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# Stimulation of Increases in Intracellular Calcium and Prostaglandin $E_2$ Generation in Chinese Hamster Ovary Cells Expressing Receptor-G$\alpha_{16}$ Fusion Proteins

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**We examined whether fusion proteins of G protein-coupled receptors with the α subunit of $G_{16}$ (G$\alpha_{16}$) could activate downstream signals. We expressed fusion proteins of $G_i$-coupled receptors, *i.e.* $CX_3C$ chemokine receptor 1 ($CX_3CR1$) and $M_2$ receptor, in Chinese hamster ovary cells. An agonist for $CX_3CR1$ induced greater increases in intracellular $Ca^{2+}$ and prostaglandin $E_2$ generation in cells expressing $CX_3CR1$-G$\alpha_{16}$ fusion protein than in cells expressing $CX_3CR1$ alone or both $CX_3CR1$ and G$\alpha_{16}$ separately. Similarly, agonist-induced prostaglandin $E_2$ generation was greater in cells expressing $M_2$-G$\alpha_{16}$ fusion protein than ones expressing $M_2$ alone or both $M_2$ and G$\alpha_{16}$ separately. In cells expressing fusion proteins with G$\alpha_{16}$ of $G_q$-coupled receptors, *i.e.* urotensin II receptor and $M_1$ receptor, the relevant agonists induced similar increases in intracellular $Ca^{2+}$ and prostaglandin $E_2$ generation as in ones expressing the receptor alone. In cells expressing urotensin II receptor-G$\alpha_{16}$ fusion protein, prostaglandin $E_2$ generation exhibited a lower $EC_{50}$ value than the intracellular $Ca^{2+}$ increase. These results indicate that agonist-stimulated receptor-G$\alpha_{16}$ fusion proteins are coupled to downstream signaling pathways, and suggest that receptor-G$\alpha_{16}$ fusion proteins may be useful for screening for ligands of orphan G protein-coupled receptors and $G_i$-coupled receptors.**

**Key words: Chinese hamster ovary (CHO) cell, G protein, G protein-coupled receptor, intracellular calcium, prostaglandin.**

Abbreviations: CHO, Chinese hamster ovary; $CX_3CR1$, $CX_3C$ chemokine receptor 1; Gα, α subunit of G protein; GPCR, G protein-coupled receptor; $PGE_2$, prostaglandin $E_2$; RT-PCR, reverse transcriptase-polymerase chain reaction; sFKN, soluble fractalkine; [$^{35}$S]GTPγS, guanosine 5′-*O*-(3-[$^{35}$S]thiotriphosphate).

G protein-coupled receptors (GPCRs) constitute one of the largest families of genes in the human genome, and are among the largest targets for drug development (*1–3*). Although a large number of GPCR genes have recently been cloned, the endogenous ligands for many of them have not yet been identified (*1–6*). Various assay systems have been employed to identify agonists or antagonists for GPCRs, but there is still no simple and general method for screening for ligands of $G_i$-coupled receptors and orphan GPCRs.

$G_{15}$ and $G_{16}$ are G proteins that are primarily found in hematopoietic cells (*7*, *8*). They have been shown to be promiscuously activated by different kinds of GPCRs and to activate phospholipase Cβ (*9*, *10*), although some GPCRs are reported not to activate $G_{15}$ or $G_{16}$ (*10*, *11*). Several ligands for orphan $G_i$-coupled receptors have been identified by using human embryonic kidney 293 cells expressing the α subunit of $G_{16}$ (G$\alpha_{16}$), indicating the usefulness of G$\alpha_{15}$ and G$\alpha_{16}$ (*12*, *13*).

Fusion proteins of GPCRs with Gα have been developed to study the interactions between GPCRs and G proteins (*14–16*). This strategy takes advantage of the physical proximity of GPCRs and Gα molecules in the cell and their 1:1 stoichiometry. GPCRs and Gα have been found to interact more efficiently when they are expressed as fused proteins than when they are separately expressed. Examples include fusion proteins of $\beta_2$-adrenoceptor with G$\alpha_s$ (*17*, *18*), IP prostanoid receptor with G$\alpha_s$ (*19*), adenosine $A_1$ receptor with G$\alpha_i$ (*20*), and Edg2 receptor with G$\alpha_{i1}$ (*21*). These findings suggested that fusion proteins of GPCR with G$\alpha_{16}$ may be utilized to screen ligands for GPCRs.

