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Chemotactic Factor-Induced Recruitment and Activation of Tec Family Kinases in Human Neutrophils. II. Effects of LFM-A13, a Specific Btk Inhibitor[1]

Caroline Gilbert,* Sylvain Levasseur,* Philippe Desaulniers,* Andrée-Anne Dusseault,* Nathalie Thibault,* Sylvain G. Bourgoin,† and Paul H. Naccache[2]*

Tyrosine phosphorylation events play major roles in the initiation and regulation of several functional responses of human neutrophils stimulated by chemotactic factors such as the bacterially derived tripeptide formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe). However, the links between the G protein-coupled receptors, the activation of the tyrosine kinases, and the initiation of neutrophil functional responses remain unclear. In the present study we assessed the effects of a Btk inhibitor, leflunomide metabolite analog (LFM-A13), on neutrophils. LFM-A13 decreased the tyrosine phosphorylation induced by fMet-Leu-Phe and inhibited the production of superoxide anions and the stimulation of adhesion, chemotaxis, and phospholipase D activity. We observed a decreased accumulation of phosphatidylinositol-3,4,5-trisphosphate in response to fMet-Leu-Phe in LFM-A13-pretreated cells even though the inhibitor had no direct effect on the lipid kinase activity of the p110γ or p85/p110 phosphatidylinositol 3-kinases or on the activation of p110γ by fMet-Leu-Phe. The phosphorylation of Akt and of extracellular signal-regulated kinases 1/2 and p38 were similarly inhibited by LFM-A13. LFM-A13 also negatively affected the translocation of Rac-2, RhoA, ADP ribosylation factor-1, Tec, Bmx, and Btk induced by fMet-Leu-Phe. The results of this study provide evidence for an involvement of Btk and possibly other Tec kinase family members in the regulation of the functional responsiveness of human neutrophils and link these events, in part at least, to the modulation of levels of phosphatidylinositol-3,4,5-trisphosphate. ***The Journal of Immunology,*** **2003, 170: 5235–5243.**

The activation of the human polymorphonuclear neutrophils by the vast majority of the agents inducing its directed migration to sites of injury or infection (chemotactic factors and chemokines) is mediated by their interaction with G protein-coupled receptors (GPCRs)[3] (1). The stimulation of these receptors leads to activation of a variety of signal transduction pathways, prominent among which are those relying on phospholipase C (2–5) and the tyrosine phosphorylation cascade(s) (6–8). While much is known about the coupling of G proteins to phospholipase C, the initial steps involved in the initiation of the tyrosine phosphorylation cascade(s) upon engagement of GPCRs remain, for the most part, undetermined. This lack of knowledge contrasts with the documented importance of tyrosine phosphorylation events in the initiation and/or regulation of several neutrophil functions established using correlative, pharmacological (7–11), and genetic approaches (12–15) .

A novel link between GPCRs and tyrosine kinases has recently been suggested (16). Occupation of the formylated peptide and IL-8 receptors on human neutrophils has been shown to result in activation of the G protein-dependent phosphatidylinositol 3-kinase isoform called p110γ (17). This response correlates closely with the previously described accumulation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) (18, 19). Of particular relevance to the subject of the present investigation, stimulation of human neutrophils by chemotactic factors has very recently been observed to be associated with the translocation and activation of the PH domain-containing tyrosine kinases of the Tec family termed Tec, Btk and Bmx (16). A causative link between these two events is strongly suggested by the high affinity of the pleckstrin homology (PH) domains of Tec and Btk for PtdIns(3,4,5)P_3 (20–22) and by the wortmannin sensitivity of the recruitment of Tec kinases (16) and of the stimulation of the tyrosine phosphorylation cascade by chemotactic factors (23).

The present study was initiated to characterize the functional significance of the activation of Tec family kinases in human neutrophils. As genetic or molecular biological approaches are very difficult, if not impossible, at present to apply to the terminally differentiated neutrophils, an alternative pharmacological approach based on the use of a recently described Btk inhibitor, leflunomide metabolite analog (LFM-A13) (24), was used. The latter was found to affect the tyrosine phosphorylation profile, adhesive, chemotactic and oxidative responses to the chemotactic factor formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe). Evidence was obtained that LFM-A13 inhibited the stimulation of phospholipase D (PLD) and the accumulation of PtdIns(3,4,5)P_3. Incubation with LFM-A13 resulted in a decreased activation of the mitogen-activated protein kinase (MAPK) and Akt pathways induced by fMet-Leu-Phe.

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[3] Abbreviations used in this paper: GPCR, G protein-coupled receptor; Arf-1, ADP ribosylation factor-1; DFP, di-isopropylfluorophosphate; Erk, extracellular signal-regulated kinase; fMet-Leu-Phe, formylmethionyl-leucyl-phenylalanine; MAPK, mitogen-activated protein kinase; PDK, 3-phosphoinositide-dependent kinase; PH, pleckstrin homology; PI, phosphoinositol; PLD, phospholipase D; PtdIns(3,4,5)P_3, phosphatidylinositol-3,4,5-trisphosphate.

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LFM-A13 also inhibited the translocation of Tec kinases (Btk, Bmx, and Tec) and small GTPases (ADP ribosylation factor-1 (Arf-1), Rac-2, and RhoA) elicited by fMet-Leu-Phe. These data provide the first evidence for a role of Tec family kinases in the regulation of the functional responsiveness of human neutrophils.

Materials and Methods

Antibodies

The anti-Bmx (SC-8873), anti-Tec (SC-1109), anti-RhoA (SC-179), and anti-Rac-2 (SC-96) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Btk (65251A) and anti-extracellular signal-regulated kinase (anti-Erk1; M12320) Abs were obtained from BD Transduction Laboratories (Mississauga, Canada). The anti-phosphotyrosine Ab (UBI 05–321, clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). The anti-phospho-Erk1/2 (44-680) was obtained from BioSource (Camarillo, CA). The anti-phospho-p38 (9216), anti-p38 (9212), anti-phospho-Akt (phospho-Ser^{473}, no. 9271; phospho-Thr^{308}, no. 9275), and anti-Akt (no. 9272) were purchased from Cell Signaling Technology (Beverly, MA). The anti-Arf-1 rabbit serum was generated in-house as previously described (25).

Reagents

Di-isopropylfluorophosphate (DFP), wortmannin, and fMet-Leu-Phe were purchased from Sigma-Aldrich (St. Louis, MO). Dextran T-500 and Ficoll-Paque were purchased from Pharmacia Biotech (Dorval, Canada). Cytochrome *c* was purchased from ICN Biomedical (Aurora, OH). LFM-A13 and its inactive analog, LFM-A11, were purchased from Calbiochem (San Diego, CA).

