A High Potency Nonformylated Peptide Agonist for the Phagocyte *N*-Formylpeptide Chemotactic Receptor

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Summary

Analysis of synthetic tri- and tetrapeptides has previously indicated that *N*-formylation is required for high biological activity when they react with the phagocyte *N*-formylpeptide receptor, suggesting that the natural ligand for the receptor is from bacterial and/or mitochondrial sources. To explore this requirement further, we synthesized the pentapeptide methionyl-norleucyl-leucyl-phenylalanyl-phenylalanine (MNleLFF) and studied the effects of different NH_2-terminal modifications on its activity. *N*-formyl-MNleLFF induced transient alterations of $[Ca^{2+}]_i$ and superoxide production in human neutrophils with 10- and 100-fold greater potency, respectively, than the prototype *N*-formylpeptide, *N*-formylmethionyl-leucyl-phenylalanine (fMLF). Surprisingly, *N*-acetyl-MNleLFF was as potent as *N*-formyl-MNleLFF. Moreover, the unacylated counterpart H-MNleLFF was also highly active, having an EC_{50} for calcium mobilization of 10 nM, and for respiratory burst activation of 100 nM. All three pentapeptides could completely desensitize calcium transients elicited by stimulation of neutrophils with fMLF, whereas the neutrophil chemoattractants C5a and interleukin 8 only weakly affected fMLF-induced transients, suggesting that they activate neutrophils via the same receptor as fMLF. Finally, all three pentapeptides activated the recombinant human *N*-formylpeptide receptor expressed in frog oocytes, but did not effectively activate related phagocyte receptors. These data broaden the potential sources of natural ligands for the *N*-formylpeptide receptor from *N*-formylated bacterial and mitochondrial products to other nonformylated endogenous peptides.

In studies designed to delineate the mechanisms by which bacterial infections induce pus formation, Schiffmann et al. (1) investigated the ability of peptides derived from supernatants of bacterial cultures to chemoattract mammalian phagocytes in vitro. The NH_2 termini of the peptides were blocked and could not be sequenced. Since the NH_2-terminal residue of all bacterial proteins is *N*-formylmethionyl, a series of *N*-formylmethionyl peptides was synthesized and these were found to activate phagocyte functions with high potency. Specific, high affinity receptors were demonstrated on phagocytes, and *N*-formylation of the tested peptides was shown to be required for high activity (2–4). Acetylated and nonacylated tri- and tetrapeptides have been reported to have little if any ability to activate phagocytes. The synthetic *N*-formylpeptide *N*-formylmethionyl-leucyl-phenylalanine (fMLF)[1] became a prototype for the study of neutrophil activation by chemoattractants.

The sequence of a high affinity human *N*-formylpeptide receptor (FPR) was deduced from cloned cDNAs in 1990 (5). Two human genes (gene symbols *FPRL1* and *FPRL2*) and their corresponding phagocyte cDNAs have been cloned that encode proteins with 69 and 56% amino acid sequence identity to FPR (6–9). The FPRL1 receptor (FPRL1R) is a low affinity receptor for fMLF (K_d = 430 nM; EC_{50} for calcium mobilization > 100 nM) (6, 10); the putative FPRL2 product has no known ligand (9). When FPR is expressed in rat basophilic leukemia cells, it mediates degranulation and cytoskeletal remodeling, responses that mimic neutrophil responses to fMLF (11). When FPR is expressed in adenovirus-transformed human embryonic kidney 293 cells, the same peptide binding selectivity is observed as for native neutrophil binding sites (12). Taken together, these findings strongly suggest that FPR is the receptor responsible for most if not all neutrophil responses to fMLF.

Despite these advances, the source of natural ligands for

[1] *Abbreviations used in this paper:* ac, acetyl; f, formyl; fMLF, *N*-formylmethionyl-leucyl-phenylalanine; FPR, human *N*-formylpeptide receptor; H-MLF, methionyl-leucyl-phenylalanine; MNleLFF, methionyl-norleucyl-leucyl-phenylalanyl-phenylalanine.

