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Novel chemoattractant peptides for human leukocytes

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Abstract

Phospholipase A_2 plays a key role in phagocytic cell functions. By screening a synthetic hexapeptide combinatorial library, we identified 24 novel peptides based on their ability to stimulate arachidonic acid release associated with cytosolic phospholipase A_2 activity in differentiated HL60 cells. The identified peptides, that contain the consensus sequence (K/R/M)KYY(P/V/Y)M, also induce intracellular calcium release in a pertussis toxin-sensitive manner showing specific action on phagocytic leukocytes, but not on other cells. Functionally, the peptides stimulate superoxide generation and chemotactic migration in human neutrophils and monocytes. Four of the tested active peptides were ligands for formyl peptide receptor like 1. Among these, two peptides with the consensus sequence (R/M)KYYYM can induce intracellular calcium release in undifferentiated HL60 cells that do not express formyl peptide receptor like 1, indicating usage of other receptor(s). A study of intracellular signaling in differentiated HL60 cells induced by the peptides has revealed that four of the novel peptides can induce extracellular signal-regulated protein kinase activation *via* shared and distinct signaling pathways, based on their dependence of phospatidylinositol-3-kinase, protein kinase C, and MEK. These peptides provide previously unavailable tools for study of differential signaling in leukocytes.

Keywords: Phospholipase A_2; Peptide; Formyl peptide receptor like 1; Chemotaxis; Superoxide; Phagocytes

1. Introduction

Neutrophils play a key role in innate immune responses. Diverse extracellular agonists modulate neutrophil functions by stimulating the activities of intracellular enzymes [1,2]. Several recent reports have demonstrated the critical involvement of phospholipases in neutrophil immune response [3–5]. Among these phospholipases, phospholipase A_2 (PLA_2) is an important enzyme that mediates important immune and inflammatory responses. PLA_2 hydrolyzes the fatty acyl group from the *sn*-2 position of phospholipid and concomitantly generates lysophospholipid [3,6]. Arachidonic acid (AA), the product of PLA_2 activity, has been implicated in the regulation of various cellular responses, including calcium influx and superoxide generation in phagocytic cells [7,8].

Mammalian cells contain several isozymes of PLA_2, namely, cytosolic PLA_2 ($cPLA_2$), calcium-independent PLA_2 ($iPLA_2$), and secretory PLA_2 [3,9]. Among the PLA_2 isozymes, $cPLA_2$ is believed to play an important role in agonist-induced AA release and in the regulation of lysophospholipid levels in cells [3]. Recently Dana *et al.* developed $cPLA_2$-deficient mice and confirmed the role of $cPLA_2$ in eicosanoid production [10]. Set against these backgrounds $cPLA_2$ is considered to be an important

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Abbreviations: PLA_2, phospholipase A_2; AA, arachidonic acid; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, calcium-independent PLA_2; PS-SPCL, positional scanning synthetic peptide combinatorial library; dHL60, differentiated HL60; FPRL1, formyl peptide receptor like 1; FPR, formyl peptide receptor; $[Ca^{2+}]_i$, intracellular calcium concentration; Fura-2/AM, fura-2 pentaacetoxymethylester; PTX, pertussis toxin; GF109203X, 2-[1-(3-dimethylamino propyl)-1*H*-indol-3-yl]-3-(1*H*-inol-3-yl)-maleimide; PD98059, 2′-amino-3′-methoxyflavone; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; ERK, extracellular signal-regulated protein kinase.

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pharmacological target for several physiological responses. With this role of PLA_2 in mind, particularly with respect to neutrophil functions, we sought to identify new ligands that modulate PLA_2 activity and to characterize their action mechanisms.

Several recent studies have reported the use of combinatorial peptide libraries to identify sequences involved in various biological responses [11–13]. A powerful yet simple method for identifying peptide sequences in certain biological reactions was developed by Dooley and Houghten [14]. This method, which uses a positional scanning synthetic peptide combinatorial library (PS-SPCL), has been used for various purposes, including the identification of human immunodeficiency virus protease inhibitors, interleukin-8-specific antagonists, the inhibitor for the nuclear factor of activated T cells, the ligands of opioid receptors, and novel peptides responsible for modulating leukocyte functions [15–19].

In the present study, we adopted the PS-SPCL method to identify the peptides that are responsible for AA release in neutrophil-like, differentiated HL60 (dHL60) cells. We found 24 different peptides that could stimulate AA release in dHL60 cells, and found that these peptides act as chemoattractants for human phagocytes. In addition, we found that several peptides bound to formyl peptide receptor like 1 (FPRL1). Some of the peptides may also bind to other receptor(s) expressed in HL60 cells. In addition, these peptides were found to be capable of stimulating shared and distinct intracellular signaling pathways.

2. Materials and methods

2.1. Reagents

Fmoc amino acids were obtained from Millipore, Rapidamide resin from Dupont, peripheral blood mononuclear cell (PBMC) separation medium (Histopaque-1077), cytochrome *c*, and *N*-formyl-methionyl-leucyl-phenylalanine (fMLF) from Sigma, fura-2 pentaacetoxymethylester (fura-2/AM) from Molecular Probes, RPMI 1640 from Invitrogen, dialyzed fetal bovine serum and supplemented bovine serum from Hyclone Laboratories Inc., pertussis toxin (PTX), GF109203X (2-[1-(3-dimethylamino propyl)-1*H*-indol-3-yl]-3-(1*H*-inol-3-yl)-maleimide), and PD98059 (2′-amino-3′-methoxyflavone) from Calbiochem. LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one), MAFP (methyl arachidonylfluorophosphonate), $AACOCF_3$ (arachidonyltrifluoromethyl ketone), and BEL (bromoenol lactone) were purchased from BIOMOL Research Laboratories, Inc.

