We investigated the coupling of the fMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine; ‘chemotactic peptide’) receptor with phosphorylation of the actin-binding protein L-plastin in neutrophils. Using two-dimensional IEF (isoelectric focusing)/PAGE and MALDI–TOF (matrix-assisted laser desorption ionization–time-of-flight)-MS, L-plastin was identified as a major phosphoprotein in fMLP-stimulated neutrophils whose phosphorylation was dependent on phosphoinositide 3-kinase, PLD (phospholipase D) and PKC (protein kinase C) activity. Two fMLP receptor subtypes were identified in neutrophils, characterized by a distinct sensitivity to fMLP and antagonistic peptides. Both receptor subtypes induced the phosphorylation of L-plastin. L-plastin phosphorylation induced by low-affinity fMLP receptors involves an action of phosphoinositide 3-kinase, PLD and PKC isotypes. In contrast, none of these intermediates are utilized by high-affinity fMLP receptors in the phosphorylation of L-plastin. However, the PKC inhibitor Ro-31-8220 inhibits L-plastin phosphorylation induced by the high-affinity fMLP receptor. Thus, an as yet unknown Ro-31-8220-sensitive kinase regulates L-plastin phosphorylation in response to the high-affinity fMLP receptor. The results suggest a model in which receptor subtypes induce a similar endpoint event through different signal-transduction intermediates. This may be relevant in the context of cell migration in which one receptor subpopulation may become desensitized in a concentration gradient of chemotactant.

Key words: L-plastin, fMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine) receptor, human neutrophil, signalling, PKC (protein kinase C) isotype.

INTRODUCTION

Polymorphonuclear leucocytes (neutrophils) play a key role in the host defence against micro-organisms. Their migration is governed by chemoattractants, the most important of which is fMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine; ‘chemotactic peptide’) [1–4]. Two functional fMLP receptors have been cloned and characterized, namely FPR (formyl peptide receptor) and FPR-L1 (FPR-like 1), with a high and a low affinity for fMLP respectively [5–9]. FMLP is a known activator of PLC (phospholipase C) [10,11], PI3K (phosphoinositide 3-kinase) [12,13] and PLD (phospholipase D) [14]. The resulting second messengers act on various intracellular kinases, including PKC (protein kinase C). PKC is a family of isoforms classified into three groups on the basis of their structure and mode of activation [15,16]: conventional PKCs (cPKCs; PKC-α, -β, -γ and -δ), novel PKCs (nPKCs; PKC-δ, -ε, -η and -θ) and atypical PKCs (aPKCs; PKC-ζ and -λ).

Direct proof for the involvement of fMLP receptors in host defence was provided with the demonstration that mice lacking FPR were more susceptible to infection than wild-type mice [9]. Furthermore, the study of FPR2, a mouse FPR-L1 orthologue that possesses a low affinity for fMLP, has suggested that distinct high- and low-affinity receptors could be used by the same chemoattractant to facilitate leukocyte migration at high concentration of chemoattractants when high-affinity receptors are desensitized [17]. However, the heterogeneity of fMLP receptor in human neutrophils has not been addressed fully, nor is it known how these different receptors couple with downstream effector molecules.

In the course of our investigation into phosphorylated effectors of fMLP receptors, we identified phosphorylation of L-plastin as a major response to fMLP receptor activation. L-plastin belongs to the fimbrin family. It is expressed exclusively in leucocytes and some transformed cells [18]. It possesses two actin-binding domains and two EF-hand calcium-binding domains [19,20]. In resting cells, most of the L-plastin is involved in cross-linking of actin fibres. Cellular stimulation increases intracellular calcium concentration, which decreases the F-actin bundling activity of L-plastin, and the concomitant L-plastin phosphorylation has been suggested to play a role in integrin activation and cell adhesion [18,20–22].

