The Journal of Immunology

RESEARCH ARTICLE | DECEMBER 15 2003

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J Immunol (2003) 171 (12): 6807–6813.
https://doi.org/10.4049/jimmunol.171.12.6807

Differential Activation of Formyl Peptide Receptor-Like 1 by Peptide Ligands[1]

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Formyl peptide receptor-like 1 (FPRL1) plays a key role in the regulation of immune responses. The activation of FPRL1 induces a complicated pattern of cellular signaling, which results in the regulation of several immune responses, such as chemotactic migration and the production of reactive oxygen species (ROS). Because some of these cellular responses are not beneficial to the host, ligands that selectively modulate these cellular responses are useful. His-Phe-Tyr-Leu-Pro-Met (HFYLPM) is a synthetic peptide that binds to FPRL1. In this study, we generated various HFYLPM analogues and examined their effects on cellular responses via FPRL1 in FPRL1-expressing rat basophilic leukemia-2H3 cells or in primary human neutrophils. Among the HXYLPM analogues, His-Arg-Tyr-Leu-Pro-Met (HRYLPM) activated a broad spectrum of cellular signaling events, including an intracellular Ca^{2+} concentration increase, phosphoinositide 3-kinase, extracellular signal-regulated kinase, and Akt activation, however, His-Glu-Tyr-Leu-Pro-Met (HEYLPM) activated only intracellular Ca^{2+} concentration and Akt but did not increase Ca^{2+}. In addition, HRYLPM was found to stimulate chemotaxis and ROS generation via phosphoinositide 3-kinase and an intracellular Ca^{2+} concentration increase, respectively, whereas HEYLPM stimulated chemotaxis but not ROS generation. With respect to the molecular mechanisms involved in the differential action of HRYLPM and HEYLPM, we found that HRYLPM but not HEYLPM competitively inhibited the binding of ^{125}I-labeled Trp-Lys-Tyr-Met-Val-D-Met-NH_2 (WKYMVm, a FPRL1 ligand) to FPRL1. This study demonstrates that the important chemoattractant receptor, FPRL1, may be differentially modulated by distinct peptide ligands. We also suggest that HRYLPM and HEYLPM may be used to selectively modulate FPRL1. ***The Journal of Immunology,*** **2003, 171: 6807–6813.**

Formyl peptide receptor-like 1 (FPRL1)[3] is mainly expressed in phagocytic cells such as neutrophils and monocytes. Moreover, FPRL1 has been reported to perform an important function in the regulation of immune responses against pathogen infection. The two critical roles of FPRL1, in the regulation of phagocytic cell activity, concern the chemotactic migration of phagocytes and the production of reactive oxygen species (ROS) (1, 2). Recruitment of phagocytes into an infected area is pivotal for the initiation of immune responses (1, 3). Recruited phagocytes in a specific site can be activated by various environmental stimuli, including FPRL1 agonists, which results in the production of ROS. The ROS produced can be used as an important weapon against invading pathogens (4). Although ROS generation is necessary for the killing of pathogens, it also causes complex responses that are not beneficial to host cells. Bearing in mind the adverse effects of ROS, an immunoregulating agent is required that elicits selective immune responses without evoking ROS generation.

Various different FPRL1 agonists have been reported from endogenous sources or by artificial synthesis (5–8). They include serum amyloid A (SAA), HIV-envelope domains (F peptide and V3 peptide), and host-derived agonist ($A\beta_{42}$) (5–7). Previously we reported upon a synthetic peptide ligand, His-Phe-Tyr-Leu-Pro-Met-NH_2 (HFYLPM) that stimulates monocytes and neutrophils, and results in the chemotaxis of the cells and ROS generation (8). We also suggested that HFYLPM binds to the formyl peptide receptor (FPR) or its related receptors on human phagocytic cells (8). In this study, we found that HFYLPM is a specific ligand for FPRL1. Moreover, because HFYLPM is a short peptide that potently induces phagocyte activation, it may be a useful material in the study of FPRL1-mediated signaling.

In this study, we generated various analogues of HFYLPM, and examined their effects on FPRL1-mediated signaling and their cellular activities in rat basophilic leukemia (RBL)-2H3 cells and human neutrophils. Finally, we found that FPRL1 is activated differentially by HFYLPM analogues.

Materials and Methods

Materials

Fmoc amino acids were obtained from Millipore (Bedford, MA). Rapid-amide resin was purchased from DuPont (Boston, MA). Cytochrome *c* was purchased from Sigma-Aldrich (St. Louis, MO). Fura 2-pentaacetoxymethylester (fura 2-AM) and BAPTA-AM were purchased from Molecular Probes (Eugene, OR). RPMI 1640 was obtained from Invitrogen (Carlsbad, CA). Dialyzed FBS and supplemented bovine serum were purchased from HyClone Laboratories (Logen, UT). PD98059 was purchased from Calbiochem (San Diego, CA). LY294002 was from Biomol (Plymouth Meeting, PA). Anti-phospho-extracellular signal-regulated

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Received for publication June 16, 2003. Accepted for publication October 7, 2003.

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[1] This work was supported by the Korea Science and Engineering Foundation through the Medical Science and Engineering Research Center for Cancer Molecular Therapy at Dong-A University and by Grant FPR02A5-43-110 of the 21C Frontier Functional Proteomics Project from the Korean Ministry of Science and Technology.

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[3] Abbreviations used in this paper: FPRL1, formyl peptide receptor-like 1; ROS, reactive oxygen species; SAA, serum amyloid A; $[Ca^{2+}]_i$, intracellular calcium concentration; PTX, pertussis toxin; FPR, formyl peptide receptor; PI3K, phosphoinositide 3-kinase; LXA_4, lipoxin A_4; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; MEK, mitogen-activated protein/ERK kinase; fura 2-AM, fura 2-pentaacetoxymethyl ester.

0022-1767/03/$02.00

kinase (ERK) Abs and anti-phospho-Akt Abs were purchased from Cell Signaling Technology (Beverly, MA).

