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The Receptor-Gα Fusion Protein as a Tool for Ligand Screening: a Model Study Using a Nociceptin Receptor-$G\alpha_{i2}$ Fusion Protein

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As a model system to screen endogenous ligands for G_i-coupled receptors, we have prepared and characterized a fusion protein of nociceptin receptor and α subunit of G_{i2}. We detected nociceptin binding to the fusion protein by measuring stimulation of [^{35}S]GTPγS binding with an EC_{50} of 2.0 nM and a gain of approximately five times. The stimulation by nociceptin of [^{35}S]GTPγS binding to the fusion protein was clearly observed in the presence of an appropriate concentration of GDP, because the affinity for GDP was decreased in the presence of agonist. Full and partial agonists differed in their effects on apparent the affinity of the fusion protein for GDP: the IC_{50} values for GDP to displace 100 pM [^{35}S]GTPγS were estimated to be 2 μM, 0.4 μM, and 0.05 μM in the presence of full agonist (nociceptin), partial agonist (F/G-NC), and antagonist (NBZH), respectively. We also detected the activity to stimulate [^{35}S]GTPγS binding to the fusion protein in the brain extract derived from 2–3 g wet weight tissue without false-positive results. The active component was identified as endogenous nociceptin itself. These results indicate that the fusion protein of GPCR and $G\alpha_i$ is useful for screening of endogenous ligands.

Key words: endogenous ligands, G protein-coupled receptor, high-throughput screening, nociceptin.

Abbreviations: CHO, Chinese hamster ovary; F/G-NC, [Phe^{1}Ψ(CH_2-NH)Gly^2]nociceptin(1–13)NH_2; $G\alpha_{i2}$, α subunit of inhibitory GTP binding regulatory protien (G_{i2}); GPCR, G protein-coupled receptor; G proten, heterotrimeric GTP binding regulatory protein; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate); MALDI-TOF, matrix-assisted laser desorption ionization; MS, mass spectrometric; MS/MS, tandem mass spectrometric; NBZH, naloxone benzoylhydrazone; NR, nociceptin receptor; NR-$G\alpha_{i2}$, a fusion protein of rat nociceptin receptor and bovine G_{i2} protein α subunit; TFA, trifluoroacetic acid.

G protein-coupled receptors (GPCRs) are integral components of the cell surface membrane that receive extracellular signals and interact with and activate G proteins. Binding of an agonist to the receptor induces formation of a complex comprising a ligand, the receptor, and a G protein, followed by nucleotide exchange from GDP to GTP on the G protein α subunits. The complex then dissociates to the GTP-bound α-subunit (α_{GTP}), βγ-complex, and receptor. The α_{GTP} and βγ subunits both interact with downstream members of the signal cascades and initiate activation or inhibition of various effector enzymes and/or ion channels (*1–4*). GPCRs constitute one of the largest protein superfamilies. Approximately 900 distinct GPCRs including 350 for endogenous ligands have been identified from human genome analysis (*5–7*). Natural ligands for many GPCRs and their functions remain unknown. These "orphan GPCRs" and their ligands are expected to be involved in unique biological events that have yet to be investigated and are potential targets for novel drug discovery. Development of new subtype-specific ligands for known GPCRs is also a key step in elucidating receptor functions and developing new drugs. Thus, screening many thousands of samples for each target GPCR is now routine practice to identify novel ligands or drugs in pharmacological research. This situation promoted the development of excellent assay systems that are suitable for high-throughput screening.

The high-throughput screening of GPCR ligands has generally been based on a cell-based assay system that monitors downstream events of signal cascades. The representative downstream events for G_s- and G_q-coupled receptors are increase in intercellular cAMP concentrations and Ca^{2+} concentration, respectively. Thus, measurements of cAMP and Ca^{2+} are now the most popular assay systems available for GPCRs. On the other hand, satisfactory high-throughput assay systems have not yet been developed for G_i/G_o-coupled receptors. Inhibition of cAMP formation by activation of G_i/G_o-coupled receptors is not very suitable for ligand screening, since the extent