In the present study, we have investigated the coupling of each of the two fMLP receptor subtypes with L-plastin phosphorylation. We characterized the two fMLP receptor subtypes in human neutrophils [23] and determined the conditions to study their individual downstream effects. We show that L-plastin phosphorylation occurs in response to either receptor and that distinct signal-transduction intermediates couple the individual receptor subtypes with L-plastin. In this way, we identified a novel pathway activated in response to low concentrations of fMLP, which does not involve PI3K, PLD or any of the known PKC isoforms. These observations are discussed in relation to the function of fMLP receptor subtypes in the detection and processing of increasing concentrations of the ligand.
EXPERIMENTAL

Materials

Dextran was purchased from ICN Biochemicals (Costa Mesa, CA, U.S.A.) and Lymphoprep™ from Nycomed Pharma AS (Oslo, Norway). [3H]fMLP (specific activity, 60 Ci/mmol) was purchased from NEN™ Life Science Products (Boston, MA, U.S.A.). [32P]Pi (PBS13) was purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.), Ro-31-8220 from Calbiochem®–CN Biosciences U.K. (Nottingham, U.K.) and LY294002 from Sigma (Poole, Dorset, U.K.). Butanol was obtained from BDH (Merck Eurolab, Leuven, Belgium). Rabbit polyclonal IgGs directed against cPKC-β1 (C-16), cPKC-βII (C-18), nPKC-δ (C-20) and aPKC-ζ (C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI, U.S.A.), t-Boc (tertbutyloxycarbonyl-Phe-Leu-Phe-Leu-Phe) was obtained from ICN Biomedicals (Aurora, OH, U.S.A.).

Neutrophil purification

Human neutrophils were isolated from heparinized venous blood of healthy volunteers by sedimentation on 1% dextran (final concentration) in 0.9% (w/v) NaCl. The leucocyte-rich fraction was collected and purified over a 20% (v/v) layer of Lymphoprep™ [24,25]. After 15 min centrifugation at 1000 g at room temperature (20 °C), the pellet was submitted to hypotonic lysis in water for 10 s, after which the buffer was adjusted to 0.9% (w/v) NaCl. After 8 min centrifugation at 500 g at room temperature, the neutrophil pellet was washed once with 0.9% (w/v) NaCl and then washed once with RPMI 1640 without sodium phosphate. Finally, neutrophils were counted and resuspended in the latter buffer at a concentration in the range 2–5 × 10⁷ cells/ml.

32P-labelling of neutrophils

Neutrophils were incubated in phosphate-free RPMI 1640 containing 0.5–1 mCi of [32P]Pi/2–5 × 10⁷ cells/ml for 1 h at 37 °C. The cells were then stimulated with fMLP at the concentrations and length of time specified in the text and Figure legends. The reaction was stopped by the addition of 5 vol. of ice-cold PBS; cells were collected by centrifugation (1 min at 14,000 g), resuspended in lysis buffer [50 mM Tris/HCl, pH 7.3/1% (w/v) Triton X-100/20 μM leupeptin/1.5 μM pepstatin/27 μM tosyllysylchloromethane (‘TLCK’)/2 mM PMSF/10 μM di-isopropyl fluorophosphate/10 mM glycercophosphate/25 mM NaF] and incubated for 15 min on ice. The resulting lysate was centrifuged at 100,000 g for 15 min at 4 °C (TLA 100.2 rotor; Beckman Instruments, Fullerton, CA, U.S.A.) to obtain a soluble and an insoluble fraction.

Binding experiments

Neutrophils (5 × 10⁶ cells/assay) were incubated in 200 μl of RPMI 1640 containing 0.5% (w/v) BSA for 15 min at 37 °C [23], in the presence of increasing concentrations of [3H]fMLP (0–6 μM). To reach concentrations higher than 8 × 10⁻⁷ M, [3H]fMLP was mixed with unlabelled fMLP. In this case, the specific binding measured was corrected with a coefficient corresponding to the percentage of [3H]fMLP in the mixture. Non-specific binding was measured in the presence of 10 or 100 μM unlabelled fMLP. At the end of the incubation, cells were filtered through Whatman GF/B filters using a Brandel cell harvester and washed six times with 2 ml of cold PBS. Filters were air-dried for 45 min and the associated radioactivity was measured by liquid-scintillation spectrometry (Topcount instrument) after the addition of 50 μl of scintillant/well. In competition experiments of fMLP and t-Boc, cells were incubated in the presence of 32 nM [3H]fMLP and increasing concentrations of t-Boc (10⁻⁵–10⁻⁴ M) for 15 min at 37 °C. Then, cells were immediately harvested, washed and radioactivity was measured as described above. Under the conditions described, the radioactivity bound to the filter in the absence of cells was < 2% of the maximum specific binding measured in the presence of cells.

