RESEARCH COMMUNICATION

Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses

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Phosphatidylinositol 3,4,5-trisphosphate (PtdInsP_3) is rapidly produced upon exposure of neutrophils to the chemoattractant N-formylmethionyl-leucylphenylalanine (fMLP), and has been proposed to act as a second messenger mediating actin polymerization and respiratory-burst activity. Here we present evidence that wortmannin, a known inhibitor of respiratory-burst activity, acts on PtdIns 3-kinase, the enzyme producing PtdInsP_3 from PtdIns(4,5)P_2. Pretreatment of ^{32}P-labelled · human neutrophils with 100 nM wortmannin totally abolished fMLP-mediated PtdInsP_3 production, raised PtdInsP_2 levels, and did not affect cellular PtdIns$P$ and PtdIns contents. The inhibitory effect on PtdInsP_3 formation in intact cells was dose-dependent, with an IC_{50} of ~ 5 nM. Similar results were obtained with PtdIns 3-kinase immunoprecipitated by antibodies against the p85 regulatory subunit: wortmannin totally inhibited PtdIns3P production in immunoprecipitates at concentrations of 10–100 nM ($IC_{50} \sim 1$ nM). These results illustrate the direct and specific inhibition of PtdIns 3-kinase by wortmannin. Since agonist-mediated respiratory-burst activation is most sensitive to wortmannin ($IC_{50} = 12$ nM), this suggests that agonist-mediated PtdInsP_3 formation is indispensable for this cell response. Neutrophils pretreated with wortmannin develop oscillatory changes in F-actin content, but actin polymerization in response to fMLP is not inhibited. This, and the absence of PtdInsP_3 under these conditions, are in agreement with a modulatory role for PtdInsP_3 in cytoskeletal rearrangements, but imply that PtdInsP_3 production is not a primary event triggering elongation of actin filaments in neutrophils.

INTRODUCTION

Neutrophils move and orientate themselves in gradients of chemotactic substances released by invading micro-organisms. At the site of infection, the cells phagocytose and digest the foreign particles by the concerted action of hydrolytic enzymes and reactive oxygen species originating from superoxide anions. The enzyme producing the latter, NADPH oxidase, has been reconstituted from cytochrome b_{558} p21^{phox} and gp91^{phox} subunits, the cytosolic factors p47^{phox}, p67^{phox} and the small GTP-binding protein rac (for a review see [1]). Although the oxidase can be assembled and stimulated in vitro by negatively charged amphiphiles such as SDS or arachidonic acid [2], one of the signals in the intact cell seems to be protein kinase C (PKC)-mediated phosphorylation of p47 [3]. Other metabolites increased by agonist stimulation, such as phosphatidic acid [4–7] or arachidonic acid [8], have also been proposed to take part in NADPH oxidase activation.

While neutrophils move towards their target, they continuously protrude and retract lamellipodia, enabling them to advance on surfaces. How the force for locomotion is generated and how cells find their way is still being discussed [9–12], but redistribution and polymerization of actin play an important role in all proposed models. It has been shown that actin polymerization/depolymerization cycles [13,14] correlate with cell shape and pseudopod volume [15] of suspended neutrophils. Control of actin polymerization was proposed to be mediated by proteins such as gelsolin, whose actin-binding and severing properties can be influenced by polyphosphoinositides and Ca^{2+} [9,16,17].

The role of PtdInsP_2 and Ca^{2+} in controlling actin polymerization, however, has been disputed. PtdInsP_2 levels fall in response to agonists and cells migrate even with artificially lowered cytosolic calcium [18] where phospholipase C-mediated PtdInsP_2 turnover is eliminated [19,20].

Traynor-Kaplan et al. [21,22] showed agonist-mediated accumulation of a novel phospholipid in neutrophils and identified it as PtdIns(3,4,5)P_3. Since the time course of relative changes in PtdIns(3,4,5)P_3 correlated with the respiratory burst and actin polymerization, PtdIns(3,4,5)P_3 was proposed to be a messenger for these cell responses [21,23]. PtdIns 3-kinase, the enzyme converting PtdIns(4,5)P_2 into PtdIns(3,4,5)P_3, consists of a regulatory (p85) [24–26] and a catalytic (p110) subunit [27]. Its activation by growth-factor receptors, for instance by the platelet-derived growth factor (PDGF) β-receptor, proceeds via autophosphorylation of two of the receptor's tyrosine residues, which are subsequently recognized by the SH2 domains contained in p85 [24,25,28–30]. Due to this translocation, p85 becomes tyrosine-phosphorylated and p110 gains access to its substrate [31,32]. PtdIns 3-kinase activation by the N-formylmethionyl-leucylphenylalanine (fMLP) receptor, however, seems to be transduced via heterotrimeric G-proteins [21] without the need for tyrosine phosphorylation of p85 [33].

