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Pharmacological analysis of protein kinases responsible for chemotaxis of rat peritoneal neutrophils

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Abstract

Several types of kinase inhibitors were used to investigate the possible signaling pathways leading to the chemotaxis of rat peritoneal neutrophils toward macrophage inflammatory protein-2, cytokine-induced neutrophil chemoattractant-1, and platelet-activating factor. The chemotaxis and shape changes induced by each of these chemoattractants were strongly inhibited by a tyrosine kinase inhibitor (herbimycin A) and protein kinase C inhibitors (H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride) and calphostin C). The formation of phosphatidyl 3,4,5-triphosphate in chemoattractant-stimulated neutrophils was completely inhibited by 100 nM of wortmannin, an inhibitor of phosphatidylinositol 3-kinase, whereas the chemotaxis toward each of these chemoattractants was partially inhibited (50% inhibition). The mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK-1) inhibitor PD 98059 did not inhibit the neutrophil chemotaxis. These findings suggest that the activation of tyrosine kinase and protein kinase C strongly participates in neutrophil chemotaxis and that the activation of phosphatidylinositol 3-kinase is partially involved, but that the activation of mitogen-activated protein kinase is not involved in neutrophil chemotaxis. The cross-linking of the signaling pathways for chemotaxis toward each chemoattractant was also examined.

Keywords: Chemotaxis; Neutrophil; Macrophage inflammatory protein-2; Cytokine-induced neutrophil chemoattractant-1; PAF (platelet-activating factor); Tyrosine kinase; Protein kinase C; MAP kinase; PI 3-kinase

1. Introduction

The recruitment of neutrophils into inflammatory loci plays a key role in host defense and also contributes to the pathogenesis of inflammation. It has been found that the activated complement components C5a (Fernandez and Hugli, 1978), the lipid chemoattractants platelet-activating factor (PAF) (Watanabe et al., 1990, 1994a,b) and leukotriene B_4 (Palmer et al., 1980), and the CXC chemokines interleukin-8 (Koch et al., 1991), growth-related gene product (Koch et al., 1995), macrophage inflammatory protein-2 (MIP-2) (Xiao et al., 1997) and cytokine-induced neutrophil chemoattractants (Shibata et al., 1995) are responsible for the activation of leukocytes and serve as chemoattractants to induce neutrophil migration.

These chemoattractants activate neutrophils through their respective G protein-coupled seven-transmembrane receptors (Honda et al., 1991; Nakamura et al., 1991; Buhl et al., 1994; Lee et al., 1995; Dunstan et al., 1996; Knall et al., 1996). Activation of G protein-coupled receptors on the surface of neutrophils by chemoattractants induces a series of biological functions including chemotaxis, respiratory burst and phagocytosis (Ben-Baruch et al., 1995; Bokoch, 1995; Frevert et al., 1995). The most well-defined classic signal transduction pathway through G protein-coupled receptors is the activation of phospholipase C, resulting in the release of diacylglycerol and inositol 1,4,5-triphosphate, which act as second messengers to activate protein kinase C and elevate cytosolic Ca^{2+}, respectively (Lu et al., 1993). Although it is widely accepted that PKCs play an important role in neutrophil activation, little is known about their intracellular mechanisms for regulating the function of neutrophils. The activation of mitogen-activated protein kinase (MAP kinase) pathways in human neutrophils by C5a, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and interleukin-8 has been suggested (Buhl et al., 1994; Worthen et al., 1994; Knall et al., 1996, 1997). In addition, the expression and activation of phos-

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phatidylinositol 3-kinase (PI 3-kinase) in human neutrophils by fMLP and interleukin-8 have been demonstrated (Vlahos et al., 1995; Knall et al., 1997). Tyrosine kinase and phospholipase D appear to be required in the respiratory burst (Morel et al., 1991; Yasui et al., 1994). However, the intracellular signaling pathways linking activated chemoattractant receptors to neutrophil chemotaxis are not well characterized.

MIP-2 and cytokine-induced neutrophil chemoattractant-1 have been described as rat homologues to human growth-related gene product (Tekamp-Olson et al., 1990) and contribute to neutrophil infiltration in several experimental models of inflammatory diseases (Feng et al., 1995; Frevert et al., 1995; Seebach et al., 1995; Schmal et al., 1996; Xiao et al., 1997). Two rat G protein-coupled receptors (CXC receptor 1 and CXC receptor 2), 70% identical to the human interleukin-8 A and B receptor subtypes, have been cloned (Dunstan et al., 1996), but only CXC receptor 2 is expressed in rat neutrophils. In previous studies, we demonstrated that MIP-2 plays an important role in neutrophil infiltration in the late phase of rat allergic inflammation (Tanabe et al., 1995; Xiao et al., 1997). In contrast, in the early phase of allergic inflammation in rats, lipophilic factors are involved in the recruitment of neutrophils (Omata et al., 1990). One candidate lipid chemoattractant is PAF (Watanabe et al., 1994a,b). The present study was performed to pharmacologically identify the types of kinase that are responsible for neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF, each of which has different receptors.