FPR remains undefined. If the formyl group is truly essential for ligand activity, the only known natural sources would be bacteria and mitochondria. In fact, Marasco et al. (13) have shown that the major chemotactic peptide for neutrophils produced by *Escherichia coli* is *N*-formylated, and is identical in sequence to the synthetic prototype fMLF. Although Carp (14) has shown that mitochondrial proteins can attract neutrophils in vitro, the requirement for *N*-formylation for activity and the receptor target have not been defined.

To further examine the requirement of *N*-formylation for activation of FPR, we synthesized the pentapeptide methionyl-norleucyl-leucyl-phenylalanyl-phenylalanine (MNleLFF) and studied the effects of different NH_2-terminal modifications on its activity. We now report that the acetylated and nonacylated forms of the peptide could potently and specifically activate both natural and recombinant sources of FPR. Thus, natural ligands for FPR may not be restricted to bacterial and mitochondrial sources.

Materials and Methods

Materials. fMLF, methionyl-leucyl-phenylalanine (H-MLF), ATP, recombinant human C5a, and superoxide dismutase were from Sigma Chemical Co. (St. Louis, MO). FURA-2 AM was from Molecular Probes, Inc. (Eugene, OR). Recombinant human 72 amino acid IL-8 was obtained from PeproTech (Rocky Hill, NJ).

Peptide Synthesis. Peptides were prepared by solid-phase peptide synthesis using the 9-fluoroenylmethoxycarbonyl (FMoc) group for protection of the amino group. Deprotection was with 25% piperidine. Formylation was carried out using carbodiimide/hydroxybenztriazole (ECD/HOBT). Acetylation was carried out using acetic anhydride. Removal from the resin was with trifluoroacetic acid. Peptides were purified using reverse-phase HPLC and were homogeneous. Lyophilized peptides were aliquoted as 10 mM stocks in DMSO, stored at −20°C, and diluted into aqueous buffers before use.

Preparation of Neutrophils. Granulocytes were isolated from heparinized human peripheral blood by Ficoll-Hypaque discontinuous density gradient centrifugation, dextran sedimentation, and hypotonic lysis. Cells were always >95% neutrophils. All experiments were conducted within 6 h of cell isolation.

Intracellular Calcium Measurements in Neutrophils. Cells (10^7/ml) were incubated in HBSS with Ca^{2+}, Mg^{2+}, and 10 mM Hepes, pH 7.4, containing 2.5 μM FURA-2 AM for 30 min at 37°C in the dark. The cells were subsequently washed twice with HBSS, and resuspended at 2×10^6 cells/ml. 2 ml of the cell suspension were placed in a continuously-stirred cuvette maintained at 37°C (DeltaScan; Photon Technology International Inc., S. Brunswick, NJ). Fluorescence was monitored at λ_{ex1} = 340 nm, λ_{ex2} = 380 nm, and λ_{em} = 510 nm, and the data presented as the relative ratio of fluorescence at 340 and 380 nm for experiments with FURA-2. Data were collected every 200 ms.

Receptor Reconstitution in Xenopus Oocytes. cDNAs and genes for FPR, FPRL1, and FPRL2 have been previously cloned, and expression constructs described (6, 9, 15). Capped sense RNA was synthesized by first linearizing plasmid constructs by digestion with an appropriate restriction endonuclease which cut the plasmid once in the 3′ polylinker region, followed by in vitro transcription with either T3 or T7 RNA polymerase using a kit and instructions from the manufacturer (Stratagene, La Jolla, CA). All cRNA transcripts were shown to migrate as single appropriately sized bands by denaturing agarose gel electrophoresis. The materials and methods used for the calcium efflux assay were as described (15) with minor modifications. 1–2 d after harvesting, defolliculated oocytes were microinjected with 10 ng of the indicated receptor cRNA with or without 200 ng of HL-60 cell poly(A)$^+$ RNA. The HL-60 RNA supplies mRNA for an undefined complementary human factor that along with FPR and the FPRL1 receptor is necessary to establish a calcium-mobilizing signal transduction pathway for fMLF in the oocyte (10, 15). Microinjection of either the cRNA or HL-60 RNA separately failed to confer fMLF sensitivity to the oocyte. Oocytes were then incubated at 20–23°C for 3–5 d in ND96 media (96 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.45). Oocytes were then incubated with $^{45}Ca^{2+}$ (100 μCi/ml) for 4 h. After 10 washes with ND96, individual oocytes were stimulated with ligands. Data are presented as the mean ± SEM of the percent of loaded $^{45}Ca^{2+}$ that was released in 20 min by individual oocytes in response to the stimulus.

