N-Formyl peptide receptor subtypes in human neutrophils activate L-plastin phosphorylation through different signal transduction intermediates

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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 chemoattractant.

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 isotypes classified into three groups on the basis of their structure and mode of activation [15,16]: conventional PKCs (cPKCs; PKC-α, -βI, -βII and -γ), 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 leucocyte 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 isotypes. These observations are discussed in relation to the function of fMLP receptor subtypes in the detection and processing of increasing concentrations of the ligand.

Abbreviations used: DTT, dithiothreitol; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine ('chemotactic peptide'); FPR, formyl peptide receptor; FPR-L1, FPR-like 1; t-Boc, terbutoxycarbonyl-Phe-Leu-Phe-Leu-Phe; IEF, isoelectric focusing; MALDI–TOF, matrix-assisted laser desorption ionization–time-of-flight; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D.

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EXPERIMENTAL

Materials

Dextran was purchased from ICN Biochemicals (Costa Mesa, CA, U.S.A.) and Lymphoprep™ from Nycomed Pharma AS (Oslo, Norway). [^{3}H]fMLP (specific activity, 60 Ci/mmol) was purchased from NEN™ Life Science Products (Boston, MA, U.S.A.). [^{32}P]P_i (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-βI (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 (terbutoxycarbonyl-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 hypo-osmotic 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 $2–5 \times 10^7$ cells/ml.

^{32}P-labelling of neutrophils

Neutrophils were incubated in phosphate-free RPMI 1640 containing 0.5–1 mCi of [^{32}P]P_i/$2–5 \times 10^7$ 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 tosyl-lysylchloromethane ('TLCK')/2 mM PMSF/10 μM di-isopropyl fluorophosphate/10 mM glycerophosphate/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 (5×10^6 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 [^{3}H]fMLP (0–6 μM). To reach concentrations higher than 8×10^{-7} M, [^{3}H]fMLP was mixed with unlabelled fMLP. In this case, the specific binding measured was corrected with a coefficient corresponding to the percentage of [^{3}H]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 [^{3}H]fMLP and increasing concentrations of t-Boc (10^{-9}–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 binding, dissociation constant (K_d) 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 % (w/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 (Volthour). 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 NH_4HCO_3 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 NH_4HCO_3 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 NH_4HCO_3 was added to the samples. NH_4HCO_3 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

and allowed to air-dry. This was overlaid with 0.5 μl of matrix solution [1 % (w/v) α-cyano-4-hydroxycinnamic acid in 50 % (v/v) acetonitrile/0.1 % (v/v) trifluoroacetic acid] and dried again. Spectra were obtained by using a Biflex III MALDI–TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). MALDI–TOF spectra were analysed by comparing a list of mass-to-charge ratios acquired for each protein digested against three separate databases [Owl, National Center for Biotechnology Information (NCBI) and SwissProt] by using three different browsers (MS-FIT at falcon.ludwig.ucl.co.uk/ucsfhtml3.2/msfit.htm, Mascot at www.matrixscience.com and Peptident at www.expasy.ch/tools/peptident.ftml). Mass tolerance was limited to 200 p.p.m. and proteins were matched according to the probability scores deduced by each browser.

PKC activation assay

PKC activity was assessed by measuring its redistribution from cytosol to the plasma membrane. Polymorphonuclear neutrophils at 1×10^8 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 $MgCl_2$ containing 20 μM leupeptin, 1.5 μM pepstatin, 27 μM tosyl-lysylchloromethane, 2 mM PMSF, 10 μM di-isopropyl 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-βI (C-16; 1:1000), cPKC-βII (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 $\pm$ 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 [^{32}P]P_i 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. Close examination