2. Materials and methods

2.1. Reagents

Herbimycin A was purchased from Funakoshi, Tokyo, Japan. PD 98059 (2′-amino-3′-methoxyflavone) was from Daiichi Pure Chemicals, Tokyo, Japan. H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride) was obtained from Seikagaku Kogyo, Tokyo, Japan. Calphostin C and LY 294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one) were purchased from Calbiochem-Novachem, La Jolla, CA, USA. Wortmannin was a gift from Eisai, Tsukuba, Japan. PAF and bovine serum albumin were from Sigma, St. Louis, MO, USA. [^{32}P]Orthophosphate (in aqueous solution) was from Daiichi Pure Chemicals. RPMI 1640 was purchased from Nissui Seiyaku, Tokyo, Japan. Rat recombinant MIP-2 was produced as described previously (Xiao et al., 1997). Cytokine-induced neutrophil chemoattractant-1 was obtained from the Peptide Institute, Osaka, Japan. H-7 was dissolved in water, and the other kinase inhibitors used were dissolved in dimethylsulfoxide. An aliquot of each solution was added to the medium, and the final concentration of the vehicle in the medium was adjusted to 0.1% (v/v). The control medium contained the same amount of the vehicle.

2.2. Preparation of rat peritoneal neutrophils

Male Sprague–Dawley strain rats, specific pathogen-free (Charles River Japan, Kanagawa, Japan) were used. The rats were treated in accordance with procedures approved by the Animal Ethics Committee of the Faculty of Pharmaceutical Sciences, Tohoku University, Sendai, Japan. Rat peritoneal neutrophils were harvested 16 h after intraperitoneal injection of 40 ml of Ca^{2+}-free Krebs–Ringer solution containing 1% casein (casein from milk, vitamin-free, Wako, Osaka, Japan) which had been sterilized by autoclaving at 120°C for 15 min. The peritoneal cells were washed twice with Ca^{2+}-free phosphate-buffered saline (PBS, pH 7.4) and finally suspended in RPMI 1640 medium containing 0.25% (w/v) bovine serum albumin at 1.6×10^7 cells/ml.

2.3. Treatment of rat peritoneal neutrophils with kinase inhibitors

Rat peritoneal neutrophils (0.8×10^7 cells/ml) were preincubated in RPMI 1640 medium containing 0.25% (w/v) bovine serum albumin and the indicated concentrations of kinase inhibitors at 37°C for 30 min (herbimycin A and PD 98059) or 10 min (other inhibitors). After this treatment, the viability of the neutrophils was examined by the Trypan blue exclusion test; no cytotoxic effect was observed.

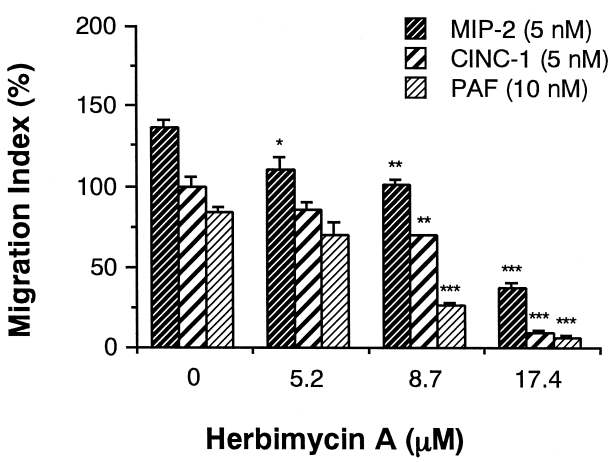

Fig. 1. Effects of herbimycin A on neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF. Rat peritoneal neutrophils were preincubated with the indicated concentrations of herbimycin A at 37°C for 30 min. The chemotactic activity of the pretreated neutrophils toward MIP-2 (5 nM), cytokine-induced neutrophil chemoattractant-1 (CINC-1) (5 nM) and PAF (10 nM) was determined. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. corresponding control.

2.4. Measurement of neutrophil chemotactic activity

The chemotactic activity of pretreated neutrophils toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF was determined using modified Boyden chambers as described previously (Watanabe et al., 1994a,b).

2.5. Western blot analysis of MAP kinase phosphorylation

After the incubation of neutrophils at 37°C for 0.25, 0.5, 1, 3, 5 or 10 min with MIP-2 (5 nM), the cells were lysed in ice-cold lysis buffer (20 mM HEPES, pH 7.3, 1% (v/v) Triton X-100, 1 mM EDTA, 50 mM NaF, 2.5 mM *p*-nitrophenylphosphate, 1 mM Na_3VO_4, 10 μg/ml leupeptin and 10% (v/v) glycerol). Proteins in the cell lysate were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The membrane was blocked in Block Ace (Wako) at room temperature for 1 h and then incubated overnight at 4°C with anti-rat MAP kinase R2 (Upstate Biotechnology, Lake Placid, NY, USA), which was diluted 1/500 in 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween 20 (TBST) containing 1% BSA. After extensive washes, the membrane was incubated with anti-rabbit immunoglobulin G alkaline phosphatase-conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) (1/5000 dilution) for 1 h at room temperature. After three washes in TBST, the alkaline phosphatase activity was developed by using a Nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate system (Promega, Madison, WI, USA).

