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Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function

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Although it is known that many stimuli can activate mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinases (PI3K) in human neutrophils, little is known concerning either the mechanisms or function of this activation. We have utilized a selective inhibitor of MAPK kinase (MEK), PD098059, and two inhibitors of PI3K, wortmannin and LY294002, to investigate the roles of these kinases in the regulation of neutrophil effector functions. Granulocyte/macrophage colony-stimulating factor, platelet-activating factor (PAF) and *N*-formylmethionyl-leucyl-phenylalanine are capable of activating both $p44^{ERK1}$ and $p42^{ERK2}$ MAPKs and phosphotyrosine-associated PI3K in human neutrophils. The activation of extracellular signal-related protein kinases (ERKs) is correlated with the activation of $p21^{ras}$ by both tyrosine kinase and G-protein-coupled receptors as measured by a novel assay for GTP loading. Wortmannin and LY294002 inhibit, to various degrees, superoxide generation, neutrophil migration and PAF release. Incubation with PD098059, however, inhibits only the PAF release stimulated by serum-treated zymosan. This demonstrates that, while neither MEK nor ERK kinases are involved in the activation of respiratory burst or neutrophil migration, inhibition of PAF release suggests a potential role in the activation of cytosolic phospholipase A_2. PI3K isoforms, however, seem to have a much wider role in regulating neutrophil functioning.

INTRODUCTION

The regulation of neutrophil function through the interaction with chemoattractants and cytokines is a critical aspect of host defence against microbial infection. Furthermore, neutrophils are implicated in the pathogenesis of a variety of inflammatory diseases associated with tissue damage [1–3]. Neutrophils express a wide range of receptors and become rapidly activated in response to a diverse array of stimuli. These receptors include tyrosine kinase-associated receptors such as granulocyte/macrophage colony-stimulating factor (GM-CSF) [4], seven-membrane-spanning receptors such as *N*-formylmethionyl-leucyl-phenylalanine (fMLP), platelet-activating factor (PAF), [5] and receptors reacting with immune complexes, for example Fc [6]. After interaction of the ligand with the receptor, neutrophils engage in migration, phagocytosis, antibody-dependent cell-mediated cytotoxicity, granule release and production of superoxide [7,8]. Interestingly, optimal activation of neutrophils occurs only in a 'primed' state induced by a prior stimulus [8]. For example, pretreatment of neutrophils with GM-CSF primes the NADPH oxidase for a subsequent challenge with fMLP without directly activating the oxidase itself. However, the mechanisms by which these factors activate or prime mature neutrophils remain to be fully defined.

Tyrosine phosphorylation is thought to be crucial to the regulation of neutrophil effector functions [9,10]. Although the identities of many of the tyrosine-phosphorylated substrates present in stimulated neutrophils remain unknown, it has been demonstrated that members of the mitogen-activated protein kinase (MAPK) family can be phosphorylated in response to fMLP, PAF and GM-CSF [9,11–16]. There are three distinct groups of MAPK: extracellular signal-related protein kinases (ERKs) activated by a large variety of agonists, stress-activated protein kinases and p38 MAPK. The latter two groups seem to be more specifically activated by environmental stress and pro-inflammatory cytokines. There is now considerable evidence supporting a role for these evolutionary conserved protein kinases in cellular growth and differentiation, although little is known of the function of these kinases in haemopoietic systems.

Evidence pointing to the existence of additional neutrophil signalling pathways has been obtained. One such pathway concerns the lipid kinase phosphatidylinositol 3-kinase (PI3K), which generates the putative signalling molecule inositol 3,4,5-trisphosphate [17]. The use of the inhibitor wortmannin has demonstrated that PI3K has a critical role in several neutrophil effector functions including respiratory burst [18–20] and assembly of the neutrophil actin cytoskeleton [21]. Neutrophils contain two classes of PI3K: the classical p85/p110 heterodimer [22,23] and a novel G-protein $\beta\gamma$ subunit-regulated PI3K [23]. The regulation of these two classes of PI3K by cytokines and chemoattractants in human neutrophils remains to be elucidated.

Several reports have suggested a role for the MAP/ERK kinases in a variety of neutrophil functions [10,11,13,16]. The

Abbreviations used: $cPLA_2$, cytosolic PLA_2; ERK, extracellular signal-related protein kinase; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; GM-CSF, granulocyte/macrophage colony-stimulating factor; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PAF, platelet-activating factor; PI3K, phosphatidylinositol 3-kinase; PLA_2, phospholipase A_2; PMN, polymorphonuclear cell; RBD, Ras-binding domain; STZ, serum-treated zymosan; TNF, tumour necrosis factor.

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aim of the present study was to analyse the mechanisms of activation and function of both $p44^{ERK1}/p42^{ERK2}$ kinases and PI3K in human neutrophils. Activation of ERKs by both tyrosine kinase-linked and G-protein-coupled receptors is correlated with activation of $p21^{ras}$. Furthermore, all stimuli are capable of activating phosphotyrosine-associated PI3K isoforms. Recently a specific inhibitor of the MAP/ERK kinase activator MAPK kinase (MEK) has become available (PD098059) [24,25]. Utilizing PD098059 and the PI3K inhibitors wortmannin or LY294002, we have investigated the potential role of these two signalling pathways in neutrophil function and priming. Results demonstrate that the activation of PI3K seems to be critical for superoxide generation, neutrophil chemokinesis and PAF release mediated by serum-treated zymosan (STZ). Inhibition of cell migration is apparent only for reagents activating granulocyte chemokinesis rather than chemotaxis. The activation of MEK or $p44^{ERK1}/p42^{ERK2}$, however, is only necessary for the STZ-induced PAF release. Interestingly, the inhibition of PAF release by PD098059, a cytosolic phospholipase A_2 ($cPLA_2$)-mediated process, suggests a possible role for ERK kinases in the activation of $cPLA_2$.

MATERIALS AND METHODS

Reagents

PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphorylcholine), fMLP and L-α-phosphatidylinositol were purchased from Sigma (St. Louis, MO, U.S.A.). Human serum albumin was from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Recombinant human GM-CSF (2.5×10^8 i.u./mg) was from Genzyme (Boston, MA, U.S.A.). Percoll was obtained from Pharmacia (Uppsala, Sweden). Polyclonal ERK2 antisera was a gift from Professor J. L. Bos (Utrecht, The Netherlands); ERK-1 (C-16) and ERK-2 (C-14) polyclonal antisera were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The anti-phosphotyrosine monoclonal antibody (4G10) and anti-p85 polyclonal sera were obtained from UBI (Lake Placid, NY, U.S.A.). Wortmannin and LY294002 were purchased from BioMol (Plymouth Meeting, PA, U.S.A.) and PD098059 was a gift from Dr. Dudley and Dr. Saltiel (Parke-Davis Pharmaceuticals, Ann Arbor, MI, U.S.A.).