2.2. Cell culture and HL60 cell differentiation

U937 (human histiocytic lymphoma cells), HL60 (human promyelocytic leukemia cells), Raw 264.7 (mouse macrophage), Jurkat (human acute T cell leukemia), PC12 (rat adrenal pheochromocytoma cells), 3Y1 (rat embryonic fibroblasts), 3T3L1 (preadipocytes), and NCI-H292 (human mucoepidermoid pulmonary carcinoma cells) were obtained from the American Type Culture Collection and maintained as recommended. FPR- or FPRL1-expressing RBL-2H3 cells were cultured as described previously [20]. Cells were maintained at about 1×10^6 cells/mL under standard incubator conditions (humidified atmosphere, 95% air, 5% CO_2, at 37°). HL60 cells were induced to differentiate into the granulocyte phenotype by adding DMSO (final concentration 1.25%, v/v) for 4 days to the culture medium, as described previously [21].

2.3. Isolation of leukocytes

Peripheral blood leukocyte concentrates were donated by the Ulsan Red Cross Blood Center (Ulsan, Korea). PBMCs were separated on a Histopaque-1077 gradient. After two washings with HBSS without Ca^{2+} and Mg^{2+}, the PBMCs were suspended in 10% FBS containing RPMI and incubated for 60 min at 37° to let the monocytes attach to the culture dish. Cells were washed five times with warmed RPMI medium to remove lymphocytes, and then the attached monocytes were collected, as described previously [22]. Human neutrophils were isolated according to standard procedures, using dextran sedimentation, hypotonic lysis of erythrocytes and using a medium lymphocyte separation gradient, as described previously [23]. Isolated human leukocytes were then used promptly.

2.4. Preparation of peptide libraries, and the synthesis and analysis of peptides

The hexapeptide libraries were prepared in the Peptide Library Support Facility of Pohang University of Science and Technology, as described previously [19]. Finally, 114 peptide pools (Cys was excluded from the library constructions) were individually dissolved in water to a final concentration of 27 nM per peptide. The peptides were synthesized by the solid-phase method described previously [19]. Briefly, peptides were synthesized on a Rapidamide support resin and assembled following the standard Fmoc/*t*-butyl strategy on an acid-labile linker. The composition of peptides was confirmed by amino acid analysis, as described previously [19].

2.5. Initial screening of the PS-SPCLs and the measurement of AA release

For the initial screening of the PS-SPCLs, we measured the AA release stimulating activity of each peptide pool. Cultured dHL60 cells (10^7 cells/mL) were pre-labeled with 0.5 μCi/mL of [^{3}H]AA in RPMI 1640 medium containing 10% FBS for 90 min at 37° in a humidified incubator supplied with 95% air and 5% CO_2, as described

previously [24]. The labeled cells were then washed twice with serum-free RPMI 1640 and incubated in RPMI 1640 medium containing 0.1% fatty acid-free BSA for 15 min at 37°. After discarding the medium, the cells were stimulated with various concentrations of peptide for the indicated times. Radioactivity in the medium and of collected cells was determined with a liquid scintillation counter. When investigating the effects of inhibitors, cells were preincubated with the indicated concentrations of each inhibitor or vehicle for 15 min prior to stimulation.

2.6. Measurement of $[Ca^{2+}]_i$

The level of $[Ca^{2+}]_i$ was determined using Grynkiewicz's method using fura-2/AM [25]. Briefly, prepared cells were incubated with 3 μM of fura-2/AM at 37° for 50 min in serum-free RPMI 1640 medium under continuous stirring. For DNP-HSA stimulation, FPR- or FPRL1-expressing RBL-2H3 cells were sensitized with 1 μg/mL mouse DNP-specific IgE overnight prior to fura-2 loading [26]. 2×10^6 cells were aliquoted for each assay in Ca^{2+} free Locke's solution (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). Fluorescence changes were measured at the dual excitation wavelength of 340 and 380 nm, and the calculated fluorescence ratio was translated into $[Ca^{2+}]_i$.

2.7. Measurement of superoxide generation

We determined superoxide anion generation by measuring cytochrome *c* reduction using a microtiter 96-well plate ELISA reader (Bio-Tek Instruments, EL312e) as described previously [26]. Human neutrophils (1×10^6 cells/100 μL of RPMI 1640 medium per well of a 96-well plate) were preincubated with 50 μM of cytochrome *c* at 37° for 1 min and then incubated with the indicated peptide concentrations. Superoxide generation was determined from change in light absorption at 550 nm over 5 min at 1 min intervals.

2.8. Chemotaxis assay

Chemotaxis assays were performed using multiwell chambers (Neuroprobe Inc.) [27]. Briefly, prepared human monocytes were suspended in RPMI at a concentration of 1×10^6 cells/mL, and 25 μL of the suspension was then placed onto the upper well of a chamber separated by a 5-μm polyhydrocarbon filter (3 μm pores size not polyvinylpyrrolidone coated, as is needed for neutrophils) from peptides or fMLF in the lower well. After incubation for 2 hr (90 min for neutrophils) at 37°, non-migrated cells were removed by scraping, and cells that migrated across the filter were dehydrated, fixed, and stained with hematoxylin (Sigma). Stained cells were counted in five randomly chosen high power fields (HPF) (400×) [27].

3. Results

3.1. Identification of peptides that stimulate AA release in dHL60 cells

We screened 114 peptide pools (around 47 million different peptides) from hexapeptide PS-SPCLs to identify those peptides that stimulate AA release in dHL60 cells. Figure 1 shows the results of the initial screening. Hexapeptides with a given amino acid in different positions induced different levels of AA release-stimulating activity. The most active peptide position combinations are: Lys (K), Met (M), or Arg (R) in the first position, Lys (K) in the second, His (H), Lys (K), or Tyr (Y) in the third, His (H), Lys (K), or Tyr (Y) in the fourth, Lys (K), Pro (P), Arg (R), Val (V), or Tyr (Y) in the fifth, and Met (M) in the sixth position.

