Phosphatidylinositol (3,4,5)-Trisphosphate Stimulates Phosphorylation of Pleckstrin in Human Platelets*

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We have reported that platelets exposed to thrombin or thrombin receptor-directed ligand activate phospholipase C and rapidly accumulate phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) and phosphatidylinositol (3,4)-bisphosphate (PtdIns(3,4)P2) as a function of the activation of phosphoinositide (PI) 3-kinases in a GTP-binding protein-dependent manner. In such platelets, serine- and threoninedirected phosphorylation of pleckstrin also occurs and has been attributed to protein kinase C activation. We now report that the phosphorylation of pleckstrin is partially dependent upon PI 3-kinase. Pleckstrin phosphorylation in response to thrombin receptor stimulation is progressively susceptible to inhibition by wortmannin, a potent and specific inhibitor of platelet PI 3-kinases. PI 3-kinase thus seems to play a gradually increasing role in promoting pleckstrin phosphorylation. The IC50 for wortmannin in inhibiting SFLLRN-stimulated 3-phosphorylated phosphoinositide accumulation is 10 nM, and that (i.e. 50% of maximum inhibition) for inhibiting pleckstrin phosphorylation is 15 nM. Synthetic PtdIns(3,4,5)P3, when added to saponin-permeabilized (but not intact) platelets, causes wortmannin-insensitive phosphorylation of pleckstrin. PtdIns(3,4,5)P3 also overcomes the inhibition by wortmannin of thrombin- or guanosine 5'-O-(thio)trisphosphate-stimulated pleckstrin phosphorylation. In contrast, PtdIns(4,5)P2 or inositol (1,3,4,5)-tetrakisphosphate are ineffective in these respects. The pattern of phosphorylation of pleckstrin activated by PtdIns(3,4,5)P3 is not distinguishable from that of pleckstrin phosphorylated in intact platelets exposed to protein kinase C-activating ρ-phorbol myristate acetate, mimicking diacylglycerol. Activation of protein kinase(s) by PtdIns(3,4,5)P3, thus offers a route for pleckstrin phosphorylation in vivo that is an alternative to activation of phospholipase C → diacylglycerol → protein kinase C.

EXPERIMENTAL PROCEDURES

Materials—α-Phosphatidyl-α-myoinositol (3,4,5)-trisphosphate was synthesized as the sn-1,2-dioctanoyl analogs (dICβ) according to the procedure of Reddy et al. (16) and dissolved in H2O or Me2SO. The dipalmitoyl analog (dIC16) was kindly provided by Dr. Roy Gigg (NIMR, London, UK) and dissolved in Me2SO. Wortmannin, PtdIns(4,5)P2, and Ins[1,3,4,5]P4, purchased from Sigma, and the former two were dissolved in Me2SO, whereas Ins[1,3,4,5]P4 was dissolved in H2O. [32P]Pi was obtained from DuPont NEN, and [γ-32P]ATP was synthesized (5). α-Thrombin was bought from Hemotech (Burlington, VT), and the thrombin receptor tethered ligand analogue SFLLRN was synthesized. PtdIns, phosphatidylinositol (locants of phosphates indicated by numbers shown in parentheses); diC8-, dioctanoyl; diC16-, dipalmitoyl; 3-PP, 3-phosphorylated phosphoinositides; PtdOH, phosphatic acid; SFLLRN, Ser-Phε-Leu-Leu-Arg-Asn peptide; GTPγS, guanosine 5'-3-O-(thio)trisphosphate; Me2SO, dimethyl sulfoxide; Ins[1,3,4,5]P4, inositol (1,3,4,5)-tetrakisphosphate.

