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N-Formyl Peptide Receptors in Human Neutrophils Display Distinct Membrane Distribution and Lateral Mobility when Labeled with Agonist and Antagonist

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Abstract. Receptors for bacterial *N*-formyl peptides are instrumental for neutrophil chemotactic locomotion and activation at sites of infection. As regulatory mechanisms for signal transduction, both rapid coupling of the occupied receptor to cytoskeletal components, and receptor lateral redistribution, have been suggested (Jesaitis et al., 1986, 1989).

To compare the distribution and lateral diffusion of the nonactivated and activated neutrophil *N*-formyl-peptide receptor, before internalization, we used a new fluorescent *N*-formyl-peptide receptor antagonist, tert-butyloxycarbonyl-Phe(D)-Leu-Phe(D)-Leu-Phe-OH (Boc-FLFLF, 0.1–1 μM), and the fluorescent receptor agonist formyl-Nle-Leu-Phe-Nle-Tyr-Lys (fnLLFnLYK, 0.1–1 μM). Fluorescent Boc-FLFLF did not elicit an oxidative burst in the neutrophil at 37°C, as assessed by chemiluminescence and reduction of p-nitroblue tetrazolium chloride, but competed efficiently both with formyl-methionyl-leucyl-phenylalanine (fMLF) and fnLLFnLYK. It was not internalized, as evidenced by confocal microscopy and acid elution of surface bound ligand.

The lateral mobility characteristics of the neutrophil fMLF receptor were investigated with the technique of FRAP. The diffusion coefficient (D) was similar for antagonist- and agonist-labeled receptors ($D \approx 5 \times 10^{-10}$ cm^2/s), but the fraction of mobile receptors was significantly lower in agonist- compared to antagonist-labeled cells, ~40% in contrast to ~60%.

This reduction in receptor mobile fraction was slightly counteracted, albeit not significantly, by dihydrocytochalasin B (dhcB, 5 μM).

To block internalization of agonist-labeled receptors, receptor mobility measurements were done at 14°C. At this temperature, confocal microscopy revealed clustering of receptors in response to agonist binding, compared to a more uniform receptor distribution in antagonist-labeled cells. The pattern of agonist-induced receptor clustering was less apparent after dhcB treatment.

To summarize, this work shows that activated *N*-formyl peptide receptors aggregate and immobilize in the plane of the neutrophil plasma membrane before internalization, a process that is affected, but not significantly reversed, by cytochalasin. The results are consistent with a model where arrested receptors are associated mainly with a cytochalasin-insensitive pool of cytoskeletal elements.

THE neutrophil granulocyte has a distinguished capacity to sense and actively move along a concentration gradient of chemotactic peptide excreted by invading bacteria (Zigmond, 1977; Gallin, 1988). When the neutrophil arrives at the site of infection, high concentrations (0.1–1 μM) of chemotactic peptide lead to cellular arrest, degranulation and activation of neutrophil bactericidal systems, e.g., production of oxidative metabolites (Snyderman and Pike, 1984; Gallin, 1988; Omann and Sklar, 1988).

The neutrophil receptor for chemotactic bacterial *N*-formyl peptides, the f-Met-Leu-Phe (fMLF)[1] receptor (Schiffman, 1975; Becker, 1979; Snyderman and Pike, 1984; Boulay, 1990*a*,*b*) and its complex signal transducing system has been studied intensively (Snyderman and Uhing, 1988; Jesaitis and Allen, 1988). The signal has been proposed to be G-protein-dependent (Jesaitis et al., 1989; Särndahl et al., 1989; Bommikanti et al., 1992), and to include activation of phospholipase C (Martin, 1989) and protein kinase C (Helf-

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1. *Abbreviations used in this paper*: Boc-FLFLF, tert-butyloxycarbonyl-Phe(D)-Leu-Phe(D)-Leu-Phe-OH; dhcB, dihydrocytochalasin B; fMLF, formyl-methionyl-leucyl-phenylalanine; fnLLFnLYK, formyl-Nle-Leu-Phe-Nle-Tyr-Lys; KRG, Krebs-Ringer glucose phosphate buffer containing 10 mM glucose and 1 mM Ca^{2+} and Mg^{2+}, pH 7.3; NBT, *p*-nitroblue tetrazolium chloride.

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man et al., 1983; Nishizuka, 1986), formation of inositolphosphates and diacylglycerols (Berridge, 1987), and calcium mobilization (Lew, 1989; Baggiolini and Wymann, 1990).

Upon activation, there is compelling biochemical evidence of a redistribution of receptors from membrane domains rich in G-proteins to areas depleted of G-proteins but rich in cytoskeletal elements, preferably actin (Jesaitis et al., 1989). Receptor-actin interaction has been proposed as a shut-off mechanism for the signal, before receptor internalization. This notion is supported by the findings that formyl peptide is a potent inducer of neutrophil actin polymerization (Jesaitis et al., 1986; Bengtsson et al., 1986).

To investigate the lateral mobility characteristics and distribution of the neutrophil formyl peptide receptor independently of receptor activation, a new fluorescent receptor antagonist was synthesized, viz. fluoresceinated tertbutyloxycarbonyl-Phe(D)-Leu-Phe(D)-Leu-Phe-OH (Boc-FLFLF; Freer et al., 1980). It was compared to a fluorescent receptor agonist (formyl-Nle-Leu-Phe-Nle-Tyr-Lys (fnLLFnLYK; Niedel et al., 1979).

Receptor lateral mobility was measured using the technique of fluorescence recovery after photobleaching (Jacobson et al., 1976; Johansson et al., 1987). The experiments were performed at 14°C, which blocks receptor internalization (Sklar et al., 1984). Receptor specificity, distribution and internalization was studied with confocal microscopy (Åslund et al., 1987; Carlsson and Åslund, 1987). The oxidative response to the ligands was determined with a chemiluminescence assay (Dahlgren, 1987) and as reduction of *p*-nitroblue tetrazolium chloride (NBT; Baehner et al., 1976).