Oxidant Production. The neutrophil suspension was maintained in PBS at 10^4 cells/μl at room temperature until use. 10 μl of the neutrophil suspension were placed in a single well of a 96-well microtiter plate. Cells in all wells were stimulated simultaneously by adding 90 μl of a cocktail containing 40 μl of HBSS, 50 μl of Diogenes chemiluminescence reagent (National Diagnostics Inc., Atlanta, GA) using a multichannel pipetter, prepared according to the instructions of the manufacturer, and the indicated agonists. The plate was immediately placed in a luminometer (Labsystems Luminoskan, Helsinki, Finland) at 37°C and the instantaneous luminescence produced by each sample was recorded every 30 s for 6 min. The lag time for data collection after activation of the cells was between 15 and 20 s, allowing measurement of the peak but not the initial rate of superoxide production.

Results

Binding of fMLF to neutrophils results in rapid, transient increases in $[Ca^{2+}]_i$ that can be monitored by calcium-sensitive dyes to follow receptor activation in real time (12). After the initial rise, $[Ca^{2+}]_i$ declines rapidly to a new sustained level that, at saturating concentrations of ligand, substantially exceeds the prestimulation baseline. When human neutrophils were stimulated with the pentapeptides *N*-formyl-MNleLFF (fMNleLFF), *N*-acetyl-MNleLFF (acMNleLFF) or the unacylated counterpart H-MNleLFF, transient elevations in $[Ca^{2+}]_i$ were observed whose kinetics were indistinguishable from those elicited with fMLF (Fig. 1 *A*). The magnitude of both the peak and sustained levels of $[Ca^{2+}]_i$ was dependent on the ligand concentration tested. Neutrophil $[Ca^{2+}]_i$ also rapidly increased upon stimulation with two other neutrophil chemoattractants, C5a and IL-8. However, $[Ca^{2+}]_i$ then declined from the peak level more rapidly than was observed with the pentapeptides and fMLF, and the sustained increase after stimulation with saturating concentrations of ligand only slightly exceeded the prestimulation baseline. This suggested that the three pentapeptides interact with a receptor or class of receptors similar to those for fMLF, but distinct from those for C5a and IL-8. The EC_{50} for the unacylated pentapeptide was ~10 nM, a value that was 10-fold higher than that for fMLF. fMNleLFF and acMNleLFF were equipotent but were ~10-fold more potent than fMLF (Fig. 1 *B*).

