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The phosphatidylinositol 3-kinase inhibitor wortmannin markedly reduces chemotactic peptide-induced locomotion and increases in cytoskeletal actin in human neutrophils

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Abstract

To define a possible role of the enzyme phosphatidylinositol 3-kinase (PI 3-kinase) in motile functions of neutrophils, we have used a potent inhibitor of this enzyme, [1*S*-(1α,6bα,9aβ,11α,11bβ)]-11-(acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(methoxymethyl)-9a,11b-dimethyl-3*H*-furo[4,3,2-de]indeno[4,5-*h*]-2-benzopyran-3,6,9-trione (wortmannin). Wortmannin markedly attenuated chemotactic peptide-induced development of polarity, locomotion and increases in cytoskeletal actin and α-actinin in human neutrophils at low, nM, concentrations (ED_{50} = 4–40 nM; 0.4–3 pmol/10^6 cells). The increase in cytoskeletal actin induced by phorbol-12-myristate-13-acetate in contrast was not affected by wortmannin (18 pmol/10^6 cells). Moreover, the increase in total F-actin induced by an incubation for 1 min with chemotactic peptide was much less sensitive to wortmannin than increases in cytoskeletal actin; 80 pmol/10^6 cells were necessary for half-maximal inhibition. Wortmannin thus appears to primarily affect F-actin organization, rather than polymerization. Inhibition of development of polarity by wortmannin correlated with inhibition of production of phosphatidylinositol 3,4,5-trisphosphate. According to our findings, activation of a wortmannin-sensitive target, very likely PI 3-kinase, is required for optimal chemotactic peptide-induced neutrophil motility. © 1997 Elsevier Science B.V.

Keywords: Neutrophils; Cell locomotion; Wortmannin; Phosphatidylinositol 3-kinase; Actin, α-Actinin

1. Introduction

Directed migration is an indispensable feature of neutrophil granulocytes in fulfilling their physiological role in host defense against pathogens. However, the molecular mechanisms of directed migration are as yet poorly understood. Certainly, a functional actin network and actin filament turnover are involved in this process (Zigmond, 1993). Upon activation with chemotactic stimuli, neutrophils rapidly extend protrusions in all directions, concomitant with a marked increase in total F-actin and cytoskeletal actin (Rao and Varani, 1982; White et al., 1983). Total cellular F-actin is defined as that fraction of actin binding NBD-phallacidin after fixation of cells, and cytoskeletal actin as that fraction of actin sedimenting at low g force (10,000–16,000 × g) after solubilization of cells with non-ionic detergents (Watts and Howard, 1993). After a few minutes of incubation with the stimulus, the cells assume a polarized morphology with a broad front lamella and a contracted tail. The newly formed F-actin accumulates in the front lamellae of polarized neutrophils (Zigmond, 1993). How signals are transferred to actin and actin-associated proteins, how localized actin polymerization, development of cell polarity and locomotion are achieved, are as yet largely unresolved questions, although a number of new chemoattractant-induced signalling pathways have been identified in neutrophils (Bokoch, 1995). Activation of heterotrimeric G-proteins is necessary for directed locomotion and for receptor-linked increases in actin polymerization (Bokoch, 1995). A rise in intracellular calcium, activation of protein kinase C and of tyrosine kinases may be relevant for chemotactic peptide-induced locomotion (Maxfield, 1993; Niggli and Keller, 1993; Gaudry et al., 1992), although these signalling pathways appear not to be relevant for actin reorganization (Bengtsson et al., 1986; Niggli and Keller, 1991; Naccache

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et al., 1990). We have obtained evidence for a role of an okadaic acid-sensitive phosphatase regulating neutrophil shape and actin organization (Kreienbühl et al., 1992).

Interesting new putative second messengers, phosphatidylinositol-3,4,5-trisphosphate (PtIns(3,4,5)P_3) and phosphatidylinositol-3,4-diphosphate (PtIns(3,4)P_2), have been shown to be produced in neutrophils stimulated by formylated chemotactic peptides with kinetics paralleling that of increased actin polymerization. The content of these lipids in neutrophils is increased by low, nM concentrations of chemotactic peptides, conditions which are optimal also for inducing locomotion and actin reorganization but not for other functions. *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) as well as the formyl-hexapeptide used in this study have been shown to be effective (Traynor-Kaplan et al., 1989; Eberle et al., 1990). Production of these lipids is due to activation of the enzyme PI 3-kinase (Stephens et al., 1991). This enzyme can be inhibited by the fungal metabolite [1*S*-(1α,6$b\alpha$,9$a\beta$,11α,11$b\beta$)]-11-(acetyloxy)-1,6*b*,7,8,9*a*,10,11,11*b*-octahydro-1-(methoxymethyl)-9*a*,11*b*-dimethyl-3*H*-furo[4,3,2-de]indeno[4,5-*h*]-2-benzopyran-3,6,9-trione (wortmannin; Nakanishi et al., 1995; Fig. 1 in Ui et al., 1995). Wortmannin has been shown to inhibit PI 3-kinase isolated from neutrophils and other cell types selectively at low (nM) concentrations, by covalent reaction with lysine 802 (Arcaro and Wymann, 1993; Wymann et al., 1996). Protein kinases A, C, G and phosphatidylinositol 4-kinase have reported to be unaffected by concentrations of wortmannin up to 1 μM (Nakanishi et al., 1995; Ui et al., 1995). Involvement of PI 3-kinase in motile responses of neutrophils is an attractive hypothesis in view of the number of findings in other cell types demonstrating the involvement of this enzyme in chemotaxis and in producing membrane ruffles enriched in F-actin (Kotani et al., 1994; Derman et al., 1996).

We have now studied the effects of wortmannin on development of polarity, locomotion and actin reorganization in human neutrophils stimulated by chemotactic peptide, and provide evidence for an important role of a wortmannin-sensitive target, most likely PI 3-kinase, in regulating these processes.