All data were analysed using the non-linear regression curve-fitting computer program Origin that allows direct calculation of the maximum specific binding, dissociation constant (Kd) and the probability that the curve-fitting model (one or two binding sites) chosen was correct.

Two-dimensional gel electrophoresis

Proteins in the 1% Triton X-100-soluble extract were precipitated for 1 h at −20 °C in the presence of 0.8 vol. of 90% (v/v) acetone, 10% (v/v) trichloroacetic acid and 20 mM DTT (dithiothreitol). The precipitate was collected by 15 min centrifugation at 15,000 g at 4 °C, washed once with acetone containing 20 mM DTT and then resuspended in 250 μl of IEF (isoelectric focusing) buffer [7 M urea, 2 M thiourea, 4% (w/v) Chaps, 1% (v/v) Triton X-100, 0.8% Pharmalyte 3-10 (Amersham Biosciences) and 1% (v/v) DTT]. Protein samples were separated according to their pI in precast Immobiline gel containing a linear gradient of pH 4–7 (Immobiline Drystrips; Amersham Biosciences). IEF was performed at 16 °C and 50 μA/strip; voltage was changed in steps. First, the strips were re-hydrated at 360 V for 360 Vh. Samples were run at 300 V for 150 Vh, followed by a step at 3000 V for 3000 Vh, then 6000 V for 3000 Vh and the last step at 7500 V for 55,000 Vh. After the first dimension, the strips were equilibrated in equilibration buffer [50 mM Tris/HCl, pH 8.8/6 M urea/30% (w/v) glycerol/2% (w/v) SDS], containing 10 mg/ml DTT for 15 min at room temperature, followed by equilibration in the same buffer containing 48 mg/ml iodoacetamide for 15 min at room temperature. Then, the samples were run on an SDS/10% (w/v) polyacrylamide gel, followed by silver staining [26]. Phosphorylation was detected by autoradiography of the dried acrylamide gels. Densitometry of the bands corresponding to the phosphorylated proteins was quantified by using the Scion image program. Results are expressed as the percentage of maximum phosphorylation obtained after stimulation with fMLP.

Sample preparation for MALDI–TOF (matrix-assisted laser desorption ionization–time-of-flight)-MS analysis

Spots of interest were cut from dried two-dimensional gels and soaked in 200 μl of 50 mM NH₄HCO₃ for 15 min at room temperature. This step was repeated twice. After removing the buffer, samples were dehydrated in 200 μl of acetonitrile for 10 min. This step was repeated three times. After removing acetonitrile, samples were dried under vacuum for 30 min, re-hydrated in 50 μl of 100 mM NH₄HCO₃ containing 10 mM DTT for 30 min at 56 °C and dehydrated again in acetonitrile as above before drying under vacuum for 30 min. Then, 400 ng of trypsin reconstituted in 5 μl of 50 mM NH₄HCO₃ was added to the samples. NH₄HCO₃ buffer (5–10 μl) was further added to avoid dehydration of the sample during the digestion. Trypsin digestion was performed overnight at 30 °C [27]. For MALDI–TOF-MS, 0.5 μl of the digestion solution was deposited on to the target disc.
PKC activation assay
PKC activity was assessed by measuring its redistribution from cytosol to the plasma membrane. Polymorphonuclear neutrophils at 1 × 10⁶ cells/ml were stimulated for the indicated times with PMA (400 nM) or fMLP (10 nM or 10 µM) at 37 °C. The reaction was stopped by the addition of an 8-fold excess of ice-cold PBS. Cells were collected and resuspended in 400 µL of 10 mM Pipes (pH 7.0), 100 mM KCl, 3 mM NaCl and 3.5 mM fluorophosphate, 10 mM glycerophosphate and 25 mM NaF. Subsequently, cells were sonicated three times for 5 s on ice, and centrifuged at 1000 × g for 15 min at 4 °C to remove nuclei and unbroken cells. This post-nuclear supernatant was layered on a 15 % (w/w) sucrose cushion, centrifuged at 150 000 × g for 75 min at 4 °C (TLS 55 rotor; Beckman Instruments) [27], and the top layer was collected (cytosol) [28] and analysed for the level of PKC isotypes by Western blotting using specific antibodies.