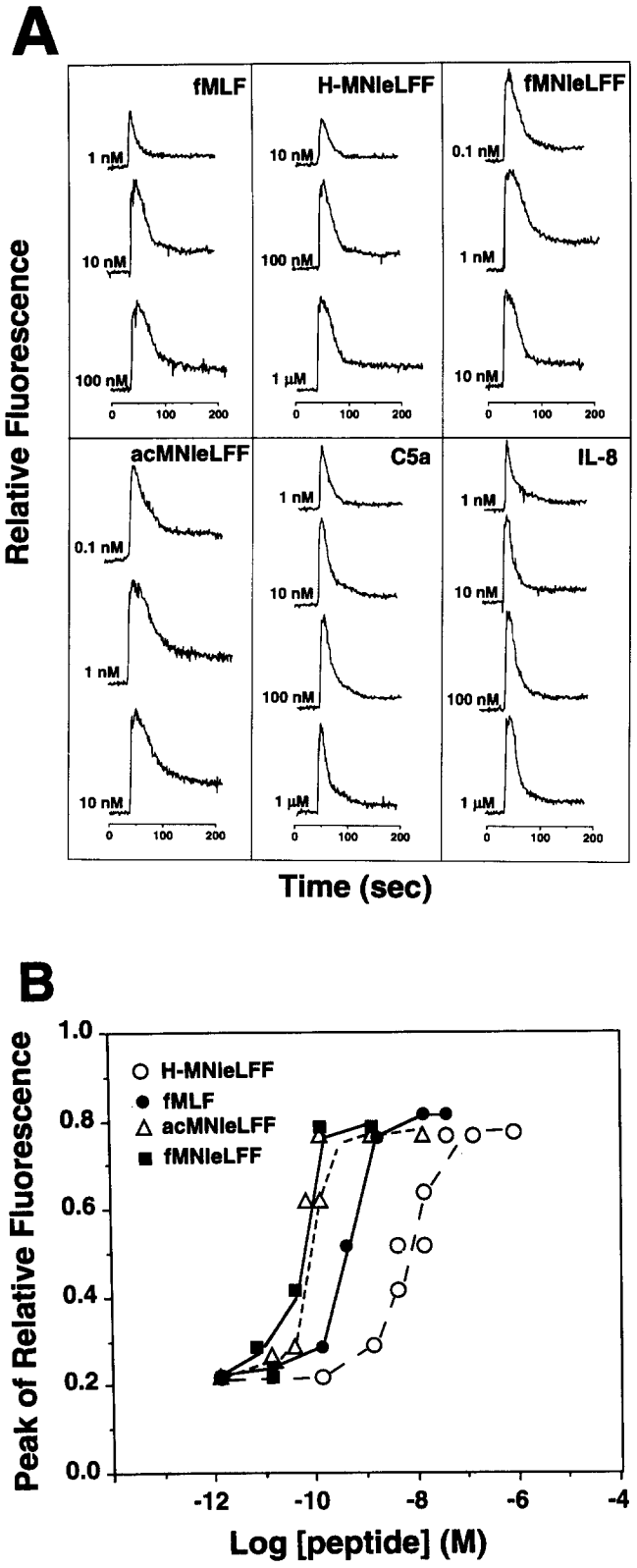

Figure 1. Calcium mobilization in human neutrophils stimulated by synthetic peptides with different NH_2-terminal modifications. (*A*) Kinetics. Ratio fluorescence was monitored from neutrophils loaded with FURA-2 before and during stimulation with the substance indicated at the *top right* of each box at the concentration indicated at the beginning of each tracing. Test ligands were added at t = 50 s. The tracings shown are from the same experiment which is representative of four experiments. (*B*) Concentration dependence. The amplitude of the peak change in fluorescence is shown as a function of peptide concentration. The data represent single determinations obtained at the indicated concentration for each peptide during the same experiment. The key for each curve is indicated at the *top left*. The data are representative of two separate experiments.

To examine the ability of the pentapeptides to activate microbicidal functions of the human neutrophil, we measured superoxide production by superoxide dismutase-inhibitable chemiluminescence. Fig. 2 shows that the *N*-formyl-, *N*-acetyl-, and unacylated pentapeptides could all stimulate superoxide formation with the same kinetics as for fMLF. Superoxide production peaked at ~30 s after stimulation, and then rapidly decreased to low but sustained levels. In contrast, the unacylated counterpart of fMLF, H-MLF, was inactive even at micromolar concentrations (Figs. 2 and 3). The rank order of potency for the peptides tested was identical to that determined for calcium mobilization (Fig. 3). Again the unacylated pentapeptide had high activity, with an EC_{50} 10-fold higher than for fMLF.