Isolation of human polymorphonuclear cells (PMNs)

Blood was obtained from healthy volunteers from the Red Cross Blood Bank (Utrecht, The Netherlands). Mixed granulocytes were isolated from the buffy coat of 500 ml of blood treated with 0.4% trisodium citrate, pH 7.4, to prevent coagulation, as previously described [26]. Mononuclear cells were removed by centrifugation over isotonic Percoll (1.078 g/ml). After lysis of the erythrocytes in isotonic ice-cold NH_4Cl solution, the granulocytes were washed and resuspended in incubation buffer [20 mM Hepes/132 mM NaCl/6 mM KCl/1 mM $MgSO_4$/1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1 mM $CaCl_2$ and 0.5% (v/v) human serum albumin]. Granulocytes were incubated for 30 min at 37 °C before stimulation.

ERK2 phosphorylation

PMNs were isolated as described above and incubated at 37 °C for 30 min in incubation buffer. After stimulation, cells were washed twice in ice-cold PBS and immediately lysed in sample buffer essentially as described previously [27]. After being heated for 5 min at 95 °C, total cell lysates were analysed on SDS/10% (w/v) polyacrylamide gels. Proteins were transferred to Immobilon-P and incubated with polyclonal ERK antiserum. Detection was by enhanced chemiluminescence (Amersham, Little Chalfont, Bucks., U.K.).

MAPK activity

PMNs were isolated as described above and incubated at 37 °C for 30 min. After stimulation, cells were washed twice in ice-cold PBS and lysed in 50 mM Tris/HCl (pH 7.5)/100 mM NaCl/50 mM NaF/5 mM EDTA/40 mM β-glycerophosphate/1 mM Na_3VO_4/1% (v/v) Triton X-100/10 μg/ml aprotinin/10 μg/ml leupeptin/1 mM PMSF. Lysates were precleared for 30 min at 4 °C with Protein A–Sepharose and MAPK was immunoprecipitated with 1 μg of ERK-1/ERK-2 polyclonal antiserum for 1 h at 4 °C on a rotating wheel. Protein A–Sepharose was then added for a further 1 h at 4 °C. After being washed twice with lysis buffer, samples were washed twice with kinase buffer without ATP. Precipitates were then incubated in 25 μl of kinase buffer [30 mM Tris/HCl (pH 8.0)/20 mM $MgCl_2$/2 mM $MnCl_2$/10 μM rATP], containing 10 μg of myelin basic protein and 0.3 μCi of [γ-^{32}P]ATP, for 20 min at 30 °C. The reaction was stopped by the addition of 5 × Laemmli sample buffer. Samples were separated by SDS/PAGE [15% (w/v) gel]. Myelin basic protein phosphorylation was detected by autoradiography.

$p21^{ras}$ activation assay utilizing Raf1 Ras-binding domain (RBD)

A fusion protein of glutathione S-transferase (GST) and RBD (residues 51–131 of Raf1) was constructed and isolated as described previously [28]. The desired amount of crude GST–RBD was incubated with glutathione–agarose beads at 4 °C for 1 h. The beads were isolated by centrifugation and washed five times with lysis buffer [50 mM Tris/HCl (pH 7.4)/150 mM NaCl/1% Nonidet P-40/10% (v/v) glycerol/0.1 μM aprotinin/1 μM leupeptin/1 mM PMSF). PMNs (10^7 cells) were lysed in 1 ml of lysis buffer at 4 °C and centrifuged to remove nuclei. Precoupled GST–RBD beads were added and the lysates were incubated for 30 min at 4 °C. Beads were pelleted by centrifugation and washed three times with lysis buffer before being resuspended in Laemmli sample buffer. Protein samples were separated by SDS/PAGE [15% (w/v) gel] and transferred to a PVDF membrane. Western blots were probed with the anti-Ras monoclonal antibody Y13-259 for 16 h at 4 °C, followed by rabbit anti-rat antiserum (2 h at 4 °C) and horseradish peroxidase-coupled goat anti-rat antiserum (Santa Cruz) (1.5 h at 4 °C). Blots were developed by enhanced chemiluminescence (New England Nuclear, Boston, MA, U.S.A.).

PI3K activity

PMNs were treated as described above. After stimulation, reactions were stopped by adding 2 vol. of ice-cold incubation buffer containing 2 mM Na_3VO_4. Subsequently the PMNs were pelleted by centrifugation at 4 °C before being resuspended for 30 min at 4 °C in lysis buffer [1% Triton X-100/20 mM Tris/HCl (pH 8.0)/100 mM NaCl/10 mM $Na_4P_2O_7$/2 mM EDTA/50 mM NaF/10% (v/v) glycerol/10 μg/ml aprotinin/10 μg/ml leupeptin/10 μg/ml soybean tryptase inhibitor/1 mM PMSF/1 mM Na_3VO_4]. Lysates were incubated for 1 h at 4 °C on a rotating wheel with 1 μg of anti-phosphotyrosine monoclonal antibody 4G10 before the addition of Protein A–Sepharose for a further 1 h. Samples were washed three times with lysis buffer and twice with 10 mM Tris/HCl, pH 7.4, containing 1 mM Na_3VO_4. PI3K

activity was measured by adding 100 μg of sonicated phosphatidylinositol and 20 μCi of [γ-^{32}P]ATP in the presence of 200 μM adenosine (to inhibit phosphatidylinositol 4-kinase activity), 30 mM $MgCl_2$ and 35 μM ATP in a total volume of 60 μl. Reactions were performed for 20 min at room temperature and stopped by the addition of 100 μl of 1 M HCl and 200 μl of chloroform/methanol (1:1, v/v). After centrifugation and removal of the upper layer, 80 μl of methanol/HCl (1:1) was added. After further centrifugation, lipids were separated on TLC plates (Merck) with a solvent system of chloroform/methanol/NH_4OH (45:35:10, by vol.). TLC plates were exposed to X-ray film at -80 °C. Immunoprecipitation with polyclonal anti-p85 antibody was used as positive control for PI3K activity.

Migration assay

PMN migration was measured with a modification of the method of Boyden [29], by using a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD, U.S.A.). Chemotaxins or incubation buffer (30 μl) were added to the lower compartments. Two filters were placed between the lower and upper compartments. The lower filter (Millipore, Bedford, MA, U.S.A.) had a pore width of 0.45 μm, whereas the upper filter (cellulose nitrate) had a pore width of 8 μm (thickness 150 μm; Sartorius, Göttingen, Germany). Before use the filters were soaked in incubation buffer. PMNs were placed in the upper compartment (25 μl, of 2×10^6 cells/ml). The chambers were subsequently incubated for 1.5 h at 37 °C. The upper filters were removed, fixed in butanol/ethanol (20:80, v/v) for 10 min and stained with Weigert solution [1% (v/v) haematoxylin in ethanol/70 mM acidic $FeCl_3$; 1:1 (v/v)]. The filters were dehydrated with ethanol, made transparent with xylene and fixed upside down. All migratory responses were quantified with an image analysis system (Quantimet 570C; Leica Cambridge) with Quantimet 570 Control Software (QUIC version 2.02) and custom software. An automated microscope (Leitz DMRXE; Leica, Weitzlar, Germany) was used to step through the filters in the z direction with 17 intervals of 10 μm. PMNs were counted at each level and the total migration to each level was calculated. The results are expressed as migratory index (μm per cell), which is calculated by the cumulative migration of all intervals (in μm) divided by the total number of cells. In this index the cells at level 0 μm were not included. The mean of four randomly chosen points on each filter spot was calculated.