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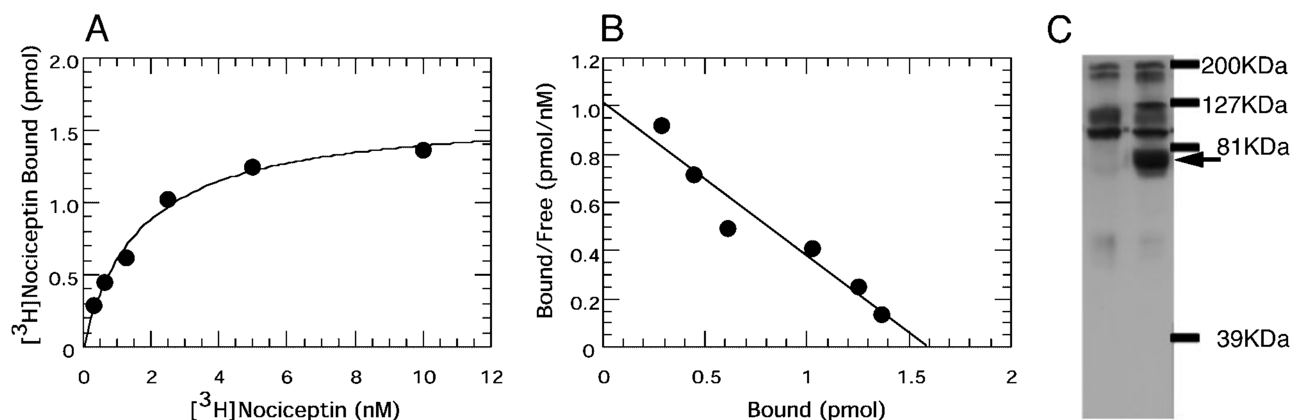

Fig. 1. **Binding of [^{3}H]nociceptin to the fusion protein (NR-Gα_{i2}).** A reaction mixture (1 ml) containing the membrane preparation expressing NR-$G_{i2}\alpha$ (100 µg protein), [^{3}H]nociceptin ranging from 0.3 to 10 nM (4.5 Ci/mmol), 50 mM Tris-HCl (pH 7.5), and 5 mM $MgCl_2$ was incubated at 30°C for 1 h. The membrane was collected on a GF/B filter and washed three times with 2 ml each of 20 mM potassium phosphate buffer (pH 7.0), then radioactivity was counted. (A) The ordinate shows the specific binding, as determined by subtracting non-specific binding from the total binding, which were measured in the absence and presence of 2 µM unlabeled nociceptin. (B) The dissociation constant (K_d) for [^{3}H]nociceptin was determined by Scatchard analysis. (C) Western blotting of the Sf9 membrane expressing NR-$G_{i2}\alpha$ using anti- $G_i\alpha$ The left and right lanes correspond to membrane fractions prepared from non-infected Sf9 cells and Sf9 cells with expression virus, respectively. The bars at right show positions of molecular weight markers. The arrow undicates an NR-$G_{i2}\alpha$ fusion protein.

of inhibition does not exceed 60%. The promiscuous G protein G_{16} and chimera of G_q and G_i (*8*) can transfer G_i-activated signals to G_q-activated signals and are utilized in ligand screening for G_i/G_o-coupled receptors (*9*, *10*). Screening systems using melanophore cells have also been proposed, in which pigmentation of cells is induced by receptor activation (*11*, *12*). These cell-based assay systems were applied to several receptors and contributed to the finding of novel ligands, nociceptin (*13*, *14*), orexin (*15*), and ghrelin (*16*). However, these cell-based assays have the disadvantage that endogenous receptors on host cells respond to their ligands resulting in false-positive signals. Assay protocols that directly estimate G protein activation by a target receptor *in vitro* may constitute a simple screening system that avoids such false-positive reactions in high-throughput screening.

Nociceptin and a nociceptin receptor (NR), which have structural similarities to opioid peptides and opioid receptors, respectively, have been found to constitute a novel neuromodulatory system (*14*, *15*). Nociceptin was shown to have the potential to modulate the spinal nociceptive event (*14*, *17*) and a possible functional role in memory modulation in brain (*18*). Although there are a few reports of non-natural ligands for NR (*19–23*), their clinical application has not been reported. NR is, therefore, now attracting the attention of many pharmacologists as a target for novel drug discovery (*24*). We planned to construct a new ligand screening system and apply it to NR. The fusion protein between a GPCR and its partner G protein α subunit has been shown to be useful for studies of receptor-G protein interactions because of their efficient coupling (*25–29*). Thus, the receptor-Gα fusion protein could be useful candidate for a new screening system.