In the context of the structure-activity relationships previously reported for *N*-formyl-tri and tetrapeptides, the high activity of acMNleLFF and H-MNleLFF was surprising. To show that the analogues activate neutrophils via the same receptors as fMLF, we first performed sequential stimulation studies with the pentapeptides, fMLF, C5a, IL-8 and ATP, all of which are able to induce transient elevations of $[Ca^{2+}]_i$ in human neutrophils. After responding to an initial stimulation with saturating concentrations of fMLF or any of the three pentapeptides, neutrophils became completely insensitive to a second stimulation with 1 nM fMLF, the EC_{50} for fMLF, given 200 s after the first stimulus (Fig. 4 *A*). In contrast, saturating concentrations of ATP, IL-8, or C5a could only weakly attenuate the calcium response to 1 nM fMLF. The desensitization patterns observed for fMLF, C5a, and ATP confirm those previously reported by Didsbury et al. (16). The rank order of potency for desensitization of the full response to 1 nM fMLF was fMNleLFF ≈ acMNleLFF > fMLF > H-MNleLFF, identical to that determined for calcium mobilization and superoxide production (Fig. 4 *B*). Moreover, any one pentapeptide given as a first stimulus could fully desensitize neutrophil responsiveness to any other pentapeptide given as a second stimulus (data not shown). Conversely, saturating concentrations (10-fold greater than the EC_{50}) of fMLF or any of the pentapeptides could only partially attenuate neutrophil responsiveness to a 1 nM dose of C5a, the EC_{50} for C5a, whereas a saturating concentration of C5a completely desensitized the cell to a second stimulation with 1 nM C5a (Fig. 5). Thus the desensitization properties of all of the pentapeptides are concordant with those of fMLF and differ from those of the other neutrophil activators tested. Taken together, these data suggest that the pentapeptides specifically activate the same neutrophil receptor or class of receptors as fMLF.

To further characterize the receptor specificity of the penta-

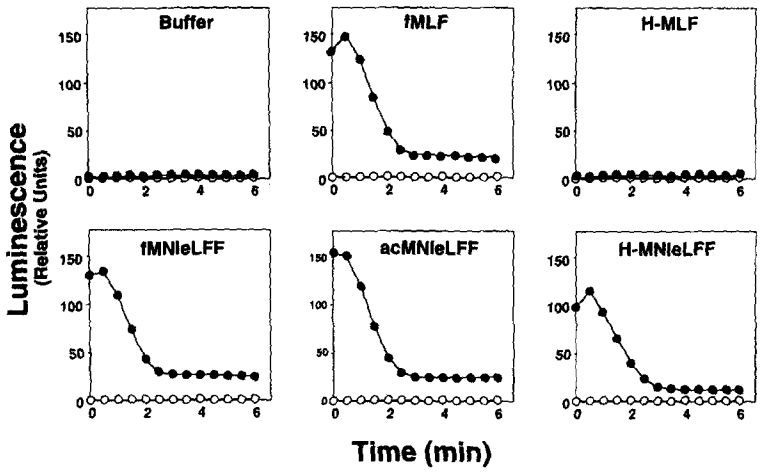

Figure 2. Activation of the neutrophil respiratory burst by synthetic peptides with different NH_2-terminal modifications: kinetics. Neutrophils were stimulated with the substance indicated at the top of each panel in the presence (*open circles*) or absence (*solid circles*) of superoxide dismutase 1 μg/ml. Peptides were tested at 500 nM. Superoxide production is quantified as relative luminescence units.

peptides, they were used to stimulate calcium release from frog oocytes expressing only a single type of human phagocyte receptor, either FPR, or the highly related receptors FPRL1R and the putative FPRL2 product, after microinjection of specific cRNA synthesized in vitro from the corresponding cloned open reading frames (Fig. 6). All three pentapeptides at 100 nM could stimulate calcium release from oocytes expressing FPR, but not from those expressing FPRL1R or from oocytes injected with the FPRL2 cRNA. In contrast, and as previously reported (10), oocytes expressing either FPR or FPRL1R could be activated by fMLF at 100 nM. The EC_{50} for calcium release from oocytes expressing FPR was 10 nM for the unacylated pentapeptide, a value similar to that measured for calcium mobilization by this peptide in neutrophils. This value was 10-fold higher than that measured in parallel experiments using fMLF (Fig. 7).