It is being appreciated increasingly that the metabolism of phosphoinositides catalyzed by phosphoinositide (PI) 3-kinase has signal-transducing consequences that rival those of phosphoinositidase C activation in a variety of cells. PtdIns(3,4,5)P3 and PtdIns(3,4)P2 are both major physiological products of activated PI 3-kinase. They have been proposed to be modulators of protein kinase(s), thereby amplifying an initial limited amount of signal (1), analogously with diacylglycerol and PKC. Indeed, several members of the PKC family have been reported recently to be stimulated by PtdIns(3,4,5)P3, including a mixture of rat brain PKC isoforms (2), PKCζ (3), and PKCδ, δ, and (by either PtdIns(3,4,5)P3 or PtdIns(3,4)P2) (4). Other protein kinases may also be stimulated. We have described the activation of two species of PI 3-kinase by a variety of agonists, including thrombin, thromboxane A2, analogue, GTPγS, and thrombin receptor-directed ligand, leading to the sequential accumulation of PtdIns(3,4,5)P3 and PtdIns(3,4)P2 in intact or permeabilized platelets (5–7). Another response to platelet activation, one of the earliest reported (8, 9), is the phosphorylation of an apparent Mr 40,000–47,000 protein known as pleckstrin (platelet and leukocyte C kinase substrate) (10) at serine and threonine. As the name implies, pleckstrin is considered to be one of the major (and is certainly one of the most readily identifiable) substrate(s) for PKC in the platelet. Recently, an inhibitory effect of Mr concentrations of the mycostatin wortmannin on pleckstrin phosphorylation, which is not attributable to inhibition of PKC, has been reported (11). Since wortmannin has since proved to be a potent and rather selective irreversible inhibitor of PI 3-kinases (12–14), blocking both the lipid kinase and intrinsic protein kinase activities of this enzyme (15), we decided to determine whether the inhibitory effects of wortmannin on pleckstrin phosphorylation were attributable to inhibition of PtdIns(3,4,5)P3 formation.

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sized by the Jefferson Cancer Institute peptide facility. Rabbit polyclonal antiserum (no. 354) was raised against a recombinant protein corresponding to pleckstrin residues Glu22808-Asp22832. Solvents and chromatographic systems were as described elsewhere (5–7).

Studies with Intact Labeled Platelets—Platelets were prepared from blood freshly drawn from normal human donors, treated with aspirin, washed, and incubated with 0.75 mM [32P]Pi, for 90 min as previously described (18). After suspension of [32P]-labeled platelets to 2 x 10^9/ml, platelets were incubated in the presence of [Ca^{2+}]_{	ext{free}} = 2 mM with MeSO or 100 nM wortmannin at 37 °C for 5 min, or with wortmannin (100 nM) for 5 min prior to washing and labeling, followed by the addition of SFLLRN (10 μM) for up to 120 s, or buffer for 0 or 120 s. In other cases, platelet incubations were with varied wortmannin concentrations (0–100 mM) for 5 min, followed by 120 s of incubation with SFLLRN (10 μM) or buffer. Incubations were terminated, and [32P]-labeled lipid extracted, digested, resolved, and quantitated as described elsewhere (6, 18). In parallel incubations, Laemmli lysis buffer was added to terminate incubations, proteins were resolved by 10% reducing SDS-polyacrylamide gel electrophoresis, and pleckstrin ("p47") was quantitated after autoradiography and scintillation spectrophotometry as reported previously (19).

Studies with Permeabilized Platelets—Platelets (unlabeled) were incubated ± 100 nM wortmannin for 5 min at 37 °C, in the absence of added Ca^{2+}, prior to washing. The washed platelets were permeabilized as described in the presence of saponin and [γ-32P]ATP, 2 x 10^9 platelets/ml. After 60 s, varied amounts of diC8 or diC16-PtdIns(3,4,5)P3 were added for different periods of time, terminating with addition of Laemmli buffer and boiling. Alternatively, GTP-S (10 μM), thrombin (5 units/ml), or buffer was added after 5 min, and incubations were terminated after 6 min. Proteins were resolved and quantitated as described above.