2.6. Determination of total cellular phosphatidylinositol 3,4,5-triphosphate (PIP_3)

PIP_3 production by neutrophils was determined according to the method described by Okada et al. (1994). Briefly, rat peritoneal neutrophils (10^8 cells/ml) were incubated with the labeling buffer (10 mM HEPES/NaOH, pH 7.4, 136 mM NaCl, 4.9 mM KCl, and 5.5 mM glucose) containing 9.25 MBq of [^{32}P]orthophosphate at 37°C for 1 h, washed, and resuspended at 1.7×10^7 cells/ml in the

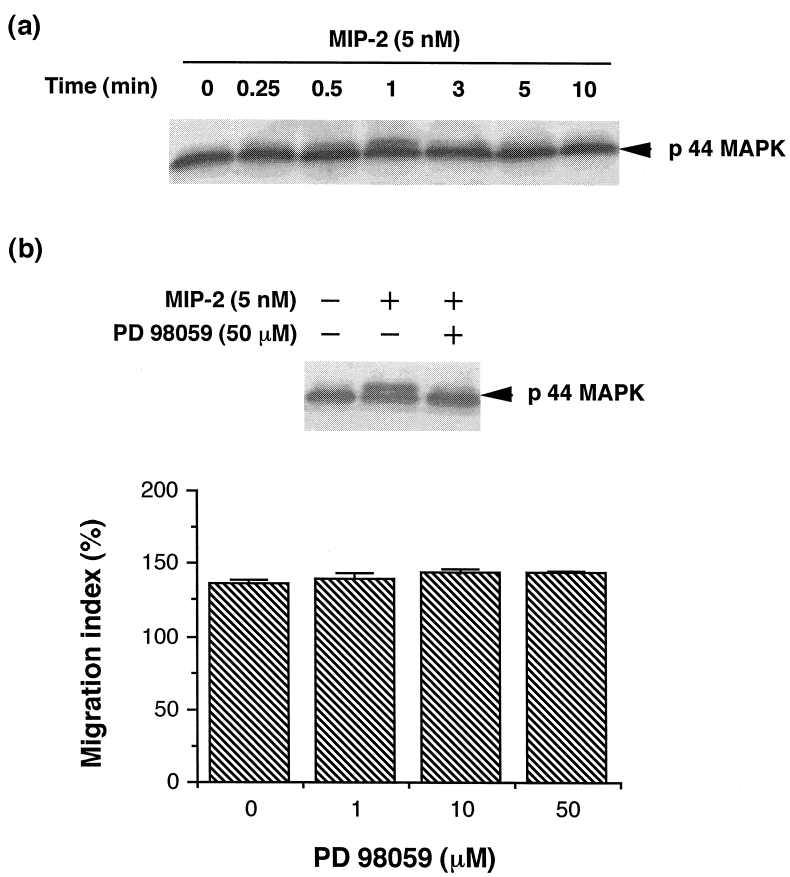

Fig. 2. Activation of MAP kinase by MIP-2 and effects of PD 98059 on neutrophil chemotaxis toward MIP-2. (a) Rat peritoneal neutrophils were incubated with 5 nM of MIP-2 for the periods indicated. The phosphorylation of p44 MAP kinase was analyzed by immunoblotting. (b) Rat peritoneal neutrophils were preincubated with 50 μM of PD 98059 at 37°C for 30 min. The cells were then stimulated with 5 nM of MIP-2 for 1 min. The effect of PD 98059 on the phosphorylation of p44 MAP kinase was analyzed by immunoblotting, and neutrophil chemotaxis toward MIP-2 was determined. Values are the means from four samples with S.E.M. shown by vertical bars.

same buffer supplemented with 1 mM $CaCl_2$. Aliquots of the labelled cells (150 μl) were incubated at 37°C for 10 min with or without wortmannin, and further incubated for the periods indicated in the presence of 100 nM of MIP-2, cytokine-induced neutrophil chemoattractant-1 or PAF. The reaction was stopped by the addition of 1.55 ml of chloroform/methanol/8% $HClO_4$ (50:100:5, v/v), and the mixture was mixed well. Then, 0.5 ml each of chloroform and 8% $HClO_4$ were added, and the mixture was centrifuged at $500 \times g$ at room temperature for 3 min to separate the organic phase, which was then washed with chloroform-saturated 1% $HClO_4$. The organic phase was dried under N_2 gas, dissolved in 20 μl of chloroform, spotted on a TLC plate (Silica Gel-60, Merck, Darmstadt, Germany) which had been impregnated with 1.2% potassium oxalate, and developed in chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14, v/v). The plate was then dried and the radioactivity was visualized by using Kodak Scientific Imaging Films (Eastman Kodak, Rochester, NY, USA).

2.7. Scanning electron microscopy analysis of chemoattractant-stimulated neutrophils

Neutrophils (1×10^7 cells/ml) were preincubated in the presence or absence of various kinase inhibitors and then stimulated by MIP-2 (5 nM), cytokine-induced neutrophil chemoattractant-1 (5 nM) or PAF (10 nM) at 37°C for 3 min. The cells were fixed overnight in 2.5% glutaraldehyde (Electron Microscopy Sciences, Washington, PA, USA). After dehydration in a series of ethanol and 2-methyl-2-propanol solutions, the cells were allowed to adhere to micro-cover glasses which had been coated with poly-L-lysine hydrobromide (Wako) prior to use. Morphological changes were examined using a scanning electron microscope (S-3200, Hitachi, lbaragi, Japan).