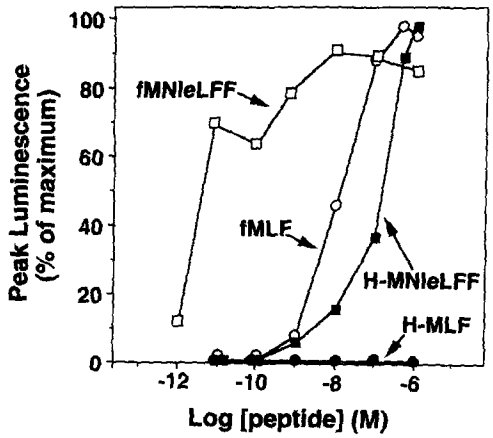

Figure 3. Activation of the neutrophil respiratory burst by synthetic peptides with different NH_2-terminal modifications: concentration dependence. The peak luminescence yield for the indicated concentration range is shown for each peptide. Data represent the means of values obtained in the following number of independent experiments: H-MNleLFF, 5; fMLF, 6; H-MLF, 2; and fMNleLFF, 5.

Discussion

The present work demonstrates that the nonformylated peptide H-MNleLFF can activate with high potency the human *N*-formylpeptide receptor. When used to stimulate neutrophils, the peptide is a potent activator of the microbicidal respiratory burst, whereas H-MLF, the unacylated form of the potent prototype fMLF, is completely inactive at concentrations as high as 1 μM.

H-MNleLFF did not activate the related human receptors FPRL1R or the FPRL2 product, which are 69 and 56% identical in amino acid sequence to FPR, respectively. These results, based on expression of the cloned receptors in frog oocytes, together with the shared patterns of desensitization revealed by sequential stimulation experiments in human neutrophils, strongly suggest that the neutrophil responses elicited by H-MNleLFF and the other pentapeptides are mediated by FPR. FPRL1R is a low affinity receptor for fMLF (K_d = 430 nM; EC_{50} for calcium mobilization > 100 nM) (6, 10). fMLF does not bind to COS cells transfected with the FPRL2 open reading frame, nor does fMLF activate oocytes injected with FPRL2 cRNA (8, 9). The negative results with FPRL2 are reasonable, as its sequence is more divergent than the FPRL1R sequence from that of FPR, yet they must be interpreted with caution since physical expression of the FPRL2 product on the plasma membrane of transfected COS cells and microinjected frog oocytes has not yet been demonstrated.

The sensitivity of FPR to *N*-formylpeptides points to bacteria and mitochondria as reasonable sources of its natural ligands. However, their importance as natural sources of ligands for FPR has not yet been demonstrated in vivo. The ability of H-MNleLFF to activate FPR now implies that natural ligands for FPR may be produced by sources other than bacteria and mitochondria. A data base search revealed several natural proteins that contain the internal sequence MLLFF. Whether this specific peptide exists in vivo and its activity relative to H-MNleLFF remain to be determined.

Previous studies of the structural requirements for binding of ligands to FPR have measured the ability of test compounds to displace labeled *N*-formylpeptides from neutrophils or from

