The Journal *of* Immunology

This information is current as of August 4, 2022.

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Kinase Pathways in Chemoattractant-Induced Degranulation of Neutrophils: The Role of p38 Mitogen-Activated Protein Kinase Activated by Src Family Kinases[1]

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The aim of the present study was to investigate the role of tyrosine phosphorylation pathways in fMLP-induced exocytosis of the different secretory compartments (primary and secondary granules, as well as secretory vesicles) of neutrophils. Genistein, a broad specificity tyrosine kinase inhibitor, blocked the exocytosis of primary and secondary granules, but had only a marginal effect on the release of secretory vesicles. Genistein also inhibited the phosphorylation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinases (MAPK), raising the possibility that inhibition of ERK and/or p38 MAPK might be responsible for the effect of the drug on the degranulation response. Indeed, SB203580, an inhibitor of p38 MAPK, decreased the release of primary and secondary granules, but not that of secretory vesicles. However, blocking the ERK pathway with PD98059 had no effect on any of the exocytic responses tested. PP1, an inhibitor of Src family kinases, also attenuated the release of primary and secondary granules, and neutrophils from mice deficient in the Src family kinases Hck, Fgr, and Lyn were also defective in secondary granule release. Furthermore, activation of p38 MAPK was blocked by both PP1 and the $hck^{-/-}fgr^{-/-}lyn^{-/-}$ mutation. Taken together, our data indicate that fMLP-induced degranulation of primary and secondary granules of neutrophils is mediated by p38 MAPK activated via Src family tyrosine kinases. Although piceatannol, a reportedly selective inhibitor of Syk, also prevented degranulation and activation of p38 MAPK, no fMLP-induced phosphorylation of Syk could be observed, raising doubts about the specificity of the inhibitor. *The Journal of Immunology,* 2000, 164: 4321–4331.

S timulation of the chemoattractant receptors of neutrophils evokes multiple effector responses, including chemotaxis, superoxide production, and degranulation. The latter response consists of the release of the different intracellular compartments (several types of granules as well as the secretory vesicle compartment) (1) to the extracellular space or into the phagosome.

The receptor for the bacterial tripeptide fMLP, the most widely studied chemoattractant of neutrophils, activates a pertussis toxin-sensitive, heterotrimeric G protein belonging to the $G_{i/o}$ family. One of the most characteristic features of fMLP signal transduction is the rapid induction of tyrosine phosphorylation of several intracellular proteins, including tyrosine kinases (2, 3), different members of the mitogen-activated protein kinase (MAPK)[3] family

(4–6), adaptor proteins (3), and components of the small G protein network (7). Despite clear evidence of phosphorylation (and activation) of these proteins, little is known about their role in triggering the fMLP-induced effector responses.

Using broad specificity tyrosine kinase inhibitors, we and others suggested that tyrosine kinases might play a role in the fMLP-induced release of primary and secondary granules (Ref. 8 and references therein). In the last few years great progress has been made in understanding the roles of the different tyrosine kinases in the regulation of neutrophil functions (9). Activation of the Src-related kinase Lyn in response to fMLP stimulation has been reported by several authors (2, 3, 9, 10). Fgr, another member of the Src kinase family, has been shown to be associated with the secondary granules and to translocate to the plasma membrane upon fMLP stimulation of the cells (11). Similarly, Hck, the third Src family member present in neutrophils, is associated with primary granules and translocates to the phagosome during phagocytosis of serum-opsonized zymosan (12). Our pharmacological and gene knockout experiments performed on murine neutrophils showed that the fMLP-induced exocytosis of secondary granules is mediated by Src-related tyrosine kinases (13). However, these reports did not provide any clue to identification of possible downstream targets of tyrosine kinases. In addition, they did not address in detail the distinctive role of tyrosine phosphorylation in regulating exocytosis of the different granule populations and of secretory vesicles.

In the present report we analyzed the fMLP-induced exocytosis of primary and secondary granules as well as secretory vesicles in response to fMLP using inhibitors with reported selectivity for different tyrosine kinases or tyrosine-phosphorylated proteins and studying mice deficient in Src family kinases expressed in neutrophils.

This study followed two major directions of investigation. First, to obtain information on the molecular identity of the kinases participating in the fMLP-induced degranulation response, we focused on 1) the ERK and p38 MAPK proteins, members of the MAP kinase family shown to become rapidly phosphorylated on adjacent Tyr and Thr residues and thereby activated upon chemoattractant stimulation (4–6, 14, 15); 2) the Src family of tyrosine kinases, the members of which have been implicated in several aspects of the degranulation process (see above); and 3) the Syk tyrosine kinase, the function of which is closely related to that of Src family kinases in several cellular systems (16–19). Our second objective was to compare the involvement of the above pathways in the fMLP-induced release of three exocytic compartments of human neutrophils: primary granules, secondary granules, and secretory vesicles. Previous reports indicated differences in calcium sensitivity (20) and inhibition by wortmannin (21) of fMLP-induced exocytosis among these three granule populations. All these differences warranted a comparative study on the role of tyrosine kinases in the release of the three exocytic compartments.

In the present paper we provide evidence that p38 MAPK, activated by Src family kinases has a central role in eliciting the fMLP-induced release of primary and secondary granules, but not in that of secretory vesicles.

Materials and Methods

Materials

Dextran T-500 and Ficoll-Paque were obtained from Pharmacia (Uppsala, Sweden). fMLP, cytochalasin B (CB), and genistein were obtained from Sigma (St. Louis, MO). PD98059, PP1, and piceatannol were obtained from Calbiochem (San Diego, CA). SB203580 was provided by SmithKline Beecham (King of Prussia, PA). Human lactoferrin (Lfr), human serum albumin (HSA), and goat anti-HSA Abs were obtained from Sigma. Rabbit anti-human Lfr Abs were either purchased from Sigma or provided by Dr. Katalin Némct (National Institute of Hematology and Blood Transfusion, Budapest, Hungary). Where necessary, Abs were labeled with HRP (22).

Unless otherwise stated, incubation of neutrophils was performed in HBSS (containing 0.5 mM CaCl$_2$ and 1 mM MgCl$_2$) supplemented with 20 mM Na-HEPES (pH 7.4) and 0.1% BSA (HBSS-HB). All incubation media were prepared using sterile and endotoxin-free water.

Preparation of human neutrophils

Venous blood was drawn from healthy volunteers. After dextran sedimentation at room temperature, neutrophils were obtained by centrifugation at 4°C through Ficoll-Paque followed by hypotonic lysis of erythrocytes (8, 13). Unless otherwise stated, cells were resuspended in ice-cold HBSS-HB and kept on ice until use. The preparations usually contained >98% polymorphonuclear cells; the viability, as determined by the erythrosin B dye exclusion test, was >98%.

Degranulation of human neutrophils

Human neutrophils at 10^6/ml were incubated in HBSS-HB with or without the indicated inhibitors and/or 10 μM cytochalasin B for 15 min on ice and for an additional 15 min at 37°C. The cells were then stimulated for 10 min with 100 nM fMLP. The reaction was stopped by cooling, and the suspension was centrifuged for 10 min at 2000 × g at 4°C. The extent of degranulation was determined by measuring the concentrations of the different granule markers in the supernatant. Addition of up to 0.2% DMSO (the maximum solvent concentration added with the inhibitors) had no influence on any of the exocytic responses tested (not shown).

The activity of the primary granule marker β-glucuronidase (β-GU) was determined by a fluorometric assay described previously (8). In some experiments the total cellular β-GU activity was also measured, using suspensions treated with 0.02% cetyl-trimethyl-ammonium bromide as detergent. The concentrations of the secondary granule marker Lfr (23) and HSA, a marker of secretory vesicles (24), were determined by ELISA. Maxisorp F96 plates (Nunc, Naperville, IL) were covered with the relevant unlabeled Abs and blocked by PBS supplemented with 0.5% BSA and 0.1% Tween 20. Plates were then incubated with diluted supernatants or known concentrations of Lfr or HSA, followed by treatment with peroxidase-labeled anti-human Lfr or anti-HSA Abs, and developed with o-phen-

ylenediamine. The OD of the samples was read using a Labsystems (Helsinki, Finland) iEMS microplate reader.