Peptide synthesis

Peptides were synthesized by the solid-phase method, as previously described (9). Briefly, the synthesis was performed on a Rapidamide support resin, and assembly using a standard Fmoc/t-butyl strategy on an acid-labile linker. Peptide compositions were confirmed by amino acid analysis, as previously described (9). In this study, we synthesized HFYLPM analogues by substituting amino acid residues with amino acids having different functionalities. Specifically, His (1) was replaced with Gly, Arg, Gln, Trp, or Tyr; Phe (2) with Gly, Trp, Arg, Glu, Gln, or His; Tyr (3) with Gly, His, Arg, Glu, Trp, or Phi; Leu (4) with Gly, His, Arg, Glu, Gln, or Trp; Pro (5) with Gly, His, Arg, Glu, Gln, or Trp; and Met (6) with Gly, Glu, Val, Arg, Trp, or His. We also synthesized a C-terminal Met-deletion peptide (HFYLP-NH_2), N-terminal deletion peptides (FYLPM-NH_2, YLPM-NH_2, and LPM-NH_2), and a peptide with both terminals deleted (FYLP-NH_2).

Cell culture

RBL-2H3 cells, FPR-, or FPRL1-expressing RBL-2H3 cells were cultured in DMEM supplemented with 20% FBS and 200 μg/ml G418, as previously described (10). PBLs were obtained from healthy young donors. Human neutrophils were isolated by dextran sedimentation, hypotonic erythrocyte lysis, and by using a lymphocyte separation medium gradient, as previously described (8). The isolated human leukocytes so obtained were then used promptly.

Ca^{2+} measurement

Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined by Grynkiewicz's Method using fura 2-AM (11). Briefly, prepared cells were incubated with 3 μM fura 2-AM at 37°C for 50 min in fresh serum free RPMI 1640 medium with continuous stirring. Ca^{2+} measurement was performed at 37°C. Fura 2-loaded cells were suspended in serum-free RPMI 1640 medium and 2×10^6 cells were taken for each measurement. After centrifugation, RPMI 1640 medium was discarded and the cells were suspended in Locke's buffer (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES, pH 7.3, 10 mM glucose, and 0.2 mM EGTA). We monitored the baseline of the fluorescence ratio for ~30 s before adding peptide. Fluorescence was measured at 500 nm at excitation wavelengths of 340 and 380 nm, and the calculated fluorescence ratio was translated into $[Ca^{2+}]_i$.

Stimulation of cells with peptides for Western blot analysis

Cultured RBL-2H3 cells, FPRL1-expressing RBL-2H3 cells, or freshly isolated human neutrophils (2×10^6) were stimulated with the indicated concentrations of His-Arg-Tyr-Leu-Pro-Met (HRYLPM) or His-Glu-Tyr-Leu-Pro-Met (HEYLPM) for predetermined lengths of time. After stimulation, the cells were washed with serum-free RPMI 1640 and lysed in lysis buffer (20 mM HEPES, pH 7.2, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Detergent insoluble materials were pelleted by centrifugation (12,000 $\times$ *g*, 15 min, at 4°C), and the soluble supernatant fraction was removed and stored at either −80°C or used immediately. Protein concentrations in the lysates were determined by Bradford protein assay.

Electrophoresis and immunoblot analysis

Protein samples were prepared for electrophoresis then separated in an 8% SDS-PAGE and the buffer system described by King and Laemmli (12). Following the electrophoresis, the proteins were blotted onto nitrocellulose membrane, which was blocked by incubating with TBST (0.05% Tween 20) containing 5% nonfat dried milk. The membranes were then incubated with anti-phospho-ERK Ab, anti-phospho-Akt Ab, or anti-ERK Ab and washed with TBS. Ag-Ab complexes were visualized after incubating the membrane with 1:5000 diluted goat anti-rabbit IgG or goat anti-mouse IgG Ab coupled to HRP by ECL.

Measurement of superoxide anion generation

Superoxide anion generation was determined by measuring cytochrome *c* reduction using a microtiter 96-well plate ELISA reader (EL312e; Bio-Tek Instruments, Winooski, VT) as previously described (8). Human neutrophils (2×10^6 cells in RPMI 1640 medium) were preincubated with 50 μM cytochrome *c* at 37°C for 1 min and then incubated with each peptide. Superoxide generation was determined by measuring light absorption changes at 550 nm over 5 min at 1-min intervals.

Chemotaxis assay

Chemotaxis assays were performed using multiwell chambers (NeuroProbe, Gaithersburg, MD) (8). Briefly, prepared human neutrophils were suspended in RPMI 1640 at 1×10^6 cells/ml, and 25 μl of this suspension was placed into the upper well of a chamber separated by a 3-μm polyhydrocarbon filter from the peptide-containing lower well. After incubation for 90 min at 37°C, nonmigrated cells were removed by scraping, and cells that had migrated across the filter were dehydrated, fixed, and stained with hematoxylin (Sigma-Aldrich). Stained cells from a particular well were then counted in five randomly chosen high power fields (×400) (8).

Ligand binding assay

Ligand binding analysis was performed as previously described (13). Radioiodinated Trp-Lys-Tyr-Met-Val-D-Met-NH_2 (WKYMVm, ^{125}I-labeled) was a gift from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). Briefly, RBL-2H3 cells or FPRL1-expressing RBL-2H3 cells were seeded at 1×10^5 cells per well into a 24-well plate and cultured overnight. Addition of various concentrations of [^{125}I] WKYMVm induced significant specific binding of the radiolabeled WKYMVm to FPRL1 within 50 pM to 1 nM (data not shown). Because the specific binding of [^{125}I] WKYMVm was at linear phase around 500 pM and ligand was in excess mole against the receptor at this ligand concentration, we selected this concentration. In addition, at this concentration we could observe sensitive competition of [^{125}I] WKYMVm to FPRL1 by cold WKYMVm. After blocking the cells with blocking buffer (33 mM HEPES, pH 7.5, 0.1% BSA in RPMI 1640) for 2 h, 500 pM labeled WKYMVm was added to the cells in binding buffer (PBS containing 0.1% BSA), in the absence or in the presence of unlabeled peptides, and incubated for 3 h at 4°C with continuous shaking. The samples were then washed five times with ice-cold binding buffer, and 200 μl of lysis buffer (20 mM Tris, pH 7.5, 1% Triton X-100) was added to each well. After 20 min at room temperature, the lysates were collected and counted using a gamma-ray counter (13).