2. Materials and methods

2.1. Reagents and suppliers

Reagents and suppliers were: *N*-formyl-L-norleucyl-L-leucyl-L-phenylalanyl-L-norleucyl-L-tyrosyl-L-lysine (fNLPNTL), Bachem, Bubendorf; human serum albumin (HSA), Behringwerke (Marburg, Germany); LY294002, Calbiochem (La Jolla, CA, USA); wortmannin, PMA, paraformaldehyde, dimethyl sulfoxide (DMSO), Sigma (St. Louis, MO, USA); diisopropylfluorophosphate, Fluka (Buchs, Switzerland); *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin (NBD-phallacidin), Molecular Probes (Junction City, OR, USA); Triton X-100 (a 10% solution in H_2O, stored under nitrogen), Pierce (Rockford, IL, USA); [^{32}P]orthophosphate (in aqueous solution, HCl-free, carrier-free), NEN DuPont (Wilmington, DE, USA). The medium contained 138 mM NaCl, 6 mM KCl, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, 20 mM Hepes, pH 7.3, without or with divalent cations, phosphate and HSA, depending on the assays (see below).

Wortmannin was prepared as a stock solution of 10 mM in DMSO and LY294002 as a stock solution of 50 mM in DMSO. Both stock solutions were stored at −20°C. DMSO alone, up to 0.4%, had no effects in our experiments.

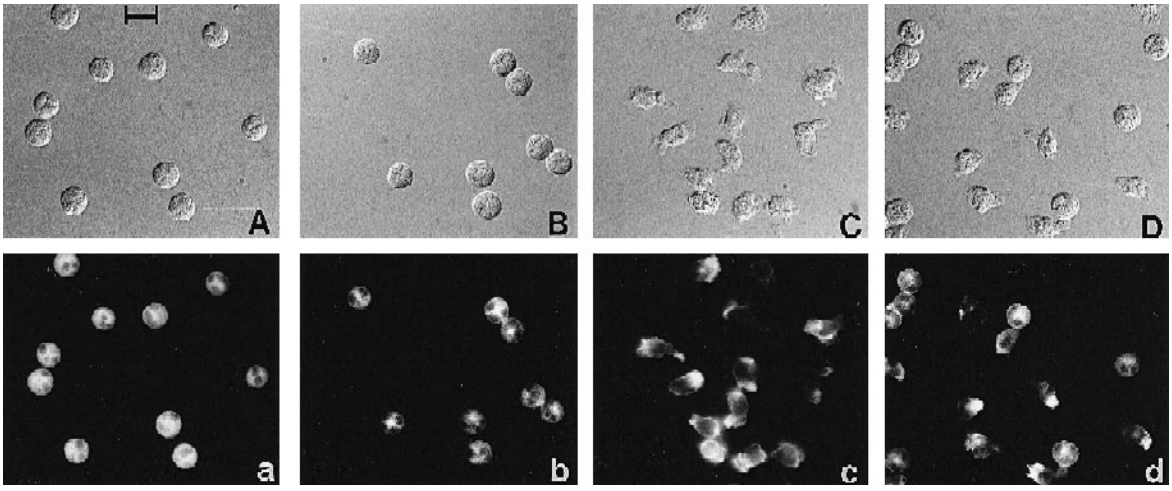

Fig. 1. Effects of wortmannin on fNLPNTL-induced changes in shape and F-actin distribution in neutrophils. Neutrophils were treated as follows (A,a) untreated controls (medium); (B,b) wortmannin, 100 nM and medium; (C,c) fNLPNTL, 10^{-9} M; (D,d) wortmannin, 100 nM and fNLPNTL, 10^{-9} M. Neutrophils were preincubated with or without wortmannin at 37°C for 10 min. Incubation was continued for another 30 min after addition of medium or fNLPNTL. Paraformaldehyde-fixed cells were stained with NBD-phallacidin. Photographs were obtained with differential interference contrast optics (A–D) or in fluorescent light (a–d). Bar 10 μm.

2.2. Proteins and antibodies

Actin was prepared from rabbit skeletal muscle (Pardee and Spudich, 1982) and α-actinin from chicken gizzard (Niggli and Jenni, 1989). A monoclonal anti-α-actinin antibody (clone BM-75.2) was obtained from Sigma. Gold-labeled second antibodies for immunoblotting (goat anti-mouse IgG/M, LM grade) were obtained from Aurion (Wageningen, Netherlands).

2.3. Isolation of human neutrophils

Neutrophils were isolated from heparinized human blood (10 units/ml). In a first step, red blood cells were removed with metrizoate-methocel (Böyum, 1968). Subsequently, mononuclear cells were removed using neutrophil isolation medium (NIM, obtained from Cardinal Associates, Santa Fe, NM, USA) which contains Hypaque and Ficoll, see Ferrante and Thong (1980). Approximately 95% of the leukocytes were neutrophils.

2.4. Analysis of shape changes

Neutrophils (12×10^6 cells/ml) were incubated in medium containing 0.1% HSA and 10 mM ethylenediaminetetraacetic acid (EDTA) in a reciprocating waterbath at 37°C without or with wortmannin and fNLPNTL, as indicated in Section 3. The reaction was stopped by fixing the cells in 1% glutaraldehyde (final concentration) for 30 min. The cells were washed and resuspended in 0.9% NaCl containing NaN_3 (1 mg/ml). Cell shape was determined using differential interference contrast microscopy (Nomarski optics) using a Zeiss IM 35 microscope with a 100× objective (NA 1.25). The shape of neutrophils was classified into the following categories as previously described: spherical, spherical with unifocal projections, polarized and non-polar cells with surface projections (Keller and Niggli, 1994).

2.5. Locomotion

For locomotion assays, a discontinuous Ficoll-metrizoate gradient (Böyum, 1968) instead of NIM was used for removal of mononuclear cells. Neutrophils (3×10^6/ml) were incubated in medium containing 100 μM EGTA, 0.1% HSA, with inhibitor and/or stimulus at 37°C in a reciprocating waterbath as indicated in Section 3. The cells were centrifuged at $300 \times g$ for 5 min and resuspended in medium containing 1.1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 μM EGTA, 2% HSA and inhibitor and/or stimulus. Neutrophils (5 μl containing 0.75×10^6 cells) were placed between a slide and a round coverslip (25 mm diameter). The slide-coverslip preparation was sealed with paraffin and placed on the heated stage (37°C) of a Zeiss IM 35 microscope with a 63× objective. The locomotor behavior of the cells (35–80 cells for each condition) was recorded for 10 min using video microscopy. The outline of the cell at the initial and final position and the path traveled during 10 min was drawn on a transparency. Cells remaining totally or partially within the outline of the initial position are defined as stationary; cells found outside after 10 min are defined as locomoting.