Neutrophil purification

Peripheral blood from healthy adult volunteers collected on isocitrate anticoagulant and the neutrophils were purified sterilely as previously described (26), except that the sedimentation of erythrocytes was performed with a 2% dextran solution diluted in HBSS containing 1.6 mM calcium and no magnesium, pH 7.4. Neutrophils were resuspended in HBSS. Cell preparations with nonspherical cell morphology (an indication of preactivation) were excluded from the study.

Tyrosine phosphorylation and immunoblotting of total cell lysates

Neutrophil suspensions (2×10^7 cells/ml) were preincubated at room temperature with 1 mM DFP and with or without the indicated concentrations of LFM-A13 or LFM-A11 for 10 min at 37°C before stimulation by fMet-Leu-Phe (100 nM) for the indicated times. The reactions were stopped by transferring 100 μl of the cell suspensions to an equal volume of boiling 2× Laemmli sample buffer (1× = 62.5 mM Tris-HCl (pH 6.8), 4% SDS, 5% 2-ME, 8.5% glycerol, 2.5 mM orthovanadate, 10 mM paranitro-phenylphosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.025% bromophenol blue) and boiled for 7 min. The samples were then subjected to 7.5–20% SDS-PAGE and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Immunoblotting was performed using the anti-phosphotyrosine mAb 4G10 (1/4,000), anti-Erk (1/2,500), anti-p38 (pThr/pY 180/182; 1/1,000), and the polyclonal anti-p38, anti-Erk1/2 (pThr/pY 180/182; 1/1,000), and anti-Akt (pSer^{473}; 1/1,000) and was revealed using the Renaissance detection system (NEN Life Science, Boston, MA) and HRP-conjugated secondary anti-mouse or anti-rabbit Abs (final dilution, 1/15,000 to 1/20,000; Jackson ImmunoResearch Laboratories, Mississauga, Canada) as previously described (27). Exceptionally, incubations with the anti-Akt (pSer^{473}) and (pThr^{308}) Abs were performed overnight in TBS, and the HRP-conjugated secondary anti-rabbit Abs were used at a dilution of 1/10,000.

Superoxide measurements

Superoxide production was measured using the reduction of cytochrome *c* assay as previously described (8). The cells (10^7 cells/ml) were preincubated with various concentrations of the inhibitors for 10 min at 37°C. Before stimulation, the cells were diluted at 10^6 cells/ml in the presence of 130 μM cytochrome *c* and stimulated for 5 min at 37°C with fMet-Leu-Phe (10^{-7} M). The tubes were kept on ice for 15 min and centrifuged for 10 min at $1500 \times g$. The amounts of superoxide anions in the supernatants were calculated from the differences between the OD readings at 550 and 540 nm using an extinction coefficient of 21.1.

PLD measurements

Neutrophils were prelabeled with 1-*O*-[^{3}H]alkyl-2-lyso-phosphatidylcholine (2 μCi/10^7 cells) for 90 min. The cells were then centrifuged, resuspended at 10×10^6 cells/ml in HBSS, and preincubated at 37°C for 10 min with the indicated concentrations of inhibitors or an equal amount of DMSO. Cytochalasin B (10 μM) was added to the cells 5 min before the addition of fMet-Leu-Phe (10^{-7} M). Ethanol (final concentration, 1.0%, v/v) was added immediately preceding the addition of fMet-Leu-Phe. The reactions were stopped after 10 min by adding 1.8 ml of cold chloroform/methanol/HCl (50/100/1, v/v/v) and unlabeled phosphatidylethanol as standard. The lipids were extracted, dried under nitrogen, and separated on prewashed (chloroform/methanol, 1/2) silica gel 60 TLC plates using a chloroform/methanol/acetic acid (65/15/2, v/v/v) solvent mixture. The lipids were visualized by Coomassie Brilliant Blue staining, and the different lipid classes were scraped off the plates. Radioactivity in phosphatidylethanol was monitored by liquid scintillation counting, and the results were corrected for background radioactivity and quenching.

Measurement of neutrophil migration

Neutrophils were resuspended in RPMI 1640 and 10% FBS at 10^7 cell/ml and were preincubated with 5 μg/ml calcein-AM (Molecular Probes, Eugene, OR) at 37°C for 60 min in the dark with constant agitation. The cells were washed twice and resuspended in RPMI/FCS at 5×10^6 cell/ml before being incubated for 10 min with the various inhibitors at 37°C. Neutrophil migration was monitored using a 96-well chemoTX disposable chemotaxis system (NeuroProbe, Gaithersburg, MD). The wells of the plate lower chamber were filled with 31 μl of fMet-Leu-Phe solutions diluted in HBSS at the appropriate concentrations. The polycarbonate filters were positioned on the plate, and neutrophils (30 μl, 60,000 cells/well) were placed on the filter and allowed to migrate for 1 h at 37°C in the presence of 5% CO_2 in the dark. The cells that had not migrated were removed by gently wiping the filters with a tissue. The fluorescence of the cells in the filters was measured with a microplate fluorescence reader (FL600; Bio-Tek Instruments, Winooski, VT; excitation and emission wavelengths, 485 and 530 nm, respectively). The fluorescence from known numbers of neutrophils was obtained by placing them into the bottom chamber. The results are expressed as the number of cells that penetrated the filters.

Fibrinogen adhesion assay

Neutrophils (10^7 cell/ml) were preincubated with calcein-AM (5 μg/ml) at 37°C for 60 min in the dark with constant agitation. The cells were washed twice and resuspended in HBSS and $MgCl_2$ (0.8 mM) (5×10^6 cell/ml). The cells were preincubated at 37°C for 10 min with the various inhibitors. fMet-Leu-Phe and Me_2SO_4 were added to the wells before transferring 100 μl of cell suspensions into the wells of fibrinogen-precoated, 96-well microplates. After 60 min of incubation, the plates were washed twice by immersion into PBS solution. The fluorescence of the adherent cells was read with a microplate fluorescence reader (FL600; Bio-Tek Instruments, Winooski, VT). Quantification of adherent cells was obtained using a standard curve of lysates of known cell numbers and their corresponding fluorescence.