For determination of adhesion-dependence of the exocytic response, the effect of withdrawal of Mg^{2+} on the release of secondary granules was tested (13, 25). Cells were resuspended in HBSS-HB without CaCl$_2$, MgCl$_2$, and BSA, supplemented with 5 mM Na-EDTA. After incubation for 10 min at room temperature, cells were pelleted and resuspended in ice-cold HBSS-HB with or without 1 mM MgCl$_2$. The fMLP-stimulated release of Lfr was then determined as described above. Simultaneously, cells were plated on tissue culture plates precoated with human fibrinogen (13) and stimulated with 20 ng/ml human TNF-α (PeproTech, Rocky Hill, NJ) for 30 min at 37°C. Spreading of the cells was assessed visually under a phase contrast microscope.

Immunoblotting of human cell lysates and kinase assays

Cells at 2.5 × 10^7/ml were pretreated exactly as described for degranulation experiments, followed by a 2-min stimulation with 100 nM fMLP at 37°C. Samples were then immersed in liquid N$_2$ to stop the reaction and were lysed by adding a Triton-based lysis buffer with protease and phosphatase inhibitors (8) from a 5× concentrated stock solution followed by a 10-min incubation on ice and spinning down at 16,000 × g for 1 min at 4°C. The Triton-soluble supernatant was used in further determinations.

For immunoblot experiments, the Triton-soluble lysate was mixed with sample buffer, boiled for 10 min, run on 12% SDS-PAGE, and blotted onto nitrocellulose sheets. Blots were processed using phospho-specific rabbit polyclonal anti-ERK or anti-p38 MAPK Abs (both from New England Biolabs, Beverly, MA) followed by a peroxidase-labeled anti-rabbit Ab (Amersham, Aylesbury, U.K.). Blots were developed using Amersham's enhanced chemiluminescence system and exposed to x-ray film, and signal intensities were quantified by a Pharmacia-LKB (Uppsala, Sweden) Ultroscan XL laser densitometer. For control blots, non-phospho-specific rabbit polyclonal anti-ERK1 and anti-ERK2 (used in combination; both from Santa Cruz Biotechnology, Santa Cruz, CA) or anti-p38 MAPK (Upstate Biotechnology, Lake Placid, NY) with peroxidase-labeled anti-rabbit Abs (Amersham) were used. It should be noted that the slight difference in the migrations of ERK1 and ERK2 could only be resolved when the acrylamide/bisacrylamide ratio of the SDS-PAGE gel was increased to 37.5:1. Even when such gels were used, we never observed any duplication of the anti-p38 MAPK immunoreactive band.

The activity of MAPK-activated protein kinase 2 (MAPKAPK2), a kinase downstream of p38 MAPK, was measured by an in vitro kinase assay (26) using recombinant heat shock protein 27 (Hsp27), a substrate of MAPKAPK2. Triton-soluble lysates were supplemented with kinase buffer (15 mM Na-HEPES (pH 7.4), 10 mM MgCl$_2$, 1 mM Na-EGTA, 2 mM DTT, 50 mM NaCl, 10 mM NaF, 5 μM sodium pyrophosphate, 20 mM β-glycerophosphate, 2 μM okadaic acid, 0.2 mM [γ-^{32}P]ATP (~200 μCi/μmol), and 20 μg/ml human Hsp27 (Stressgen, Victoria, Canada)) from a 2× concentrated stock solution and incubated for 20 min at room temperature. The reaction was stopped by adding sample buffer, followed by boiling for 10 min. Samples were then run on a 12% SDS-PAGE, dried, and exposed to x-ray film or quantitated using a Bio-Rad (Hercules, CA) GS-525 PhosphorImager. To determine the incorporation of radioactivity into endogenous cellular proteins, parallel measurements without Hsp27 were also performed. Radioactivity in these samples was then subtracted from values obtained from Hsp27-containing tubes.

For the in vitro ERK kinase activity measurements (27), 100 μl Triton-soluble cell lysates were incubated for 3 h with 3 μg of rabbit polyclonal anti-ERK2 Abs (provided by Dr. Jackie R. Vandenheede, Catholic University of Leuven, Leuven, Belgium). Immunocomplexes were captured with protein A-TSK gel and were washed three times with PBS containing 1% Nonidet P-40, and twice with kinase buffer containing 20 mM MOPS (pH 7.6), 2 mM EGTA, 20 mM MgCl$_2$, 1 mM DTT, 1 mM Na$_3$VO$_4$, and 0.1% Triton X-100. The washed beads were resuspended in 30 μl of kinase reaction mixture containing kinase buffer supplemented with 40 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 50 μg myelin basic protein as kinase substrate. After incubation at 30°C for 15 min, the reaction was terminated by addition of sample buffer and heating at 100°C for 3 min. The proteins were separated on 11% SDS-PAGE, dried, and exposed to x-ray film. The myelin basic protein band was then cut out, and the incorporated radioactivity was quantified using a Wallac (Turku, Finland) 1409 liquid scintillation counter.

Determination of Tyr phosphorylation of Syk

Tyr phosphorylation of Syk was determined by immunoprecipitation followed by immunoblotting with anti-phosphotyrosine (DFP) Abs. To prevent the degradation of Syk, these experiments were performed on neutrophils pretreated with 0.2 μl/ml di-isopropyl fluorophosphate at the end

FIGURE 1. Effect of genistein on neutrophil degranulation. Human neutrophils were preincubated with or without 100 μM genistein and/or 10 μM CB, followed by stimulation for 10 min with 100 nM fMLP. Release of the primary granule marker β-GU (*A*; *n* = 8), the secondary granule marker Lfr (*B*; *n* = 9–10), and the secretory vesicle marker HSA (*C*; *n* = 10) is shown. Data represent the mean $\pm$ SD of results from the indicated numbers of experiments. *, Significantly different ($p < 0.05$) from corresponding release in the absence of genistein. See the text for details on presentation of data.
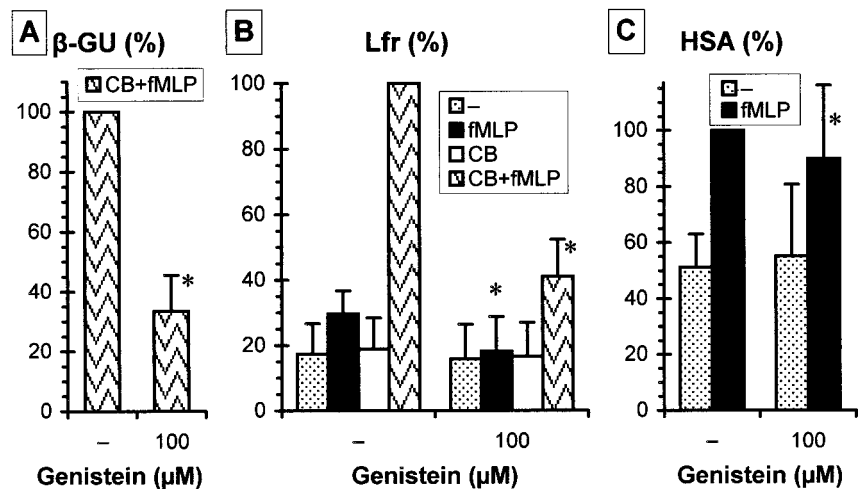
of the cell preparation. Cells were stimulated with 100 nM fMLP or by hyperosmotic shock (addition of an extra 120 mM NaCl; Ref. 28) for 2 min. The reaction was stopped by the addition of an equal volume of ice-cold PBS, followed by lysing the cells with a 5× concentrated lysis buffer. Triton-soluble fractions were then prepared as described above and precleared by incubating for 60 min with protein G-Sepharose beads (Pharmacia). The precleared lysates were incubated for 60 min with 5 μg/sample mouse monoclonal anti-human Syk Abs (Santa Cruz Biotechnology) and for a further 60 min with protein G-Sepharose beads. The beads were washed three times with PBS containing 0.1% Triton X-100 and 1 mM Na_3VO_4 and were boiled for 10 min in sample buffer. An aliquot of the supernatant was run on an 8% SDS-PAGE and immunoblotted using mouse monoclonal anti-phosphotyrosine Abs (clone 4G10 from Upstate Biotechnology). The Tyr phosphorylation of Syk was quantitated by densitometric analysis. The amounts of Syk immunoprecipitated from the different samples were compared by immunoblotting aliquots of the immunoprecipitates with the precipitating Abs.