Measurement of PAF release

After incubation with or without GM-CSF (100 pM) for 30 min, STZ (0.5 mg/ml) was added to the PMNs (10^6 cells/ml) for a subsequent incubation of 30 min at 37 °C with vigorous shaking. Cells and zymosan particles were then removed by centrifugation (at 4 °C and 200 ***g*** for 15 min) and the supernatants were collected and stored at -80 °C. PAF was measured in these samples with a competitive radioimmunoassay (New England Nuclear).

Measurement of superoxide production

Superoxide was measured by cytochrome c reduction by the method of Pick and Mizel [30], with modifications. Briefly, PMNs (4×10^6 cells/ml) were preincubated for 5 min at 37 °C in incubation buffer. Inhibitors and/or cytokines were then added for the indiated periods; the cells (200 μl) were transferred to a microtitre plate in a thermostat-controlled microtitre plate reader (340 ATTC; SLT LabInstruments, Salzburg, Austria), mixed with cytochrome c (75 μM) and the incubation was continued for 5 min. The plates were shaken every 3 s. The cells were then stimulated with fMLP (1 μM) and cytochrome c reduction was measured every 12 s as an increase in A_{550} ($\epsilon_{550} = 21 \times 10^3$ $M^{-1} \cdot cm^{-1}$). Results are expressed as the maximal rate of superoxide production produced by 10^7 cells/min.

RESULTS

Activation of MAPKs by PMN stimulants

We analysed the ability of various reagents to induce both the phosphorylation and activity of the MAPKs $p44^{ERK1}$ and $p42^{ERK2}$. Phosphorylation of MAPK on threonine and tyrosine residues results in a mobility shift in SDS/PAGE. As shown in Figure 1(A), ERK2 was phosphorylated in extracts of cells stimulated by both cytokines and chemoattractants. Interestingly, all stimulants provided different activation profiles. The phosphorylation was strongest and more transient after stimulation by ligands of serpentine receptors fMLP and PAF, whereas stimulation with GM-CSF, whose action is mediated by a single transmembrane receptor, was of much longer duration but less potent. The addition of tumour necrosis factor α (TNF-α) to PMNs resulted in a very weak and extremely transient activation of ERK2 phosphorylation. The very weak activation of $p42^{ERK2}$ by TNF-α is in agreement with Waterman and Sha'afi [14], who found no activation of $p44^{ERK1}$ or $p42^{ERK2}$ in human neutrophils. To analyse the activity of ERK kinases in response to these stimuli we performed immune-complex kinase assays utilizing immunoprecipitating antibodies to both $p44^{ERK1}$ and $p42^{ERK2}$. The results shown in Figure 1(B) corroborate the phosphorylation results described above. Taken together, they demonstrate that the MAPKs $p44^{ERK1}$ and $p42^{ERK2}$ can be activated by both cytokines and chemoattractants in human neutrophils and that their regulation by fMLP and PAF is distinct from that by GM-CSF.

Activation of $p21^{ras}$ is correlated with activation of ERK by GM-CSF, PAF and fMLP

Although the activation of ERKs by tyrosine kinase-linked receptors is $p21^{ras}$-dependent, this correlation has not been widely established for other receptor families such as G-protein-coupled receptors. Although fMLP has been reported to activate GTP-loading of $p21^{ras}$ [31], activation of $p21^{ras}$ was not observed for PAF [32]. We have utilized a Raf1-RBD as an activation-specific probe for $p21^{ras}$ GTP-loading after stimulation of neutrophils by GM-CSF, TNF-α, fMLP and PAF. This technique is based on the principle that Raf1 interacts only with GTP-bound $p21^{ras}$. Thus a GST fusion protein containing the minimal RBD of Raf1 (residues 51–131) is used to 'pull down' GTP-bound $p21^{ras}$ [28]. Although not quantitative, this method provides results on the ability and kinetics of activation. PMNs were stimulated and $p21^{ras}$ was precipitated with GST–RBD bound to glutathione–agarose beads and identified by Western blotting with a monoclonal antibody against $p21^{ras}$. As shown in Figure 2, TNF-α, which failed to activate $p44^{ERK1}$ or $p42^{ERK2}$, also failed to induce $p21^{ras}$ activation, whereas GM-CSF, which produced a slow but sustained activation of ERKs, also resulted in an identical activation profile for $p21^{ras}$. Furthermore, activation of the G-protein-coupled receptors for PAF and fMLP resulted in a very rapid and short-lived $p21^{ras}$ activation perfectly correlated with their activation of ERKs. Thus it seems that all neutrophil stimuli that activate ERKs also activate $p21^{ras}$ with the same kinetics, suggesting that $p21^{ras}$ activation is necessary for signal propagation to ERKs.

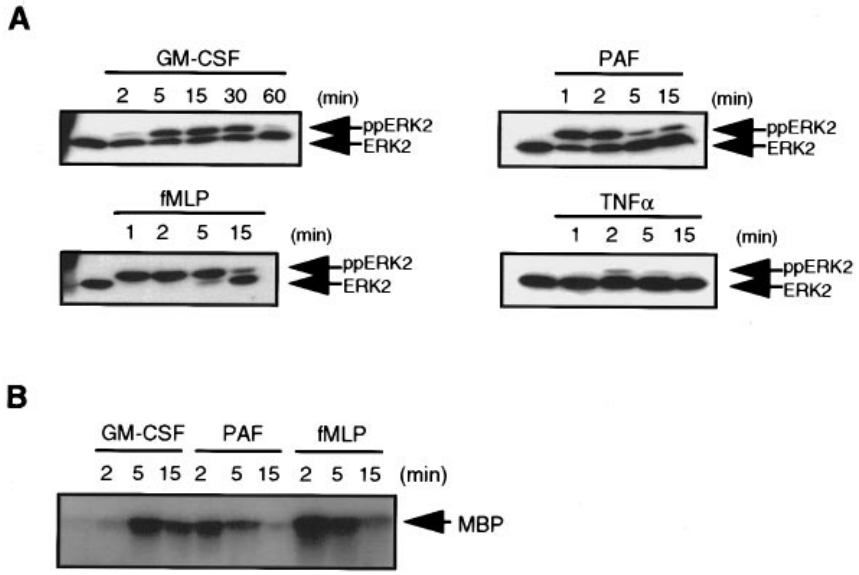

Figure 1 Cytokines and chemoattractants stimulate ERK phosphorylation and activity

(**A**) Isolated neutrophils were incubated for 30 min at 37 °C in incubation buffer before stimulation. GM-CSF (100 pM), PAF (1 μM), fMLP (1 μM) and TNF-α (100 i.u./ml) were incubated for the times indicated. After stimulation, cells were immediately lysed in Laemmli sample buffer and samples were analysed by ERK2 Western blotting as described in the Materials and methods section. (**B**) Cells were stimulated for the times indicated. After stimulation, samples were lysed and immunoprecipitated with a mixture of $p44^{ERK1}$ and $p42^{ERK2}$ antibodies. Kinase activity was analysed as described in the Materials and methods section.