The results show that human neutrophil *N*-formyl peptide receptors occupied with active ligand aggregate and become immobilized in the neutrophil plasma membrane before internalization; a process that is affected, but not significantly reversed, by cytochalasin.

To our knowledge, this is the first study using fluorescence recovery after photobleaching and confocal microscopy to address this question.

Materials and Methods

Chemicals

Tert-butyl-oxycarbonyl-Phe(D)-Leu-Phe(D)-Leu-Phe-OH (Boc-FLFLF) was obtained from Sigma Chemical Co. (St Louis, MO) and conjugated to FITC by Molecular Probes Inc. (Eugene, OR). Molecular Probes Inc. also provided fluoresceinated formyl-Nle-Leu-Phe-Nle-Tyr-Lys (fnLLFnLYK). Dihydrocytochalasin B (dhCB), Luminol, NBT and fMet-Leu-Phe (fMLF) were purchased from Sigma Chemical Co. HRP and BSA were obtained from Boehringer Mannheim GmbH (Mannheim, Germany).

The peptides were stored frozen as stock solutions in small portions, Boc-FLFLF and fnLLFnLYK at −70°C and fMLF at −20°C. Before use, they were diluted in Krebs-Ringer phosphate buffer pH 7.3, containing 10 mM glucose and 1 mM Ca^{2+} and Mg^{2+} (KRG).

Cells

Human neutrophils were obtained by two methods. Fingertip blood (two drops) was allowed to coagulate at 37°C for 30 min on a clean glass microscope slide with wells (Novakemi, Sweden), placed in a wet chamber. The blood clot was carefully washed off with KRG. At least 95% of the glass-adherent cells were neutrophils. Alternatively, neutrophils were purified from heparinized venous blood by sedimentation, first on Macrodex (LKB-Pharmacia Biotechnology, Uppsala, Sweden) at room temperature and then on a Ficoll-Paque gradient according to Böyum (1968). The cells were washed twice in Ca^{2+} and Mg^{2+}-free KRG by centrifugation at 4°C and resuspended in KRG. 50 μl of 5 × 10^6 cells/ml were allowed to adhere to BSA (0.5%, wt/wt) coated glasses for 30 min at 37°C in a wet chamber. The glasses were finally washed gently in KRG.

N-formyl Peptide Receptor Labeling

50 μl of ice-cold fluorescent agonist (fnLLFnLYK) and antagonist (Boc-FLFLF) at 1 or 0.1 μM in KRG was added to the glass-adherent neutrophils, precooled on ice for 10 min in a humid chamber. A coverslip was placed over the well with the cells, forming a chamber which was sealed on ice with a hot wax-vaseline mixture (1:1). Receptor mobility measurements and confocal microscopy started within 5 min after labeling including equilibration to the desired temperature, and were completed within 30 min (confocal microscopy) or 45–60 min (FRAP).

Cytochalasin Treatment

Before labeling, the neutrophils were preincubated for 10 min at 37°C in a humid chamber with 5 μM dhCB in KRG. The fluorescent fMLF agonist fnLLFnLYK was diluted in buffer containing 5 μM dhCB as a final concentration. Control cells were preincubated with buffer only.

Acid Elution of Surface-bound Ligand

A method where low extracellular pH elutes surface-bound ligand, while leaving intracellular ligand intact (Sklar et al., 1982; O'Shea et al., 1985; Bengtsson et al., 1991) was used to estimate the degree of receptor internalization. KRG was adjusted to pH 3.5 by addition of 5 M HCl ($KRG_{pH3.5}$). After labeling with fluorescent peptide at 4°C and a 15 min incubation at 14 or 37°C, the glass-adherent cells were mounted in $KRG_{pH3.5}$ and examined with the confocal microscope.

Chemiluminescence Measurements

Chemiluminescence was measured in a six-channel Biolumat LB9505 (Berthold Co., Wildbad, FRG). Briefly, purified neutrophils were suspended in KRG to 0.25 × 10^6/ml with 0.1 mg/ml Luminol and 4 U/ml HRP and preheated to 37°C for 5 min. The reaction mixture (1 ml) was then subjected to fMLF (0.1 μM, final concentration), fluoresceinated fnLLFnLYK (0.01 μM) or fluoresceinated Boc-FLFLF (0.1 μM). Furthermore, to ensure that the antagonist and the agonists competed for the same receptor, Boc-FLFLF (0.1–1 μM) was added at maximal fMLF and fnLLFnLYK-induced chemiluminescence. Light emission was recorded continuously (Dahlgren, 1987).

Reduction of p-Nitroblue Tetrazolium Chloride

To estimate the degree of activation of the neutrophil oxidase in the experimental set-ups, reduction of NBT was used (Baehner et al., 1976). In the presence of oxidative metabolites, NBT (yellow) is reduced to formazan (blue). Boc-FLFLF (0.1 μM) or fnLLFnLYK (0.1 μM) in an 0.85 mg/ml NBT solution (PBS) was added to glass-adherent cells equilibrated to 14 and 37°C, respectively, and the cells were further incubated for 30 min. KRG, fMLF (0.1 μM) and PMA (0.1 μM) were used as controls.

Fluorescence Recovery after Photobleaching

The equipment used has been described in detail elsewhere (Johansson et al., 1987). Briefly, it is computer menu-driven and allows measurements of either of continuous fluorescence microphotolysis (Peters et al., 1981) or FRAP (Peters et al., 1974; Jacobson et al., 1976; Axelrod et al., 1976). A SLOW-FRAP protocol suitable for protein diffusion studies was used (Johansson et al., 1987).