We show here that wortmannin blocks fMLP-mediated PtdInsP_3 synthesis as well as PtdIns 3-kinase activity in vitro at nM concentrations. Wortmannin has been found to inhibit agonist-induced respiratory burst [34,35] and phospholipase D activity [4,5,7], myosin light-chain kinase at μM concentrations [36] and pleckstrin phosphorylation in platelets [37]. The inhibitor's target at low concentrations, however, has remained unknown. Wortmannin will be a valuable tool to elucidate the importance of PtdInsP_3 signalling in numerous receptor-mediated cell responses. Resulting consequences for two neutrophil responses, respiratory-burst activation and actin polymerization, are presented below.

MATERIALS AND METHODS

Materials

Phosphoinositides, PtdIns, fMLP and butylated hydroxytoluene were purchased from Sigma. PtdIns(4,5)P_2 was from Boehringer Mannheim. Precoated t.l.c. plates (silica-gel 60, 0.2 mm thick) and potassium oxalate were from Merck. N-Ethylmaleimide, iodoacetic acid, benzamidine, leupeptin, pepstatin, EDTA, dimethyl sulphoxide (DMSO), $NH_4H_2PO_4$ and methylamine/ethanol were from Fluka, Buchs, Switzerland. Anti-(p85 PtdIns 3-kinase) polyclonal antibodies were from UBI. [γ-^{32}P]ATP (3000 Ci/mmol) and [^{32}P]P$_i$ (carrier-free) were from Amersham. The Partisphere SAX (10 μm, 4.6 mm × 250 mm) h.p.l.c. column was from Alltech, Deerfield, IL, U.S.A. Lymphoprep and Protein A–Sepharose CL 4-B were from Pharmacia. Wortmannin was kindly given by Dr. T. G. Payne, Preclinical Research, Sandoz, Basle, Switzerland.

Neutrophil isolation

Neutrophils were isolated from acid-citrate/dextrose-decoagulated buffy coats (provided by the Swiss Red Cross Laboratory, Fribourg, Switzerland) according to Böyum [38]. Remaining erythrocytes in the sediments of Lymphoprep gradients were lysed by isotonic NH_4Cl treatment, as described in detail elsewhere [39].

Phospholipid labelling and extraction

Neutrophils (5×10^7 cells/ml) were incubated with [^{32}P]P$_i$ (500 μCi/ml) in Hepes test buffer (30 mM Hepes, pH 7.4, 110 mM NaCl, 10 mM KCl, 1 mM MgCl$_2$ and 10 mM glucose) supplemented with 2 mg/ml fatty-acid-free BSA for 60 min at 37 °C. After removal of unincorporated [^{32}P]P$_i$, 5×10^6 cells were incubated in 0.5 ml of Hepes test buffer (without BSA, but with 1.5 mM CaCl$_2$) in the presence of the indicated concentrations of wortmannin or vehicle (DMSO) only for 10 min at 37 °C. Neutrophils were then stimulated with 100 nM fMLP and the reaction was stopped by addition of 3 ml of chloroform/methanol (1:2, v/v, with 0.63 mg/ml butylated hydroxytoluene and 10 μg/ml carrier phosphoinositides) at various times. Phospholipids were extracted and analysed by t.l.c. on potassium oxalate-impregnated silica-gel 60 plates, as described previously [21]. Labelled phospholipids were detected by autoradiography on Kodak X-Omat films or quantified with an automatic t.l.c. linear analyser (Berthold LB 2842). Radioactive bands were scraped from the plates and subjected to deacylation.

Deacylation of phospholipids

Deacylation was essentially performed as described in [40]. Methylamine reagent [25% (v/v) methylamine solution in ethanol/water (10:3, v/v)]/methanol/butan-1-ol (43:46:11, by vol.) was added directly to silica gel or extracted lipids and incubated at 53 °C for 1 h. The samples were dried in a Speed-Vac apparatus, resuspended in water and extracted with 2 × 1 vol. of butan-1-ol/light petroleum (b.p. 50–70 °C)/ethyl formate (20:4:1, by vol.). The aqueous phase was subjected to h.p.l.c.