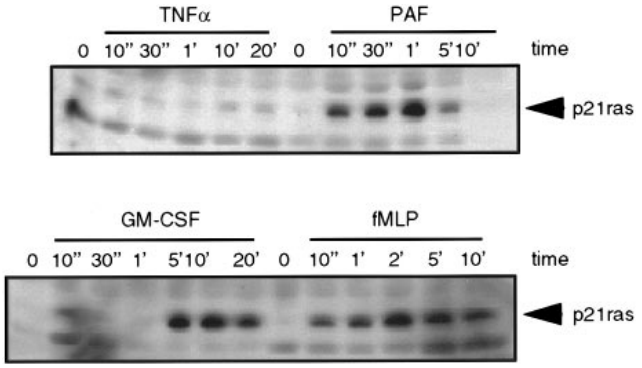

Figure 2 GM-CSF, fMLP and PAF stimulate $p21^{ras}$ activation in neutrophils

PMNs were stimulated as indicated (TNF-α, 100 i.u./ml; GM-CSF, 100 pM; PAF, 1 μM; fMLP, 1 μM) before lysis and incubation with GST–RBD to bind GTP-bound $p21^{ras}$ as described in the Materials and methods section. Samples were analysed by SDS/PAGE and immunoblotting with a monoclonal antibody against $p21^{ras}$. The position of immunoprecipitated $p21^{ras}$ is marked by an arrowhead.

PD098059 inhibits activation of PMN $p44^{ERK1}$/$p42^{ERK2}$

To analyse the role of ERK activation in neutrophil effector functions we utilized the recently identified inhibitor PD098059 [24,25]. This compound has been described to specifically inhibit the upstream MAPK kinases (MEK1 and MEK2) in several cell lines. To determine whether PD098059 could inhibit ERK phosphorylation and activation by cytokines and chemoattractants in human PMNs we peformed both mobility-shift and kinase assays on cells pretreated for 20 min with or without PD098059. As shown in Figure 3(A), increasing concentrations of PD098059 resulted in a decrease in ERK2 phosphorylation with a half-maximal inhibition of approx. 10 μM. To analyse the effect on both $p44^{ERK1}$ and $p42^{ERK2}$ we utilized immune-complex kinase assays. The results shown in Figure 3(B) demonstrate a similar pattern for kinase activity to that observed for mobility-shift analysis. Thus it seems that all three stimulants, fMLP, PAF and GM-CSF, require MEK1 or MEK2 for activation of ERK isoforms in human PMNs. Furthermore, PD098059 at concentrations of 10–50 μM can be utilized to block MEK/ERK activity completely in human neutrophils.

GM-CSF, PAF and fMLP induce phosphotyrosine-associated PI3K activity in PMNs

The mechanism of activation of PI3K in human neutrophils has not been thoroughly investigated. To determine whether cytokines and chemoattractants activate the classical p85-linked PI3K we measured the phosphatidylinositol-phosphorylating activity present in phosphotyrosine immunoprecipitates from stimulated neutrophils. After immunoprecipitation and kinase assay, phosphorylated lipids were separated by TLC (as described in the Materials and methods section). As shown in Figure 4(A), GM-CSF induces a rapid induction of PI3K activity. Interestingly, a similar rapid induction was observed for the serpentine receptor agonists fMLP and PAF (Figure 4B). This clearly demonstrates that these G-protein-coupled receptors are also capable of activating phosphotyrosine-associated PI3K activity, in contrast with other reports [22]. To inhibit PI3K activity we utilized both the fungal metabolite wortmannin and the synthetic inhibitor LY294002 [19,33]. Pretreatment of cells with 30 nM wortmannin for 20 min was sufficient to inhibit more than 90 % of phosphotyrosine-associated PI3K activity (Figure 4B). This was true of all stimulants (GM-CSF, fMLP and PAF) tested. These concentrations of wortmannin had no effect on ERK activation in PMNs (results not shown). Importantly this demonstrates that GM-CSF as well as the serpentine re-

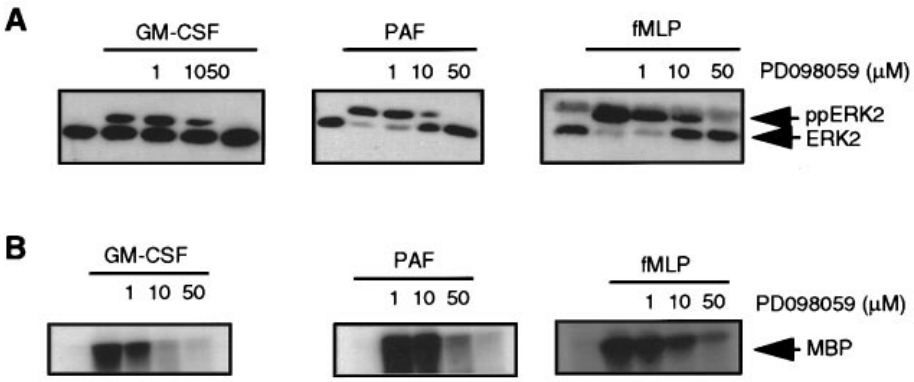

Figure 3 PD098059 inhibits ERK phosphorylation and activation by both cytokines and chemoattractants

(**A**) Isolated neutrophils were incubated with DMSO or increasing concentrations of PD098059 for 20 min and then stimulated as indicated. Phosphorylation of $p42^{ERK2}$ was monitored by SDS/PAGE and Western blotting as described in the Materials and methods section. (**B**) Cells were treated as described above and MAPKs were immunoprecipitated with a mixture of $p44^{ERK1}$ and $p42^{ERK2}$ antibodies before being analysed in kinase assays as described above.

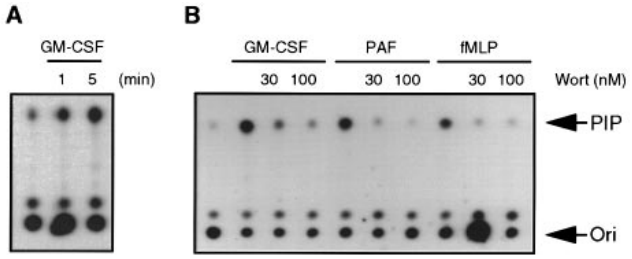

Figure 4 Cytokines and chemoattractants activate a phosphotyrosine-associated PI3K activity that is inhibited by wortmannin

(**A**) Isolated neutrophils were stimulated with GM-CSF (100 pM) for the times indicated. After lysis, phosphotyrosine-associated PI3K activity was immunoprecipitated with 4G10 monoclonal antibody and lipid kinase assays *in vitro* were performed as described in the Materials and methods section. The position of phosphorylated phosphatidylinositol is marked by PIP. (**B**) Neutrophils were preincubated with DMSO alone or with 30 or 100 nM wortmannin (Wort) for 15 min. Samples were harvested and PI3K assays were performed as described above. Ori, origin.

ceptor agonists fMLP and PAF are capable of activating phosphotyrosine-associated PI3K (p85/p110) activity.