Experiments on murine neutrophils

The $hck^{-/-}fgr^{-/-}lyn^{-/-}$ triple knockout mice deficient in all three Src family kinases present in neutrophils have been described previously (29). Preparation and stimulation of wild-type (C57BL/6) or $hck^{-/-}fgr^{-/-}lyn^{-/-}$ bone marrow neutrophils as well as determination of the release of the secondary granule marker Lfr were performed as previously described (13), using 1 μM fMLP as stimulus. Exocytosis of mouse serum albumin (MSA) was measured by ELISA, using rabbit anti-MSA Abs (unlabeled or peroxidase labeled for capturing and developing, respectively; both from Nordic Immunology, Tilburg, The Netherlands). The assay was calibrated using purified MSA (Sigma). Phosphorylation of murine p38 MAPK was determined as described for human neutrophils, except that 1 μM fMLP was used to induce a well measurable phosphorylation of the protein.

Presentation of data and statistical analysis

Degranulation assays were performed in triplicate or quadruplicate. The concentration of granule markers in the supernatants was expressed as a percentage of values of fMLP-stimulated samples of human cells without kinase inhibitors or of wild-type murine cells, in the presence (primary and secondary granules) or the absence (secretory vesicles) of CB. The 100% values for human cells corresponded to 29 $\pm$ 16% of the total cellular β-GU content in the case of primary granules (CB present; *n* = 8), 3.4 $\pm$ 1.3 μg Lfr/10^6 cells in the case of secondary granules (CB present; *n* = 19), and 189 $\pm$ 77 ng HSA/10^6 cells in the case of secretory vesicles (CB absent; *n* = 26). Further information on presentation of results of degranulation experiments is provided at the beginning of *Results*. Data for phosphorylation and kinase activity are also expressed as a percentage of the fMLP-stimulated values without kinase inhibitors, except for the Syk phosphorylation experiments, where data are expressed as the fold increase over values obtained from nonstimulated samples.

Because of the floating zero point of the fluorometer used, the results of the β-GU measurements reflect the differences between but not the absolute values of the β-GU activity of the samples. For this reason, only fMLP-stimulated values of β-GU (i.e., after subtraction of values in nonstimulated samples) are provided. None of the inhibitors used had any considerable effect on β-GU activity in supernatants of nonstimulated cells (not shown).

For statistical analysis throughout this paper, nonstimulated values were subtracted from fMLP-stimulated ones, and the obtained data were expressed as a percentage of similarly calculated values in the absence of kinase inhibitors or the $hck^{-/-}fgr^{-/-}lyn^{-/-}$ mutation. The mean $\pm$ SD of these values from the indicated number of experiments are provided. The difference in these normalized values from 100% was then analyzed using Student's paired two-population *t* test. Values of $p < 0.05$ at $n \geq 3$ were considered statistically significant.

Results

CB enhances the fMLP-induced release of primary and secondary granules, but not that of secretory vesicles of human neutrophils

In this report we investigated the signaling mechanisms leading to exocytosis of three different intracellular compartments of neutrophils: the primary and secondary granules as well as the secretory vesicles. The microfilament disrupting agent CB has long been known to enhance certain responses of neutrophils, including the release of primary and secondary granules (30). To determine optimal conditions for studying the exocytic activity of neutrophils we first investigated the effect of CB on the release of the three exocytic compartments tested. In accordance with previous findings (30), while fMLP induced a substantial release of the primary granule marker β-GU from human neutrophils pretreated with 10 μM CB, no exocytosis of β-GU was observed in the absence of CB (not shown). CB strongly increased fMLP-induced release of the secondary granule marker Lfr, but exocytosis of Lfr from CB-untreated neutrophils could also be easily observed (for example, see Fig. 1*B*). In contrast, the exocytosis of HSA, a constituent of the secretory vesicles, was maximal even in the absence of CB, and no further increase was observed after pretreating the cells with CB (not shown). On the basis of these findings, only degranulation of primary granules from CB-pretreated cells and release of secretory vesicles from CB-untreated cells are shown throughout this paper (Figs. 1, 3, 4, 5, and 7). However, data on Lfr release are provided in both the presence and the absence of CB. Comparing the effects of kinase inhibitors on Lfr release in the presence and the absence of CB allowed us to speculate about whether the drugs act on fMLP signaling or on the cytoskeletal changes induced by CB.
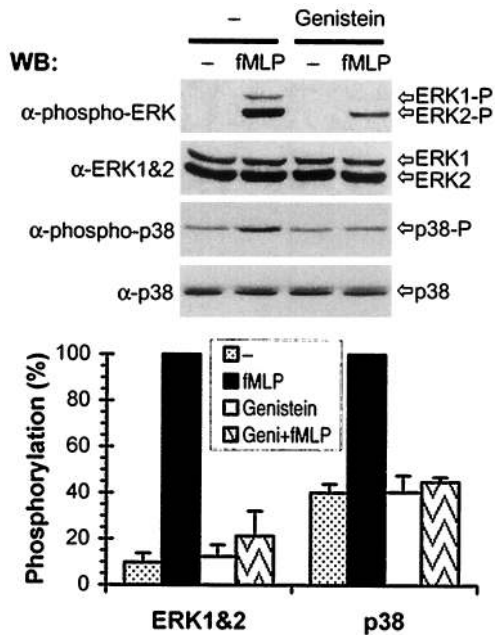
FIGURE 2. Effects of genistein on ERK and p38 MAPK phosphorylation. Human neutrophils were preincubated with or without 100 μM genistein (Geni), followed by stimulation for 2 min with 100 nM fMLP. Total cell lysates were subjected to immunoblotting (WB) using the specified Abs. The diagram represents the mean $\pm$ SD of results of densitometric analysis of two experiments.

The tyrosine kinase inhibitor genistein inhibits exocytosis of primary and secondary granules and phosphorylation of ERK and p38 MAPK

As shown in Fig. 1, 100 μM genistein, a broad specificity tyrosine kinase inhibitor, caused a strong inhibition of the fMLP-induced exocytosis of primary (67 $\pm$ 12% inhibition) and secondary (71 $\pm$ 14% inhibition) granules from CB-treated neutrophils. The fact that genistein also decreased the release of the secondary granule

marker Lfr from CB-untreated neutrophils (77 $\pm$ 17% inhibition) suggests that the drug affects a signaling pathway initiated by fMLP itself, instead of acting on the cytoskeletal changes induced by CB. On the other hand, the release of HSA, a marker of secretory vesicles, was only moderately influenced by 100 μM genistein (29 $\pm$ 22% inhibition). Taken together, these data suggest that tyrosine kinases are strongly involved in the fMLP-induced degranulation of primary and secondary granules, but to only a lesser extent in that of secretory vesicles.

Exocytosis of the secondary granules can be induced in an integrin-dependent (adherent) manner, and we have recently shown that this adherent exocytosis is mediated by tyrosine kinases (13). To exclude the possibility that adhesive (e.g., intercellular) interactions were also involved in the fMLP-induced exocytosis by suspended neutrophils, we exploited the evidence that integrin function requires the presence of Mg^{2+} ions in the extracellular medium (13, 25). Withdrawal of Mg^{2+} ions from the incubation medium had no effect on the fMLP-induced release of Lfr (CB absent; 102 $\pm$ 26% remaining exocytosis; $n = 4$), while it prevented the TNF-α-induced spreading of neutrophils over fibrinogen-coated surface (not shown), a response known to involve integrin activation (25). Thus, in the assay conditions that we employed, fMLP triggered the exocytosis of secondary granules in an integrin-independent manner.