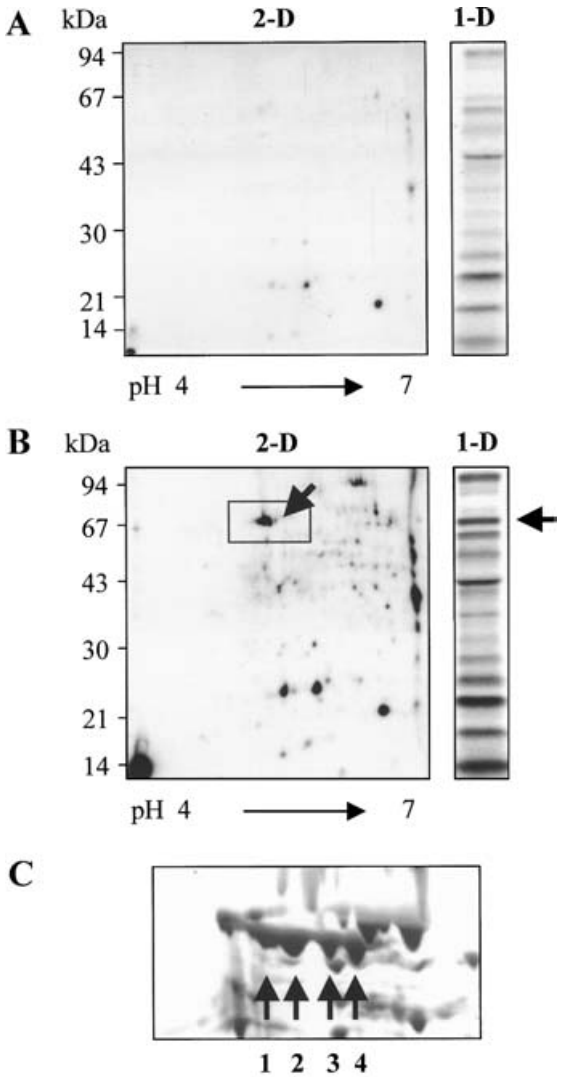

Figure 1 fMLP-induced phosphorylations in neutrophils

Proteins contained in the 1 % Triton X-100-soluble extract (5×10^6 cell equivalent/gel) were precipitated, washed and resuspended in 250 μl of IEF buffer. Proteins were separated according to their pI in precast Immobiline gel containing a linear gradient of pH 4–7. Details of the IEF method are described in the Experimental section. After the first dimension, the samples were run on SDS/10 % (w/v) polyacrylamide gel, followed by silver staining. Phosphorylation was detected by autoradiography of the dried acrylamide gels. (**A**) Autoradiograms of the control experiment without fMLP: autoradiograms of the two-dimensional gel (left panel) and the corresponding one-dimensional gel (right panel). (**B**) Autoradiograms of the soluble extract after stimulation with 1 μM fMLP for 5 min at 37 °C: autoradiograms of the two-dimensional gel (left panel) and the corresponding one-dimensional gel (right panel). The arrow indicates the proteins predominantly phosphorylated in response to 1 μM fMLP. (**C**) Silver-stained two-dimensional gel corresponding to the 70 kDa spots phosphorylated after 1 μM fMLP stimulation (**B**, arrow on the left panel). Four spots (1–4) were clearly separated on the gel.

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

Tryptic peptides observed in the MALDI–TOF spectrum of the four spots corresponding to the 70 kDa protein phosphorylated in response to fMLP (Figures 1B and 1C). The observed mass 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).

Spots (pH 4–7)	Peptide mass		Position	Peptide
	Experimental	Calculated		
1–4	1116.53	1116.59	40–49	AACLPLPGYR
1–4	1876.89	1876.85	52–68	EITENLMATGDLDQDGR
1, 2	997.46	997.51	69–76	ISFDEFIK
1–4	1474.71	1474.66	77–88	IFHGLKSTDVAK**
1–4	2224.22	2224.05	124–141	YAFVNWINKALENDPDCR
1–4	1501.71	1501.74	166–178	MINLSVPDTIDER
1–4	1232.62	1232.68	234–244	IGLFADIELSR
1–4	1011.56	1011.61	245–253	NEALIALLR
1, 3, 4	2296.30	2296.09	254–272	EGESLEDLMKLSPEELLLR*†
1–4	1068.57	1068.62	264–272	LSPEELLLR
1–4	1575.65	1575.70	273–285	WANYHLENAGCNK
1–4	1742.87	1742.85	310–326	GDEEGVPAVVIDMSGLR
1–4	1234.53	1234.56	334–343	AECMLQQAER
1–4	1134.55	1134.60	348–357	QFVTATDVVR
1	1404.74	1404.79	362–373	LNLAFIANLFNR
1–4	2538.40	2538.21	374–395	YPALHKPENQDIDWGALEGET
1–4	1286.57	1286.62	402–412	NWMNSLGVNPR
1	1382.61	1382.58	402–412	NWMNSLGVNPR†
1	2366.37	2366.22	413–432	VNHLYSDLSDALVIFQLYEK
1–4	1125.56	1125.63	433–441	IKVPVDWNR
1–3	884.44	884.45	435–441	VPVDWNR
1–4	1674.84	1674.83	473–488	FSLVGIGGQDLNEGNR
1–4	1798.95	1798.92	516–530	VNDDIIVNWVNETLR
1–4	841.50	841.42	535–542	SSSISSFK
1, 2	1430.69	1430.67	580–591	LNNAKYAISMAR*
1–4	1584.77	1584.84	597–610	VYALPEDLVEVNPK

