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Activation of p38 mitogen-activated protein kinase by formyl-methionyl-leucyl-phenylalanine in rat neutrophils

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Abstract

The signaling pathways leading to p38 mitogen-activated protein kinase (MAPK) activation in formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated rat neutrophils were examined. Immunoblot analysis with antibodies against a phosphorylated form of p38 MAPK showed that fMLP-stimulated p38 MAPK activation was dependent on a pertussis toxin-sensitive G protein. Two phosphatidylinositol 3-kinase inhibitors, wortmannin and 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), did not affect the p38 MAPK activation. Phosphorylation of p38 MAPK was concentration dependently attenuated by a tyrosine kinase inhibitor, genistein, and by a Ca^{2+}-dependent protein kinase C inhibitor, 13-cyanoethyl-12-methyl-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-7-one (Gö6976). However, the protein kinase C inhibitors with a broader spectrum, 2-[1-(3-dimethylaminopropyl)-5-methoxy-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (Gö6983) and 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF109203X), had no inhibitory effect. fMLP-stimulated p38 MAPK phosphorylation was also reduced in cells pretreated with a phospholipase C inhibitor, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione (U73122), or preloaded with an intracellular Ca^{2+} chelator, 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA). We conclude that phosphorylation of p38 MAPK by fMLP stimulation in rat neutrophils is dependent on $G_{i/o}$ protein, nonreceptor tyrosine kinase, phospholipase C/Ca^{2+}, and probably Ca^{2+}-dependent protein kinase C pathways. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Neutrophils, rat; p38 Mitogen-activated protein kinase; Tyrosine kinase; Protein kinase C; Phospholipase C; Ca^{2+}, cytosolic-free

1. Introduction

Neutrophils play a pivotal role in inflammatory reactions and constitute the first line of host defense. In response to a variety of soluble and particulate stimuli, activated neutrophils display cytoskeletal rearrangement, chemotaxis, phagocytosis, granule secretion, and release of superoxide anion (Borregaard, 1988). The signal transduction events pertaining to these specific functions remain elusive. However, it has been shown that chemotactic activation is mediated by a seven-transmembrane-spanning receptor coupled to the heterotrimeric G protein, resulting in transduction of signals to the interior of the cells and phosphorylation of multiple polypeptides (Grinstein et al., 1994). Increased phosphorylation of several proteins has been found to correlate with the stimulation of neutrophil effectors (Niessen and Verhoeven, 1994). Although serine and threonine residues are the main targets of protein kinases in stimulated neutrophils, tyrosine phosphorylation is also increased in chemoattractant-treated cells (Gomez-Cambronero et al., 1989).

The identity of many phosphorylated substrates present in activated neutrophils remains unknown. Studies on neutrophils stimulated with chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP) have led to the identification of mitogen-activated protein kinases (MAPKs) as targets of phosphorylation (Krump et al., 1997). Three distinct mammalian MAPKs have been identified, including extracellular signal-regulated kinase (ERK or p42/44 MAPK), p38 MAPK, and c-Jun N-terminal kinase (JNK). MAPKs are a family of serine/threonine kinases that are activated by a cascade of protein kinase