The cell membrane was illuminated and photobleached (×100–1,000 intensity) with an Argon laser (Type 2020-03, Spectra Physics, Mountain View, CA) at 488 nm through a 160-μm circular aperture of a Zeiss Universal microscope (Carl Zeiss, Oberkochen, FRG) equipped with an epifluorescence condenser for FITC-activation and an ×63 oil-immersion plan achromatic objective, which gives an estimated bleach spot radius (w) of 0.89 μm at $1/e^2$ intensity.

The lateral diffusion coefficient (D) was calculated according to Axelrod et al. (1976) and the percent recovery (R), reflecting the proportion of mo-

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bile receptors, was determined according to Jacobson et al. (1976). The SLOW-FRAP protocol used allowed us to first measure the fluorescence in the spot before bleaching (F_i), bleach (150–300 ms) and then sample the recovery of fluorescence in the bleached spot at time intervals of increasing length (e.g., from 1 to 10 s with a final measurement, F_∞, at 60 s).

To avoid cell locomotion and prevent internalization of the fMLF-receptor (Sklar et al., 1984), glass-adherent cells were kept at 14°C during the experiments using a temperature-regulated microscope stage.

Confocal Microscopy

A Sarastro 1000 microscope (Molecular Dynamics, Sunnyvale, CA) (Åslund et al., 1987; Carlsson and Åslund, 1987) equipped with an Argon laser and a ×100, high numeric aperture (1.4) objective (Nikon, Japan) was used.

Neutrophils, isolated and prepared as for FRAP measurements, were transported on ice and were, for the study, kept at 14 or 37°C, using a temperature-regulated (Peltier-type) microscope stage (Detrona AB, Linköping, Sweden). Cells were chosen randomly in white light and optical sectioning made in fluorescent light (488 nm line). To localize the cells after low pH elution of ligand, pictures were made using the white light source of the microscope.

Statistical Methods

Estimates of p-values were obtained using t test (Kirkwood, 1991). A significant difference between two means has a two-sided p-value ≤ 0.05.

Results

Receptor Specificity and Capacity of the Antagonist and Agonist to Activate an Oxidative Burst

The *N*-formyl peptide receptor specificity of fluoresceinated fnLLFnLYK is well established (Niedel et al., 1979). Incidentally, this compound is a more potent activator of the neutrophil oxidative burst than fMLF (Sklar et al., 1985; Table I and Fig. 1).

Preservation of the receptor specificity of the antagonist Boc-FLFLF (Freer et al., 1980) after FITC-conjugation was shown in two ways. First, a rapid, distinct reduction of the oxidative response was observed after addition of fluoresceinated Boc-FLFLF to either fMLF- or fnLLFnLYK-stimulated cells at maximal chemiluminescence (Fig. 1). Second, addition of a 10-fold excess of fMLF to cells labeled with 0.1 μM of the fluoresceinated antagonist obliterated cell-associated fluorescence at 14°C (Fig. 2).

Table I. Production of Oxidative Metabolites in Glass-adherent Human Neutrophils Measured as Reduction of p-Nitroblue Tetrazolium Chloride

Stimulus	Temperature	
	14°C	37°C
none	–	–
Boc-FLFLF 0.1 μM	–	–
fMLF 0.1 μM	–	+(+)
fnLLFnLYK 0.1 μM	–	++
fnLLFnLYK 0.1 μM + dhcB 5 μM	–	++
PMA 0.1 μM	–	+++

Glass-adherent human neutrophils were subjected to 100 μl of 0.1 μM Boc-FLFLF, fnLLfnLYK, fMLF, or PMA (positive control) together with 0.85 mg/ml p-nitroblue tetrazolium chloride (NBT) and incubated at the desired temperature for 30 min. DhcB-treated cells were preincubated with 5 μM dhcB for 10 min at 37°C. The reaction was stopped by addition of 4% paraformaldehyde (PFA) at 4°C. The degree of activation of the respiratory burst was estimated from the percentage of blue cells due to formazan formation (NBT-positive cells). +++, >95% positive cells; –, <5% positive cells. At least 200 cells were counted for each preparation.

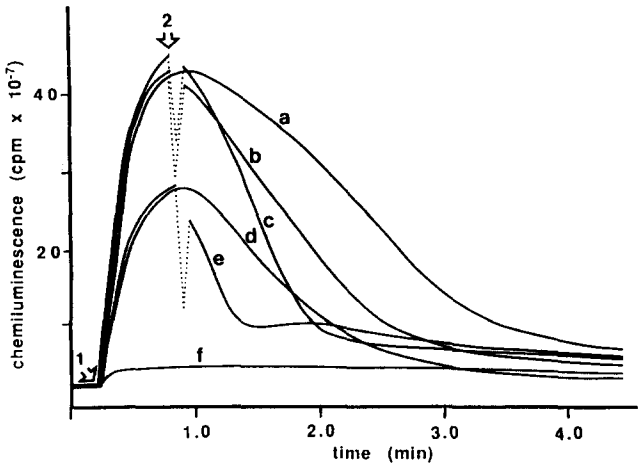

Figure 1. Production of oxidative metabolites in non-adherent human neutrophils upon binding of agonists (fMLF, fnLLFnLYK) and antagonist (Boc-FLFLF) to the *N*-formyl peptide receptor. Luminol-amplified chemiluminescence in human neutrophils (37°C) in response to 0.01 μM fluoresceinated receptor agonist fnLLFnLYK (*a*, *b*, and *c*), to 0.1 μM fMLF (*d* and *e*) and to 0.1 μM fluoresceinated receptor antagonist Boc-FLFLF (*f*), added at point 1. The fluoresceinated antagonist (Boc-FLFLF), at 0.1 μM (*b*) or 1 μM (*c* and *e*) was also added at maximal agonist-induced chemiluminescence (point 2). The figure shows a set of curves from one representative experiment of a total of 5–10.

