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Identification of novel peptide agonists from a random peptide library for a 5-oxo-ETE receptor, a receptor for bioactive lipids

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Abstract: A combinatorial peptide library contains an enormous combination of amino acid sequences and drug candidates, but an effective screening strategy to identify a variety of bioactive peptides has yet to be established. In this article, a random hexapeptide library was screened to identify novel peptide ligands for a 5-oxo-ETE receptor (OXER), which is a G-protein-coupled receptor for bioactive lipids, by using an OXER-Gi1α fusion protein. We successfully identified 2 hexapeptides – Ac-HMQLYF-NH_2 and Ac-HMWLYF-NH_2 – that exhibited agonistic activity. Although the corresponding affinities were relatively low (EC_{50} values of 146 and 6.7 μM, respectively), the activities were confirmed by other independent cell-based assay methods, namely, intracellular calcium mobilization and cell chemotaxis. This study demonstrates that a combinatorial peptide library may be screened using a [^{35}S]GTPγS binding assay with G-protein-coupled receptor (GPCR)–Gα fusion proteins, in general, and that of peptide ligands can be obtained even for nonpeptide receptors. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: random peptide library; G-protein-coupled receptor; 5-oxo-ETE; GPCR–Gα fusion proteins

INTRODUCTION

Combinatorial chemistry has gained widespread acceptance for the rapid preparation of an enormous number of chemical compounds and drug candidates. This is because synthetic combinatorial libraries are composed of possible combinations of the building blocks used in the syntheses. Peptide synthesis is a well-established technique and is one of the most suitable methods that can be used in combinatorial chemistry. Solid-phase peptide chemistry has been employed in many studies of mixture-based combinatorial libraries and has been used to generate diverse peptides attached to a solid support [1]. It was reported that combinatorial peptide libraries were used to discover novel ligands for GPCRs [2]. In particular, Doory *et al.* identified and characterized hexapeptides that exhibited high affinity toward a nociceptin receptor from a combinatorial library containing more than 52 million different hexapeptides by using [^{3}H]-labeled nociceptin and a displacement assay [3]. Their binding studies indicated that these peptides exhibited an affinity for a nociceptin receptor in the nanomolar range as well as nociceptin, which is an endogenous peptide ligand that comprises 17 amino acids. Their strategy is very efficient at identifying novel ligands for receptors; however, its application is still limited to GPCRs in general, since highly specific radioligands are not yet available for some GPCRs. The development of not only reaction schemes and chemical libraries but also screening systems is imperative for the application of combinatorial chemistry to drug development. In order to expand the uses of a random hexapeptide library to ligand screening for GPCRs in general, the following three questions were investigated: (i) What is the type of screening method suitable for identifying peptide ligands without the use of a specific radioligand? (ii) Does a peptide activate a nonpeptide receptor? (iii) Is the diversity of a hexapeptide library sufficient for identifying ligands for GPCRs in general?

The high-throughput screening of GPCR ligands has usually been based on cell-based assay systems that monitor the downstream events of signal cascades, such as intercellular calcium concentrations. However, these cell-based assays have a disadvantage: the endogenous receptors on host cells respond to their corresponding ligands, yielding false-positive signals. To develop a cell-free assay method to detect ligand-induced responses on the targeted GPCRs, the fusion proteins of various GPCRs with α subunits of G proteins (GPCR–Gα fusion proteins) have been prepared using the baculovirus-Sf9 expression system [4–6]. The [^{35}S]GTPγS binding assay with Sf9 membranes that

Abbreviations: 5-oxo-ETE; 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; GPCR; G-protein-coupled receptor; OXER; 5-oxo-ETE receptor.

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expressed GPCR–Gα fusion proteins has been used to screen agonists and antagonists. Compared to screening methods that used cultured cells transfected with GPCR genes, the GPCR–Gα fusion protein assay exhibited fewer false-positive reactions because the level of expression of the fusion proteins was extremely high [5]. The use of GPCR–Gα fusion proteins for ligand screening has other advantages. It allows easy preparation of a large amount of Sf9 membranes that express the fusion proteins, which can be stocked in deep freezers, and a large number of ligands can be assayed with the same batch of membrane proteins. Moreover, the GPCR–Gα fusion protein assay is simple and suitable for high-throughput screening with the use of 96-well microplates. Hence, GPCR–Gα fusion proteins have been used to identify several endogenous and/or surrogate ligands [4–6].

