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Differential Effects of Tyrosine Kinase Inhibitors and an Inhibitor of the Mitogen-activated Protein Kinase Cascade on Degranulation and Superoxide Production of Human Neutrophil Granulocytes

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ABSTRACT. The effects of two different tyrosine kinase inhibitors (genistein and erbstatin analog) and an inhibitor (2′-amino-3′-methoxyflavone; PD98059) of the mitogen-activated protein (MAP) kinase kinase on the primary granule exocytosis and superoxide (O_2^-) production of human neutrophil granulocytes were compared. The effector responses induced by stimulation of the chemotactic receptors by formyl-methionyl-leucyl-phenylalanine and platelet-activating factor were blocked both by genistein and erbstatin analog. In contrast, degranulation and O_2^- production triggered by the activation of protein kinase C with phorbol-12-myristate-13-acetate were reduced by erbstatin analog but not by genistein. This inhibitory pattern was observed in both effector responses, but the sensitivity of O_2^- production toward tyrosine kinase inhibition was markedly higher than that of degranulation. PD98059 caused no considerable effect on any of the above responses. The data presented indicate that tyrosine kinases are involved not only in the respiratory burst but also in the organization of the degranulation response of neutrophil granulocytes. It is suggested that several tyrosine kinases of different inhibitor sensitivity may participate in the transduction of extracellular signals. However, activation of the MAP kinase cascade does not appear to be involved in either of the investigated biological responses of the neutrophils. BIOCHEM PHARMACOL **54**;7:781–789, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. neutrophil granulocytes; superoxide production; exocytosis; tyrosine kinases; MAP kinase; signal transduction

Stimulation of neutrophil granulocytes either by physiological agents through membrane receptors or by nonphysiological means, e.g. with phorbol-12-myristate-13-acetate (PMA§), results in the induction of multiple effector responses including superoxide (O_2^-) production and exocytosis of various intracellular granules (degranulation) [1–3]. Initial steps such as the activation of phospholipase C and elicitation of the Ca^{2+} signal or the activation of protein kinase C (PKC) represent a common signal transduction pathway, but the specific processes leading to the assembly of the NADPH oxidase complex or to initiation of the exocytotic machinery are poorly understood (for review see [1, 4]).

In recent years, a considerable amount of data has been accumulated as to the participation of tyrosine kinases in signal transduction in neutrophil granulocytes: 1) stimulation of the cells with different agents resulted in increased phosphorylation of several proteins on tyrosine residues [5–14]; 2) tyrosine kinase activity of mitogen-activated protein (MAP) kinase/extracellular signal regulated kinase kinase (MEK) [15–18] and several *src*-type tyrosine kinases [19–20] was increased during activation of the cells; 3) various inhibitors of tyrosine kinases were reported to inhibit O_2^- production triggered by different agonists [5, 12, 13, 21–24]; and 4) an increase in phosphotyrosine phosphatase activity in the plasma membrane resulted in impaired O_2^- generation induced by the bacterial chemotactic factor formyl-methionyl-leucyl-phenylalanine (fMLP) [21].

Although the involvement of tyrosine kinases in the induction of O_2^- production is generally accepted [5, 12, 13,

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§ *Abbreviations:* erbstatin-A, erbstatin analog (2,5-dihydroxymethylcinnamate); fMLP, formyl-methionyl-leucyl-phenylalanine; fura-2/AM, {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pentaacetoxymethyl ester}; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/extracellular signal regulated kinase kinase; O_2^-, superoxide anion; PAF, platelet-activating factor; PD98059, 2′-amino-3′-methoxyflavone; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine.

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21–24], the role of these signaling proteins in the organization of the degranulation response is poorly understood [22–24]. Furthermore, despite the evidence of the activation of the MAP kinase cascade in response to neutrophil stimulation, no direct evidence has been provided concerning the possible involvement of this pathway in any of the above effector functions of the cells.

To clarify the relation of signaling pathways provoking the two effector responses, we carried out a systematic investigation of the effect of two widely used, unrelated tyrosine kinase inhibitors and an inhibitor of MEK on degranulation and O_2^- production in human neutrophils. Our results indicate that tyrosine kinases are involved in both effector functions of the cells. However, activation of the MAP kinase cascade does not seem to play a major role in the signaling events leading to degranulation or O_2^- production of human neutrophils.

MATERIALS AND METHODS

Materials

Dextran T500 and Ficoll-Paque were obtained from Pharmacia (Uppsala, Sweden), genistein, 2,5-dihydroxymethylcinnamate (erbstatin-A), ionomycin, and {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pentaacetoxymethylester} (fura-2/AM) were from Calbiochem (San Diego, CA, USA), and cytochalasin B, ferricytochrome c, fMLP, platelet-activating factor (PAF), and PMA were from Sigma (St. Louis, MO, USA). 2′-Amino-3′-methoxyflavone (PD98059) was kindly provided by Dr. Julian Downward (Imperial Cancer Research Fund, London, UK). All other reagents were of research grade.

The routinely used extracellular medium (H-medium) contained 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.8 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose, pH 7.4. All incubations were carried out at 37°.

Cell Isolation

Human neutrophils and lymphocytes from healthy volunteers were prepared by dextran sedimentation followed by Ficoll-Paque gradient centrifugation as described [25]. Contaminating erythrocytes were removed from the neutrophil preparation by hypotonic lysis. The neutrophil preparation contained >95% polymorphonuclear cells, while the lymphocyte preparation contained >97% mononuclear cells. Cells were stored at a concentration of 10^7/mL at room temperature until use. The viability of the cells (as determined by Erythrosin B dye exclusion) was >97% in both preparations and was not influenced by any of the applied inhibitors.

