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Differential Activation of Formyl Peptide Receptor Signaling by Peptide Ligands

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ABSTRACT

Formyl peptide receptor (FPR) and formyl peptide receptor like 1 (FPRL1) play important roles in inflammation and immunity. Stimulation of FPR and FPRL1 initiates a cascade of signaling events, leading to activation of various phagocyte responses, including chemotaxis, superoxide generation, and exocytosis. Trp-Lys-Tyr-Met-Val-D-Met-NH_2 (WKYMVm) is a synthetic peptide that binds to and activates FPR and FPRL1. To develop agonists that selectively activate phagocyte functions and therefore protect host from unwanted tissue damage, we generated various WKYMVm analogs and examined their effects on cellular responses in FPR- or FPRL1-expressing RBL-2H3 cells. Analogs with substitution at the third position such as WKGMVm, WKRMVm, as well as analogs with substitution at the sixth D-Met, selectively altered calcium mobilization in cells expressing FPRL1 but not in cells expressing FPR. Whereas binding of WKYMVm to FPR activates a broad spectrum of cellular signaling events, including phospholipase C-mediated intracellular calcium concentration ($[Ca^{2+}]_i$) mobilization and activation of extracellular signal-regulated kinase (ERK) and Akt, WKGMVm and WKRMVm could only activate ERK and Akt but did not induce $[Ca^{2+}]_i$ mobilization. With respect to phagocyte functions, WKYMVm could induce both chemotaxis and exocytosis, but the two analogs WKGMVm and WKRMVm could only induce chemotaxis but not exocytosis. This study demonstrates that a major phagocyte chemoattractant receptor FPR may be activated differentially by distinct peptide ligands. Our results suggest that WKGMVm and WKRMVm may be useful model for further development of pharmacological agents that selectively activate FPR-mediated functions.

Formyl peptide receptor (FPR) and formyl peptide receptor like 1 (FPRL1) are chemoattractant receptors expressed in phagocytic cells, such as neutrophils and monocytes, and play an important role in host defense against pathogen infection. The receptors are known to couple to pertussis toxin (PTX)-sensitive Gi proteins (Le et al., 2000, 2001a). Activation of FPR and FPRL1 induces the dissociation of G$\beta\gamma$ subunits from Gαi subunit, which then mediate the activation of phospholipase Cβ (PLCβ) and phosphoinositide 3-kinase (PI3K) (Le et al., 2000, 2001a). The activation of these effector molecules induces complicated downstream signaling events, which leads to diverse cellular responses, including chemotactic migration, degranulation, and superoxide generation.

Most chemoattractants activate multiple signaling pathways that evoke complex immune responses. Many of the induced immune responses are essential for the proper functioning of host cells and for eliminating invading pathogens. However, some immune responses are not beneficial under certain conditions. For the development of therapeutic agents, it is important to enhance the beneficial effects and reduce the adverse effects. To attain this goal, many research groups have attempted to develop selective immune response activator and selective receptor antagonists (Zagorski and Wahl, 1997; White et al., 1998).

A larger number of FPR and FPRL1 agonists have been identified either from endogenous sources or by artificial synthesis (Le et al., 2000, 2001a). They include bacterial-derived peptides (fMLF), HIV-envelope protein fragments (T20, T21, F, and V3), and host-derived agonists (Annexin I and Aβ_{42}) (Prossnitz and Ye, 1997; Su et al., 1999; Walther et al., 2000; Le et al., 2001b). We have previously characterized a synthetic peptide ligand, Trp-Lys-Tyr-Met-Val-D-Met-NH_2 (WKYMVm)

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ABBREVIATIONS: FPR, formyl peptide receptor; FPRL1, formyl peptide receptor like 1; PTX, pertussis toxin; PLC, phospholipase C; PI3K, phosphoinositide 3-kinase; fMLF, formyl-methionyl-leucyl-phenylalanine; WKYMVm, Trp-Lys-Tyr-Met-Val-D-Met-NH_2; AM, acetoxymethyl ester; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; PD98059, 2′-amino-3′-methoxyflavone; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; ERK, extracellular signal-regulated kinase; BSA, bovine serum albumin; RPMI, Roswell Park Memorial Institute; MEK, mitogen-activated protein kinase kinase.

that activates leukocytes such as monocytes and neutrophils (Baek et al., 1996; Seo et al., 1997; Bae et al., 1999a,b). In addition, Le et al. (1999) demonstrated that WKYMVm binds to FPR and FPRL1. Because WKYMVm is a short peptide and has high affinity for FPR and FPRL1, it may be used as a useful tool to study FPR- and FPRL1-mediated signaling.

In this study, we generated various analogs of WKYMVm and examined their effects on FPR- and FPRL1-mediated signaling pathways and the related cellular activities in transfected RBL-2H3 cells. We found that FPR can be activated differentially by these peptide ligands.

Materials and Methods

Materials. Fmoc amino acids were obtained from Millipore (Bedford, MA). Rapidamide resin was purchased from PerkinElmer Life Sciences (Boston, MA). fMLF and *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide were 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 medium was obtained from Invitrogen (Carlsbad, CA). Dialyzed fetal bovine serum and supplemented bovine serum were purchased from Hyclone Laboratories (Logan, UT). PD98059 was purchased from Calbiochem (San Diego, CA). LY294002 was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Anti-phospho-ERK antibodies and anti-phospho-Akt antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA).