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

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 four spots of 70 kDa corresponded to a single protein known as L-plastin (Table 1) [20,21], which has a calculated pI of 5.3. The four spots were distinguished by a different pI value; on the basis of the Immobilines used for two-dimensional electrophoresis, we calculated their pI values to be 5.2, 5.3, 5.4 and 5.5.

fMLP-induced L-plastin phosphorylation is regulated by multiple signal-transduction intermediates

To identify the signal-transduction intermediates involved in the coupling of the fMLP receptor with L-plastin phosphorylation, we employed a number of well-characterized inhibitors. Cells were incubated for 30 min at 37 °C with 50 μM LY294002 (PI3K inhibitor), 50 mM butanol (PLD modulator) and/or 10 μM Ro-31-8220 (PKC inhibitor) [31] before stimulation with 1 μM fMLP. PI3K or PLD inhibition decreased fMLP-induced L-plastin phosphorylation by approx. 50–60 % (Figure 2). In the presence of Ro-31-8220, L-plastin phosphorylation was inhibited by approx. 90 % (Figure 2). Combinations of inhibitors led to a complete absence of L-plastin phosphorylation (Figure 2). Thus, fMLP receptors engage multiple signal-transduction intermediates to drive the phosphorylation of L-plastin, including PI3K and PLD. On the basis of the inhibition by Ro-31-8220, PKC is probably a kinase involved in L-plastin phosphorylation.

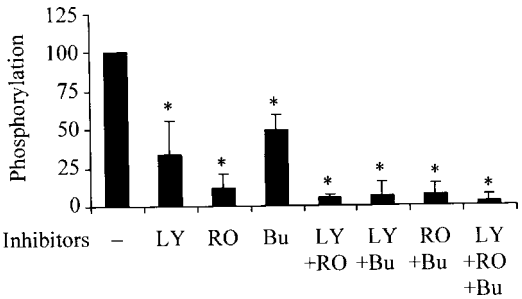

Figure 2 Involvement of PI3K, PLD and PKC in fMLP-induced L-plastin phosphorylation in neutrophils

Neutrophils preloaded with [^{32}P]P_i 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. Results are expressed as the means $\pm$ S.D. from 3–5 experiments. *Statistically significant ($P < 0.05$) from the control.

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/mmol). 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 ($K_d = 1.5$ μM) and a high-affinity receptor ($K_d \approx 60$ 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 overexpressed in Sf9 cells [35] and the endogenous FPR in neutrophils [36], both of which have a high affinity for fMLP. At 32 nM [^{3}H]fMLP, specific binding to neutrophils was completely inhibited in the presence of concentrations of t-Boc above 1×10^{-5} 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

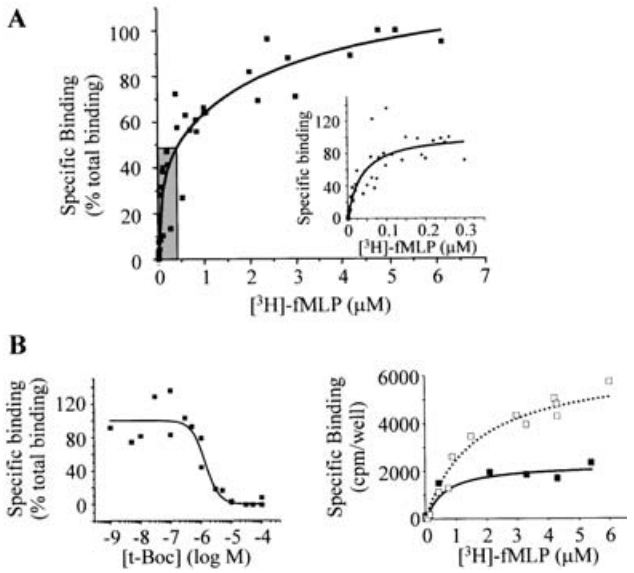

Figure 3 fMLP receptor heterogeneity in human neutrophils

(**A**) Aliquots of neutrophils (5×10^6 cells/200 μl) were incubated for 15 min at 37 °C, in the presence of increasing concentrations of [^{3}H]fMLP (0–6 μM; specific activity, 2 Ci/mmol). Non-specific binding was measured in the presence of 100 μM unlabelled fMLP. Inset shows the binding curve for 0–300 nM [^{3}H]fMLP (specific activity, 60 Ci/mmol). Results were obtained from 3–5 (inset) independent experiments and are expressed as a percentage of the maximal binding level obtained in each individual experiment. Experiments for each concentration were performed in triplicate. (**B**) Inhibition of the fMLP binding to high-affinity FPRs in the presence of t-Boc. Cells were incubated with 32 nM [^{3}H]fMLP and increasing concentrations of t-Boc (10^{-9}–10^{-4} M) for 15 min at 37 °C. Non-specific binding was measured in the presence of 10 μM unlabelled fMLP (left panel). Results are representative of two independent experiments performed in triplicate for each concentration. Results are expressed as percentage of the maximal binding level obtained in each individual experiment. In a second experiment (right panel), cells were incubated first for 15 min with 3×10^{-5} M t-Boc (which corresponds to a concentration that inhibits totally the binding of fMLP to high-affinity FPRs) (solid line) or with 0.3 % DMSO in RPMI 1640 for the control experiment (dotted line), and then incubated for another 15 min with increasing concentrations of [^{3}H]fMLP (0–6 μM). Non-specific binding was determined in the presence of 100 μM unlabelled fMLP. Specific binding is expressed in c.p.m./well. Results are representative of two independent neutrophil preparations, performed in triplicate for each concentration.