Effect of PI3K or MEK inhibition on respiratory burst

Stimulation of neutrophils with the chemotactic peptide fMLP induces the rapid formation of microbicidal oxidants, a process dependent on prior priming of cells with cytokines, chemoattractants or lipopolysaccharide [34–36]. Activation is a result of the rapid assembly of the NADPH oxidase system, a process that *in vivo* is dependent on the phosphorylation of oxidase components, e.g. $p47^{phox}$. Previous reports have suggested roles for PI3K in this process [18,20,33,37], whereas a role for $p44^{ERK1}$ and $p42^{ERK2}$ MAPKs has also been proposed, although indirectly [38–40]. We therefore analysed the effect of preincubation of human neutrophils with various concentrations of PD098059 or wortmannin/LY294002 on fMLP-induced respiratory burst, continuously measured by cytochrome *c* reduction (as described in the Materials and methods section). Neutrophils were then pretreated with the priming agents GM-CSF, PAF and TNF-α. As shown in Figure 5(A), treatment of cells with different concentrations of PD098059 for 20 min before stimulation had no effect on fMLP-induced respiratory burst. Even at 50 μM PD098059, a concentration that we have shown to inhibit all ERK activity (Figure 2), there was no effect on superoxide production. We have analysed the effect of these inhibitors on cytokine-primed respiratory burst and thus it seems that activation of MEK/ERK kinases is not required for either the activation or the priming of the NADPH oxidase system, in contrast with other suggestions [38–40]. Other reports [20,33,37] have demonstrated that inhibition of PI3K results in a decrease in fMLP-induced respiratory burst. As shown in Figure 5(B), increasing concentrations of wortmannin or LY294002 do indeed inhibit superoxide production. The addition of 30–100 nM wortmannin or 10 μM LY294002 to GM-CSF-primed neutrophils results in an almost complete inhibition of O_2^- production, as measured by cytochrome *c* reduction. This correlates well with the inhibition of PI3K as measured by immune-complex kinase assays (Figure 4B). Interestingly, although wortmannin inhibits the PAF-primed respiratory burst, the concentration of wortmannin required is higher than that needed to abolish PI3K activity in immune-complex kinase assays from PAF- or fMLP-stimulated cells (Figure 4B). This can be explained by the fact that the kinase assays measure only phosphotyrosine-associated PI3K activity, e.g. p85–p110α. It has been reported that neutrophils also express p110γ, a G-protein-activated PI3K isoform [23]. The p110γ PI3K isoform is inhibited by much higher concentrations of wortmannin in intact cells (IC_{50} 200 nM) than p110α [23]. This suggests that for the PAF- and TNF-α-primed respiratory bursts different isoforms of PI3K are utilized in the activation of the NADPH oxidase system from that for GM-CSF.

Effect of wortmannin and PD098059 on PMN migration

The influx of inflammatory cells into tissues involves processes such as cell rolling, attachment to endothelial cells, spreading, trans-endothelial diapedesis and directed migration to the inflammatory site or chemotaxis. To determine whether MEK-ERK or PI3K might have a role in neutrophil migration we utilized a modification of the method described by Boyden ([29]; see the Materials and methods section) with a 48-well microchemotaxis chamber. Cytokine or chemoattractant was placed in the lower chamber and migratory activity was measured. As can be

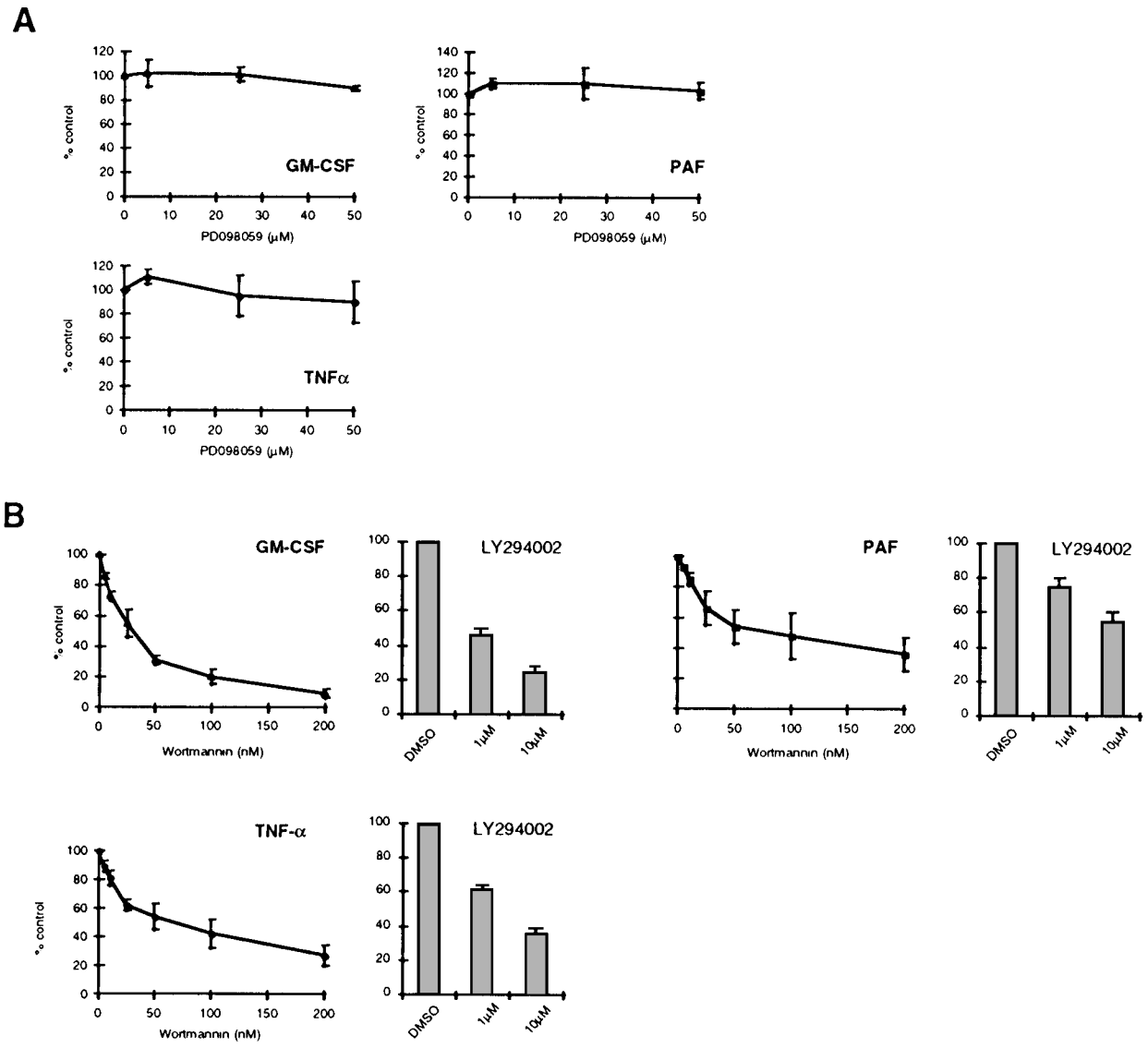

Figure 5 Effect of inhibitors of PI3K and MEK on fMLP-induced neutrophil respiratory burst

Neutrophils were preincubated with DMSO, increasing amounts of PD098059 for 20 min (**A**) or wortmannin for 15 min (**B**), before the addition of priming agents. GM-CSF (100 pM; 30 min), PAF (1 μM; 2 min) or TNF-α (100 i.u./ml; 30 min) was added before stimulation with fMLP. Superoxide production was monitored continuously by measurement of cytochrome *c* reduction as described in the Materials and methods section. Results are represented as percentages of burst relative to control cells and are means $\pm$ S.E.M. ($n = 4$). Control rates: GM-CSF, 19.2 $\pm$ 1.5 nmol of O_2^-/min per 10^7 cells; PAF, 23.6 $\pm$ 1.5 nmol/min per 10^7 cells; TNF-α, 16.5 $\pm$ 1.5 nmol/min per 10^7 cells.

seen in Figure 6(A), all three stimuli provoked an apparent migratory activity to different degrees, fMLP being the most potent and GM-CSF the weakest.