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reactions (Kyriakis and Avruch, 1996). Investigation of human neutrophils has suggested that p38 MAPK is involved in an intracellular kinase cascade that regulates stress-activated signal transduction (Nick et al., 1996). p38 MAPK can phosphorylate transcription factors, thereby regulating gene expression (Wang and Ron, 1996), and it can also phosphorylate other proteins to stimulate NADPH oxidase activity, adhesion, and chemotaxis (Zu et al., 1998). Our recent report has indicated that p38 MAPK is also involved in fMLP-stimulated superoxide anion generation in rat neutrophils (Wang et al., 1999a). However, little is known about the signaling pathways responsible for fMLP-stimulated p38 MAPK activation. The activation of Rac and/or Cdc42 by upstream signals leads to increased activity of p21-activated kinase (PAK), which has been suggested to be involved in the p38 MAPK activation of HeLa cells and monkey kidney COS-7 (SV40 transformed) cell line (Zhang et al., 1995). PAK does not directly phosphorylate p38 MAPK, which requires dual phosphorylation on both tyrosine and threonine residues within the Thr-Gly-Tyr motif by MAPK kinase (Derijard et al., 1995). Thus, the mechanisms by which p38 MAPK activation occurs in response to external stimuli remain to be determined. In this study, to understand the upstream regulators involved in fMLP-stimulated p38 MAPK activation, the effects of pharmacologic inhibitors of several signaling pathways on p38 MAPK activation, as determined by immunoblot analysis with an antibody against the phosphorylated form of p38 MAPK, were examined.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except for the following: dextran T-500 (Pharmacia Biotech, Uppsala, Sweden); Hanks' balanced salt solution (HBSS) (Gibco Life Technologies, Grand Island, NY, USA); pertussis toxin (Research Biochemicals International, Natick, MA, USA); U73122 (1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione) and LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one) (Biomol Research, Plymouth Meeting, PA, USA); BAPTA-AM (1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester) (Molecular Probes, Eugene, OR, USA); GF109203X (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide), Gö6976 (13-cyanoethyl-12-methyl-6,7,12,13-tetrahydroindolo[2,3*a*]-pyrrolo[3,4-*c*]carbazole-7-one), Gö6983 (2-[1-(3-dimethylaminopropyl)-5-methoxy-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide) and wortmannin (Calbiochem-Novabiochem, San Diego, CA, USA); enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Buchinghamshire, UK); polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA); rabbit polyclonal antibodies to p38 MAPK and to phospho-p38 MAPK (New England Biolabs, Beverly, MA, USA). Inhibitors were dissolved in dimethylsulfoxide (DMSO). No significant effects of vehicle were observed at the final concentrations of DMSO $\leq$ 0.3%.

2.2. Isolation of neutrophils

Rat blood was collected from the abdominal aorta and the neutrophils were purified by dextran sedimentation, centrifugation through Ficoll-Hypaque, and hypotonic lysis of erythrocytes (Wang et al., 1995). Purified neutrophils containing $>$ 95% viable cells were resuspended in HBSS containing 10 mM HEPES, pH 7.4, and 4 mM $NaHCO_3$, and kept in an ice-bath before use.

2.3. Immunoblotting analysis

Cells were preincubated with test drugs at 37°C for the indicated time before stimulation with 0.1 μM fMLP plus 5 μg/ml dihydrocytochalasin B. One minute later, reactions were quenched by addition of stopping solution (20% trichloroacetic acid, 1 mM phenylmethylsulphonyl fluoride, 2 mM *N*-ethylmaleimide, 10 mM NaF, 2 mM Na_3VO_4, 2 mM *p*-nitrophenyl phosphate, 7 μg/ml of leupeptin and pepstatin). Proteins were electrophoresed on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels (PAGE) and then transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat dried milk in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20) and probed with rabbit polyclonal anti-phospho-p38 MAPK antibodies (1:2500 dilution in TBST buffer with 1% bovine serum albumin) (Wang et al., 1999b). To standardize protein loading in each lane, blots were stripped by incubation in Tris buffer (62.5 mM Tris–HCl, pH 6.8, 100 mM 2-mercaptoethanol, 2% SDS) at 50°C for 30 min. Then, the blots were washed extensively followed by reprobing with antibody against p38 MAPK (1:1000 dilution in TBST buffer with 1% non-fat dried milk). Detection was done with enhanced chemiluminescence reagent (Amersham). Quantification was by densitometry.

2.4. Statistical analysis

Statistical analyses were performed using the Bonferroni *t*-test method after analysis of variance. A P value of less than 0.05 was considered significant for all tests. Analysis of the regression line was used to calculate the IC_{50} values. Data are expressed as means ± S.E.M.

3. Results

3.1. Roles of G protein and phosphatidylinositol 3-kinase in p38 MAPK activation by fMLP

Exposure of rat neutrophils to 0.1 μM fMLP plus 5 μg/ml of dihydrocytochalasin B for 1 min induced the activation of p38 MAPK, as shown by immunoblot analysis with anti-phospho-p38 MAPK antibodies. Pretreatment of rat neutrophils for 2 h with 1 μg/ml of pertussis toxin, which ribosylates the α subunit of $G_{i/o}$, significantly attenuated (about 52% inhibition, $P < 0.01$) the phosphorylation of p38 MAPK (Fig. 1A,B). Under the same conditions, there was a more than 80% inhibition of fMLP-stimulated superoxide anion generation and $[Ca^{2+}]_i$ changes (Wang and Chen, 1998; Wang et al., 1999a). Two distinct pharmacologic inhibitors of phosphatidylinositol 3-kinase, wortmannin and LY294002 (Powis et al., 1994; Vlahos et al., 1994), were used to examine the role of phosphatidylinositol 3-kinase in p38 MAPK activation by fMLP in rat neutrophils. Neither wortmannin (10 μM) nor LY294002 (10 μM), at the concentrations which significantly attenuated the phosphorylation of ERK by fMLP in rat neutrophils (Chang and Wang, 1999), affected basal (data not shown) or fMLP-stimulated p38 MAPK phosphorylation (Fig. 1A,B).