Translocation assays

Neutrophils (4×10^7 cell/ml) were treated with 1 mM DFP for 10 min at room temperature. The cell suspensions were diluted in HBSS at 10^7 cell/ml. The cells were preheated for 5 min at 37°C and then preincubated with the indicated concentrations of inhibitors for 10 min at 37°C before the addition of fMet-Leu-Phe (10^{-7} M) in the presence of 10 μM cytochalasin B. The incubations were stopped by diluting the cells 4-fold with ice-cold HBSS, and membranes were prepared as previously described (28). Briefly, the cell suspensions were centrifuged and resuspended at 1.3×10^7 cell/ml in ice-cold KCl/HEPES relaxation buffer (100 mM KCl, 50 mM HEPES, 5 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 2.5 mM PMSF (pH 7.2)). The suspensions were sonicated for 20 s, then centrifuged for 7 min at $700 \times g$. The supernatants were ultracentrifuged at $180,000 \times g$ for 45 min in a TL-100 ultracentrifuge using a TL-100.4 rotor (65,000 rpm, 4°C; Beckman Coulter, Fullerton, CA). The membrane pellets were washed once and resuspended in a small volume of solubilization buffer containing 250 mM Na_2HPO_4, 300 mM NaCl, 2.5% (w/v) SDS, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, and 2.5 mM PMSF. Aliquots were assayed for protein content with the Coomassie Brilliant Blue protein assay (Pierce, Rockford, IL). The protein samples (20 μg) were then subjected to 7.5–20% SDS-PAGE and transferred to Immobilon polyvinylidene difluoride membranes. Immunoblotting was performed using the rabbit anti-Arf-1 (1/2,500), anti-RhoA (1/500), anti-Rac-2 (1/250), and anti-Btk (1/1,000) Abs and the polyclonal

goat anti-Bmx (1/750) and anti-Tec (1/750) Abs. The proteins were revealed using HRP-conjugated secondary anti-rabbit (1/20,000), anti-mouse (1/20,000), or anti-goat (1/10,000) Abs and revealed using the Renaissance detection system.

PtdIns(3,4,5)P_3 formation

Neutrophils (5×10^7 cells/ml) were incubated with 0.5 mCi/ml of [^{32}P]orthophosphate (Perkin-Elmer, Norwalk, CT; 1000 Ci/mmol) for 1 h at 37°C in HBSS without Ca^{2+}. Unincorporated radioactivity was discarded, and the cells were washed twice in HBSS. The cells were resuspended at 2×10^7 cells/ml in HBSS with Ca^{2+} and preincubated with inhibitors or diluent for 10 min at 37°C. The cells were stimulated with 100 nM fMet-Leu-Phe for the indicated times. The reactions were stopped by transfer of 500 μl of the cells into 400 μl of chloroform-methanol (1/1, v/v) and were rapidly vortexed (15–30 s). The samples were centrifuged for 3 min at 13,000 $\times$ *g*, and the organic phase (lower phase) was transferred into borosilicate tubes. The samples were applied to oxalate-treated silica gel 60 plates (Merck, Rahway, NJ), which were developed in chloroform-acetone-methanol-acetic-acid-H_2O (80/30/26/24/14, v/v/v/v/v) for 3 h. The plates were dried, and the products were visualized by exposure of x-ray films at −80°C or with a bioimaging analyzer (FujiFilm BAS-1800).

Phosphoinositol (PI) 3-kinase sssays

Lipid kinase activity was evaluated as described by Naccache et al. (17). Briefly, 8×10^7 cells/ml were preincubated for 10 min at room temperature with or with out 25 μM LFM-A13. Five hundred cells stimulated, or not, were diluted in ice-cold buffer I (PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 100 μM Na_3VO_4). The samples were centrifuged and then washed twice in cold buffer II (50 mM HEPES (pH 7.4), 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2 mM Na_3VO_4). The pellet were lysed quickly in 1 ml of ice-cold buffer III [1% (v/v) of Nonidet P-40, 50 mM HEPES (pH 7.4), 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 μM Na_3VO_4, 10% (v/v) glycerol, 2 mM PMSF, 1 mM EDTA, 100 mM NaF, 10 mM $Na_2P_2O_4$, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and were left on ice for 5 min before being centrifuged for 10 min at 13,000 $\times$ *g* at 4°C. The supernatants were incubated for 1 h at 4°C on a rotating wheel with the anti-p110γ serum generated as previously described (17). The immune complexes were precipitated after incubation with 50 μl of protein A-Sepharose for 1 h. The beads were then washed three times with buffer IV (PBS, 1% (v/v) Nonidet P-40, and 100 μM Na_3VO_4), three times with buffer V (100 μM Tris-HCl (pH 7.5), 500 mM LiCl, and 100 μM Na_3VO_4), and finally twice with buffer VI (10 μM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4). LFM-A13 or LFM-A11 (25 μM) were added to the beads for 10 min at 37°C. Ten microliters of PtdIns (2 mg/ml phosphatidylinositol in 10 mM Tris-HCl (pH 7.5) and 1 mM EGTA) was sonicated for 10 min, added to 50 μl of buffer VI and 10 μl of 100 mM $MgCl_2$, and mixed with the beads for 10 min on ice. The reactions were initiated by adding 10 μl of 440 μM ATP containing 30 μCi of [γ-^{32}P]ATP (Perkin-Elmer; 3000 Ci/mmol) and incubated for 15 min at 37°C. Twenty microliters of 1 N HCl were added to stop the reactions, and PtdIns(3)P_3 was extracted by the addition of 200 μl of chloroform/methanol (1/1, v/v) was spun at 15–30 s. The samples were centrifuged, and 75 μl of lower organic phase were applied to oxalate-treated silica gel 60 plates which were developed in 2-propanol-2-*N*-acetic acid (2:1, v/v) for 8 h. The plates were dried, and the products of the kinase reactions were visualized by exposure of x-ray films at −80°C or with a bioimaging analyzer (FujiFilm BAS-1800).

Results

Inhibition of phosphorylation events by LFM-A13

As an initial characterization of the effects of LFM-A13 on human neutrophils, we first examined its impact on stimulation of the profile of tyrosine phosphorylation induced by fMet-Leu-Phe. In these experiments, the cells were preincubated with increasing concentrations of LFM-A13 or its inactive analog (LFM-A11), and the levels of tyrosine phosphorylation induced by fMet-Leu-Phe were monitored as described in *Materials and Methods*. The characteristic tyrosine phosphorylation response to fMet-Leu-Phe (45 s of stimulation) (29) is illustrated in Fig. 1*A* (*left panel*). LFM-A13 decreased the fMet-Leu-Phe-induced tyrosine phosphorylation of proteins in a concentration-dependent manner (*middle panel*). Slight inhibitory effects were noted at concentrations as low as 1 μM, and major reductions in the levels of tyrosine phosphorylation were evident at 25 and 50 μM. On the other hand, LFM-A11 had no significant effect even at the highest concentration tested (50 μM; Fig. 1*A*, *right panel*). The effects of LFM-A13 were agonist specific in that it did not significantly affect the responses to monosodium urate monohydrate crystals or to cross-linking of CD32, which were monitored on the same cell suspensions (data not shown).

