Exposure of neutrophils to a variety of agonists including chemoattractant peptides and cytokines induces degranulation and activation of the oxidative burst which are required for bacterial killing. The signaling pathways regulating these important functions are incompletely characterized. Mitogen-activated protein (MAP) kinases, which include the extracellular signal-regulated kinases (ERKs), are activated rapidly in neutrophils, suggesting that they may regulate cell activation. We found that neutrophils express two isoforms of MAP/ERK kinase (MEK), mixed-function kinases that are responsible for phosphorylation and activation of ERK. Like MEK-1, MEK-2 was found to reside in the cytosol both before and after stimulation. Studies were undertaken to define the relative abundance and functional contribution of MEK-1 and MEK-2 in neutrophils and to characterize the signaling pathways leading to their activation. Although the abundance of the two isoforms was similar, the activity of MEK-2 was at least 3-fold greater than that of MEK-1. A rise in cytosolic [Ca^{2+}] was insufficient for MEK stimulation, and blunting the [Ca^{2+}] change with intracellular chelators failed to prevent receptor-activated activation of either isoform, implying that cytosolic Ca^{2+} transients are not necessary. In contrast, both MEK-1 and MEK-2 were activated by exposure of cells to protein kinase C (PKC) agonists. Conversely, PKC antagonists inhibited the chemotactic stimulation of both isoforms, suggesting that PKC was required for their activation. Despite these similarities, clear differences were also found in the pathways leading to activation of the MEK isoforms. In particular, MEK-2 was considerably more sensitive than MEK-1 to the phosphatidylinositol 3-kinase inhibitor wortmannin. Phosphorylation and activation of ERK-1 and ERK-2 were also reduced by this inhibitor. In summary, MEK-2 is stimulated in formyl-methylionyl-leucyl-phenylalanine-treated neutrophils, where it appears to be functionally the predominant isoform. The time course and inhibitor sensitivity of MEK-2 activation parallel those of several components of the microbicidal response, suggesting a signaling role of the MEK-ERK pathway.

The main function of neutrophils is host protection, which they accomplish by destruction of invading microorganisms and removal of inflammatory debris. To this end, neutrophils have evolved a variety of rapid and coordinated responses to reach sites of inflammation, where they mount a microbicidal response. After recognition and ingestion (phagocytosis) of the microorganisms, production of reactive oxygen intermediates by NADPH oxidase and degranulation ensue (for review, see Smith and Curnutte, 1991; Borregaard et al., 1993), resulting in destruction of pathogens. The importance of these effectors in host defense is illustrated by the marked propensity of patients deficient in one or more of these responses to develop infections (Wolff et al., 1972; Smith and Curnutte, 1991). Paradoxically, inappropriate release of these compounds into the extracellular milieu by neutrophils contributes to inflammatory tissue damage. A more complete understanding of the regulation of these functions is crucial for prevention or amelioration of inflammatory tissue injury while preserving important host defense functions.

Neutrophils express a variety of plasma membrane receptors that trigger the responses to a variety of compounds including bacterial products, components of the complement and clotting cascades, and soluble factors such as cytokines released by other cells. Activation of neutrophils by chemoattractants such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), CSa, and interleukin-8 is mediated by serpine receptors that are linked to heterotrimeric GTP-binding proteins (for review, see Gerard and Gerard, 1994). Platelet-derived growth factor receptors possess intrinsic tyrosine kinase activity (Westmark et al., 1990), and others such as granulocyte-macrophage colony stimulating factor (GM-CSF), and Fcγ receptors are linked to cytoplasmic tyrosine kinases (Quelle et al., 1994; Hamada et al. 1996).

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These seemingly diverse stimuli have remarkably similar effects on neutrophils, leading to priming or activation of effector responses.