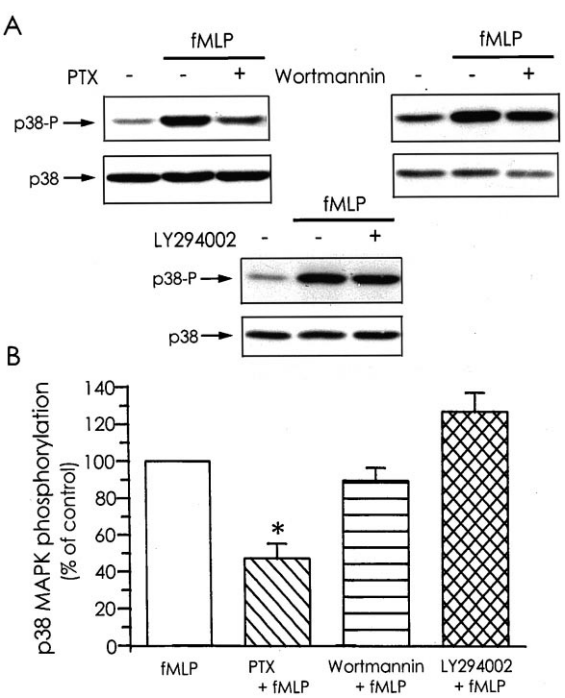

Fig. 1. Effects of pertussis toxin, wortmannin and LY294002 on fMLP-stimulated p38 MAPK phosphorylation. Cells were preincubated for 2 h at 37°C with 1 μg/ml of pertussis toxin (PTX), or for 10 min with vehicle, 10 μM wortmannin or 10 μM LY294002, either before stimulation with 0.1 μM fMLP plus 5 μg/ml dihydrocytochalasin B or without stimulation. One minute later, phosphorylation of p38 MAPK was detected by immunoblot analysis using anti-phospho-p38 MAPK antibodies. The total p38 MAPK was measured by reprobing the above blots with anti-p38 MAPK antibody. (A) represents the results of Western blot analysis, and (B) shows means ± S.E.M. for p38 MAPK phosphorylation in three independent experiments. * $P < 0.01$ compared with fMLP/dihydrocytochalasin B alone (as control).

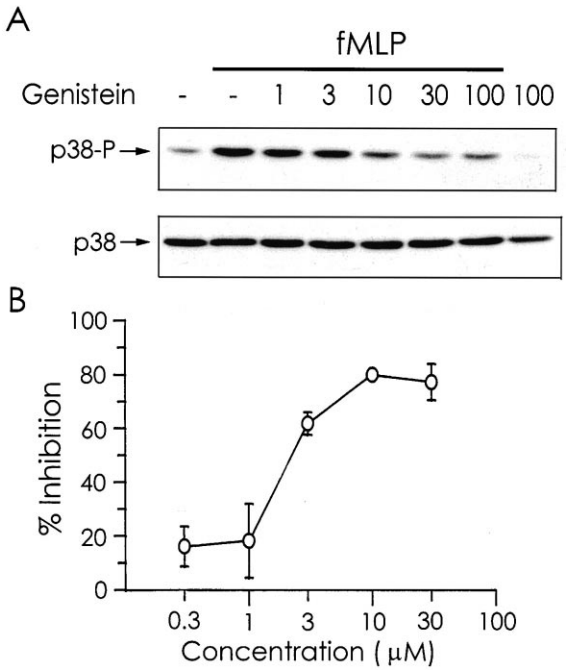

Fig. 2. Effect of genistein on fMLP-stimulated p38 MAPK phosphorylation. Cells were pretreated with the vehicle or indicated concentrations (μM) of genistein for 30 min at 37°C, either before stimulation with 0.1 μM fMLP plus 5 μg/ml dihydrocytochalasin B or without stimulation. One minute later, phosphorylation of p38 MAPK was detected by immunoblot analysis using anti-phospho-p38 MAPK antibodies. The total p38 MAPK was measured by reprobing the above blot with anti-p38 MAPK antibody. (A) represents the results of Western blot analysis, and (B) shows means ± S.E.M. for % inhibition of p38 MAPK phosphorylation in three to four separate experiments.