2.6. Localization and determination of F-actin

The intracellular localization and relative amounts of F-actin were determined by NBD-phallacidin-staining of paraformaldehyde-fixed cells as described (Howard and Meyer, 1984), with modifications (Keller and Niggli, 1995). Neutrophils (10^6 cells/ml) were incubated in medium containing 0.1% HSA and 1.1 mM EDTA with or without wortmannin and fNLPNTL as indicated in Section 3. The cells were then fixed in 4% paraformaldehyde and stained with NBD-phallacidin as described (Keller and Niggli, 1995). Cellular fluorescence was visualized using a Zeiss Axioplan microscope with a 100× Plan Neofluar objective (NA 1.30). Photographs were taken with a Pentax camera and a Kodak Tmax 400 film. The relative amount of F-actin was determined based on NBD-phallacidin binding by means of flow cytometry (Epics Profile II, Coulter Corporation, Hialeah, FL) using an excitation wavelength of 488 nm and a 525 nm band pass filter. The median channel number (linear fluorescence scaling) was determined. The difference between cells stained with NBD-phallacidin and the corresponding unstained controls was considered as specific uptake of NBD-phallacidin. The value for stained, unstimulated controls was set as 1 and the increase over unstimulated control values was calculated.

2.7. Determination of cytoskeletal actin

The percentage of cytoskeletal actin was determined as described (White et al., 1983), with some modifications (Niggli and Keller, 1991). After treatment with diisopropylfluorophosphate in order to block endogenous proteases (Amrein and Stossel, 1980), neutrophils were resuspended in medium, pH 7.3, with 0.1% HSA, containing in addition 10 mM EDTA (12×10^6 cells/ml). Aliquots of this suspension (450 μl) were incubated with different stimuli, with or without wortmannin, as described in Section 3, followed by isolation of cytoskeletal fractions (Niggli and Keller, 1991) The resulting pellets, corresponding to the cytoskeletal fractions, and the supernatants, corresponding to the Triton-soluble fractions, were analyzed on 5–15% gradient gels (Laemmli, 1970), together with known amounts of purified rabbit skeletal muscle actin. Actin present in both fractions was quantified by scanning of the Coomassie Blue-stained gels using a Camag TLC scanner at 590 nm. In some experiments, cytoskeletal fractions, obtained as described above, were subjected to SDS-PAGE together with known amounts of purified actin and α-actinin. The lower part of the gel, containing actin, was

stained with Coomassie Blue, whereas the upper part, containing α-actinin, was transblotted to nitrocellulose using a mini-genie blotter from Idea Scientific (Minneapolis, MN), for 70 min at 24 V. Blots were decorated with a monoclonal anti-α-actinin antibody as described (Niggli and Jenni, 1989).

2.8. Phospholipid labelling and extraction

The production of PtIns(3,4,5)P_3 was measured essentially according to the method described by Traynor-Kaplan et al. (1989). Isolated neutrophils were washed 3 × with a phosphate- and HSA-free buffer (138 mM NaCl, 6 mM KCl, 20 mM Hepes, pH 7.3, 5.5 mM glucose) and incubated with [^{32}P]orthophosphate (0.2 mCi/ml) for 1 h at 37°C in a shaking waterbath (20×10^6 cells/ml). The cells were washed twice with 5 ml of this buffer and resuspended at 12×10^6 cells/ml. Aliquots of the labelled cells (225 μl) were incubated with or without wortmannin and fNLPNTL, as indicated. The reaction was stopped with 1.5 ml chloroform/methanol (1:2, v/v) containing 0.63 mg/ml butylated hydroxyethylene and phosphoinositides (Sigma, 60 μg/ml) as cold carrier. 0.23 mM $CaCl_2$ and 0.15 mM $MgSO_4$ (final) were also added to the extracts, followed by vortexing. Another 1 ml of chloroform and 1 ml of 2.4 M HCl were added. The upper, aqueous phase was washed twice with 1 ml chloroform. The combined lower phases were washed once with 1 ml of methanol, 1 M HCl (1:1, v/v), dried under nitrogen and resuspended in 30 μl of chloroform/methanol (2:1, v/v) for thin layer chromatography (TLC). Precoated TLC plates (Silica gel-60, 0.25 mm thick, Merck) were impregnated with 1.2% potassium oxalate in methanol/water (2:3, v/v) over night. Plates were activated for 50 min at 110°C before spotting. The plates were developed in chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14, v/v/v/v/v). Radioactivity was detected using Trimax XM films (3 M AG, Rüschlikon), and with the phosphoimager Storm (Molecular Dynamics). Spots were quantitated after background subtraction using Image Quant (Molecular Dynamics) after a 24–48 h exposure.

2.8.1. Statistical analysis of data

Differences between data were analyzed with the Student's t test for paired data, with a P value of < 0.05 considered significant.