Degranulation Measurements

Degranulation of primary granules was measured after preincubation of the cells (10^6/mL) with 10 μM cytochalasin B. Where indicated, genistein, erbstatin-A, or PD98059 was added to the preincubation medium 5 min after cytochalasin B, and the cells were incubated for a further 10 min (genistein and erbstatin-A) or 30 min (PD98059). At the end of the preincubation, the stimulating agents were added and the cells were incubated for 10 min. Cells were then pelleted (8000 × *g* for 1 min at 4°), and the β-glucuronidase activity in the supernatant was determined using the fluorigenic substrate 4-methylumbelliferyl-β-D-glucuronide (Sigma), essentially as described [26]. The β-glucuronidase activity in unstimulated cell supernatants was subtracted, and the enzyme release was expressed in percent of the total cellular enzyme content (enzyme activity in the supernatant of samples treated with 0.02% cetyltrimethylammonium-bromide as detergent). The myeloperoxidase and elastase activity of the same supernatants was measured photometrically using 3,3′,5,5′-tetramethylbenzidine (Sigma; [27]) and *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (Calbiochem; λ = 405 nm) as substrates, respectively.

Measurement of Superoxide Production

Superoxide production was measured in 96-well microplates as the superoxide dismutase sensitive reduction of ferricytochrome c. Cells (10^6/mL) were preincubated for 10 min (genistein and erbstatin-A) or 30 min (PD98059) with 150 μM ferricytochrome c with or without the indicated inhibitor. The cells were then stimulated, and the absorption change was followed at 550 nm. Absorption of unstimulated samples was subtracted, and O_2^- production was calculated using an absorption coefficient of 21.1 mM^{-1} cm^{-1}.

Preparation of membrane and cytosolic components of the porcine cell-free NADPH oxidase system and measurement of its activity were carried out as described [28].

Measurement of Protein Kinase C Activity

PMA-induced PKC activity in human neutrophil lysates was determined using two selective oligopeptide substrates of PKC: H1-peptide, resembling the phosphorylation site of H1 histone (AAASFKAKK-amide) [29, 30], or delta-peptide (PKC-delta pseudosubstrate site like peptide substrate, MNRRRGSIKQAKI) [31]. The method described in [29] was slightly modified. Briefly, 10^7 cells were sonicated in 2 mL of a lysis buffer containing 250 mM sucrose, 20 mM Tris-HCl (pH 7.5), 0.2% Triton X-100, 2 mM EDTA, 5 mM EGTA, 0.25 mg/mL phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 2 μM pepstatin-A, 40 mU/mL aprotinin, and 1 mM benzamidine. 50-μL samples of the lysates were incubated in a reaction mixture (400 μL, final volume) in the presence of 0.7 mg/mL of H1-peptide or 0.5 mg/mL delta-peptide substrate, 10 μM [γ-^{32}P]ATP (ca. 7 × 10^5 cpm per reaction mixture), 10 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.5). Where indicated, 100 nM PMA and 140 μg/mL phosphatidylserine (PS) were also added to the

incubation medium, or the free Ca^{2+} concentration was set to 0.75 mM by $CaCl_2$. After 5 min of incubation at 37°, the reaction was stopped by 120 μL of glacial acetic acid, and the samples were centrifuged. The supernatant was applied onto small phosphocellulose columns, and ^{32}P incorporation into the oligopeptide substrate was determined as described.

Measurement of Ca^{2+} Signal

The Ca^{2+} signal induced by 1 μM fMLP was followed using fura-2/AM-loaded human neutrophils as previously described [25].

Determination of Protein Tyrosine Phosphorylation

Tyrosine-phosphorylated proteins were detected by the immunoblot technique using antiphosphotyrosine antibodies. Cells at 10^7/mL were preincubated for 10 min with or without genistein or erbstatin-A followed by stimulation with 1 μM fMLP or 200 nM PMA. After a 1-min incubation, the reaction was stopped quickly by the addition of twice the volume of 0° H-medium. The samples were then centrifuged at 16000 × *g* for 10 s at 4°, the pellet was resuspended in lysis buffer (100 mM NaCl, 30 mM Na-HEPES, 20 mM NaF, 1 mM Na-EGTA, 10 mM benzamidine, 1% Triton-X-100, pH 7.5, freshly completed with 10 μg/mL aprotinin, 10 μM leupeptin, 2 μM pepstatin A, 1 mM Na_3VO_4, and 1 mM PMSF), incubated on ice for 10 min, and centrifuged at 16000 × *g* for 5 min at 4°. Four times concentrated sample buffer was then added to the supernatants, and the samples were boiled for 10 min. 10^6 cell equivalents per lane were run on a 10% SDS-PAGE [32], and the proteins were transferred onto nitrocellulose sheets. After blocking with 2% BSA in Tris-buffered saline (0.5 M NaCl, 20 mM Tris-OH, pH 7.5), blots were processed using mouse monoclonal antiphosphotyrosine primary antibody (Upstate Biotechnology, Lake Placid, NY, USA; clone 4G10) and peroxidase-labeled affinity-purified sheep antimouse secondary antibody (Amersham, Buckinghamshire, UK). The blots were then developed by Amersham's enhanced chemiluminescence system according to the manufacturer's instructions.