To determine the effect of inhibiting $p44^{ERK1}$ and $p42^{ERK2}$ MAPKs on neutrophil migration we preincubated PMNs with various concentrations of PD098059 before performing Boyden-chamber migration assays (Figure 6B). It is clearly seen that even at concentrations of 50 μM, enough to inhibit all ERK activity totally, cell migration is more than 90 % of control values for all stimuli measured. This demonstrates that although it has been previously postulated that activation of MAPKs might have a role in granulocyte migration [10], there is no apparent role for either $p44^{ERK1}$ or $p42^{ERK2}$.

We next analysed the potential role of PI3K in neutrophil migratory responses. As is seen in Figure 6(C), there is a clear distinction between the effect of wortmannin on unstimulated or GM-CSF-stimulated cells compared with fMLP or PAF. Whereas 100 nM wortmannin inhibits migration by 50 % for buffer or GM-CSF, for PAF and fMLP even 200 nM results in less than 10 % inhibition. We have recently reported that for migration of eosinophilic granulocytes, the increased locomotion induced by GM-CSF is due to enhanced chemokinesis of cells rather than chemotaxis [41]. In contrast, PAF-induced migration is mainly gradient-dependent and is thus chemotaxis. As shown in Figure 6(D), the same distinction can apparently be made for neutrophils. Whereas PAF acted as a true chemoattractant, GM-CSF stimulated chemokinesis rather than chemotaxis, as measured by its ability to stimulate migration if present in either the upper or the lower chamber, e.g. non-gradient-directed. Thus for PMNs, wortmannin seems to inhibit only those agonists responsible for

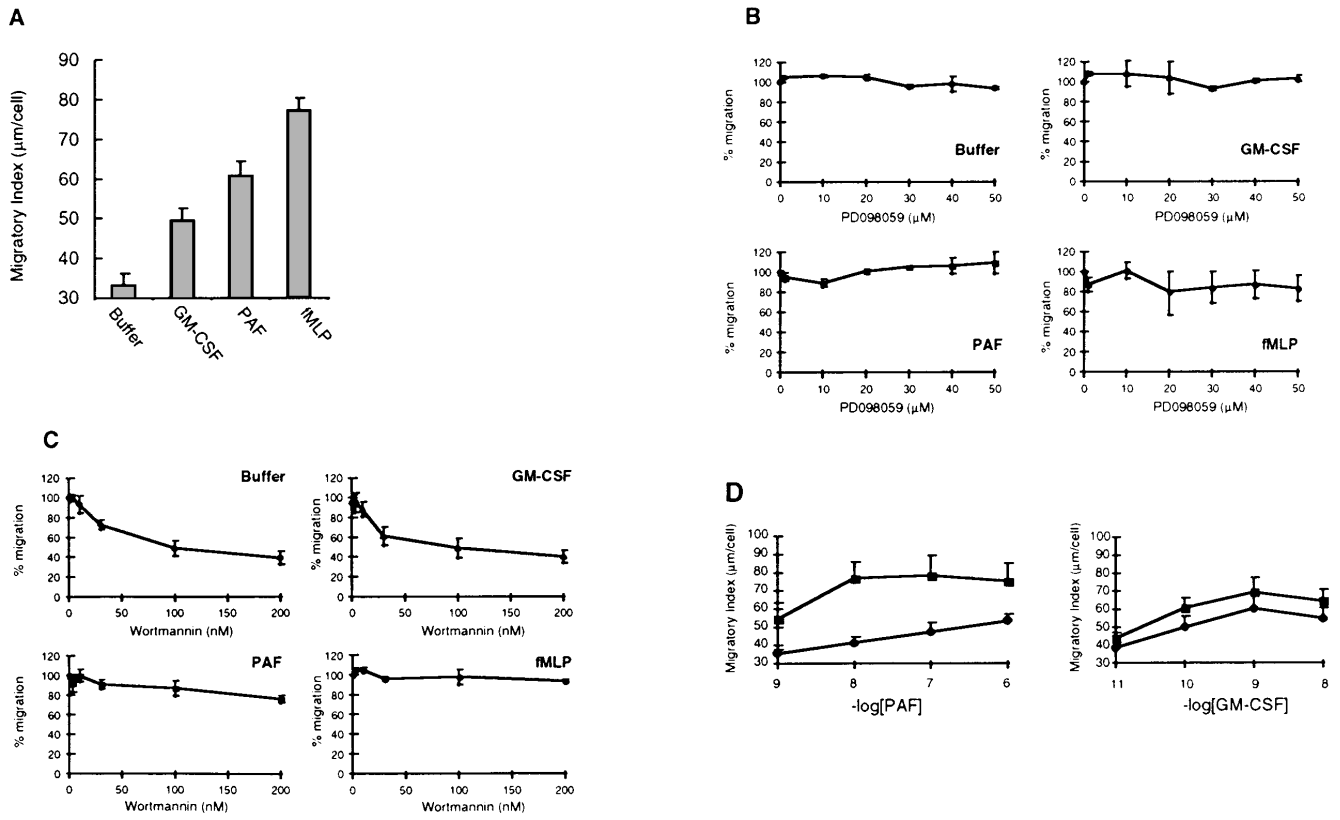

Figure 6 Effect of PD098059 and wortmannin on neutrophil migration

(**A**) Migration of neutrophils was monitored in microchemotaxis Boyden chambers in response to buffer, GM-CSF (100 pM), PAF (10 nM) or fMLP (10 nM) as described in the Materials and methods section. Cytokines or chemoattractants were placed in the lower compartment and cells were left to migrate for 1.5 h at 37 °C. Results are expressed as migratory index (μm per cell) and are means $\pm$ S.E.M. ($n = 4$). Neutrophils were preincubated with DMSO, increasing amounts of PD098059 for 20 min (**B**) or wortmannin for 15 min (**C**), before analysis in microchemotaxis Boyden chambers as described above. In (**D**), cells were stimulated with increasing concentrations of either PAF or GM-CSF in either the upper chamber (■) or the lower chamber (●). Results are expressed as percentage migrations relative to control cells not preincubated with inhibitor ($n = 4$).