Results

The synthetic peptide, HFYLPM, is an agonist for FPRL1

In our previous report, we demonstrated that HFYLPM elicited an intracellular calcium increase and the chemotactic migration of human monocytes in a pertussis toxin (PTX)-sensitive manner (8). Furthermore, pretreatment of HFYLPM before *N*-formyl-methionylleucylphenylalanine (fMLF) stimulation in human monocytes was found to inhibit fMLF-induced $[Ca^{2+}]_i$ release (8). In this study, we investigated whether HFYLPM stimulates FPR or FPRL1. HFYLPM at concentrations up to 3 μM did not affect $[Ca^{2+}]_i$ release in vector-transfected or FPR-expressing RBL-2H3 cells (Fig. 1*A*). When FPRL1-expressing RBL-2H3 cells were stimulated with 3 μM HFYLPM, a transient and potent $[Ca^{2+}]_i$ release was induced (Fig. 1*A*). We also examined the concentration dependency of $[Ca^{2+}]_i$ release by HFYLPM in FPRL1 cells. HFYLPM-induced $[Ca^{2+}]_i$ release was significantly elicited from 100 nM, and showed maximal activity at 3 μM (Fig. 1*B*). These results indicate that HFYLPM is an agonist of FPRL1 and not of FPR.

Effect of several HFYLPM analogues on $[Ca^{2+}]_i$ release in FPRL1 cells

Because HFYLPM is a specific agonist of FPRL1, we used it as a starting point to develop the analogues needed to study the relation between the structure and function of FPRL1 agonists. Therefore, we synthesized various kinds of HFYLPM analogues as listed in Table I. The effect of the peptides on the regulation of FPRL1 was examined by monitoring $[Ca^{2+}]_i$ increases. The EC_{50} of HFYLPM in terms increasing $[Ca^{2+}]_i$ activity in FPRL1 cells was 0.9 μM (Table I). Among the HFYLPM analogues, RFYLPM, WFYLPM, YFYLPM, HWYLPM, HRYLPM, HFYWPM, and HFYLWM showed more activity for FPRL1 than the mother peptide, and the other analogues were not as active (Table I). In particular, several analogues including HEYLPM did not cause a $[Ca^{2+}]_i$ increase at up to 20 μM in FPRL1-expressing RBL-2H3 cells (Table I). We also checked the effect of HFYLPM and its analogues in terms of $[Ca^{2+}]_i$ increasing activity in vector-transfected RBL-2H3 cells and confirmed that the peptides were inactive in these cells (data not shown). Of the HFYLPM analogues, although HRYLPM stimulated $[Ca^{2+}]_i$ increase, HEYLPM did not induce $[Ca^{2+}]_i$ increase

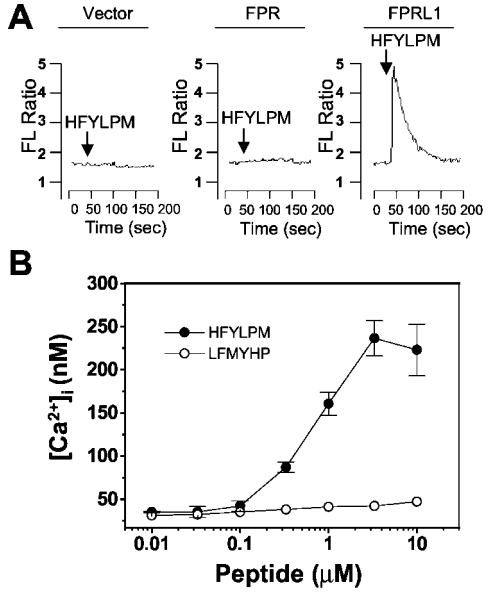

FIGURE 1. The effects of HFYLPM on $[Ca^{2+}]_i$ in FPRL1-expressing RBL-2H3 cells. *A*, Vector transfected RBL-2H3 cells, FPR-, or FPRL1-expressing RBL-2H3 cells were stimulated with 5 μM HFYLPM. $[Ca^{2+}]_i$ was determined fluorometrically using fura 2-AM as described in *Materials and Methods*. Data are representative of three independent experiments (*A*). *B*, FPRL1-expressing RBL-2H3 cells were stimulated by various concentrations of HFYLPM or the scrambled inactive peptide, LFMYHP. The peak level of $[Ca^{2+}]_i$ was recorded. Results are presented as mean ± SE of three independent experiments (*B*).

via FPRL1 (Fig. 2*A*). Preincubation of FPRL1-expressing RBL-2H3 cells with PTX (100 ng/ml) for 24 h caused complete inhibition of HRYLPM-induced $[Ca^{2+}]_i$ increase, suggesting the involvement of Gi/o proteins (Fig. 2*A*). For HRYLPM, the peptide stimulated $[Ca^{2+}]_i$ increase in a concentration-dependent manner in FPRL1-expressing RBL-2H3 cells (Fig. 2*B*). HRYLPM-induced $[Ca^{2+}]_i$ increase was apparent from 100 nM and showed maximal activity at 3.3 μM (Fig. 2*B*).