Activation of neutrophils is associated with a marked increase in the phosphorylation of multiple polypeptides on serine, threonine, and to a lesser extent tyrosine residues (Babior, 1988). Early studies concentrated on the activation of protein kinase C (PKC) and other Ca2+-dependent kinases (Tauber, 1987). However, subsequent investigations uncovered a more complex network of signaling pathways which included a cascade of phosphorylation-dependent reactions. Some of the major substrates of these phosphorylation reactions are the MAP kinases, which require phosphorylation on both threonine and tyrosine residues for activation. Three families of MAP kinases have been described: the ERKs, c-Jun NH2-terminal kinase (JNK or SAPK) and p38 (Derijard et al., 1995). Several isoforms of ERK have been described, and at least two of them, ERK-1 (p44MAP kinase) and ERK-2 (p42MAP kinase), are expressed in neutrophils (Torres et al., 1993). ERKs are serine/threonine kinases thought to participate not only in the control of growth and differentiation (Lange-Carter et al., 1993; Blenis, 1994), but also in cytoskeletal remodeling (Crews and Erikson, 1993) and in activation of phospholipase A2 (Durston et al., 1994), two aspects of great relevance to the microbicidal response of neutrophils. Additionally, it has been suggested that ERKs may participate in the activation of the oxidative burst (EI Benna et al., 1994) in part because p47

isoforms of MEK, termed MEK-1 to -3, have been described (Zheng et al., 1993; Seger et al., 1995). Functionally, however, two types of MEK activity can be discerned in neutrophils (Thomson et al., 1994), which are activated by bacterial peptides (Grinstein et al., 1994). Neutrophils were counted using a model ZM Coulter counter, resuspended in Hepes-buffered medium RPMI 1640 at 106 cells/ml, and maintained in this medium at room temperature with gentle mixing until use. To minimize proteolysis following extraction, the cells (107/ml) were pretreated with 2.5 mm diisopropylfluorophosphate for 30 min at room temperature. Where specified, the cells were metabolically labeled with [32P]orthophosphate by incubation with 0.5 µCi/ml (≈285 µCi/mg phosphate) of the isotope in nominally phosphate-free medium for 3 h at 37°C.

To study the role of phosphatidylinositol-3-kinase (PI-3-kinease), the cells were treated with the specified concentrations of wortmannin for 5 min before addition of the agonist. To assess the role of PKC, the cells were incubated for 30 min with 2 µM bis-indolylmaleimide or for 1 h with either 1 µM calphostin C or 10 µM chelerythrin, prior to stimulation with either FMLP or PMA. All incubations were at 37°C.

Neutrophil fractionation was performed as described in Borregaard et al. (1993) and references therein. Briefly, cells were disrupted by nitrogen cavitation in a buffer containing 10 mM KCl, 3 mM NaCl, 1 mM Na3ATP, 3.5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM PIPES, pH 7.2. Nuclei and unbroken cells were removed by centrifugation at 400 x g for 15 min, and the resulting postnuclear supernatant was applied to a BioRad A Percol gradient ranging from 1.05 to 1.21 g/ml. Centrifugation at 37,000 x g for 30 min yielded four separable bands. Three of these correspond to the primary, secondary, and tertiary granules; a fourth band contains both secretory vesicles and plasma membrane (sv/pm fraction), and the top layer is the cytosolic fraction. The gradient was collected into 1-ml fractions, and each was assayed for markers of the above subcellular compartments. The fraction profile was then plotted according to the content of myeloperoxidase (primary granules, α-band), lactoferrin (secondary granules, β-band), gelatinease (tertiary granules, β2-band), and histocompatibility leukocyte antigen and albumin (plasma membranes and secretory vesicles, γ-band), all measured by enzyme-linked immunosorbent assay as described (Borregaard et al., 1993). Percoll was removed by centrifugation, and the biological material was mixed with boiling 2 x concentrated Laemmli sample buffer and subjected to SDS-PAGE and immunoblotting (see below).

Immunoblotting and Immunoprecipitation—Immunoblotting was performed essentially as described (Grinstein et al., 1994), after separation of the proteins by SDS-PAGE. MEK-1 was detected using a 1:20,000 dilution of the primary antisera and MEK-2 with a 1:5,000 dilution. Phosphoryosine was detected using a 1:5,000 dilution of monoclonal anti-phosphotyrosine antibodies (4G10 hybridoma, from Upstate Biotechnology, Inc.). Immunoprecipitation of phosphotyrosine-containing proteins was done under denaturing conditions using monoclonal anti-phosphotyrosine antibodies (4G10 hybridoma)
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**RESULTS**