Gel electrophoresis and immunoblotting
Triton (1 %)-soluble extracts or cytosolic fractions were submitted to SDS/PAGE [10 or 12 % gel respectively, containing 0.2 % (w/v) SDS and a 5 % stacking gel] [29]. For Western blotting, proteins were electrotransferred on to nitrocellulose at 100 mA for 1 h [30]. Nitrocellulose was incubated for 1 h in 0.05 % (w/v) TBS–Tween containing 2 % (w/v) low-fat milk proteins, and then incubated overnight with polyclonal antibodies directed against cPKC-βII (C-16; 1:1000), cPKC-βIII (C-18; 1:1000), nPKC-δ (C-20; 1:2000) and PKC-ζ (C-20; 1:500). The immunocomplexes were detected with goat anti-(rabbit Ig) Ig secondary antibody conjugated with peroxidase. The bound peroxidase activity was detected using ECL® (enhanced chemiluminescence) reagents (Amersham Biosciences).

Statistical analysis
Results are expressed as means ± S.D. Statistical analysis was performed using the unpaired t test. The results significantly different (P < 0.05) from the control are indicated.

RESULTS
L-plastin is a major phosphoprotein observed after fMLP stimulation of neutrophils
Neutrophils preloaded with [³²P]Pi, were incubated for 5 min with 1 µM fMLP, collected by centrifugation and lysed. A Triton-soluble extract was submitted to SDS/PAGE, followed by autoradiography. fMLP stimulation consistently induced the phosphorylation of a prominent 70 kDa phosphoprotein (Figure 1). To identify this protein, cell extracts were analysed by two-dimensional IEF/SDS/PAGE, followed by autoradiography of the gels. A prominent phosphoprotein spot was observed in the 70 kDa region with a pI of approx. 5.3 (Figure 1B). Since no other major phosphoproteins were observed over this molecular-mass range, we concluded that this spot represented the 70 kDa phosphoprotein observed on SDS/polyacrylamide gels.

![Figure 1: fMLP-induced phosphorylations in neutrophils](image-url)
of the two-dimensional autoradiograms suggested that the 70 kDa protein separated into multiple spots (Figure 1B). Over-laying of the two-dimensional autoradiogram and the silver-stained two-dimensional gel revealed four protein spots that corresponded to the phosphoprotein spots on the autoradiogram (Figure 1C). The four protein spots were taken from the gel and trypsinized, and the trypsin digests were analysed by MALDI–TOF-MS. The spectra were compared with three different databases of predicted peptide profiles. The first column indicates in which of the four spots the peptide was found (e.g. 1–4, the peptide was found in all four spots). Spot 1 corresponds to the most acidic spot and spot 4 to the most alkaline spot (Figure 1C).

Table 1: MALDI–TOF spectrum analysis of the 70 kDa protein

<table>
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<tr>
<th>Spots (pH 4–7)</th>
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<th>Calculated</th>
<th>Position</th>
<th>Peptide</th>
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<td>1116.59</td>
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<tr>
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<td>1584.84</td>
<td>597–610</td>
<td>YVAPLEDLVEVNPK</td>
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</table>

*Phosphorylation of one residue, either serine, threonine or tyrosine.
† Oxidation of one residue, methionine.

The four spots of 70 kDa corresponded to a single protein known as L-plastin. The observed mass of Ro-31-8220 (PKC inhibitor), 50 mM butanol (Bu; PLD modulator) and/or 10 µM Ro-31-8220 (RO; PKC inhibitor) before stimulation with 1 µM fMLP. The Triton-soluble fractions were prepared as described previously and subjected to SDS/PAGE and radiography. Densitometric analysis of L-plastin phosphorylation in response to 1 µM fMLP stimulation for 5 min at 37 °C. Phosphorylation is expressed as a percentage of the control obtained in the absence of inhibitor. Results are expressed as the means ± S.D. from 3–5 experiments. *Statistically significant (P < 0.05) from the control.