Determination of MAP Kinase Phosphorylation

Phosphorylation of MAP kinase was monitored on the basis of the decrease in electrophoretic mobility due to phosphorylation of the protein. Cells at 10^7/mL were preincubated for 30 min with or without 30 μM PD98059 and then stimulated with 1 μM fMLP for 1 min. The reaction was stopped by adding twice the volume of ice-cold medium followed by rapid pelleting of the cells at 16000 × *g* for 10 s. The pellet was then resuspended in hot sample buffer and boiled for 10 min. 10^6 cell equivalents per lane were run on a 15% SDS-PAGE and blotted onto nitrocellulose sheets. Immunoblotting was performed as described for determination of tyrosine phosphorylation, except that the primary antibody was a mouse monoclonal anti-MAP kinase antibody recognizing $p42^{mapk}$ (anti-ERK2 from Transduction Laboratories, Lexington, KY, USA: dilution, 1:1000). Identical results were obtained with another mouse monoclonal anti-MAP kinase antibody reacting with both $p42^{mapk}$ and $p44^{mapk}$ (anti-pan-ERK from the same supplier; dilution, 1:2000), confirming the finding of Thompson *et al.* [15] that $p42^{mapk}$ is the predominant MAP kinase isoform in human neutrophils. Densitometric analysis was carried out with an Ultroscan XL laser densitometer (Pharmacia-LKB, Uppsala, Sweden).

Statistical Analysis

Measurements were carried out at the indicated times in duplicate or triplicate. Data are given as mean ± S.D. Statistical analysis was performed using Student's paired *t* test.

RESULTS

Effect of Tyrosine Kinase Inhibitors and PD98059 on the Degranulation of Primary Granules

To investigate the possible role of tyrosine kinases and the MAP kinase cascade in the activation of the exocytic response of neutrophils, we tested the effect of tyrosine kinase inhibitors and of an inhibitor of MEK on the degranulation of primary granules in the presence of cytochalasin B. Under these conditions, 1 μM fMLP induced the release of 34.8 ± 4.8% ($n = 24$) of the total cellular content of the primary granule enzyme marker β-glucuronidase, whereas 50 nM PMA evoked 9.8 ± 3.6% ($n = 14$) degranulation. Degranulation could be induced by 100 ng/mL PAF (17.6 ± 4.6%; $n = 5$) or by the simultaneous addition of 50 nM PMA and 1 μM ionomycin (15.9 ± 5.3%; $n = 5$), while 1 μM ionomycin alone exerted only a marginal effect (lower than 5% release). When cytochalasin B was omitted, neither of the stimuli caused significant enzyme release (data not shown).

Preincubation of the cells with genistein, a tyrosine kinase inhibitor competing at the ATP-binding site, caused a concentration-dependent inhibition of fMLP-induced degranulation. As shown in Fig. 1A, in the presence of 25 μM and 100 μM genistein, the liberated enzyme activity was decreased by 62% ($n = 13$) and 92% ($n = 4$), respectively. Fifty percent inhibitory concentration (IC_{50}) was in the 15–20 μM range (data not shown). On the other hand, genistein caused no significant inhibition of PMA-induced degranulation even when used at 100 μM concentration ($p > 0.5$, $n = 3$).

Another family of tyrosine kinase inhibitors is competitive at the peptide-binding site of the enzymes. We also tested the effect of one member of this group, erbstatin-A (a stable analog of erbstatin [33]), on the degranulation response of human neutrophils. After preincubating the cells with 25–100 μM erbstatin-A, both fMLP- and PMA-

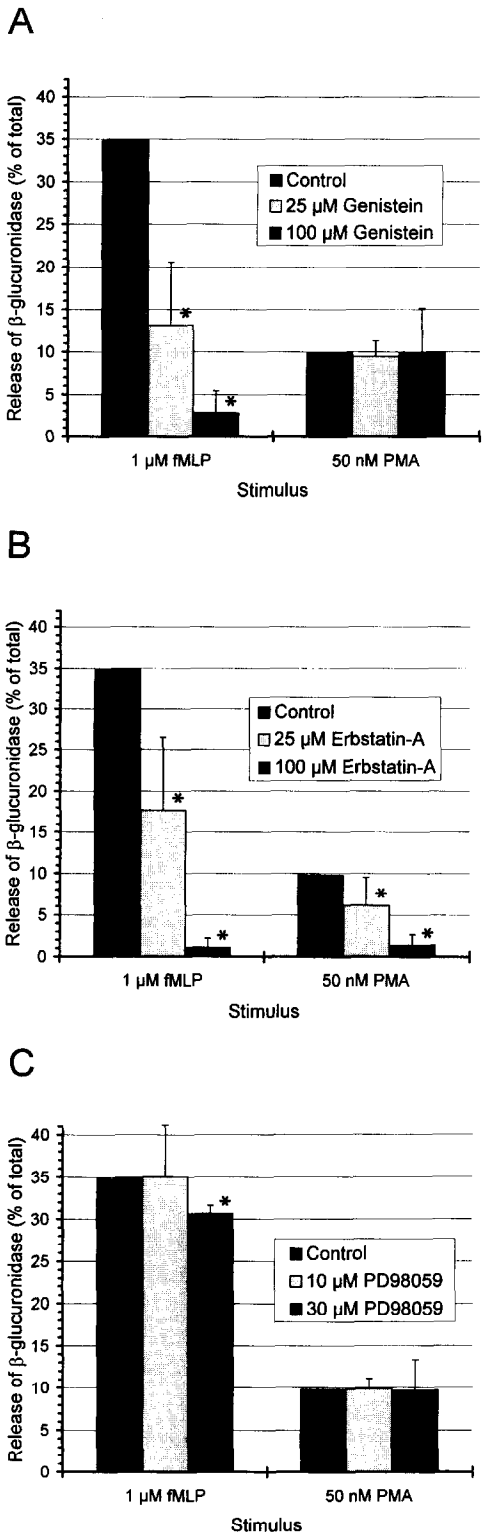

FIG. 1. Effect of tyrosine kinase inhibitors and PD98059 on the degranulation of primary granules of human neutrophils. Cytochalasin B-treated human neutrophils were preincubated with or without genistein (A), erbstatin-A (B), or PD98059 (C) at the indicated concentrations followed by stimulation with 1 μM fMLP or 50 nM PMA. The release of the primary granule enzyme marker β-glucuronidase into the extracellular space during a 10-min incubation was determined as detailed in "Materials and Methods." Each bar represents the mean ± S.D. of normalized data of 3 to 13 independent experiments. *, significantly different ($p < 0.05$) from control.

induced degranulation was strongly inhibited (Fig. 1B). At a concentration of 25 μM, erbstatin-A inhibited the fMLP- and PMA-evoked response by 49% ($n = 9$) and 37% ($n = 7$), respectively. The IC_{50} values of erbstatin-A in inhibiting degranulation evoked by fMLP or by PMA (25 and 30 μM, respectively) were not significantly different.