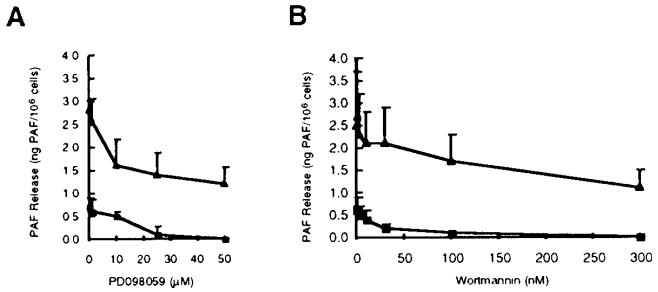

Figure 7 PD098059 and wortmannin each inhibit STZ-stimulated PAF release in human neutrophils

Isolated neutrophils were incubated for 20 min with PD098059 (**A**) or for 15 min with wortmannin (**B**) and then additionally for 30 min with (▲) or without (■) GM-CSF (100 pM). STZ (0.5 mg/ml) was then added for a further 30 min before supernatants were collected and concentrations of PAF were measured by radioimmunoassay as described in the Materials and methods section. Results are expressed as PAF release (ng of PAF per 10^6 cells) and are means $\pm$ S.E.M. ($n = 4$).

increased granulocyte chemokinesis. This suggests that activation of PI3K isoforms is critical for enhanced random movement of cells rather than gradient-directed migration.

PD098059 and wortmannin both inhibit STZ-induced PAF release

Neutrophils are potently stimulated by opsonized particles such as STZ coated with, for example, IgG and iC3b, resulting in the activation of the respiratory burst and the production of lipid mediators such as PAF and leukotriene-C_4 [42]. The process of lipid release is up-regulated or primed by pretreatment of cells with GM-CSF. Relatively little is understood about the mechanisms involved in this response, although a role for $cPLA_2$ has been defined [43]. To determine whether the activation of MEK-ERK or PI3K signal-transduction pathways might have a role in this process we analysed the release of PAF from GM-CSF-primed STZ-stimulated PMNs. Supernatants were collected from stimulated cells and PAF (ng/10^6 cells) was analysed by competitive radioimmunoassay. As can be seen in Figure 7(A), the addition of increasing concentrations of PD098059 inhibits STZ-stimulated PAF release with a profile that is well correlated with the inhibition of ERK activity (Figure 2). Priming of PMNs by pretreatment with GM-CSF (100 pM) before treatment with STZ results in a much greater release of PAF, as has been previously described (Figure 7A). PAF release induced in this primed system is also inhibited by incubation with PD098059; however, 50 μM PD098059 decreases this only to 50% of the control value. Thus, whereas STZ seems to require functional MEK for the generation of PAF, GM-CSF-primed cells are able to short-circuit this requirement, presumably by the induction of

other downstream protein kinases. A similar reponse was observed for the effect of wortmannin on both primed and unprimed STZ-mediated PAF release (Figure 7B). Unprimed PMNs were very sensitive to wortmannin, with 50% inhibition occurring at approx. 30 nM. The effect of inhibitor on GM-CSF-primed PMNs was, however, much less marked, with 100 nM wortmannin resulting in only a 20% decrease in PAF release compared with control values. This again suggests that cytokine-mediated priming of neutrophils results in the activation of distinct signalling pathways capable of enhancing or replacing those utilized in the unprimed reponse.

DISCUSSION

In this paper we have analysed the activation of both $p44^{ERK1}$/$p42^{ERK2}$ MAPKs and phosphotyrosine-associated PI3K in human neutrophils. Furthermore, we have compared for the first time the effects of inhibiting these two signal transduction cascades on various aspects of neutrophil effector functioning. A role for the $p44^{ERK1}$/$p42^{ERK2}$ MAPKs in neutrophil functioning has previously been proposed by several studies [10–14,44]. The MEK inhibitor PD098059 has been recently utilized to confirm a role for both $p44^{ERK1}$/$p42^{ERK2}$ MAPKs in fibroblast cell growth and differentiation [24,25], as well as in platelet functioning [45]. By adding this synthetic inhibitor to human neutrophils we have been able to determine the effect of blocking MEK-ERK signalling on downstream effector functions.

Activation of $p21^{ras}$ involves the exchange of GDP for GTP and the subsequent binding of various Ras-effector molecules such as Raf1 and PI3K. We have utilized this observation to analyse $p21^{ras}$ activation by its ability to bind to Raf1-RBD only when bound to GTP [28]. We have demonstrated the activation of $p21^{ras}$ by a variety of receptors in intact human neutrophils (Figure 2). This is the first time that this technique has been utilized in primary cells and provides a powerful method for characterizing $p21^{ras}$ activation. Most interesting is the activation kinetics observed with the various stimuli. Whereas TNF-α is incapable of activating $p21^{ras}$, GM-CSF stimulates a slow activation of relatively long duration (more than 20 min). In contrast, the G-protein-coupled receptor agonists PAF and fMLP induce a very rapid (less than 10 s) and short-lived (less than 5 min) $p21^{ras}$ activation. This activation is correlated with the activation of $p44^{ERK1}$ and $p42^{ERK2}$ for all stimuli in neutrophils, suggesting either that $p21^{ras}$ must be bound to GTP to maintain ERK activation or that these signals are down-regulated at the same time. It has recently been reported that fMLP stimulates the tyrosine phosphorylation and subcellular redistribution of p125 GAP, a $p21^{ras}$ GTP-activating protein [46], providing a potential down-regulatory mechanism.

Activation of PI3K can be measured by using anti-phosphotyrosine immunoprecipitates and lipid kinase assays *in vitro*. We have clearly demonstrated that GM-CSF, PAF and fMLP all stimulate phosphotyrosine-associated PI3K activity that is inhibited by low concentrations of wortmannin (Figures 4A and 4B). This is in direct contrast with a study by Vlahos and Matter [22], who demonstrated fMLP-inducible PI3K activity in neutrophils but observed no PI3K activity in anti-phosphotyrosine immunoprecipitates. Furthermore they observed no tyrosine phosphorylation of p85. Although it is possible that p85 is not tyrosine phosphorylated but associates with one or more tyrosine-phosphorylated proteins, it is difficult to reconcile the differences between this study and our own, but differences in phosphotyrosine antibodies might be responsible.