Based on the results of the first round screening of the peptide libraries, we generated, by reiterative synthesis, peptide pools containing, 15 individual hexapeptides (H, K, or Y for the 4th position mixture; K, P, R, V, or Y for the 5th position mixture) or 9 individual hexapeptides (K, M, or R, for the 1st position mixture; H, K, or Y for the 3rd position mixture). We then tested the effectiveness of these peptide pools for AA release-stimulating activity in dHL60 cells using the same methods as used in the initial screening (Fig. 2A and B). After this second round screening, we found that KKHXXX, KKYXXX, RKYXXX, MKYXXX, XXXHKM, XXXHVM, XXXYKM, XXXYPM, XXXYVM, or XXXYYM were most active (Fig. 2A and B). Finally, we synthesized the 24 different peptides listed in Table 1 and measured their effects on AA release in dHL60 cells. All of these 24 novel peptides stimulated AA release at a concentration of 10 μM (Table 1), and (K/R/M)KYY(P/V/Y)M (P10, P11, P12, P16, P17, P18, P22, P23, and P24), (R/M)KYHVM (P14, P20) and MKYYKM (P21) were the most effective at this concentration (Table 1).

3.2. Effect of isozyme-specific inhibitors of PLA_2 on the novel peptides-stimulated AA release

We chose four representative peptides (P14, RKYHVM, P18, RKYYYM, P21, MKYYKM, and P24, MKYYYM) for further analysis, based on the differential sequence of amino acids having distinct characteristics among peptides which showed higher AA release-stimulating activity. To address the question as to which isoform of PLA_2 is responsible for the peptide-induced AA release, we introduced several isoform-specific inhibitors of PLA_2. The four representative peptides were found to stimulate AA release at 1 μM in dHL60 cells (Fig. 3). Pretreatment of these cells with the $cPLA_2$-specific inhibitors, $AACOCF_3$ and MAFP blocked the induction of AA by the four of the novel peptides, P14, P18, P21, and P24 (Fig. 3). MAFP or $AACOCF_3$ at 10 μM almost completely prevented AA release induced by the four peptides, whereas another

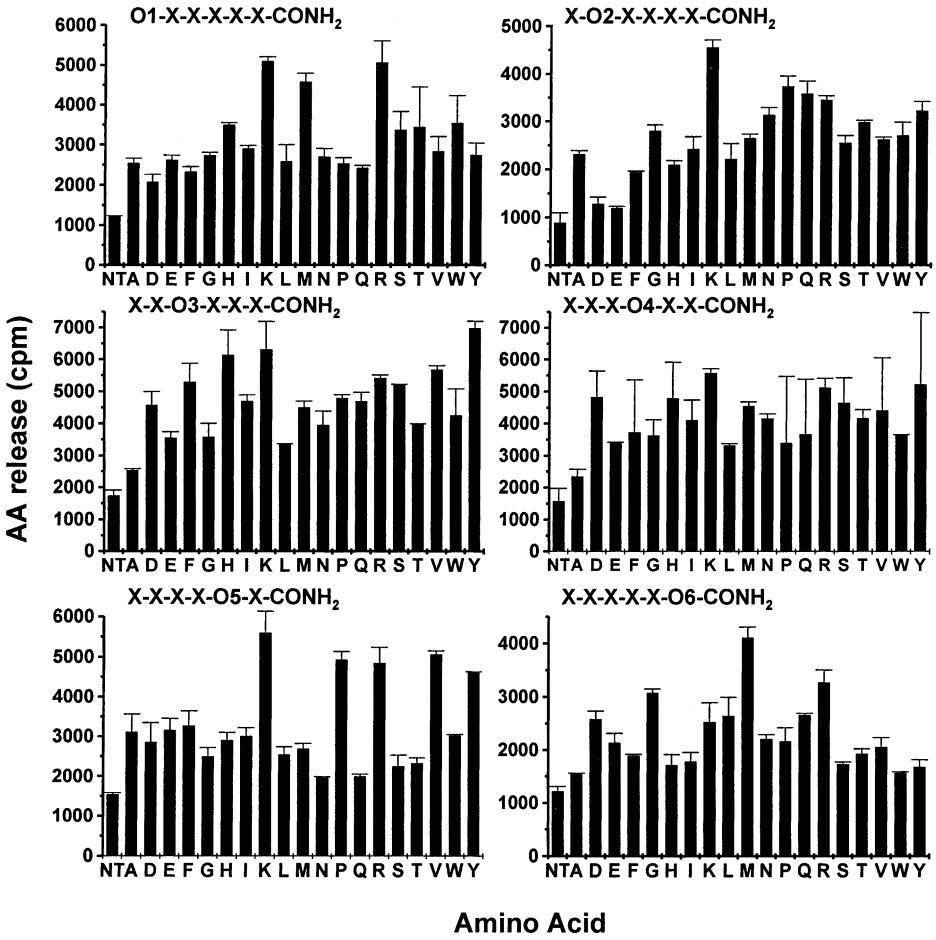

Fig. 1. Initial screening of the PS-SPCLs for peptides stimulating AA release in dHL60 cells. Each panel shows the results obtained with peptide pools containing known amino acids at each of the six positions of the hexapeptide. The six positions were individually defined (O1, O2, etc.) by one of the 19 L-amino acids. The remaining five positions consist of mixtures (X) of the 19 L-amino acids (except cysteine). [^{3}H]AA-labeled differentiated HL60 cells (1×10^6 cells/100 μL) were used for each assay. AA release was measured as described in Section 2. The results are of representative experiments, which were conducted in quadruplicate.

PLA_2 inhibitor, BEL, known to be specific for $iPLA_2$, did not interfere with peptide-induced AA release (Fig. 3). AA release stimulated by these peptides was also inhibited by chelating of intracellular Ca^{2+} with BAPTA/AM, which also supports the involvement of $cPLA_2$ activation (data not shown). These results, therefore, indicate that the four peptides evoke AA release by stimulating $cPLA_2$ but not $iPLA_2$ in dHL60 cells.