Peptide Synthesis. Peptides were synthesized by the solid-phase method, described previously (Baek et al., 1996; Seo et al., 1997). Briefly, peptides were synthesized on a Rapidamide support resin and assembled using a standard Fmoc/*t*-butyl strategy on an acid-labile linker. The compositions of the peptides were confirmed by amino acid analysis, as described previously (Baek et al., 1996). Earlier, we reported that the replacement of each amino acid with glycine in the mother peptide of WKYMVm, MKYMPM, caused a loss of phosphatidylinositol hydrolysis-stimulating activity (Seo et al., 1997). In particular, the substitution of Lys^2, Tyr^3, Pro^5, or Met^6 with Gly- in WKYMVm resulted in a dramatic loss of activity (Seo et al., 1997). In this study, we synthesized WKYMVm analogs and replaced Lys^2, Tyr^3, Val^5, or D-Met^6 with other amino acids. Specifically, Lys^2 was replaced with Gly, Arg, Glu, His, or Asp; Tyr^3 was replaced with Gly, His, Glu, Trp, Arg, Asp, or Phe; Val^5 was substituted with Gly, Phe, Trp, or Tyr; and D-Met^6 was substituted with Gly, Glu, Val, Arg, or Trp. We also synthesized a C-terminal D-Met-deleted peptide (WKYMV-NH_2), N-terminal-deleted peptides (KYMVm-NH_2, YMVm-NH_2, and MVm-NH_2), and a peptide with both terminals deleted (KYMV-NH_2).

Cell Culture. RBL-2H3 cells transfected with the human FPR cDNA, the FPRL1 cDNA or vector were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum and 200 μg/ml G418, as described previously (He et al., 2000).

Ca^{2+} Measurement. Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined by the method of Grynkiewicz et al. (1985) using fura-2/AM. Briefly, prepared cells were incubated with 3 μM fura-2/AM at 37°C for 50 min in serum-free RPMI 1640 medium with continuous stirring. Approximately 2×10^6 cells were aliquoted for each assay into 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 was measured at 500 nm at excitation wavelengths of 340 and 380 nm. The fluorescence ratio was then used for calculation of $[Ca^{2+}]_i$.

Ligand Binding Assay. Ligand binding assay was performed as described previously (Hu et al., 2001). Radioiodinated WKYMVm (^{125}I-labeled) was a gift from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Briefly, RBL-2H3 cells or FPR-expressing RBL-2H3 cells were seeded at 1×10^5 cells/well into a 24-well plate and cultured overnight. After treating the cells with blocking buffer (33 mM HEPES, pH 7.5, 0.1% BSA in RPMI 1640 medium) for 2 h, 50 pM labeled WKYMVm was added to the cells in binding buffer (phosphate-buffered saline containing 0.1% BSA) in the absence or presence of unlabeled peptides. The mix was then incubated for 3 h at 4°C with continuous agitation. The samples were then washed 5 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 incubation for 20 min at room temperature, the lysates were collected and the associated radioactivity was determined using a gamma-ray counter (Hu et al., 2001).

Stimulation of Cells with Peptides. Cultured RBL-2H3 cells or FPR-expressing RBL-2H3 cells (2×10^6) were stimulated with the indicated concentrations of WKYMVm or its analogs for the predetermined lengths of time. After stimulation, the cells were washed with serum-free RPMI 1640 medium 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 phenylmethylsulfonyl fluoride). Detergent-insoluble materials were pelleted by centrifugation (12,000*g*, 15 min, at 4°C), and the soluble fraction (supernatant) was removed and stored at either −80°C or used immediately. Protein concentrations in the lysates were determined using Bradford protein assay reagent.

Electrophoresis and Immunoblot Analysis. Protein samples were subject to electrophoresis using 10% SDS-polyacrylamide gel and the buffer system described by Laemmli (King and Laemmli, 1971). After the electrophoresis, the protein samples were blotted onto nitrocellulose membrane, which was blocked by incubating with Tris-buffered saline, 0.05% Tween 20 containing 5% nonfat dried milk. The membranes were then incubated with anti-phospho-ERK antibody, anti-phospho-Akt antibody, or anti-Akt antibody and washed with Tris-buffered saline. After incubating the membrane with a 1:5,000 diluted goat anti-rabbit IgG or goat anti-mouse IgG antibody conjugated to horseradish peroxidase, the antigen-antibody complexes were visualized using the enhanced chemiluminescence detection system.

β-Hexosaminidase Secretion Assay. The amount of released β-hexosaminidase was measured as described previously (Haribabu et al., 1999). Briefly, RBL-2H3 cells or FPR-expressing RBL-2H3 cells (2×10^5/well) were cultured overnight in 24-well tissue culture plates. The cells were then washed twice with Tyrode's buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.6 mM glucose, 2.7 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 0.1g/100 ml BSA, and 25 mM HEPES, pH 7.4) and stimulated with individual peptides. The reaction was terminated 20 min after stimulation by placing the plate on ice. The amount of β-hexosaminidase secreted into the medium was determined by incubating 50 μl of supernatant or cell lysate with 25 μl of 5 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide in 0.1 M sodium citrate buffer, pH 3.8, at 37°C for 2 h. At the end of the incubation, 50 μl of 0.4 M Na_2CO_3 was added. Absorbance was monitored at 405 nm. Values (means ± S.E.) are expressed as a percentage of the total β-hexosaminidase present in the cells.

Chemotaxis Assay. Chemotaxis assays were performed using multiwell chambers (Neuro Probe, Gaithersburg, MD) as described previously (He et al., 2000). Briefly, polycarbonate filters (8-μm pore size) were precoated with 50 μg/ml rat type I collagen (Collaborative Research, Bedford, MA) in HEPES-buffered RPMI 1640 medium. A dry coated filter was placed on a 96-well chamber containing different concentrations of peptides. RBL-2H3 cells expressing FPR were suspended in RPMI at a concentration of 1×10^6 cells/ml, and 25 μl of the cell suspension was placed onto the upper well of the chamber. After incubation for 4 h at 37°C, nonmigrating cells were removed by scraping, and cells that migrated across the filter were dehydrated, fixed, and stained with hematoxylin (Sigma-Aldrich). The stained cells in five randomly chosen high-power fields (400×) were then counted for each well.