H.p.l.c. analysis of glycerophosphoinositol phosphates

This was carried out on a Partisphere SAX 10 μm column as described in [41]. A gradient from buffers A (water) and B (1 M NH$_4$H$_2$PO$_4$, pH 3.8) was developed as follows: 0–10 min 100% A, to 25% B within 60 min and to 100% B over 50 min,

followed by 100% B for 10 min. Eluates from the column were mixed 1:3 (v/v) with FLO-Scint IV (Packard) and examined on-line with a FLO-ONE A500 β-detector (Packard). Elution times of resolved peaks were compared with those of ^{3}H-labelled standards [Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 from NEN] and deacylation products from ^{32}P-labelled PtdIns3P and PtdIns(3,4,5)P_3. These two compounds were produced with purified PtdIns 3-kinase ([42]; kindly provided by Dr. C. L. Carpenter, Harvard Medical School, Boston, MA, U.S.A.).

Immunoprecipitation and assays in vitro of PtdIns 3-kinase

PtdIns 3-kinase was immunoprecipitated from lysed resting neutrophils with rabbit polyclonal antibodies against the p85 subunit and Protein A–Sepharose as described previously [33]. Washed immunoprecipitates were resuspended in 500 μl of reaction buffer (20 mM Hepes, pH 7.4, 10 mM MgCl$_2$) and continuously agitated in the presence of wortmannin or DMSO for 10 min at room temperature. Immunoprecipitates were then centrifuged and resuspended in 40 μl of reaction buffer supplemented with 10 μM ATP and 20 μCi of [γ-^{32}P]ATP. The reaction was started by addition of 10 μl of a PtdIns/phosphatidylserine suspension (final concn. 200 μg/ml each, sonicated in reaction buffer). The samples were frequently mixed for 10 min at room temperature and then stopped with 40 μl of 1 M HCl. Lipids were recovered from the lower organic phase after addition of 80 μl of chloroform/methanol (1:1, v/v). Samples were spotted on t.l.c. plates and developed in chloroform/methanol/water/aq. NH$_3$ (90:70:17:3, by vol.). Radioactivity was quantified with the t.l.c. linear analyser, and radioactive bands were scraped off and deacylated for identification by h.p.l.c.

Respiratory-burst measurements

Respiratory-burst activity was measured by chemiluminescence originating from horseradish-peroxidase-catalysed luminol oxidation [43]. Neutrophils (10^6 cells/ml in Hepes test buffer supplemented with 9 units/ml horseradish peroxidase and 20 μM luminol) were preincubated with the indicated concentrations of wortmannin for 10 min at 37 °C before they were stimulated with 100 nM fMLP or 2.5 nM phorbol 12-myristate 13-acetate (PMA).

Determination of filamentous actin

Filamentous actin was stained with fluorescein-labelled phalloidin according to Howard and Oresajo [44] with modifications described elsewhere [15]. Aliquots of 10^6 fMLP-stimulated cells in 100 μl of Hepes test buffer were injected into 100 μl of staining solution (8% formaldehyde, 200 μg of lysophosphatidylcholine/ml and 0.33 μM fluorescein–phalloidin in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$, pH 7.4) precooled on ice. Samples were assayed for bound fluorescence after overnight incubation at 4 °C.

RESULTS AND DISCUSSION

When stimulated with the chemoattractant fMLP, neutrophils rapidly form PtdInsP_3. On t.l.c., PtdInsP_3 appears as a band migrating close to PtdInsP_2 (Figure 1; [21,22]). PtdInsP_3 is produced transiently, reaches a maximum at 10–20 s and returns to initial levels within 3–4 min. When neutrophils were preincubated with increasing concentrations of wortmannin, however, agonist-stimulated PtdInsP_3 formation was progress-
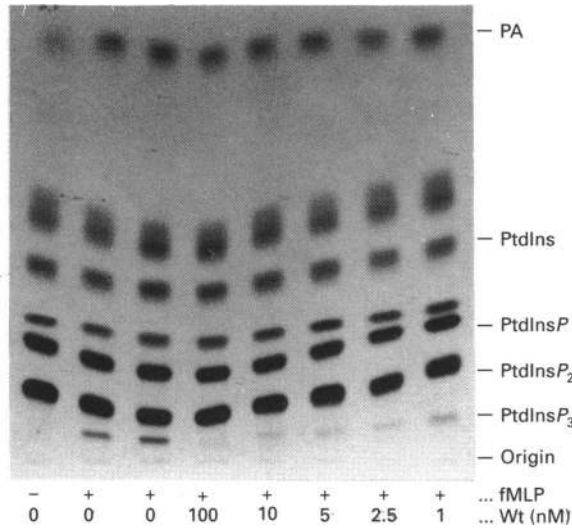
Figure 1   Effects of wortmannin on fMLP-induced [^{32}P]PtdInsP_3 formation and [^{32}P]phospholipid content

Neutrophils were preincubated with the indicated concentrations of wortmannin (Wt) and subsequently stimulated with 100 nM fMLP for 30 s where marked (+). ^{32}P-labelled lipids were then extracted, separated by t.l.c. and exposed for autoradiography as described in the Materials and methods section. Standards for PtdInsP_2, PtdIns and phosphatidic acid (PA) were stained by iodine vapour, and PtdInsP_3 and PtdIns$P$ were deacylated and analysed as described.