Table I. *Effect of peptides modified from HFYLPM-NH_2 on intracellular calcium increase in FPRL1-expressing RBL-2H3 cells*[a]

Sequence	EC_{50} (μM)	Sequence	EC_{50} (μM)
HFYLPM-NH_2	0.9 ± 0.05	HFYEPM-NH_2	Inactive
GFYLPM-NH_2	5.5 ± 0.2	HFYQPM-NH_2	8.3 ± 0.4
RFYLPM-NH_2	0.27 ± 0.07	HFYWPM-NH_2	0.42 ± 0.03
QFYLPM-NH_2	3.1 ± 0.1	HFYLGM-NH_2	Inactive
WFYLPM-NH_2	0.08 ± 0.01	HFYLHM-NH_2	2.7 ± 0.2
YFYLPM-NH_2	0.18 ± 0.03	HFYLRM-NH_2	Inactive
HGYLPM-NH_2	6.5 ± 0.1	HFYLEM-NH_2	Inactive
HWYLPM-NH_2	0.18 ± 0.02	HFYLQM-NH_2	Inactive
HRYLPM-NH_2	0.32 ± 0.04	HFYLWM-NH_2	0.57 ± 0.1
HEYLPM-NH_2	Inactive	HFYLPG-NH_2	Inactive
HQYLPM-NH_2	6.4 ± 0.2	HFYLPE-NH_2	Inactive
HHYLPM-NH_2	3.7 ± 0.1	HFYLPV-NH_2	Inactive
HFGLPM-NH_2	Inactive	HFYLPR-NH_2	Inactive
HFHLPM-NH_2	13 ± 3	HFYLPW-NH_2	Inactive
HFRLPM-NH_2	18 ± 5	HFYLPH-NH_2	Inactive
HFELPM-NH_2	Inactive	FYLPM-NH_2	Inactive
HFWLPM-NH_2	0.7 ± 0.04	HFYLP-NH_2	4.3 ± 0.2
HFFLPM-NH_2	5.8 ± 0.4	FYLP-NH_2	Inactive
HFYGPM-NH_2	Inactive	YLPM-NH_2	15.2 ± 1.2
HFYHPM-NH_2	Inactive	LPM-NH_2	Inactive
HFYRPM-NH_2	7.6 ± 0.7	LFMYHP-NH_2	Inactive

[a] Intracellular calcium increase was monitored in fura 2-loaded cells and each peptide was tested up to 30 μM.

The effects of HRYLPM or HEYLPM on ERK phosphorylation in FPRL1-expressing RBL-2H3 cells

The activation of FPRL1 by several agonists has been reported to induce various intracellular signaling pathways (1, 3, 5). Because two of the HFYLPM analogues, HRYLPM and HEYLPM, produced different responses in terms of $[Ca^{2+}]_i$ increase in FPRL1-expressing RBL-2H3 cells (Fig. 2*A*), we examined their effects on other signaling events (i.e., ERK and Akt), which are independent of intracellular calcium. The stimulation of these cells with various concentrations of HRYLPM or HEYLPM for 5 min induced ERK activation in a concentration-dependent manner (Fig. 3*A*). Moreover, HRYLPM-induced ERK activation was apparent within 5–10 μM concentration in these cells (Fig. 3*A*). Although HEYLPM did not increase cytosolic calcium activity, it did stimulate ERK phosphorylation (Fig. 3*A*). HEYLPM-induced ERK activation was concentration-dependent: it induced significant activity at 5–10 μM (Fig. 3*A*). Stimulation of FPRL1-expressing RBL-2H3 cells with 10 μM HRYLPM or 10 μM HEYLPM caused the transient activation of ERK and maximal activity 5–10 min after stimulation (Fig. 3*B*). To investigate the signaling pathway leading to ERK activation by HRYLPM or HEYLPM via FPRL1, we used several different types of enzyme inhibitor. When FPRL1-expressing RBL-2H3 cells were pretreated with PTX or PD98059 before HRYLPM or HEYLPM stimulation, ERK activation was found to be sensitive to both, indicating that ERK activation is PTX-sensitive G protein or mitogen-activated protein/ERK kinase (MEK)-dependent (Fig. 3*C*). Pretreatment with a calcium chelator (BAPTA-AM) did not affect these peptide-induced ERK activations (Fig. 3*C*), suggesting that ERK activation is independent of a $[Ca^{2+}]_i$

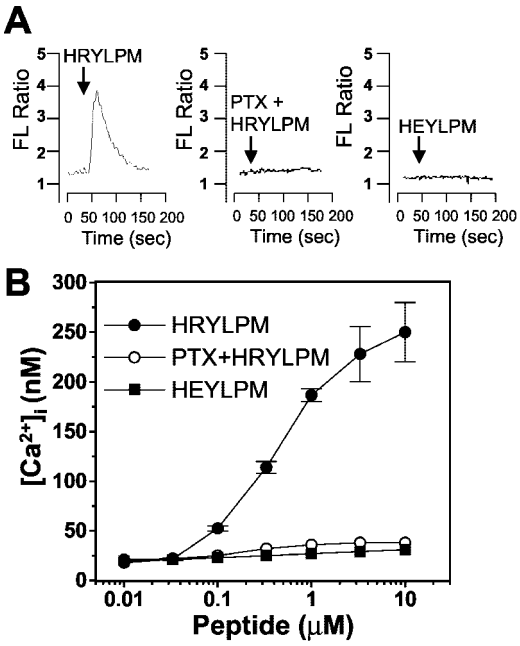

FIGURE 2. The effects of HRYLPM and HEYLPM on $[Ca^{2+}]_i$ in FPRL1-expressing RBL-2H3 cells. FPRL1-expressing RBL-2H3 cells were stimulated with 5 μM HRYLPM or HEYLPM, and $[Ca^{2+}]_i$ was determined fluorometrically using fura 2-AM as described in *Materials and Methods*. To examine the effect of PTX on the HRYLPM-induced $[Ca^{2+}]_i$ increase, the cells were preincubated with 100 ng/ml PTX for 24 h before $[Ca^{2+}]_i$ measurement. *A*, The peak level of $[Ca^{2+}]_i$ was recorded. Data are representative of three independent experiments (*A*). *B*, FPRL1-expressing RBL-2H3 cells were stimulated by various concentrations of HRYLPM or HEYLPM. The peak level of $[Ca^{2+}]_i$ was recorded. Results are presented as the mean ± SE of three independent experiments (*B*).