3. Results

3.1. Wortmannin markedly reduces the fraction of polarized cells in human neutrophils stimulated by chemotactic peptide

Neutrophils isolated from a healthy donor showed mainly a spherical shape with a smooth surface and a diffuse F-actin distribution (Fig. 1A,a). Upon stimulation with chemotactic peptide the cells developed within one minute protrusions in all directions (not shown). This non-polar shape was gradually changed into a polar shape with front lamellae and contracted tail upon longer times of incubation (Fig. 1C). F-actin was enriched in the front and in the tail of polarized cells (Fig. 1C,c). Preincubation of cells with 100 nM wortmannin (9 pmol/10^6 cells) did not markedly affect resting cell shape and F-actin distribution (Fig. 1B,b) and the first rapid phase of protrusion formation (not shown), but it significantly reduced the fraction of polarized cells normally observed after a 30 min incubation with chemotactic peptide. The cells assumed a spherical shape with diffusely distributed F-actin. A fraction of the cells formed one or several small protrusions (Fig. 1D,d). Cells with several protrusions were counted as 'non-polar'. However, shape changes in these cells were less extensive than the extensive surface ruffling seen in neutrophils treated with PMA or diacylglycerol (Zimmermann et al., 1988). A quantitative evaluation of the effects of wortmannin on cell shape is shown in Fig. 2. The amount of polarized cells was significantly ($P < 0.005$) reduced by $44 \pm 14\%$ ($n = 4$) at 100 nM wortmannin (9 pmol/10^6 cells), by $56 \pm 15\%$ ($n = 4$) at 200 nM wortmannin (18 pmol/10^6 cells) and by $59 \pm 17\%$ ($n = 6$) at 1 μM (90 pmol/10^6 cells). Half-maximal effects of wortmannin on cell polarity were observed at 14–32 nM (1–3 pmol/10^6 cells). In parallel with a decrease in the amount of polarized cells, the amount of nonpolar cells with minor surface projections and the spherical cells increased (Fig. 2). At lower wortmannin concentrations (10–40 nM) the nonpolar cells increased, at higher (100–200 nM) concentrations a substantial fraction of the total cell population

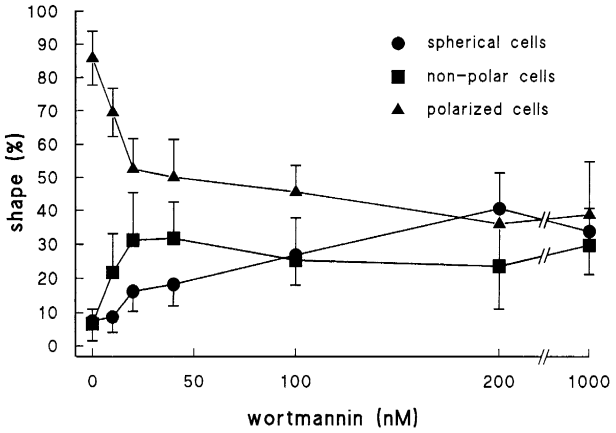

Fig. 2. Concentration-dependent effects of wortmannin on neutrophil shape changes. (●) Spherical cells; (■) cells with small surface projections; (▲) polarized cells. Neutrophils were preincubated in medium containing 10 mM EDTA and 0.1% HSA with either 0.2% DMSO or increasing concentrations of wortmannin at 37°C for 10 min. The incubation was continued for another 30 min after the addition of 10^{-9} M fNLPNTL. At the end of an incubation period of a total of 40 min, cells were fixed with glutaraldehyde and examined using Nomarski optics. Mean ± S.D. of 3–7 experiments.

($41 \pm 9\%$, $n = 5$) was spherical, comparable to resting cells. As shown in Figs. 1 and 2, a fraction of the cells ($36 \pm 11\%$, $n = 4$) remained polarized in the presence of 200 nM wortmannin. LY294002, a structurally different PI 3-kinase inhibitor (Vlahos et al., 1995) similarly markedly reduced the fraction of polarized cells, consistent with inhibition of PI 3-kinase ($ED_{50} = 11 \pm 5$, mean $\pm$ S.D., $n = 5$, data not shown). Comparable to the findings with wortmannin, development of polarity was only partially inhibited by LY294002, $48 \pm 4\%$ ($n = 4$) of the cells remaining polarized in the presence of 70 μM of the drug, as compared to $80 \pm 8\%$ ($n = 4$) of the cells assuming a polarized morphology in the control samples.

3.2. Wortmannin inhibits stimulated locomotion of neutrophils

As shown in Figs. 1 and 2, wortmannin significantly inhibited development of polarity in fNLPNTL-activated neutrophils. Front-tail polarity is thought to be a prerequisite for locomotion (Devreotes and Zigmond, 1988). We have therefore studied the effect of wortmannin on fNLPNTL-stimulated locomotion of neutrophils in the absence of a gradient (= chemokinesis). Cells incubated in the absence of fNLPNTL did not show significant migration. 0% of the cells migrated under these conditions. Preincubation with 10^{-9} M fNLPNTL induced migration in 55–89% of the cells. As shown in Table 1, preincubation of cells with 20 nM wortmannin induced a very substantial inhibition of migration and 50–100 nM wortmannin (17–33 pmol/10^6 cells) reproducible inhibited fNLPNTL-induced chemokinesis by 100%. Cells stimulated in the absence of wortmannin migrated with a speed of 9–16 μm/min; that population of cells which still migrated in the presence of 20 nM wortmannin, with a comparable speed of 8–16 μm/min. The remaining cells were spherical and non-motile, with occasional protrusion of unifocal pseudopods, or several minor pseudopods. In the presence of 50–100 nM wortmannin, a few percent still remained polarized under the conditions of the migration assay, but did not locomote. Inhibition of polarity is thus much more pronounced under these conditions than under those used in the experiments shown in Figs. 1 and 2. This may be due to differences in the assay conditions: in the migration assays, divalent cations (1.1 mM Ca^{2+} and 1 mM Mg^{2+}) were added, whereas the experiments shown in Figs. 1 and 2 were carried out in the presence of 10 mM EDTA in order to avoid cell aggregation. Indeed, when cells, isolated with the Ficoll–Metrizoate method (Section 2.5) which is optimal for migration assays, were incubated in suspension with 100 nM wortmannin in the presence of 10 mM EDTA, prior to addition of chemotactic peptide, 18–19% of the cells remained polarized, corresponding to 76–79% inhibition of development of polarity. When aliquots of the same population of cells were incubated in the presence of divalent cations, as described for the migration assays (Section 2.5), with 100 nM wortmannin and fNLPNTL, only 3–5% of the cells remained polarized, corresponding to 94–97% of inhibition of development of polarity (results of 2 experiments).