Fluorescent Boc-FLFLF (0.1 μM) induced no chemiluminescence and no reduction of NBT at 37°C, confirming the antagonistic nature of the labeled compound (Table I and Fig. 1).

Reduction of NBT in glass-adherent cells further revealed that no activation of the neutrophil oxidase occurred in glass-adherent cells at 14°C in response to 0.1 μM fnLLFnLYK, fMLF or PMA, respectively (Table I).

N-formyl Peptide Receptor Distribution and Internalization

For neutrophils fluorescently labeled with the receptor antagonist Boc-FLFLF (0.1 μM) an almost uniform membrane distribution of *N*-formyl peptide receptors was observed. The receptor distribution did not significantly change when the temperature was increased from 14 to 37°C, and no internalization was detected (Figs. 4, 5, and 6).

A strikingly different lateral distribution of the receptor was seen at 14°C in cells fluorescently labeled with 0.1 μM of the receptor agonist fnLLFnLYK. It was evident that the agonist induced a lateral clustering of the receptors in the neutrophil plasma membrane at temperatures suboptimal for oxidase activation and *N*-formyl peptide receptor internalization (Figs. 4 and 5). At 37°C, receptor internalization was obvious in agonist-labeled cells (Fig. 6).

Neutrophils pretreated with and maintained in 5 μM dihydrocytochalasin B (dhcB) still displayed a patchy distribution of agonist-labeled receptors at 14°C. However, the receptor clusters in dhcB-treated cells (Fig. 4, *g–i*) appeared less well developed compared to cells not treated with dhcB, which displayed a less diffuse, but more highly punctate

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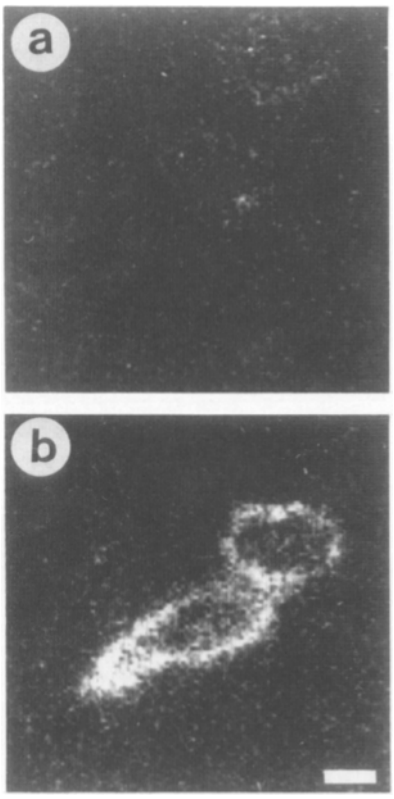

Figure 2. Competitive inhibition of the binding of fluorescent *N*-formyl peptide receptor antagonist by fMLF. Glass-adherent neutrophils, labeled at 4°C with 0.1 μM of the fluorescent receptor antagonist Boc-FLFLF, were mounted in buffer containing 1 μM fMLF (*a*), or in buffer only (*b*) and examined in the confocal microscope at 14°C. Bar, 5 μM.

receptor distribution (Fig. 4, *d–f*). Moreover, at 37°C, along with large fluorescent clusters, agonist-labeled cells showed more internal diffuse fluorescence (Fig. 6 *d*) compared to cells treated with dhcB (Fig. 6 *g*).

Table II. Lateral Mobility at 14°C of the N-formyl Peptide Receptor in Individual Human Neutrophils Labeled with Antagonistic and Agonistic Fluorescent Peptides

Receptor label		*n*	*D* $\times 10^{-10}$ cm²/s	*R* %
Boc-FLFLF	1 μM	12	5.4 ± 0.3 (5.2)	63 ± 3 (65)
	0.1 μM	29	4.7 ± 0.5 (4.8)	57 ± 5* (57)
fnLLFnLYK	0.1 μM	16	5.5 ± 0.3 (6.0)	40 ± 3* (38)
	0.1 μM + dhcB 5 μM	21	5.6 ± 0.3 (5.6)	44 ± 2 (46)

* $p < 0.001$. Glass-adherent human neutrophils were fluorescently labeled at 4°C with either a N-formyl peptide receptor antagonist (Boc-FLFLF) or an agonist (fnLLFnLYK). Measurements of receptor lateral mobility were done at 14°C using the FRAP technique. The effect of 5 μM dihydrocytochalasin B (dhcB) on the lateral mobility of the agonist-labeled fMLF-receptor was also assessed. D is the diffusion coefficient, R the fraction of mobile receptors. Mean values are given ± SEM with median values in parenthesis. *n* is the number of cells. Two-sided p-values were obtained with *t* test.

Lateral Mobility Characteristics of the N-formyl Peptide Receptor

Around 60% of the receptors for fMLF appeared laterally mobile in neutrophils labeled with antagonist, i.e., 40% of the receptors were immobile. The mobile fraction of receptors, *R*, decreased significantly, to ~40%, in cells labeled with the agonist fnLLFnLYK.