To test the possible role of the MAP kinase pathway in neutrophil degranulation, we measured the effect of PD98059, a recently described MEK inhibitor [34, 35], on the degranulation response. Fig. 1C shows that PD98059 at 10 μM or 30 μM concentrations caused no inhibition of PMA-induced β-glucuronidase release and exerted only a marginal effect (12% inhibition at 30 μM) on the fMLP-induced response.

Degranulation induced by 100 ng/mL PAF was inhibited both by genistein and erbstatin-A in the same concentration range as that induced by fMLP. On the other hand, degranulation evoked by the simultaneous addition of 50 nM PMA + 1 μM ionomycin was sensitive only to erbstatin-A. PD98059 caused no inhibition of PAF-induced degranulation (data not shown).

Neither genistein nor erbstatin-A affected β-glucuronidase itself, as they did not decrease the activity of the enzyme in the supernatant of detergent-treated samples. The results presented with the β-glucuronidase release were confirmed by measuring the release of two other primary granule enzyme markers, elastase and myeloperoxidase, from the same samples (data not shown).

Effect of Tyrosine Kinase Inhibitors and PD98059 on the Respiratory Burst

In view of the remarkable difference in the effect of the two tyrosine kinase inhibitors and PD98059 on the degranulation process, we tested whether another effector response, the respiratory burst, was also differentially sensitive to the applied drugs.

Superoxide anion production was measured by the cytochrome c reduction test in the absence of cytochalasin B. Kinetic analysis showed that fMLP-induced O_2^- production developed rapidly within the first minute and was completed during the first 3 to 4 min, whereas the PMA-evoked respiratory burst needed 2 to 3 min to reach the maximal rate but continued for more than 15 min. We chose the amount of O_2^- produced during the first 5-min period as the measure of the respiratory burst activity. Under these conditions, 1 μM fMLP and 50 nM PMA induced a O_2^- production of 14.4 ± 5.2 ($n = 14$) and 25.8 ± 6.3 ($n = 14$) nmol/5 min/10^6 cells, respectively.

The data obtained with the two tyrosine kinase inhibitors and with PD98059 are summarized in Fig. 2. In the range of 0.03–100 μM, genistein caused a concentration-dependent decrease in fMLP-induced O_2^- production. Half-maximal inhibition was attained at ≈0.3 μM. It should be recalled that half-maximal inhibition of degranulation evoked by the same stimulus needed an almost two order of magnitude higher (15–20 μM) genistein concentration

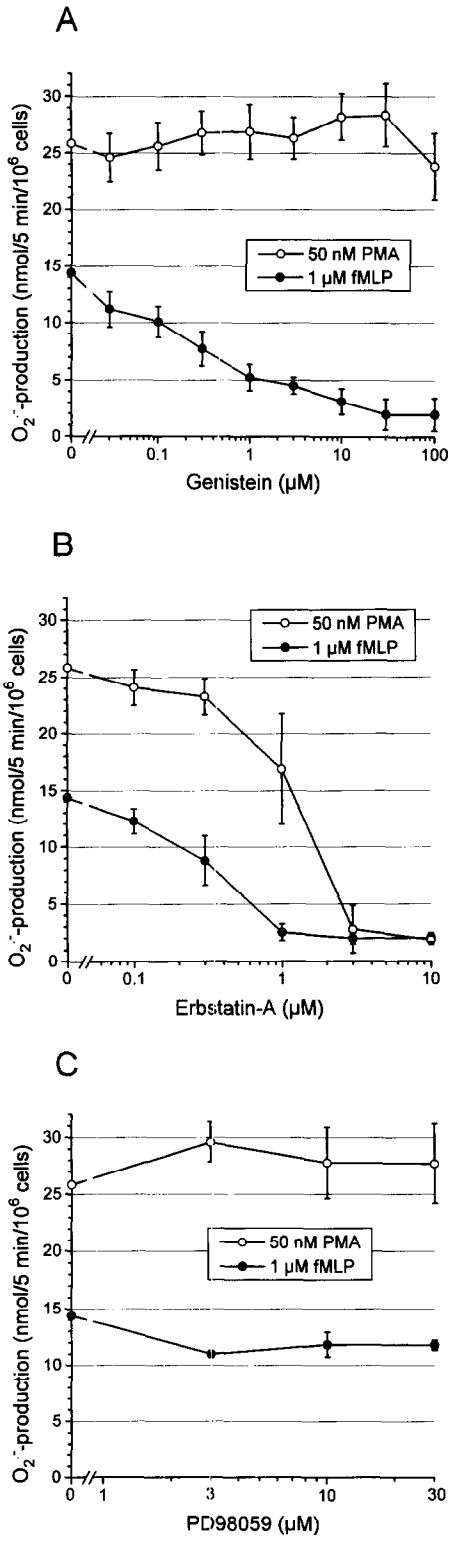

FIG. 2. Effect of tyrosine kinase inhibitors and PD98059 on the respiratory burst of human neutrophils. Human neutrophils were preincubated with or without genistein (A), erbstatin-A (B), or PD98059 (C) at the indicated concentrations in the absence of cytochalasin B followed by stimulation with 1 μM fMLP or 50 nM PMA. The O_2^- production during a 5-min incubation period was determined as detailed in "Materials and Methods." Each point represents the mean ± S.D. of normalized data of three to five independent experiments.