**MEK-2 Is Present in Neutrophils**—The presence of MEK-2 and its abundance relative to MEK-1 were assessed in human neutrophils. Cell extracts were immunoblotted using isoform-specific antibodies and compared with defined amounts of purified recombinant MEK-1 or MEK-2, generated as GST fusion proteins (Fig. 1). The content of the MEK isoforms was then quantified by interpolation. In three separate experiments, the MEK-1 content of human neutrophils averaged 126 ± 14 ng/10^6 cells; MEK-2 was not significantly different, averaging 138 ± 38 ng/10^6 cells. It is noteworthy that the reactivity of the antibodies was not altered by stimulation of the cells, although the electrophoretic mobility of MEK-1, and to a lesser extent that of MEK-2, was reduced in cells stimulated with the chemotactant fMLP.

The subcellular location of MEK-2 was defined next. Neutrophils were disrupted by cavitation and fractionated on a Percoll gradient, yielding five major fractions that have been characterized extensively before (Borregaard et al., 1993). When these were analyzed by immunoblotting, MEK-2 was found to be absent from the primary, secondary, or tertiary granules and the fraction containing both secretory vesicles and plasma membranes (not illustrated). By contrast, MEK-2 was clearly detectable in the cytosolic fraction, where it was enriched relative to the whole cell extract. Similar results were obtained following stimulation of the cells with TPA, excluding the possibility that MEK-2 became redistributed during activation, as has been reported for other serine/threonine kinases.

**MEK-2 Is Activated during Neutrophil Stimulation**—The activity of the MEK isoforms was compared next. For this purpose, MEK-1 and MEK-2 were immunoprecipitated from resting and chemotactant-stimulated cells and incubated with radiolabeled ATP in the presence of a GST fusion of ERK-1 (Crews et al., 1992), the putative substrate of MEK in vivo. A mutated form of ERK-1 devoid of kinase activity was used, to preclude the occurrence of autophosphorylation. Consistent with earlier findings, MEK-1 was not detectably active in resting cells, but displayed both autophosphorylation and the ability to phosphorylate GST-ERK following stimulation with fMLP. Similarly, MEK-2 was inactive before, but clearly active after stimulation (Fig. 2A). It was capable of autophosphorylation, but its activity toward exogenous substrate was much more evident (Fig. 2A, inset). At 37°C, the activity of MEK-2 was detectable within 1 min of stimulation, peaked after approximately 2 min, and decayed thereafter, reaching near basal levels by 10 min (Fig. 2B). This profile resembles the time course of activation and subsequent deactivation of several effectors in chemotactant-stimulated neutrophils (for review, see Sha'afi and Molski (1988)).

The availability of GST fusions of the two MEK isoforms enabled us to quantify the amount of immunoprecipitated kinase and thereby establish the relative activity of MEK-1 versus MEK-2. The results of three such experiments are summarized in Fig. 2A. The basal activity of both isoforms was negligible. In fMLP-stimulated cells, MEK-2 was at least 3-fold more active per unit of protein than MEK-1. These findings are consistent with those reported by Zheng and Guan (1993) using purified recombinant MEKs activated by stimulated cell lysates. The higher activity of MEK-2, together with its comparable level of expression (Fig. 1), implies that this isoform is the

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Covalently bound to agarose (Upstate Biotechnology, Inc.). In all cases, detection was made using the enhanced chemiluminescence (ECL) system from Amersham Corp.

For immunoprecipitation, 1–2 × 10^6 cells were suspended in 1 ml of lysis buffer (150 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM peptatin, 10% glycerol, and 50 mM Tris-Cl, pH 7.5). The lysate was sonicated and sedimented in an Eppendorf Microfuge for 5 min. The resulting supernatant was incubated with primary antibody for 2 h followed by the addition of protein A-Sepharose beads (100 μl of 50% suspension) and incubated further for 1–2 h. For MEK-1, 3 μl of primary antiserum was used per sample, whereas for MEK-2 we used 10 μl of the monoclonal antibody. Next, the beads were washed six times with lysis buffer and were either used for kinase determinations (see below) or were boiled for 5 min in Laemmli sample buffer, sedimented rapidly, and the supernatant used for SDS-PAGE.