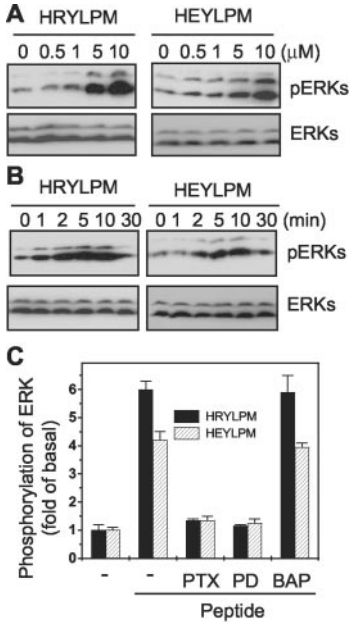

FIGURE 3. The effects of HRYLPM and HEYLPM on ERK phosphorylation in FPRL1-expressing RBL-2H3 cells. *A*, FPRL1-expressing RBL-2H3 cells were stimulated with various concentrations of HRYLPM or HEYLPM for 10 min. *B*, Cells were stimulated with 10 μM HRYLPM or HEYLPM for various lengths of time. *C*, Cells were preincubated with vehicle or 100 ng/ml PTX (24 h), 10 μM BAPTA-AM (60 min), or 50 μM PD98059 (60 min) before being treated with HRYLPM (10 μM) or HEYLPM (10 μM) for 10 min. Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and phosphorylated ERK was determined by immunoblot analysis using anti-phospho-ERK Ab (*A* and *B*). ERK phosphorylation was quantified by densitometry (*C*). Results are presented as the mean ± SE of three independent experiments (*C*).

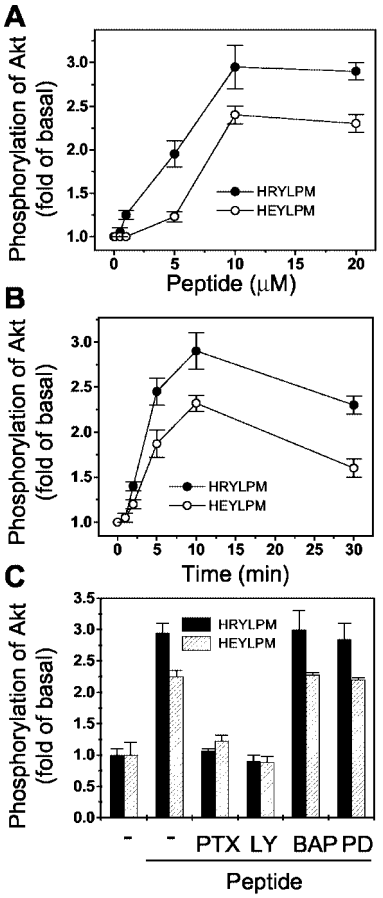

FIGURE 4. The effects of HRYLPM and HEYLPM on Akt phosphorylation in FPRL1-expressing RBL-2H3 cells. *A*, FPRL1-expressing RBL-2H3 cells were stimulated with various concentrations of HFYLPM or HEYLPM for 10 min. *B*, FPRL1-expressing RBL-2H3 cells were stimulated with HRYLPM (10 μM) or HEYLPM (10 μM) for various lengths of time. The cells were preincubated with vehicle or 100 ng/ml PTX (24 h), 50 μM LY294002 (15 min), 10 μM BAPTA-AM (60 min), or 50 μM PD98059 (60 min) before being treated with HRYLPM (10 μM) or HEYLPM (10 μM) for 10 min (*C*). Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and phosphorylated Akt was determined by immunoblot analysis using anti-phospho-Akt Ab. Akt phosphorylation was quantified by densitometry. Results are presented as the mean ± SE of three independent experiments.

increase. Therefore, it appears that HRYLPM induces $[Ca^{2+}]_i$ increase and ERK activation via independent signaling pathways. Bearing in mind the fact that HEYLPM did not affect the $[Ca^{2+}]_i$ increase, this result is very interesting because HEYLPM caused ERK phosphorylation in FPRL1-expressing RBL-2H3 cells. Separate experiments using vector-transfected RBL-2H3 cells confirmed that none of the tested peptides affected ERK activity (data not shown).

Effect of HRYLPM and HEYLPM on Akt phosphorylation in FPRL1-expressing RBL-2H3 cells

It is well established that the activation of chemoattractant receptors induces Akt activation via phosphoinositide 3-kinase (PI3K) (14). We also observed that the stimulation of FPRL1-expressing RBL-2H3 cells with HRYLPM or HEYLPM induced Akt phosphorylation in a concentration-dependent manner (Fig. 4*A*). Akt activation was significantly induced by HRYLPM at 1–10 μM in FPRL1-expressing RBL-2H3 cells (Fig. 4*A*), and HEYLPM-induced Akt activation was significant at 5–10 μM (Fig. 4*A*). HRYLPM- or HEYLPM-induced Akt activation was transient showing maximal activity at 10 min after stimulation (Fig. 4*B*). Moreover, HRYLPM- or HEYLPM-induced Akt activation was sensitive to PTX and to LY294002, but not to BAPTA-AM or PD98059, indicating involvement of PTX-sensitive G proteins and PI3K (Fig. 4*C*). Because ERK and Akt activation induced by the two analogues was mediated by PI3K activation, it appears that HRYLPM and HEYLPM successfully induce PI3K-mediated signaling downstream of activated FPRL1. We confirmed that none of the tested peptides affected Akt activity in vector-transfected RBL-2H3 cells (data not shown).

Effect of HRYLPM and HEYLPM on intracellular signaling in human neutrophils

Because two of the HFYLPM analogues, HRYLPM and HEYLPM, stimulated FPRL1-expressing RBL-2H3 cells in a distinct manner in terms of $[Ca^{2+}]_i$ increase and ERK/Akt activation, we investigated their effects in primary human neutrophils that express FPRL1. As expected, stimulation of human neutrophils with various concentrations of HRYLPM elicited $[Ca^{2+}]_i$ increase in a concentration-dependent manner showing maximal activity at 1 μM (Fig. 5*A*). However, when we stimulated human neutrophils with various concentrations of HEYLPM, we did not observe any significant $[Ca^{2+}]_i$ increase (Fig. 5*A*). At 1 μM, HRYLPM but not HEYLPM induced a dramatic $[Ca^{2+}]_i$ increase in neutrophils (Fig. 5*A*, *inset*).