Table 2 Saturation curve analysis of fMLP binding

fMLP-binding experiments were performed as described in the Experimental section. Briefly, neutrophils (5×10^6 cells/200 μl) were incubated for 15 min at 37 °C [27], in the presence of increasing concentrations of [^{3}H]fMLP (0–6 μM) having low (approx. 2 Ci/mmol) or high (60 Ci/mmol) specific activity. Non-specific binding was measured in the presence of 10 or 100 μM unlabelled fMLP. At the end of the incubation, cells were filtered 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. SA, specific activity; P, probability of correctness of the model.

	K_d (μM)	P
Low SA tracer		
One-site model	0.36	0.90
Two-site model	1.5, 0.067	0.93
High SA tracer		
One-site model	0.036	0.99

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

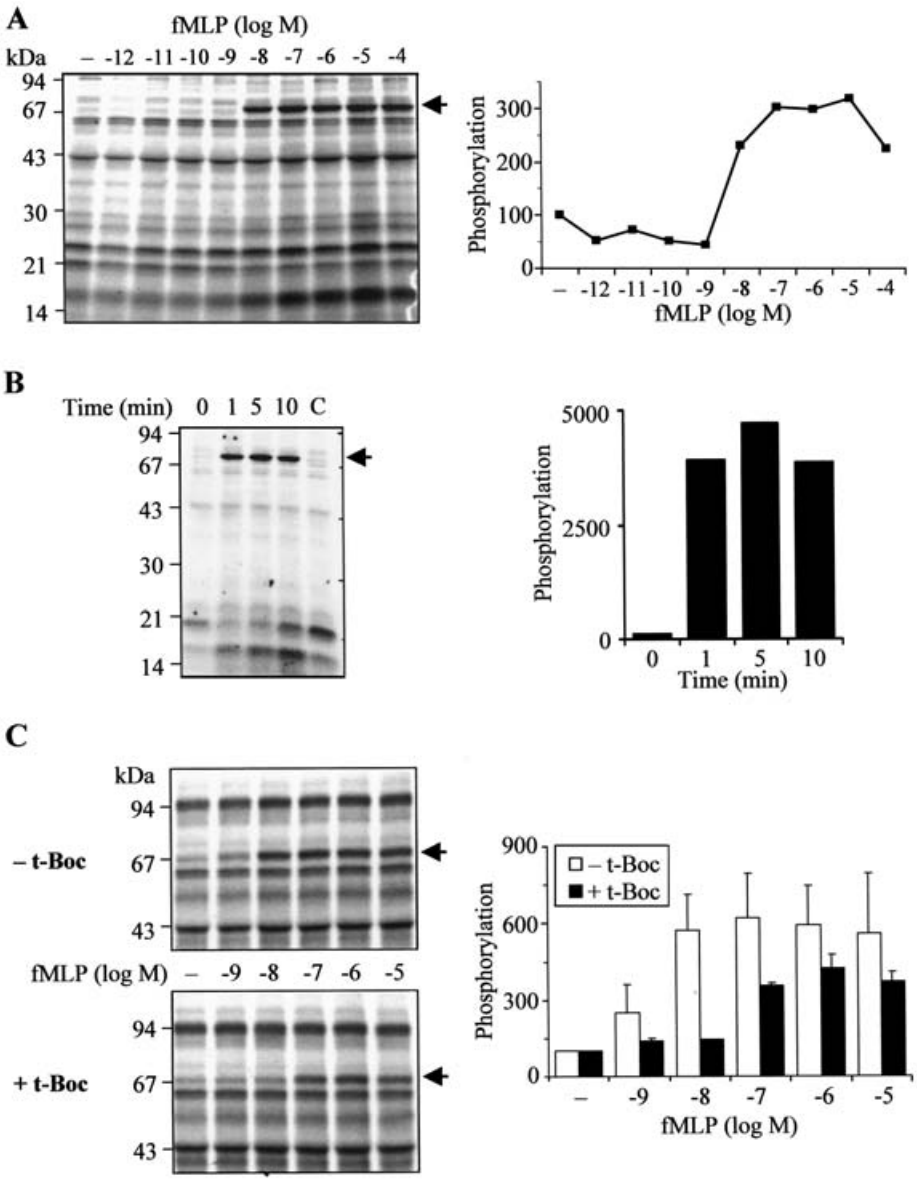

Figure 4 L-plastin phosphorylation by individual fMLP receptor isotypes in neutrophils