Kinase Assays—The kinase assay used was adapted from Alessandrini et al. (1992). MEK-1 or MEK-2 immunoprecipitates were obtained as above, and following the washes with lysis buffer the beads were washed twice more with kinase assay buffer (3 mM magnesium acetate, 1 mM EDTA, 0.1 mg/ml ovalbumin, and 50 mM Tris-Cl, pH 8.0). The reaction was initiated by suspending the beads in 30 μl of kinase assay buffer containing 50 μM ATP, 5 μCi of [γ-32P]ATP, 5 mM dithiothreitol, with or without 1.75 μg of kinase-inactive GST-ERK, and the samples were incubated at 30°C for 20 min with vigorous shaking in a Thermomixer. Kinase activity was then terminated by the addition of 30 μl of hot Laemmli sample buffer and boiling for 5 min. The samples were then analyzed by SDS-PAGE (10% acrylamide), and the radioactivity incorporated into GST-ERK and MEK was determined using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The kinase activity of ERK-1 and -2 immune-complexes was assayed by the method of Bashey et al. (1994) using myelin basic protein as a substrate.

Other Methods—[Ca2+]i was measured fluorometrically using indo-1, as described (Grinstein et al., 1994). Radioactivity incorporated into MEK, GST-ERK, or myelin basic protein was quantified by Phosphorimaging. Dried gels or blots were exposed to a Molecular Dynamics phosphor screen, and images were obtained using the PhosphorImager and analyzed with the ImageQuant software. For reproduction, the images acquired with ImageQuant were saved as 16-bit TIFF files. Such images were next cropped using Adobe Photoshop version 3.0, saved as EPS files, and labeled using Adobe Illustrator version 5.5. Graphs were generated using Cricket Graph 3, saved as PICT files, and labeled using Illustrator. For illustrations, immunoblots were scanned using a Hewlett-Packard J et Scan II cx using Desk Scan II version 2.1 software. For quantification, immunoblots were scanned using a model DNA 35 high resolution flatbed scanner and analyzed using the PDI one-dimensional gel analysis software. Results are presented as typical radiograms or fluorescence traces or as the means ± S.E. of the indicated number of replicates. All results are presented as the mean ± 1 S.E.
measured as in Fig. 1. Data are the means ± S.E. of three experiments.

Panel B, time course of MEK-2 activation by fMLP. Cells were stimulated with $10^{-7}$ M fMLP for the periods indicated and the samples processed as in panel A.

Phosphorylation of MEK-2—The transition between the inactive and active forms of MEK is believed to result from phosphorylation at one or more sites (Gardner et al., 1994; Zheng and Guan, 1994a; Yan and Templeton, 1994; Huang et al., 1995). In support of this notion we found that in cells metabolically labeled with $^{32}$P orthophosphate, MEK-1 phosphorylation increased upon chemoattractant stimulation (Grinstein et al., 1994). Similarly, MEK-2 became phosphorylated when the cells were activated by fMLP (not illustrated). When normalized per amount of immunoprecipitated kinase, the extent of MEK-2 phosphorylation was lower than that of MEK-1 ($2,100$ cpm/ng of protein for MEK-1), despite the fact that the total amount of MEK immunoprecipitated was estimated by immunoblotting and compared with the appropriate GST-MEK standards, as in Fig. 1. Data are the means ± S.E. of three experiments. Panel B, time course of MEK-2 activation by fMLP. Cells were stimulated with $10^{-7}$ M fMLP for the periods indicated and the samples processed as in panel A.

Phosphorylation of two sites, Ser218 and Ser222, is universally recognized to result in activation of MEK-1 (Zheng and Guan, 1994; Yan and Templeton, 1994; Huang et al., 1995). There is, however, some discrepancy as to whether maximal activation requires phosphorylation of only one or both serine residues.

Fig. 2. Activation of MEK-1 and MEK-2 by chemoattractant.
Panel A, inset, the kinase activity of MEK-1 (left) or MEK-2 (right) immunoprecipitated from control (C) and stimulated (fMLP; $10^{-7}$ M for 2 min) neutrophils was tested in vitro using GST-ERK as substrate. The locations of the fusion protein and of autophosphorylated MEK-1 and -2 are indicated by open and solid arrowheads, respectively. Main panel, summary of quantitation of in vitro activity of MEK-1 and MEK-2 immunoprecipitated from control and stimulated cells ($10^{-7}$ M fMLP for 2 min). The amount of MEK immunoprecipitated was estimated by immunoblotting and compared with the appropriate GST-MEK standards, as in Fig. 1. Data are the means ± S.E. of three experiments. Panel B, time course of MEK-2 activation by fMLP. Cells were stimulated with $10^{-7}$ fMLP for the periods indicated and the samples processed as in panel A.