The aim of this paper is to examine if the receptor-Gα fusion protein can be used for screening of endogenous ligands for orphan GPCRs. The capability of the system may depend on the level of endogenous ligand, the sensitivity and selectivity of the system, and the level of endogenous substances causing false positive responses. There is no way to examine these factors for orphan GPCRs, for which endogenous or surrogate ligands are not known. Using a nociceptin receptor-Gα_{i2} fusion protein (NR-Gα_{i2}) as a model system, we have examined whether this system is sensitive enough to detect endogenous nociceptin and specific enough for possible false positive responses to be neglected. Here we successfully detected a nanomolar range of endogenous nociceptin in only 2–3 g of wet brain without appreciable false-positive response. These results suggest the potential usefulness and advantage of receptor-Gα fusion proteins for screening of endogenous ligands for orphan GPCRs.

EXPERIMENTAL PROCEDURES

Expression of NR-Gα_{i2}—The cDNAs encoding rat NR and bovine $G_{i2}\alpha$ subunit were kindly provided by Prof. Takeshima (Tohoku University) (*30*) and Dr. Nukada (Tokyo Institute of Psychiatry) (*31*), respectively. To amplify and fuse both genes, three steps of PCR were performed with KOD polymerase (Toyobo, Osaka) as follows. First, to amplify nociceptin receptor and Gα_{i2} genes, 20 cycles of PCR reaction was performed with primers 5′-GCTCTAGAATGGAGTCCCTCTTTCCTGCT-3′ and 5′-TGCTGGCCGTGGTACTGTC-3′ for an NR gene, and 5′-<u>CTTCTGAGACAGTACCACGGCCAGCA</u>ATGGGCTGCA-CCGTGAGC-3′ and 5′-CCGCGTCGACTCAGAAGAGGC-CGCAGTCCT-3′ for a $G_{i2}\alpha$ gene. Temperature program was 94°C for 30 s, 50°C for 30 s, and 74°C for 60 s. The forward primer for a Gα_{i2} gene contained 26 base pairs coding the C-terminus of NR, which is underlined above. Next, to fuse both genes, 5 cycles of reactions were carried out with the PCR products obtained above but without primers. Temperature program was 94°C for 30 s, 60°C for 30 s, and 74°C for 60 s. Finally, a fused gene was

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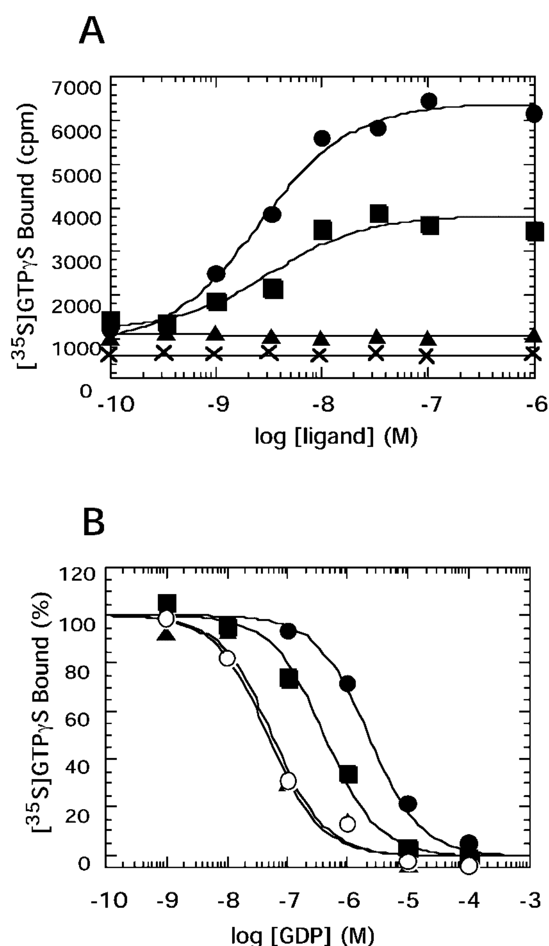

Fig. 2. **(A) Stimulation by nociceptin and other ligands of [^{35}S]GTPγS binding to NR-Gα_{i2}.** The membrane preparations expressing NR-Gα_{i2} (20 µg of protein) were incubated with indicated concentrations of ligands [circles, nociceptin (full agonist), squares, F/G-NC (partial agonist), triangles, NBZH (antagonist)] 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 each of cold 20 mM potassium phosphate buffer (pH 7.0). Radioactivity was then counted with liquid scintillation counter. The same assay in the presence of nociceptin using membrane fractions prepared from non-infected Sf9 cells with expression virus showed no stimulation (crosses). (B) Displacement by GDP of [^{35}S]GTPγS binding to NR-$G_{i2}\alpha$. The same assay was performed in the presence of 1 µM nociceptin (closed circles), F/G-NC (squares), NBZH (triangles), and in the absence of ligand (open circles) under the same conditions as in (A) except for the concentrations of guanine nucleotides.