Immunoprecipitations and Cyanogen Bromide Digestions—Permeabilized wortmannin-treated platelets were incubated in triplicate, as above, with buffer or diC8-PtdIns(3,4,5)P3, for 5 min and lysed with an equal volume (250 μl) of ice-cold lysis buffer (2% Triton X-100, 10 mM Tris, pH 7.6, 100 mM NaCl, 60 mM NaPPi, 10 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 0.2% aprotinin, 2 mM sodium vanadate, and 50 μM leupeptin). After 30 min on ice, lysates were cleared by centrifugation at 14,000 rpm for 30 min at 4 °C, and supernatants were mixed with equal volumes of 0.5 x lysis buffer and exposed to immunoprecipitating antiserum 354 + protein A-Sepharose. Protein in each immunoprecipitate was resolved by SDS-polyacrylamide gel electrophoresis as above and visualized by autoradiography to confirm the presence of 32P-labeled pleckstrin, free of other 32P-labeled protein. Pleckstrin was eluted as described elsewhere (20). In addition, [32P]-labeled platelets (see "Intact Labeled Platelets," above) were spun in the presence of 1 μM prostaglandin E1, at 500 x g for 15 min, resuspended in incubation buffer ( -Ca^{2+}), and stimulated with 50 nM PMA at room temperature for 5 min. Incubations were terminated with lysis buffer, as above, and pleckstrin was immunoprecipitated, resolved, and eluted. Cyanogen bromide mapping of immunoprecipitated pleckstrin was performed as described previously (20).

RESULTS AND DISCUSSION

We present evidence that wortmannin inhibits the accumulation of 3-PPI and phosphorylation of pleckstrin (p47) in response to thrombin, thrombin receptor-directed peptide, or GTPyS, and that the inhibition of pleckstrin phosphorylation can be overcome in permeabilized platelets by diC8- or diC16-PtdIns(3,4,5)P3, but not by PtdIns(4,5)P2 or Ins(1,3,4,5)P4. These findings implicate a PtdIns(3,4,5)P3-activated protein kinase, rather than the intrinsic protein kinase activity of PI-3-kinase. Phosphorylation of pleckstrin can be initiated by PtdIns(3,4,5)P3 in a time and concentration-dependent manner, and does not lead to accumulation of 32P-PtdOH or further accumulation of 32P-3-PPI. The pattern of phosphorylation of pleckstrin achieved in platelets in response to PtdIns(3,4,5)P3 is not distinguishable from that resulting from exposure to PMA, although this does not constitute proof that PKC is being activated by PtdIns(3,4,5)P3.

Fig. 1 indicates the dose-dependence for wortmannin's inhibitory effects on both 3-PPI accumulation (Panel A) and p47 phosphorylation (Panel B). These are seen to be quite similar, with IC_{50} values of 10–15 nM, where maximum inhibition of p47 (pleckstrin) phosphorylation is 50–60%.

As shown in Fig. 2, phosphorylation of pleckstrin occurs rapidly in platelets exposed to SFLLRN, and net phosphorylation is inhibited only slightly by 100 nM wortmannin within the first 15 s. In contrast, net accumulation of PtdIns(3,4,5)P3 peaks by 30 s, followed by sustained accumulation of PtdIns(3,4,5)P3, and the levels of both are inhibited completely by 100 nM wortmannin in this period (not shown; IC_{50} ~ 10 nM). Accumulation of 32P-PtdOH (not shown), indicative of phosphoinositide C and DG kinase activation in platelets (21), is unaffected by wortmannin. With time, however, following accumulation of the 3-PPIs, the inhibitory effects of wortmannin on 32P-p47 levels become more pronounced, reaching a maximum of about 50% by 60 s, and decreasing by 120 s. The delayed nature of wortmannin-inhibitable phosphorylation of p47 is emphasized by the broken line in this figure. Similar effects are observed if platelets are exposed to wortmannin for 5 min and then washed prior to stimulation. Thus, the delayed effects of wortmannin on p47 phosphorylation are not due to a time-dependent direct inhibition by wortmannin of pleckstrin-directed protein kinase.

The involvement of PtdIns(3,4,5)P3 in this event is made clear in Figs. 3 and 4. When added to permeabilized platelets, PtdIns(3,4,5)P3 causes phosphorylation of p47 in a time- and dose-dependent manner (Fig. 3), achieving close to maximum effects after a 5-min exposure to 4 μM diC8-PtdIns(3,4,5)P3.
Approximately twice that concentration of diC16-PtdIns(3,4,5)P3 is required to attain the same results, attributable to the different solubility characteristics of these isoforms (not shown). Both isoforms of PtdIns(3,4,5)P3 overcome the inhibitory effects of 100 nM wortmannin on p47 phosphorylation induced by GTPγS (Fig. 4), implicating PtdIns(3,4,5)P3 in regulating pleckstrin phosphorylation in response to a variety of agonists that are dependent upon GTP-binding proteins. Exogenous diC8- or diC16-PtdIns(3,4,5)P3 is not acting via contaminant diC8-DG or diC16-DG, or via phosphoinositidase C action leading to diC8-DG or diC16-DG, since intact platelets are not affected, no increase in 32P-pleckstrin occurred when intact platelets, labeled as in Fig. 2, were exposed to PtdIns(3,4,5)P3. Results are the averages of duplicates, included within symbols.