This study attempted to discover peptide ligands for a lipid receptor in order to confirm the agonistic ability of a peptide for a lipid receptor and the diversity of the hexapeptide library. Several lipid mediators, including the products of arachidonic acid metabolism, are potent stimulators of eosinophils. The chemoattractant 5-oxo-ETE is a metabolite of arachidonic acid and plays a role in inflammatory response by stimulating eosinophil migration [7]. This lipid has attracted the attention of molecular pharmacologists since the recent identification of its receptor, the OXER [8–10]. An OXER is a member of the family of GPCRs and couples with $G_{i/o}$ and $G_{12/13}$ proteins, which initiate or attenuate the chemotaxis of an OXER-expressing cell toward 5-oxo-ETE, respectively [11]. Unfortunately, more detailed study of OXER functions has been limited by the lack of good pharmacological tools or specific high-affinity ligands for an OXER. The development of specific ligands against an OXER is also required to evaluate the potential of an OXER as a new therapeutic target for anti-inflammation [12]. Recently, the development of novel ligands for an OXER has been discussed in order to suppress the infiltration of leukocytes, thus providing several new approaches for allergy and asthma.

Here, the strategies of combinatorial peptide libraries and GPCR–Gα fusion protein screening were extended to identify novel peptide ligands for a nonpeptide receptor. A combinatorial library of 64 000 000 hexapeptides (in theory) in the positional scanning format was employed in OXER–Gi1α fusion protein screening. The results yielded information about the functional amino acid sequences at each position, leading to the identification of two novel agonistic peptides for an OXER.

MATERIALS AND METHODS

Materials

A pack of 1200 types of random hexapeptides was obtained from American Peptide Co. This library consists of three separate sublibraries, each containing 400 mixtures and having two defined positions (O) and four mixture positions (X) as follows: Ac-$O_1O_2X_3X_4X_5X_6$-NH_2, Ac-$X_1X_2O_3O_4X_5X_6$-NH_2, and Ac-$X_1X_2X_3X_4O_5O_6$-NH_2. The positions labeled 'O' are individually defined by the 20 natural L-amino acids (i.e. AA, AC, AD, ..., YV, YW, and YY, yielding $20 \times 20 = 400$ combinations) and the positions labeled 'X' consist of mixtures of the 20 natural L-amino acids. It should be noted that this library can possibly contain a total of 64 000 000 ($= 20^6$) hexapeptides. For a screening of this random peptide library, approximately 2 mg of each peptide mixture was dissolved with 44 µl of dimethyl sulfoxide (DMSO), and 10 µl was used in a total reaction mixture of 100 µl (see '[^{35}S]GTPγS binding assay' below for details).

Peptide Synthesis and Purification

The peptides were synthesized using Fmoc (*N*-(9-fluorenyl) methoxycarbonyl)/*t*-butyl chemistry and a solid-phase method on a tandem reaction mixer (Peti-Syzer Model PSP-510, Hipep Laboratory, Kyoto, Japan) by using 50 mg of Rink Amide AM Resin (0.57 mmol/g). The yielded peptides were subjected to and purified using reverse-phase HPLC (pump: PU-2089plus; UV detector: UV-2075plus; JASCO, Tokyo, Japan) on a C18 column (6.0 × 250 mm: YMC-pack ODS R&D, Kyoto, Japan), which was eluted with a linear gradient of aqueous CH_3CN (1% CH_3CN/min) in the presence of 0.1% TFA at a flow rate of 1 ml/min. The purified peptide fractions were analyzed using a Perkin-Elmer Sciex API-100 electrospray-ionization mass spectrometer, and their chemical structures were confirmed as follows: Ac-HMQLYF-NH_2: found $m/z = 879.4$ $[M + H]+$, $C_{42}H_{59}N_9O_9S$, requires 879.4; $t_R = 17.4$ min (20–45% aqueous CH_3CN), Ac-HMWLYF-NH_2: found $m/z = 937.4$ $[M + H]+$, $C_{48}H_{61}N_{10}O_8S$, requires 937.4; $t_R = 15.3$ min (30–55% aqueous CH_3CN), Ac-HMQLDF-NH_2: found $m/z = 831.3$ $[M + H]+$, $C_{37}H_{55}N_{10}O_{10}S$, requires 831.4; $t_R = 15.3$ min (20–45% aqueous CH_3CN).

GTPγS Binding Assay

[^{35}S]GTPγS (1250 Ci/mmol) was obtained from Perkin-Elmer (Boston) and 5-oxo-ETE was obtained from BIOMOL (Pennsylvania). The preparation of the Sf9 membrane fractions expressing an OXER–Gi1α fusion protein and GTPγS binding assay were performed as described previously [5,13]. The Sf9 cells were grown at 27 °C and infected with the recombinant virus carrying an OXER–Gi1α fusion gene at a density of 3×10^6 cells/ml. After 48 h of infection, the cells were harvested and homogenized in a homogenizing buffer (50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)-KOH (pH 7.0), 1 mM EDTA, and 10 mM $MgCl_2$) with a protease inhibitor cocktail (Sigma, Boston). The plasma membrane was precipitated by centrifugation at $10\,000 \times g$ for 2 h (SCP70H ultracentrifugator and SW28 rotor; Hitachi), and the precipitate was resuspended in the homogenizing buffer described above. The membrane expressing an OXER–Gi1α fusion protein (20 µg of the total protein) and 10 µl of peptides dissolved in DMSO were incubated in 20 mM of HEPES-KOH (pH 8.0), 1 mM of EDTA, 160 mM of NaCl, 1 mM of dithiothreitol (DTT), 100 pM of [^{35}S]GTPγS, 1 µM of guanosine diphosphate (GDP), and 10 mM of $MgCl_2$ with a final volume of 100 µl.