3.2. Effect of genistein on p38 MAPK activation

Pretreatment of neutrophils with a tyrosine kinase inhibitor, genistein (Akiyama et al., 1987), for 30 min resulted in the inhibition of p38 MAPK phosphorylation by fMLP in a concentration-dependent manner (Fig. 2A,B). Significant inhibition was observed at concentrations of genistein ≥ 3 μM. The IC_{50} value was about 2.9 ± 1.0 μM.

3.3. Role of phospholipase C / Ca^{2+} in p38 MAPK activation by fMLP

The role of cellular free Ca^{2+} in p38 MAPK activation by fMLP was evaluated. Neutrophils were preincubated for 1 h with 10 μM BAPTA-AM, an intracellular Ca^{2+} chelator (Kessels et al., 1991), to prevent the fMLP-induced

$[Ca^{2+}]_i$ transient measured in the fura-2 fluorescence ratio mode (data not shown). This treatment greatly attenuated (about 98% inhibition, $P < 0.01$) the phosphorylation of p38 MAPK by fMLP (Fig. 3A,B). In addition, pretreatment of cells with 10 μM U73122, a phospholipase C inhibitor (Smith et al., 1990), significantly reduced (about 52% inhibition, $P < 0.01$) fMLP-stimulated p38 MAPK phosphorylation (Fig. 3A,B).

3.4. Role of protein kinase c in p38 MAPK activation by fMLP

To determine whether the intracellular signaling pathway leading to p38 MAPK activation by fMLP involves protein kinase C, we tested the effects of three distinct pharmacologic inhibitors of protein kinase C, Gö6976, Gö6983, and GF109203X (Martiny-Baron et al., 1993; Gschwendt et al., 1996). Pretreatment of cells with Gö6976 effectively attenuated p38 MAPK phosphorylation in a concentration-dependent manner (Fig. 4B,D). Significant inhibition was observed at concentrations of Gö6976 ≥ 3 μM. The IC_{50} value was about 5.2 ± 1.6 μM. However,

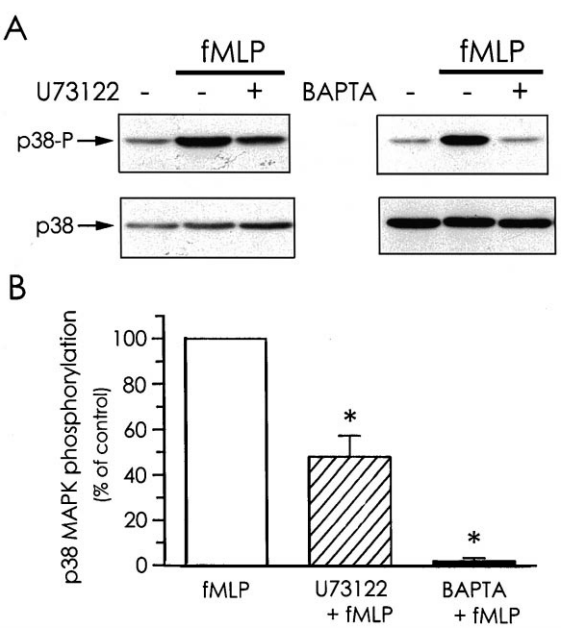

Fig. 3. Effects of U73122 and BAPTA on p38 MAPK activation by fMLP. Cells were stimulated with 0.1 μM fMLP plus 5 μg/ml dihydrocytochalasin B or its diluent for 1 min. Where indicated, the cells were pretreated for 10 min at 37°C with the vehicle or 10 μM U73122, or were loaded with BAPTA by incubation with 10 μM BAPTA-AM for 1 h. After stimulation, phosphorylation of p38 MAPK was detected by immunoblot analysis using anti-phospho-p38 MAPK antibodies. The total p38 MAPK was measured by reprobing the above blots with anti-p38 MAPK antibody. (A) represents the results of Western blot analysis, and (B) shows means ± S.E.M. for p38 MAPK phosphorylation in three independent experiments. $^*P < 0.01$ compared with fMLP/dihydrocytochalasin B alone (as control).