2.8. Homologous and heterologous desensitization of neutrophil chemotaxis

Neutrophils were preincubated with 30 nM of MIP-2, cytokine-induced neutrophil chemoattractant-1 or PAF at 37°C for 20 min. The cells were washed twice with ice-cold RPMI 1640 medium containing 0.25% (w/v) bovine serum albumin, and resuspended at a concentration of 0.8×10^7 cells/ml. The chemotaxis of the pretreated neutrophils toward MIP-2 (5 nM), cytokine-induced neutrophil chemoattractant-1 (5 nM) or PAF (10 nM) was examined using Boyden chambers, which were incubated at 37°C for 40 min under 5% CO_2.

2.9. Statistical analysis

The results are presented as means ± S.E.M. from at least four samples. Comparisons were performed by Student's unpaired *t*-test.

3. Results

3.1. Effects of tyrosine kinase inhibitors on neutrophil chemotaxis

The pretreatment of neutrophils with herbimycin A, a tyrosine kinase inhibitor, for 30 min at 37°C inhibited chemotaxis toward MIP-2 (5 nM), cytokine-induced neutrophil chemoattractant-1 (5 nM), and PAF (10 nM) in a concentration-dependent manner (Fig. 1). At 17.4 μM of herbimycin A, neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF was inhibited 73%, 91% and 90%, respectively. These findings suggested that tyrosine kinase plays a crucial role in

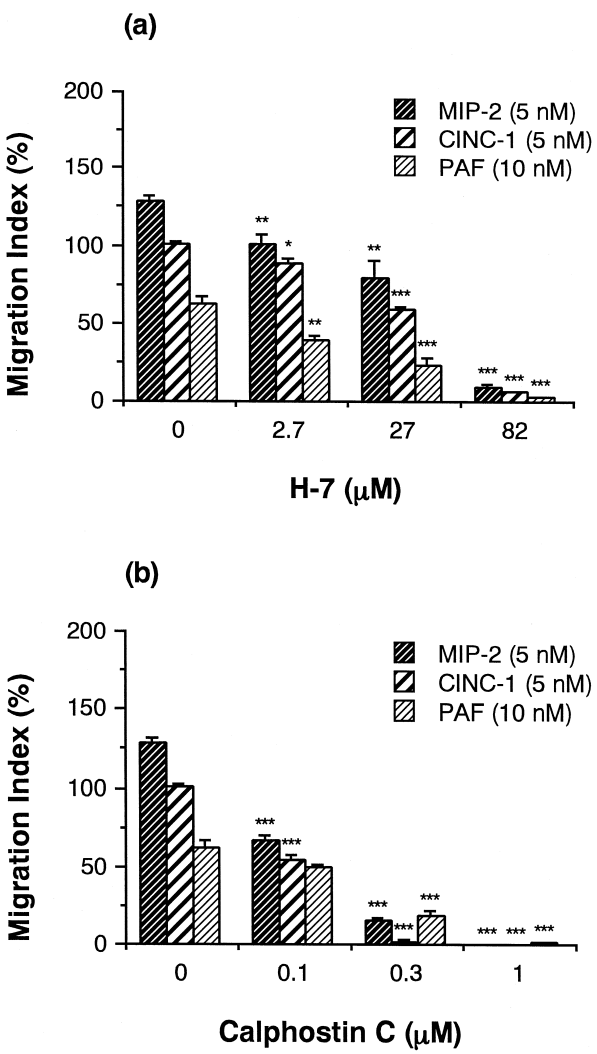

Fig. 3. Effects of H-7 and calphostin C on neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF. Rat peritoneal neutrophils were preincubated with the indicated concentrations of (a) H-7 and (b) calphostin C at 37°C for 10 min. The chemotactic activity of the pretreated neutrophils toward MIP-2 (5 nM), cytokine-induced neutrophil chemoattractant-1 (CINC-1) (5 nM) and PAF (10 nM) was determined. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $* P < 0.05$, $** P < 0.01$, $*** P < 0.001$ vs. corresponding control.

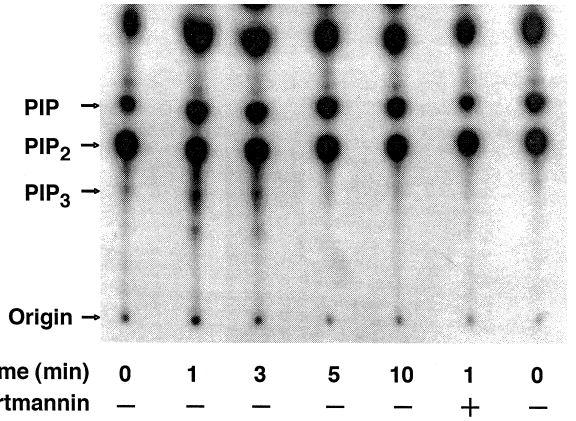

Fig. 4. Inhibition of MIP-2-induced production of PIP_3 by wortmannin. ^{32}P-Labeled rat peritoneal neutrophils were incubated with (+) or without (−) 100 nM wortmannin at 37°C for 10 min and stimulated with 100 nM MIP-2 for the periods indicated. Phospholipids were extracted and separated on an oxalate-impregnated TLC plate as described in Section 2.6. An autoradiogram of the TLC plate is shown. PIP, phosphatidylinositol 4-phosphate; PIP_2, phophatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-triphosphate.

neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF.