Several proteins are labeled with 32P in permeabilized platelets at “rest” or in response to exogenous PtdIns(3,4,5)P3. However, p47 is the protein most conspicuously phosphorylated in response to physiological platelet agonists or β-phorbol esters. As seen in Fig. 5A, which shows results with pleckstrin-directed immunoprecipitations, 32P-labeled pleckstrin accumulates in platelets exposed to PtdIns(3,4,5)P3, and other labeled proteins do not co-immunoprecipitate appreciably. Upon CNBr digestion, the pattern of phosphorylation, which is alkali-labile,

S. E. Rittenhouse, unpublished results.

**Fig. 2.** Accumulation of 32P-labeled 3-PPI and pleckstrin in platelets exposed to SFLLRN. Labeled platelets were exposed to 100 nM wortmannin or Me2SO for 5 min prior to the addition of SFLLRN (10 µM) or buffer for varied periods. In some studies, platelets were exposed to wortmannin prior to washing and labeling. Lipids and proteins were extracted, resolved, and quantitated as described. Data are the average ± range of duplicates (some ranges are included within symbols) and are representative of two experiments. 3-PPI are presented as a percent of the activity (basal) in the absence of SFLLRN: PtdIns(3,4,5)P3 – wortmannin (☐) and PtdIns(3,4,5)P2 – wortmannin (○). For p47 (pleckstrin): – wortmannin (○); + wortmannin (●); (– wortmannin) (+ wortmannin) (△) in disintegrations/min. Wortmannin inhibited completely 3-PPI accumulation at all times, but did not affect basal 32P-pleckstrin, which did not change in the absence of SFLLRN.

**Fig. 3.** Phosphorylation of pleckstrin in platelets incubated with PtdIns(3,4,5)P3. Permeabilized platelets were exposed to varied concentrations of diC8-PtdIns(3,4,5)P3 for 5 min (A) or to 2 µM diC8-PtdIns(3,4,5)P3 for various periods (B) after an initial 60-s labeling period (1) with [γ-32P]ATP + saponin, and incubations were terminated with Laemmli SDS-reducing buffer and boiling, followed by SDS-polyacrylamide gel electrophoresis and quantitation of 32P in p47 protein, as above. Similar results were obtained using diC16-PtdIns(3,4,5)P3, which was about half as efficient. No accumulation of labeled PtdOH or 3-PPI was observed in response to exogenous PtdIns(3,4,5)P3. No increase in 32P-pleckstrin occurred when intact platelets, labeled as in Fig. 2, were exposed to PtdIns(3,4,5)P3. Results are the averages ± ranges of duplicates, included within symbols.