Figure 2: Involvement of PI3K, PLD and PKC in fMLP-induced L-plastin phosphorylation in neutrophils

Neutrophils preloaded with 32P Pi were incubated for 30 min at 37 °C with 50 µM LY294002 (LY; PI3K inhibitor), 50 mM butanol (Bu; PLD modulator) and/or 10 µM Ro-31-8220 (RO; PKC inhibitor) before stimulation with 1 µM fMLP. The Triton-soluble fractions were prepared as described previously and subjected to SDS/PAGE and autoradiography. Densitometric analysis of L-plastin phosphorylation in response to 1 µM fMLP stimulation for 5 min at 37 °C. Phosphorylation is expressed as a percentage of the control obtained in the absence of inhibitor.

Characterization of fMLP receptors on neutrophils

Although it is clear that L-plastin is phosphorylated in response to fMLP stimulation of neutrophils, two fMLP receptor subtypes are known to exist with different affinities for fMLP [17]. Under the conditions of phosphorylation employed above, either of these could be involved in L-plastin phosphorylation. To define fMLP receptor heterogeneity in intact human neutrophils, we have performed binding assays. Figure 3 shows the saturation binding curve for fMLP, obtained using a fMLP tracer of low specific activity (approx. 2 Ci/mm mol). In a one-site fit, the calculated K d was approx. 360 nM (Figure 3A); however, a better statistical fit was obtained with a two-binding-site model, suggesting the existence of a low-affinity receptor (K d1 = 1.5 µM) and a high-affinity receptor (K d2 = 60 nM) for fMLP (Table 2). To define clearly the second K d, the experiment was performed using an fMLP tracer of high specific activity (60 Ci/mmol). The binding followed a saturating curve with a calculated K d of 36 nM (Figure 3A, inset; Table 2). These results were well within the range of what was observed previously at low temperatures [23]. Fabbri et al. [23] reported two fMLP-binding sites on neutrophils with K d1 = 25 nM and K d2 = 1.5 µM in assays performed at 4 °C.

To differentiate between the two receptors, a peptide antagonist, t-Boc, was used [32–34]. t-Boc is known to block the FPR over-expressed in S9 cells [35] and the endogenous FPR in neutrophils [36], both of which have a high affinity for fMLP. At 32 nM [3H]fMLP, specific binding to neutrophils was completely inhibited in the presence of concentrations of t-Boc above 1 × 10−7 M (Figure 3B, left panel). Conversely, saturable binding of fMLP was observed under conditions of blocking the high-affinity receptor with t-Boc (Figure 3B, right panel). As expected, the level of saturation was less than that obtained without t-Boc. Taken together, our results indicate that t-Boc can differentiate between a high- and a low-affinity fMLP receptor on neutrophils, and the high-affinity receptor is most probably FPR. The low-affinity receptor may be FPR-L1, based on the fact that its mouse
orthologue (FPR2) has a low affinity for fMLP [17] and on the observation that activation of FPR-L1 by a synthetic peptide agonist is insensitive to t-Boc [36]. By this method, we have studied the intracellular events associated with activation of these receptors in human neutrophils.