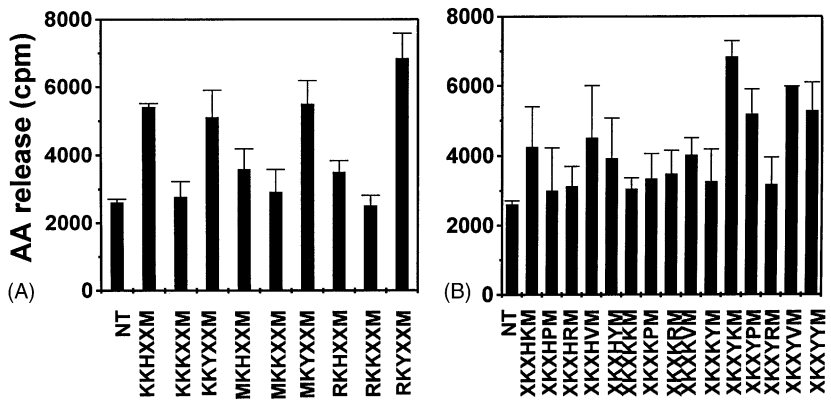

Fig. 2. Effect of several candidate peptides synthesized on the basis of the screening results of the PS-SPCLs with respect to AA release in dHL60 cells. [^{3}H]AA-labeled differentiated HL60 cells were stimulated with 1 μM concentrations of several peptides and AA release was measured. The results are presented as means ± SE of three independent experiments.

Table 1
Effect of novel peptides on AA release in differentiated HL60 cells[a]

Peptide	Sequence	Folds of increase (% of total)	Peptide	Sequence	Folds of increase (% of total)
P1	KKHHKM-NH_2	1.25 ± 0.168	P13	RKYHKM-NH_2	1.47 ± 0.220
P2	KKHHVM-NH_2	1.23 ± 0.153	P14	RKYHVM-NH_2	2.23 ± 0.403
P3	KKHYKM-NH_2	1.45 ± 0.306	P15	RKYYKM-NH_2	1.71 ± 0.214
P4	KKHYPM-NH_2	1.41 ± 0.247	P16	RKYYPM-NH_2	2.57 ± 0.450
P5	KKHYVM-NH_2	1.68 ± 0.390	P17	RKYYVM-NH_2	2.82 ± 0.210
P6	KKHYYM-NH_2	1.52 ± 0.296	P18	RKYYYM-NH_2	2.61 ± 0.295
P7	KKYHKM-NH_2	1.42 ± 0.226	P19	MKYHKM-NH_2	1.68 ± 0.221
P8	KKYHVM-NH_2	1.30 ± 0.170	P20	MKYHVM-NH_2	2.55 ± 0.271
P9	KKYYKM-NH_2	1.49 ± 0.268	P21	MKYYKM-NH_2	2.86 ± 0.426
P10	KKYYPM-NH_2	2.27 ± 0.199	P22	MKYYPM-NH_2	2.95 ± 0.668
P11	KKYYVM-NH_2	2.49 ± 0.023	P23	MKYYVM-NH_2	3.05 ± 0.401
P12	KKYYYM-NH_2	2.58 ± 0.168	P24	MKYYYM-NH_2	2.93 ± 0.323

[a] AA release was measured in [^{3}H]AA-labeled cells stimulated with 10 μM of peptide.

3.3. Effect of the novel peptides on $[Ca^{2+}]_i$ rise in dHL60 cells

It is well known that intracellular calcium ($[Ca^{2+}]_i$) elevation is required for the activation of $cPLA_2$ [3]. The finding that the peptide-stimulated AA release is inhibited by the $cPLA_2$ inhibitor, MAFP, led us to investigate whether the novel peptides affect $[Ca^{2+}]_i$ increase. When dHL60 cells were stimulated with 1 μM of the individual peptide, all were found to increase $[Ca^{2+}]_i$ activity, except P1 and P7 (data not shown). Most of these peptides caused an increase in $[Ca^{2+}]_i$ in dHL60 cells in a concentration-dependent manner. We calculated the EC_{50} values of the novel peptides based on calcium increase in differentiated HL60 cells (Table 2). P16, P17, P18, P22, P23, and P24 showed higher potency than others (Tables 2 and 3). A number of reports have demonstrated that many extracellular ligands modulate cellular activities *via* PTX-sensitive G-protein(s) in human leukocytic cells [28,29]. To investigate the possible involvement of PTX-sensitive G-proteins in the increase in $[Ca^{2+}]_i$ by the novel peptides, dHL60 cells were treated with PTX (150 ng/mL) for 20 hr prior to the addition of each of the 24 novel peptides. As shown in Fig. 4, induction of $[Ca^{2+}]_i$ rise by each active

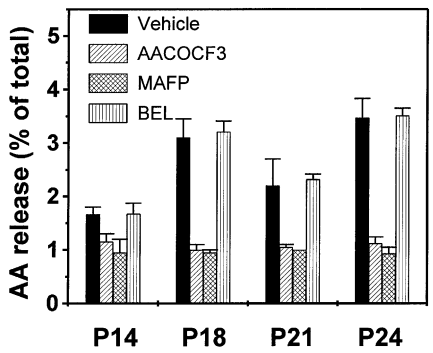

Fig. 3. Peptide-induced AA release was derived from $cPLA_2$ activation. The dHL60 cells were suspended in HBSS containing 0.1% fatty acid-free BSA, incubated for 15 min in the presence or absence of 10 μM of MAFP ($cPLA_2$ inhibitor), $AACOCF_3$ ($cPLA_2$ inhibitor), and BEL ($iPLA_2$ inhibitor) at 37°, and stimulated for 30 min with 1 μM of each peptide or vehicle as control. Release of [^{3}H]AA into the extracellular medium was determined with a liquid scintillation counter. Results are expressed as percentages of total cellular radioactivity, mean values ±SE (N = 6) are shown.