Statistics. The results are expressed as means ± S.E. The Student's *t* test was used to compare individual treatments with their respective control values. A probability of $p < 0.05$ was accepted as a significant difference.

Results

The Effect of WKYMVm Analogs on $[Ca^{2+}]_i$ Increase in FPR- and FPLR1-Expressing RBL-2H3 Cells. The activities of WKYMVm and of its analogs on $[Ca^{2+}]_i$ release were measured in FPR- or FPRL1-expressing RBL-2H3 cells. The mother peptide, WKYMVm, induced a $[Ca^{2+}]_i$ increase in a concentration-dependent manner in FPR-expressing RBL-2H3 cells, showing maximal activity at approximately 300 nM (data not shown). The EC_{50} value for WKYMVm-induced $[Ca^{2+}]_i$ mobilization in FPR cells was 47 nM (Table 1). In the case of the WKYMVm analogs, WHYMVm, WKWMVm, and WKFMVm showed higher potency for FPR than the mother peptide, and the other analogs were not as active (Table 1). In particular, WKGMVm, WKYMGm, and sixth D-Met-substituted analogs did not cause $[Ca^{2+}]_i$ increase at up to 30 μM in FPR-expressing RBL-2H3 cells (Table 1; Fig. 1A). The N- or C-terminal-truncated analogs were inactive with respect to $[Ca^{2+}]_i$-increasing activity in FPR cells (Table 1). These results suggest that Tyr^3 and D-Met^6 are critically important for activation of FPR leading to $[Ca^{2+}]_i$ increase.

The effect of WKYMVm and its analogs on $[Ca^{2+}]_i$ increase was checked in FPRL1-expressing RBL-2H3 cells. In these cells, WKYMVm showed maximal activity at 10 nM (data not shown). The EC_{50} value of WKYMVm for the $[Ca^{2+}]_i$-increasing activity in FPRL1 cells is 0.6 nM (Table 2). Unlike in FPR cells, all the peptide analogs were active in inducing $[Ca^{2+}]_i$ increase in FPRL1 cells (Table 2). Some of the analogs such as WRYMVm, WKWMVm, WKFMVm, WKYMYm, and WKYM(F/W)m displayed higher potency to FPRL1 (Table 2). WKGMVm-, WKYMGm-, and sixth D-Met-substituted analogs, which did not affect $[Ca^{2+}]_i$ in FPR cells, also showed $[Ca^{2+}]_i$-increasing activity in FPRL1 cells, although with lower potency than the mother peptide (Table 2; Fig. 1B). The N- or C-terminal-truncated analogs also stimulated $[Ca^{2+}]_i$ increase in FPRL1 cells (Table 2). We also checked the effect of WKYMVm and its analogs on cytosolic Ca^{2+} increase in vector-transfected RBL-2H3 cells and confirmed that the peptides had no such effect in these cells (Fig. 1C). These results indicate that Tyr^3 and D-Met^6 are less critical for the activation of FPRL1 for $[Ca^{2+}]_i$ increase compared with FPR.

Effect of WKYMVm Analogs on ^{125}I-WKYMVm Binding to FPR. Our finding that some of the WKYMVm analogs (e.g., WKGMVm-, WKRMVm-, and D-Met^6-substituted WKYMVm analogs) could not induce cytosolic calcium increase prompted us to check whether the analogs are able to bind to FPR. FPR-expressing RBL-2H3 cells were incubated with [^{125}I] WKYMVm in the absence or presence of increasing amounts of unlabeled WKYMVm or its analogs. As shown in Fig. 2, unlabeled WKYMVm, WKGMVm, and WKRMVm competed with the binding of ^{125}I-WKYMVm in a concentration-dependent manner. The K_d values for the interaction of FPR with WKYMVm and its analogs were determined from the IC_{50} values using the equation: $K_d = IC_{50} -$ [radioligand used] (DeBlasi et al., 1989). Determined K_d values were 0.095, 7.4, and 9.6 μM for WKYMVm, WKGMVm, and WKRMVm, respectively. The control peptide LFMYHP, previously reported not to affect the activation of phagocytes that express FPR, did not compete for the binding of WKYMVm (Fig. 2). Moreover, ^{125}I-WKYMVm did not bind to

TABLE 1
Effect of peptides modified from WKYMVm-NH_2 on intracellular calcium increase in FPR-expressing RBL-2H3 cells
Intracellular calcium increase was monitored from fura-2-loaded cells as described under *Materials and Methods*.

Sequence	EC_{50}
	nM
WKYMVm-NH_2	47 ± 11
WKGMVm-NH_2	Inactive
WKYMGm-NH_2	Inactive
WKYMVG-NH_2	Inactive
WRYMVm-NH_2	55 ± 8
WEYMVm-NH_2	323 ± 29[a]
WHYMVm-NH_2	32 ± 3[a]
WDYMVm-NH_2	99 ± 18[a]
WKHMVm-NH_2	282 ± 36[a]
WKEMVm-NH_2	1,304 ± 89[a]
WKWMVm-NH_2	19 ± 5[a]
WKRMVm-NH_2	Inactive
WKDMVm-NH_2	1,302 ± 207[a]
WKFMVm-NH_2	18 ± 5[a]
WKYMYm-NH_2	671 ± 82[a]
WKYM(F/W)m-NH_2	57 ± 10
WKYMVE-NH_2	Inactive
WKYMVV-NH_2	Inactive
WKYMVR-NH_2	Inactive
WKYMVW-NH_2	Inactive
WKYMV-NH_2	Inactive
KYMVm-NH_2	383 ± 33[a]
KYMV-NH_2	Inactive
YMVm-NH_2	567 ± 63[a]
MVm-NH_2	Inactive
wkymvm-NH_2	Inactive

[a] $p < 0.05$ compared with WKYMVm.