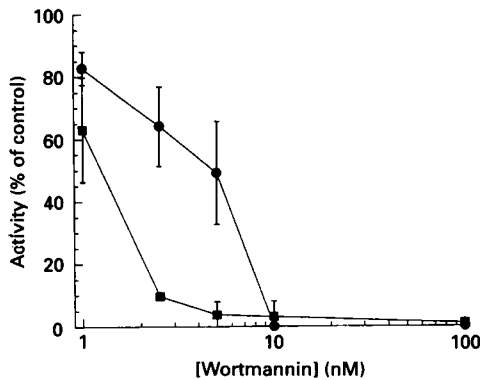**Figure 2   Dose-dependent inhibition of PtdIns 3-kinase activity by wortmannin in neutrophils (●) or in anti-p85 immunoprecipitates (■)**

[^{32}P]P$_i$-labelled neutrophils (●) were pretreated with various concentrations of wortmannin or DMSO (control) and subsequently stimulated with 100 nM fMLP for 30 s. Lipids were extracted and PtdInsP_3 was quantified as described. PtdIns 3-kinase immunoprecipitated from resting cells (■) was incubated with wortmannin or DMSO (control) before determination of PtdIns3P production from PtdIns and [γ-^{32}P]ATP. Results are means ± S.D. ($n = 3$).

ively inhibited. Figure 1 illustrates the effect of wortmannin on the PtdInsP_3 band, which is prominent in cells stimulated for 30 s with fMLP, but hardly detectable in wortmannin-treated or resting cells. Determination of the remaining radioactivity in PtdInsP_3 obtained from neutrophils exposed to wortmannin before stimulation with fMLP yielded a dose–response curve with an IC$_{50}$ of about 5 nM (Figure 2).

In principle, PtdInsP_3 formation could be aborted by effects of wortmannin on any step of phosphoinositide phosphorylation, thus removing the substrate for PtdIns 3-kinase. The pathway of
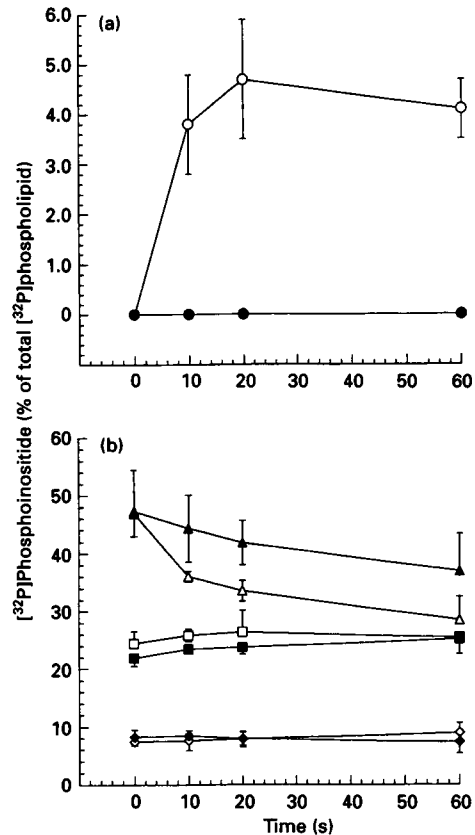

Figure 3   Effects of wortmannin on fMLP-induced changes in [^{32}P]phosphoinositides

Neutrophils were pretreated with 100 nM wortmannin (closed symbols) or DMSO (open symbols) before stimulation with 100 nM fMLP at $t = 0$. PtdInsP_3 (a, ○, ●), PtdInsP_2 (b, ▲, △), PtdIns$P$ (b, □, ■) and PtdIns (b, ◆, ◇) are displayed as percentage of total ^{32}P-labelled lipid extracted from cell suspensions at the indicated times ($n = 3$, means ± S.D.). PtdInsP_2 levels in stimulated cells ($t = 10, 20, 60$ s) were always enhanced in the presence of wortmannin as compared with their untreated counterparts.

fMLP-induced PtdInsP_3 synthesis in neutrophils has been analysed by Stephens et al. [45] in detail: PtdIns is converted into PtdIns4P and this into PtdIns(4,5)P_2, from which PtdIns 3-kinase finally produces PtdInsP_3. Later, PtdInsP_3 can be metabolized by a membrane-associated PtdIns(3,4,5)P_3 5-phosphomonoesterase to PtdIns(3,4)P_2. Dephosphorylations via PtdIns3P close the cycle and form PtdIns [45].