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The incubation was performed at 30 °C for 30 min on 96-well microplates. [^{35}S]GTPγS bound to the membrane was trapped on a GF/B glass fiber filter (Whatman, UK). The GF/B filter was washed three times with 300 µl of cold 20 mM potassium phosphate buffer solution (pH 7.0) and then counted with a liquid scintillation counter (LC6500, Beckman Coulter, California).

Calcium Mobilization Assays

The stock solution of Hepes Tyrode's bovine albumin buffer (HTB) contains 125 mM of Hepes/NaOH (pH 7.4), 700 mM of NaCl, 13.5 mM of KCl, 60 mM of $NaHCO_3$, 2 mM of NaH_2PO_4, 2.5 mM of $MgCl_2$, 5 mM of $CaCl_2$, 28 mM of D-glucose, and 0.05% bovine serum albumin (BSA). This stock solution was diluted five times just before use for Chinese hamster ovary (CHO) cells. The CHO cells were grown in Ham's F-12 supplemented with 10% fetal bovine serum and 5% CO_2 at 37 °C. The CHO cells that stably expressed an OXER [11] were maintained in the presence of 0.1 mg/ml of G418 (Invitrogen, California). The intracellular calcium ion levels were determined by measuring the fluorescence of Fura-2. Cells expressing an OXER were suspended at a final concentration of $1–5 \times 10^6$ cells/ml in a loading buffer containing 2.5 µM of Fura-2/AM (Dojin, Kumamoto, Japan), 1.25 mM of probenecid (Wako, Osaka, Japan) to inhibit anion transporters, and 0.02% of F-127 (Molecular Probes, Oregon) to avoid the aggregation of Fura-2/AM; this mixture was incubated at 37 °C in 5% CO_2 for 1 h to load Fura-2. After the cells were washed with HTB, 6 µl of ligand solution was added to 600 µl of cell suspensions, and then the intracellular calcium ion levels were measured using a RF-5300PC spectrofluorometer system (Shimadzu, Kyoto, Japan).

Chemotaxis Assay

A polycarbonate filter with 5-µm pores (Neuroprobe, Maryland) was coated with 10 µg/ml of fibronectin (Wako, Osaka, Japan) in phosphate-buffered saline (PBS) for 30 min and was placed on a 96-well Boyden chamber (Neuroprobe, Maryland). Ligand droplets (34 µl/well) were added to the bottom wells and CHO cells (200 µl, 8×10^4 cells/well) were added to the top wells of the chamber. The ligand solution and cell suspension were prepared in a serum-free Ham's F-12 medium supplemented with 0.1% fatty-acid-free BSA (Serologicals, California). After incubation at 37 °C in 5% CO_2 for 4 h, the Boyden chamber was disassembled. The cells on the filter were fixed with methanol and stained with a Diff-Quick staining kit (International Reagent Corporation, Kobe, Japan). The top of the filter was scraped to remove cells. The number of cells that migrated to the underside of the filter was determined by measuring optical densities at 595 nm using a 96-well microplate reader (Bio-Rad Model 680).

RESULTS

A random hexapeptide library containing two defined positions was screened using an OXER–Gi1α fusion protein and [^{35}S]GTPγS binding assay. The authentic agonist, 5-oxo-ETE, yielded 5 nM for EC_{50} in this assay system [8]. This library consists of three subsets of 400 mixtures each that are packed in 96-well microplates. Upon screening, each well was found to contain 4.5 mg/ml of peptide, leading to an estimate of 40 nM for each peptide in a mixture comprised of 160 000 hexapeptides (in theory). In this screening, the background count in the absence of any ligands was 2706 ± 311 cpm and the count for the activated state in the presence of 1 µM of 5-oxo-ETE was 5211 ± 422 cpm for 96 measurements. In contrast, the average count was 3347 ± 639 cpm in the presence of Ac-OOXXXX-NH_2, 3686 ± 882 in that of Ac-XXOOXX-NH_2, and 3590 ± 613 in that of Ac-XXXXOO-NH_2. The distributions of peptide activity yielded at least two peaks: one corresponding to nonagonistic and the other corresponding to agonistic peptides (Figure 1). Based on these results, the top 40 amino acid combinations were initially selected. These were compared to the results of independent screenings performed thrice and their corresponding top 40 candidates, after which reproducible sequences were selected: 10 amino acid combinations for Ac-OOXXXX-NH_2, 6 for Ac-XXOOXX-NH_2, and 9 for Ac-XXXXOO-NH_2 (Table 1). These sequences were employed to design agonist hexapeptide candidates.