(compare Figs. 1A and 2A). Up to a concentration of 100 μM, PMA-induced O_2^- generation was not affected by genistein.

In contrast to the effect of genistein, erbstatin-A clearly inhibited both the fMLP- and the PMA-induced respiratory burst. The drug was slightly but reproducibly more effective on the fMLP-elicited response, the concentrations required for half-maximal inhibition in the case of fMLP and PMA being ≈0.3 μM and ≈1 μM, respectively. These concentrations are 30–100 times lower than those required for inhibition of the degranulation response (25–30 μM; compare Figs. 1B and 2B).

The conditions of measurement of degranulation and O_2^- production differed in the presence or absence of cytochalasin B. To test whether this difference was responsible for the observed shift in the sensitivity toward tyrosine kinase inhibitors, we investigated the effect of genistein and erbstatin-A on the respiratory burst after preincubating the cells with 10 μM cytochalasin B. These experiments showed that cytochalasin B did not significantly influence the IC_{50} values of either of the two drugs (data not shown).

As shown in Fig. 2C, PD98059 did not inhibit O_2^- production of PMA-activated neutrophils in concentrations up to 30 μM. The fMLP-induced respiratory burst was only slightly reduced by PD98059, resulting in 18% inhibition at the highest concentration tested.

Investigation of the respiratory burst revealed the same pattern of inhibitor sensitivity as detected in the degranulation response. However, there was a significant (30–100-fold) difference in the effective concentration of both genistein and erbstatin-A, indicating that O_2^- generation is markedly more sensitive toward tyrosine kinase inhibition than the release of primary granules.

Control of Possible Unspecific Actions of Genistein and Erbstatin-A

In a previous paper, the tyrosine kinase inhibitor ST638 was shown to interfere with the binding of fMLP to its receptor [23]. To exclude a similar action of the inhibitors applied in this study, the Ca^{2+} signal elicited by chemotactic receptor stimulation was investigated using fura-2/AM-loaded human neutrophils. Neither genistein nor erbstatin-A, applied at a concentration of 25 μM, had any influence on the Ca^{2+} signal induced by 1 μM fMLP (data not shown).

Although erbstatin was originally described to be a specific inhibitor of tyrosine kinases that did not influence PKC [33], one group reported inhibition of rat brain PKC activity by erbstatin [36]. As the effects of genistein and erbstatin-A differed only in the inhibition of PMA-induced responses by the latter compound, the possibility arose that this drug exerted its effect through inhibition of PKC activity. Therefore, we investigated the effect of erbstatin-A on the PKC activity of human neutrophil cell lysate using two different substrates: H1-peptide, which is selective for the Ca^{2+}-sensitive conventional isoforms [29, 30],

TABLE 1. Effect of erbstatin-A on the PMA-induced PKC activity of human neutrop lysate

Activator	^{32}P incorporation (pmol/5 min/10^6 cells)		
	—	Erbstatin-A 25 μM	100 μM
		H1-Peptide	
—	363	379	374
PMA/PS	412	414	393
PMA/PS + Ca^{2+}	573	549	578
		Delta-Peptide	
—	368	336	344
PMA/PS	541	480	548
PMA/PS + Ca^{2+}	671	630	668

Human neutrophil lysates were incubated for 5 min with or without erbstatin-A at the indicated concentrations in the presence of [γ-^{32}P]ATP and the PKC-specific substrate H1-peptide or delta-peptide in a Ca^{2+}-free medium, and the ^{32}P incorporation into the substrate peptides was determined as detailed in "Materials and Methods." Where indicated, the following additions were made: PMA/PS. 100 nM PMA + 140 μg/mL PS; Ca^{2+}, free Ca^{2+} concentration set to 0.75 mM by $CaCl_2$. Mean values of duplicates from a representative experiment out of three (H1-peptide) or two (delta-peptide) similar experiments are shown.

and delta-peptide, which better reflects the activity of Ca^{2+}-insensitive isoforms of PKC [31].

As shown in Table 1, neutrophil lysates contained a relatively high phosphotransferase activity in the absence of PMA/PS and Ca^{2+}, which was probably due to the intensive proteolytic cleavage of PKC that occurred even in the presence of protease inhibitors and that gave rise to an unregulated catalytic subunit [29]. Addition of PMA and PS to the neutrophil lysate in the absence of Ca^{2+} caused an increase in the PKC activity representing the PMA-dependent, Ca^{2+}-independent PKC isoforms. Addition of Ca^{2+} to this reaction mixture caused a further activation, demonstrating the presence of PMA- and Ca^{2+}-dependent PKC activity. As seen in Table 1, erbstatin-A at 25 μM or 100 μM concentration caused no significant inhibition of any of the PKC fractions detected by either the H1-peptide or the delta-peptide substrate.

These results show that inhibition of degranulation and O_2^- production by erbstatin-A cannot be ascribed to a direct action of the drug on PKC activity itself.

The inhibitory action of erbstatin-A on the O_2^- production induced by the applied stimuli could be explained by a direct effect of the drug on the respiratory burst oxidase itself. To test this possibility, we measured the effect of the drug on the assembled NADPH oxidase in the cell-free activation system. Erbstatin-A caused no significant inhibition of the enzyme at concentrations that completely blocked O_2^- production in intact cells (data not shown). Thus, the inhibitory action of the drug on the respiratory burst in intact cells is probably not due to a direct influence on the respiratory burst oxidase.