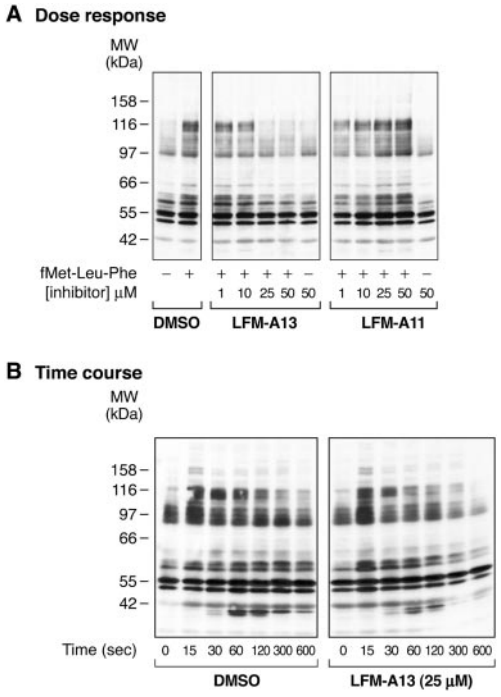

FIGURE 1. Inhibition of the global profile of tyrosine phosphorylation induced by fMet-Leu-Phe. *A*, Dose response to LFM-A13. Neutrophils (2×10^7/ml) were pretreated with 1 mM DFP and the indicated concentrations of LFM-A13 and LFM-A11 (37°C, 10 min). The cells were stimulated with fMet-Leu-Phe (100 nM for 45 s) and analyzed as described in *Materials and Methods*. *B*, Time course of fMet-Leu-Phe stimulation. Neutrophils were preincubated with 25 μM LFM-A13 for 10 min at 37°C and stimulated with fMet-Leu-Phe (100 nM). The reactions were stopped and analyzed as described in *Materials and Methods*. The data shown are representative of at least three independent experiments performed with two different lots of LFM-A13 on separate cell preparations.

As the tyrosine phosphorylation responses to fMet-Leu-Phe are transient, we tested next whether the above effects of LFM-A13 were caused by a shift in the time course of the response. Neutrophil suspensions were preincubated with 25 μM LFM-A13 for 10 min and stimulated with 10^{-7} M fMet-Leu-Phe for the indicated times, and the levels of tyrosine phosphorylation were monitored. As shown in Fig. 1*B*, LFM-A13 reduced the duration of the tyrosine phosphorylation response induced by fMet-Leu-Phe.

The viability of the cells (as monitored by trypan blue exclusion) was not affected at any concentration of LFM-A13 (data not shown).

Effect of LFM-A13 on the functional responses of human neutrophils

Having observed that LFM-A13 had profound effects on the levels of tyrosine phosphorylation induced by fMet-Leu-Phe, we next examined the effects of this inhibitor on several of the functional tyrosine kinase-dependent responses elicited by this chemotactic factor. These included stimulation of directed locomotion (chemotaxis) (10), adhesiveness to fibrinogen-coated surfaces (30), and production of bactericidal oxygen free radicals (8).

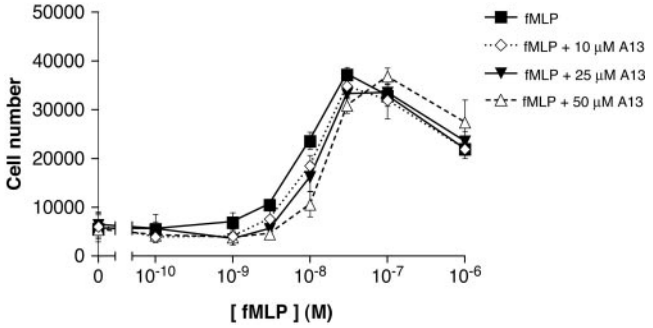

FIGURE 2. Effects of LFM-A13 on the chemotactic response of human neutrophils to fMet-Leu-Phe. Neutrophils (10^7 cells/ml) were preincubated with or without the indicated concentrations of LFM-A13 for 10 min. The assay was performed as described in *Materials and Methods*. The results presented are representative of at least three independent experiments performed in duplicate on separate cell preparations. Two different lots of LFM-A13 were tested.

The ability of neutrophils to migrate to sites of injury or infection is an integral element of their defense-oriented functions. The locomotory behavior of these cells can be monitored by a number of in vitro chemotactic assays, one of the better established being based on the Boyden chamber (and its variants). The effects of LFM-A13 on the chemotactic responses of human neutrophils to complete concentration-response curves of fMet-Leu-Phe were tested. As shown in Fig. 2, the chemotactic response of untreated cells exhibited a classical bell-shaped curve. Preincubation with LFM-A13 resulted in a shift to the right of this curve in a concentration-dependent manner (10–50 μM), an effect that was not shared by LFM-A11 (data not shown).

Neutrophils perform most of their functions in close contact with various cells and extracellular matrixes. These interactions are known to be dependent on and modulated by the state of activation of the cells, which alters the functional status of the integrins involved. In an initial attempt to examine the potential effects of LFM-A13 on the adhesive properties of human neutrophils, we examined the effects of this inhibitor on the ability of fMet-Leu-Phe to stimulate the interaction of neutrophils with fibrinogen-coated surfaces, a response thought to be mediated in part by changes in the affinity and/or avidity of β_2 integrins (30). The results of these experiments, summarized in Fig. 3, show that LFM-A13 inhibited in a

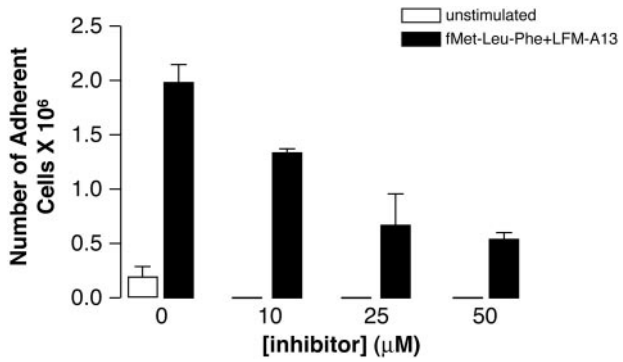

FIGURE 3. Effects of LFM-A13 on stimulation of the adhesion of neutrophils to fibrinogen-coated surfaces induced by fMet-Leu-Phe. Neutrophils (10^7 cells/ml) were preincubated with or without LFM-A13 at the indicated concentrations for 10 min at 37°C. The cells were stimulated with fMet-Leu-Phe (100 nM), and adhesion was monitored as described in *Materials and Methods*. The results are the average of three independent experiments conducted on cells from separate donors in quadruplicate.