At this point, there were 540 ($= 10 \times 6 \times 9$) candidates for the sequence combination. Among these possibilities, 41 hexapeptides were designed based on the reliability and reproducibility of the first screening and in light of designed sequence varieties. These were synthesized as a second screening process and their agonistic activity was evaluated by [^{35}S]GTPγS binding assay (Table 2). Out of 41 of these synthesized peptides, only two peptides – Ac-HMQLYF-NH_2 and

Table 1 Selected candidates for amino acid combinations from the first screening. The library consists of three separate sublibraries, each containing 400 mixtures and having two defined positions (O) and four mixture positions (X) as follows: (A) Ac-$O_1O_2X_3X_4X_5X_6$-NH_2, (B) Ac-$X_1X_2O_3O_4X_5X_6$-NH_2, and (C) Ac-$X_1X_2X_3X_4O_5O_6$-NH_2. The positions labeled 'O' are defined by the 20 acids (i.e. AA, AC, AD, …, YV, YW, and YY, yielding $20 \times 20 = 400$ combinations) and the positions labeled 'X' consist of mixtures of the 20 amino acids

Ac - O_1O_2 XXXX - NH_2	Ac - XXO_3O_4 XX - NH_2	Ac - $XXXXO_5O_6$ - NH_2
YM	PK	SG
PV	IK	DF
FF	RM	IN
QR	QL	RS
HM	FI	CF
IC	WL	YF
RP		YS
TN		HK
YF		KI
EV		

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Ac-HMWLYF-NH_2 – resulted in activity to stimulate [^{35}S]GTPγS incorporation for cell membranes expressing an OXER–Gi1α fusion protein; this activity was concentration-dependent, with the two peptides exhibiting EC_{50} values of 146 and 6.7 μM, respectively (Figure 2). According to this [^{35}S]GTPγS assay, Ac-HMQLYF-NH_2 functioned as a full agonist. In contrast, Ac-HMWLYF-NH_2 functioned as a partial agonist and increased [^{35}S]GTPγS incorporation to up to 60% of the value provided by 5-oxo-ETE. The remaining 39 peptides did not exhibit any agonistic activity even at a final concentration of 10 mM.

The two peptides mentioned above were further characterized with two different cell-based assays in