In neutrophils, differentiation to macrophages results in a predominant MEK in neutrophils activated by chemotactic peptides.

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when the effect of TPA is maximal (Fig. 4B). The possibility that PKC also mediates the stimulation of MEK-2 by fMLP was investigated pharmacologically. Because of the existence of multiple isoforms of PKC and due to the imperfect specificity of the currently available antagonists, we compared the effectiveness of three different inhibitors. The agents chosen, bis-indolylmaleimide, chelerythrin, and calphostin, are among the most selective PKC inhibitors reported to date. Moderate doses of the blockers were chosen so that, while inhibition of PKC was incomplete, secondary effects on other systems were minimized. As anticipated, all three inhibitors depressed the activation of MEK-2 induced by TPA (Fig. 4, C and D). More importantly, the stimulation by fMLP was also inhibited. The extent of inhibition of the TPA and fMLP responses by calphostin and chelerythrin was virtually identical, whereas bis-indolylmaleimide reduced the TPA response slightly more than the chemotactic peptide response (Fig. 4D). Jointly, these experiments suggest that activation of PKC by the fMLP receptor is an important contributor to the activation of MEK-2.

Several aspects of the microbicidal response, including the activation of the respiratory burst, are precluded by pretreatment of neutrophils with wortmannin or with compound LY294002, two potent and selective inhibitors of PI 3-kinase (Vlahos et al., 1995). To evaluate the possible role of MEK in these responses we compared the sensitivity of the two isoforms to increasing concentrations of wortmannin (Fig. 5). The PI 3-kinase antagonist had no effect on MEK activity measured and its kinase activity measured. Panel B, comparison of the time courses of activation of MEK-2 by fMLP and TPA. Data were normalized to the maximal value reached with each agonist. Panel C, effect of inhibitors on the fMLP and TPA responses. The cells were preincubated without or with bis-indolylmaleimide (BIM), chelerythrin (CHE), and calphostin (CAL) as described under "Experimental Procedures" and then stimulated with either fMLP or TPA. MEK-2 was next immunoprecipitated and its activity determined. Panel D, summary of the effects on the fMLP and TPA activity, calculated from two or three experiments like that in panel C. Data were normalized to facilitate comparison among agonists.

FIG. 4. Assessment of the role of PKC in MEK-2 activation. Panel A, time course of activation by TPA. Cells were stimulated with either TPA (10⁻⁷ M) or fMLP (10⁻⁷ M) for the indicated times. MEK-2 was immunoprecipitated and its kinase activity measured. Panel B, comparison of the time courses of activation of MEK-2 by fMLP and TPA. Data were normalized to the maximal value reached with each agonist. Panel C, effect of inhibitors on the fMLP and TPA responses. The cells were preincubated without or with bis-indolylmaleimide (BIM), chelerythrin (CHE), and calphostin (CAL) as described under "Experimental Procedures" and then stimulated with either fMLP or TPA. MEK-2 was next immunoprecipitated and its activity determined. Panel D, summary of the effects of inhibitors on MEK-2 activity, calculated from two or three experiments like that in panel C. Data were normalized to facilitate comparison among agonists.

FIG. 5. Inhibition of MEK-1 and MEK-2 activation by the PI 3-kinase inhibitor wortmannin. Cells were pretreated for 5 min with the dose of wortmannin (wtmn) indicated and then stimulated for 2 min with fMLP in the continued presence of wortmannin. Lysates were prepared and used to immunoprecipitate MEK-1 (panel A) or MEK-2 (panel B). Kinase activity was assessed in vitro. Panel C, concentration dependence of the effect of wortmannin on MEK-1 (diamonds) or MEK-2 (squares). Data are the means of two to four experiments. Maximal activities were normalized, to facilitate comparison.

to the concentration required for 50% inhibition of PI 3-kinase in these cells (Vlahos et al., 1995).