Neutrophils ($2–5 \times 10^7$ cells/ml) were incubated in phosphate-free RPMI 1640 containing 0.5–1 mCi of [^{32}P]P_i for 1 h at 37 °C. (**A**) Cells were stimulated with fMLP ($0–1 \times 10^{-4}$ M) for 5 min at 37 °C. The cells were solubilized in lysis buffer and the Triton-soluble extract was collected after centrifugation at 100 000 ***g*** for 15 min at 4 °C and subjected to SDS/PAGE and autoradiography (3×10^5 cell equivalent/lane) (left panel). Densitometric analysis of phosphorylation profile of L-plastin (right panel). (**B**) Time-course experiment. Cells were stimulated with 1 μM fMLP for 0–10 min at 37 °C and lysed as described in (**A**). Samples (3×10^5 cell equivalent/lane) were analysed by SDS/PAGE. Left panel: the autoradiogram representative of two different neutrophil preparations. Lane C, the extract from cells incubated without the stimulus for 10 min at 37 °C. Right panel: densitometric quantification of the phosphorylation of L-plastin (arrow on the left panel). (**C**) Neutrophils ($2–5 \times 10^7$ cells/ml) were incubated in phosphate-free RPMI 1640 containing 0.5–1 mCi of [^{32}P]P_i, for 45 min at 37 °C. Then, 3×10^{-5} M t-Boc (final concentration) or 0.5 % DMSO in phosphate-free RPMI 1640 was added and the incubation was performed for 15 min. The cells were then stimulated with fMLP ($10^{-9}–10^{-5}$ M) for 5 min at 37 °C and the soluble fraction was collected. Autoradiogram of phosphorylation experiments (left panel) in the absence (– t-Boc) or presence of 3×10^{-5} M t-Boc (+ t-Boc). Right panel: histogram representing the autoradiogram analysis by densitometry of L-plastin phosphorylation in fMLP-stimulated neutrophils in the absence (open bars) or presence of 3×10^{-5} M t-Boc (black bars). Results are expressed as the means ± S.D. from two independent experiments.

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-βI, -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

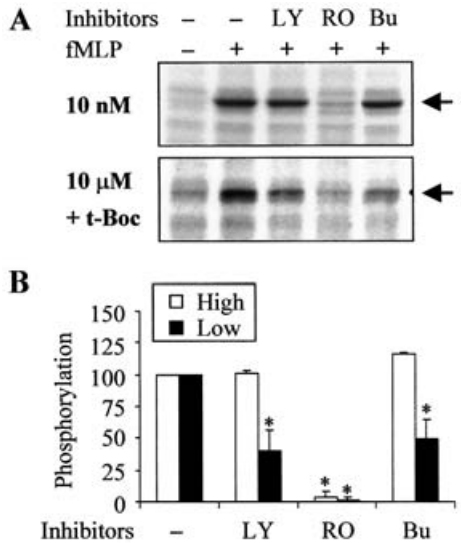

Figure 5 Pharmacology of signal-transduction pathways involved in L-plastin phosphorylation by fMLP receptor subtypes

Cells were incubated with the inhibitors described in Figure 2 and stimulated either with 10 nM fMLP for 5 min to activate only the high-affinity receptors, or first with 3×10^{-5} M t-Boc for 15 min and then with 10 μM fMLP for 5 min to activate only the low-affinity receptors. (**A**) Autoradiograms corresponding to these experiments. The arrows indicate the migration of L-plastin. (**B**) Densitometric analyses of L-plastin phosphorylation induced by the activation of the high- (open bars) or low- (black bars) affinity receptors. Results are expressed as the means ± S.D. from at least three experiments.