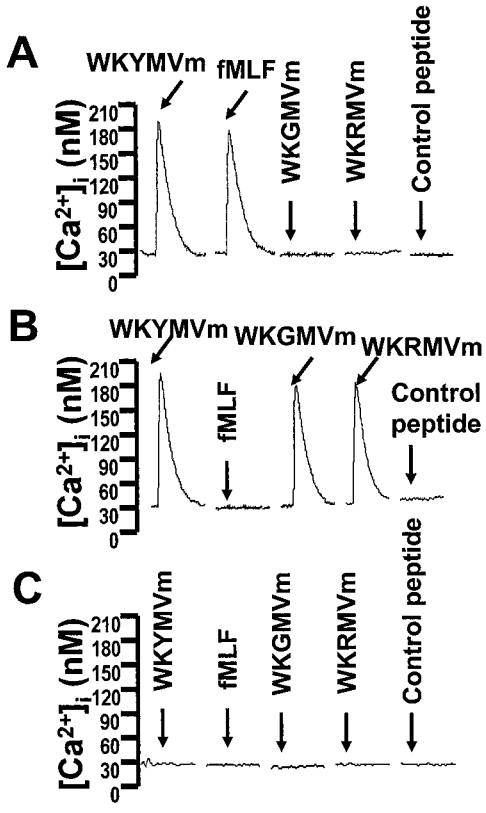

Fig. 1. Effect of WKYMVm analogs or fMLF on $[Ca^{2+}]_i$ in FPR- or FPRL1-expressing RBL-2H3 cells. FPR(A)-, FPRL1(B)-expressing RBL-2H3 cells or vector-transfected RBL-2H3 cells (C) were stimulated with 10 μM concentrations of each peptide, and $[Ca^{2+}]_i$ was determined fluorometrically using fura-2/AM, as described under *Materials and Methods*. The peak level of $[Ca^{2+}]_i$ was recorded. Data are representative of three independent experiments.

vector-transfected RBL-2H3 cells (data not shown). These results indicate that although WKGMVm and WKRMVm are able to bind to FPR and may use the same or overlapping receptor-binding domains, none of these analogs could induce cytosolic calcium increase in the FPR-expressing RBL cells.

Effect of the WKYMVm Analogs on ERK Phosphorylation in FPR-Expressing RBL-2H3 Cells. Although some analogs were able to bind to FPR, they failed to stimulate PLC-mediated calcium increase. Therefore, we examined their effects on other signaling events (e.g., activation of ERK and Akt), which are independent of changes in intracellular calcium concentration. Stimulation of FPR-expressing RBL-2H3 cells with 1 μM WKYMVm induced transient ERK activation, which peaked by 2 min after peptide treatment (Fig. 3A). When FPR-expressing RBL-2H3 cells were pretreated with several inhibitors before WKYMVm stimulation, the WKYMVm-induced ERK activation was found to be sensitive to PTX and PD98059, indicating that this event is mitogen-activated protein kinase kinase (MEK)-dependent and mediated by PTX-sensitive G proteins (Fig. 3B). As expected, pretreatment with a calcium chelator (BAPTA/AM) did not affect WKYMVm-induced ERK activation (Fig. 3B). Therefore, it seems that WKYMVm induces $[Ca^{2+}]_i$ increase and ERK activation via independent signaling pathways. In this study, we checked the effect of WKYMVm analogs on ERK activation by Western blot analysis using anti-phospho-ERK antibody. Although they did not increase cytosolic calcium activity, the WKYMVm analogs (WKGMVm, WKRMVm, WKYMVR, and WKYMVE) stimulated ERK phosphorylation in FPR-expressing RBL-2H3 cells (Fig. 3C). This result is of potential interest because these analogs did not affect the PLC-mediated $[Ca^{2+}]_i$. Separate experiments confirmed that none of the tested peptides affected ERK activity in vector-transfected RBL-2H3 cells (Fig. 3D).

Effect of the WKYMVm Analogs on Akt Phosphorylation in FPR-Expressing RBL-2H3 Cells. It is well established that agonist stimulation of chemoattractant receptors induces Akt activation via PI3K (Kampen et al., 2000; Li et al., 2000). We also observed that WKYMVm stimulation induced Akt phosphorylation in a time-dependent manner in FPR-expressing RBL-2H3 cells (Fig. 4A). Moreover, WKYMVm-induced Akt phosphorylation was sensitive to PTX and to the PI3K inhibitor LY294002, indicating PTX-sensitive G protein(s) and PI3K dependence (Fig. 4B). The induced Akt phosphorylation was not sensitive to BAPTA/AM or PD98059. Stimulation of FPR-expressing RBL-2H3 cells by WKYMVm and by its analogs (WKGMVm, WKRMVm, WKYMVE, and WKYMVR) caused Akt phosphorylation (Fig. 4C). Because WKYMVm-induced activation of ERK and Akt was mediated by PI3K activation, it seems that WKGMVm, WKRMVm, WKYMVE, and WKYMVR can successfully induce PI3K-mediated signaling events resulting from FPR activation. We confirmed that none of the tested peptides affected Akt activity in vector-transfected RBL-2H3 cells (Fig. 4D).