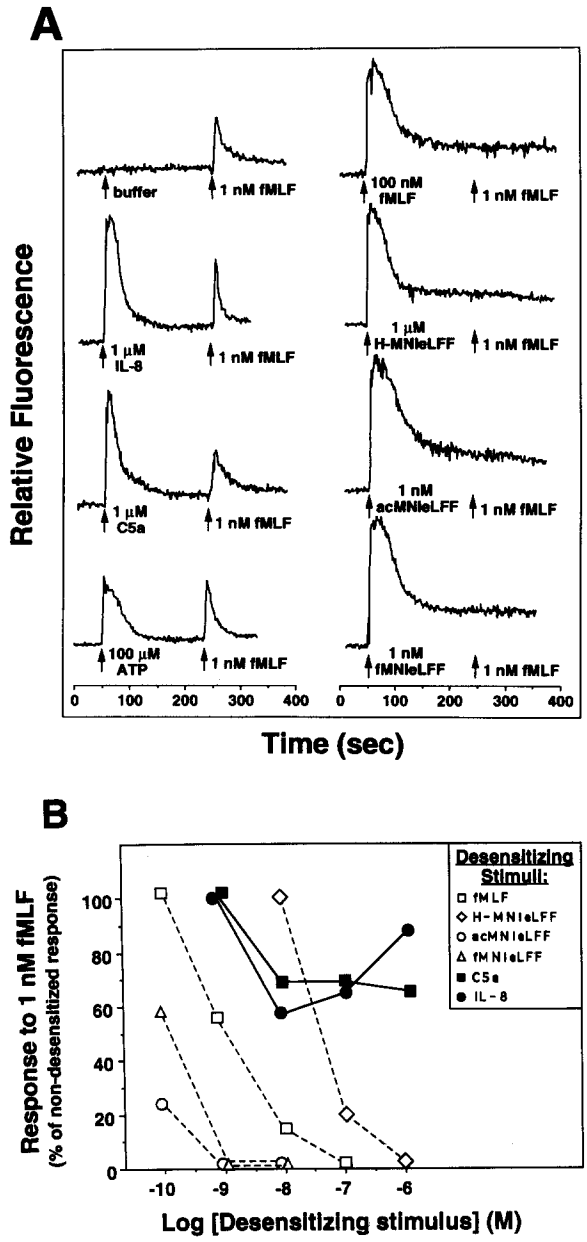

Figure 4. Receptor selectivity of MNleLFF analogs: desensitization of calcium transients elicited by fMLF. (*A*) Kinetics. Ratio fluorescence was monitored from neutrophils loaded with FURA-2 before and during sequential addition of test substances at the times indicated by the *arrows*. The concentrations and identities of each stimulus are indicated to the right of each arrow. The concentration of the first agonist is saturating in each case, whereas the concentration of the second stimulus, 1 nM fMLF, is the EC_{50} for calcium mobilization. The tracings shown are from the same experiment which is representative of three experiments. (*B*) Concentration dependence. The amplitude of the peak change in fluorescence in response to 1 nM fMLF was plotted as a function of the concentration of the agonists indicated in the box at the *top right* of the panel, in each case added 200 s before the addition of 1 nM fMLF. The data, presented as percent of the full non-desensitized response to 1 nM fMLF, represent single determinations obtained at the indicated concentration for each peptide during the same experiment, and are representative of two separate experiments. *Open symbols* and *dashed lines* are for agonists capable of complete desensitization; *solid* symbols and *solid lines* are for agonists capable of partial desensitization.

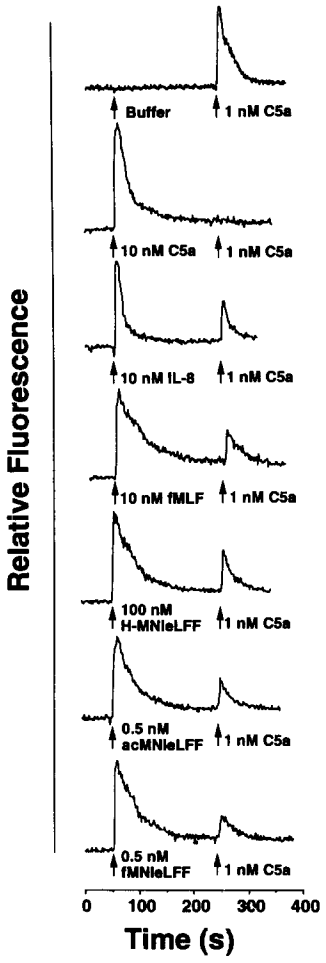

Figure 5. Receptor selectivity of MNleLFF analogs: desensitization of calcium transients elicited by C5a. The experimental design is as indicated in the legend to Fig. 4. The concentrations of the first agonist are 10-fold greater than the corresponding EC_{50} in each case. The tracings shown are from the same experiment which is representative of two experiments.