Previously we expressed fusion proteins with G$\alpha_{16}$ of muscarinic acetylcholine receptor $M_1$, $M_2$ subtypes ($M_1$ and $M_2$ receptors), and $\beta_2$-adrenoceptor, which are coupled to $G_q$, $G_i$, and $G_s$ family G proteins, respectively, in

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insect Sf9 cells as model systems, and examined the effects of agonists on these fusion proteins in membrane preparations (*22*). An agonist-dependent increase in guanosine 5′-*O*-(3-[$^{35}$S]thiotriphosphate) ([$^{35}$S]GTPγS) binding activity was detected for these GPCR-G$\alpha_{16}$ fusion proteins, but the extent of the effect was much less than that for fusion proteins with G$\alpha_{i1}$ or G$\alpha_{i2}$ of $G_i$-coupled receptors (*22* and unpublished data *41*). In the present study, we have examined if cells expressing GPCR-G$\alpha_{16}$ fusion proteins could be used as ligand screening systems. We constructed cDNAs encoding fusion proteins with G$\alpha_{16}$ for four GPCRs–$CX_3C$ chemokine receptor 1 ($CX_3CR1$), $M_2$ receptor, urotensin II receptor, and $M_1$ receptor. $CX_3CR1$ and $M_2$ receptor are GPCRs that are coupled to $G_i$ family G proteins (*23–26*), while urotensin II receptor and $M_1$ receptor are coupled to $G_q$ family G proteins (*25*, *27*, *28*). Since it is usually difficult to identify the species of G proteins coupled to a given orphan GPCR (*1*), a fusion protein of it with G$\alpha_{16}$ should be particularly useful for orphan GPCRs.

Agonist-stimulated increases in the intracellular $Ca^{2+}$ concentration in cultured cells expressing the relevant GPCRs are most commonly utilized to screen for ligands of GPCRs. $G_{16}$ or a $G_{q/i}$ chimera is coexpressed for $G_i$-coupled receptors, because intracellular $Ca^{2+}$ increases through endogenous $G_i$ family G proteins are usually difficult to detect (*2*, *3*, *5*). We have transfected plasmids encoding GPCR-G$\alpha_{16}$ fusion proteins into Chinese hamster ovary (CHO) cells, and assayed the agonist-induced intracellular $Ca^{2+}$ increases in these cells. We compared the results with those obtained by transfecting plasmids encoding the individual proteins. In addition, we measured agonist-stimulated prostaglandin $E_2$ ($PGE_2$) generation, since this assay method has been reported to be more sensitive as to the agonist concentration than agonist-stimulated inositoltrisphosphate formation (*29*).

## EXPERIMENTAL PROCEDURES

*cDNA Constructs*—A cDNA fragment encoding full-length $CX_3CR1$ (GenBank accession No. U04808) was isolated by hybridization screening of a rat brain cDNA library, and cloned into pBluescript SK(–) using a Lambda ZAP II Vector/Gigapack Cloning Kit (Stratagene, La Jolla, CA) according to standard techniques (*30*) and the manufacturer's instructions. To clone full-length urotensin II receptor cDNA (GenBank accession No. AB012210), rat urinary bladder poly $A^+$ RNA was prepared using TRIzol (Life Technologies, Grand Island, NY) and oligotex-dT 30 (Takara Shuzo, Shiga), reverse transcribed, and then amplified by PCR, and the PCR product was cloned into pCR2.1 using a TA cloning kit (Invitrogen, Carlsbad, CA).

Each of the cDNAs encoding $CX_3CR1$, urotensin II receptor, and G$\alpha_{16}$ was subcloned into the mammalian expression vector pEF-BOS (*31*) and the baculovirus transfer vector pFastBac1 (Life Technologies).

Plasmids encoding the fusion proteins $CX_3CR1$-G$\alpha_{i2}$ and $CX_3CR1$-G$\alpha_{16}$ were constructed through three PCR steps (*32*). The cDNA encoding rat $CX_3CR1$ from which the stop codon had been removed was PCR-amplified from pBluescript-$CX_3CR1$. Bovine G$\alpha_{i2}$ and human G$\alpha_{16}$ cDNAs were PCR-amplified separately by adding to their 5′ ends a 24-bp sequence corresponding to the 3′ end of the $CX_3CR1$ cDNA from which the stop codon had been removed. $CX_3CR1$ cDNA was subsequently fused to G$\alpha_{i2}$ or G$\alpha_{16}$ cDNA in a second PCR reaction, and the fusion products were amplified in a third PCR reaction. The PCR products were subcloned into pEF-BOS and the baculovirus transfer vector pBacPAK8 (CLONTECH Laboratories, Palo Alto, CA).