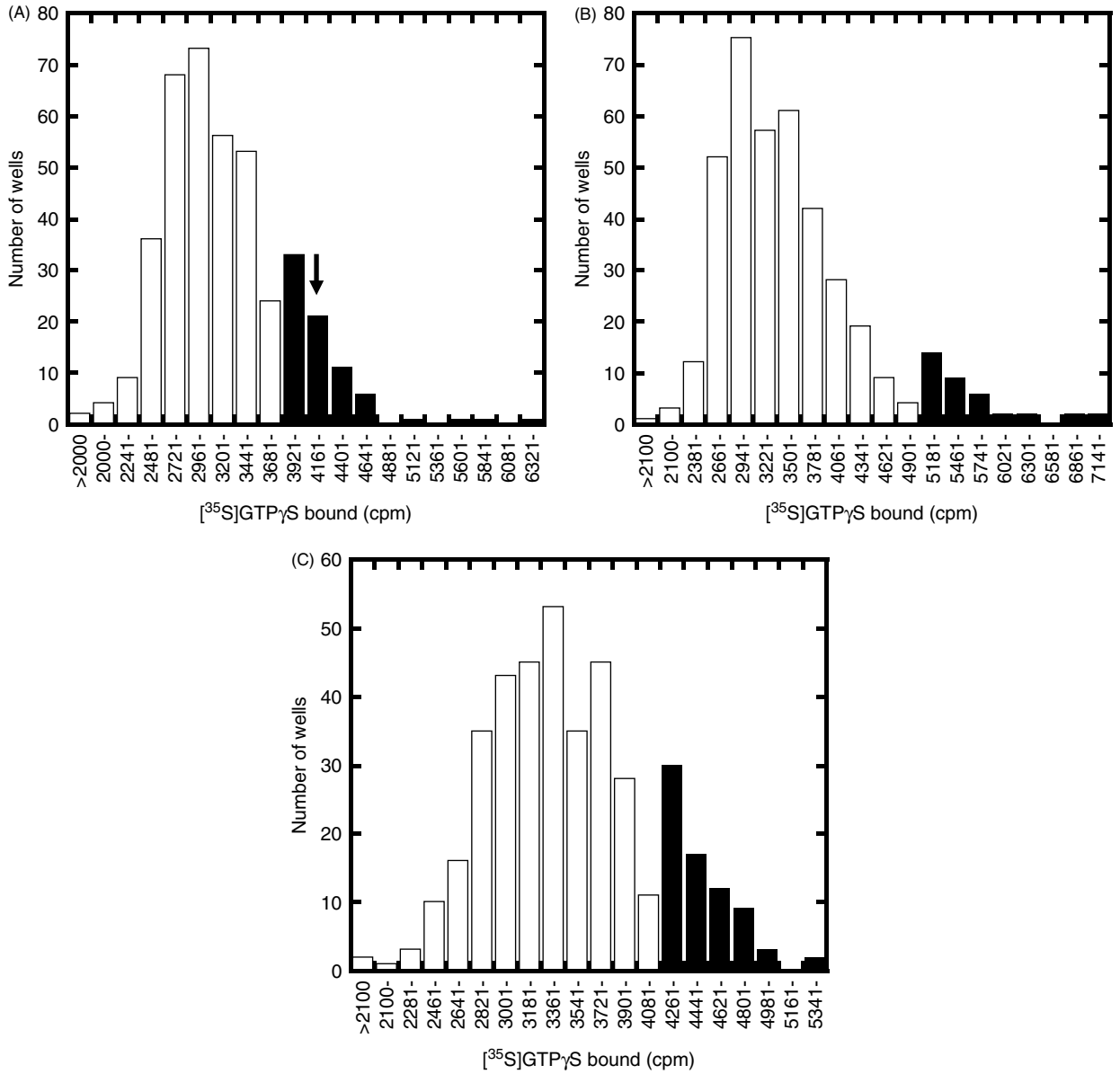

Figure 1 Frequency distribution for results of the first screening using an OXER–Gi1α fusion protein and [^{35}S]GTPγS binding assay. For example, the arrowed bar in (A) corresponds to the number of wells that yielded 4161–4400 cpm. The black bars represent agonistic activity. Upon screening, each well contained 40 nM of each peptide from a mixture of 160 000 ($= 20^4$) hexapeptides (in theory). The library consists of three separate sublibraries, each containing 400 mixtures and having two defined positions (O) and four mixture positions (X) as follows: (A) Ac-$O_1O_2X_3X_4X_5X_6$-NH_2, (B) Ac-$X_1X_2O_3O_4X_5X_6$-NH_2, and (C) Ac-$X_1X_2X_3X_4O_5O_6$-NH_2. The positions labeled 'O' are defined by the 20 amino acids (i.e. AA, AC, AD, ..., YV, YW, and YY, yielding $20 \times 20 = 400$ combinations) and the positions labeled 'X' consist of mixtures of the 20 amino acids. The background count in the absence of any ligands was approximately 2706 ± 311 cpm, and the count for the activated state in the presence of 1 μM of 5-oxo-ETE was around 5211 ± 422 cpm for 96 measurements. Means and standard deviations for nonactivated wells (white bars in the panels) are 3117 ± 396 cpm for (A), 3473 ± 570 cpm for (B), and 3400 ± 457 cpm for (C); the same values for activated wells (black bars in the panels) are 4337 ± 464 cpm for (A), 5767 ± 660 cpm for (B), and 4634 ± 394 cpm for (C).

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Table 2 Synthesized peptides and their EC_{50} values for the [^{35}S]GTPγS binding assay. The crosses indicate the absence of agonist activity

Peptide sequence	[^{35}S]GTPγS binding EC_{50} (μM)
1 Ac-YMPKRS-NH_2	X
2 Ac-PVPKRS-NH_2	X
3 Ac-FFRMRS-NH_2	X
4 Ac-YMWLRS-NH_2	X
5 Ac-YMFIRS-NH_2	X
6 Ac-FFWLRS-NH_2	X
7 Ac-FFPKRS-NH_2	X
8 Ac-YMIKSG-NH_2	X
9 Ac-YMIKRS-NH_2	X
10 Ac-QRIKDF-NH_2	X
11 Ac-QRIKYF-NH_2	X
12 Ac-QRQLDF-NH_2	X
13 Ac-QRQLYF-NH_2	X
14 Ac-QRPKYF-NH_2	X
15 Ac-HMIKDF-NH_2	X
16 Ac-HMIKYF-NH_2	X
17 Ac-HMQLDF-NH_2	X
18 **Ac-HMQLYF-NH_2**	**146**
19 Ac-HMPKYF-NH_2	X
20 Ac-RPDEYS-NH_2	X
21 Ac-RPDEHK-NH_2	X
22 Ac-RPIKYS-NH_2	X
23 Ac-RPIKHK-NH_2	X
24 Ac-RPDEIN-NH_2	X
25 Ac-TNDEYS-NH_2	X
26 Ac-TNIKYS-NH_2	X
27 Ac-TNIKHK-NH_2	X
28 Ac-TNDEIN-NH_2	X
29 Ac-YFQLYF-NH_2	X
30 Ac-YMQLYF-NH_2	X
31 Ac-EVQLYF-NH_2	X
32 **Ac-HMWLYF-NH_2**	**6.7**
33 Ac-HMVDYF-NH_2	X
34 Ac-HMRMYF-NH_2	X
35 Ac-HMQLIN-NH_2	X
36 Ac-HMQLKI-NH_2	X
37 Ac-HMQLHK-NH_2	X
38 Ac-ICWLRS-NH_2	X
39 Ac-YMWLCF-NH_2	X
40 Ac-YMWLRS-NH_2	X
41 Ac-ICWLCF-NH_2	X

order to confirm their agonistic activities with respect to an OXER using both cell membranes as well as living cells. Increases in intracellular calcium concentrations were observed with stimulation by 100 μM of Ac-HMQLYF-NH_2 or Ac-HMWLYF-NH_2. Although Ac-HMQLDF-NH_2 had a very similar sequence to Ac-HMQLYF-NH_2, it did not produce changes in intracellular calcium concentrations (Figure 3) nor did it increase [^{35}S]GTPγS incorporation (Figure 2). Therefore, the cause of these changes in calcium concentrations