**L-plastin phosphorylation by specific fMLP receptor subtypes**

To analyse the contribution of the two different fMLP receptor subtypes to L-plastin phosphorylation, the effect of a wide concentration range of fMLP was tested. The autoradiogram presented in Figure 4(A) shows that L-plastin phosphorylation occurred at low concentrations of fMLP (between 1 and 10 nM) and continued over the whole concentration range. Phosphorylation of L-plastin was time-dependent and maximal at 5 min of incubation (Figure 4B). The results suggest that at least the high-affinity fMLP receptor induces L-plastin phosphorylation, but a contribution of the low-affinity receptor could not be excluded. Therefore we investigated whether one or both of the two fMLP receptor subtypes govern L-plastin phosphorylation. The contribution of the high-affinity receptor to phosphorylation was assessed in neutrophils preincubated with 3 × 10⁻⁵ M t-Boc for 15 min at 37 °C before stimulation with a low concentration of fMLP (10 nM). As shown in Figure 4(C), t-Boc completely blocked L-plastin phosphorylation at 10 nM fMLP, indicating that the high-affinity receptor directs phosphorylation of L-plastin (Figure 4C). t-Boc treatment had no effect on basal phosphorylation in unstimulated cells (Figure 4C). At high concentrations of fMLP (e.g. 10 μM), L-plastin phosphorylation is decreased by t-Boc, but not blocked entirely. At these t-Boc concentrations, the high-affinity receptor is blocked completely (Figure 3B); therefore this t-Boc-insensitive L-plastin phosphorylation must be directed by the low-affinity receptor (Figure 4C). On the basis of the levels of L-plastin phosphorylation driven by each receptor, their individual contributions are not additive, suggesting the existence of a common limiting component. Thus, both receptors (the high- and low-affinity fMLP receptors) were capable of inducing the phosphorylation of L-plastin.

The two fMLP receptor subtypes use a different set of signal-transduction intermediates to drive L-plastin phosphorylation

We investigated the involvement of PI3K, PLD and PKC in the phosphorylation of L-plastin by the two fMLP receptor subtypes. Cells were stimulated either with 10 nM fMLP without t-Boc to activate the high-affinity receptor, or with 10 μM fMLP in the presence of t-Boc to activate the low-affinity receptor. As shown in Figure 5, activation of the low-affinity receptor led to the phosphorylation of L-plastin, which was strongly inhibited by Ro-31-8220, the PKC inhibitor. This phosphorylation was also sensitive to LY294002 and butanol (Figure 5A, lower panel; Figure 5B, black bars), suggesting that PI3K and PLD are coupled with the low-affinity receptor. In contrast, stimulation of the high-affinity receptor, by using 10 nM fMLP without t-Boc, induced L-plastin phosphorylation that was insensitive to LY294002 and butanol, suggesting that, in this case, phosphorylation was PI3K- and PLD-independent, but sensitive to the PKC inhibitor Ro-31-8220 (Figure 5A, upper panel; Figure 5B, open bars). Thus, different signal-transduction pathways are involved in the regulation of L-plastin phosphorylation by fMLP receptor subtypes in neutrophils. PI3K and PLD are not involved in high-affinity receptor signalling, but do couple with the low-affinity receptor. A Ro-31-8220-sensitive component is involved in the transduction of both receptor signals.

**PKC isotype responses to high- and low-affinity fMLP receptors**

PKC is not a single molecular entity, but defines a family of isotypes, which share their sensitivity to Ro-31-8220. It is possible
that individual isotypes couple differently with fMLP receptor subtypes, thus explaining the sensitivity of either receptor to Ro-31-8220. To assess PKC activation by fMLP receptor subtypes, we performed a cytosol-depletion assay (Figure 6). Human neutrophils were stimulated for 5 min with either 10 nM fMLP (high-affinity receptor) or 10 µM fMLP in the presence of t-Boc (low-affinity receptor) at 37 °C, and cytosolic fractions were prepared. A control for cell exposure to t-Boc alone was included, as was a positive control for PKC activation (the phorbol ester PMA). Compatible with the sensitivity of individual isotypes, a decrease in the cytosol level of PKC-β1, -βII and -δ but not PKC-ζ was observed after PMA stimulation. Activation of all PKC isotypes was observed after the stimulation of receptors with a low affinity for fMLP (Figure 6). Hence, it is probable that PKC isotypes are involved in L-plastin phosphorylation by the low-affinity fMLP receptor, thus explaining the effect of Ro-31-8220.
of sensitivity of our assay system and an inability to pick up translocation of a small proportion of the total PKC. Alternatively, PKC isotypes may not couple with the high-affinity fMLP receptor. Since Ro-31-8220 inhibits L-plastin phosphorylation by this receptor, the latter results would imply that an as yet unidentified Ro-31-8220-sensitive pathway impinges on L-plastin. Altogether, our results indicate that the different fMLP receptor subtypes induce L-plastin phosphorylation through different signal-transduction intermediates.