We then examined the effects of HRYLPM and HEYLPM on ERK activation in human neutrophils. When human neutrophils were stimulated with various concentrations of HRYLPM for 5 min ERK activation was apparent at 0.5–10 μM in human neutrophils (Fig. 5*B*). HEYLPM also stimulated ERK phosphorylation in the cells (Fig. 5*B*), although it did not elicit a $[Ca^{2+}]_i$ increase

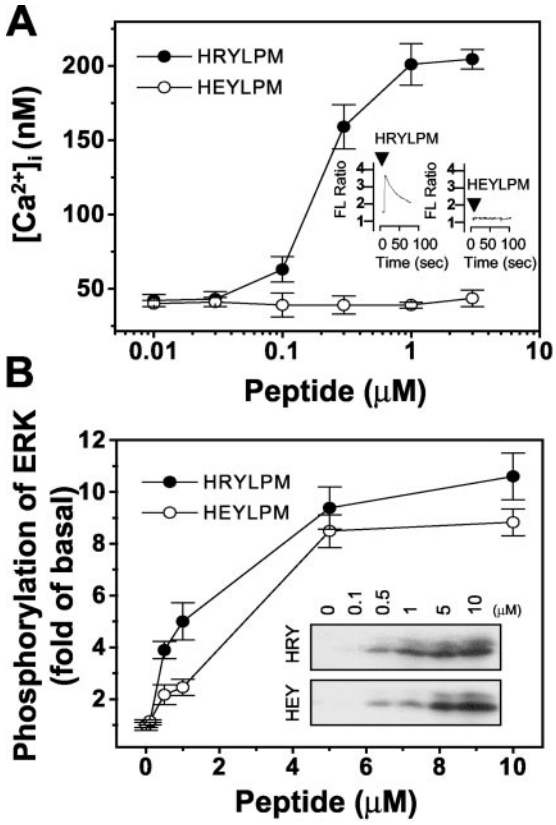

FIGURE 5. The effects of HRYLPM and HEYLPM on $[Ca^{2+}]_i$ and ERK activation in human neutrophils. Freshly isolated human neutrophils were stimulated with various concentrations of HRYLPM or HEYLPM, and $[Ca^{2+}]_i$ was determined fluorometrically using fura 2-AM. The peak level of $[Ca^{2+}]_i$ was recorded. Results are presented as the mean ± SE of three independent experiments (*A*). Fura 2-loaded human neutrophils were stimulated with HRYLPM (1 μM) or HEYLPM (1 μM), and the fluorescence (FL) ratio (340:380 nm) was monitored (*A*, *inset*). *B*, Human neutrophils were stimulated with various concentrations of HRYLPM or HEYLPM for 10 min. Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and phosphorylated ERK was determined by immunoblot analysis using anti-phospho-ERK Ab (*B*, *inset*). ERK phosphorylation was quantified by densitometry. Results are presented as the mean ± SE of three independent experiments (*B*).

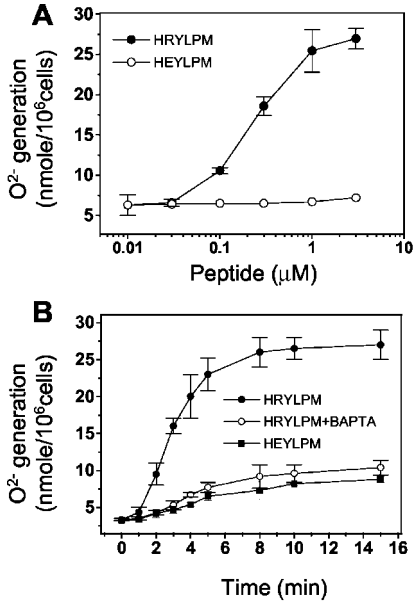

FIGURE 6. The effects of HRYLPM and HEYLPM on superoxide generation in human neutrophils. *A*, Isolated human neutrophils (2×10^6 cells/ml/assay) were preincubated for 1 min at 37°C with 50 μM cytochrome *c* before being stimulated with various concentrations of HRYLPM or HEYLPM for 5 min. *B*, Human neutrophils were stimulated with 1 μM HRYLPM or 1 μM HEYLPM in the absence and in the presence of 10 μM BAPTA-AM for various lengths of time. Cytochrome *c* reduction was monitored as a change in absorption at 550 nm at 1 min intervals over 15 min, as described in *Materials and Methods*. Superoxide anion generated is expressed as mean ± SE (*A* and *B*).

in human neutrophils. Moreover, this HEYLPM-induced ERK activation was concentration-dependent and significant activity was obtained at 0.5–10 μM (Fig. 5*B*). These results parallel the differential signaling induced by HRYLPM and HEYLPM in FPRL1-expressing RBL-2H3 cells.

Effect of HRYLPM and HEYLPM on superoxide generation in human neutrophils

The differential effects of HRYLPM and HEYLPM in terms of $[Ca^{2+}]_i$ increase in human neutrophils led us to check the possibility of differential modulation of neutrophil functions in which calcium increase is essential. Previously we and others have demonstrated that superoxide generation is absolutely dependent on $[Ca^{2+}]_i$ increase in human phagocytic cells, such as monocytes and neutrophils (15, 16). Therefore, we checked the effects of HRYLPM and HEYLPM on superoxide generation by measuring cytochrome *c* reduction, as previously described (8). The stimulation of human neutrophils with various concentrations of HRYLPM was found to generate superoxide in a concentration-dependent manner (Fig. 6*A*), and this activity was maximal at 1 μM HRYLPM (Fig. 6*A*). Treatment of human neutrophils with different concentrations of HEYLPM (10 nM-5 μM) did not affect superoxide generation (Fig. 6*A*). Fig. 6*B* shows that HRYLPM stimulates superoxide generation in a time-dependent manner, showing maximal activity within 5 min of stimulation. These results are in complete agreement with our previous results, namely, that HEYLPM cannot induce a $[Ca^{2+}]_i$ increase in human neutrophils (Fig. 5*A*). To confirm the critical role of intracellular calcium on HRYLPM-induced superoxide generation in human neutrophils, we pretreated human neutrophils with 10 μM BAPTA-AM before peptide stimulation. As shown in Fig. 6*B*, preincubation of human neutrophils with BAPTA-AM abrogated HRYLPM-induced superoxide generation (Fig. 6*B*), strongly suggesting that HRYLPM stimulates superoxide generation via an intracellular calcium-dependent signaling pathway.