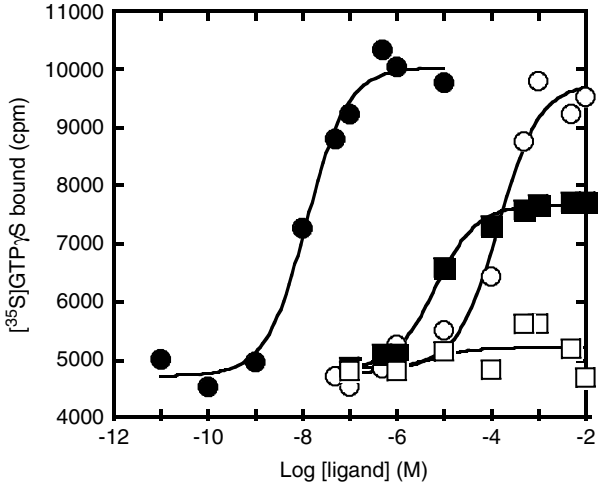

Figure 2 Simulation of [^{35}S]GTPγS binding to an OXER–Gi1α fusion protein by the peptides obtained and 5-oxo-ETE. The membrane expressing an OXER–Gi1α (20 μg of protein) was incubated with the indicated concentrations of ligands (closed circles, 5-oxo-ETE; open circles, Ac-HMQLYF-NH_2; closed squares, Ac-HMWLYF-NH_2; open squares, Ac-HMQLDF-NH_2) at 30 °C for 30 min in 100 μl of 20 mM HEPES-KOH (pH 8.0), 1 mM EDTA, 160 mM NaCl, 1 mM DTT, 100 pM [^{35}S]GTPγS, 1 μM GDP, and 10 mM $MgCl_2$ on 96-well microplates. The membranes were trapped on a GF/B glass filter, which was washed three times with 300 μl of cold 20 mM potassium phosphate buffer (pH 7.0). Radioactivity was then measured with a liquid scintillation counter (LC6500, Beckman Coulter). The EC_{50} values of Ac-HMQLYF-NH_2 and Ac-HMWLYF-NH_2 were estimated to be 6.7 μM and 146 μM, respectively.

can be attributed to the activation of an OXER. The chemotaxis of the CHO cells expressing an OXER was also examined using the Boyden chamber. Cell migration was induced by not only 100 nM of 5-oxo-ETE but also 100 μM of Ac-HMQLYF-NH_2 or Ac-HMWLYF-NH_2 (Figure 4). The cells did not migrate to Ac-HMQLDF-NH_2. This confirms that the migration of cells on the filter represents the chemotaxis toward the attractants mediated by an OXER.

DISCUSSION

The efficient technical development of GPCR ligand identification is crucial to identifying novel clinical drugs. Peptides are nonhazardous endogenous chemical compounds that can exhibit a wide diversity. Since methods of peptide synthesis have already been firmly established, peptides are suitable for the preparation of a combinatorial library. Doory *et al.* reported all of the D-amino acid opioid peptides in 1994, and also published several reports on the identification of novel peptide ligands for opioid-related receptors [14]. Their peptide library and screening strategy were very powerful and had great potential for a wide range of applications. They also showed

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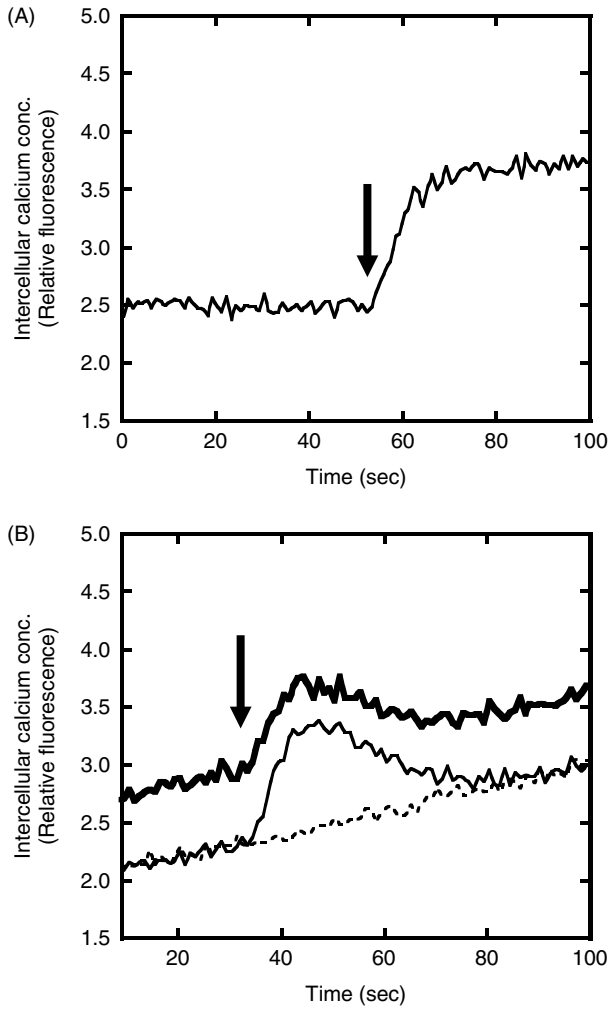