Effect of PD98059 on the Phosphorylation of MAP Kinase

PD98059 was originally described as a specific inhibitor of platelet-derived growth factor-induced MEK activation in Swiss 3T3 fibroblasts [34]. We tested whether the inhibitory action of the drug on MEK activation was also exerted in human neutrophils. Phosphorylation of MAP kinases by MEK induces a decrease in the electrophoretic mobility of the MAP kinase protein. As shown in Fig. 3A, treatment of the neutrophils with 1 μM fMLP shifted the p42^{mapk} immunoreactive band to a higher apparent molecular weight, and this shift was prevented by pretreating the cells with 30 μM PD98059. Densitometric analysis (Fig. 3B)

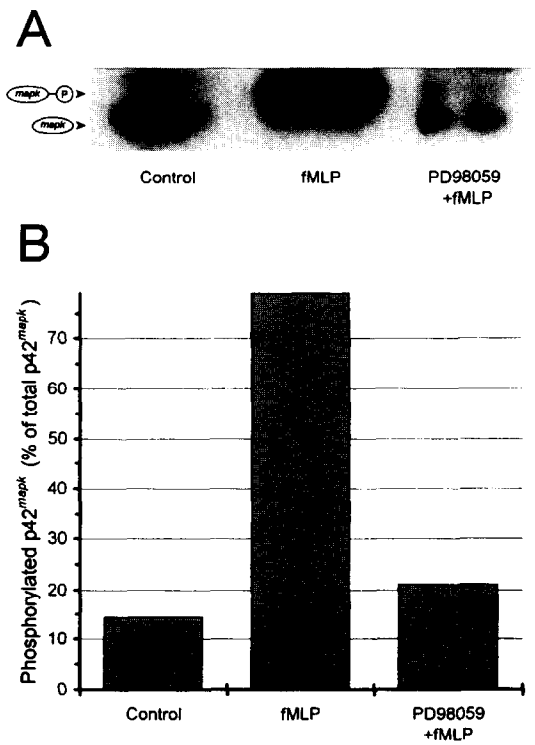

FIG. 3. Effect of PD98059 on fMLP-induced MAP kinase phosphorylation. Human neutrophils were preincubated with or without 30 μM PD98059 followed by stimulation with 1 μM fMLP. The electrophoretic mobility of p42^{mapk} kinase was detected and quantified as detailed in "Materials and Methods." A representative immunoblot (A) and the result of densitometric analysis of p42^{mapk} mobility (B) are shown. In B, each bar represents the mean of results from two independent experiments.

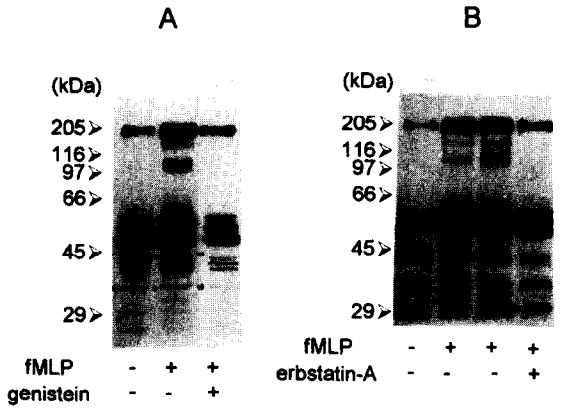

FIG. 4. Inhibition of tyrosine phosphorylation by genistein and erbstatin-A. Human neutrophils (10^7/mL) were preincubated with genistein (A) or erbstatin-A (B) followed by stimulation with 1 μM fMLP, as indicated below the pictures. The arrows on the left show the position of the molecular weight markers.

revealed that PD98059 reduced the MAP kinase phosphorylation by 90%. Thus, similarly to previously investigated cell types, PD98059 exerted its strong inhibitory effect on MEK activity in human neutrophils as well.

Tyrosine Phosphorylation of Proteins of Neutrophil Granulocytes

Activation of neutrophil granulocytes was shown to induce the phosphorylation of several proteins on tyrosine residues, although the phosphorylation patterns reported by different groups are not completely consistent [5–14]. In our experiments, activation of the cells by 1 μM fMLP resulted in an increase in the tyrosine phosphorylation of protein bands at ≈42 kDa, ≈58 kDa, ≈64 kDa, ≈110 kDa, ≈150 kDa, and ≈200 kDa (Fig. 4). The phosphorylation of these bands was clearly inhibited both by genistein (Fig. 4A) and by erbstatin-A (Fig. 4B) in concentrations comparable to the concentration where the biological responses were abolished. Similar results were obtained when the neutrophils were stimulated by PMA (data not shown). Thus, the inhibitory action of the two drugs on the investigated effector responses may be the consequence of inhibition of tyrosine kinase activity.

DISCUSSION

In the experiments described above, the two investigated effector functions of neutrophil granulocytes were inhibited both by genistein and erbstatin-A (Figs. 1 and 2). The effective concentration of the drugs corresponds to the concentration previously shown to inhibit tyrosine kinases in neutrophils and in other cell types [9, 11, 22, 33]. Neither of the drugs influenced the Ca^{2+} signal induced by the chemotactic receptor agonist fMLP, suggesting that their site of action was not upstream to elicitation of the Ca^{2+} signal. The activity of protein kinase C was not impaired by erbstatin-A, and both compounds effectively inhibited tyrosine phosphorylation of several proteins upon cell stimulation (Fig. 4). All these data are in accord with the presumption that the two agents exerted the observed effects through inhibition of tyrosine kinases in neutrophil granulocytes.