One of the earliest events of fMLP signal transduction is the tyrosine phosphorylation of several intracellular proteins, including ERK (ERK1 and ERK2) and p38 MAPK proteins. As shown in Fig. 2, phosphorylation of ERK (90 $\pm$ 7% inhibition) and p38 MAPK (94 $\pm$ 9% inhibition) proteins was blocked by 100 μM genistein, raising the possibility that the inhibition of ERKs and/or that of p38 MAPK might mediate the effect of genistein on the degranulation response. To test this possibility, we next investigated the effect of pharmacological inhibition of the ERK and p38 MAPK pathways in fMLP-induced degranulation of neutrophils.

The p38 MAPK, but not the ERK, pathway is involved in the release of primary and secondary granules

Despite evidence of the rapid activation of the ERK cascade in response to fMLP stimulation (4, 14, 15) and the intensive search
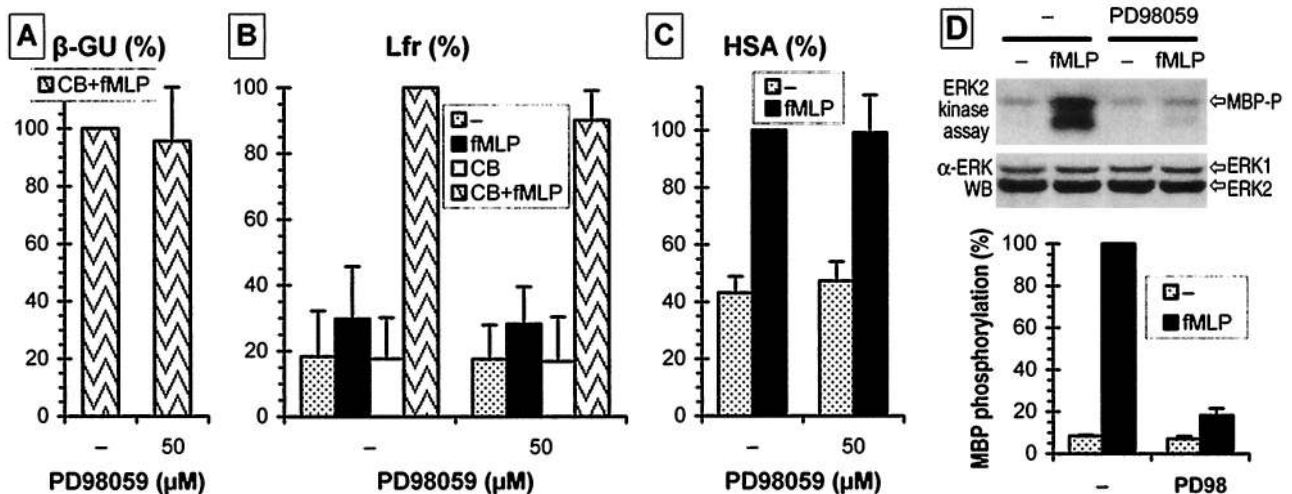
FIGURE 3. Effect of PD98059 on neutrophil degranulation and ERK activation. Human neutrophils were preincubated with or without 50 μM PD98059 and/or 10 μM CB, followed by stimulation for 10 (*A–C*) or 2 min (*D*) with 100 nM fMLP. Release of the primary granule marker β-GU (*A*; $n = 3$), the secondary granule marker Lfr (*B*; $n = 4$), and the secretory vesicle marker HSA (*C*; $n = 4$) is shown. Data represent the mean $\pm$ SD of results from the indicated numbers of experiments. See the text for details on presentation of data. *D*, Total cell lysates were subjected to in vitro ERK2 kinase assay or immunoblotting with a combination of anti-ERK1 and anti-ERK2 Abs (α-ERK WB). The diagram represents the mean $\pm$ SD of results from the ERK2 kinase assay in two experiments.
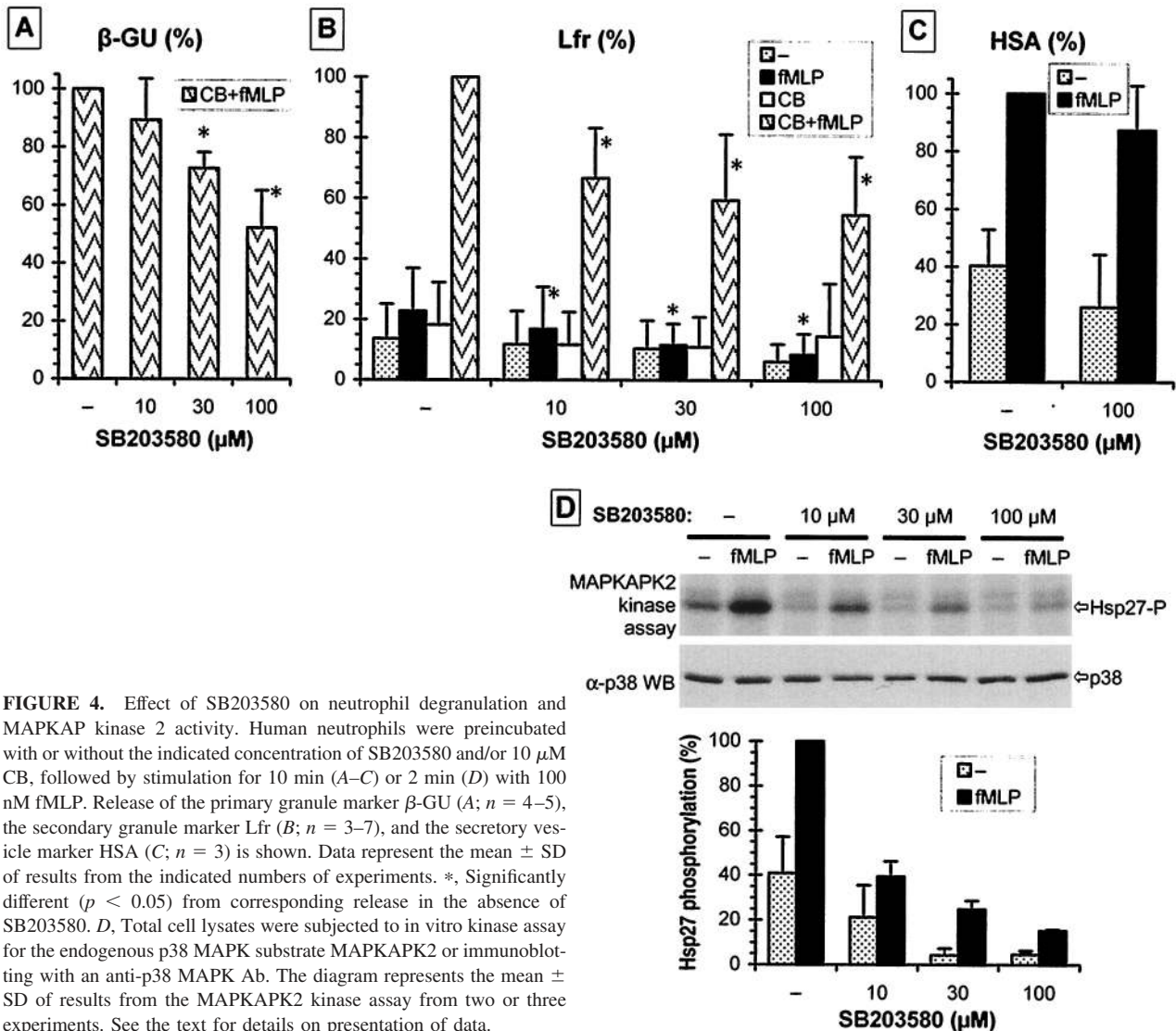
FIGURE 4. Effect of SB203580 on neutrophil degranulation and MAPKAP kinase 2 activity. Human neutrophils were preincubated with or without the indicated concentration of SB203580 and/or 10 μM CB, followed by stimulation for 10 min (A–C) or 2 min (D) with 100 nM fMLP. Release of the primary granule marker β-GU (A; n = 4–5), the secondary granule marker Lfr (B; n = 3–7), and the secretory vesicle marker HSA (C; n = 3) is shown. Data represent the mean $\pm$ SD of results from the indicated numbers of experiments. *, Significantly different ($p < 0.05$) from corresponding release in the absence of SB203580. D, Total cell lysates were subjected to in vitro kinase assay for the endogenous p38 MAPK substrate MAPKAPK2 or immunoblotting with an anti-p38 MAPK Ab. The diagram represents the mean $\pm$ SD of results from the MAPKAPK2 kinase assay from two or three experiments. See the text for details on presentation of data.