Table 2
Effect of novel peptides on intracellular calcium increase in differentiated HL60 cells

Peptide	EC_{50} (μM)	Peptide	EC_{50} (μM)
P1	Inactive	P13	>10
P2	Inactive	P14	3.0 ± 0.46
P3	>10	P15	1.3 ± 0.15
P4	>10	P16	0.09 ± 0.012
P5	8.9 ± 0.59	P17	0.07 ± 0.0058
P6	5.6 ± 0.48	P18	0.05 ± 0.0095
P7	Inactive	P19	>10
P8	>10	P20	0.1 ± 0.037
P9	3.2 ± 0.093	P21	0.08 ± 0.0032
P10	0.7 ± 0.12	P22	0.06 ± 0.0062
P11	0.1 ± 0.035	P23	0.05 ± 0.011
P12	0.07 ± 0.0057	P24	0.04 ± 0.0073

Table 3
Effect of peptides on the leukocyte activities

	Peptide			
	P14	P18	P21	P24
Activated PLA_2				
$cPLA_2$	++	+++	++	+++
$iPLA_2$	−	−	−	−
Chemoattractant for				
Monocytes	+	+++	+	+++
Neutrophils	+	+++	++	+++
Ca^{2+} increase in				
FPR/RBL-2H3	−	−	−	−
FPRL1/RBL-2H3	++	+++	++	+++
HL60	−	++	−	+++
ERK activation *via*				
PI3K	+++	−	+++	+
PKC	++	++	+	−
MEK	+++	−	+++	+++

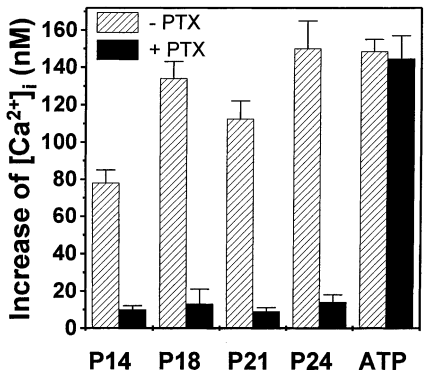

Fig. 4. Effect of PTX on peptide-induced $[Ca^{2+}]_i$ rise in dHL60 cells. dHL60 cells were incubated in the presence or absence of PTX (150 ng/mL) for 20 hr and the cells were loaded with fura-2. The fura-2-loaded dHL60 cells were stimulated with 1 μM of each peptide or 500 μM of ATP. The change in 340/380 nm was monitored. Results are representative of four independent experiments. Data are presented as means ± SE of four independent experiments.

peptide was almost completely inhibited by PTX. $[Ca^{2+}]_i$ increase induced by ATP, which does not rely on PTX-sensitive G proteins, was not inhibited by the toxin (Fig. 4). These results indicate that the novel peptides stimulate $[Ca^{2+}]_i$ release *via* a receptor coupled to a PTX-sensitive G-protein in dHL60 cells.

3.4. Cell type specificity of the novel peptides

Since the synthesized novel peptides stimulated neutrophil-like dHL60 cells, we examined their effects on neutrophils. Stimulation of neutrophils with one of the novel peptides, P24, resulted in $[Ca^{2+}]_i$ rise (Fig. 5). Monocytes and U937 cells were also activated by P24 (Fig. 5), but Raw 264.7 and Jurkat cells were not activated by this peptide (Fig. 5). Next, we examined the effects of P24 on $[Ca^{2+}]_i$ rise in several non-leukocytic cell lines. None of the cells surveyed, which include 3Y1, PC12, NCI-H292, and HUVEC, showed response to P24 in terms of $[Ca^{2+}]_i$ rise (Fig. 5 and data not shown). This result suggests that the peptide effects are neutrophil and monocyte-specific. The other active peptides showed similar results in terms of their leukocyte-specificities (data not shown).

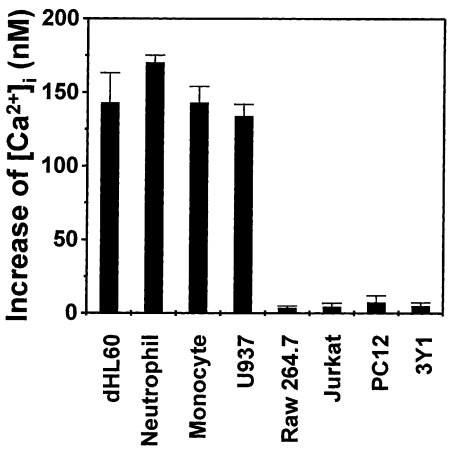

Fig. 5. Effect of P24 on $[Ca^{2+}]_i$ rise in cells of various origins. Each cell was loaded with fura-2 for 50 min. The cells were stimulated with 10 μM of P24 and $[Ca^{2+}]_i$ increase was monitored. Data are presented as means ± SE of three independent experiments.

3.5. Effect of the novel peptides on superoxide generation

Superoxide generation is one of the important steps in the host's defense mechanism by phagocytes [30]. We tested the effect of the four representative peptides (P14, P17, P21, and P24) on superoxide generation in human neutrophils. These four peptides were found to stimulate superoxide generation in a concentration-dependent manner in human neutrophils (Fig. 6). The EC_{50} for superoxide generation were >10,000 nM, 4320 ± 370 nM, 183 ± 13 nM, and 87 ± 4 nM for P14, P18, P21, and P24, respectively. P24 was most potent in terms of stimulating superoxide generation in human neutrophils.