Effect of WKYMVm Analogs on Exocytosis. Granule secretion is one of the most important functions of mast cells (Tapper, 1996; Borregaard and Cowland, 1997; Jin et al., 1997). We checked the effect of WKYMVm on granule secretion by measuring β-hexosaminidase release, as described previously (Haribabu et al., 1999). The stimulation of FPR-expressing RBL-2H3 cells with various concentrations of WKYMVm was found to cause β-hexosaminidase release in a concentration-dependent manner (Fig. 5A). Maximal activity was obtained when stimulating FPR-expressing RBL-2H3 cells with 100 nM of WKYMVm (Fig. 5A). In a series of control experiments, we confirmed that none of the tested peptides stimulated β-hexosaminidase release in vector-transfected RBL-2H3 cells (data not shown). It has been reported that cytosolic calcium increase is critical for the

TABLE 2
Effect of peptides modified from WKYMVm-NH_2 on intracellular calcium increase in FPRL1-expressing RBL-2H3 cells
Intracellular calcium increase was monitored from fura-2-loaded cells as described under *Materials and Methods*.

Sequence	EC_{50}
	nM
WKYMVm-NH_2	0.60 ± 0.090
WKGMVm-NH_2	21.32 ± 2.104[a]
WKYMGm-NH_2	18.11 ± 1.308[a]
WKYMVG-NH_2	5,945.8 ± 176.100[a]
WRYMVm-NH_2	0.12 ± 0.010[a]
WEYMVm-NH_2	5.23 ± 0.196[a]
WHYMVm-NH_2	0.72 ± 0.075
WDYMVm-NH_2	14.28 ± 1.225[a]
WKHMVm-NH_2	1.94 ± 0.268[a]
WKEMVm-NH_2	28.30 ± 1.354[a]
WKWMVm-NH_2	0.16 ± 0.027[a]
WKRMVm-NH_2	2.06 ± 0.256[a]
WKDMVm-NH_2	8.73 ± 1.210[a]
WKFMVm-NH_2	0.23 ± 0.042[a]
WKYMYm-NH_2	0.29 ± 0.061[a]
WKYM(F/W)m-NH_2	0.12 ± 0.015[a]
WKYMVE-NH_2	502.87 ± 64.965[a]
WKYMVV-NH_2	1,259.15 ± 95.750[a]
WKYMVR-NH_2	177.52 ± 26.035[a]
WKYMVW-NH_2	194.48 ± 19.210[a]
WKYMV-NH_2	917.85 ± 45.610[a]
KYMVm-NH_2	3.01 ± 0.232[a]
KYMV-NH_2	>30,000
YMVm-NH_2	17.15 ± 0.889[a]
MVm-NH_2	>30,000
wkymvm-NH_2	Inactive

[a] $p < 0.05$ compared with WKYMVm.

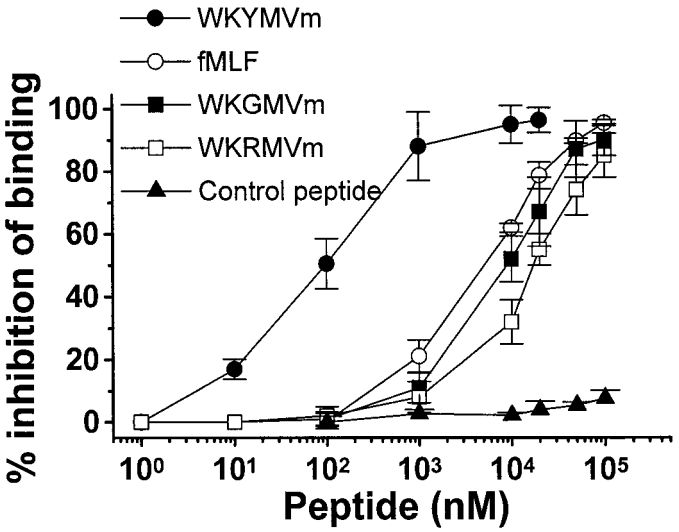

Fig. 2. Replacement of ^{125}I-labeled WKYMVm binding to FPR by WKYMVm analogs. ^{125}I-Labeled WKYMVm was incubated for 3 h at 4°C with FPR-expressing RBL-2H3 cells in the presence of various concentrations of each unlabeled peptide. The quantity of bound ^{125}I-labeled WKYMVm was determined using a gamma-ray counter.

secretion of granules in mast cells and the rat basophilic leukemia cell line RBL-2H3 (Haribabu et al., 1999). We confirm that chelation of intracellular free calcium by BAPTA/AM treatment before peptide stimulation almost completely inhibited WKYMVm-induced granule secretion (Fig. 5B). Our new finding that cytosolic calcium release was induced by WKYMVm and many of its analogs, but not by others (WKGMVm-, WKRMVm-, and D-Met^6-substituted analogs), led us to check the effect of the analogs on granule secretion in RBL cells. When the FPR-expressing RBL-2H3 cells were stimulated with the WKYMVm analogs, granule secretion was not induced by by WKGMVm-, WKRMVm-, or D-Met^6-substituted analogs (Fig. 5A; data not shown). These results are in complete agreement with our previous results, namely, that WKGMVm-, WKRMVm-, and the D-Met^6-substituted analogs cannot induce cytosolic calcium release in FPR expressing RBL-2H3 cells (Fig. 1; data not shown).