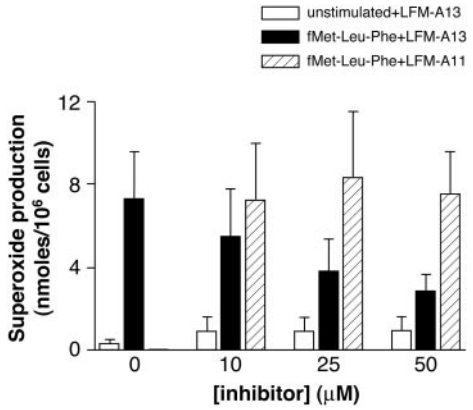

FIGURE 4. Effects of LFM-A13 on the production of superoxide anions in fMet-Leu-Phe-stimulated neutrophils. Neutrophils (10^7 cells/ml) were preincubated with DMSO (□), LFM-A13 (■), or LFM-A11 (▨) at the indicated concentrations for 10 min at 37°C. The cells were stimulated with fMet-Leu-Phe (100 nM) for 5 min, and superoxide production was evaluated as described in *Materials and Methods*. The results are from three independent experiments conducted in duplicate on cells from separate donors. The same results were obtained with three lots of inhibitors.

concentration-dependent manner the fMet-Leu-Phe-induced adhesion of neutrophils to fibrinogen-coated plates.

Activation of the tyrosine kinase pathway is closely linked to that of the NADPH oxidase in human neutrophils (8). The results presented in Fig. 4 show that LFM-A13 inhibited the production of superoxide anions induced by fMet-Leu-Phe in a concentration-dependent manner (from 10 to 50 μM), while its inactive analogue LFM-A11 was without effect. This effect was not due to a direct inhibition of the NADPH oxidase or of the assay, as stimulation of the production of superoxide anions induced by monosodium urate monohydrate crystals was unaffected by LFM-A13 and LFM-A11 (data not shown).

Effect of LFM-A13 on stimulation of PLD activity

PLD represents a signaling pathway shown to be implicated in regulation of the functional responsiveness of human neutrophils

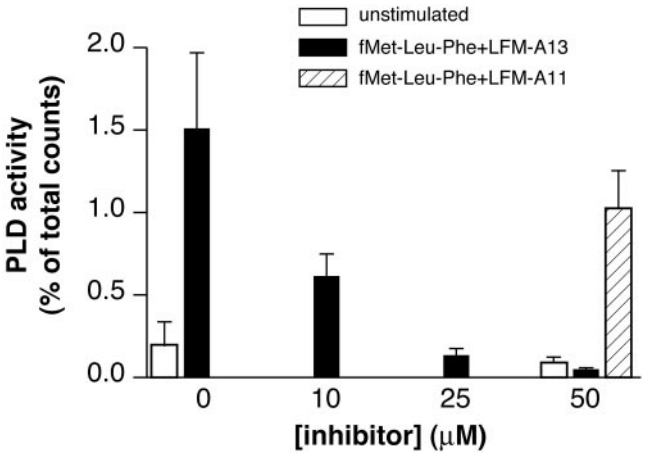

FIGURE 5. Effects of LFM-A13 on the stimulation of PLD activity induced by fMet-Leu-Phe. Neutrophils (10^7 cells/ml) were preincubated with DMSO (□), LFM-A13 (■), or LFM-A11 (▨) at the indicated concentrations for 10 min at 37°C. Cytochalasin B (10 μM) was added to the cells 5 min before stimulation with fMet-Leu-Phe (100 nM) for 60 s. Stimulation of PLD activity was evaluated as described in *Materials and Methods*. The results are from three independent experiments performed in duplicate on cells from separate donors. Two different lots of LFM-A13 were tested.

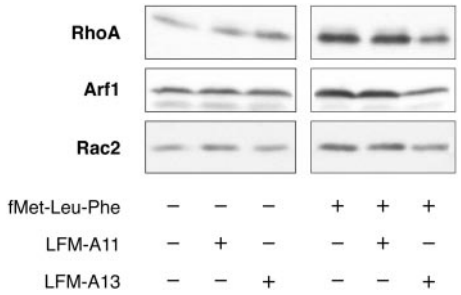

FIGURE 6. Inhibition of the stimulated translocation of activation cofactors for PLD and NADPH oxidase. Neutrophils (10^7 cells/ml) were preincubated with DMSO, LFM-A13 (25 μM), or LFM-A11 (25 μM) for 10 min at 37°C. Cytochalasin B (10 μM) was added to the cells 5 min before stimulation with fMet-Leu-Phe (100 nM) for 60 s, and membrane fractions were prepared as described in *Materials and Methods*. The membranes were blotted with the respective Abs as described in *Materials and Methods*. The results are representative of at least two independent experiments conducted on cells from separate donors.

(31). Previous studies have provided evidence that stimulation of the activity of PLD by chemotactic factors was tyrosine kinase dependent (31), although the specific tyrosine kinases involved have yet to be identified. LFM-A13 was found to be a potent inhibitor of the stimulation of PLD activity by fMet-Leu-Phe in human neutrophils (Fig. 5). Preincubation of the cells with LFM-A13, but not LFM-A11, resulted in a concentration-dependent inhibition of the activation of PLD induced by fMet-Leu-Phe. This response was particularly sensitive to LFM-A13, with inhibitory effects being detectable at concentrations as low as 1 μM (data not shown).

The activation of PLD is regulated in part by the membrane recruitment of Arf-1 and small GTPases (RhoA and Rac-2) (25, 32, 33). The assembly of a functional NADPH oxidase is influenced by Rac-2 (34). RhoA is known to influence the reorganization of the actin cytoskeleton, thereby indirectly influencing cell locomotion (35, 36). Houle et al. (33) has shown that membrane recruitment of the above cofactors was tyrosine kinase dependent in fMet-Leu-Phe-stimulated neutrophils. In the next series of experiments the effects of LFM-A13 on the membrane recruitment of Arf-1, RhoA, and Rac-2 induced by fMet-Leu-Phe were tested, and the results are presented in Fig. 6. These data show that LFM-A13 decreased the amounts of RhoA, Arf-1, and Rac-2 recovered in the membrane fractions following stimulation by fMet-Leu-Phe (compare *lanes 1* and *4* (effect of fMet-Leu-Phe) and *lanes 4–6* (effect of LFM-A13)) This effect was concentration dependent between 10 and 25 μM LFM-A13 and was not observed when 25 μM LFM-A11 was used.