Urotensin II receptor cDNA was fused to G$\alpha_{i2}$ or G$\alpha_{16}$ cDNA according to the same protocol. These PCR products were also subcloned into pEF-BOS and pBacPAK8. cDNAs encoding the $M_1$-G$\alpha_{16}$ and $M_2$-G$\alpha_{16}$ fusion proteins were PCR-amplified from pFastBac1-$M_1$-G$\alpha_{16}$ and pFastBac1-$M_2$-G$\alpha_{16}$ (*22*), respectively, and then ligated into pEF-BOS. All constructs were verified by restriction endonuclease mapping and DNA sequencing using an ABI PRISM 377 (PE Applied Biosystems, Foster City, CA).

*Transfection and Cell Culture*—CHO-K1 cells (Health Science Research Resources Bank, Osaka) were maintained in Ham's F-12 medium (Sigma, St. Louis, MO), supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS) and penicillin/streptomycin (50 U/ml and 50 μg/ml, respectively; Life Technologies), at 37°C under a humidified atmosphere of 5% $CO_2$/95% air. For transient transfection, cells and plasmids were incubated with LipofectAMINE 2000 (Invitrogen) for 42–48 h, after which the stimulants were added. For stable transfection, the constructed plasmids were cotransfected with pEF-neo, which confers neomycin resistance on the cells, using a TransFast kit (Promega, Madison, WI). After 48 h, the cells were incubated in medium containing 400 μg/ml G418 (Nacalai Tesque, Kyoto) for 10 days, and G418-resistant clones were screened for expression of the relevant mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR), and selected according to intracellular $Ca2^+$ mobilization induced by the appropriate agonist. Of these clones, those exhibiting the highest $Ca^{2+}$ increases were used for the subsequent assay.

*Sf9 Membrane Preparation and [$^{35}$S]GTPγS Binding Assay*—Sf9 cells were infected with recombinant baculoviruses, cultured for 72 h, and then harvested as previously described (*22*). Membrane fractions were prepared by homogenization in 20 mM HEPES-KOH buffer (pH 8.0), 1 mM EDTA, 2 mM $MgCl_2$, 0.5 mM phenylmethylsulphonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 5 mM benzamidine, using a Dounce homogenizer, and then centrifuged at 150,000 ×*g* for 30 min at 4°C. The pellets were homogenized in the same buffer and stored in aliquots at –80°C. Binding assays were performed as described with some modifications (*22*). Briefly, membrane fractions containing 10 μg protein were incubated with 100 pM [$^{35}$S]GTPγS (DuPont-New England Nuclear, Boston, MA), various concentrations of GDP, and relevant agonists at 30°C for 30 min in 0.1 ml of 20 mM HEPES-NaOH buffer (pH 7.4), 1 mM EDTA, 1 mM DTT, 100 mM NaCl, and 10 mM $MgCl_2$. Reactions were terminated by filtration through GF/B Unifilter plates (Packard, Meriden, CT), followed by rinsing three times with cold 20 mM potassium phosphate buffer (pH 7.0) in a cell harvester. Radioactivity was measured with a liquid scintillation counter (TopCount; Packard).

*Intracellular $Ca^{2+}$ Measurement*—Confluent CHO cells in a 96-well plate (Packard) were incubated in Hank's balanced salt solution (Life Technologies) containing 5 μM fura-2/AM (Molecular Probes, Eugene, OR) for 2 h at room temperature in the dark. After rinsing with Hank's balanced salt solution, the cells were incubated with an appropriate agonist, and the changes in intracellular $Ca^{2+}$-dependent fluorescence were recorded using a fluorescence imaging plate reader (FDSS; Hamamatsu Photonics, Shizuoka). Fura-2 fluorescence was measured with excitation at 340 nm and 380 nm, and emission at 510 nm. The fluorescence intensity ratio, $I_{340}/I_{380}$, was utilized to determine intracellular $Ca^{2+}$ mobilization.

*$PGE_2$ Determination*—Measurement of $PGE_2$ generation was performed as previously described (*33*). Briefly, confluent CHO cells grown in 24-well plates (Costar, Corning, NY) were washed once with 0.5 ml of culture medium. The experiments were initiated by adding 0.19 ml of culture medium containing various stimulants. The cells were incubated for 4 h or the times indicated at 37°C under a humidified atmosphere of 5% $CO_2$/95% air. Subsequently, supernatants of the cells were collected and $PGE_2$ generation was measured using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

*Semi-Quantitative RT-PCR*—Total cellular RNA was isolated using TRIzol, followed by treatment with DNase I. The RNA was reverse-transcribed using an oligo (dT) primer and then incubated with *Tth* RNaseH. For PCR amplification, $CX_3CR1$ and $G\alpha_{16}$ primers were designed to amplify the full-length coding region. As controls, we utilized primers for glyceraldehyde-3-phosphate dehydrogenase (sense, 5′-ATGACCCCTTCATTGACCT-3′; and antisense, 5′-GTTGGGGGTAGGAACAC-3′). PCR was performed for 20, 25, 30 and 35 cycles. Aliquots of the PCR products were analyzed by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. The intensity of the bands at various cycle numbers was compared for each primer pair.