The observation that the degranulation response of neutrophil granulocytes is impaired by two different tyrosine kinase inhibitors (Figs. 1 and 3) clearly indicates the involvement of tyrosine kinases in the organization of the exocytosis of primary granules. The inhibitory effect of erbstatin-A on fMLP-induced degranulation is in apparent contradiction with previously published data [22]. However, it should be considered that in those experiments cells were preincubated with erbstatin for 1 h at 37° and then washed free of the inhibitor before testing, whereas in our experiments cells were incubated with a more stable analog of erbstatin for only 10 min, and the inhibitor was not removed. Degradation of erbstatin [33] and the reversible nature of the inhibition may account for the different results obtained under the differing experimental conditions.

Although both genistein and erbstatin-A were able to inhibit the degranulation response under certain conditions, a significant difference was detected in their effectiveness depending on the nature of the stimulating agent (Fig. 1). Degranulation induced by chemotactic receptor agonists (fMLP and PAF) was inhibited both by genistein and erbstatin-A. In contrast, exocytosis evoked by activation of protein kinase C by phorbol ester was only blocked by erbstatin-A. This finding suggests that different tyrosine kinases (or different sets of tyrosine kinases) may participate in the signal transduction pathways triggered by receptor stimulation or by protein kinase C activation.

Investigation of degranulation and the respiratory burst revealed the same pattern of inhibition by genistein and erbstatin-A. However, O_2^- production seemed to be markedly more sensitive to the effect of the two drugs, even when the two responses were measured under identical conditions. This difference raises the possibility that distinct sets of tyrosine kinases may be involved in the organization of the two effector responses or that a different level of activity of the same kinases is required for degranulation and NADPH oxidase activation.

The best characterized signaling protein shown to become tyrosine phosphorylated during neutrophil activation is MAP kinase, phosphorylated on tyrosine and threonine residues by the dual specificity kinase MEK. PD98059, a recently described inhibitor of MEK, did in fact inhibit the fMLP-induced phosphorylation of MAP kinase in neutrophil granulocytes. However, the drug caused no considerable inhibition of any of the effector responses tested, suggesting that the MAP kinase cascade does not play a major role in the mediation of the signals leading to the respiratory burst or primary granule exocytosis in human neutrophils.

Taken together, the data presented in this paper clearly indicate that, in addition to the respiratory burst, tyrosine

kinases are also involved in the organization of the degranulation response of neutrophil granulocytes. On the basis of our pharmacological data, the possibility is raised that several different tyrosine kinases may participate in the transduction of the signals originating from the cell environment. Furthermore, our results argue against the involvement of the MAP kinase cascade in the signaling pathways coupling stimulation of chemotactic receptors to the respiratory burst or primary granule exocytosis in human neutrophil granulocytes.