Effect of LFM-A13 on the phosphorylation of specific substrates induced by fMet-Leu-Phe

Stimulation of human neutrophils by fMet-Leu-Phe leads to the phosphorylation of several substrates, such as p38 kinase (37), Erk1/2 kinase (38), MAPK, and Akt (39). In the next series of experiments we evaluated the effects of LFM-A13 on the phosphorylation levels of these substrates. Briefly, the cells were preincubated with or without inhibitors, and fMet-Leu-Phe (10^{-7} M) was subsequently added. The reactions were stopped by transfer of aliquots (100 μl) in boiling 2× sample buffer, followed by immunoblotting with the appropriate Abs.

The samples were first analyzed with specific Abs against phospho-Erk1/2 (Fig. 7*A*) and phospho-p38 (Fig. 7*B*), which recognize dual threonine and tyrosine phosphorylation sites as described in *Materials and Methods*. The results of these experiments are presented in Fig. 7. The *left panels* illustrate the time course of stimulation of phosphorylation of the MAPKs induced by fMet-Leu-Phe (38). The phosphorylation of Erk1/2 and p38 was decreased by LFM-A13 at all times tested (*middle panels*). The inactive analog LFM-A11 had no significant effect on the levels of phosphorylation of Erk1/2 and p38 (*right panels*). Reprobing the membranes with the respective Abs to the nonphosphorylated forms of these proteins revealed equal amounts of Erk1/2 and p38 in each sample.

Activation of human neutrophils by fMet-Leu-Phe results in the phosphorylation and activation of Akt on Thr^{308} and Ser^{473} (39, 40) by 3-phosphoinositide-dependent kinase-1 and -2 (PDK1 and -2), respectively (40). PDK1 has been cloned, but the precise identity of PDK2 remains to be unambiguously characterized. A recent report provided evidence that the p38 kinase-dependent MAPK-activated protein kinase 2 possessed PDK2 activity in human neutrophils (40). In view of the above-described effects of LFM-A13 on the stimulated phosphorylation of p38 (Fig. 7), we examined next the effects of LFM-A13 on stimulation of phosphorylation of Akt induced by fMet-Leu-Phe at both Thr^{308} and Ser^{473} using specific phospho-Akt Abs. As shown in Fig. 8, preincubation with LFM-A13 drastically reduced the stimulation of the Thr^{308} as well as Ser^{473} phosphorylation of Akt induced by fMet-Leu-Phe. LFM-A11 was without effect. These results suggest that LFM-A13 inhibited the activation of both PDK1 and PDK2 induced by fMet-Leu-Phe in human neutrophils.

PtdIns(3,4,5)P_3 formation and PI 3-kinase activity

The activation of PI 3-kinases represents another major signaling pathway in neutrophils stimulated by chemotactic factors (17, 18, 41). In the next series of experiments we assessed the effects of

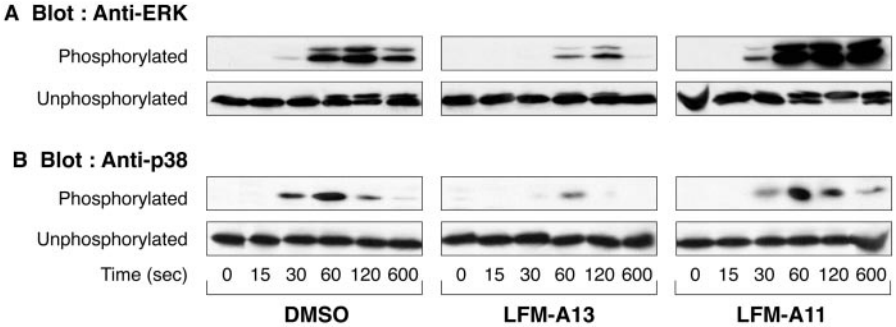

FIGURE 7. Effect of LFM-A13 on the phosphorylation of Erk1/2 and p38 induced by fMet-Leu-Phe. Neutrophils (2×10^7/ml) were pretreated with 1 mM DFP (37°C, 10 min) and DMSO (*left panels*), LFM-A13 (25 μM; *middle panels*), or LFM-A11 (25 μM; *right panels*). The cells were stimulated upon transfer to tubes containing fMet-Leu-Phe (100 nM) for the indicated times. The reactions were stopped as described in *Materials and Methods*. The samples were analyzed by immunoblotting with specific anti-phospho Abs to Erk1/2 (pThr/pY182/184; *A*) and p38 (pThr/pY 180/182; *B*). The data shown are representative of three independent experiments conducted on cells from separate donors and with two different lots of LFM-A13.

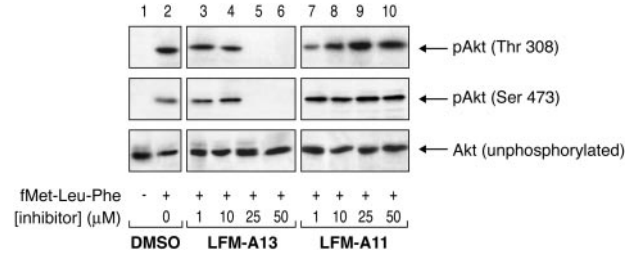

FIGURE 8. Effect of LFM-A13 on the phosphorylation of Akt induced by fMet-Leu-Phe. Neutrophils (2×10^7/ml) were pretreated with 1 mM DFP and the indicated concentrations of LFM-A13 and LFM-A11 (37°C, 10 min). Stimulation began when the cells were transferred into microcentrifuge tubes containing fMet-Leu-Phe (100 nM) and was conducted for 45 s. The reactions were stopped by direct transfer of cell aliquots into boiling modified Laemmli sample buffer (SB2×). The proteins in the samples were then separated by electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were probed with anti-phospho Akt-1 (Ser^{473}, Thr^{308}) and anti-Akt Abs. The data shown are representative of at least three independent experiments conducted on cells from separate donors.

LFM-A13 on the formation of PtdIns(3,4,5)P_3 in response to fMet-Leu-Phe. The results obtained (Fig. 9*A*) show that LFM-A13 inhibited the accumulation of PtdIns(3,4,5)P_3 in response to fMet-Leu-Phe, an effect that was not shared with LFM-A11 (data not shown). The PtdIns(3,4,5)P_3 accumulation in response to GM-CSF was not affected by LFM-A13 (data not shown).