3.2. Effects of the mitogen-activated protein kinase / extracellular signal-regulated kinase kinase (MEK-1) / inhibitor PD 98059 on neutrophil chemotaxis

The treatment of rat neutrophils with MIP-2 (5 nM) induced a time-dependent activation of p44 MAP kinase. The maximal mobility shift of p44 MAP kinase was observed at 1 min and returned to near the basal level at 10 min (Fig. 2a). We therefore examined the effects of PD 98059, a selective inhibitor of MEK-1, to see if MAP kinase is involved in neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF. As shown in Fig. 2b, PD 98059 at concentrations of 1–50 μM did not inhibit neutrophil chemotaxis toward MIP-2 (5 nM), although the MIP-2-induced phosphorylation of MAP kinase in rat neutrophils was completely inhibited by 50 μM of PD 98059. Neutrophil chemotaxis toward cytokine-induced neutrophil chemoattractant-1 (5 nM) and PAF (10 nM) was also unaffected by treatment with PD 98059 (1–50 μM) (data not shown), indicating that the activation of MAP kinase by these chemoattractants does not play a significant role in chemoattractant-induced neutrophil chemotaxis.

3.3. Effects of protein kinase C inhibitors on neutrophil chemotaxis

Rat peritoneal neutrophils were preincubated with the protein kinase C inhibitor, H-7 or calphostin C, for 10 min at 37°C, and the effects of these protein kinase C inhibitors on neutrophil chemotaxis were examined. As shown in Fig. 3a, neutrophil chemotaxis toward MIP-2 (5 nM), cytokine-induced neutrophil chemoattractant-1 (5 nM) and PAF (10 nM) was significantly inhibited by H-7 at 2.7 μM and almost completely inhibited at 82 μM. Because H-7 inhibits not only protein kinase C but also protein kinase A and myosin light chain kinase, we examined the effects of calphostin C, a more specific protein kinase C inhibitor. As shown in Fig. 3b, neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF was inhibited by the pretreatment with calphostin C in a concentration-dependent manner. The same results were

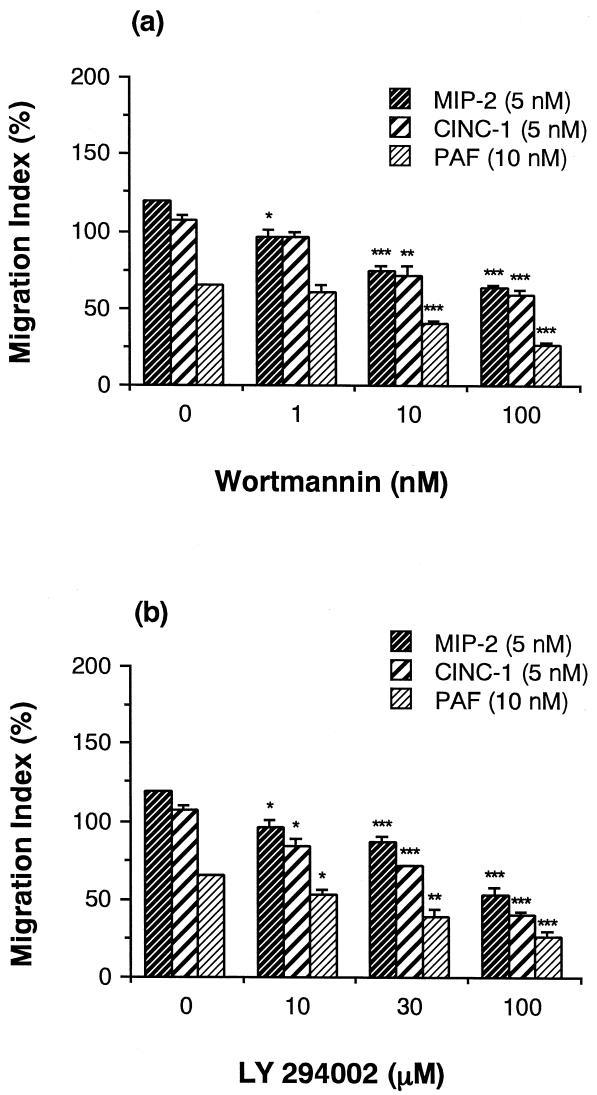

Fig. 5. Effects of wortmannin and LY 294002 on neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF. Rat peritoneal neutrophils were preincubated with the indicated concentrations of (a) wortmannin and (b) LY 294002 at 37°C for 10 min. The chemotactic activity of the pretreated neutrophils toward MIP-2 (5 nM), cytokine-induced neutrophil chemoattractant-1 (CINC-1) (5 nM) and PAF (10 nM) was determined. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $* P < 0.05$, $** P < 0.01$, $*** P < 0.001$ vs. corresponding control.

obtained when the cells were preincubated for 30 min at 37°C with Ro 318425, another protein kinase C inhibitor, at 1–10 μM (data not shown). These findings suggested that the activation of protein kinase C is also required for neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF.