Neutrophils treated with 100 nM wortmannin and stimulated with fMLP for various durations did not show detectable levels of PtdInsP_3 at any time, whereas in their untreated counterparts [^{32}P]PtdInsP_3 rose to about 5 % of the total ^{32}P-labelled lipid (Figure 3a). On the other hand, the time courses of [^{32}P]PtdIns and [^{32}P]PtdIns$P$ were indistinguishable in normal and wortmannin-treated cells (Figure 3b). [^{32}P]PtdInsP_2 was found to be increased (two-way analysis of variance, $P < 0.0001$), when wortmannin was present. Since phosphatidylinositols phosphorylated on position D-3 of the inositol ring contribute less than 3 % to the radioactivity found in PtdIns$P$ and PtdInsP_2 [45], the kinetics in Figure 3(b) represent the relative amounts of PtdIns4P and PtdIns(4,5)P_2. One can thus conclude that the chain of substrate formation for PtdIns 3-kinase is intact, and PtdIns 4- and 5-kinases are not affected by wortmannin. In response to agonists, both phospholipase C and PtdIns 3-kinase

deplete the pool of PtdIns(4,5)P_2. Direct inhibition of PtdIns 3-kinase by wortmannin is therefore well suited to explain the observed decrease in PtdInsP_2 degradation.

The PtdIns 3-kinase complex, composed of a regulatory (p85) [24–26] and a catalytic (p110) subunit [27], can be immuno-precipitated with antibodies directed against p85. When immunoprecipitated from resting cells and incubated with wortmannin, PtdIns 3-kinase showed an even greater sensitivity towards wortmannin compared with the activity in the cellular system: production of PtdIns3P from PtdIns and ATP was already decreased to 50% in the presence of 1 nM inhibitor. Because activity *in vitro* does not depend on agonist-mediated activation, these results illustrate the direct action of wortmannin on PtdIns 3-kinase.

In neutrophils, the receptor-coupled mechanisms leading to PtdIns 3-kinase activation are not as well understood as its interaction with growth-factor receptors. It has been proposed that PtdIns 3-kinase activation by the fMLP receptor involves heterotrimeric GTP-binding proteins [21], which is in agreement with the finding that fMLP-induced PtdIns 3-kinase activation is not correlated with tyrosine phosphorylation of p85 [33]. It is not clear, however, if this interaction is direct or transduced through an adapter protein, as is the case for the insulin receptor substrate-1 [46,47].

We have shown recently (M. P. Wymann and A. Arcaro, unpublished work) that wortmannin inhibits PDGF-stimulated PtdInsP_3 production without affecting PDGF-receptor-mediated tyrosine phosphorylation of p85, which only occurs after PDGF-receptor activation, autophosphorylation and translocation of p85. This, and the fact that wortmannin at high concentrations (μM) does not interfere with fMLP-induced Ca^{2+} release from internal stores [35] [depending on functional heterotrimeric G-proteins and phospholipase C producing Ins(1,4,5)P_3], also indicates that not the signalling pathway, but PtdIns 3-kinase itself, is the target of wortmannin.

The production of recombinant p110 in the absence of p85 by Hiles et al. [27] has shown clearly that p110 contains the catalytic site of PtdIns 3-kinase. One could argue that wortmannin does not interact with p110, but leads to the dissociation of p110 from p85. PtdIns 3-kinase would thus be lost from immunoprecipitates, and in cells p110 would eventually not be translocated to the membrane. To test this hypothesis, immunoprecipitates obtained with anti-p85 antibodies were treated with 1 μM wortmannin for 15 min. After such a treatment, no activity was left in the immunoprecipitates (see Figure 2). Subsequent denaturing gel electrophoresis and detection of p110 by immunoblotting revealed that the p85–p110 complex remained unaffected by wortmannin (results not shown). Because p110 is present, but inactive, it is likely that wortmannin binds to p110. Such a view is supported by preliminary experiments showing that high concentrations of ATP, included during incubations with wortmannin, can partly protect the enzyme from inactivation (M. P. Wymann and A. Arcaro, unpublished work). One can thus speculate that the binding site of wortmannin on p110 might lie close to the ATP-binding site of the lipid kinase.