3.3. Correlation of inhibition of fNLPNTL-induced development of neutrophil polarity by wortmannin with inhibition of PtIns(3,4,5)P_3 production

The effects of wortmannin on PI 3-kinase activity and on cellular functions such as the respiratory burst have been reported to be considerably affected by concentrations of ATP and proteins, incubation time, sequence of addition, pH, temperature and cell density (Baggiolini et al., 1987; Nakanishi et al., 1995). We have therefore assessed inhibition of chemotactic peptide-induced polarity and PtIns(3,4,5)P_3 production on aliquots of the same cell population. The cells used for these assays were washed in HSA- and phosphate-free buffer and preincubated for 1 h at 37°C, part of the cells being equilibrated with [^{32}P]orthophosphate. fNLPNTL (10^{-9} M) induced a substantial, 2.7 ± 1.0-fold (mean $\pm$ S.D., $n = 3$) increase in neutrophil PtIns(3,4,5)P_3 levels after 1 min incubation. In Fig. 3, the concentration dependence of the relative inhibition of development of cell polarity and PtIns(3,4,5)P_3 production are shown. Half-maximal effects on both processes were observed at 4 nM wortmannin, considering only that population of cells sensitive to wortmannin. PtIns(3,4,5)P_3 production was inhibited maximally by 95–100%, whereas development of polarity was inhibited by $64 \pm 19\%$ ($n = 3$). A variable subpopulation of cells appears thus to be able to develop polarity independently of PI 3-kinase

Table 1
Effect of wortmannin on locomotion of human neutrophils

Additions	Migrating cells (%)	Inhibition (%)
fNLPNTL (10^{-9} M)	74 ± 17	0
Wortmannin (20 nM) and fNLPNTL (10^{-9} M)	14 ± 20	84 ± 22
Wortmannin (50 nM) and fNLPNTL (10^{-9} M)	0 ± 0	100 ± 0
Wortmannin (100 nM) and fNLPNTL (10^{-9} M)	0 ± 0	100 ± 0

Neutrophils (3×10^6/ml) were preincubated for 10 min with 0.4% DMSO or with 20, 50 or 100 nM wortmannin at 37°C, followed by addition of 10^{-9} M fNLPNTL and a further incubation for 30 min (for details see Section 2.5). Subsequently locomotor activity was determined in slide coverslip preparations at 37°C using video microscopy. Mean $\pm$ S.D. of 3 independent experiments (50 nM wortmannin: 2 experiments).

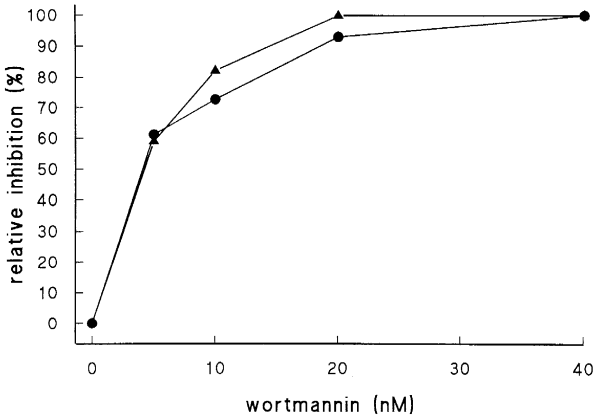

Fig. 3. Correlation of inhibition of fNLPNTL-induced development of cell polarity by wortmannin with effects on PIP_3 production. Neutrophils, after preincubation with [^{32}P]orthophosphate, were exposed to 0.4% DMSO, or to various concentrations of wortmannin. After incubation of the cells at 37°C for 10 min, fNLPNTL (10^{-9} M) was added, followed by a further incubation for 1 min for the determination of the PtIns(3,4,5)P_3 production (●). For the analysis of development of cell polarity (▲) cells of the same population were preincubated for 10 min in the presence of 0.4% DMSO or wortmannin, followed by a further incubation for 30 min in the presence of 10^{-9} M fNLPNTL. Values given on the *y*-axis correspond to the relative inhibition of the two processes by wortmannin, considering only that population of cells sensitive to the drug. Maximal inhibition of PtIns(3,4,5)P_3 production by 40 nM wortmannin was 95%; maximal inhibition of cell polarity was 53% in this experiment.

activity. Comparable results were obtained in two other independent experiments (ED_{50} for inhibition of PtIns(3,4,5)P_3 production, 6 ± 5 nM; for inhibition of polarity, 7 ± 3 nM, mean $\pm$ S.D., $n = 3$). These results thus reinforce a major role of PI 3-kinase in motile responses of neutrophils.

3.4. Wortmannin markedly reduces chemotactic peptide-induced increases in cytoskeletal actin in human neutrophils

The actin network is thought to be crucial for neutrophil locomotion. We therefore assessed possible effects of wortmannin on actin reorganization in neutrophils, which could lead to defective motility. In agreement with previous work (Arcaro and Wymann, 1993), we could not detect a significant effect of wortmannin up to 40 nM (40 pmol/10^6 cells) on increases in total F-actin induced by incubation with 10^{-9} M fNLPNTL for 1 min. At higher concentrations (100 pmol/10^6 cells) wortmannin partially inhibited the increase by $39 \pm 14\%$ ($n = 3$). 1 μM wortmannin (1000 pmol/10^6 cells) induced a more substantial inhibition of $70 \pm 10\%$ ($n = 3$). Half-maximal effects were obtained at 80–100 nM wortmannin (80–100 pmol/10^6 cells; data not shown).