Activation of ERK-1 and ERK-2—Considering this differential sensitivity of the MEK isoforms to wortmannin, it was of interest to compare the effects of wortmannin on the putative MEK substrates, ERK-1 (p44MAP kinase) and ERK-2 (p42MAP kinase), both of which are known to be activated by fMLP stimulation (Torres et al., 1993). Fig. 6A illustrates that although fMLP induced tyrosine phosphorylation of both ERK-1 and ERK-2, the inhibitory effects of wortmannin were somewhat greater on ERK-1 than on ERK-2. On average, wortmannin inhibited tyrosine phosphorylation of ERK-1 by 56% and that of ERK-2 by only 27%. The stimulation of ERK by fMLP and the inhibitory effects of wortmannin were confirmed using an antibody that recognizes only the phosphorylated form of the ERKs (Fig. 6B). As phosphorylation of ERK-1 and -2 is only an indirect measure of their activation, we quantitated ERK activity by immune complex assays. ERK-1 and ERK-2 were immunoprecipitated with isofrom-specific antibodies, and phosphorylation of myelin basic protein was quantified in vitro. A typical experiment is illustrated in Fig. 6C, and the results of three similar experiments are summarized in 6D. In accordance with the phosphorylation results, both ERK-1 and -2 were activated by fMLP, and they were partially inhibited by pretreatment with wortmannin. The differential inhibition of ERK-1 and -2, though not as marked as in Fig. 6, A and B, was also noted by this method. Given the variability of the assays, however, this difference was not statistically significant.
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**Fig. 6. Inhibition of ERK-1 and ERK-2 phosphorylation and activation by wortmannin.** Panel A, tyrosine-phosphorylated proteins were immunoprecipitated from cell lysates prepared from control or fMLP-stimulated cells, separated by SDS-PAGE, and immunoblotted with anti-ERK-1 (left) or anti-ERK-2 (right) antibodies. Where indicated, cells were pretreated for 10 min with 100 nM wortmannin (wtmm) and then stimulated for 2 min with fMLP in the continued presence of wortmannin. Panel B, lysates from cells treated with or without fMLP in the presence or absence of wortmannin were blotted with an antibody that recognizes only the phosphorylated form of ERK (New England Biolabs). Panel C, lysates from cells treated with or without fMLP in the presence or absence of wortmannin were immunoprecipitated with ERK-1 or -2 antibodies, as indicated. The kinase activity of the precipitates was assayed using myelin basic protein as a substrate. Panel D, three experiments like that in panel C were quantified by PhosphorImaging. The background, obtained omitting the primary antibody, was subtracted, and the results were normalized and are summarized as the mean ± S.E.

**DISCUSSION**

The experiments using immunoblotting and immunoprecipitation reported above demonstrated the presence of both MEK-1 and MEK-2 in human neutrophils. Quantitation of their relative abundance by comparison with standard recombinant proteins revealed that the content of the two isoforms was comparable (Fig. 1). This contrasts with earlier findings in the brain, where MEK-1 was reported to be the predominant isoform. Although the abundance of the two isoforms is comparable in neutrophils, phosphorylation of MEK-1 is more pronounced when the cells are stimulated by fMLP. The increased phosphorylation is manifested as a reduction in the electrophoretic mobility of the kinase, an effect that is greater for MEK-1 than for MEK-2. That phosphorylation is responsible for the anomalous mobility of MEK-1 was shown earlier, where the electrophoretic shift of the stimulated kinase was reversed by treatment with alkaline phosphatase (Gristein et al., 1994).

Despite its comparable abundance and reduced phosphorylation, the activity of MEK-2 in chemoattractant-stimulated cells was at least 3-fold greater than that of MEK-1 (Fig. 2). This finding is qualitatively consistent with the in vitro experiments of Zheng and Guan (1993), who found MEK-2 to be nearly seven times more active than MEK-1. It is conceivable that the lower degree of MEK-2 phosphorylation reflects incomplete activation by fMLP. If this were the case, the activity of fully stimulated MEK-2 relative to MEK-1 could be even higher than specified above, possibly approaching the factor of seven determined in vitro. On the other hand, excess phosphorylation of MEK-1 could reflect phosphorylation of inhibitory (Rossomando et al., 1994) or nonstimulatory sites, such as the threonine residues targeted by ERK (Gardner et al., 1994). In any event, it is clear that in terms of catalytic activity, MEK-2 is the predominant isoform stimulated by chemotactic peptides in neutrophils.