Effect of the WKYMVm Analogs on Cell Chemotaxis. WKYMVm has been reported to induce the chemotactic migration of phagocytic cells, such as monocytes and neutrophils (Bae et al., 1999b). Le et al. (2000) demonstrated that WKYMVm induced cellular chemotaxis by binding to FPR. As expected, in the present study WKYMVm induced chemotactic migratory activity with a bell-shaped dose-response curve in FPR-expressing RBL-2H3 cells (Fig. 6A). In addition, WKYMVm-induced cell chemotaxis was found to be sensitive to LY294002 and PD98059 (Fig. 6B), suggesting that WKYMVm-induced cell chemotaxis is PI3K- and MEK-dependent. We also examined the effect of the WKYMVm analogs on cell chemotaxis in FPR-expressing RBL-2H3 cells. As with WKYMVm, these analogs (WKGMVm, WKRMVm, WKYMVE, and WKYMVR) induced cell chemotaxis (Fig. 6A; data not shown). The concentrations of the WKYMVm analogs required to induce chemotaxis were higher than that of WKYMVm (Fig. 6A). In terms of the signaling pathways involved in WKYMVm analog-induced chemotaxis, we investigated the possible involvement of PI3K and MEK-mediated signaling. Treatment with LY294002 or PD98059 almost completely inhibited cell migration induced by the WKYMVm analogs (Fig. 6B).

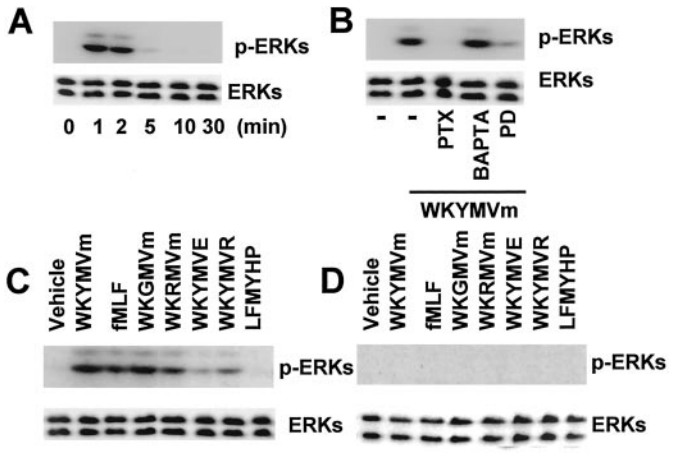

Fig. 3. Effect of WKYMVm analogs on ERK phosphorylation in FPR-expressing RBL-2H3 cells. FPR-expressing RBL-2H3 cells were stimulated with 1 μM WKYMVm for various times (A). The cells were preincubated with vehicle or 100 ng/ml PTX (24 h), 10 μM BAPTA/AM (60 min), or 50 μM PD98059 (60 min) before being treated with 1 μM WKYMVm (B). FPR-expressing RBL2H3 cells (C) or vector-transfected RBL-2H3 cells (D) were stimulated with 10 μM WKYMVm analogs for 2 min. Each sample (30 μg of protein) was subjected to 10% SDS-polyacrylamide gel electrophoresis, and phosphorylated ERK was determined by immunoblot analysis using anti-phospho-ERK antibody. Western blot analysis was also performed with anti-ERK2 antibody to confirm that the amounts of samples used were similar. Results are representative of three independent experiments.

Discussion

In this study, we demonstrate that FPR can be activated by distinct peptide ligands, leading to differential cellular signaling and different functional consequences. To demonstrate ligand-specific activation of FPR, we generated diverse analogs of WKYMVm, a potent ligand of FPR. Among these analogs, WKGMVm-, WKRMVm-, and the D-Met^6-substituted analogs bound to FPR and induced PI3K-mediated Akt activation and MEK-mediated ERK activation, which resulted in chemotactic cell migration. However, these analogs did not affect cytosolic calcium increase. Because the mother peptide WKYMVm could induce both ERK activation and cytosolic calcium increase, and stimulated chemotaxis and degranulation, our experimental data suggest differential activation of FPR by distinct ligands.

Several recent reports have demonstrated that certain G protein-coupled receptors can be differentially activated by their ligands (Palanche et al., 2001; Seifert et al., 2001). These binding events induce distinct conformational changes of G protein-coupled receptors during the process of ligand-specific activation of the receptor and induce selective coupling of the receptor with certain effector molecules and G

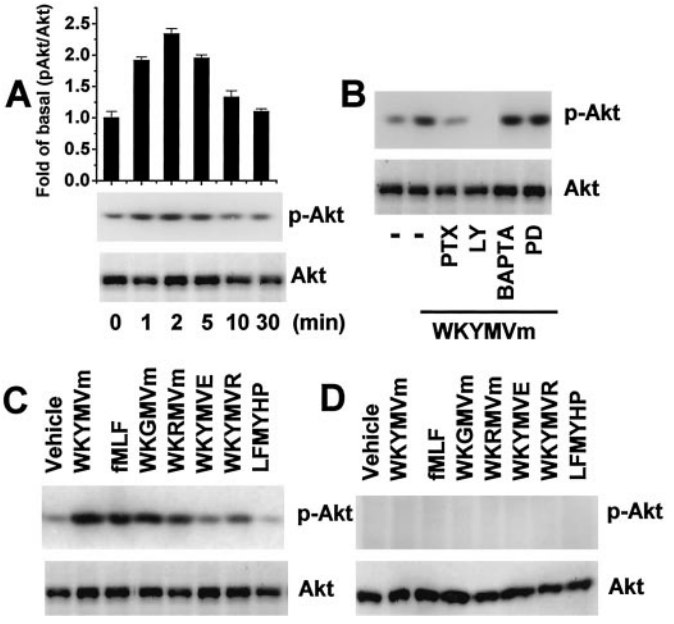

Fig. 4. Effect of WKYMVm analogs on Akt phosphorylation in FPR-expressing RBL-2H3 cells. FPR-expressing RBL-2H3 cells were stimulated with 1 μM of WKYMVm for various times (A). Levels of *p*-Akt as percentage of total Akt were quantified using densitometer and presented the data as means ± S.E. of three independent experiments (A). 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 1 μM WKYMVm (B). FPR-expressing RBL-2H3 cells (C) or vector-transfected RBL-2H3 cells (D) were stimulated with 10 μM WKYMVm analogs for 2 min. Each sample (30 μg of protein) was subjected to 10% SDS-polyacrylamide gel electrophoresis, and phosphorylated Akt was determined by immunoblot analysis with anti-phospho-Akt antibody. Western blot analysis with anti-Akt antibody confirmed that the amounts of samples used were similar. Results are representative of three independent experiments.