Treatment with 5 μM dhcB did not significantly affect the

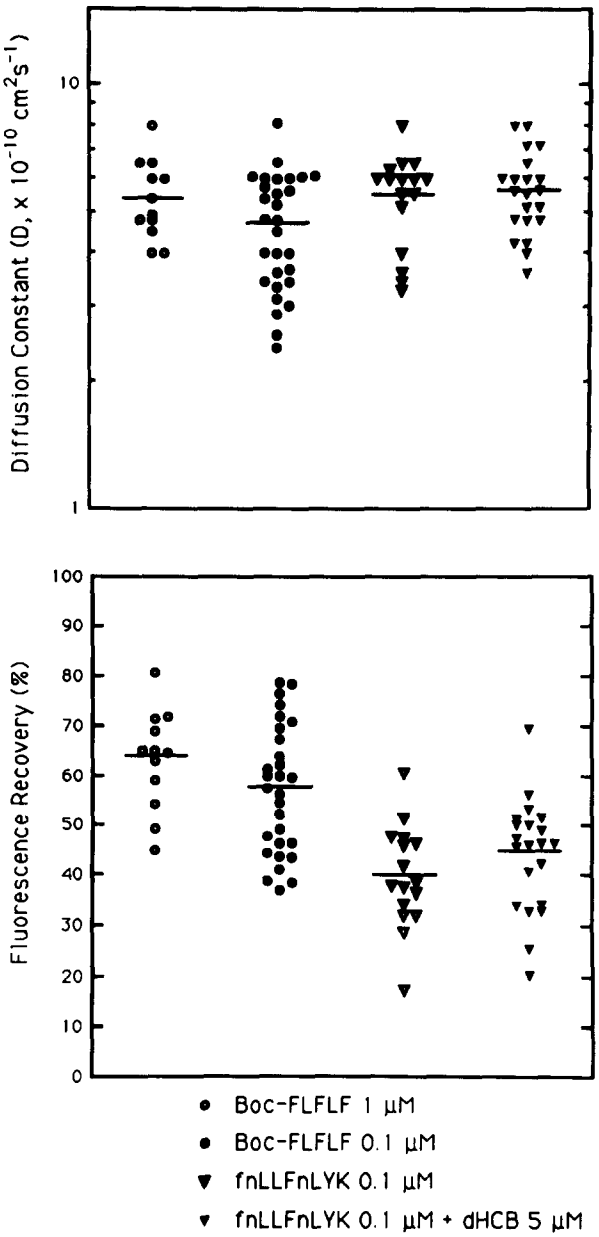

Figure 3. Lateral mobility of antagonist and agonist-labeled *N*-formyl peptide receptor in individual neutrophils, effect of cytochalasin. Diffusion constant, D, and mobile fraction, R, of the *N*-formyl peptide (fMLF) receptors in individual human neutrophils measured with fluorescence recovery after photobleaching (FRAP) at 14°C. Neutrophils were labeled at 4°C with 1 or 0.1 μM of receptor antagonist (Boc-FLFLF) or 0.1 μM of receptor agonist (fnLLnLYK). The agonist-labeled cells in the last lane were further treated with 5 μM dihydrocytochalasin B (dhcB). Solid line indicates mean value (Table II).

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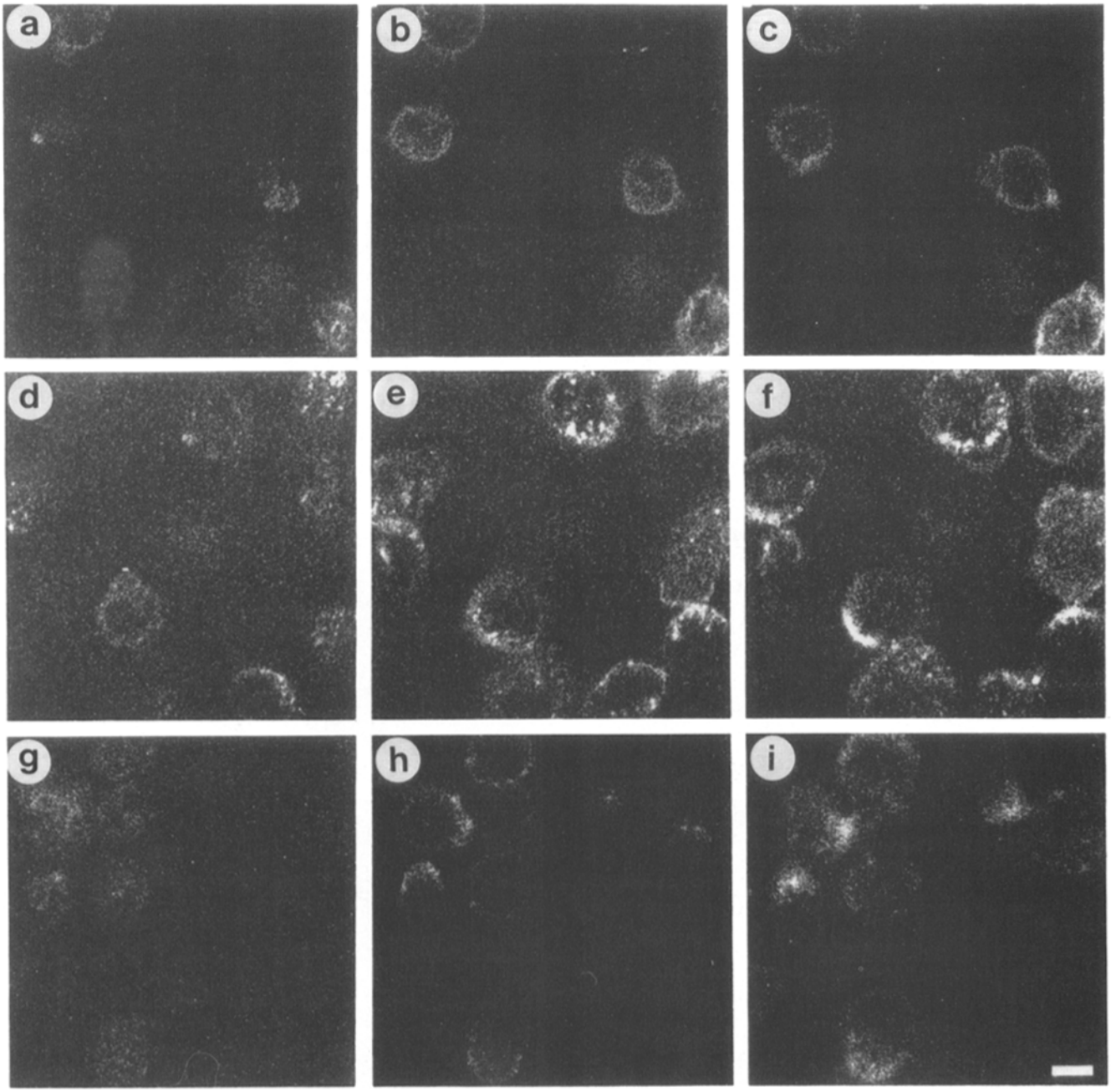