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References

1. Thelen M, Dewald B and Baggiolini M, Neutrophil signal transduction and activation of the respiratory burst. *Physiol Rev* **73:** 797–821, 1993.
2. Borregaard N, Lollike K, Kjeldsen L, Sengeløv H, Bastholm L, Nielsen MH and Bainton DF, Human neutrophil granules and secretory vesicles. *Eur J Haematol* **51:** 187–198, 1993.
3. Sengeløv H, Kjeldsen L and Borregaard N, Control of exocytosis in early neutrophil activation. *J Immunol* **150:** 1535–1543, 1993.
4. Bokoch GM, Chemoattractant signaling and leukocyte activation. *Blood* **86:** 1649–1660, 1995.
5. Gomez-Cambronero J, Huang C-K, Bonak VA, Wang E, Casneille JE, Shiraishi T and Sha'afi RI, Tyrosine phosphorylation in human neutrophils. *Biochem Biophys Res Commun* **162:** 1478–1485, 1989.
6. Berkow RL and Dodson RW, Tyrosine-specific protein phosphorylation during activation of human neutrophils. *Blood* **75:** 2445–2452, 1990.
7. Huang C-K, Laramee GR and Casneille JE, Chemotactic factor-induced tyrosine phosphorylation of membrane-associated proteins in rabbit peritoneal neutrophils. *Biochem Biophys Res Commun* **151:** 794–801, 1988.
8. Grinstein S and Furuya W, Chemoattractant-induced tyrosine phosphorylation and activation of microtubule-associated protein kinase in human neutrophils. *J Biol Chem* **267:** 18122–18125, 1992.
9. Brumell JH and Grinstein S, Serine/threonine kinase activation in human neutrophils: relationship to tyrosine phosphorylation. *Am J Physiol* **267:** C1574–C1581, 1994.
10. Naccache PH, Caon AC, Gilbert C, Gaudry M, Roberge CJ, Poubelle PE and Bourgoin S, Inhibition of tyrosine phosphorylation by wortmannin in human neutrophils: dissociation from its inhibitory effects on phospholipase D. *Lab Invest* **69:** 19–23, 1993.
11. Dusi S, Donini M and Rossi F, Tyrosine phosphorylation and activation of NADPH oxidase in human neutrophils: a possible role for MAP kinases and for a 75 kDa protein. *Biochem J* **304:** 243–250, 1994.
12. Azuma EK, Kitagawa S, Yuo A, Mizoguchi H, Umezawa K, Takaku F and Saito M, Activation of the respiratory burst and tyrosine phosphorylation of proteins in human neutrophils: no direct relationship and involvement of protein kinase C-dependent and -independent signaling pathways. *Biochim Biophys Acta* **1179:** 213–223, 1993.
13. Torres M, Hall FL and O'Neill K, Stimulation of human neutrophils with formyl-methionyl-leucyl-phenylalanine induces tyrosine phosphorylation and activation of two distinct mitogen-activated protein-kinases. *J Immunol* **150:** 1563–1577, 1993.
14. Rollet E, Caon A, Roberge CJ, Liao NW, Malawista SE, McColl SR and Naccache PH, Tyrosine phosphorylation in activated human neutrophils: comparison of the effects of different classes of agonists and identification of the signaling pathways involved. *J Immunol* **153:** 353–363, 1994.
15. Thompson HL, Shiroo M and Saklatvala J, The chemotactic factor *N*-formylmethionyl-leucyl-phenylalanine activates microtubule-associated protein 2 (MAP) kinase and a MAP kinase kinase in polymorphonuclear leucocytes. *Biochem J* **290:** 483–488, 1993.
16. Thompson HL, Marshall CJ and Saklatvala J, Characterization of two different forms of mitogen-activated protein kinase kinase induced in polymorphonuclear leukocytes following stimulation by *N*-formylmethionyl-leucyl-phenylalanine or granulocyte-macrophage colony-stimulating factor. *J Biol Chem* **269:** 9486–9492, 1994.
17. Downey GP, Butler JR, Brumell J, Borregaard N, Kjeldsen L, Sue-A-Quan AK and Grinstein S, Chemotactic peptide-induced activation of MEK-2, the predominant isoform in human neutrophils: inhibition by wortmannin. *J Biol Chem* **271:** 21005–21011, 1996.
18. Grinstein S, Butler JR, Furuya W, L'Allemain G and Downey GP, Chemotactic peptides induce phosphorylation and activation of MEK-1 in human neutrophils. *J Biol Chem* **269:** 19313–19320, 1994.
19. Ptasznik A, Traynor-Kaplan A and Bokoch GM, G protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase-Shc adapter protein signaling complexes. *J Biol Chem* **270:** 19969–19973, 1995.
20. Yan SR, Fumagalli L and Berton G, Activation of *src* family kinases in human neutrophils: evidence that $p58^{c\text{-}fgr}$ and $p53/56^{lyn}$ redistributed to a Triton X-100-insoluble cytoskeletal fraction, also enriched in the caveolar protein caveolin, display an enhanced kinase activity. *FEBS Lett* **380:** 198–203, 1996.
21. Cui Y, Harvey K, Akard L, Jansen J, Hughes C, Siddiqui RA and English D, Regulation of neutrophil responses by phosphotyrosine phosphatase. *J Immunol* **152:** 5420–5428, 1994.
22. Nacchache PH, Gilbert C, Caon AC, Gaudry M, Huang C-K, Bonak VA, Umezawa K and McColl SR, Selective inhibition of human neutrophil functional responsiveness by erbstatin, an inhibitor of tyrosine protein kinase. *Blood* **76:** 2098–2104, 1990.
23. Berkow RL, Dodson RW and Kraft AS, Human neutrophils contain distinct cytosolic and particulate tyrosine kinase activities: possible role in neutrophil activation. *Biochim Biophys Acta* **997:** 292–301, 1989.
24. Dryden P, Duronio V, Martin L, Hudson AT and Salari H, Inhibition of human neutrophil responses by alpha-cyano-3,4-dihydroxythiocinnamamide: a protein-tyrosine kinase inhibitor. *Br J Pharmacol* **106:** 656–664, 1992.
25. Geiszt M, Káldi K, Szeberényi JB and Ligeti E, Thapsigargin inhibits Ca^{2+} entry into human neutrophil granulocytes. *Biochem J* **305:** 525–528, 1995.
26. Dewald B and Baggiolini M, Methods for assessing exocytosis by neutrophil leukocytes. *Methods Enzymol* **132:** 267–277, 1986.
27. Menegazzi R, Zabucchi G, Knowles A, Cramer R and Patriarca P, A new, one-step assay on whole cell suspensions for

peroxidase secretion by human neutrophils and eosinophils. *J Leuk Biol* **52:** 619–624, 1992.
28. Ligeti E, Doussiere J and Vignais PV, Activation of the O_2^--generating oxidase in plasma membrane from bovine polymorphonuclear neutrophils by arachidonic acid, a cytosolic factor of protein nature, and nonhydrolyzable analogues of GTP. *Biochemistry* **27:** 193–200, 1988.
29. Buday L, Seprödi J, Farkas G, Mészáros G, Romhányi T, Bánhegyi G, Mandl J, Antoni F and Faragó A, Proteolytic activation of protein kinase C in the extracts of cells treated for a short time with phorbol ester. *FEBS Lett* **223:** 15–19, 1987.
30. Romhányi T, Seprödi J, Antoni F, Mészáros G, Buday L and Faragó A, The assay of the activity of protein kinase C with the synthetic oligopeptide substrate designed for histone kinase II. *Biochim Biophys Acta* **888:** 325–331, 1986.
31. Ways DK, Qin W, Garris TO, Chen J, Hao E, Cooper DR, Usala SJ, Parker PJ and Cook PP, Effects of chronic phorbol ester treatment on protein kinase C activity, content, and gene expression in the human monoblastoid U937 cell. *Cell Growth Differ* **5:** 161–169, 1994.
32. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
33. Umezawa K and Imoto M, Use of erbstatin as protein-tyrosine kinase inhibitor. *Methods Enzymol* **201:** 379–385, 1991.
34. Dudley DT, Pang L, Decker SJ, Bridges AJ and Saltiel AR, A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci USA* **92:** 7686–7689, 1995.
35. Alessi DR, Cuenda A, Cohen P, Dudley DT and Saltiel AR, PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J Biol Chem* **270:** 27489–27494, 1995.
36. Bishop WR, Petrin J, Wang L, Ramesh U and Doll RJ, Inhibition of protein kinase C by the tyrosine kinase inhibitor erbstatin. *Biochem Pharmacol* **40:** 2129–2135, 1990.