proteins. With respect to FPR, annexin I, a recently identified FPR ligand, can partially activate certain FPR-mediated signaling pathways (Walther et al., 2000). However, the mechanism by which annexin I induces FPR activation is not clear, nor is the structural requirements for a ligand to differentially activate this chemoattractant receptor. Ligands with substantially different structures, including formyl peptides, peptides derived from the HIV-1 Env protein and annexin I, have been reported to bind FPR (Le et al., 2000). Moreover, Le et al. (1999) reported that WKYMVm could also bind to FPR. In the present study, we demonstrate that the substitution of Tyr^3 or D-Met^6 with other amino acids selectively abolishes FPR-mediated cytosolic calcium increase through PLC (Fig. 1). Table 1 and Fig. 1 show that WKGMVm-, WKRMVm-, and D-Met^6-substituted analogs do not stimulate PLC-mediated cytosolic calcium increase in FPR-expressing RBL-2H3 cells. However, these WKYMVm analogs can stimulate ERK and Akt phosphorylation in the same cells (Figs. 3C and 4C). For WKYMVm and its analogs, cytosolic calcium increase is induced by the hydrolysis of phosphatidylinositol through PLCβ activation, whereas ERK and Akt phosphorylation is mediated by the activation of MEK and PI3K, respectively. Because the WKYMVm analogs WKGMVm and WKRMVm and the D-Met^6-substituted analogs cannot induce cytosolic calcium increase yet can stimulate Akt phosphorylation via PI3K, it seems that the peptide analogs bind to FPR and selectively induce conformational changes required for PI3K activation and MEK-mediated ERK activation, resulting in chemotaxis of RBL-2H3 cells. Our result indicates that Tyr^3 and D-Met^6 are critical structural determinants for the activation of PLC by FPR.

Interestingly, site-directed mutagenesis of FPR has produced mutant receptors with phenotypes similar to the ones observed in this study using the WKYMVm analogs (Miettinen et al., 1997, 1999). Miettinen et al. (1999) have con-

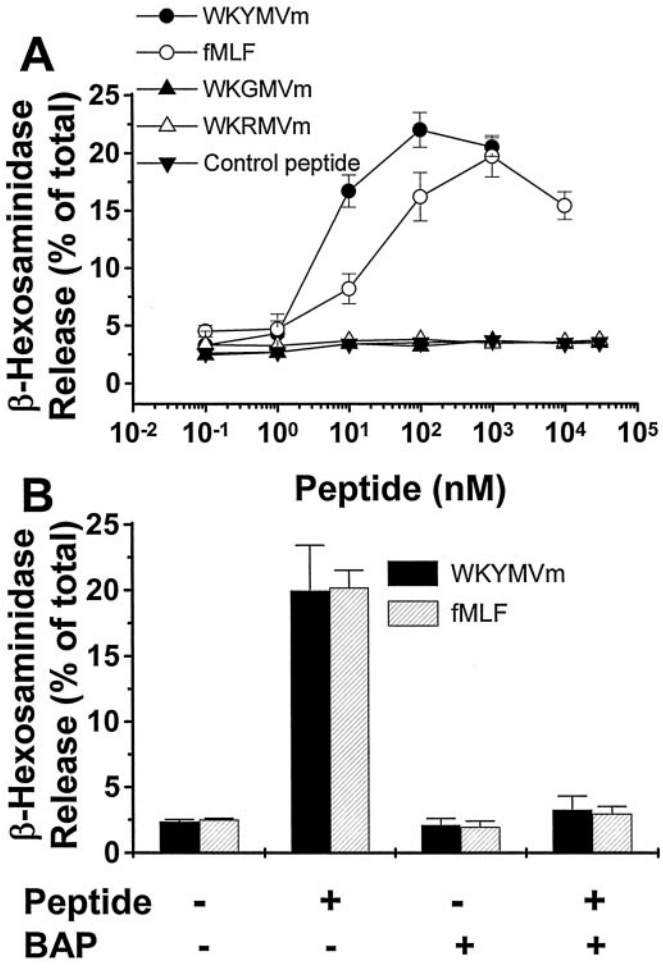

Fig. 5. Effect of WKYMVm analogs on exocytosis. Various concentrations of WKYMVm, fMLF, or of the WKYMVm analogs were administered to FPR-expressing RBL-2H3 cells (A). WKYMVm (1 μM) or 1 μM fMLF was then added to the cell lines in the absence and in the presence of 10 μM BAPTA/AM (B). The peptide-induced secretion of β-hexosaminidase was determined as described under *Materials and Methods*. Data are means ± S.E. of three experiments performed in triplicate.