for its function in the fMLP-induced responses (8, 31, 32), no clear data supporting such a role has been presented. To determine the involvement of the ERK cascade in the exocytic activity of neutrophils, we used PD98059, an inhibitor of MEK1 and MEK2, the kinases responsible for phosphorylation of the ERK1 and ERK2 proteins (33). As shown in Fig. 3, 50 μM PD98059 had no considerable effect on the release of β-GU (96 $\pm$ 19% remaining degranulation), Lfr (109 $\pm$ 37 and 89 $\pm$ 9% remaining degranulation in the absence and the presence of CB, respectively), or HSA (91 $\pm$ 24% remaining activity). Under identical conditions, 50 μM PD98059 strongly inhibited fMLP-induced activation of the ERK cascade as determined using an in vitro ERK2 kinase assay (Fig. 3D; 88 $\pm$ 2% inhibition) or by immunoblotting using anti-phospho-ERK Abs (not shown). These data suggest that the ERK pathway, although clearly activated by fMLP, plays no role in triggering the exocytic responses of neutrophils.

In contrast to the ERK pathway, the p38 MAPK cascade has been implicated in fMLP-induced superoxide production and chemotaxis (6, 26) as well as in several responses triggered by TNF or LPS stimulation (26, 34) in neutrophils. We tested the role of p38 MAPK in the fMLP-induced exocytic activity of neutrophils using SB203580, an inhibitor of this kinase (35, 36). As shown in Fig. 4, SB203580 decreased the exocytosis of primary and secondary

granules from CB-pretreated neutrophils, reaching 48 $\pm$ 13 and 51 $\pm$ 19% inhibition, respectively, at a 100 μM inhibitor concentration. The release of Lfr from CB-untreated neutrophils was also reduced by the drug (62 $\pm$ 5% inhibition at 100 μM SB203580). However, 100 μM SB203580 had no effect (102 $\pm$ 27% remaining exocytosis) on the release of the secretory vesicle marker HSA. The effect of SB203580 on p38 MAPK activity was determined using an in vitro kinase assay for MAPKAPK2, a known in vivo substrate of p38 MAPK (5). Fig. 4D shows that SB203580 blocked the activation of MAPKAPK2 in a concentration-dependent manner, reaching 85 $\pm$ 1% inhibition at 100 μM. Taken together, these data indicate that the p38 MAPK pathway is involved in the fMLP-induced release of primary and secondary granules, but not in that of secretory vesicles. It should be noted, however, that while a major part of the inhibition of secondary granule release and MAPKAPK2 activation was attained by 10 μM SB203580, a moderately higher concentration of the drug was required to prevent the exocytosis of primary granules. This observation points to a possible difference between the signaling of the two granule populations.

It should also be noted that in addition to the classical p38 MAPK (also known as p38α or SAPK2a), a novel isoform of p38
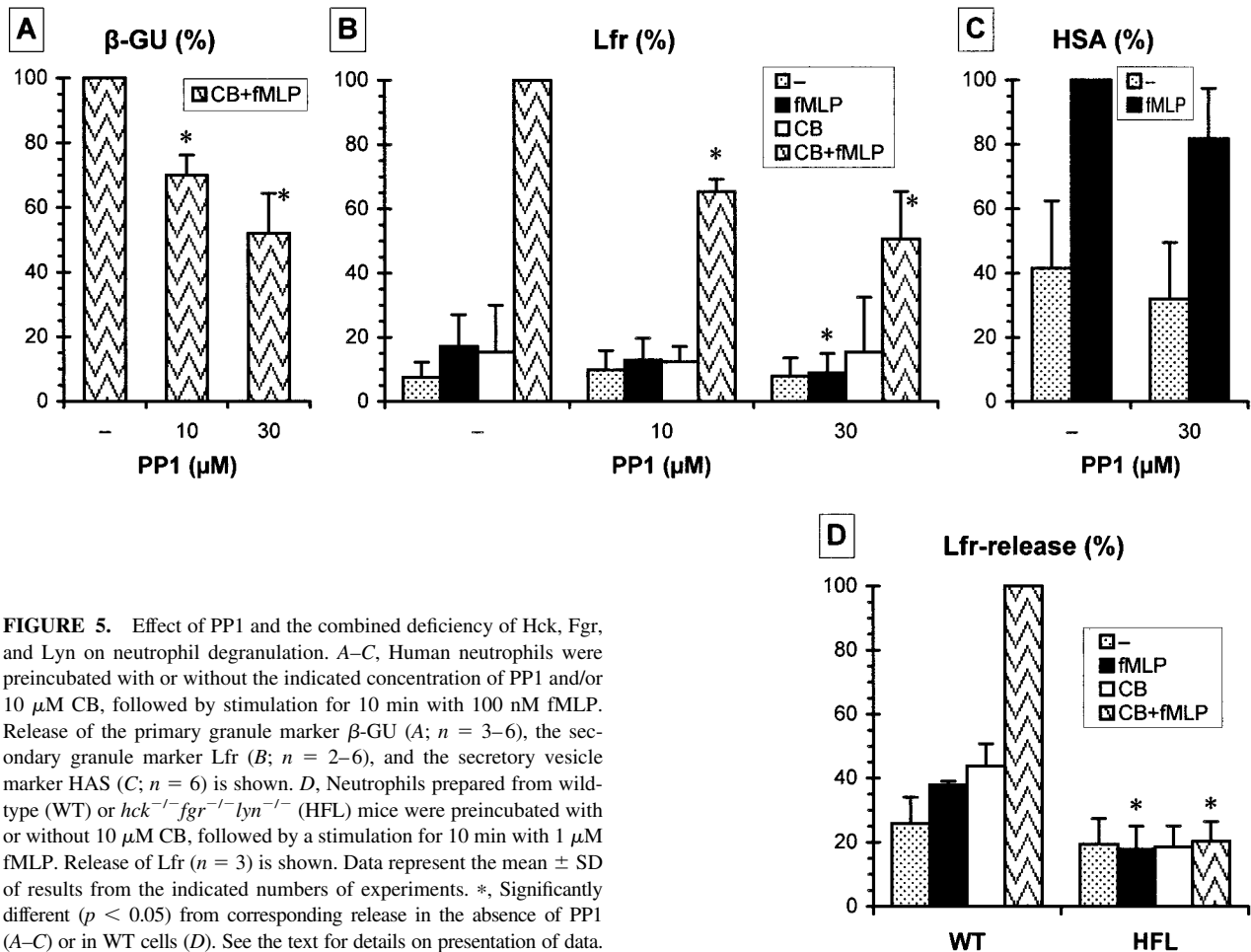
FIGURE 5. Effect of PP1 and the combined deficiency of Hck, Fgr, and Lyn on neutrophil degranulation. *A–C,* Human neutrophils were preincubated with or without the indicated concentration of PP1 and/or 10 μM CB, followed by stimulation for 10 min with 100 nM fMLP. Release of the primary granule marker β-GU (*A; n = 3–6*), the secondary granule marker Lfr (*B; n = 2–6*), and the secretory vesicle marker HAS (*C; n = 6*) is shown. *D,* Neutrophils prepared from wild-type (WT) or $hck^{-/-}fgr^{-/-}lyn^{-/-}$ (HFL) mice were preincubated with or without 10 μM CB, followed by a stimulation for 10 min with 1 μM fMLP. Release of Lfr (*n = 3*) is shown. Data represent the mean $\pm$ SD of results from the indicated numbers of experiments. ∗, Significantly different (*p < 0.05*) from corresponding release in the absence of PP1 (*A–C*) or in WT cells (*D*). See the text for details on presentation of data.