Effect of HRYLPM and HEYLPM on neutrophil chemotaxis

HFYLPM has been reported to induce chemotactic migration of phagocytic cells, such as monocytes and neutrophils (8). In the present study, we investigated the effects of HRYLPM and HEYLPM on neutrophil chemotaxis. Both HRYLPM and HEYLPM showed chemotactic migratory activity and a bell-shaped concentration dependency in human neutrophils (Fig. 7*A*). Previous reports demonstrated that several chemoattractants induce neutrophil chemotaxis via the Gi/o, PI3K, and MEK signaling pathway (17–20). We found that treatment with HRYLPM or HEYLPM caused transient ERK activation in a PTX- and PD980592-sensitive manner (Fig. 3*C*). In the present study, we investigated the effects of PTX, LY294002, or PD98059 on the neutrophil chemotaxis induced by the two peptides, and we found that both peptides are sensitive to PTX, LY294002, or PD98059 (Fig. 7*B*). These results suggest that HRYLPM- and HEYLPM-induced neutrophil chemotaxis is mediated by the Gi/o, PI3K, and MEK-dependent signaling

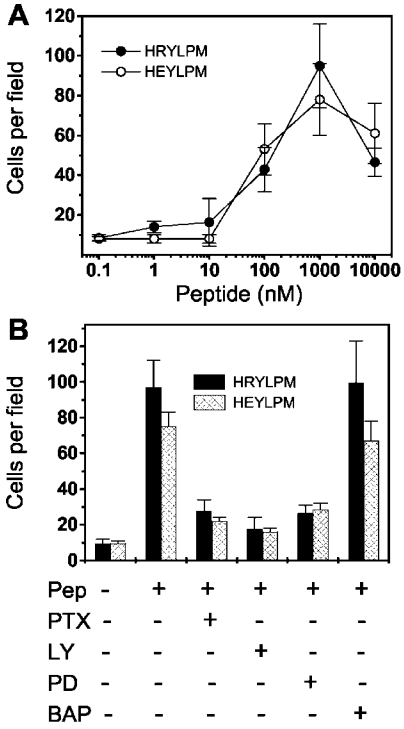

FIGURE 7. The effect of HRYLPM or HEYLPM on neutrophil chemotaxis. Assays were performed using a modified Boyden chamber assay, as described in *Materials and Methods*. Freshly isolated human neutrophils (1×10^6 cells/ml of serum-free RPMI 1640) were added to the upper wells of a 96-well chemotaxis chamber, and migration across a 3-μm pore size polycarbonate membrane was assessed after incubating at 37°C for 90 min. Various concentrations of each peptide were used for the chemotaxis assay (*A*). Vehicle, 1 μg/ml PTX (90 min), 50 μM LY294002 (15 min), 50 μM PD98059 (60 min), or 10 μM BAPTA-AM (60 min) pretreated cells were subjected to the chemotaxis assay with 1 μM HRYLPM or 1 μM HEYLPM (*B*). The numbers of migrated cells were determined by counting in a high power field (×400). Data are presented as the mean ± SE of three independent experiments performed in duplicate.

pathway. Preincubation of neutrophils with BAPTA-AM, an intracellular calcium chelator, did not affect neutrophil chemotaxis by HRYLPM or HEYLPM, which suggests that $[Ca^{2+}]_i$ increase is not essentially required for neutrophil chemotaxis by these peptides.

Effect of HFYLPM analogues on [^{125}I] WKYMVm binding to FPRL1

The finding that FPRL1 stimulation by HRYLPM caused $[Ca^{2+}]_i$ increase and ERK activation, resulting in ROS generation and chemotactic migration by human neutrophils, and that FPRL1 stimulation by HEYLPM only caused ERK activation but not a $[Ca^{2+}]_i$ increase, resulting in chemotaxis but not ROS generation, led us to check whether HRYLPM and HEYLPM share the same binding site on FPRL1. Thus, we incubated FPRL1-expressing RBL-2H3 cells with [^{125}I] WKYMVm in the absence and in the presence of increasing amounts of unlabeled WKYMVm, HRYLPM, or HEYLPM. As shown in Fig. 8, unlabeled WKYMVm competed with [^{125}I] WKYMVm binding in a concentration-dependent manner, and HRYLPM, also competed with WKYMVm binding in a concentration dependent manner (Fig. 8). However, HEYLPM did not affect [^{125}I] WKYMVm binding to FPRL1 (Fig. 8). These results indicate that HRYLPM, but not HEYLPM, interacts with the same FPRL1 binding site as WKYMVm.

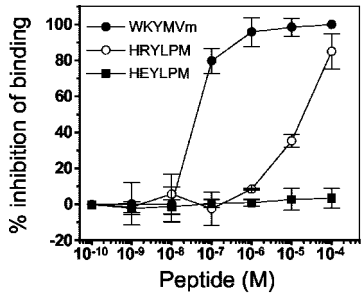

FIGURE 8. Replacement of ^{125}I-labeled WKYMVm binding to FPRL1 by HRYLPM but not by HEYLPM. ^{125}I-labeled WKYMVm was incubated for 3 h at 4°C with FPRL1-expressing RBL-2H3 cells in the presence of various concentrations of unlabeled WKYMVm, HRYLPM, or HEYLPM. The quantity of bound ^{125}I-labeled WKYMVm was determined using a gamma-ray counter. Results are presented as the mean ± SE of three independent experiments.