PtdInsP_3 was proposed to be a novel messenger molecule in the activation pathway of the respiratory burst and actin polymerization, since the time course of its agonist-induced elevation correlated with these cell responses [21–23]. Indeed, fMLP-mediated NADPH oxidase activity can be inhibited by nM concentrations of wortmannin (Figure 4, $IC_{50} = 12$ nM; see also [34]), whereas the PMA-mediated response is resistant to μM concentrations of the inhibitor. It has also been shown that wortmannin does not influence NADPH oxidase activity in a cell-free system [34]. Because PtdInsP_3 is also produced in Ca^{2+}-
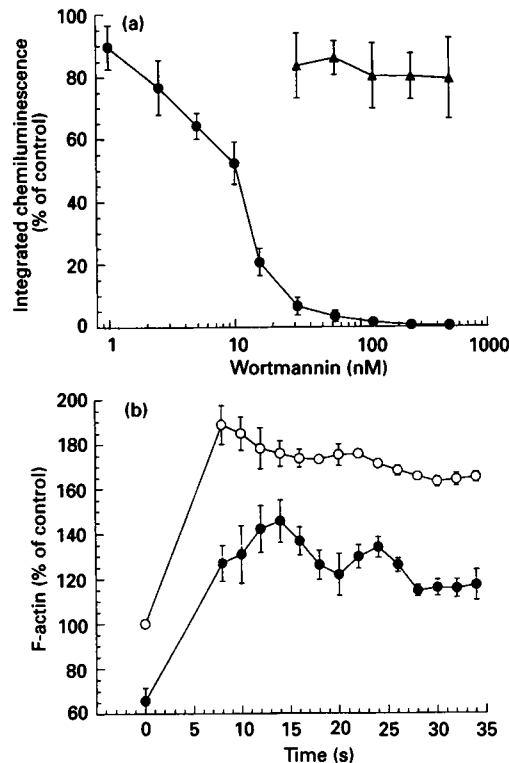

Figure 4   Differential sensitivity of neutrophil responses to wortmannin

(a) Cells were treated with the indicated concentrations of wortmannin before 100 nM fMLP (●) or 2.5 nM PMA (▲) was added, and respiratory-burst activity was determined by luminol-dependent chemiluminescence. The response curves were integrated and values were compared with those obtained from cells stimulated in the absence of wortmannin ($n = 3$, means ± S.D.). (b) fMLP-induced changes in relative filamentous-actin content in neutrophils treated with 1 μM wortmannin (●) or control cells (DMSO only; ○). At the indicated times, cells were fixed and stained for filamentous actin. Values at $t = 0$ represent unstimulated controls. Bound phalloidin fluorescence is expressed as percentage of unstimulated untreated controls. The progress curves are means for three experiments (± S.D.; error bars omitted were smaller than the symbols).

depleted cells [21], it might provide an element for the presumed Ca^{2+}-independent activation pathway [35,39,48], which was proposed to act in parallel with PKC-mediated phosphorylations of the 47 kDa cytosolic factor of the oxidase (for a review see [49]).

Prolonged activation of PKC by PMA seems to bypass the need for PtdInsP_3 synthesis in NADPH oxidase activation. Phosphorylation might here replace the action of the anionic amphiphile PtdInsP_3, as SDS, phosphatidic acid and arachidonic acid replace the need for phosphorylations in cell-free systems [2]. The way PtdInsP_3 actually mediates assembly and activation of the oxidase system will have to be determined by experiments in cell-free systems.

In contrast with the respiratory burst, fMLP-induced actin polymerization was not inhibited, although influenced, by μM concentrations of wortmannin. Since PtdInsP_3 is totally absent under these conditions, it cannot provide the repetitive signal that generates the observed oscillations in filamentous-actin content (Figure 4b). PtdInsP_3, however, seems to have a modulatory role in the rearrangement of the actin cytoskeleton. Oscillations occur under conditions where no or little PtdInsP_3 is produced: in the presence of wortmannin [13,15] or low concentrations of agonist [14,50].

We have found recently that 50 nM wortmannin totally abolished the PDGF-induced formation of filamentous-actin-rich circular membrane ruffles in human foreskin fibroblasts (M. P. Wymann and A. Arcaro, unpublished work). Why actin rearrangements are abolished in one cell type, but not in the other, is at present not clear. Explanations might arise from the different cytoskeletal structures and actin compositions present in fibroblasts and leucocytes.