Figure 4. Distribution of antagonist and agonist-labeled *N*-formyl peptide receptors in human neutrophils at 14°C, effect of cytochalasin. Glass-adherent human neutrophils were examined in the confocal fluorescence microscope at 14°C after labeling at 4°C with 0.1 μM receptor antagonist (Boc-FLFLF; *a–c*), or 0.1 μM receptor agonist (fnLLFnLYK; *d–i*). The cells in *g–i* were preincubated, and maintained, in 5 μM dihydrocytochalasin B (dhcB) during labeling and examination. Optical sections at increasing depth are shown in *a–c*, *d–f* and *g–i*. Distance between sections was 2 μm. Bar, 5 μm.

size of the mobile fraction in agonist-labeled cells (Table II). However, there was evidence of a shift in the distribution of R values of the cytochalsin-treated cells towards the mean value of antagonist-treated cells (Fig. 3). This effect was even more evident from the median values (Table II).

The lateral diffusion coefficient (*D*) of the mobile fraction of *N*-formyl peptide receptors was similar regardless of whether the cells had been labeled with receptor agonist or antagonist, and was found to be around 5×10^{-10} cm²/s. The diffusion constant was not significantly affected by dhcB treatment (Table II, Fig. 3).

Discussion

This is, to our knowledge, the first conclusive study of the *N*-formyl peptide receptor distribution and lateral mobility in the neutrophil membrane (compare Bültmann et al., 1987; McKay et al., 1991), using FRAP and confocal microscopy.

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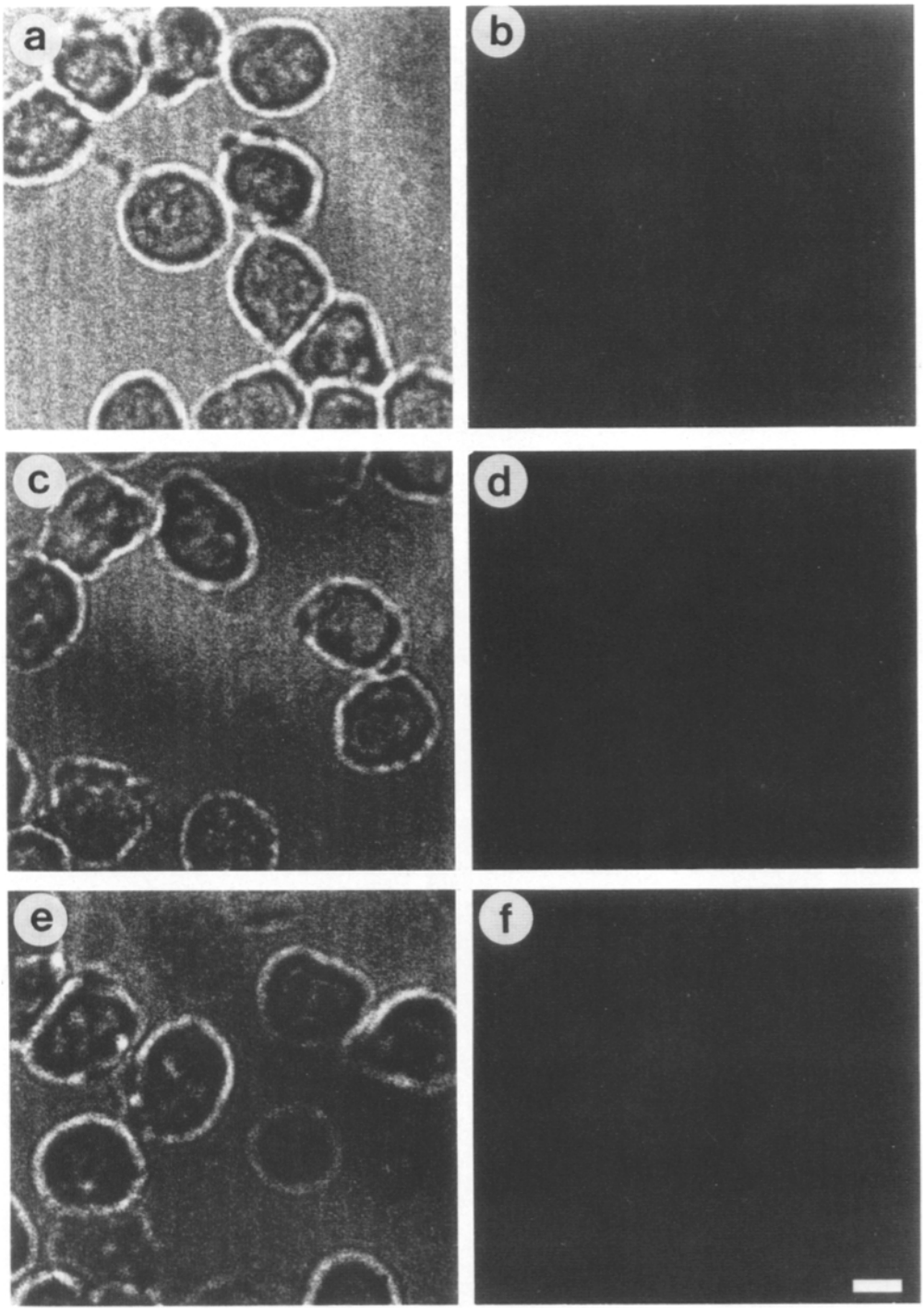