The signaling pathway leading to MEK activation was also investigated. Experiments using PAF and thapsigargin suggested that a rise in cytosolic Ca\(^{2+}\) is insufficient for stimulation. Moreover, blunting the [Ca\(^{2+}\)] transient with permeant chelators failed to prevent activation of MEK (not shown), implying that the Ca\(^{2+}\) transient is not necessary. On the other hand, PKC agonists mimicked the response, whereas three different antagonists inhibited the activations induced by fMLP and TPA to a comparable extent. These observations suggest that PKC is involved in MEK-2 activation. It is unlikely, however, that MEK is directly phosphorylated by PKC. Instead, it is thought that either ras or a MEK kinase lie immediately upstream of MEK (Mindell et al., 1994). Although PKC could potentially phosphorylate these enzymes, in vitro experiments failed to show phosphorylation of c-raf-1 by certain PKC isozymes (MacDonald et al., 1993). Nevertheless, other isoforms of PKC, ras, or MEK kinase could be involved in the process. In fact, B-ras was recently shown to be present and active in neutrophils (Worthen et al., 1994). Alternatively, PKC may act more than one step upstream of ras or MEK kinase.

Not only was the extent of activation of MEK-1 and MEK-2 different, but the signaling route also appears to differ. This was highlighted by the differential susceptibility of the two isoforms to inhibition by wortmannin. MEK-2 proved to be considerably more sensitive than MEK-1 to the PI 3-kinase inhibitor (Fig. 5). Differential signaling of the MEK isoforms has been suggested previously in experiments using cells transfected with v-ras, which displayed greatly activated MEK-1 while MEK-2 was only modestly stimulated. In accordance with this finding, MEK-1, but not MEK-2, was found to form a complex with ras and c-raf-1 (J. elinek et al., 1994). In view of these findings, it appears that the coexistence of two MEK isoforms in the same cell type is not so much a reflection of redundancy as an indication that varying stimuli can use distinct signaling pathways.

Further support that the two MEK isoforms perform different functions is provided by the studies illustrated in Fig. 6 which indicate that phosphorylation of ERK-1 is more sensitive to wortmannin than is ERK-2. Because ERKs are the substrates of MEKs, these data may be interpreted as evidence that MEK-2 is the preferential activator of ERK-1, whereas MEK-1 may preferentially activate ERK-2. Moreover, as only certain neutrophil effector functions, notably the oxidative burst (Vlahos et al., 1993) and granule secretion (Dewald et al., 1988), are known to be sensitive to wortmannin, we speculate that MEK-2 may be involved in the signaling pathway leading to activation of these functions. These diverging pathways may provide a mechanism for selective regulation of the individual cellular responses, as appropriate for the set of stimuli encountered in any particular biological microenvironment.

MEK activation was not only susceptible to inhibition by wortmannin, but also by inhibitors of PKC. These seemingly incongruous findings can be explained by the recent observation that human neutrophils express predominantly PKC\(\xi\) (Dang et al., 1994). This atypical isoform of PKC is activated by phosphatidylinositol 3,4,5-trisphosphate (Nakanishi et al., 1993), a product of PI 3-kinase which is the principal target of wortmannin.

In summary, MEK-2 is stimulated in fMLP-treated neutrophils on Thr\(^{296}\) and Thr\(^{292}\). It is believed presently that phosphorylation of Thr\(^{296}\) inhibits MEK-1 activity, but there is some discrepancy regarding the consequences of Thr\(^{292}\) phosphorylation (Rossomando et al., 1994; Gardner et al., 1994).
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phils, where it appears to be the predominant isoform. The time course of MEK-2 activation is similar to that of several components of the microbicidal response, consistent with a signaling role of the MEK-ERK pathway. In this regard, it is noteworthy that consensus sites for phosphorylation by ERK have been identified in p47phox, a critical component of NADPH oxidase which mediates the respiratory burst. These considerations are in good accordance with the reported inhibitory effects of wortmannin and LY294002 on superoxide generation and on secretion of activated neutrophils (Vlahos et al., 1995).

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