In contrast, increases in cytoskeletal actin, a stable fraction of F-actin sedimenting at low speed after extraction of cells with 0.1% Triton X-100, were much more sensitive to wortmannin. We have studied the effect of preincubating cells for 10 min with various concentrations

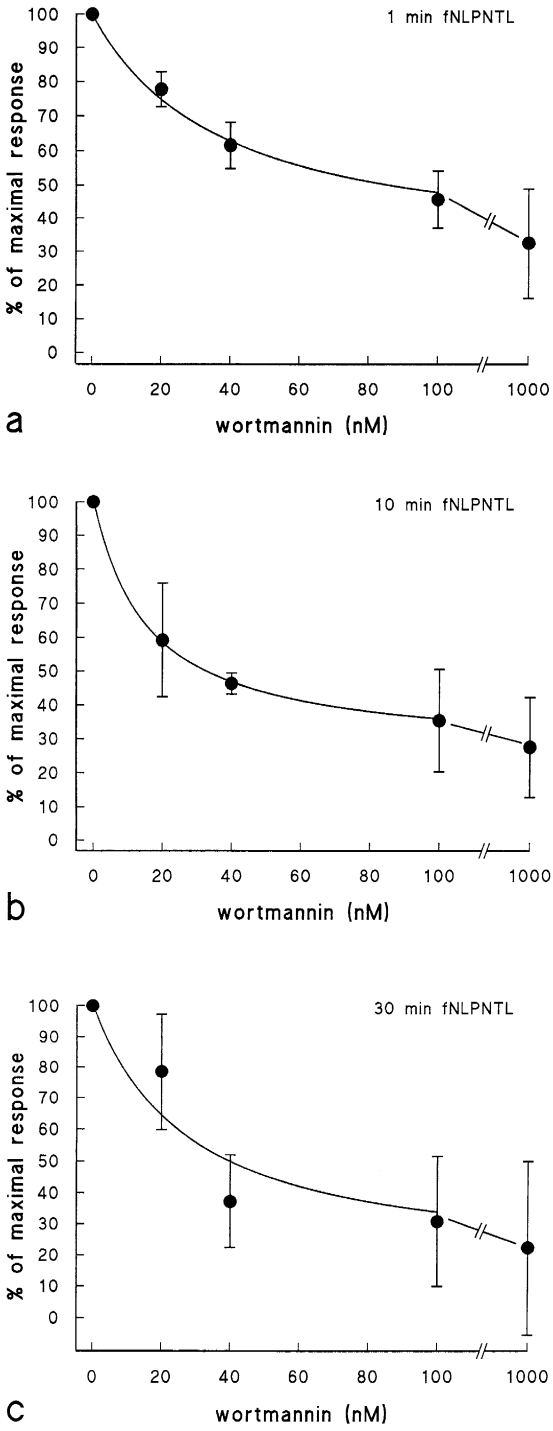

Fig. 4. Inhibition of fNLPNTL-induced increases in cytoskeletal actin by wortmannin. Neutrophils were preincubated for 10 min at 37°C in medium containing 0.1% HSA and 10 mM EDTA, in the presence of DMSO (0.2%) or increasing concentrations of wortmannin, as indicated. Cells were then exposed for 1 min (a), 10 min (b) or 30 min (c) to 10^{-9} M fNLPNTL and cytoskeletal actin was determined as described in Section 2. Values given on the *y*-axis correspond to % of maximal responses obtained in the absence of wortmannin. The best fit of the data points is shown. In control cells, 4–14% of total actin was recovered in the cytoskeletal fraction. This value was 2–4-fold increased for cells incubated for 1 min with fNLPNTL; 1.8–3.2-fold for a 10 min stimulation and 1.5–2.0-fold for a 30 min stimulation. Mean ± S.D. of 3 independent experiments.

of wortmannin on fNLPNTL-induced increases in cytoskeletal actin. In control cells, $10 \pm 4\%$ (mean $\pm$ S.D., $n = 3$) of total cellular actin was recovered in the cytoskeletal fraction. This value was increased to $29 \pm 6\%$ ($n = 3$) by incubation of the cells for 1 min with 10^{-9} M fNLPNTL. This corresponds to a 3.1 ± 1.1-fold (mean $\pm$ S.D., $n = 3$) increase in cytoskeletal actin. Upon longer incubation, cytoskeletal actin decreased to a lower level, which was still significantly (1.7 ± 0.2-fold, $n = 3$, $P < 0.025$) increased above controls within 30 min after addition of stimulus (data not shown; see Kreienbühl et al., 1992). Wortmannin inhibited the increase observed after 1 min, 10 and 30 min of addition of stimulus in a concentration-dependent manner (Fig. 4). Wortmannin (100 nM; 9 pmol/10^6 cells) significantly ($P < 0.005$–0.025) inhibited the increase elicited by incubation of cells for 1 min with 10^{-9} M fNLPNTL by $55 \pm 9\%$ (mean $\pm$ S.D., $n = 3$), that by a 10 min incubation by $65 \pm 15\%$ ($n = 3$) and that by a 30 min incubation by $69 \pm 21\%$ ($n = 3$). Half-maximal effects were obtained, for 1 min of stimulation, at 36 ± 4 nM (3 pmol/10^6 cells) wortmannin, for 10 min at 15 ± 1 nM (1.2 pmol/10^6 cells) and for 30 min at 27 ± 14 nM (2.2 pmol/10^6 cells). These concentrations are in the range of those suppressing development of polarity (Fig. 2). Comparable results were obtained with three different batches of wortmannin. Wortmannin did not change the level of cytoskeletal actin in resting cells: cytoskeletal actin amounted to 0.97 ± 0.15 (mean $\pm$ S.D., $n = 4$) in cells treated for 40 min with 200 nM (18 pmol/10^6 cells) wortmannin, as compared to control cells. In order to confirm a role of PI 3-kinase in increases in cytoskeletal actin, we also tested the effect of LY294002. This inhibitor similarly attenuated the increase in cytoskeletal actin resulting from a 10 min incubation with 10^{-9} M fNLPNTL with half-maximal effects observed at 8 ± 2 μM ($n = 5$), concentrations consistent with inhibition of PI 3-kinase (Vlahos et al., 1995). Maximal inhibition by 100 μM LY294002 was somewhat lower than that induced by wortmannin ($46 \pm 8\%$, $n = 5$, $P < 0.0005$), but this may be explained by the observation, that 100 μM LY294002 itself, in the absence of stimuli, induced a 1.24 ± 0.15-fold ($n = 5$) increase in cytoskeletal actin.