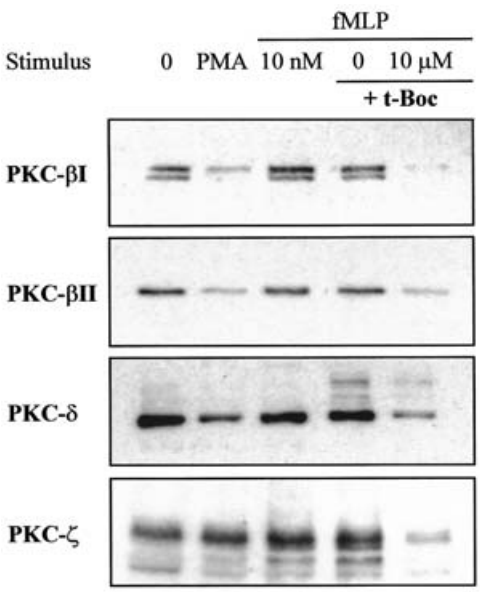

Figure 6 PKC activation by fMLP receptor subtypes

Western-blot analysis of cytosolic fractions isolated from neutrophils either stimulated for 5 min at 37 °C with 10 nM fMLP or first incubated with 3×10^{-5} M t-Boc for 15 min at 37 °C and then with 10 μM fMLP for 5 min at 37 °C. Purified fractions were isolated after centrifugation at 200 000 ***g*** for 75 min at 4 °C on a discontinuous gradient as described in the Experimental section. Cytosolic proteins (20 or 10 μg for PKC-δ) were loaded on to SDS/polyacrylamide gel, followed by immunoblotting with specific polyclonal antibodies as described in the Experimental section. The immunocomplexes were detected by ECL®.

on L-plastin phosphorylation by this receptor. However, in our assay system, PKC isotypes were not activated in response to high-affinity fMLP receptor stimulation. This may be due to a lack 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 $\alpha_M\beta_2$-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 both high- and low-affinity receptors is completely blocked by Ro-31-8220. Since our biochemical results clearly show the activation of PKC isotypes by the low-affinity receptor, these may represent the Ro-31-8220-sensitive component of L-plastin phosphorylation through this receptor. PKC isotypes themselves are controlled by PLC, PLD and/or PI3K [43]. PKC has been shown to be downstream of PLD, at least in a pathway leading to activation of the NADPH oxidase activation in response to fMLP [14,39,44]. Activation of PKC isotypes by the products of PI3K has also been suggested, although the mechanism is as yet elusive [45]. The lack of PKC activation in response to the high-affinity receptor may reflect a lack of sensitivity of our assay system, but could also indicate that PKC is not involved in L-plastin phosphorylation through this receptor. It should also be noted that, unlike other PKC isotypes, redistribution of PKC-ζ may not be a good measure of its activation (although redistribution occurs in response to high fMLP concentrations). Therefore, formally, PKC-ζ may still mediate this response [46]. Altogether, an as yet unknown Ro-31-8220-sensitive component may regulate L-plastin phosphorylation in response to low concentrations of fMLP.

Our results suggest an attractive model in which sequential activation of fMLP receptors, through different signal-transduction intermediates, may govern a chemotactic response to fMLP. When neutrophils detect low concentrations of chemoattractant, Ro-31-8220-sensitive pathways are initiated by the high-affinity receptor. With increasing concentrations of formyl peptides and possible desensitization of the high-affinity receptor, a second, low-affinity receptor would detect higher concentrations of the chemoattractant. This leads to activation of additional intracellular pathways, coupling with the same biological output (L-plastin phosphorylation). This model extends observations on mouse neutrophils, where genetic deletion of the FPR does not lead to complete abrogation of the fMLP responses, but rather results in responses to higher concentrations of fMLP [17]. These mechanisms of sequential activation of receptor subtypes may be particularly relevant to detection of and response to a gradient of ligand.