DISCUSSION

We have studied phosphorylation events induced in human neutrophils after fMLP stimulation. Our results indicate that human neutrophils express at least two fMLP receptor subtypes, with a high and a low affinity for fMLP. Multiple fMLP receptors have been cloned, including the FPR and its homologue FPR-L1, which share 69% amino acid sequence identity [9]. The pharmacology established in transfected cells indicates that FPR binds fMLP with a high-affinity, whereas FPR-L1 binds fMLP with a low affinity [5–8], suggesting that these two receptors represent the ones measured in the present study. The identity of the high-affinity receptor was further defined using t-Boc, which discriminates between the two receptors.

When studying the phosphorylation events associated with receptor activation, we observed a major phosphorylated band at approx. 70 kDa, which was the most consistently observed fMLP-driven phosphorylated protein. To identify this phosphoprotein, we made use of recent advances in micro-mass spectrometry methods that now make it possible to identify proteins even when only very small amounts of material are available [37]. To obtain a pure protein sample for MS, we performed two-dimensional gel separation of the protein complement and retrieved the protein from the gel. In this way, we could identify L-plastin as the 70 kDa protein phosphorylated in response to fMLP (Table 1). L-plastin belongs to the fimbrin family of actin-binding proteins [20]. It possesses two actin-binding domains and two EF-hand calcium-binding domains [19], is subject to phosphorylation/dephosphorylation [20] and has actin bundling activity [38]. L-plastin phosphorylation on serine residues has been implicated in α5β1-mediated neutrophil adhesion [18,21], possibly through the regulation of the actin cytoskeleton.

A major finding of the present study is that L-plastin phosphorylation is not driven by one but both fMLP receptor subtypes. This is of interest since physiologically fMLP is a chemoattractant, which governs the migratory behaviour of the cell over a range of concentrations [17]. The existence of receptor subtypes with different ligand affinities is an obvious tool in the detection of such a gradient. The fact that these receptors have a common output is in keeping with the required common biological response over the wide range of concentrations presented. Similar differences in fMLP receptor subtypes have been suggested in mouse neutrophils where two distinct optima of chemoattractant concentration have been shown to induce chemotaxis.

A second finding has been that the two receptor subtypes appear to use a different complement of signalling molecules to elicit the phosphorylation of L-plastin. Using pharmacological interference, we showed that PI3K and PLD mediate the low-but not the high-affinity receptor signal. To our knowledge, this is the first example illustrating a single event that occurred in response to stimulation of two receptor subtypes, and via two different signalling pathways. Although their activation has not formally been assessed for either receptor, existing evidence
confirms that both PI3K and PLD are activated in response to fMLP concentrations above 100 nM, i.e. through the low-affinity receptor [39,40]. Studies involving PI3K-γ knockout mice showed that this isoform was the sole PI3K coupled with the fMLP receptor in neutrophils [11,41]. In these knockout mice, chemotaxis was decreased by approx. 50% in response to fMLP, suggesting that a PI3K-γ-independent pathway exists. Our observation that pharmacological interference with PI3K does not fully inhibit l-plastin phosphorylation in response to fMLP also suggests a PI3K-independent pathway. This may be represented by PLD, as indicated by the effect of butanol and the combination of LY294002 compound and butanol. A contribution of PLC may also be considered [42]; however, it should be noted that the PLC pathway is not required for chemotactic activity in fMLP-stimulated neutrophils (in fact, it appears to be a negative regulator of chemotaxis), as demonstrated using PLC knockout mice [11]. Our own preliminary observations, using a PLC inhibitor, also indicated that PLC is not involved in mediating l-plastin phosphorylation by fMLP (results not shown).

Although pharmacological interference clearly differentiated between the two receptors in their use of PI3K and PLD to regulate l-plastin phosphorylation, the interpretation of the effects of Ro-31-8220 is less straightforward. L-plastin phosphorylation by fMLP (results not shown).

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L-Plastin phosphorylation in fMLP-stimulated neutrophils


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