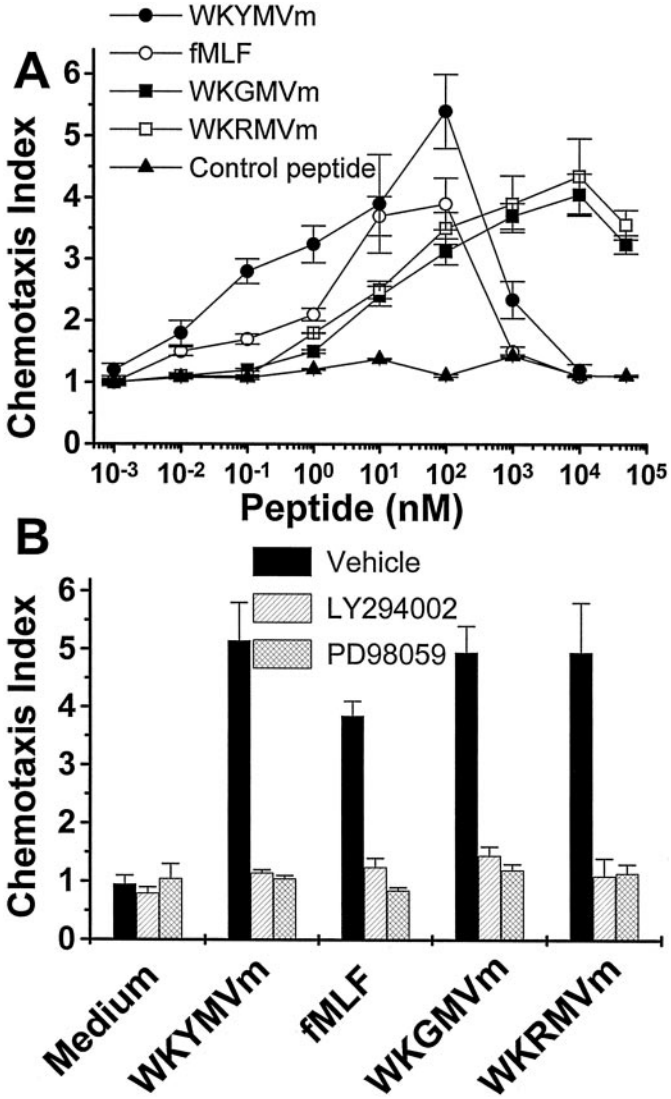

Fig. 6. Effect of WKYMVm analogs on chemotaxis. Assays were performed using a modified Boyden chamber assay, as described under *Materials and Methods*. FPR-expressing RBL-2H3 cells (1×10^6 cells/ml of serum-free RPMI) were added to the upper wells of a 96-well chemotaxis chamber and migration across an 8-μm pore size polycarbonate membrane was assessed after 4-h incubation at 37°C. Various concentrations of each peptide were used for the chemotaxis assay (A). Vehicle, 50 μM LY294002 (15 min), or 50 μM PD98059 (60 min) pretreated cells were subjected to the chemotaxis assay with 100 nM WKYMVm, 100 nM fMLF, or 1 μM WKYMVm analog (B). The number of migrated cells was determined by counting in a high-power field (400×). Data are presented as means ± S.E. of three independent experiments performed in duplicate.

structed 35 mutant FPRs and checked the effect of individual mutations on the G protein coupling and cellular signaling of FPR. They found that Ser63, Asp71, Arg123, and Cys124/126 are important for FPR coupling to G proteins (Miettinen et al., 1999). Among the mutants compared, the R123A mutant, which is unable to mediate calcium mobilization, is nevertheless able to induce ERK phosphorylation by fMLF (Miettinen et al., 1999). It has also been postulated that Asp122 and Arg123, which form the conserved (D/E)RY motif (DRC in FPR), participate in a hydrogen-bonding network that stabilizes the inactive form of the receptor (Miettinen et al., 1999). A possible mechanism could be that ligand binding to the receptor alters the hydrogen-bonding network and that certain amino acid residues, for example, arginine in the DRY motif then become exposed to interact with G proteins (Miettinen et al., 1999). This coincides with our finding that the WKYMVm analog WKGMVm could induce ERK phosphorylation but not calcium mobilization. It is postulated that ligation of WKYMVm or WKGMVm to FPR induces different changes in receptor conformation. For example, WKYMVm can induce a conformational change of FPR that includes altering the hydrogen bonding of the DRY motif, whereas WKGMVm causes conformational changes of the receptor without affecting DRY hydrogen bonding. Other binding pockets may also be involved in peptide binding, and it is important that the residues involved in the differential binding of WKYMVm and WKGMVm to FPR be identified in future studies.

FPR mediates critical functions in leukocytes and is known to activate phagocyte functions, including adhesion, emigration, and chemotaxis, and bacteriocidal activities of phagocytes such as respiratory burst, degranulation, and phagocytosis (Le et al., 2000, 2001a). FPR also activates cytokine gene expression in phagocytes and local cytokine network, and it facilitates the recruitment of additional leukocytes to sites of inflammation (Le et al., 2000, 2001a). Regulation of these phagocyte functions requires activation of various and sometimes overlapping signaling pathways. For example, the activity of PI3Kγ seems to be required for both chemotaxis and degranulation. These phagocyte functions may also depend on specific signaling events. In this study, we demonstrate that FPR-mediated exocytosis by WKYMVm and fMLF is absolutely dependent on cytosolic Ca^{2+} increase (Fig. 5B). However, FPR-mediated chemotaxis does not require an increase in cytosolic Ca^{2+} (data not shown). The PI3K- and MEK-mediated signaling pathways play a critical role in chemotaxis induced by FPR stimulation (Fig. 6B). Our results strongly suggest that FPR-induced chemotaxis and exocytosis are differentially regulated and require different intracellular signaling events in addition to sharing PI3Kγ, as evidenced by the abilities of WKGMVm and WKRMVm to stimulate chemotaxis but not exocytosis. These findings are potentially important for the development of selective immunomodulators. For example, a compound that specifically activates signaling events, such as cytosolic calcium increase or ERK activation, may selectively stimulate chemotaxis but not exocytosis. Proof of this concept with the two peptides tested in the current study may not only help future development of synthetic ligands but also facilitate the identification of natural ligands that can selectively activate phagocyte functions through differential modulation of FPR.

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