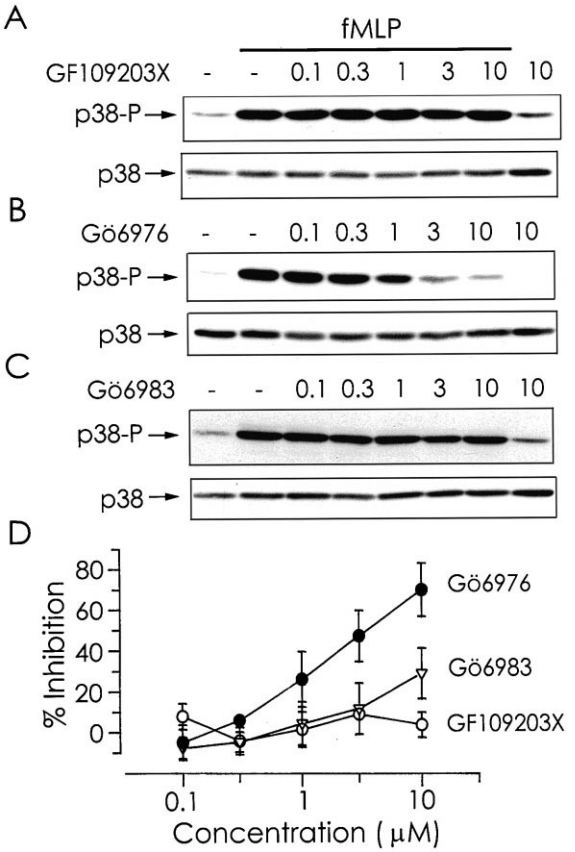

Fig. 4. The role of protein kinase C in fMLP-stimulated p38 MAPK phosphorylation. Cells were pretreated with the vehicle or indicated concentrations (μM) of GF109203X (A), Gö6976 (B), or Gö6983 (C) for 10 min at 37°C, either before stimulation with 0.1 μM fMLP plus 5 μg/ml dihydrocytochalasin B or without stimulation. One minute later, phosphorylation of p38 MAPK was detected by immunoblot analysis using anti-phospho-p38 MAPK antibodies. The total p38 MAPK was measured by reprobing the above blots with anti-p38 MAPK antibody. (A–C) represent the results of Western blot analysis, and (D) shows means ± S.E.M. for % inhibition of p38 MAPK phosphorylation in three to four separate experiments.

GF109203X and Gö6983 (up to 10 μM) had no significant effect on fMLP-induced responses (Fig. 4A,C,D).

4. Discussion

Immunoblot analysis with anti-phospho-p38 MAPK antibodies showed that there was a rapid induction of p38 MAPK activation by fMLP in rat neutrophils. Our findings are in agreement with previous reports on human neutrophils (Krump et al., 1997). Heterotrimeric G protein-coupled receptors are able to induce a variety of responses including cell proliferation, differentiation, and activation of several intracellular kinase cascades. It has been reported that p38 MAPK can be activated by βγ subunits of G protein in a pathway involving RhoA, Rac1, and Cdc42 (Lopez-Ilasaca, 1998). Formyl peptide receptor couples to pertussis toxin-sensitive G proteins (G_{i2} and G_{i3}) to acti-

vate chemotaxis, exocytosis, and superoxide anion generation in neutrophils (Lad et al., 1986; Lund-Johansen and Olweus, 1992; Nath et al., 1994). The results that pertussis toxin greatly attenuated the phosphorylation of p38 MAPK in rat neutrophils are in contrast with those for human neutrophils (Nick et al., 1997). This variation may arise from the difference in species.

Activation of phosphatidylinositol 3-kinase by G protein-coupled receptors in neutrophils has been associated with functional events (Arcaro and Wymann, 1993). However, the downstream events have not yet been defined. The results that neither wortmannin nor LY294002, two distinct phosphatidylinositol 3-kinase inhibitors (Powis et al., 1994; Vlahos et al., 1994), affected basal or fMLP-stimulated p38 MAPK phosphorylation are in contrast with a recent report which showed that wortmannin reduces fMLP-stimulated p38 MAPK activity and that LY294002 alone stimulates p38 MAPK activity in human neutrophils (Krump et al., 1997). The concentrations of phosphatidylinositol 3-kinase inhibitors used in this study were high enough to significantly attenuate the phosphorylation of ERK by fMLP in rat neutrophils (Chang and Wang, 1999). The stimulatory effect of LY294002 has been assumed to be independent of its inhibition of phosphatidylinositol 3-kinase but stress-related because of the comparatively high (100 μM) concentrations of LY294002 needed (Krump et al., 1997). The reasons for this discrepancy are not clear but might be attributed to differences in species or assay methods. The present results suggest that phosphatidylinositol 3-kinase does not participate in fMLP-stimulated p38 MAPK phosphorylation in rat neutrophils.