MAPK, p38δ (or SAPK4) has been shown to be present in neutrophils (37, 38). Although LPS stimulation of neutrophils activates only p38α, treatment of the cells with H_2O_2 activates both p38α and p38δ (37). At present it is not known which of the two isoforms becomes activated in response to fMLP stimulation. In contrast to p38α, p38δ is not sensitive to SB203580 (39, 40) and does not phosphorylate MAPKAPK2 (39–41). Thus, the above experiments provide information only on the role of the classical isoform, p38α.

The role of Src family kinases in neutrophil degranulation and activation of p38 MAPK

In the last few years great progress has been made in understanding the role of the different tyrosine kinases in the regulation of neutrophil functions (9). Several reports implicated the Src kinase family in different aspects of the fMLP-induced degranulation process (2, 3, 9–12). These findings together with the effect of genistein on the degranulation response prompted us to investigate the role of Src-related kinases in the exocytic activity of the different granules and secretory vesicles of neutrophils.

As shown in Fig. 5, PP1, a selective inhibitor of Src family kinases (42, 43) diminished the exocytosis of primary and secondary granules from CB-pretreated human neutrophils (48 $\pm$ 12 and 58 $\pm$ 11% inhibition, respectively, at 30 μM PP1). PP1 also prevented the release of Lfr from CB-untreated neutrophils (83 $\pm$ 11% inhibition at 30 μM PP1), but had no considerable effect on the release of the secretory vesicle marker HSA (92 $\pm$ 26% remaining activity at 30 μM PP1).

To confirm that the effect of PP1 on granule release was in fact due to its action on Src family kinases, we investigated the fMLP-induced release of Lfr from neutrophils prepared from mice deficient in all three Src family kinases (Hck, Fgr, and Lyn) present in neutrophils (29). As shown in Fig. 5*D*, exocytosis of Lfr was strongly decreased in $hck^{-/-}fgr^{-/-}lyn^{-/-}$ triple mutant neutrophils compared with wild-type cells (97 $\pm$ 2 and 119 $\pm$ 14% inhibition in the presence and the absence of CB, respectively). This finding was in accordance with our previous observation that in the presence of CB, fMLP-induced release of Lfr was significantly decreased in $hck^{-/-}fgr^{-/-}$ double-mutant or PP1-treated wild-type murine neutrophils (13) and confirmed that Src family kinases are in fact involved in the exocytic activity of neutrophils. The more pronounced effect of the $hck^{-/-}fgr^{-/-}lyn^{-/-}$ mutation compared with that of PP1 probably reflects the complete absence of any Src-related kinase activity in the $hck^{-/-}fgr^{-/-}lyn^{-/-}$ neutrophils, while some residual activity might be present in the PP1-treated cells.

Unfortunately, we were unable to study the fMLP-induced exocytosis of primary granules in the $hck^{-/-}fgr^{-/-}lyn^{-/-}$ mice because murine neutrophils do not release their intracellular β-GU enzymes under conditions inducing strong release of β-GU from human or of Lfr from murine cells (13). Surprisingly, we could not observe consistent release of MSA from wild-type or mutant cells in response to 1 μM fMLP, i.e., under conditions causing maximal release of HSA from human neutrophils (data not shown). While this fact did not allow us to study fMLP-induced release of MSA from $hck^{-/-}fgr^{-/-}lyn^{-/-}$ neutrophils, we did consistently observe
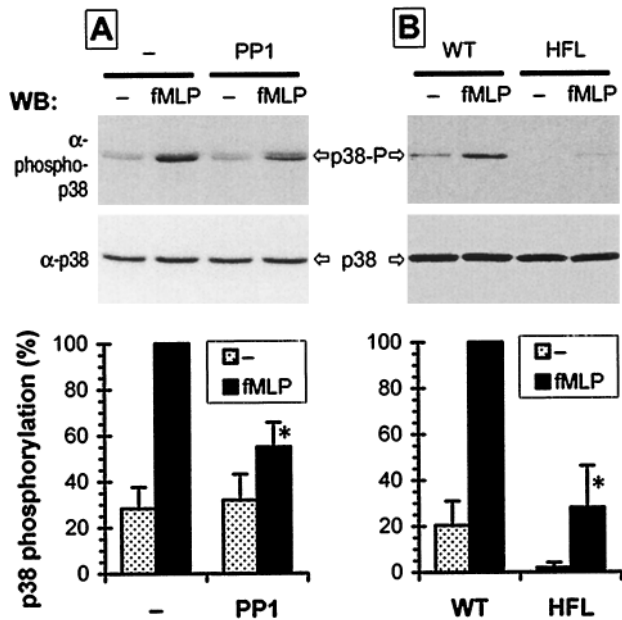
FIGURE 6. Effect of PP1 and the combined deficiency of Hck, Fgr, and Lyn on phosphorylation of p38 MAPK. Total cell lysates of fMLP-stimulated neutrophils were subjected to immunoblotting (WB) using the specified Abs. *A*, Results obtained with human neutrophils pretreated with or without 30 μM PP1, followed by a 2-min stimulation with 100 nM fMLP. *B*, Results obtained with neutrophils form wild-type (WT) or $hck^{-/-}fgr^{-/-}lyn^{-/-}$ (HFL) mice stimulated for 1 min with 1 μM fMLP. The diagrams represent the mean $\pm$ SD of results from densitometric analysis in three experiments. *, Significantly different ($p < 0.05$) from corresponding values in the absence of PP1 (*A*) or in WT cells (*B*).

that, similar to the effect of PP1 on HSA release (Fig. 5*C*), the unstimulated release of MSA was significantly lower in the triple mutant than in the wild-type cells (data not shown).

Several reports have implicated Src family kinases in the activation of the ERK pathway by G proteins (44–46), but very little is known of their role in the activation of p38 MAPK. This fact together with our observation that both the p38 MAPK pathway and Src family kinases seem to be involved in degranulation of neutrophils prompted us to examine the possible involvement of Src family kinases in the fMLP-induced activation of p38 MAPK.

As shown in Fig. 6*A*, 30 μM PP1 exerted a strong (67 $\pm$ 17%) inhibition on the fMLP-induced phosphorylation of p38 MAPK in human neutrophils. Similarly, phosphorylation of p38 MAPK was strongly decreased in $hck^{-/-}fgr^{-/-}lyn^{-/-}$ triple-mutant murine neutrophils (Fig. 6*B*; 67 $\pm$ 23% decrease in phosphorylation). These observations suggest that Src family kinases play a major role in linking the G_i-coupled fMLP receptor to the p38 MAPK pathway.

Taken together, our observations presented in Figs. 4–6 suggest that the fMLP-induced release of primary and secondary granules is mediated by p38 MAPK activated by a Src family kinase-dependent mechanism.

Piceatannol inhibits both degranulation and activation of p38 MAPK

Besides Src family tyrosine kinases, Syk, a member of the Syk/ ZAP-70 kinase family is also present in neutrophils (47). Activation of Syk by agonists of neutrophils (19, 47, 48) and the closely related functions of the Src and Syk/ZAP-70 kinase families in several cell types (16–19) prompted us to investigate the role of Syk in the signaling mechanisms initiated by fMLP in neutrophils.

As shown in Fig. 7, piceatannol, a reportedly selective inhibitor of Syk (49), exerted a strong inhibition of the fMLP-induced release of primary granules (69 $\pm$ 8% inhibition at 100 μM). The drug also prevented the exocytosis of secondary granules in both the presence and the absence of CB (71 $\pm$ 6 and 83 $\pm$ 4% inhibition, respectively, at 100 μM), but exerted a statistically nonsignificant effect on the release of secretory vesicles (49 $\pm$ 30% inhibition at 100 μM). Furthermore, as shown in Fig. 7*D*, piceatannol prevented the activation of p38 MAPK triggered by fMLP (74 $\pm$ 21% inhibition). These data resembled the effects of genistein and PP1 as well as the responses observed in $hck^{-/-}fgr^{-/-}lyn^{-/-}$ neutrophils and raised the possibility that Syk, similar to its role in several other cellular systems, participates in a signaling pathway closely related to the Src family of tyrosine kinases.