amplified with primers 5′-GCTCTAGAATGGAGTCCCT-CTTTCCTGCT-3′ (a forward primer for an NR gene) and 5′-CCGCGTCGACTCAGAAGAGGCCGCAGTCCT-3′ (a reverse primer for a $G_{i2}\alpha$ gene), with 20 cycles of 94°C for 30 s, 50°C for 30 s, and 74°C for 60 s. The NR-Gα_{i2} fusion gene was inserted in pBacPAK9 (Clontech, Palo Alto, USA) using *Xba*I and *Sal*I restriction sites and used to prepare recombinant baculovirus. The transfer vector constructed above and linearized baculovirus DNA were co-transfected into Sf9 cells, and the recombinant baculovirus produced was cloned by plaque isolation. Sf9 cells were grown at 28°C to a density of approximately 2–3 × 10^9 cells/liter culture, and then infected with the recombinant virus. After 48 h from infection, Sf9 cells were harvested and homogenized in a solution containing 50 mM HEPES-KOH (pH 7.0), 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 1 mM EDTA. Plasma membrane was precipitated by centrifugation at 50,000 rpm for 30 min (Type 50Ti, Hitachi, Tokyo) and suspended in the homogenizing buffer. The membrane suspension was stored at –80°C. Protein concentration was determined by using protein assay kits (Bio-Rad, Hercules, USA).

Ligand Binding Assay—[^{3}H]Nociceptin (45 Ci/mmole) was purchased from Amersham (Buckinghamshire, UK). Synthetic nociceptin was purchased from Peptide Institute (Osaka). The binding reaction was performed at 30°C for 1 h with the membrane suspension (15 µg of total protein) in a solution (1 ml) containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and various concentrations of [^{3}H]nociceptin (4.5 Ci/mmol). After incubation, membranes were collected on a glass fiber filter (GF/B, Whatman, Kent, UK) and washed with 20 mM potassium phosphate buffer (pH 7.0), then radioactivity was measured using a liquid scintillation counter (LS6500, Pachard, Meriden, USA). Non-specific binding was measured in the presence of an excess amount of unlabeled nociceptin (2 µM) and subtracted from total binding to obtain specific binding. The dissociation constant (K_d) for [^{3}H]nociceptin was determined by Scatchard analysis, and the apparent inhibitory constant (K_i) for non-labeled ligand was estimated from displacement of [^{3}H]nociceptin binding (3 nM) according to the equation $K_i = IC_{50}/(1 + S)$, where S is (concentration of [^{3}H]nociceptin)/(K_d for [^{3}H]nociceptin), and IC_{50} is the concentration of the ligand giving 50% displacement.

GTPγS Binding Assay—[^{35}S]GTPγS (1250 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, USA). The membrane preparation expressing NR-Gα_{i2} (20 µg of total protein) was incubated 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 $MgCl_2$ at 30°C for 30 min on 96-well microplates. In some experiments, the concentrations of GDP, membrane preparation, or ligands were varied. [^{35}S]GTPγS bound to the membrane was trapped on a GF/B glass fiber filter. The GF/B filter was washed three times with 300 µl each of cold 20 mM potassium phosphate buffer (pH 7.0), and counted with liquid scintillation counter (Top-count, Pachard).

Identification of Endogenous Activator of NR-Gα_{i2} in Porcine Brain Extract—Porcine brain extracts were prepared from 2 kg of the tissue as reported previously (*32*). In brief, 2 kg of porcine brain tissue other than cerebellum was minced and boiled in 10 liters of water for 10 min. After cooling, the boiled tissue was homogenized with a Polytron homogenizer, then acetic acid was added to final concentration of 1 M. This homogenized tissue was centrifuged at 7,000 rpm for 30 min, then filtered through a membrane with an exclusion limit of 30 K (Pellicon cassette PLTK, Millipore, Bedford, USA) to remove proteins. The filtrate was condensed by passage through a membrane with an exclusion limit of 1 K (Pellicon cassette PLAC, Millipore). The condensed filtrate was loaded onto a C_{18} column (1 liter), washed with 10% ace-