Figure 5. Acid elution of membrane-bound ligand at 14°C in human neutrophils labeled with antagonist and agonist to the *N*-formyl peptide receptor, effect of cytochalasin. Glass-adherent human neutrophils, mounted in $KRG_{pH3.5}$, were examined in the confocal microscope at 14°C after labeling at 4°C with 0.1 μM receptor antagonist (Boc-FLFLF; *a–b*), or 0.1 μM receptor agonist (fnLLFnLYK; *c–f*). The cells in *e–f* were preincubated, and maintained, in 5 μM dihydrocytochalasin B (dhcB) during labeling and examination. *a*, *c*, and *e* were recorded using the white light source of the confocal microscope. *b*, *d*, and *f* show the corresponding confocal fluorescence image to *a*, *c*, and *e*, respectively. Bar, 5 μm.

Moreover, and in contrast to the agonist fnLLFnLYK (Niedel et al., 1979; Sklar et al., 1982, 1984; Seligmann et al., 1984; Walter and Marasco, 1987; Bültmann et al., 1987; Schmitt and Bültmann, 1990), the antagonist Boc-FLFLF (Freer et al., 1980) has not previously been used as a fluorescent receptor label.

The combination of Boc-FLFLF and fnLLFnLYK provided a straightforward way to follow the effects of activation on the *N*-formyl peptide receptor lateral mobility and distribution. An advantage with these small-sized labels was also that they would, theoretically, not by themselves restrain receptor lateral diffusion.

FnLLFnLYK is a potent inducer of the neutrophil respiratory burst, both in cells in solution (Sklar et al., 1982; Fig. 1) and cells attached to glass (Table I). At 37°C it is rapidly internalized together with the *N*-formyl peptide receptor. Below 15°C, internalization is blocked (Sklar et al., 1984; Figs. 4 and 5). At this temperature, there is also no activation of the neutrophil oxidase, resulting in production of oxidative metabolites (Jesaitis et al., 1986; Table I).

At 15°C the *N*-formyl peptide receptor nevertheless undergoes an activation-specific change in ligand affinity (Jesaitis and Allen, 1988) and there is evidence that the receptors shift from areas rich in G-proteins to membrane domains in

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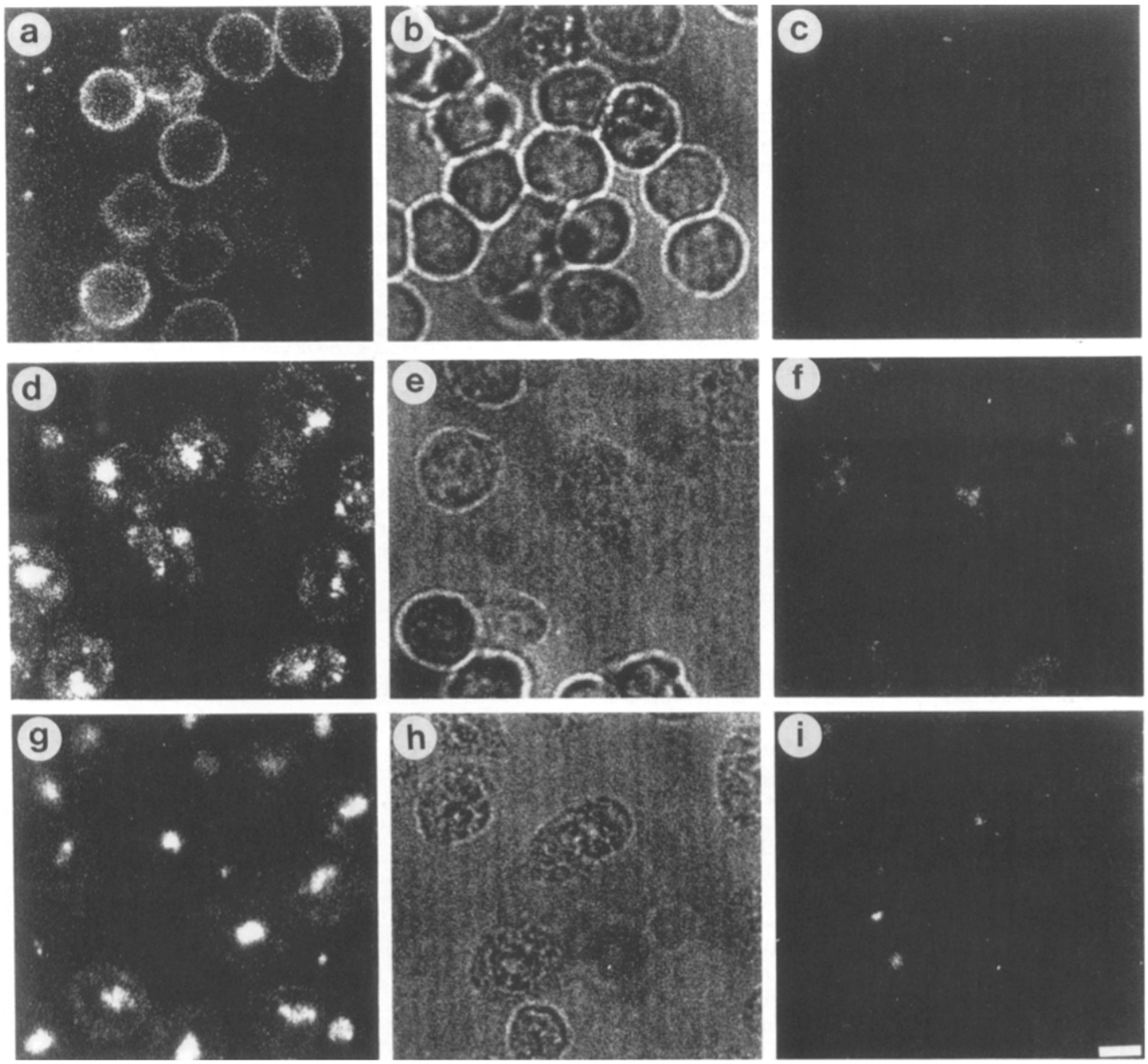