Next we investigated the possible activation of Syk by fMLP in neutrophils. These experiments had two purposes. First, we planned to reveal whether Syk, similar to the immunoreceptor signaling pathways (16–18), is activated by an Src family-dependent mechanism in fMLP-treated neutrophils. Second, since a recent report raised the possibility of a nonspecific effect of piceatannol (50), we aimed to determine whether the effect of the drug on the degranulation and p38 MAPK activation correlated with that on the activity of Syk. Because the activity of Syk is closely related to its tyrosine phosphorylation, we investigated the tyrosine phosphorylation status of Syk in fMLP-stimulated neutrophils. Unexpectedly, the tyrosine phosphorylation of Syk precipitated from lysates of neutrophils stimulated for 2 min by 100 nM fMLP did not differ from that of unstimulated cells (94 $\pm$ 6% remaining phosphorylation; Fig. 7*E*), while a strong (2.7 $\pm$ 1.8-fold) increase in the Tyr phosphorylation of Syk was observed in neutrophils subjected to a hyperosmotic shock (addition of an extra 120 mM NaCl for 2 min) (28), showing that our method was capable of determining changes in the tyrosine phosphorylation status of Syk. We did not observe any increase in Syk Tyr phosphorylation when the cells were treated for a shorter (30 to 60 s) period, stimulated with 1 μM fMLP, preincubated with 10 μM CB or when pretreatment with DFP was omitted (data not shown). We did not observe any fMLP-induced increase in Syk activity when the immunoprecipitates were subjected to an in vitro kinase assay (data not shown). Although these data do not exclude the involvement of Syk in the signaling mechanisms initiated by fMLP in neutrophils, they point to the need for more specific approaches to define the role of this enzyme in the fMLP-induced neutrophil responses.

Discussion

Our results indicate that tyrosine phosphorylation plays an important role in the signaling events leading from stimulation of fMLP receptors to exocytosis of the primary and secondary granules of human neutrophils. The release of these two granule populations displayed a similar sensitivity to the inhibitors we used, whereas significant differences were observed when studying the exocytosis of the secretory vesicles (Figs. 1, 4, 5, and 7). In fact, tyrosine kinase activity does not seem to play a decisive role in the release of the secretory vesicles upon chemotactic stimulation.

The use of inhibitors acting on different tyrosine phosphorylation-dependent signaling pathways as well as the study of neutrophils genetically deficient in Src family kinases revealed that several types of kinases are involved in the exocytic process of primary and secondary granules. The following observations indicate that phosphorylation and consequent activation of p38 MAPK represents a key element: 1) the rapid activation of p38 MAPK, following the ligand binding to fMLP receptors, precedes or coincides with the degranulation response (5); 2) the decrease in
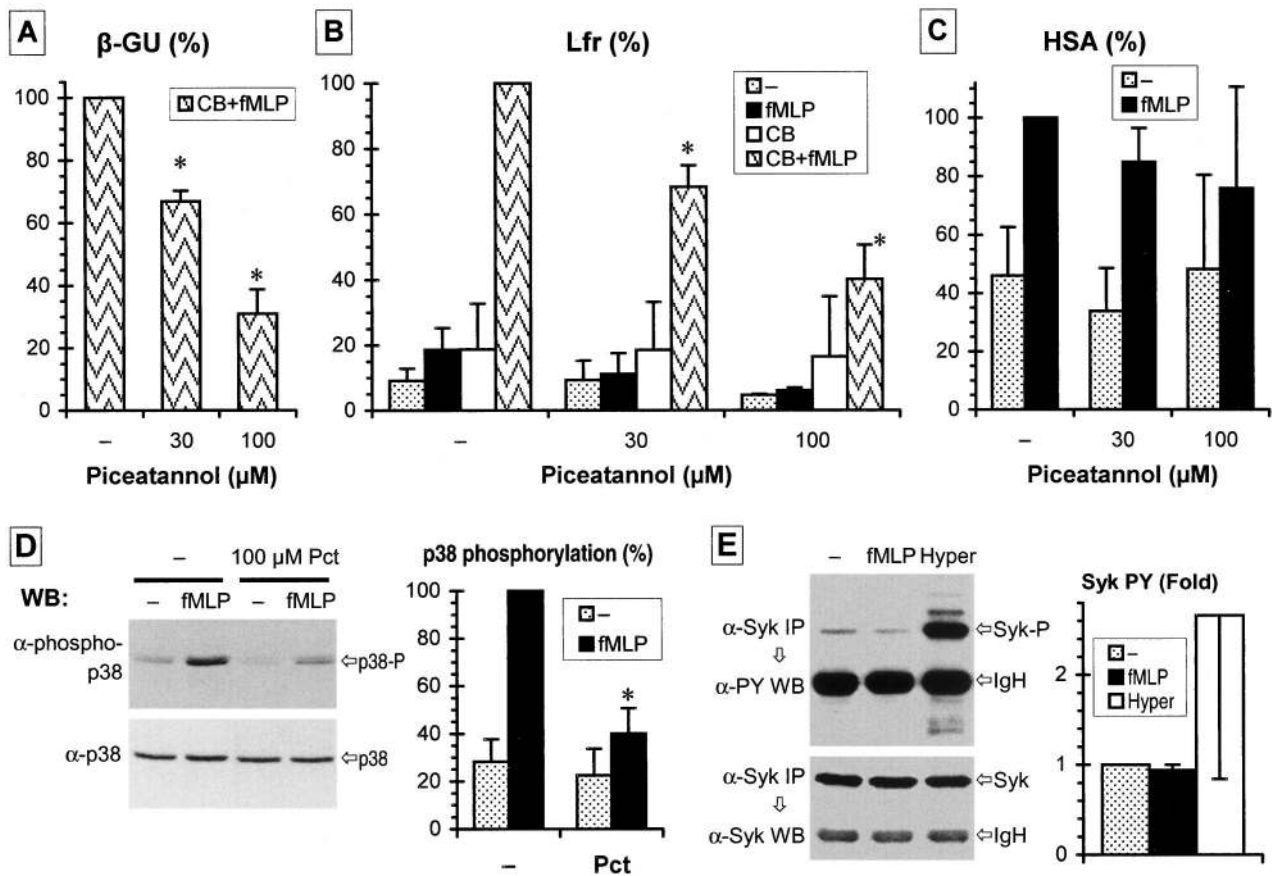
FIGURE 7. Effect of piceatannol on neutrophil responses and tyrosine phosphorylation of Syk. Human neutrophils were preincubated with or without the indicated concentration of piceatannol (Pct) and/or 10 μM CB, followed by stimulation for 10 (A–C) or 2 min (D and E) with 100 nM fMLP or by hyperosmotic shock (addition of an extra 120 mM NaCl; Hyper). Release of the primary granule marker β-GU (A; n = 3–4), the secondary granule marker Lfr (B; n = 2–4), and the secretory vesicle marker HSA (C; n = 3–4) is shown. Data represent the mean ± SD of results from the indicated numbers of experiments. D and E, Total cell lysates (D) or Syk immunoprecipitates (IP; E) were subjected to immunoblotting (WB) using the specified Abs. Diagrams represent the mean ± SD of results of densitometric analysis from three experiments. PY, phospho-tyrosine; IgH, Ig heavy chain. ∗, Significantly different ($p < 0.05$) from corresponding release in the absence of piceatannol. See the text for details on presentation of data.

degranulation in response to treatment with genistein, PP1, and piceatannol or in the $hck^{-/-}fgr^{-/-}lyn^{-/-}$ neutrophils (Figs. 1, 5, and 7) correlates with a similar decrease in the phosphorylation of p38 MAPK (Figs. 2, 6, and 7); and, most importantly, 3) direct inhibition of p38 MAPK activity by SB203580 impairs the degranulation response (Fig. 4). To our knowledge this is the first demonstration of the involvement of p38 MAPK in exocytosis from neutrophils. However, it should be noted that the effective inhibition of p38 MAPK by SB203580 results in only ~50% inhibition of the release of granule contents. This finding points to the existence of a signaling pathway that does not involve the p38 MAPK cascade (or proceeds through p38δ, the p38 MAPK isoform of neutrophils not sensitive to SB203580). The fact that genistein had a more profound effect on degranulation than SB203580 (Figs. 1 and 4), while both drugs strongly inhibited the p38 MAPK pathway (Figs. 2 and 4), suggests that some of the pathways bypassing the SB203580-sensitive p38 MAPK also require tyrosine kinase activity.