3.4. Effects of PI 3-kinase inhibitors on neutrophil chemotaxis

The participation of PI 3-kinase in neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF was investigated by using the PI 3-kinase inhibitors, wortmannin and LY 294002. Because PIP_3 is an intracellular product of PI 3-kinase, we measured PIP_3 formation in neutrophils after MIP-2, cytokine-induced neutrophil chemoattractant-1 or PAF stimulation. As shown in Fig. 4, stimulation of neutrophils with 100 nM of MIP-2 caused a rapid rise in $[^{32}P]PIP_3$, which reached a maximum at 1–3 min and then declined to the level before stimulation. Pretreatment with 100 nM wortmannin for 10 min completely inhibited $[^{32}P]PIP_3$ production. The same results were obtained by pretreatment with 100 nM wortmannin in neutrophils stimulated with 100 nM cytokine-induced neutrophil chemoattractant-1 or PAF. Although PIP_3 production was completely inhibited by pretreatment with 100 nM wortmannin for 10 min, neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF was only inhibited by 50% (Fig. 5a). LY 294002, a competitive inhibitor of PI 3-kinase, also inhibited neutrophil chemotaxis by 50% at a concentration of 100 μM (Fig. 5b), suggesting that PI 3-kinase is partially involved in neutrophil chemotaxis.

3.5. Chemoattractant-induced morphological changes of neutrophils and their suppression by kinase inhibitors

Neutrophils stimulated with MIP-2 (5 nM), cytokine-induced neutrophil chemoattractant-1 (5 nM) and PAF (10 nM) at 37°C for 3 min showed almost the same extent of polarization of cell shape (Fig. 6). Pretreatment of neutrophils with the tyrosine kinase inhibitor herbimycin A (17.4 μM), the PI 3-kinase inhibitor LY 294002 (100 μM), and the protein kinase C inhibitor calphostin C (1 μM), which significantly inhibited neutrophil chemotaxis (Figs. 1, 3 and 5), inhibited the chemoattractant-induced shape changes (Fig. 6).

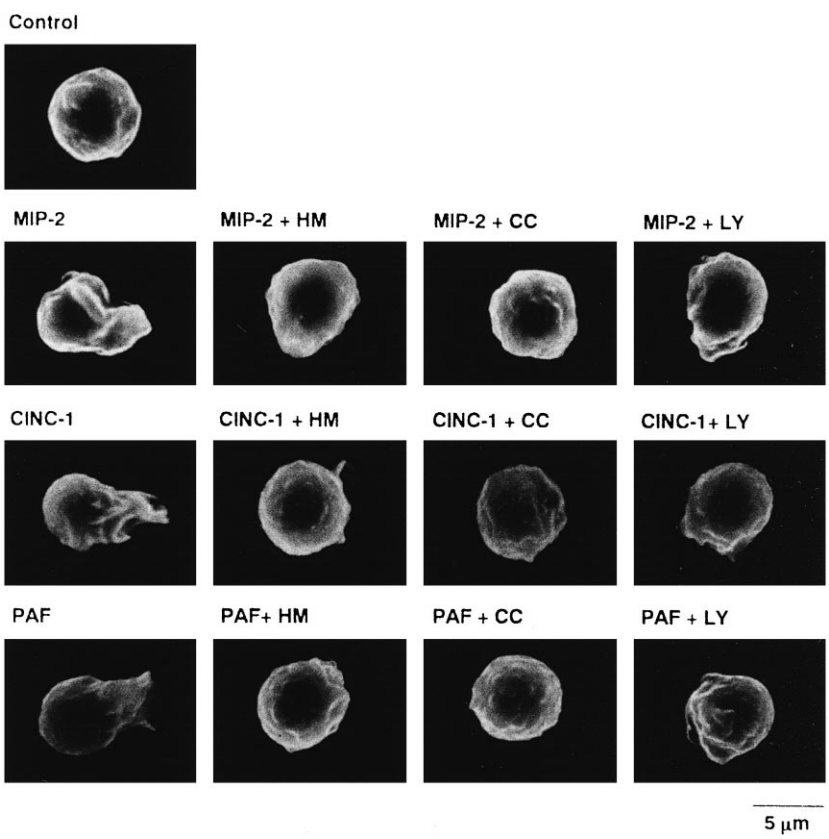

Fig. 6. Effects of kinase inhibitors on the chemoattractant-induced changes in neutrophil morphology. Rat peritoneal neutrophils were preincubated with 17.4 μM of herbimycin A (HM) for 30 min, 1 μM of calphostin C (CC), or 100 μM of LY 294002 (LY) at 37°C for 10 min. The cells were then stimulated with MIP-2 (5 nM), cytokine-induced neutrophil chemoattractant-1 (CINC-1) (5 nM) or PAF (10 nM) for 3 min, fixed with 2.5% glutaraldehyde, and analyzed by scanning electron microscopy. Each representative cell was photographed. A bar indicates 5 μm.

3.6. Homologous and heterologous desensitization of neutrophil chemotaxis

To clarify whether the intracellular signaling pathways for neutrophil migration through the activation of each chemoattractant receptor are different from each other or overlap, homologous or heterologous desensitization to MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF was examined. When neutrophils were preincubated at 37°C for 20 min with MIP-2 (30 nM), chemotaxis toward MIP-2 (5 nM) and cytokine-induced neutrophil chemoattractant-1 (5 nM) was almost completely inhibited, but that toward PAF (10 nM) was partially inhibited (about 50% inhibition) (Fig. 7). In contrast, preincubation with cytokine-induced neutrophil chemoattractant-1 (30 nM) at 37°C for 20 min completely inhibited neutrophil chemotaxis toward cytokine-induced neutrophil chemoattractant-1 (5 nM), but only partially inhibited that toward MIP-2 (5 nM) and PAF (10 nM) (about 51.8% and 47.5% inhibition, respectively). Pretreatment with PAF (30 nM) at 37°C for 20 min did not produce a complete inhibition of neutrophil chemotaxis toward PAF (10 nM) (about 69.6% inhibition), MIP-2 (about 51.1% inhibition) or cytokine-induced neutrophil chemoattractant-1 (about 73.9% inhibition) (Fig. 7). These findings suggested that the signal transduction pathways for neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF are cross-linked to some extent.