A previous study indicated that tyrosine kinase is involved in ERK activation by formyl peptide (Luttrell et al., 1996). However, the role of tyrosine kinase in p38 MAPK activation by fMLP in neutrophils is not clear. Genistein, a tyrosine kinase inhibitor (Akiyama et al., 1987), has been reported to inhibit tyrosine phosphorylation of p38 MAPK in rabbit platelets (Rho et al., 1997) and p38 MAPK activity in human neutrophils (McLeish et al., 1998). In this study, genistein reduced the phosphorylation of p38 MAPK by fMLP, suggesting that the nonreceptor tyrosine kinases are required for p38 MAPK activation in rat neutrophils.

Stimulation of neutrophils by formyl peptide activates phospholipase C with the formation of inositol trisphosphate and diacylglycerol, which increase $[Ca^{2+}]_i$ and protein kinase C activity, respectively (Berridge, 1987). Previous studies have demonstrated that exposure of human neutrophils to a Ca^{2+}-ionophore, ionomycin, induces p38 MAPK activation (Zu et al., 1998). It is plausible that Ca^{2+} plays a role in p38 MAPK activation by fMLP. The finding that cells preloaded with BAPTA had greatly attenuated phosphorylation of p38 MAPK by fMLP confirms the results obtained with human neutrophils (Krump et al., 1997). In addition, the observation that a phospholipase C inhibitor U73122 (Smith et al., 1990), which prevents $[Ca^{2+}]_i$ elevation, reduced the fMLP-stimulated p38 MAPK phosphorylation suggests that the phosphorylation of p38 MAPK by fMLP is dependent on the phospholipase C/Ca^{2+} pathway in rat neutrophils.

Protein kinase Cs form a family of phospholipid-dependent serine/threonine protein kinases that play a central role in signal transduction and are involved in the control of numerous cellular processes. Exposure of human neutrophils to phorbol 12-myristate 13-acetate (PMA) stimulates the activation of p38 MAPK, and a protein kinase C inhibitor GF109203X (Gschwendt et al., 1996) decreases PMA-induced responses (Zu et al., 1998). Rat neutrophils express protein kinase C isoforms α, β, δ, ε, θ, μ, ι/λ, and ζ, although λ and ζ are barely detected (Tsao and Wang, 1997). To determine the role of protein kinase C in p38 MAPK activation by fMLP, we used three distinct protein kinase C inhibitors. Gö6976 preferentially inhibits protein kinase C isoforms α, β, and μ, Gö6983 inhibits isoforms α, β, γ, δ, and ζ, and GF109203X inhibits isoforms α, β, δ, ε, ζ, and μ in an in vitro kinase assay (Martiny-Baron et al., 1993; Gschwendt et al., 1996). Pretreatment of cells with Gö6976, but not GF109203X or Gö6983, effectively attenuated p38 MAPK phosphorylation. A previous report has demonstrated that GF109203X partially inhibits tyrosine phosphorylation of p38 MAPK upon fMLP stimulation in human neutrophils (Krump et al., 1997). It is unclear at present whether differences in species or experimental conditions are responsible for the different results. The inhibitory effect of Gö6976 was significantly more pronounced than that of Gö6983 and GF109203X on fMLP-stimulated ERK phosphorylation in rat neutrophils (Chang and Wang, 1999). Alternatively, the Ca^{2+}-dependent protein kinase C probably act as the major isoform in the regulation of fMLP-stimulated p38 MAPK phosphorylation in rat neutrophils. Whether inhibition of novel and/or atypical protein kinase C isoforms eliminates the inhibitory effect on p38 MAPK phosphorylation awaits further investigation. There are at least four members of the p38 MAPK family, including α, β, γ, and δ forms (Li et al., 1996; Jiang et al., 1997), in which the γ and δ forms of p38 MAPK are regulated by protein kinase C (Kumar et al., 1997). It remains to be determined which subtypes of p38 MAPK are activated by fMLP in neutrophils.

In conclusion, the upstream regulators used by fMLP to activate p38 MAPK phosphorylation in rat neutrophils include $G_{i/o}$ protein, nonreceptor tyrosine kinase, phospholipase C/Ca^{2+}, and probably Ca^{2+}-dependent protein kinase C.

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