The actions of wortmannin on neutrophil responses have been attributed mostly to its effect on phospholipase D [4,5,7]. Because wortmannin does not inhibit PMA-induced phospholipase D activity [4], and even enhances the production of phosphatidic acid in some cell types [51], phospholipase D cannot be the molecular target for the inhibitor.

When tested at μM concentrations, wortmannin did not interfere with activities of PKC, cyclic-GMP-dependent protein kinase, calmodulin-dependent protein kinase II, cyclic-AMP-dependent protein kinase [36], PDGF β-receptor protein tyrosine kinase (M. P. Wymann and A. Arcaro, unpublished work) and agonist-mediated Ca^{2+} release [35]. Nakanishi et al. [36] have shown that wortmannin can interfere at μM concentrations with myosin light-chain kinase.

The results presented here clearly demonstrate the inhibition of PtdIns 3-kinase by wortmannin and offer a reasonable explanation for the effects of the inhibitor on cellular responses at nM concentrations. As a potent and specific inhibitor, wortmannin can be used to exploit the dependency of further signals and responses on PtdInsP_3 production.

REFERENCES

1 Segal, A. W. and Abo, A. (1993) Trends Biochem. Sci. **18**, 43–47
2 Uhlinger, D. J., Tyagi, S. R., Inge, K. L. and Lambeth, J. D. (1993) J. Biol. Chem. **268**, 8624–8631
3 Tauber, A. I. (1987) Blood **69**, 711–720
4 Bonser, R. W., Thompson, N. T., Randall, R. W., Tateson, J. E., Spacey, G. D., Hodson, H. F. and Garland, L. G. (1991) Br. J. Pharmacol. **103**, 1237–1241
5 Reinhold, S. L., Prescott, S. M., Zimmerman, G. A. and McIntyre, T. M. (1990) FASEB J. **4**, 208–214
6 Rossi, F., Grzeskowiak, M., Della Bianca, V., Calzetti, F. and Gandini, G. (1990) Biochem. Biophys. Res. Commun. **168**, 320–327
7 Gelas, P., von Tscharner, V., Record, M., Baggiolini, M. and Chap, H. (1992) Biochem. J. **287**, 67–72
8 Maridonneau Parini, I. and Tauber, A. I. (1986) Biochem. Biophys. Res. Commun. **138**, 1099–1105
9 Stossel, T. P. (1989) J. Biol. Chem. **264**, 18261–18264
10 Devreotes, P. N. and Zigmond, S. H. (1988) Annu. Rev. Cell Biol. **4**, 649–686
11 Omann, G. M., Allen, R. A., Bokoch, G. M., Painter, R. G., Traynor, A. E. and Sklar, L. A. (1987) Physiol. Rev. **67**, 285–322
12 Cunningham, C. C. (1992) Cancer Metastasis Rev. **11**, 69–77
13 Wymann, M. P., Kernen, P., Deranleau, D. A., Dewald, B., von Tscharner, V. and Baggiolini, M. (1987) Biochem. Biophys. Res. Commun. **147**, 361–368
14 Omann, G. M., Porasik, M. M. and Sklar, L. A. (1989) J. Biol. Chem. **264**, 16355–16358
15 Wymann, M. P., Kernen, P., Bengtsson, T., Andersson, T., Baggiolini, M. and Deranleau, D. A. (1990) J. Biol. Chem. **265**, 619–622
16 Janmey, P. A., Iida, K., Yin, H. L. and Stossel, T. P. (1987) J. Biol. Chem. **262**, 12228–12236
17 Howard, T., Chaponnier, C., Yin, H. and Stossel, T. (1990) J. Cell Biol. **110**, 1983–1991
18 Zigmond, S. H., Slonczewski, J. L., Wilde, M. W. and Carson, M. (1988) Cell Motil. Cytoskeleton **9**, 184–189
19 Della Bianca, V., Grzeskowiak, M., Cassatella, M. A., Zeni, L. and Rossi, F. (1986) Biochem. Biophys. Res. Commun. **135**, 556–565
20 Bengtsson, T., Rundquist, I., Stendahl, O., Wymann, M. P. and Andersson, T. (1988) J. Biol. Chem. **263**, 17385–17389
21 Traynor-Kaplan, A. E., Thompson, B. L., Harris, A. L., Taylor, P., Omann, G. M. and Sklar, L. A. (1989) J. Biol. Chem. **264**, 15668–15673