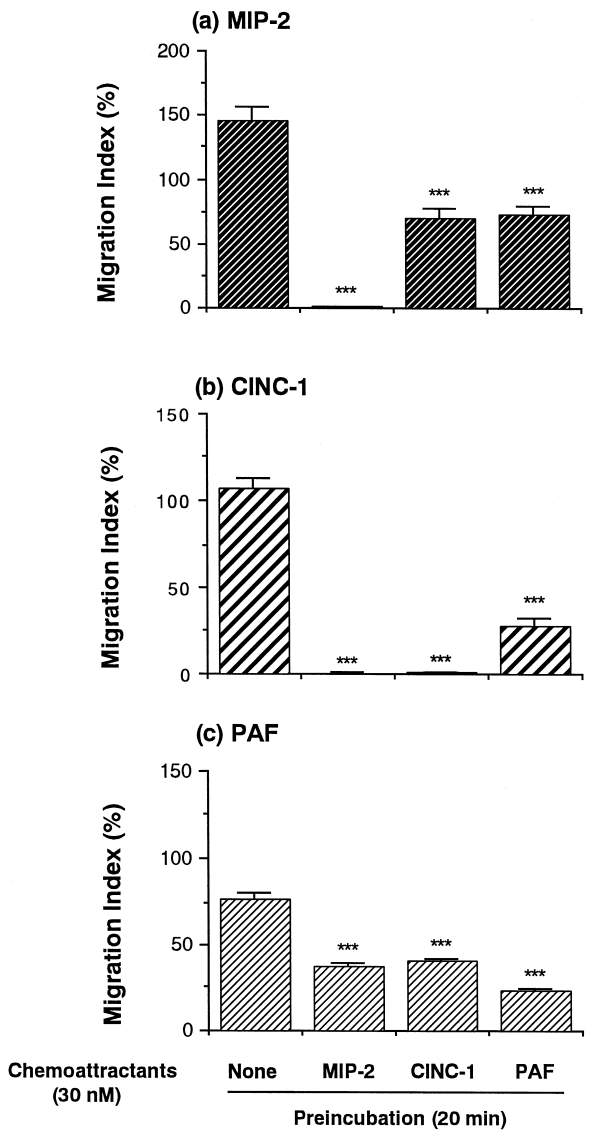

Fig. 7. Homologous and heterologous desensitization of neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF. Rat peritoneal neutrophils were preincubated with 30 nM of MIP-2, cytokine-induced neutrophil chemoattractant-1 (CINC-1) or PAF at 37°C for 20 min. The cells were washed twice with ice-cold phosphate-buffered saline and resuspended in RPMI 1640 medium containing 0.25% bovine serum albumin. The chemotaxis toward (a) MIP-2 (5 nM), (b) cytokine-induced neutrophil chemoattractant-1 (CINC-1) (5 nM) and (c) PAF (10 nM) was then determined. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $***P < 0.001$ vs. corresponding control.

4. Discussion

In human and rabbit neutrophils, stimulation with fMLP, leukotriene B_4, 12-*O*-tetradecanoylphorbol 13-acetate or calcium ionophore A 23187 induces phosphorylation of the tyrosine residues in a series of intracellular proteins (Berkow and Dodson, 1990; Huang et al., 1990). In addition, the activation of tyrosine kinase is implicated in the respiratory burst induced by fMLP or immunoglobulin G in human neutrophils (Kusunoki et al., 1992; Torres et al., 1993). In the present study, we showed that herbimycin A, a tyrosine kinase inhibitor, inhibited rat neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF in a concentration-dependent manner (Fig. 1). Inhibition of fMLP-induced chemotaxis by the tyrosine kinase inhibitors, erbstatin and herbimycin A, has also been reported in human neutrophils (Yasui et al., 1994). It is reported that the 40–42 kDa proteins that are phosphorylated at the tyrosine residues by several stimuli are the activated form of MAP kinases (Grinstein and Furuya, 1992; Torres et al., 1993). In addition, in human neutrophils, Kuroki and O'Flaherty (1997) suggested that interleukin-8-induced neutrophil chemotaxis is dependent on MAP kinase activation, but Knall et al. (1997) reported that it is independent of MAP kinase activation. The present study demonstrated that the treatment of rat neutrophils with MIP-2, cytokine-induced neutrophil chemoattractant-1 or PAF activates MAP kinase. However, the MEK-1 inhibitor PD 98059 did not inhibit the MIP-2-, cytokine-induced neutrophil chemoattractant-1- or PAF-induced chemotaxis, suggesting that neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 or PAF is independent of MAP kinase activa-