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tonitrile containing 0.1% trifluoroacetic acid (TFA), and eluted with 60% acetonitrile containing 0.1% TFA. The effluent was evaporated, then loaded on an SP-Sephadex C-25 column (5 × 25 cm, Amersham). Materials adsorbed on the column were successively eluted with 2 column volumes each of 1 M acetic acid, 2 M pyridine, and 2 M pyridine-acetate (pH 5.0), yielding respectively fractions SP-I, SP-II and SP-III. A weak activity to activate NR-$G\alpha_{i2}$ was detected in SP-II by [^{35}S]GTPγS binding assay, while a strong activity was found in SP-III. To clarify whether the activities in SP-II and SP-III were due to different substance, the two fractions were mixed and subjected to further purification. After lyophilization, half of the combined SP-II and SP-III fractions was applied to a Sephadex G-50 column (5 × 145 cm, Amersham). The apparent molecular size of eluates was estimated based on the elution positions of C-type natriuretic peptide (CNP)-53 and CNP-22, which were detected using radioimmunoassay. The peptide fractions with molecular weight of less than approximately 6K were pooled, lyophilized and applied to a Sephadex G-25 column (5 × 145 cm, Amersham). In all purification steps, an aliquot of each fraction was subjected to the [^{35}S]GTPγS binding assay with membranes expressing NR-$G\alpha_{i2}$. The fractions with the activity to stimulate [^{35}S]GTPγS binding were sequentially applied to ion exchange HPLC and reverse phase HPLCs (C_{18} and diphenyl). The ion exchange HPLC was performed on a TSK gel SP-2SW column (4.6 × 250 mm, Tosoh, Tokyo), which was eluted with a linear gradient of ammonium formate (pH 3.8) from 10 mM to 1 M in the presence of 10% acetonitrile at a flow rate of 1 ml/min over 120 min, and fractions were collected every 2 min. Active fractions obtained from the ion exchange HPLC were subjected to reverse phase HPLC on a C_{18} column (Symmetry 300A 5 μm C18, 4.6 × 250 mm, Waters, Milford, USA), which was eluted with a linear gradient of acetonitrile from 10% to 60% in the presence of 0.1% TFA at a flow rate of 1 ml/min over 60 min, and fractions were collected every 1 min. Finally, the active fractions obtained from the C_{18} reverse phase HPLC were subjected to reverse phase HPLC on a diphenyl column (219TP5115, 1.0 × 150 mm, Vydac, Hesperia, USA), which was eluted with a linear gradient of acetonitrile from 5% to 60% in the presence of 0.1% TFA at a flow rate of 50 μl/min over 60 min, and fractions were collected every 20 s. Aliquots of separated fractions (0.5 μl/fraction), which were equivalent to10 g of porcine brain tissue, were mixed with a solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/water 50:50 (v/v) containing 0.1% TFA on a matrix-assisted laser desorption ionization (MALDI) target plate, and submitted to MALDI time-of-flight (TOF) mass spectrometric (MS) analysis