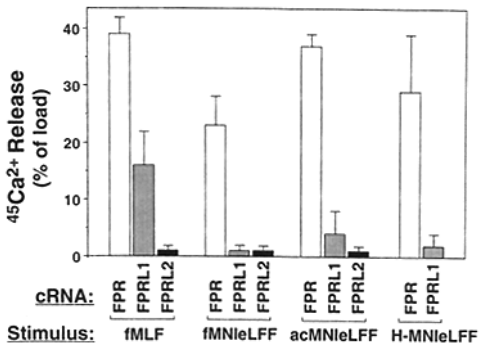

Figure 6. Delineation of the receptor for fMNleLFF, acMNleLFF and H-MNleLFF. *Xenopus* oocytes were injected with a solution containing 200 ng of HL-60 poly(A)$^+$ RNA (an RNA that lacks transcripts for FPR, FPRL1, and FPRL2, but that contains transcripts for a complementary human factor required to link FPR and FPRL1R to signal transduction processes in the oocyte [10, 15]), and 10 ng of the cRNA indicated below each column. 3 d after injection, oocytes were treated with the indicated stimulus at 100 nM and calcium release was measured. The data are from a single experiment representative of two additional experiments. Each data point represents the mean ± SEM from 4 to 6 oocytes. Basal amounts of calcium efflux and calcium uptake were similar for all experimental conditions.

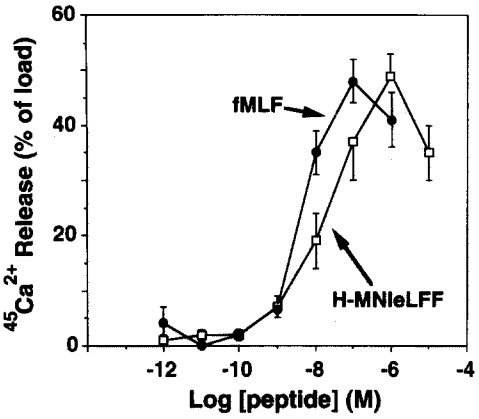

Figure 7. Relative sensitivity of FPR to fMLF and H-MNleLFF. *Xenopus* oocytes were injected with 200 ng of HL-60 poly(A)$^+$ RNA and 10 ng of FPR cRNA. 3 d after injection, oocytes were stimulated with either fMLF (*solid* circles) or H-MNleLFF (*open boxes*) at the indicated concentrations and calcium release was measured. Each data point represents the mean ± SEM from 4–6 oocytes. The data are from a single experiment representative of two separate experiments. Basal amounts of calcium efflux and calcium uptake were similar for all experimental conditions.

FPR expressed in heterologous cell types, and to activate cellular responses. Based on biochemical studies of human neutrophils using real-time quenching of fluorescein-labeled *N*-formylpeptides of different length, Sklar (17) has proposed that the ligand-binding site of FPR can accommodate a tetrapeptide, and is composed of hydrophobic and proton-donating subdomains on the extracellular face of the receptor. The hydrophobic subdomain may involve several of the seven membrane-spanning hydrophobic domains of the receptor inferred from the deduced amino acid sequence; His90 in the proposed first extracellular loop could be a proton donor. In this model, the formyl group resides at limited depth in the interhelical channel, and the COOH terminus of the peptide contacts determinants in the extracellular loops. Analysis of chimeric FPR:FPRL1R receptors has suggested that differences in all three of the extracellular loops and possibly the NH_2-terminal segments account for the large difference in affinity for fMLF observed for FPR and FPRL1R (10, 18). The importance of the transmembrane domains for fMLF binding and signaling has not yet been addressed by mutagenesis studies.

The decreased potency of H-MNleLFF relative to fMNleLFF supports the importance of *N*-formylation for stabilizing the binding interaction, whereas the small diminution of potency of H-MNleLFF relative to fMLF suggests that differences in the COOH-terminal sequence can compensate for the loss of the formyl group. However, *N*-acetyl-MLF exhibits the same loss of activity as does the unacylated tripeptide (2), whereas there is very little diminution of activity of *N*-acetyl-MNleLFF compared with its formylated analogue. This suggests that the added COOH-terminal sequence causes changes in the nature of the binding of the NH_2-terminal amino acid(s) compared with that of the tripeptide.

Despite 20 yr of study, the biological role and importance of FPR have not been established, in part because of uncertainties regarding the identity of its natural ligands. The present findings increase this uncertainty, in that *N*-formylation is no longer a conceptual constraint for a peptide to have high activity for FPR. The demonstration that *N*-formylation is not necessary for a peptide to potently activate FPR permits the existence of a nonformylated endogenous ligand in eukaryotic organisms to be more confidently considered.

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