tion. Therefore, MAP kinase might not be the target of tyrosine kinases which contribute to neutrophil chemotaxis. It has been reported that the treatment of neutrophils with wortmannin (100 nM) or LY 294002 (100 μM) completely inhibits PI 3-kinase activity in fMLP-stimulated human neutrophils (Arcaro and Wymann, 1993; Okada et al., 1994; Vlahos et al., 1995). In the present study, although MIP-2-, cytokine-induced neutrophil chemoattractant-1-, or PAF-induced PIP_3 production was completely inhibited by 100 nM of wortmannin (Fig. 4), the chemotaxis of rat neutrophils toward MIP-2, cytokine-induced neutrophil chemoattractant-1 or PAF was not completely inhibited (about 50% inhibition) (Fig. 5). These findings suggested that PI 3-kinase is not a major component responsible for neutrophil chemotaxis when compared with the contribution of tyrosine kinase or protein kinase C. In human neutrophils, two types of PI 3-kinases (p85/p110 PI3K and PI3Kγ) are activated by interleukin-8 (Knall et al., 1997). Ptasznik et al. (1996) reported that the activation of human neutrophils by fMLP induces the coupling of the Src-related tyrosine kinase Lyn to the p85/p110 form of PI 3-kinase but not to the G protein βγ subunit-regulated PI3Kγ. Therefore, Lyn is considered to be a candidate tyrosine kinase that is involved in neutrophil chemotaxis through the activation of PI 3-kinase. However, it is also possible that another type of tyrosine kinase that is not influenced by PI 3-kinase is involved in neutrophil chemotaxis. PI 3-kinase activates protein kinase C (Toker et al., 1994) and MAP kinase (Ptasznik et al., 1995; Knall et al., 1996), and PI 3-kinase is upstream or downstream of Rac (Ptasznik et al., 1995; Bokoch et al., 1996; Rodriguez-Viciana et al., 1997), a low-molecular-weight GTP-binding protein of the Rho family that accounts for membrane ruffling (Ridley et al., 1992). In addition, PI 3-kinase binds to α-actinin and regulates cytoskeletal reorganization (Shibasaki et al., 1994). Therefore, it is possible that PI 3-kinase is involved in neutrophil chemotaxis through the activation of certain isoforms of protein kinase C, or low-molecular-weight GTP-binding proteins.

Several isoforms of protein kinase C in human neutrophils have been identified, including three conventional protein kinases C (PKC-α, PKC-βI and PKC-βII), the novel protein kinase C (PKC-δ), and the atypical protein kinase C (PKC-ζ) (Berkow et al., 1987; Benna et al., 1994; Lopez et al., 1995; Kent et al., 1996). However, the involvement of protein kinase C in neutrophil chemotaxis is poorly understood. Yasui et al. (1994) reported that protein kinase C is not involved in fMLP-stimulated neutrophil chemotaxis. In contrast, over-expression of protein kinase C-α in rat capillary endothelial cells enhances the basal and stimulated migration of these cells (Harrington et al., 1997). It is also reported that protein kinase C is implicated in chemoattractant-induced shape changes and locomotion of eosinophils (Gilbert et al., 1994). Because protein kinase C has complex regulatory effects on neutrophil function due to the presence of multiple isoforms, we investigated the effect of protein kinase C inhibitors on neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF using H-7 and calphostin C, which inhibit protein kinase C via different mechanisms (Berkow et al., 1987; Mizuno et al., 1995; Beltman et al., 1996). As shown in Fig. 3, pretreatment of neutrophils with H-7 or calphostin C almost completely inhibited neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF. These findings suggested that protein kinase C is a common signal pathway shared by MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF for neutrophil chemotaxis. Characterization of the protein kinase C isoforms involved in neutrophil chemotaxis is under investigation in our laboratory.

Chemotaxis requires highly developed motile responses involving actin polymerization/depolymerization, adhesion events mediated by integrins, and changes in cell shape. Because the treatment of neutrophils with herbimycin A (a tyrosine kinase inhibitor), LY 294002 (a PI 3-kinase inhibitor) and calphostin C (a protein kinase C inhibitor), which significantly inhibited neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF (Figs. 1, 3 and 5), suppressed the shape polarization of neutrophils caused by these chemoattractants (Fig. 6), it is suggested that the chemoattractant-induced shape changes are involved in the recruitment and migration of neutrophils into inflammatory sites.

The experiments on the homologous desensitization of chemoattractant receptors revealed that although chemotaxis toward cytokine-induced neutrophil chemoattractant-1 was completely desensitized by pretreatment with MIP-2 (Fig. 7b), chemotaxis toward MIP-2 was not completely desensitized by cytokine-induced neutrophil chemoattractant-1 (Fig. 7a). These observations suggested the existence of another type of MIP-2-specific receptor, in addition to the common receptor for both MIP-2 and cytokine-induced neutrophil chemoattractant-1 (Shibata et al., 1995). The finding that MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF caused heterologous desensitization of neutrophil chemotaxis to each other suggested that the signal transduction pathways leading to chemotaxis toward each chemoattractant in rat neutrophils are cross-linked to each other.

In conclusion, the present study suggested that tyrosine kinase and protein kinase C play important roles in rat neutrophil chemotaxis toward MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF. PI 3-kinase is partially involved, but the participation of MAP kinase in neutrophil migration is negligible. The chemoattractant-induced shape changes were also inhibited by kinase inhibitors that inhibit neutrophil chemotaxis. Finally, the signaling pathways for chemotaxis stimulated by MIP-2, cytokine-induced neutrophil chemoattractant-1 and PAF are cross-linked to each other in rat neutrophils.

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