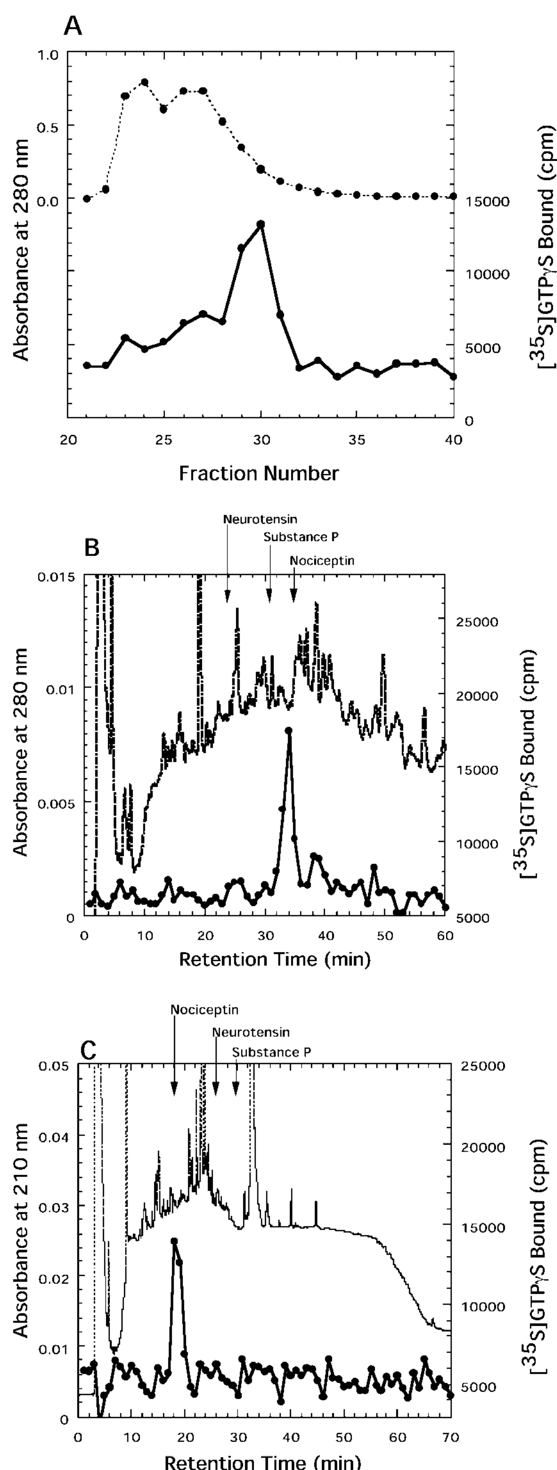

Fig. 3. **Identification of the active substance that stimulates [^{35}S]GTPγS binding to the NR-$G_{i2}\alpha$ in fractions of (A) Sephadex G-25 column chromatography (5 cm × 145 cm), (B) ion exchange chromatography (TSKgel SP-2SW, 4.6 mm × 250 mm, Tosoh), and (C) reversed-phase C_{18} HPLC (Symmetry 300 A 5μ C18, 4.6 × 250 mm, Waters).** For [^{35}S]GTPγS binding assay, a part of each fraction was lyophilized with 50 μg of bovine serum albumin, then dissolved in 100 μl of 20 mM HEPES-KOH (pH 8.0), 1 mM EDTA, and 160 mM NaCl. An aliquot (50 μl) of each fraction was assayed for [^{35}S]GTPγS binding under the conditions described in the legend to Fig. 2, using eluates from the column instead of synthetic ligands. The solvents used were 1 M acetic acid for the Sephadex G-25 column; 10 mM ammonium formate (pH3.8)/10% acetonitrile to 1 M ammonium formate (pH 3.8)/10% acetonitrile for the TSKgel SP-2SW column; and 10% acetonitrile/0.1% TFA to 60% acetonitrile/0.1% TFA, for the Symmetry 300A C_{18} column. CNP-53 and CNP-22 were detected in fraction 26 and 30 in (A), respectively, by radioimmunoassay. Retention times for authentic peptides (neurotensin, nociceptin, substance P) are inserted in (B) and (C). Solid and dotted lines are corresponded to [^{35}S]GTPγS binding activity and absorbance, respectively.

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with a Voyager-DE Pro mass spectrometer (Applied Biosystems, Foster City, USA). High-energy tandem mass spectrometric (MS/MS) analysis was performed with a 4700 Proteomics Analyzer (Applied Biosystems) in a TOF MS/MS reflector mode.

RESULTS

Characterization of NR-Gα_{i2}—NR-Gα_{i2} was expressed in cultured insect cells (Sf9 cells) using baculovirus. Membrane preparations expressing NR-Gα_{i2} were examined for binding of [^{3}H]nociceptin and [^{35}S]GTPγS. NR-Gα_{i2} was capable of binding [^{3}H]nociceptin, and Scatchard analysis yielded K_d of 1.6 ± 0.2 nM (Fig. 1), which was comparable to the value reported for NR expressed in CHO cells (*33*). Scatchard analysis also showed that Sf9 cells expressed NR-Gα_{i2} with a relatively high yield of 16 pmol/mg of total membrane protein. In [^{3}H]nociceptin displacement assay with cold ligands, K_i values for nociceptin (full agonist), [Phe$^{1}\Psi$(CH_2-NH)Gly^{2}]nociceptine(1-13)NH_2 (F/G-NC; partial agonist), and naloxone benzoylhydrazone (NBZH; antagonist) were 1.9 nM ± 0.4, 3.1 nM ± 0.8 and 9.2 nM ± 3.5, respectively, [average ± standard deviation (n = 3), data not shown]. Figure 2A shows effect of nociceptin and other ligands on the binding of [^{35}S]GTPγS to the membrane expressing NR-Gα_{i2} in the presence of 1 μM GDP. The extent of [^{35}S]GTPγS binding was approximately 5 times greater in the presence of nociceptin than in its absence. The EC_{50} value for nociceptin was estimated to be 2.0 ± 0.7 nM, which was essentially the same as the dissociation constant for [^{3}H]nociceptin, 1.6 nM. We also observed a stimulatory effect of F/G-NC with EC_{50} of 2.7 ± 0.8 nM, although the maximum response was smaller than that by nociceptin. NBZH did not stimulate the [^{35}S]GTPγS binding. These results are consistent with the previous reports that F/G-NC is a partial agonist (*34*) and NBZH an antagonist for nociceptin receptor (*33*).