Figure 6. Distribution of antagonist and agonist-labeled *N*-formyl peptide receptors in human neutrophils at 37°C, effect of cytochalasin. Acid elution of membrane-bound ligand. Glass-adherent human neutrophils were examined in the confocal microscope at 37°C after labeling at 4°C with 0.1 μM receptor antagonist (Boc-FLFLF; *a–c*), or 0.1 μM receptor agonist (fnLLFnLYK; *d–i*). The cells in *g–i* were preincubated, and maintained in 5 μM dihydrocytochalasin B (dhcB) during labeling and examination. *b–c*, *e–f* and *h–i* show cells mounted in $KRG_{pH3.5}$. *b*, *e*, and *h* were recorded using the white light source of the confocal microscope. *c*, *f*, and *i* show the corresponding confocal fluorescence images to *b*, *e*, and *h*, respectively. Bar, 5 μm.

close contact with cytoskeletal elements, preferably actin (Jesaitis et al., 1989; Klotz, K. N., D. Siemsen, and A. J. Jesaitis. 1991. *J. Cell Biol.* 115:3*a*. [Abstr.]).

In this study we observed a distinct clustering of *N*-formyl peptide receptors at 14°C induced by the receptor agonist FnLLFnLYK (Fig. 4). This finding was paralleled by a significant reduction of the fraction of mobile receptors, *R*, in the membrane (Table II, Fig. 3).

This reduction of *R* was reversed, albeit not significantly, towards the *R* values of antagonist-labeled cells (Table II, Fig. 3) by dihydrocytochalasin B (dhcB), which normally blocks actin polymerization (Lin et al., 1977, 1982). In addition, the receptor clusters formed in dhcB-treated cells at 14°C appeared less well developed compared to agonist-labeled controls (Fig. 4). Moreover, at 37°C dhcB-treated cells displayed less internal diffuse fluorescence than controls (Fig. 6).

These findings could suggest that dhcB has an effect on the internalization rate of the agonist-labeled receptor and possibly on the rate of formation of clusters. They would be in accordance with the results reported by Jesaitis and Allen (Jesaitis and Allen, 1988), where formation of receptor high affinity Triton X-100 insoluble complexes was slowed, but not completely inhibited, by dhcB-treatment. The kinetics of

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cluster formation and receptor internalization under different circumstances remains to be examined in a separate study.

We found that the diffusion constant, D, of the mobile fraction of receptors was $\approx 5 \times 10^{-10}$ cm^2/s at 14°C (Table II, Fig. 3), which is typical for an intrinsic membrane protein (Jacobson, 1983). The diffusion constant was not significantly influenced by receptor occupation with active ligand (Table II, Fig. 3). This suggests that the proposed interaction between activated *N*-formyl peptide receptors and the cytoskeleton, turning off the signal (Jesaitis et al., 1986), is an all-or-nothing effect, immobilizing receptors completely.

Incidentally, when discussing the relationship between receptor mobility and cellular activation, Jesaitis and Allen (1988) assumed a theoretical *N*-formyl peptide receptor diffusion constant of between 1 and 10×10^{-10} cm^2/s. We can now substantiate these calculations, by assigning a value, $\approx 5 \times 10^{-10}$ cm^2/s, to the rate of diffusion of the neutrophil *N*-formyl peptide receptor.

Taken together, the results indicate that before receptor internalization, and in the absence of activation of the neutrophil oxidase, *N*-formyl peptide receptors occupied by active ligand are laterally redistributed, aggregated and immobilized in the neutrophil plasma membrane by a process apparently affected by, but not entirely dependent on, actin polymerization.

There is, however, evidence of a cytochalasin-insensitive pool of actin in neutrophils (Cassimeris et al., 1990). Our results may indicate that this pool of actin, or other cytoskeletal components, are involved in receptor immobilization. Incidentally, there are recent data that suggest that the interaction between *N*-formyl peptide receptors and the neutrophil cytoskeleton is in fact mainly cytochalasin-insensitive (K. N. Klotz and A. J. Jesaitis, personal communication; Klotz, K. N., D. Siemsen, and A. J. Jesaitis. 1991. *J. Cell Biol.* 115:3*a*. [Abstr.]).

The response to *N*-formyl peptide in suspended neutrophils (Sklar et al., 1984; Jesaitis et al., 1986; Bengtsson et al., 1986; Jesaitis and Allen, 1988; Omann and Sklar, 1988; Jesaitis et al., 1989) might, in addition, differ from cells attached to glass. Cytokines, such as TNF and CSF-GM, actually fail to evoke an oxidative response in nonadherent cells (Nathan et al., 1987, 1989*a*,*b*). This absolute dependency on adherence is not true for the *N*-formyl peptide receptor, but modifications of the cellular response cannot be ruled out.

Finally, we anticipate that the method of receptor labeling outlined in this work, using pairs of fluorescent agonists and antagonists, is applicable to studies of lateral mobility and distribution of receptors in a variety of cell systems.

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