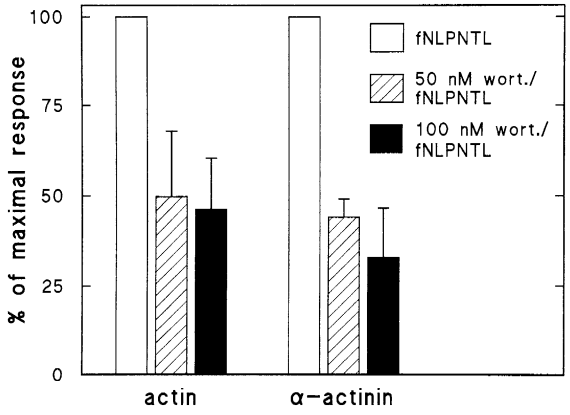

Fig. 5. Inhibition of fNLPNTL-induced increases in cytoskeletal α-actinin by wortmannin. Neutrophils were preincubated for 10 min at 37°C in medium containing 0.1% HSA and 10 mM EDTA, with either 0.4% DMSO or 50 or 100 nM wortmannin, as indicated. Cells were subsequently either incubated without or with 10^{-9} M fNLPNTL for 1 min. Cytoskeletal fractions from 4.5×10^6 cells were isolated as described in Section 2, followed by analysis of the amount of actin and α-actinin present. The maximal response corresponds to the fNLPNTL-induced increase in the amount of α-actinin and actin present in cytoskeletons isolated from 4.5×10^6 cells. Cytoskeletal actin was increased 2–3-fold and cytoskeletal α-actinin 4–7-fold by incubation for 1 min with fNLPNTL in these experiments. Data correspond to mean $\pm$ S.D. of 3 independent experiments.

Table 2
Comparison of the effects of wortmannin on fNLPNTL- and PMA-induced increases in cytoskeletal actin

Additions	% of maximal response
fNLPNTL, 1 min	100
200 nM wortmannin/fNLPNTL, 1 min	33 ± 11
fNLPNTL, 30 min	100
200 nM wortmannin/fNLPNTL, 30 min	28 ± 28
PMA, 30 min	100
200 nM wortmannin/PMA, 30 min	101 ± 12

Cells were preincubated in medium with 10 mM EDTA at 37°C for 10 min with 0.2% DMSO or 200 nM wortmannin, as indicated. Incubation was then continued for 1 or 30 min in the presence of 10^{-9} M fNLPNTL or for 30 min with 10^{-8} M PMA. Cytoskeletal actin was determined as described in Section 2. Maximal increases in cytoskeletal actin induced by fNLPNTL were 2.4 ± 0.7 ($n = 3$) relative to controls for 1 min incubation with fNLPNTL; 1.5 ± 0.2 ($n = 3$) for a 30 min incubation with fNLPNTL, and 1.5 ± 0.2 ($n = 3$) for a 30 min incubation with PMA. Data represent mean $\pm$ S.D, of 3 independent experiments.

We have shown previously, that chemotactic peptide induces a rapid and marked recruitment of the actin-cross-linking protein α-actinin to the cytoskeleton, in parallel with the increase in cytoskeletal actin (Niggli and Jenni, 1989). As shown in Fig. 5, this increase in cytoskeletal α-actinin induced by incubation of cells with 10^{-9} M fNLPNTL for 1 min was also markedly and significantly ($P < 0.05$) reduced by 50 and 100 nM wortmannin. Inhibition was almost maximal at 50 nM of the drug (Fig. 5). In contrast to its marked effects on fNLPNTL-induced increases in cytoskeletal actin and α-actinin, wortmannin (18 pmol/10^6 cells) did not significantly affect the rise in cytoskeletal actin induced by incubation of the cells for 30 min with 10^{-8} M PMA, as shown in Table 2.

4. Discussion

4.1. Specificity of inhibitory effects of wortmannin on motile responses of human neutrophils

We describe here a significant reduction by wortmannin of chemotactic peptide-induced increases in cytoskeletal actin and development of polarity, and that wortmannin

inhibits stimulated locomotion. Half-maximal effects of wortmannin were observed at concentrations of 4–40 nM. PMA-induced increases in cytoskeletal actin and resting cell morphology and cytoskeletal actin were not affected, showing that the inhibition is selective and that the drug does not interfere unspecifically with the assay for cytoskeletal actin. As reported previously, half-maximal effects of wortmannin on the respiratory burst were obtained at 7–8 pmol per 10^6 neutrophils (Dewald et al., 1988). Half-maximal effects in this study on development of cell polarity and cytoskeletal actin were obtained at 0.4–3 pmol per 10^6 cells, values even lower than those obtained for inhibition of the respiratory burst. In contrast, half-maximal effects on F-actin were obtained at 80 pmol per 10^6 cells; 27–200-fold higher amounts.

In order to strengthen the evidence for the involvement of PI-3-kinase, we have quantified fNLPNTL-induced PtIns(3,4,5)P_3 production in intact neutrophils, and its concentration-dependent inhibition by wortmannin, using the same cell density as for the assessment of cytoskeletal actin and cell shape. We have obtained a good correlation between inhibition of PtIns(3,4,5)P_3 production and inhibition of development of cell polarity. For these measurements we used aliquots of the same cell population in an identical phosphate- and HSA-free buffer (Fig. 3), and considered the population of cells sensitive to wortmannin.

Our findings thus strongly indicate a role of PI 3-kinase in initiating locomotion and controlling actin reorganization in neutrophils. Wortmannin inhibits neutrophil PI 3-kinase in vitro with half-maximal effects obtained at 1–28 nM and it has been reported to prevent production of PtIns(3,4,5)P_3 in FMLP-stimulated human neutrophils with an IC_{50} of approximately 5 nM (0.5 pmol/10^6 cells), comparable to our data (Arcaro and Wymann, 1993). Wortmannin (10 pmol/10^6 cells) and an analogue, 17-hydroxywortmannin, do not affect chemotactic peptide-induced production of diacylglycerol and the increase in cytosolic Ca^{2+} in human neutrophils (Dewald et al., 1988; Reinhold et al., 1990). Other enzymes involved in signalling, such as phospholipase D and MAP-kinase, are not directly affected by the inhibitor (Nakanishi et al., 1995; Ui et al., 1995). At concentrations up to 100 nM wortmannin is therefore thought to act specifically on PI 3-kinase and closely related enzymes. Only one report contradicts this consensus view. Wortmannin has been shown recently to inhibit bombesin-induced stimulation of phospholipase A_2 in swiss 3T3 cells with an IC_{50} of 2 nM, possibly via affecting signal transduction pathways unrelated to PI 3-kinase (Cross et al., 1995). However, as mentioned by Thelen et al. (1995), wortmannin does not inhibit phospholipase A_2 in human neutrophils. According to Thelen et al. (1995), 100 nM wortmannin does not affect neutrophil chemotaxis induced by either interleukin 8 or chemotactic peptide, as measured in Boyden-type chambers. A possible explanation for this discrepancy could be differences in the assay conditions (chemokinesis determined as migration on glass in the presence of albumin versus chemotaxis determined as migration on filters in Boyden-type chambers). However, it has been shown in a recent report, which appeared after submission of our data, that 100 nM wortmannin almost completely inhibits interleukin 8-induced chemotaxis and chemokinesis, also using Boyden-type chambers (Knall et al., 1997). The latter findings are similar to our findings with fNLPNTL.