We also examined effects of ligands on displacement of [^{35}S]GTPγS binding by guanine nucleotides. The displacement curves by GDP shifted to right in the presence of agonist, indicating that the affinity for GDP of NR-Gα_{i2} decreased by agonist binding (Fig. 2B). The full agonist, partial agonist, and antagonist differed in their effects on the apparent affinity of the fusion protein for GDP. The IC_{50} for GDP to displace [^{35}S]GTPγS was estimated to be 2, 0.4, and 0.05 μM in the presence of nociceptin (full agonist), F/G-NC (partial agonist), and NBZH (antagonist), respectively. These results indicated that the nociceptin-bound NR-Gα_{i2} had a lower affinity for GDP than the ligand-free or antagonist-bound NR-Gα_{i2}, and that the affinity for GDP of F/G-NC-bound NR-Gα_{i2} was intermediate. A similar shift was also observed in the displacement curves by GTP in the presence of full and partial agonists, although the extent of the shift was smaller than in the displacement by GDP. No shifts were observed for displacement by GTPγS with and without ligands (data not shown). These results indicate that the NR-Gα_{i2} provides a useful means to characterize ligand–receptor–Gα-guanine nucleotide interactions.

Detection of Endogenous Activator of NR-Gα_{i2} in Brain Extract—Activity to stimulate [^{35}S]GTPγS binding to the NR-Gα_{i2} was detected in the crude brain extract, and this activity decreased with the serial dilution of the extract (data not shown). From these results, 1.5 g of porcine brain (wet weight) was estimated to contain activity equivalent to approximately 3 pmol of nociceptin.

To identify the active substance, the peptide fraction was prepared from the porcine brain. This fraction was successively applied to gel filtration, ion exchange HPLC, and reverse phase HPLCs. The activity to stimulate binding of [^{35}S]GTPγS to the NR-Gα_{i2} was detected in several fractions corresponding to the relative molecular mass of nociceptin in the Sephadex G-25 column chromatography (Fig. 3A). This activity was clearly detected in fractions in which authentic nociceptin was eluted in the ion exchange and reverse HPLC results (Fig. 3, B and C). Finally, the active fraction was subjected to MS analysis. By the MALDI-TOF MS analysis, its molecular mass was determined to be 1808.09, which was almost the same as that determined for the synthetic nociceptin (FGGFTGARKSARKLANQ; 1807.98). By the high energy MS/MS analysis of the MS peak (M+H: 1809.09) of the purified fraction, a series of b ions (b2 corresponding to phenylalaninyl-glycine (FG): 205.10 (m/z); b3 corresponding to FGG: 262.12; b4 corresponding to FGGF: 409.33), y ions (y8 corresponding to SARKLANQ: 887.51 (m/z); y9 corresponding to KSARKLANQ: 1015.60; y10 corresponding to RKSARKLANQ: 1171.70; y11 corresponding to ARKSARKLANQ: 1242.74; y12 corresponding to GARKSARKLANQ: 1,299.76), and immonium ions [A: 44.0 (m/z); T: 74.06; F: 120.68, R: 129.11] were clearly observed in addition to several internal fragment ions specific for nociceptin. The synthetic nociceptin also yielded MS analysis data comparable to that of the natural peptide. Thus, the substance that stimulated [^{35}S]GTPγS binding was concluded to be nociceptin itself. These results also suggested that NR is activated only by nociceptin in the brain extract. The above estimate that 1.5 g of brain contained 3 pmol of nociceptin indicated the yield of the original purification procedure for nociceptin (*14*) was approximately 3%.

DISCUSSION

In this paper, we have reported the sensitivity and specificity of NR-Gα_{i2} in screening of ligands for NR. Many orphan GPCRs have recently been identified as the result of genetic analysis. Increasing efforts are being made to discover endogenous ligands for orphan GPCRs, not only because of thier biological interest but alsofor their potential as targets of drugs. At the same time, the development of subtype-specific ligands for known GPCRs is also required as a means to determine the physiological function of each subtype and for use as drugs with low side effects. Thus, several protocols have already been developed to find ligands for GPCRs and successfully applied to identify endogenous ligands for orphan GPCRs. For example, the activity of nociceptin in the brain extract was monitored by measuring the inhibition of forskolin-stimulated cAMP accumulation in cells expressing the receptor (*13*, *14*). This protocol is sensitive with EC_{50} of 1 nM for NR. A reporter gene assay was also applied to NR with EC_{50} of 0.8 nM (*35*). One of the most popular methods for ligand screening is measurement of intracellular Ca^{2+} concentrations in cells expressing a