The activation of respiratory burst is a metabolic event during which phagocytes generate antimicrobicidal oxidants by the reduction of oxygen to superoxide. Activation of this effector response requires prior priming of the cells with cytokines, chemoattractants or lipopolysaccharide. The NADPH oxidase itself is a multicomponent enzyme whose components are distributed between cytosol and plasma membrane. After stimulation of cells, cytosolic components migrate to the plasma membrane, where they associate with cytochrome components to generate the active oxidase [34–36]. Associated with oxidase activation is the phosphorylation of the cytosolic component $p47^{phox}$ [39,47,48]. Regions surrounding the Ser-345 and Ser-348 phosphorylation sites in $p47^{phox}$ contain the motif Pro-Xaa-Ser-Pro, which is a consensus phosphorylation site for MAPKs [49,50]. It has therefore been suggested that phosphorylation of $p47^{phox}$ by MAPKs might also have a role in the translocation of this oxidase component to the plasma membrane and activation of the respiratory burst [39]. Our results on the effect of PD098059 on superoxide production demonstrate that this is not so (Figure 5A). The inhibition of $p44^{ERK1}$ and $p42^{ERK2}$ activation does not affect fMLP-induced respiratory burst in cells primed with GM-CSF, TNF-α or PAF. Clearly the activation of the ERK signalling pathway does not have a crucial role in any aspect of NADPH oxidase activation. In contrast with the effect of PD098059, the addition of wortmannin or LY294002 inhibited fMLP-induced respiratory burst for all three priming stimuli (Figure 5B). It has also been demonstrated that both wortmannin and LY294002 can inhibit fMLP-stimulated oxidant production [20,33,37]. These studies did not, however, investigate in detail the effect of various priming agents on this response. In Figure 5(B) it is clear that wortmannin inhibits fMLP-stimulated superoxide production in GM-CSF-, PAF- and TNF-α-primed cells. Interestingly, GM-CSF-primed cells are inhibited at much lower concentrations of wortmannin than PAF-primed cells. It has previously been described that human neutrophils contain both a tyrosine kinase-linked PI3K, p110α, as well as a G-protein-linked PI3K, p110γ [23]. Interestingly, whereas p110α-mediated PtdIns(3,4,5)P_3 accumulation is inhibited by relatively small amounts of wortmannin in intact cells ($IC_{50} < 50$ nM), p110γ requires much higher concentrations ($IC_{50} > 200$ nM). This is correlated with the differential effects of wortmannin described here (Figure 5B). Whereas wortmannin inhibited GM-CSF-primed oxidant production with an IC_{50} of less than 30 nM, the IC_{50} for PAF was more than 100 nM. This suggests that whereas GM-CSF priming might involve p110α, PAF priming probably also utilizes p110γ. It has previously been shown that TNF-α can prime the neutrophil respiratory burst [38,51,52] and that this can be inhibited by tyrosine kinase inhibitors. For TNF-α priming, the IC_{50} of wortmannin inhibition was approx. 50 nM, suggesting a role for p110α *in vivo* (Figure 5B). GM-CSF, PAF and TNF-α all demonstrate distinct inhibition profiles, suggesting that different receptors can potentially utilize different PI3K isoforms or combinations of isoforms in the priming and activation of the NADPH oxidase complex. Recently several protein kinase activities present in human neutrophils have been shown to be inhibited by wortmannin [53]. These same kinases have also been shown to phosphorylate $p47^{phox}$ *in vitro*; furthermore, wortmannin has been shown to inhibit $p47^{phox}$ phosphorylation *in vivo* [53]. These results suggest that a serine/threonine protein kinase downstream of PI3K might be involved in the activation of the NADPH oxidase. One such kinase could be protein kinase B, whose activity is inhibited by antagonizing PI3K activation [54,55]. We have shown that protein kinase B is indeed activated in response to both cytokines (interleukin 5, GM-CSF and interleukin 4) and chemoattractants (PAF, fMLP and C5a) in human granulocytes (P. J. Coffer, R. C. Schweizer and L. Koenderman, unpublished work). Recently it was demonstrated

that the introduction of a constitutively active form of PI3K into a monoblastic phagocyte line (GM-1) caused constitutive activation of protein kinase B and also phosphorylation of $p47^{phox}$ [56].

Activation of phospholipase A_2 (PLA_2) results in the production of lyso-PAF, the precursor for the synthesis of PAF [57]. Incubation of neutrophils with opsonized particles results in the activation of the respiratory burst as well as the release of PAF [42]. We have found that preincubating neutrophils with PD098059 completely inhibits STZ-stimulated PAF release (Figure 7A). Furthermore PD098059 greatly decreases the GM-CSF-primed STZ-stimulated PAF release (60% inhibition; Figure 7A). Studies have demonstrated that $cPLA_2$ is inducibly serine-phosphorylated and that it is a substrate for $p42^{ERK2}$ *in vitro* [58]. Furthermore, it has recently been shown that GM-CSF promotes the phosphorylation and activation of $cPLA_2$ in human neutrophils [15]. Our results suggest, although indirectly, that the activation of MEK-ERK might indeed be a requirement for the activation of $cPLA_2$ in human neutrophils. The fact that PD098059 does not completely inhibit GM-CSF-primed STZ-stimulated PAF release suggests that priming can somehow override the requirement for MEK-ERK activation of $cPLA_2$, possibly by activation of a secondary signalling pathway. Indeed, TNF-α does not activate $p44^{ERK1}$/$p42^{ERK2}$ but does cause the phosphorylation of $cPLA_2$, demonstrating a mitogen-activated protein kinase (MAPK)-independent pathway (Figure 1A) [14]. Furthermore, a recent study has demonstrated a role for p38 MAPK in the phosphorylation of $cPLA_2$ in platelets [59]. As shown in Figure 7B, wortmannin also quite markedly inhibits the release of PAF in unprimed cells. Thus it is probable that different stimuli might activate different combinations of $cPLA_2$-phosphorylating/activating kinases.

The ability of leucocytes to move along a chemotactic gradient is crucial to their accumulation at sites of injury or infection (reviewed in [7]). To determine the effect of MEK-ERK inhibition on neutrophil motility we utilized a microchemotaxis chamber (Figure 6). The addition of PD098059 to cells before the assay resulted in no change in the migratory behaviour of the cells in response to buffer, GM-CSF, PAF or fMLP (Figure 6B). This demonstrates that there is apparently no requirement for MEK-ERK activation in the migratory movement of neutrophils, or in the detection of chemotactic gradients. More interestingly, wortmannin inhibited neutrophil chemokinesis but not chemotaxis (Figure 6C). Products of PI3K-induced phosphorylation of membrane lipids, e.g. PtdIns(3,4,5)P_3, have been suggested to mediate cytoskeletal changes, especially in Rac-mediated membrane 'ruffling' [21,60–62]. It has been demonstrated that PI3K is necessary for the activation by platelet-derived growth factor (PDGF) of Rac-mediated rearrangements of the actin cytoskeleton, possibly by regulating GTP loading on Rac [62–64]. It is therefore tempting to speculate that the activation of Rac in neutrophils by PI3K might be involved in GM-CSF-stimulated cell chemokinesis in much the same way as PDGF causes ruffling in fibroblasts [62,64]. However, it seems that this signalling pathway is not required for chemotaxis. which cannot be inhibited by high concentrations of wortmannin.

Taken together, our results demonstrate that, whereas the activation of PI3K in human neutrophils seems to have an important role in granulocyte effector functions, the MEK-ERK signalling cascade is more restricted to processes such as active lipid release. Another function of MEK-ERK stimulation is the activation of gene transcription via the potentiation of transcription factor transactivation, protein stability or increased binding of DNA (reviewed in [65,66]). It could be that in neutrophils $p44^{ERK1}$ and $p42^{ERK2}$ also have a role in gene induction responsible, for example, for the up-regulation of adhesion molecules such as Mac-1/LFA-1 or selectins, necessary for neutrophil migration *in vivo*. Complex cross-talk between the sigalling pathways described here will determine the eventual outcome of agonist-induced downstream responses. The activation of the $p21^{ras}$–ERK signal transduction pathway thus probably allows fine-tuning of the regulation of these downstream effects in response to cellular activation.

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