3.6. Chemotactic effect of novel peptides on leukocytes

Since the four novel peptides were found to stimulate superoxide generation and $[Ca^{2+}]_i$ increase in human phagocytic cells, we next checked whether the peptides exhibited chemotactic activity on human monocytes or neutrophils. The four active novel peptides induced migration of human neutrophils (Fig. 7A). The EC_{50} for neutrophil chemotaxis were >10,000 nM, 3045 ± 726 nM, 560 ± 72 nM, and 336 ± 23 nM for P14, P18, P21, and P24, respectively. The maximal cellular migration-inducing activity mediated by the novel peptides was more than 200% of that induced by 1 μM of fMLF (Fig. 7A). The four peptides (P14, P17, P21, and P24) also induced cellular chemotaxis in human monocytes (Fig. 7B). Moreover, the four peptides caused monocyte chemotaxis in a

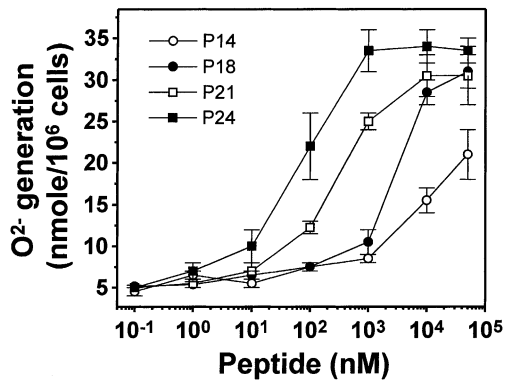

Fig. 6. Effect of peptides on superoxide generation in human neutrophils. Isolated human neutrophils (2×10^6 cells/mL per assay) were preincubated for 1 min at 37° with 50 μM of cytochrome *c* before being stimulated with various concentrations of peptides for 5 min. Cytochrome *c* reduction was monitored as a change in absorption at 550 nm, as described in Section 2. Superoxide anion generated is expressed as means ± SE of three independent experiments each performed in duplicate.

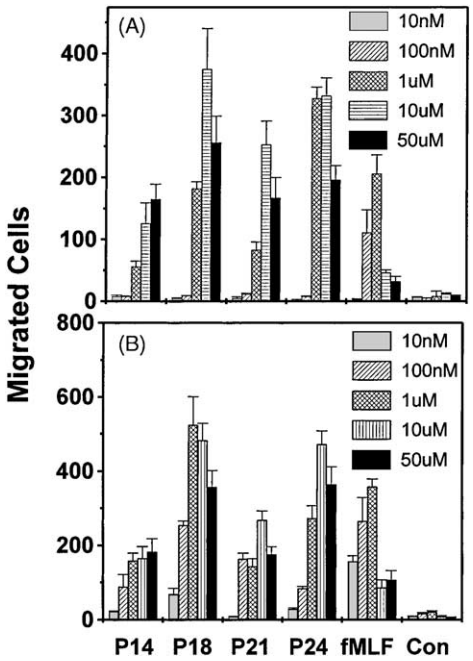

Fig. 7. Chemotactic effect of peptides. Assays were performed using a modified Boyden chamber assay, as described in Section 2. Isolated human neutrophils (A) or monocytes (B) (1×10^6 cells/mL in serum free RPMI) were added to the upper wells of a 96-well chemotaxis chamber and migration across a 3-μm pore size (5 μm for monocytes) polycarbonate membrane was assessed after 90 min (2 hr for monocytes) incubation at 37°. LFMYHP was used as an inactive control peptide (Con). The numbers of migrated cells were determined by counting them in a high power field (400×). Results are presented as means ± SE of three independent experiments each performed in duplicate.

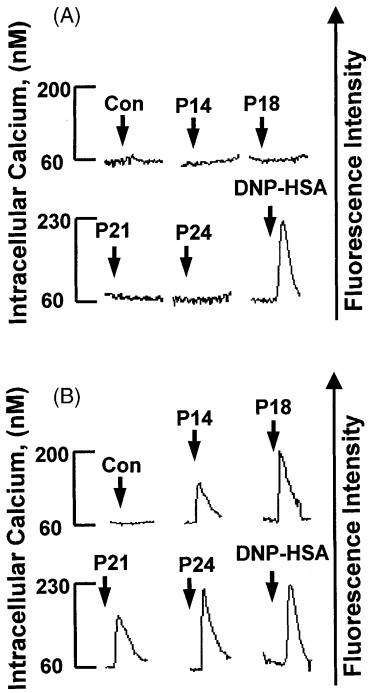

Fig. 8. Effect of peptides on $[Ca^{2+}]_i$ rise in FPR- or FPRL1-expressing RBL-2H3 cells. Fura-2 loaded FPR- (A), or FPRL1- (B) expressing RBL-2H3 cells were stimulated with 10 μM of each peptide or 10 μM of LFMYHP, an inactive control peptide (Con). FPR- or FPRL1-expressing RBL-2H3 cells were sensitized with DNP-specific IgE, loaded with fura-2, and activated with the antigen DNP-HSA (1 μg/mL). $[Ca^{2+}]_i$ increase was monitored and the traces shown are from a single experiment representative of at least three independent experiments.

concentration range of 0.01–10 μM (Fig. 7B). An inactive control peptide, LFMYHP, did not induce cellular chemotaxis in neutrophils or monocytes at concentrations up to 10 μM (Fig. 7A and B). In four experiments with independently prepared leukocytes, the four peptides showed similar cellular migration-inducing activity.

3.7. Receptor specificity of the novel peptides: effect on FPRL1

The novel peptide induced phagocyte activation was found to be very similar to that induced by several known peptide chemoattractants. Formyl peptide receptor (FPR), and FPRL1 are well-known chemoattractant receptors in neutrophils [31,32]. To examine whether the novel peptides bind to FPR or FPRL1 we investigated the effect of the peptides on $[Ca^{2+}]_i$ increase in FPR- or FPRL1-expressing RBL-2H3 cells. No peptide was found to affect $[Ca^{2+}]_i$ in FPR-expressing RBL-2H3 cells (Fig. 8A). However, several peptides including four peptides (P14, P18, P21, and P24) induced calcium increase in FPRL1-expressing RBL-2H3 cells (Fig. 8B and data not shown). An inactive control peptide (LFMYHP) was found not to be able to induce calcium increase in FPRL1 cells (Fig. 8). Among the active peptides, the potency of calcium increasing activities was found to be different for each peptide. These results indicate that the four peptides (P14, P18, P21, and P24) are ligands for FPRL1 but not for FPR.