Discussion

In the present study, we demonstrate for the first time that an important chemoattractant receptor FPRL1 is modulated by distinct peptide ligands, and that this modulation leads to differential cellular signaling and different functional consequences in human neutrophils. As shown in Figs. 6 and 7, although HRYLPM stimulated superoxide generation and chemotaxis, HEYLPM stimulated chemotaxis only and not superoxide generation in human neutrophils.

To study the relation between the structure and function of FPRL1 agonists, we generated various HFYLPM analogues as listed in Table I. Some of the HFYLPM analogues, including WFYLPM and YFYLPM, showed high affinity for FPRL1 than the parent peptide (Table I). In our original screening of hexapeptide libraries, we used human peripheral blood monocytes (8). Because human monocytes express various cell surface receptors including FPR, FPRL1, and FPRL2, superoxide generation by stimulation of the cells with peptide libraries may be induced from the activation of multiple receptors. The mixed pool of cell surface receptors in human monocytes may cause different potency of the parent peptide, so we could not find those more potent derivatives of the parent peptide in the original screening. In this study we focused on one receptor (FPRL1) and examined the effects of HFYLPM and its analogues on stably FPRL1-transfected RBL-2H3 cells. Because vector-transfected RBL-2H3 cells do not respond to HFYLPM and its analogues, all the cellular responses are mediated by the one receptor, FPRL1. This approach allows us to test the derivatives of the parent peptide.

We have previously reported on the potent synthetic FPRL1 agonist WKYMVm (9, 21). When comparing the new peptides listed in Table I with WKYMVm, we found that tyrosine in third position, proline in fifth position, and methionine in final position are essential for the proper activation FPRL1 resulting in $[Ca^{2+}]_i$ increase. In a previous report, we also demonstrated that amino acid in first position determines the receptor-specificity of peptides (8). For activation of FPRL1, aromatic residues such as tryptophan and tyrosine in first position are important (Table I). The results correlate well with our previous report that WKYMVm is the most potent agonist for FPRL1 (9, 21).

Previously, many have hypothesized upon the role of ROS, such as the superoxide anion, in inflammatory tissue injury (22, 23). Administrations of the antioxidants superoxide dismutase or catalase were found to effectively attenuate tissue injury and inflammation in experimental models of ischemia and reperfusion, arthritis, and immune complex-induced pulmonary injury (24).

Because the major sources of ROS under these inflammatory conditions are the several types of tissue-infiltrating phagocytic cells, i.e., neutrophils, monocytes, and macrophages, agents that reduce the production of ROS in phagocytic cells are urgently required. In addition to antioxidants, molecules that selectively activate phagocytic cells without evoking ROS production should be considered as valuable immunomodulating agents. The FPR family, which includes FPR and FPRL1, is one of the most important classes of cell surface receptors to regulate neutrophil and other phagocytes functioning (1–3). In our study, we developed a synthetic peptide that selectively stimulates immune responses, such as chemotactic migration via FPRL1 without inducing ROS generation in human neutrophils. Bearing in mind that ROS is a major cause of tissue damage, HEYLPM could be used as an important agonist stimulator of neutrophils without inflicting tissue damage.

G protein-coupled receptor (GPCR)-induced intracellular signaling is initiated by interaction between an extracellular agonist and its specific cell surface receptor. Several previous reports have demonstrated that quite distinct intracellular signals can be induced by distinct GPCR conformations (25, 26). Because direct binding of a ligand to its specific receptor causes a conformational change in the receptor, it is reasonable to assume that HRYLPM and HEYLPM may bind to different binding sites of FPRL1. As shown in Fig. 8, the incubation of excess of unlabeled HRYLPM competed with [^{125}I] WKYMVm for binding to FPRL1. However, HEYLPM did not compete in this manner. This result suggests that, although both HRYLPM and HEYLPM interact with FPRL1 as vector-transfected RBL-2H3 cells were unresponsive to these peptides, they bind to different sites on FPRL1. Comparing the amino acid sequences of the two peptides with WKYMVm, it is evident that HEYLPM differs from the other two in terms of the second amino acid. WKYMVm and HRYLPM have a positively charged amino acid (Lys or Arg, respectively) in the second position, whereas HEYLPM has a negatively charged Glu in this position. Based on our results, we suggest that a positively charged amino acid (Lys or Arg) is critically involved in the binding of the peptide ligand to its specific receptor, FPRL1.

Previously, He et al. (10) reported that two different ligands, lipoxin A_4 (LXA_4) and SAA, differentially stimulated FPRL1 in human neutrophils. Although SAA stimulated both of $[Ca^{2+}]_i$ increase and ERK activation, LXA_4 only stimulated $[Ca^{2+}]_i$ increase without affecting ERK activation in human neutrophils (10). In terms of the molecular mechanism involved in the activation of FPRL1 by LXA_4, Chiang and colleagues (27) demonstrated the different structural requirements for LXA_4 interaction with FPRL1, as compared with a peptide ligand such as MMK-1. In this study, we demonstrated that FPRL1 could be differentially activated by two different peptide ligands, HRYLPM and HEYLPM. Taken together, our results indicate that FPRL1 can be activated in at least three different ways: 1) FPRL1 can be activated fully by SAA or HRYLPM, resulting in a $[Ca^{2+}]_i$ increase and PI3K-mediated ERK activation, 2) FPRL1 can be partially activated by LXA4, resulting in a $[Ca^{2+}]_i$ increase without PI3K-mediated ERK activation, and 3) FPRL1 can also be activated partially by HEYLPM, resulting in PI3K-mediated ERK activation without affecting a $[Ca^{2+}]_i$ increase.

Our findings are potentially important for the development of selective immunomodulators. For example, a molecule that specifically activates signaling events, such as cytosolic calcium or ERK, may selectively stimulate chemotaxis but not superoxide generation or vice versa. The proof of this concept by the two peptides tested in this study will not only help in the future development of synthetic ligands but hopefully will facilitate the identification of natural ligands that can similarly activate the activities of FPRL1.

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