org/)

**Fig. 6. Phorbol ester can overcome wortmannin inhibition of irreversible platelet aggregation.** Gel-filtered platelets were pre-
incubated 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 $\mu$m fibrinogen, aggregation was started by the addi-
tion of 25 $\mu$m 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.
Phosphoinositides and DAG may act synergistically to modulate GP1b-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 Ptdlns(3,4,5)-P3, whose product correlates with sustained platelet aggregation. These processes could sustain the activation of PKC.

Acknowledgments—We thank Sophia Kung and Lance Taylor for excellent technical assistance in these studies.

REFERENCES

1. Kapeller, R., and Cantley, L. C. (1994) BioEssays 16, 565–576
2. 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
3. Lips, D. L., Majerus, P. W., Gorga, F. R., Young, A. T., and Benjamin, T. L. (1989) J. Biol. Chem. 264, 8757–8763
4. Liscovitch, M., and Cantley, L. C. (1995) Cell 81, 659–662
5. Chung, J., Grammer, T. C., Lemon, K. P., Kazlauskas, A., and Blenis, J. (1994) Nature 370, 71–75
6. 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
7. Burgering, B. M., and Coffer, P. J. (1995) Nature 376, 599–602
8. Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Aneja, R., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C. (1994) J. Biol. Chem. 269, 23288–23297
9. Nakanishi, H., Brewer, K. A., and Exton, J. H. (1993) J. Biol. Chem. 268, 13–16
10. Sultman, C., Breton, M., Maucou, G., Grondin, P., Plantavid, M., and Chap, H. (1990) Biochem. J. 269, 831–834
11. Kucera, G. L., and Rittenhouse, S. E. (1990) J. Biol. Chem. 265, 5345–5348
12. Kovacsovics, T. J., Bachelder, C., Toker, A., Vilagos, C. J., Duckworth, B., Cantley, L. C., and Hartwig, J. H. (1993) J. Biol. Chem. 268, 11358–11366
13. Hartwig, J. H., Bokoch, G., Carpenter, C., Jarmey, P., Taylor, L., Toker, A., and Stossel, T. P. (1995) Cell 82, 643–653
14. Nishizuka, Y. (1995) FASEB J. 9, 485–496
15. Gailani, D., Fisher, T. C., Mills, D. C., and Macfarlane, D. E. (1990) Br. J. Haematol. 74, 192–202
16. Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Anega, R., Anega, S., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C. (1994) J. Biol. Chem. 269, 23288–23297
17. Nakanishi, S., Kikita, S., Takahashi, J., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H., and Matsuda, Y. (1992) J. Biol. Chem. 267, 2157–2163
18. Powis, G., Bonjuklian, R., Berggren, M. M., Gallegos, A., Abraham, R., Ashendel, C., Zailkow, L., Matter, W. F., Dodge, J., Grindey, G., and Vlahos, C. J. (1994) Cancer Res. 54, 2419–2423
19. Hashimoto, Y., Togo, M., Tsukamoto, K., Horie, Y., Watanabe, T., and Kurokawa, K. (1994) Biochim. Biophys. Acta 1222, 56–62
20. Nakanishi, S., Catt, J. K., and Balla, T. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 5317–5321
21. Grabarik, J., Raychowdhury, M., Ravid, K., Kent, K. C., Newman, P. J., and Ware, J. A. (1992) J. Biol. Chem. 267, 10011–10017
22. Grabarik, J., and Ware, J. A. (1993) J. Biol. Chem. 268, 5543–5549
23. Pelech, S. L., Samiel, M., Charest, D. L., Howard, S. L., and Salari, H. (1991) J. Biol. Chem. 266, 8696–8705
24. Khan, W. A., Blibs, G., Haipern, A., Taylor, W., Wetsel, W. C., Burns, D., Loomis, C., and Hannun, Y. A. (1993) J. Biol. Chem. 268, 5063–5068
25. Fukami, M., and Holmsen, H. (1995) Eur. J. Biochem. 228, 579–586
26. Kröll, M. H., and Heilmann, H. (1995) Eur. J. Biochem. 228, 579–586
27. Jackson, S. P., Schoenwaelder, S., Yuan, Y., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 27093–27099
28. Abrams, C. S., Wu, H., Zhao, W., Belmonte, E., White, D., and Brass, L. F. (1995) J. Biol. Chem. 270, 14485–14492
29. Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 10217–10220
30. Toiranta, T., Mann, E., 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
Alex Toker, Christilla Bachelot, Ching-Shih Chen, J. R. Falck, John H. Hartwig, Lewis C. Cantley and Tibor J. Kovacsovics

J. Biol. Chem. 1995, 270:29525-29531.
doi: 10.1074/jbc.270.49.29525

Access the most updated version of this article at http://www.jbc.org/content/270/49/29525

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 27 of which can be accessed free at http://www.jbc.org/content/270/49/29525.full.html#ref-list-1