**Fig. 4.** The effects of wortmannin on GTPγS- or PtdIns(3,4,5)P3-induced 32P-pleckstrin accumulation in permeabilized platelets. Platelets were exposed to wortmannin (100 nM) or Me2SO for 5 min at 37°C before washing and permeabilization as above. Wortmannin did not affect basal levels of 32P-pleckstrin. Subsequent incubations were with diC8-PtdIns(3,4,5)P3 (2 µM) or PtdIns(4,5)P2 (4 µM) for 6 min, or with these agents for 6 min + GTPγS (10 µM) for the final 1 min. Similar effects were observed when thrombin (5 units/ml) was substituted for GTPγS. Results (A) are the averages ± range of duplicate determinations, counted from the gels at 47 kDa whose autoradiograph is shown in (B), corresponding to samples 1–8. Results are representative of three experiments.
We have observed\(^6\) that addition of PtdIns(3,4,5)\(_3\) to permeabilized platelets, in addition to promoting pleckstrin phosphorylation, potentiates the increase in active \(\gamma\)-PKC\(_\beta\) formed in response to SFLLRN. Recent studies in which pleckstrin has been expressed in COS-1 or HEK-293 cells indicate that it can inhibit the activation of phosphoinositidase C\(_\beta\) and phosphoinositidase C\(_\gamma\) initiated by several receptors, including that for thrombin, and the findings are consistent with an interaction between pleckstrin and PtdIns(4,5)\(_2\) (20). Given its two pleckstrin homology domains, pleckstrin may also play a part in regulating PI 3-kinase (\(\gamma\)) activity by binding to G\(_{\beta\gamma}\) subunits (7), a function currently under investigation in our laboratory. Phosphorylation of pleckstrin in response to PtdIns(3,4,5)\(_3\) offers an alternate route to that provided by the activation of phosphoinositidase C, formation of DG, and activation of PKC. This may serve a redundant function, allowing, e.g. a phosphoinositidase C deficiency to have less severe consequences for protein phosphorylation under certain circumstances, or it may affect the duration of the PKC response, permitting a more sustained activation after DG is metabolized, or it may be relevant to the localization of PKC activation. In any case, our data constitute unique evidence that PtdIns(3,4,5)\(_3\) plays a second messenger role in activating any of the pleckstrin homology domains, pleckstrin may also play a part in the reorganization of integrin

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Fig. 5. Immunoprecipitation of \(^32\)P-pleckstrin and CNBr digestion. Pleckstrin from permeabilized platelets preincubated with 100 nM wortmannin and subsequently labeled with \(\gamma\)-\(^32\)P-ATP \(\pm\) diC8-PtdIns(3,4,5)\(_3\) was extracted and immunoprecipitated as described. An autoradiograph of the immunoprecipitated \(^32\)P-labeled material is shown (A), where the first lane is the immunocube from incubated platelets, and the subsequent three lanes are from triplicate incubations with diC8-PtdIns(3,4,5)\(_3\). The autoradiograph of CNBr-digested and undigested immunoprecipitate, electrophoresed on a tricine polyacrylamide gel, is shown (B, right) in comparison with a digest of immunoprecipitated \(^32\)P-pleckstrin from labeled platelets exposed to PMA (B, left). The numbers shown indicate molecular mass (kDa).

is very similar to that achieved in response to PKC-activating PMA (Fig. 5B). Phosphorylation by PKC of pleckstrin appears to occur primarily on three residues: Ser\(^113\), Thr\(^114\), and Ser\(^117\),\(^13\),\(^14\) and it appears that this same preference is maintained for PtdIns(3,4,5)\(_3\)-activated protein kinase. It is thus possible that PKC(s) is(are) activated by PtdIns(3,4,5)\(_3\) in platelets, consistent with the observation that PKC species, notably PKC\(_\varepsilon\), are activated by PtdIns(3,4,5)\(_3\) or PtdIns(3,4)\(_3\). In fact, both species of phosphoinositide may be present when PtdIns(3,4,5)\(_3\) is added to permeabilized platelets, since 5-phosphatase activity capable of acting on this substrate is present in platelets.\(^5\) As is true for SFLRRN-stimulated intact platelets (Fig. 2), however, p47 phosphorylation is transient, whereas PtdIns(3,4)\(_3\) is increasing (Fig. 3), therefore only PtdIns(3,4,5)\(_3\) may be effective here. Of further interest, the cytoskeleton of activated platelets, with which PI 3-kinases become associated (7), also contains increased amounts of PKC\(_\varepsilon\), as detected by Western blotting,\(^6\) constituting a potential locus for the pleckstrin phosphorylation observed. At present, the function of pleckstrin (or phosphorylated pleckstrin) in activated platelets is unknown. It may play a role in the reorganization of integrin \(\alpha\)\(_IIb\)\(\beta\)\(_3\) whose active conformation binds fibrinogen and is involved in platelet aggregation.

\(^4\) C. S. Abrams, W. Zhao, E. Belmonte, and L. F. Brass, manuscript submitted for publication.

\(^5\) J. Zhang and S. E. Rittenhouse, unpublished results.

\(^6\) J. Zhang, Shattil, M. C. Cunningham, J. R. Falck, K. K. Reddy, and S. E. Rittenhouse, manuscript submitted for publication.
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