4.2. Possible targets of PI 3-kinase involved in modulating neutrophil motility

4.2.1. Tyrosine kinases, protein kinase C

Chemotactic peptide-induced activation of tyrosine kinases in neutrophils has been reported to be inhibited by low (nanomolar) concentrations of wortmannin, suggesting a role of PI 3-kinase (Naccache et al., 1993). As the tyrosine kinase inhibitor erbstatin has also been reported to suppress neutrophil locomotion (Gaudry et al., 1992), inhibition of neutrophil migration by wortmannin could be due to suppression of receptor-linked tyrosine kinase activation. However, development of polarity and increases in F-actin are not affected by tyrosine kinase inhibitors (Naccache et al., 1990; Lepidi et al., 1995). Moreover, we did not find any effect of the non-specific tyrosine kinase inhibitor staurosporine on fNLPNTL-induced increases in cytoskeletal actin (Niggli and Keller, 1991). It is thus unlikely that our findings are due to inhibition of tyrosine kinases by wortmannin.

PtIns(3,4,5)P_3 could also act by regulating specific isoforms of protein kinase C (Nakanishi et al., 1995; Ui et al., 1995). Protein kinase C isoforms very likely play no role in modulating increases in cytoskeletal actin in neutrophils (Niggli and Keller, 1991), but a protein kinase C inhibitor suppresses development of chemotactic peptide-induced polarity and locomotion of neutrophils (Niggli and Keller, 1993).

4.2.2. Cytoskeletal proteins

PI 3-kinase and its lipid products may also regulate the function of cytoskeletal proteins. PtIns(4,5)P_2 has been shown to interact with a number of cytoskeletal proteins (gelsolin, profilin, ezrin etc; Isenberg and Goldmann, 1995). As recently reported, a product of PI 3-kinase, PtIns(3,4)P_2, binds to profilin with an even ten-fold higher affinity than PtIns(4,5)P_2 (Lu et al., 1996). Interestingly, PI 3-kinase co-immunoprecipitates with α-actinin in NIH/3T3 cells (Shibasaki et al., 1994). Moreover, PtIns(4,5)P_2 has been reported to bind to α-actinin and enhance its crosslinking activity (Fukami et al., 1992). We have provided evidence in this work, that wortmannin attenuates receptor-linked increases in association of α-actinin with the cytoskeleton (Fig. 5). This could be due to a functional regulation of α-actinin by lipid products of PI 3-kinase in neutrophils. Interestingly, according to a recent report, 100 nM wortmannin completely prevents fMLP-

but not PMA-induced dephosphorylation of the actin-binding protein cofilin in human neutrophils (Okada et al., 1996). As cofilin, whose function is regulated by reversible phosphorylation, has been implicated in modulating the actin network, this finding also supports a role of PI 3-kinase in neutrophil actin reorganization.

4.3. Possible mechanisms of inhibition of neutrophil actin reorganization and motility by wortmannin

In wortmannin-treated cells, chemotactic peptide still induces formation of pseudopods enriched in F-actin within the first minutes of activation, but the percentage of cells with front-tail polarity developing later on is markedly reduced and migration is suppressed. Impairment of migration and development of polarity may either be due to an inhibitory affect of the drug on assembly of cytoskeletal actin and α-actinin, which could lead to formation of instable protrusions (Condeelis, 1993), or to other factors independent of the actin network, such as inhibition of e.g. a specific protein kinase C isoform, as discussed above, or to a combination of these different effects. Inhibition of CD18-mediated adhesion by wortmannin (Metzner et al., 1997) may also contribute to suppression of migration. We show that wortmannin markedly attenuates that fraction of actin which sediments at low speed after solubilization of cells with nonionic detergents. This fraction is thought to be strongly crosslinked and relatively stable (Watts and Howard, 1993). Treatment with wortmannin appears to reduce this stable fraction of F-actin. This in turn may result in the formation of unstable protrusions leading to impaired morphology and migration.

As shown in Figs. 1 and 2, in the presence of 10 mM EDTA, $36 \pm 11\%$ of the cells were refractory to the inhibitory action of wortmannin and maintained polarity even at higher concentrations (9–90 pmol$/10^6$ cells). Similarly, inhibition of fNLPNTL-induced increases in cytoskeletal actin, determined in the presence of 10 mM EDTA in order to block Ca^{2+}-dependent proteases, was in the range of 42–82% at 9 pmol$/10^6$ cells, whereas inhibition of production of PtIns(3,4,5)P_3 was almost complete at 40 nM wortmannin. However, under the conditions optimal for migration, in the presence of divalent cations, development of polarity was inhibited by 94% and migration by 100% in the presence of 100 nM wortmannin. Possibly, in the absence of divalent cations, unknown pathways in addition to activation of PI 3-kinase may be involved in chemotactic peptide-induced shape changes and changes in actin organization. Derman et al. (1996) have reported that epithelial chemotaxis is only partly inhibited by wortmannin. They find that both PI 3-kinase and phospholipase C contribute to the chemotactic response initiated by hepatocyte growth factor.

In summary we have identified a wortmannin-sensitive pathway, most likely involving PI 3-kinase, important for chemotactic peptide-induced motile responses of human neutrophils. This pathway is selectively required for receptor-linked events, but not for phorbol ester-induced actin reorganization.

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