It should be noted that SB203580 has recently been reported to affect the Raf protein kinase. Paradoxically, while in vitro treatment with SB203580 inhibited the activity of Raf, the in vivo treatment of the cells led to an activation of the kinase (51, 52). Nevertheless, the inhibitor did not influence the signaling pathways known to be upstream or downstream of Raf (51, 52). These

uncertainties together with the fact that the ERK cascade, the main downstream effector of Raf, does not seem to be involved in the degranulation process (Fig. 3), suggest that the effect of SB203580 on the release of primary and secondary granules is not mediated by its influence on Raf.

Inhibition of the ERK cascade by PD98059 had no effect on the fMLP-induced exocytosis of secretory vesicles, and in accordance with previous findings (8, 31, 32), it did not affect the release of primary or secondary granule marker proteins either (Fig. 3). It should be noted that despite its immediate and profound activation, clear evidence for a functional role of ERKs in any of the fMLP-induced responses of neutrophils is still lacking.

fMLP has also been shown to activate Src family kinases in neutrophils (2, 3, 9, 10). These kinases seem to participate in the signaling of the exocytic process, because application of the Src family-selective inhibitor PP1 or the genetic deficiency of the Src family kinases expressed in neutrophils significantly decreased the fMLP-induced granule release (Fig. 5). This is in accord with our previous findings suggesting a role for Src family kinases in the fMLP-induced degranulation of the secondary granules in murine neutrophils (13).

The observation that both PP1 and the genetic deficiency of Src family kinases prevented activation of p38 MAPK (Fig. 6) places the site of action of Src-related kinases between the receptor and

p38 MAPK. These data together with those presented in Figs. 4 and 5 suggest that the exocytosis of primary and secondary granules proceeds through p38 MAPK activated by a Src family kinase-dependent mechanism.

In addition to providing information on neutrophil functions, some of the experiments presented in this paper may be relevant to G protein signal transduction in other cell types as well. In the last years great effort has been made to elucidate the mechanisms linking G protein-coupled receptors to the MAP kinase cascades (for review, see Ref. 53). It has been shown that nonreceptor tyrosine kinases activated by G proteins impinge on the Ras-Raf-ERK pathway through tyrosine phosphorylation of Shc and recruitment of Grb2 and Sos to the plasma membrane. The tyrosine kinases implicated in the G protein-mediated activation of ERK include members of the Src family as well as Syk, Btk, Csk, and Pyk2 (44–46, 54). On the other hand, despite several observations showing activation of the p38 MAPK pathway by G protein-coupled receptors, very little is known about the role of tyrosine kinases in this process. To our knowledge, the only relevant information on this topic was provided by Nagao et al. (55), who described the involvement of Src family kinases in the activation of p38 MAPK by a constitutively active form of $G_{\alpha 11}$. Our finding that Src-related kinases participate in the activation of the p38 MAPK by fMLP in neutrophils (Fig. 6) raises the possibility that members of the Src family have a more general role in linking G protein-coupled receptors to the p38 MAPK pathway in different cell types of the organism.

In this study we also tried to gain more information about the role of the Syk tyrosine kinase in the fMLP signaling of neutrophils. Piceatannol, a reportedly selective inhibitor of Syk, prevented the fMLP-induced release of primary and secondary granules as well as the activation of p38 MAPK (Fig. 7). The effect of the drug on granule release is in accordance with previous reports showing that Syk plays a major role in degranulation of mast cells and basophilic leukemia cells mediated by $F_C\epsilon$-receptor activation (49, 56). Furthermore, the pattern of inhibition of neutrophil responses by piceatannol resembles the effect of PP1 and that of Src kinase deficiency, raising the possibility that Syk might participate in the same pathway linking fMLP receptors to the degranulation response via Src family kinases and p38 MAPK.

Surprisingly, the concentration of piceatannol effectively inhibiting the fMLP-induced degranulation of CB-treated neutrophils (30–100 μM) is an order of magnitude higher than that recently described to inhibit spreading and H_2O_2 release of adherent neutrophils (3–10 μM) (57). Interestingly, a similar difference between adhesion-dependent responses and degranulation in suspension exists in the case of genistein and PP1 as well. Although concentrations of genistein as low as 2–10 μM are sufficient to block the adhesion-dependent superoxide release of neutrophils (58), 25–100 μM of the drug is required to block granule release in suspension (Fig. 1 and Ref. 8). PP1 (10 μM) almost completely abolishes the adhesion-dependent Lfr release of human and murine neutrophils (13), whereas 20–30 μM was required to cause a partial decrease in the degranulation of suspended cells (Fig. 5) (13). In light of the fact that degranulation in suspension is defective in both $hck^{-/-}fgr^{-/-}$ (13) and $hck^{-/-}fgr^{-/-}lyn^{-/-}$ neutrophils (Fig. 5D), it is highly unlikely that the inhibition of degranulation in suspension by the latter two compounds was due to a nonspecific effect. Furthermore, the effective concentration of piceatannol in inhibiting the granule release in suspension falls within the range (50–100 μM; i.e., 12–24 μg/ml) required for inhibiting several cellular responses as well as the in vivo activation of Syk or ZAP-70 in other cell types (59–62). Thus, the above-mentioned

differences can reflect the extreme sensitivity of the adhesion-dependent neutrophil responses to tyrosine kinase inhibitors, rather than the nonspecific action of all three compounds. This high sensitivity of the adhesion-dependent functions might be related to tyrosine kinases involved in pathways (e.g., integrin signaling or the cytoskeletal rearrangement required for cell spreading) used by adhesion-dependent, but not by fMLP-induced, activation mechanisms.

However, while initial studies reported piceatannol to be highly selective toward Syk (49), a recent observation showed the inhibition of other tyrosine kinases by high concentrations of the drug (50). Although Src family kinases are not or are only minimally sensitive to piceatannol (49, 50, 59, 60), the compound inhibited focal adhesion kinase (Fak) at moderately high concentrations (50). The fact that neutrophils contain both Fak (57, 63, 64) and Pyk2 (28, 65, 66), a kinase closely related to Fak, raises the possibility that, in addition to Syk, piceatannol might affect other kinases present in neutrophils. Our finding that despite the effects of piceatannol on degranulation and p38 MAPK activation, no increase in the tyrosine phosphorylation of Syk in fMLP-stimulated neutrophils could be observed (Fig. 7E) supports the possibility of a nonspecific action of the drug. All these data point to the need for more specific approaches to determine whether Syk is in fact involved in fMLP signaling in neutrophils (e.g., by having a permissive effect or being activated only in a minor compartment of the cell) or whether piceatannol exerts its effect by inhibiting other cellular targets (e.g., members of the Fak kinase family). Solving this question is hampered by the fact that deficiency of the *syk* gene leads to perinatal lethality in mice (67, 68), making studies of $syk^{-/-}$ neutrophils difficult to attain.

Taken together, our observations based on pharmacological and gene knockout approaches suggest that the binding of fMLP to its G_i-coupled seven-transmembrane receptor on the surface of neutrophils leads to the phosphorylation of p38 MAPK by a mechanism involving Src family kinases. p38 MAPK, in turn, plays a role in dictating, by an as yet unidentified mechanism, the release of both primary and secondary granules of the cells. The possible involvement of Syk in the above processes requires further consideration.

Acknowledgments

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