22 Traynor-Kaplan, A. E., Harris, A. L., Thompson, B., Taylor, P. and Sklar, L. A. (1988) Nature (London) **334**, 353–356
23 Eberle, M., Traynor-Kaplan, A. E., Sklar, L. A. and Norgauer, J. (1990) J. Biol. Chem. **265**, 16725–16728
24 Skolnik, E. Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A., Ullrich, A. and Schlessinger, J. (1991) Cell **65**, 83–90
25 Otsu, M., Hiles, I. D., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J., Courtneidge, S. A., Parker, P. J. and Waterfield, M. D. (1991) Cell **65**, 91–104
26 Escobedo, J. A., Navankasattusas, S., Kavanaugh, W. M., Milfay, D., Fried, V. A. and Williams, L. T. (1991) Cell **65**, 75–82
27 Hiles, I. D., Otsu, M., Volinia, S., Fry, M. J., Gout, I., Dhand, R., Panayotou, G., Ruiz Larrea, F., Thompson, A. and Totty, N. F. (1992) Cell **70**, 419–429
28 Panayotou, G., Bax, B., Gout, I., Federwisch, M., Wroblowski, B., Dhand, R., Fry, M. J., Blundell, T. L., Wollmer, A. and Waterfield, M. D. (1992) EMBO J. **11**, 4261–4272
29 Kazlauskas, A. and Cooper, J. A. (1989) Cell **58**, 1121–1133
30 Fantl, W. J., Escobedo, J. A., Martin, G. A., Turck, C. W., Del Rosario, M., McCormick, F. and Williams, L. T. (1992) Cell **69**, 413–423
31 Kaplan, D. R., Whitman, M., Schaffhausen, B., Pallas, D. C., White, M., Cantley, L. C. and Roberts, T. M. (1987) Cell **50**, 1021–1029
32 Susa, M., Keeler, M. and Varticovski, L. (1992) J. Biol. Chem. **267**, 22951–22956
33 Vlahos, C. J. and Matter, W. F. (1992) FEBS Lett. **309**, 242–248
34 Baggiolini, M., Dewald, B., Schnyder, J., Ruch, W., Cooper, P. H. and Payne, T. G. (1987) Exp. Cell Res. **169**, 408–418
35 Dewald, B., Thelen, M. and Baggiolini, M. (1988) J. Biol. Chem. **263**, 16179–16184
36 Nakanishi, S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H., Matsuda, Y., Hashimoto, Y. and Nonomura, Y. (1992) J. Biol. Chem. **267**, 2157–2163
37 Yatomi, Y., Hazeki, O., Kume, S. and Ui, M. (1992) Biochem. J. **285**, 745–751
38 Böyum, A. (1993) Scand. J. Clin. Lab. Invest. **97**, 77–89
39 Wymann, M. P., von Tscharner, V., Deranleau, D. A. and Baggiolini, M. (1987) J. Biol. Chem. **262**, 12048–12053
40 Clarke, N. G. and Dawson, R. M. C. (1981) Biochem. J. **195**, 301–306
41 Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P. and Cantley, L. C. (1989) Cell **57**, 167–175
42 Carpenter, C. L., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S. and Cantley, L. C. (1990) J. Biol. Chem. **265**, 19704–19711
43 Wymann, M. P., von Tscharner, V., Deranleau, D. A. and Baggiolini, M. (1987) Anal. Biochem. **165**, 371–378
44 Howard, T. H. and Oresajo, C. O. (1985) Cell Motil. **5**, 545–557
45 Stephens, L. R., Hughes, K. T. and Irvine, R. F. (1991) Nature (London) **351**, 33–39
46 Backer, J. M., Myers, M. G., Jr., Shoelson, S. E., Chin, D. J., Sun, X.-J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J. and White, M. F. (1992) EMBO J. **11**, 3469–3479
47 Sun, X.-J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J. and White, M. F. (1991) Nature (London) **352**, 73–77
48 Grzeskowiak, M., Della Bianca, V., Cassatella, M. A. and Rossi, F. (1986) Biochem. Biophys. Res. Commun. **135**, 785–794
49 Baggiolini, M. and Wymann, M. P. (1990) Trends Biochem. Sci. **15**, 69–72
50 Wymann, M. P., Kernen, P., Deranleau, D. A. and Baggiolini, M. (1989) J. Biol. Chem. **264**, 15829–15834
51 Kanoh, H., Ohbayashi, H., Matsuda, Y., Nonomura, Y. and Nozawa, Y. (1992) Biochem. Biophys. Res. Commun. **188**, 510–515