The effects of LFM-A13 on the formation of PtdIns(3,4,5)P_3 could be explained by a nonspecific interference of LFM-A13 with the activity of PI 3-kinases. To directly test this possibility, PI 3-kinases were immunoprecipitated from resting cells using anti-p110γ, the isoform most responsive to fMet-Leu-Phe (17), and anti-p85 antisera. The in vitro PI 3-kinase activity of the precipitates toward an exogenous substrate (PtdIns) was tested in the absence or the presence of 25 μM LFM-A13. The results of these experiments, shown in Fig. 9*B*, indicate that LFM-A13 had no direct inhibitory activity toward p110γ or p85/p110 PI 3-kinases in this in vitro lipid kinase assay. In a final series of experiments aimed at ascertaining the specificity and site of action of LFM-A13, we examined whether it affected stimulation of p110γ activity induced by fMet-Leu-Phe. In these experiments cells were stimulated for varying times with fMet-Leu-Phe, after which they were lysed, p110γ was immunoprecipitated, and its activity was monitored in an in vitro PI 3-kinase assay as described in *Materials and Methods*. The results obtained with three separate cell preparations are shown in Fig. 9*C*. The data in the *left panel* illustrate the stimulation of the activity of p110γ that follows the stimulation of human neutrophils by fMet-Leu-Phe. The data in the *right panel* clearly show that LFM-A13 had no significant effect on this response, thereby indicating that it did not inhibit the in situ activation of p110γ by fMet-Leu-Phe.

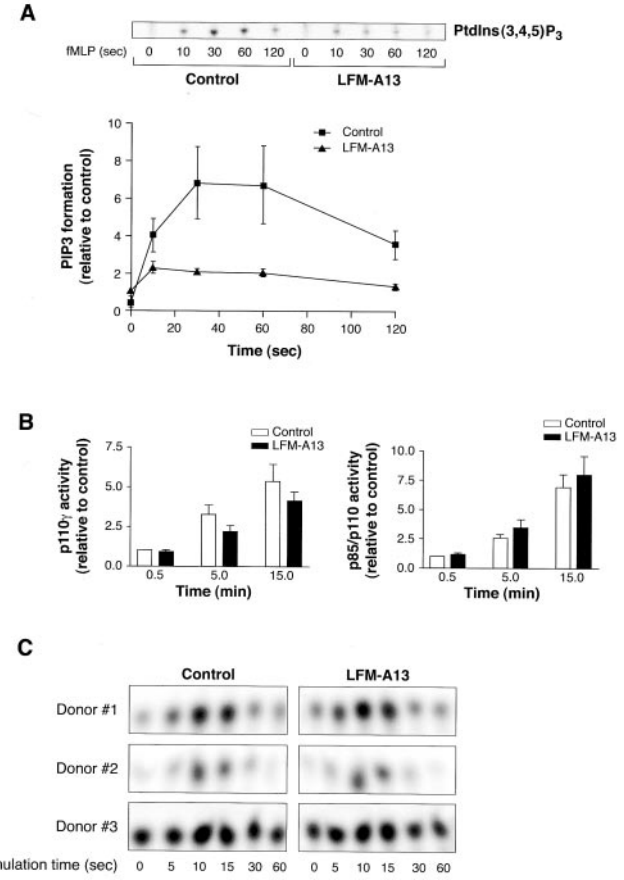

FIGURE 9. *A*, Inhibition of the accumulation of PtdIns(3,4,5)P_3 by LFM-A13. ^{32}P-labeled neutrophils (10^7 cells/ml) were preincubated with DMSO or LFM-A13 (25 μM). The cells were stimulated with fMet-Leu-Phe (100 nM) for the indicated times. *A*, Autoradiogram of the levels of PtdIns(3,4,5)P_3. *B*, Results of five independent experiments conducted on cells from separate donors and analyzed by densitometry. At least two different lots of LFM-A13 were used in this series of experiments. *B*, Lack of effect of LFM-A13 on PI 3-kinase activity. Neutrophils (10^7 cells/ml) were lysed, and the p110γ (*A*) or p85/p110 (*B*) PI 3-kinases were precipitated and submitted to a lipid kinase assay as described in *Materials and Methods*. The precipitates were incubated with DMSO (□) or LFM-A13 (■; 25 μM) for 10 min at 37°C. The times represent the duration of the lipid kinase assay. The results are from four independent experiments conducted on cells from separate donors. None of the differences between the untreated and LFM-13-treated samples were statistically significant. *C*, Lack of effect of LFM-A13 on p110γ activity. Neutrophils (10^7 cells/ml) were lysed after LFM-A13 preincubation and fMLP stimulation, and p110γ kinase was precipitated and submitted to a lipid kinase assay as described in *Materials and Methods*. The data represent the kinetics of the formation of PtdInsP following stimulation with fMLP. The data show an autoradiogram representative of three independent experiments conducted on cells from separate donors.

Inhibition of translocation of Tec family kinase

In the final series of experiments, we examined whether the PI 3-kinase kinase-dependent translocation of the Tec kinases induced by fMet-Leu-Phe (16) was itself sensitive to LFM-A13. Neutrophils were therefore preincubated with LFM-A13 (25 μM) or LFM-A11 (25 μM) and stimulated with fMet-Leu-Phe (10^{-7} M). Membrane fractions were then prepared, and the amounts of Tec family kinases (Tec, Btk, and Bmx) present in these fractions were monitored by immunoblotting. The results illustrated in Fig. 10 provide evidence that the translocation of all three Tec family kinases induced by fMet-Leu-Phe (*lanes 1* and *4*, and *lanes 4* and *6*) was inhibited by LFM-A13, but not by LFM-A11. Inhibition of translocation of Tec kinases was also observed at 10 μM LFM-A13 (data not shown).

Discussion

In a previous study (16) we provided evidence that Tec family kinases were recruited and activated upon stimulation of human neutrophils by chemotactic factors. The results of the present investigation corroborate these findings and indicate that interference with Tec family kinases through the use of the Btk inhibitor LFM-A13 profoundly affected multiple signaling pathways and

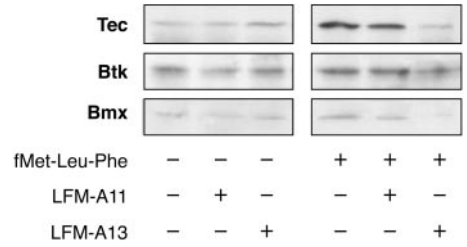

FIGURE 10. Effects of LFM-A13 on stimulation of translocation of Tec kinases. Neutrophils (10^7 cells/ml) were preincubated with DMSO, LFM-A13 (25 μM), or LFM-A11 (25 μM) for 10 min at 37°C. Cytochalasin B (10 μM) was added to the cells 5 min before stimulation with fMet-Leu-Phe (100 nM) for 60 s. Membrane fractions were prepared and blotted with the respective Abs as described in *Materials and Methods*. The results are representative of at least two independent experiments conducted on cells from separate donors.

functional responses in chemotactic factor-stimulated neutrophils. These data are the first to implicate Tec family kinases in the initiation and regulation of the functional responsiveness of human neutrophils.