3.8. Differentiation status specificity of the four peptides in HL60 cells

In Fig. 5, we showed that the novel peptides acted on leukocytic cells but not on non-leukocytic cells. Many extracellular ligands have been reported to have cellular differentiation status specificity [33,34]. We investigated whether the novel peptides showed such differentiation status specificity in myelocytes by checking the effect of these peptides on $[Ca^{2+}]_i$ increase in undifferentiated and differentiated HL60 cells. As shown in Table 2, the four peptides stimulated $[Ca^{2+}]_i$ increase in dHL60 cells. When undifferentiated HL60 cells were stimulated with the four novel peptides, $[Ca^{2+}]_i$ was found to be dramatically induced by P18 and P24 (Fig. 9). The other two peptides, P14 and P21, did not affect $[Ca^{2+}]_i$ increase in HL60 cells (Fig. 9). Unlike neutrophils or dHL60 cells, undifferentiated HL60 cells do not express FPR or FPRL1 on the cell surface [35]. We also confirmed that fMLF (a FPR-specific ligand) or lipoxin A4 (a FPRL1-specific ligand) did not affect $[Ca^{2+}]_i$ increase in HL60 cells, indicating that the undifferentiated HL60 cells do not express functional FPR or FPRL1. These results suggest that P18 and P24 may

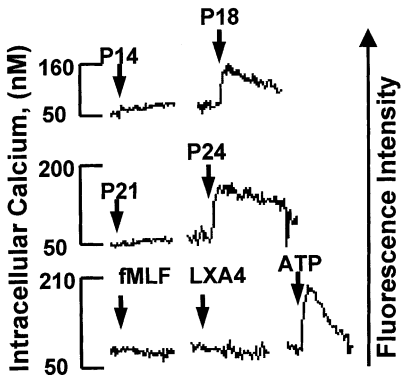

Fig. 9. Effect of peptides on $[Ca^{2+}]_i$ increase in HL60 cells. Fura-2 loaded HL60 cells were stimulated with 10 μM of each peptide, 1.4 μM of LXA4, or 500 μM of ATP. $[Ca^{2+}]_i$ increase was monitored and the traces shown are from a single experiment representative of at least three independent experiments.

activate receptors other than FPRL1 in undifferentiated HL60 cells.

3.9. Comparison of intracellular signaling by the four peptides

Extracellular signal-regulated protein kinase (ERK) is a well-known intracellular enzyme that mediates diverse cellular responses [36]. Many reports have demonstrated that chemoattractants stimulate ERK activity, and that this may result in several pivotal stages in the modulation of leukocytic cells [37,38]. In the present study, we found that stimulation of dHL60 cells with the four novel peptides (P14, P18, P21, and P24) caused a dramatic increase in the phosphorylation level of ERK (Fig. 10). Moreover, this peptides-induced ERK activation was time-dependent, with a maximal activity 5 min after stimulation (data not shown). To compare intracellular signaling involving these four peptides, dHL60 cells were pretreated either with LY294002 (50 μM), GF109203X (5 μM), or PD98059 (50 μM) or left untreated as a control. After incubation for the indicated periods (15 min for LY294002 and GF109203X, 60 min for PD98059), the cells were stimulated with 1 μM of each peptide for 5 min. As shown in Fig. 10, P14-induced ERK phosphorylation was blocked by LY294002, GF109203X, and PD98059, indicating that the peptide-induced ERK activation is phosphatidylinositol-3-kinase (PI3K), protein kinase C (PKC), or MEK-dependent. P18-induced ERK phosphorylation was completely blocked by GF109203X, partially blocked by PD98059, but not affected by LY294002 (Fig. 10). P21 also caused ERK phosphorylation in a PI3K- and MEK-dependent manner (Fig. 10), and P24-induced ERK phosphorylation was partially blocked by LY294002 but not by GF109203X (Fig. 10). These results suggest that the four peptides stimulate overlapping and non-overlapping intracellular signaling pathways possibly *via* different receptors activation, which result in the activation of ERK in dHL60 cells.

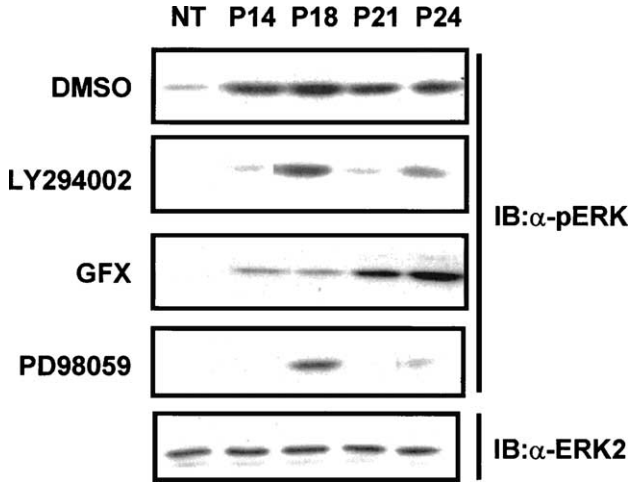

Fig. 10. Regulation of each peptide-stimulated ERK phosphorylation in dHL60 cells. dHL60 cells were preincubated with vehicle or 50 μM of LY294002, 5 μM of GFX, or 50 μM of PD98059 for 15 min (60 min for PD98059) prior to treatment with 1 μM of each peptide or vehicle alone for 5 min. Each sample (30 μg of protein) was subjected to 8% SDS–PAGE, and phosphorylated ERK was quantified by immunoblot analysis with anti-phospho-ERK antibody. Western blot analysis was also performed with anti-ERK2 antibody to confirm that the amounts of samples used were similar. The results shown are from a single experiment representative of at least three independent experiments.

4. Discussion

In this study, we screened hexapeptide combinatorial peptide libraries containing more than 47 million different peptide sequences, and identified 24 novel hexapeptides that could stimulate AA release in dHL60 cells. In terms of their physiological roles, the novel peptides were found to enhance superoxide generation and the chemotactic migration of phagocytic cells. Through experiments on the receptor specificity or the signaling specificity of the peptides, we found that the novel peptides may induce either overlapping or distinct intracellular signals *via* a common receptor, FPRL1, or *via* an unidentified receptor in leukocytic cells.