Figure 3 Intracellular calcium ion levels were measured using the calcium ion-sensitive fluorescence dye Fura-2. CHO cells stably expressing an OXER were suspended in 1.25 mM of probenecid, 0.02% F-127, and 2.5 μM of Fura-2/AM at a final concentration of $1–5 \times 10^6$ cells/ml and incubated at 37°C in 5% CO_2 for 1 h. The cell suspension (600 μl) was stimulated by adding 6 μl of 5-oxo-ETE (A; final concentration of 1 μM), Ac-HMQLYF-NH_2 (B; bold line, 100 μM), Ac-HMWLYF-NH_2 (B; thin line, 100 μM), and Ac-HMQLDF-NH_2 (B; dotted line, 100 μM) at the time denoted by the arrow. Then, the fluorescence was measured using a RF-5300PC spectrofluorometer (Shimadzu). The mock transfected CHO cells did not produce any signals with addition of these three peptides at the same concentrations (data not shown).

that a hexameric peptide library included various potential ligand candidates and had sufficient diversity to provide interesting ligands for several GPCRs [2]. Therefore, the same type of hexapeptide library was used here to obtain OXER peptide agonists since this hexapeptide library system has already provided valuable results in previous experiments [2].

Several reports successfully found new bioactive peptides, but their strategies were adapted to limited GPCRs. The most serious problem with such methods is the requirement for a highly specific radioligand that has a very low nonspecific binding. In the methods used in those reports, peptide activity was measured by decreasing the radio count resulting from the inhibition of authentic radioligand binding for a target GPCR. For authentic peptide ligands, radioligands labeled using ^3H or ^{125}I are usually easy to prepare. However, useful and specific radioactive compounds for an authentic amine or lipid ligand are not generally available. For example, radioactive 5-oxo-ETE could not be obtained commercially, so displacement assays for an OXER using synthetic peptides were not possible. In contrast, GPCR–Gα fusion proteins and [^{35}S]GTPγS binding assay were shown to be very powerful at identifying bioactive peptides from a random peptide library without using a specific radioligand. In other words, the peptide library and screening system are applicable for general GPCRs when using GPCR–Gα fusion proteins.

In the results of the first screening, the frequency distribution of activity revealed at least two peaks. The peak for the agonistic peptides (black bars in Figure 1) includes 75 fractions (18.75%) for Ac-OOXXXX-NH_2, 37 fractions (9.25%) for Ac-XXOOXX-NH_2, and 73 fractions (18.25%) for Ac-XXXXOO-NH_2. This result indicates that a remarkable number of peptides exhibited agonistic activity. In contrast, the second screening yielded only 2 peptides among the 41 synthesized peptides, namely, Ac-HMQLYF-NH_2 and Ac-HMWLYF-NH_2, which activated an OXER. Based on these results, there are assumed to be several sequence categories for agonist peptides, and sequences in the same category might share several residues and allow amino acid replacements. Therefore, each category may consist of several hundred sequence varieties and exhibit agonistic activity with a mixture of several different peptides in the first screening. A peptide was also assumed to consist of amino acid combinations consisting of sequences from different categories that were unable to express agonist activity, and accurate amino acids combinations were successfully matched only for Ac-HMQLYF-NH_2 and Ac-HMWLYF-NH_2 in the second screening.