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REFERENCES

1 Boulay, F., Naik, N., Giannini, E., Tardif, M. and Brouchon, L. (1997) Phagocyte chemoattractant receptors. Ann. N.Y. Acad. Sci. **832**, 69–84
2 Schiffmann, E., Corcoran, B. A. and Wahl, S. M. (1975) *N*-formylmethionyl peptides as chemoattractants for leucocytes. Proc. Natl. Acad. Sci. U.S.A. **72**, 1059–1062
3 Carp, H. (1982) Mitochondrial *N*-formylmethionyl proteins as chemoattractants for neutrophils. J. Exp. Med. **155**, 264–275
4 Marasco, W. A., Phan, S. H., Krutzsch, H., Showell, H. J., Feltner, D. E., Nairn, R., Becker, E. L. and Ward, P. A. (1984) Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by *Escherichia coli*. J. Biol. Chem. **259**, 5430–5439
5 Boulay, F., Tardif, M., Brouchon, L. and Vignais, P. V. (1990) The human *N*-formylpeptide receptor. Characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. Biochemistry **29**, 11123–11133
6 Murphy, P. M., Ozcelik, T., Kenney, R. T., Tiffany, H. L., McDermott, D. and Francke, U. (1992) A structural homologue of the *N*-formylpeptide receptor. Characterization and chromosome mapping of a peptide chemoattractant receptor family. J. Biol. Chem. **267**, 7637–7643
7 Ye, R. D., Cavanagh, S. L., Quehenberger, O., Prossnitz, E. R. and Cochrane, C. G. (1992) Isolation of a cDNA that encodes a novel granulocyte *N*-formyl peptide receptor. Biochem. Biophys. Res. Commun. **184**, 582–589
8 Quehenberger, O., Prossnitz, E. R., Cavanagh, S. L., Cochrane, C. G. and Ye, R. D. (1993) Multiple domains of the *N*-formyl peptide receptor are required for high-affinity ligand binding. Construction and analysis of chimeric *N*-formyl peptide receptors. J. Biol. Chem. **268**, 18167–18175
9 Gao, J. L., Lee, E. J. and Murphy, P. M. (1999) Impaired antibacterial host defense in mice lacking the *N*-formylpeptide receptor. J. Exp. Med. **189**, 657–662
10 Wu, G., La Rosa, G. J. and Simon, M. I. (1993) G protein-coupled signal transduction pathways for interleukin-8. Science **261**, 101–103
11 Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A. V. and Wu, D. (2000) Roles of PLC-β2 and -β3 and PI3Kγ in chemoattractant-mediated signal transduction. Science **287**, 1046–1049
12 Stoyanov, B., Volinia, S., Hanck, T., Rubio, I., Loubtchenkov, M., Malek, D., Stoyanova, S., Vanhaesebroeck, B., Dhand, R. and Nurnberg, B. (1995) Cloning and characterization of a G-protein-activated human phosphoinositide-3 kinase. Science **269**, 690–693
13 Stephens, L. R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A. S., Thelen, M., Cadwallader, K., Tempst, P. and Hawkins, P. T. (1997) The G $\beta\gamma$ sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. Cell (Cambridge, Mass.) **89**, 105–114
14 Rossi, F., Grzeskowiak, M., Della Bianka, V., Calzetti, F. and Gandini, G. (1990) Phosphatidic acid and not diacylglycerol generated by phospholipase D is functionally linked to the activation of the NADPH oxidase by fMLP in human neutrophils. Biochem. Biophys. Res. Commun. **168**, 320–327
15 Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science **258**, 607–614
16 Dekker, L. V. and Parker, P. J. (1994) Protein kinase C – a question of specificity. Trends Biochem. Sci. **19**, 73–77
17 Hartt, J., Barish, G., Murphy, P. M. and Gao, J. L. (1999) *N*-formylpeptides induce two distinct concentration optima for mouse neutrophil chemotaxis by differential interaction with two *N*-formylpeptide receptor (FPR) subtypes: molecular characterization of FPR2, a second mouse neutrophil FPR. J. Exp. Med. **190**, 741–747
18 Jones, S. L. and Brown, E. J. (1996) FCγRII-mediated adhesion and phagocytosis induce L-plastin phosphorylation in human neutrophils. J. Biol. Chem. **271**, 14623–14630
19 Lin, C. S., Aebersold, R. H., Kent, S. B., Varma, M. and Leavitt, J. (1988) Molecular cloning and characterization of plastin, a human leukocyte protein expressed in transformed human fibroblasts. Mol. Cell. Biol. **8**, 4659–4668
20 Jones, S. L., Wang, J., Turck, C. W. and Brown, E. J. (1998) A role for the actin-bundling protein L-plastin in the regulation of leukocyte integrin function. Proc. Natl. Acad. Sci. U.S.A. **95**, 9331–9336
21 Wang, J. and Brown, E. J. (1999) Immune complex-induced integrin activation and L-plastin phosphorylation require protein kinase A. J. Biol. Chem. **274**, 24349–24356
22 Wang, J. and Brown, E. J. (2001) L-plastin peptide activation of $\alpha_v\beta_3$-mediated adhesion requires integrin conformational change and actin filament disassembly. J. Biol. Chem. **276**, 14474–14481
23 Fabbri, E., Spisani, S., Barbin, L., Biondi, C., Buzzi, M., Traniello, S., Zecchini, G. P. and Ferretti, M. E. (2000) Studies on fMLP–receptor interaction and signal transduction pathway by means of fMLP-OMe selective analogues. Cell. Signal. **12**, 391–398
24 Segal, A. W. and Jones, O. T. (1980) Absence of cytochrome *b* reduction in stimulated neutrophils from both female and male patients with chronic granulomatous disease. FEBS Lett. **110**, 111–114