On investigating the receptor specificity of the peptides, we found that four novel peptides could stimulate $[Ca^{2+}]_i$ increase in FPRL1-expressing RBL-2H3 cells but not in FPR-expressing RBL-2H3 cells (Fig. 8). Of the four peptides, only two stimulated undifferentiated HL60 cells in Ca^{2+} mobilization assay (Fig. 9). Since undifferentiated HL60 cells do not express FPRL1, the target receptors for these two peptides (P18 and P24) could not be FPRL1. From experiments on the effects of PTX on peptide-induced $[Ca^{2+}]_i$ increase, we found that PTX pretreatment of dHL60 cells completely inhibited the novel peptide-induced calcium increase, however, PTX partially inhibited the calcium signaling stimulated by P18 or P24 in undifferentiated HL60 cells (data not shown). These results suggest that the receptors of novel peptides in dHL60 cells are coupled to PTX-sensitive G-proteins, and that the peptide receptors in undifferentiated HL60 cells might

be coupled to PTX-insensitive G-proteins. These results support our notion that the receptors of the novel peptides in undifferentiated HL60 cells are not the same as those in dHL60 cells.

Previously, various agonists for FPRL1 have been reported from endogenous and exogenous sources [39–44]. They include serum amyloid A (SAA), HIV-envelope domains (F peptide and V3 peptide), host-derived agonist ($A\beta_{42}$), and *Helicobacter pylori*-derived peptide, Hp (2–20) [39–44]. Because the novel peptides in this study were identified by screening artificially synthesized peptides, we looked for sequence similarities between the novel peptides and known proteins, including endogenous FPRL1 ligands by searching the SWISS-PROT and TrEMBL databases. We were unable to find a protein carrying the same sequence as any of the synthetic peptides. However, several viral proteins such as the major capsid protein of the pseudorabies virus contain the X(F/K)Y(L/M)(V/P)M sequence. Although there is no clear information on the relationship between our novel peptides and these viral proteins, it would be interesting to determine whether such viral proteins can bind to FPRL1 or its related cell surface receptors which can be occupied by our novel peptides. In terms of the novel peptide receptor in undifferentiated HL60 cells, recently, Dahlgren and co-workers suggested the involvement of an unknown receptor, i.e. not FPRL1 or FPRL2, on LXA4-induced ERK activation in undifferentiated HL60 cells [45]. They found that LXA4 stimulated undifferentiated HL60 cells causing ERK activation but not Ca^{2+} increase. Since LXA4 has been reported to bind to FPRL1, the unknown receptor may also be occupied by our novel peptides, for example, P24. Several recent reports have demonstrated that GPCRs, including FPRL1, can be differentially activated by distinct agonists [45–47]. It is also notable that stimulation of unknown receptor by our novel peptides but not by LXA4 will elicit Ca^{2+} increase.

Through study of intracellular signaling pathways with our novel peptides, we demonstrated that P14 induced ERK activation *via* PI3K and PKC, and that P18 induced ERK activation *via* PKC (Fig. 10). In terms of the role of MEK, the three peptides, but not P18, caused ERK activation in a MEK-dependent manner (Fig. 10). Figure 8 shows that the four novel peptides stimulated $[Ca^{2+}]_i$ increase in FPRL1-expressing RBL-2H3 cells. Since dHL60 cells also express FPRL1, the four peptides may bind to FPRL1 in dHL60 cells. However, the result that P18, but not the other peptides, induced ERK is PI3K- or MEK-independent suggests the involvement of another receptor in P18-mediated signaling. We found that P18 and P24, but not P14 or P21 stimulated $[Ca^{2+}]_i$ increase in undifferentiated HL60 cells (Fig. 9). Taken together it is reasonable to speculate that P14 and P21 bind to one receptor, FPRL1, but that P18 and P24 bind to at least two receptors, which include FPRL1 in leukocytic cells. The differential regulation of P18- and P24-induced ERK activation may be attributed to the different receptors involved in their signaling. Previously, we reported several chemoattractant peptides for human leukocytes and two other groups demonstrated that two of the synthetic peptides, WKYMVm and WKYMVM, are ligands for FPRL1 [19,23,48,49]. Since WKYMVM stimulated undifferentiated HL60 cells, resulting in inositol phosphates formation [19], it appears that WKYMVM also has another receptor in addition to FPRL1. At this point, it is not clear whether WKYMVM and the novel peptides described in this study share the same unknown receptor or not. On the cell signaling pathways involved in the activation of ERK, we have already demonstrated that WKYMVM stimulates ERK activation *via* PI3K- and MEK-dependent but not PKC-dependent mechanism [50]. We also observed that stimulation of differentiated HL60 cells with 1 μM of WKYMVM caused ERK activation *via* PI3K- and MEK-dependent but not PKC-dependent mechanism (data not shown). In this study, although signaling pathway for p21- and p24-stimulated ERK activation overlaps with that of WKYMVM-induced pathway, p14 and p18 stimulated ERK activation with a unique mechanism (Fig. 10). The result suggests that different receptors may be involved in the differential regulation of ERK activation by distinct peptide ligands.

Although chemoattractants are important immune-modulators and various chemoattractants (including chemokines) have been identified, only a few short peptides acting on human leukocytes have been identified. fMLF is a well-known short chemotactic peptide that has been widely used for probing the phagocyte signaling pathways [51,52]. Because our novel peptides stimulate human phagocytic cells, such as neutrophils and monocytes, these peptides can also be used as tools for the study of phagocytic cell functions. In the area of undifferentiated myeloma cell activation and signaling, no report has yet been published on small peptides acting on these cells. Because two of our novel peptides stimulate undifferentiated HL60 cells, they may be useful tools for the characterization of undifferentiated myeloma cell activation.

Acknowledgments

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