LFM-A13 has been designed by docking procedures to the catalytic site of the Tec family kinase Btk. It has been shown to be significantly more active toward Btk than toward Src family kinases (Hck), Janus kinases 1 and 3, or epidermal growth factor kinase (24). Its activity toward other members of the Tec family, however, has not been characterized to date, and therefore, it is not possible at present to determine whether the effects of LFM-A13 described in the present investigation are mediated by its effects on Btk alone or by interference with a combination of Tec family members. It should be pointed out that Tec, Btk, and Bmx have all previously been found to be recruited to membranes and activated in response to fMet-Leu-Phe (16). Previous studies have shown that LFM-A13 inhibits the tyrosine phosphorylation of STAT5A in B cells induced by anti-IgM stimulation (42, 43) and decreases calcium influx in platelets while increasing PKCθ activity in platelets in response to alboaggregin A (44).

LFM-A13 inhibited multiple signaling events in human neutrophils stimulated by the chemotactic factor fMet-Leu-Phe. It significantly reduced stimulation of the global tyrosine profile induced by fMet-Leu-Phe, thereby indicating that LFM-A13-sensitive tyrosine kinases play prominent roles in the generation of the latter. Src kinases have previously been shown to be similarly involved (39, 45). The respective contributions of Src and Tec kinases remain to be determined. It should be pointed out that tight interrelationships between these two families of kinases are likely, as there is evidence indicating that the optimal activation of Tec kinases depends on their phosphorylation by Src kinases (46, 47). In any event, the inhibitory effects of LFM-A13 clearly show that Btk and possibly other Tec kinases are critically involved in the initiation of the tyrosine phosphorylation-dependent signaling cascades stimulated by fMet-Leu-Phe in human neutrophils.

Activation of Btk and the other Tec kinases is mediated in part by the high affinity of their PH domains for PtdIns(3,4,5)P_3 (20–22), which is generated subsequent to the activation of one or more PI 3-kinases. In the case of human neutrophils stimulated by fMet-Leu-Phe, previous evidence points to p110γ as being the responsive PI 3-kinase isoform (17). The inhibition by LFM-A13 of the increases in PtdIns(3,4,5)P_3 induced by fMet-Leu-Phe is an unexpected finding that may explain, in part at least, several of the effects of LFM-A13 observed in the present study, including the reduced translocation and activation of Tec kinases and the subsequent inhibition of the various signaling events and functional responses.

The addition of LFM-A13 resulted in the inhibition of activation of the MAPK and Akt pathways as well as of PLD. Recently, a link between the p38 kinase and Akt was suggested when it was found that p38 kinase-dependent MAPK activated protein kinase 2 possessed PDK-2 activity (40). The inhibition of p38 activation by LFM-A13 may thus underlie the observed inhibition of Akt. On the other hand, the inhibition of the phosphorylation of Akt on both Thr^{308} and Ser^{473} by LFM-A13 indicates that the activation of both PDK1 and -2 is under the control of Tec family kinases.

The stimulation of PLD activity represented another signaling pathway that was particularly sensitive to inhibition by LFM-A13. This signaling pathway is known to be downstream of PI 3-kinases (or of the accumulation of PtdIns(3,4,5)P_3) (48). It is therefore likely that the decreased accumulation of PtdIns(3,4,5)P_3 observed in the presence of LFM-A13 underlies the inhibition of stimulation of PLD activity induced by fMet-Leu-Phe. The optimal activation of PLD is also known to require the recruitment of several cofactors, including the small m.w. G proteins Arf and RhoA (25, 32) the activation of which has previously been shown to be at least in part dependent on the activity of PI 3-kinases (49). Our results indicate that the membrane recruitment of Arf and RhoA induced by fMet-Leu-Phe was inhibited by LFM-A13, an effect that may also be related to the reduced accumulation of PtdIns(3,4,5)P_3. Regardless of the precise site of action at which LFM-A13 inhibits the membrane recruitment of the small molecular G proteins, these results provide an additional mechanistic basis for the reduced PLD activity observed under these conditions.

LFM-A13 also inhibited Rac-2 recruitment to the membranes, an essential cofactor for the assembly of a fully functional NADPH oxidase system (34), the activation of which in response to fMet-Leu-Phe, like that of Rac-2 itself (49), is dependent on PI 3-kinase activity (as judged by its sensitivity to wortmannin (50), presumably p110γ (17). This together with the inhibition of PLD in turn may provide an explanation for the ability of LFM-A13 to inhibit the generation of superoxide anions in human neutrophils.

Preincubation with LFM-A13 induced a close to 3-fold shift to the right of the chemotactic concentration-response curves induced by fMet-Leu-Phe. It should be noted, however, that the magnitude of the chemotactic response induced by optimal concentrations of fMet-Leu-Phe was not significantly affected by the Btk inhibitor. This contrasts to some extent with the characteristics of the inhibitory activity of LFM-A13 toward the signaling pathways and toward the generation of superoxide anions, which were all inhibited at optimal concentrations of fMet-Leu-Phe (see above). The underlying reasons for these differences are not known at present. These results, however, are in agreement with the phenotype of p110$\gamma^{-/-}$ knockout, in which a defective, although not abolished, neutrophil recruitment was observed (51, 52). Of potential relevance to the present study, administration of the parent compound of LFM-A13, leflunomide, to rheumatoid arthritis patients was reported to result in reduced neutrophil migration (53).

The inhibition of the accumulation of PtdIns(3,4,5)P_3 by LFM-A13 that appears to underlie several of the downstream effects of this inhibitor was not due to a direct effect on PI 3-kinases or to an inhibition of the activation of p110γ by fMet-Leu-Phe (see Fig. 9). These results suggest that the inhibition of the accumulation of PtdIns(3,4,5)P_3 by LFM-A13 could result from an increased activity of phosphoinositide-specific phosphatases (Src homology 2-containing inositol phosphatase, phosphatase and tensin homologue deleted on chromosome 10) controlled by Tec kinases. We are currently investigating this hypothesis.

The results of the present study establish for the first time the functional significance of Tec family kinases in the signaling mechanisms associated with the occupancy of the fMet-Leu-Phe

receptors. In so doing, they confirm and extend previous observations documenting the recruitment and activation of Tec kinases upon the addition of this chemoattractant. The magnitude and extent of the inhibitory effects of LFM-A13 provide compelling evidence that Tec family kinases (and Btk in particular) play critical roles in the initiation and regulation of the functional responsiveness of human neutrophils. These results represent an extension of the previously described functional relevance of Tec kinases, which were only linked to the functions of tyrosine kinase-dependent receptor systems and in particular to the B cell receptor. Whether the functional implications of the p110γ/Tec kinase link suggested by the present results is similarly implicated in the signaling mechanisms of other GPCRs and in cells other than those of the hemopoietic system remains to be examined.

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