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target receptor. EC_{50} for NR was 8 nM (*36*) by measuring nociceptin-stimulated Ca^{2+} increase in cells expressing a promiscuous G protein, G_{16}, or $G_{q/i}$ chimera that may connect G_i/G_o-coupled receptor to G_q-coupled signal cascades (*8*, *9*). The lower sensitivity of this method than the cAMP monitoring method may be due to weak coupling between NR and G_{16} or $G_{q/i}$. The most sensitive system, pH sensing of cells expressing NR, is reported to detect nociceptin with EC_{50} of 0.06 nM (*35*). Cells that express greater amounts of receptors can respond to lower concentrations of ligands, because effectors may be fully activated when a smaller fraction of receptors is bound with agonist. The EC_{50} of 2 nM for the [^{35}S]GTPγS binding assay using the NR-$G\alpha_{i2}$ is between those of the cAMP assay and Ca^{2+} assay. Our results indicate that NR-$G\alpha_{i2}$ fusion protein requires only 2–3 g of wet brain to detect stimulation of [^{35}S]GTPγS binding and is sensitive enough to be applicable for endogenous ligand identification.

The most important advantage of [^{35}S]GTPγS binding assay using the fusion protein over cell-based assays is that false-positive reactions are negligible. The cell-based assay systems are affected by different circumstances causing false-positive responses by endogenous receptors. As shown in Fig. 3 and results of MS analysis, the activity in the brain extract to stimulate [^{35}S]GTPγS binding was assigned to nociceptin itself. These results indicate that the receptor–Gα fusion protein system is useful for endogenous ligand screening because of its high specificity. Another benefit of the receptor–Gα fusion protein system is the high signal-noise ratio of roughly five in the presence and absence of agonist (Fig. 2). In contrast, the cAMP assay method has a low signal-ratio in the presence and absence of agonist, which rarely exceeds 50–100%. This feature may derive from the high expression of receptors in Sf9 cells. The density of NR-$G\alpha_{i2}$ in Sf9 cell membrane is 16 pmol/mg protein, which is approximately eight times higher than that of nociceptin receptors expressed in CHO cells (*33*). In addition, a large amount of membranes expressing a fusion protein can be prepared easily by using the baculovirus-Sf9 system. One liter of cultured Sf9 cells is estimated to be enough for more than 100,000 assays. It is, therefore, not difficult to use the same batch of membrane preparation with the same specific activity from start to end of a large-scale ligand screening for receptors. The [^{35}S]GTPγS binding assay for the receptor–Gα fusion protein system is simple and amenable to high-throughput screening by using 96-well microplates, as in cell-based ligand screening systems. In fact, the receptor for 5-oxo-eicosatetraenoic acid has been identified using the receptor-$G\alpha_i$ fusion system by both Hosoi *et al.* and us independently (*37*, *38*). We have also identified several surrogate ligands for orphan receptors by using receptor-Gα fusion protein from authentic chemical compound libraries (*39*). The [^{35}S]GTPγS assay using fusion protein may, therefore, have wide application for GPCR ligand screening.

Receptor-Gα fusion protein is also useful to analyze the interaction of a receptor and Gα. As shown in Fig. 2B, NR-$G\alpha_{i2}$ shows low, intermediate, and high affinity for GDP when it is bound with full agonist, partial agonist, and antagonist, respectively. It is reasonable to assume that the agonist-bound receptor in the fusion protein accelerates the dissociation of GDP from Gα, and that the partial agonist dose so to a lesser extent. This phenomenon was also observed for the fusion protein with $G\alpha_{i1}$ of muscarinic M_2 receptor, as described in detail in an accompanying paper (*40*).

In several receptor-G_i or -G_s fusion proteins, the agonist-bound receptors have been reported to effectively stimulate Gα (*26*, *27*, *41–46*). We have reported that the [^{35}S]GTPγS binding activity of Sf9 membrane preparations expressing receptor-$G\alpha_q$ or receptor-$G\alpha_{16}$ fusion proteins could not be effectively stimulated by agonists (*47*). But receptor-$G\alpha_{16}$ fusion proteins still appeared to be useful as means of ligand screening when they were expressed in cultured cells and the agonist-induced increase in intracellular Ca^{2+} or prostaglandin E_2 concentration was used as a monitor, as described in detail in annother accompanying paper (*48*).

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