A similar finding would be possible based on the EC_{50} results of Ac-HMQLYF-NH_2 and Ac-HMWLYF-NH_2. The EC_{50} value of Ac-HMQLYF-NH_2 was 146 μM, and the concentration of each peptide in the first screening was 40 nM. This result indicated that approximately 3650 types (146 μM/40 nM) of peptides similar to Ac-HMQLYF-NH_2 exhibited agonistic activity in the mixture in the first screening. There would also be approximately 167 types (6.7 μM/40 nM) of peptides that are similar to Ac-HMWLYF-NH_2. These results suggest that several hundred or thousand similar peptides could exhibit ligand activity in each sequence category. Furthermore, the entire discussion

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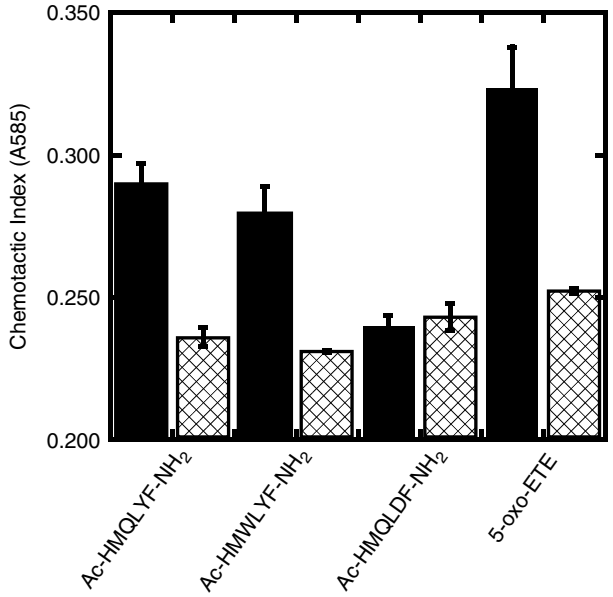

Figure 4 The cell migration was measured by using a Boyden chamber and a polycarbonate filter with 5 μm pores coated with fibronectin in the presence of 5-oxo-ETE (100 nM), Ac-HMQLYF-NH_2 (100 μM), and Ac-HMWLYF-NH_2 (100 μM) in the bottom chamber. The cells were incubated at 37 °C in 5% CO_2 for 4 h for migration. Then, the cells in the filter were stained, and their densities were estimated by measuring the optical densities at 595 nm using a 96-well microplate reader (Model 680, Bio-Rad). The black and hatched bars correspond to measurements using CHO cells stably expressing an OXER and wild-type CHO cells, respectively.

described above suggests the possibility that there would be other agonist peptide categories composed of totally different sequences than Ac-HMQLYF-NH_2 and Ac-HMWLYF-NH_2 since the first screening yielded several candidates for amino acid combinations with wide sequence varieties (Table 1). At the same time, amino acid sequence variations enabled the possibility of various ligand characteristics: full agonist for Ac-HMQLYF-NH_2, partial agonist for Ac-HMWLYF-NH_2, and no ligand activity for Ac-HMQLDF-NH_2 (Figure 2). An OXER is involved in chemotaxis for 5-oxo-ETE. The peptides Ac-HMQLYF-NH_2 and Ac-HMWLYF-NH_2 were confirmed to induce calcium mobilization (Figure 3) and cell migration toward an OXER (Figure 4) using stably expressing CHO cells. This calcium mobilization might be induced by $\beta\gamma$-subunits as well as several other Gi-coupled receptors. All the three independent assays provided direct evidence for the activity of these peptides. For the moment, the mechanisms by which these peptides activate an OXER are unknown. One possibility is that the agonistic peptides may mimic the 5-oxo-ETE structure and bind to an OXER similar to that of 5-oxo-ETE. Another possibility is that there is no single OXER activation mechanism and that agonistic peptides activated OXER via a different mechanism than 5-oxo-ETE. While the details on these activation mechanisms are still unclear, an undeniable fact is that the peptide library has a wide range of applications in the development of unique ligands.

Computer modeling of GPCRs and ligand-bound structures is currently proving useful in understanding the molecular events during receptor recognition and in designing new pharmacological ligands. Ishiguro proposed four ligand-bound structures for cationic biogenic amine receptors, namely, form I (putative inverse agonist bond), form II (antagonist bond), form III (partial agonist bond), and form IV (full agonist bond). These forms correspond to the structural models of the photointermediate, i.e. metarhodopsins I, Ib (opsin), I_{380} (a rhodopsin mutant), and II, respectively [15]. This modeling strategy was also extended to peptide receptors, and ligand-bound nociceptin receptor structures have been modeled [16]. A nociceptin receptor is potently activated not only by nociceptin but also by a synthetic peptide, i.e. Ac-RYYRIK-NH_2, that was identified by Doorey *et al.* using a random peptide library [3]. These structural models indicate that nociceptin and Ac-RYYRIK-NH_2 form similar three-dimensional shapes in receptor-bound conformations, although their amino acid sequences are completely different [16]. This result clarifies why both nociceptin and Ac-RYYRIK-NH_2 can potently activate a nociceptin receptor. In a future study, a similar strategy will be applied to an OXER in order to understand ligand–receptor interactions. Improved ligands could be designed with higher affinity than Ac-HMQLYF-NH_2 and Ac-HMWLYF-NH_2 *in silico* and with a plane to synthesize new nonpeptide ligands for an OXER. GPCRs for fatty acid derivatives (for example, GPR40 and S1P receptors) can now be modeled and discussed as part of computer-aided drug design [17,18]. The current investigation should prove helpful in developing a general method for drug design.

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