25 Dekker, L. V., Leitges, M., Altschuler, G., Mistry, N., McDermott, A., Roes, J. and Segal, A. W. (2000) Protein kinase C-β contributes to NADPH oxidase activation in neutrophils. Biochem. J. **347**, 285–289
26 Wray, W., Boulikas, T., Wray, V. P. and Hancock, R. (1981) Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118**, 197–203
27 Lopez-Lluch, G., Bird, M., Canas, B., Godovac-Zimmerman, J., Ridley, A., Segal, A. W. and Dekker, L. V. (2001) Protein kinase C-δ C2-like domain is a binding site for actin and enables actin redistribution in neutrophils. Biochem. J. **357**, 39–47
28 Tsao, L. T. and Wang, J. P. (1997) Translocation of protein kinase C isoforms in rat neutrophils. Biochem. Biophys. Res. Commun. **234**, 412–418
29 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
30 Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
31 Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. **351**, 95–105
32 Schiffmann, E., Aswanikumar, S., Venkatasubramanian, K., Corcoran, B. A., Pert, C. B., Brown, J., Gross, E., Day, A. R., Freer, R. J., Showell, A. H. and Becker, E. L. (1980) Some characteristics of the neutrophil receptor for chemotactic peptides. FEBS Lett. **117**, 1–7
33 Freer, R. J., Day, A. R., Radding, J. A., Schiffmann, E., Aswanikumar, S., Showell, H. J. and Becker, E. L. (1980) Further studies on the structural requirements for synthetic peptide chemoattractants. Biochemistry **19**, 2404–2410
34 Kanaho, Y., Kermode, J. C. and Becker, E. L. (1990) Comparison of stimulation by chemotactic formyl peptide analogs between GTPase activity in neutrophil plasma membranes and granule enzyme release from intact neutrophils. J. Leukoc. Biol. **47**, 420–428
35 Wenzel-Seifert, K., Hurt, C. M. and Seifert, R. (1998) High constitutive activity of the human formyl peptide receptor. J. Biol. Chem. **273**, 24181–24189
36 Dahlgren, C., Christophe, T., Boulay, F., Madianos, P. N., Rabiet, M. J. and Karlsson, A. (2000) The synthetic chemoattractant Trp-Lys-Tyr-Met-Val-Dmet activates neutrophils preferentially through the lipoxin A4 receptor. Blood **95**, 1810–1818
37 Mann, M. (1999) Quantitative proteomics? Nat. Biotechnol. **17**, 954–955
38 Rosales, C., Jones, S. L., McCourt, D. and Brown, E. J. (1994) Bromophenacyl bromide binding to the actin-bundling protein L-plastin inhibits inositol triphosphate-independent increase in Ca^{2+} in human neutrophils. Proc. Natl. Acad. Sci. U.S.A. **91**, 3534–3538
39 Yasui, K. and Komiyama, A. (2001) Roles of phosphatidylinositol 3-kinase and phospholipase D in temporal activation of superoxide production in FMLP-stimulated human neutrophils. Cell. Biochem. Funct. **19**, 43–50
40 Naccache, P. H., Levasseur, S., Lachance, G., Chakravarti, S., Bourgoin, S. G. and McColl, R. M. (2000) Stimulation of human neutrophils by chemotactic factors is associated with the activation of phosphatidylinositol 3-kinase γ. J. Biol. Chem. **275**, 23636–23641
41 Hirsch, E., Katanaev, V. L., Garlanda, C., Azzolino, O., Pirola, L., Silengo, L., Sozzani, S., Mantovani, A., Altruda, F. and Wymann, M. P. (2000) Central role for G-protein-coupled phosphoinositide 3-kinase γ in inflammation. Science **287**, 1049–1053
42 Ferretti, M. E., Nalli, M., Biondi, C., Colamussi, M. L., Pavan, B., Traniello, S. and Spisani, S. (2001) Modulation of neutrophil phospholipase C activity and cyclic AMP levels by fMLP-OMe analogues. Cell. Signal. **13**, 233–240
43 Newton, A. C. (1997) Regulation of protein kinase C. Curr. Opin. Cell Biol. **9**, 161–167
44 Lopez, I., Burns, D. J. and Lambeth, J. D. (1995) Regulation of phospholipase D by protein kinase C in human neutrophils. Conventional isoforms of protein kinase C phosphorylate a phospholipase D-related component in the plasma membrane. J. Biol. Chem. **270**, 19465–19472
45 Palmer, R. H., Dekker, L. V., Woscholski, R., Le Good, J. A., Gigg, R. and Parker, P. J. (1995) Activation of PRK1 by phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. A comparison with protein kinase C isotypes. J. Biol. Chem. **270**, 22412–22416
46 Ways, D. K., Cook, P. P., Webster, C. and Parker, P. J. (1992) Effect of phorbol esters on protein kinase C-ζ. J. Biol. Chem. **267**, 4799–4805

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