*Ligand Binding Assaying of $CX_3CR1$*—Confluent cells cultured in 24-well plates were washed twice with Dulbecco's phosphate-buffered saline (Sigma) and then incubated with 1–100 nM [$^{125}$I] soluble fractalkine (sFKN) (specific activity, 2,000 Ci/mmol; [$^{125}$I] human fractalkine chemokine domain; Amersham Pharmacia Biotech, Buckinghamshire, England) in 50 mM HEPES-NaOH buffer (pH 7.4), 150 mM NaCl, 5 mM $MnCl_2$, and 0.01% (w/v) BSA, for 1 h at 37°C. After washing twice with phosphate-buffered saline, 1% Triton X-100 and 1 M NaOH were added sequentially to lyse the cells. The radioactivity bound to the cells was counted with a γ-counter (Cobra; Packard). Specific binding was determined by subtracting the binding of parental CHO cells from that of transfected CHO cells.

*Materials*—Plasmids pEF-BOS-human $M_1$ and pEF-BOS-human $M_2$ were donated by Drs. A. Fukuzaki and H. Tsuga, respectively (*34*). $G\beta_1\gamma_2$ recombinant viruses were donated by Dr. F. Nakamura (*35*). Recombinant human sFKN and human urotensin II were purchased from R&D Systems (Minneapolis, MN) and the Peptide Institute (Osaka), respectively. Enzymes for construction of cDNAs were purchased from Toyobo (Osaka), Takara Shuzo, Promega, New England Biolabs (Beverly, MA), Life Technologies, and Boehringer Mannheim (Mannheim, Germany). Oligonucleotide primers were purchased from Japan Bio Service (Saitama).

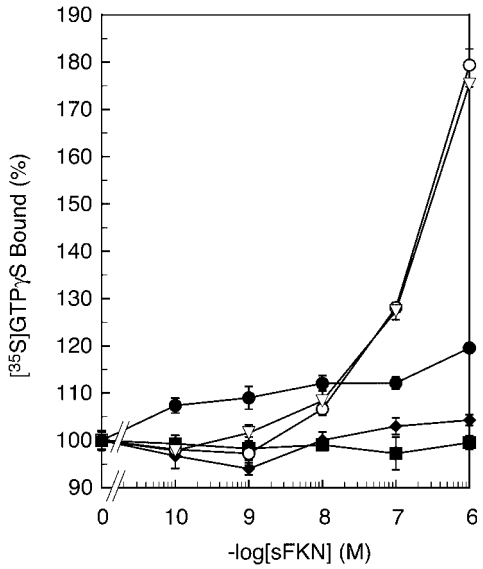


Fig. 1. **sFKN-induced [$^{35}$S]GTPγS binding to Sf9 cell membranes.** [$^{35}$S]GTPγS binding to membranes expressing $CX_3CR1$ (closed squares), $CX_3CR1$-$G\alpha_{i2}$ (open circles), $CX_3CR1$-$G\alpha_{16}$ (closed circles), and $CX_3CR1$-$G\alpha_{i2}$ plus $G\beta_1\gamma_2$ (inverted open triangles), and parental Sf9 membranes (closed diamonds) was measured in the presence of $10^{-6}$ M GDP as described under "EXPERIMENTAL PROCEDURES." Changes in [$^{35}$S]GTPγS binding are shown relative to the respective [$^{35}$S]GTPγS binding in the absence of the agonist. In the absence of the agonist, [$^{35}$S]GTPγS binding to membranes expressing $CX_3CR1$, $CX_3CR1$-$G\alpha_{i2}$, $CX_3CR1$-$G\alpha_{16}$ or $CX_3CR1$-$G\alpha_{i2}$ plus $G\beta_1\gamma_2$, and parental Sf9 membranes was 1,499 ± 17 cpm, 1,836 ± 39 cpm, 1,155 ± 13 cpm, 4,465 ± 86 cpm, and 2,369 ± 44 cpm, respectively. Each point represents the mean ± SEM of three determinations ($n$ = 3), and is representative of at least three independent experiments.

## RESULTS

*$CX_3CR1$-$G\alpha_{16}$ Fusion Protein*—We utilized PCR to ligate $CX_3CR1$ cDNA to $G\alpha_{i2}$ or $G\alpha_{16}$ cDNA, and expressed $CX_3CR1$-$G\alpha_{i2}$ and $CX_3CR1$-$G\alpha_{16}$ fusion proteins in Sf9 cells. The interaction of GPCRs with Gα was determined by measuring the agonist-induced binding of nonhydrolyzable GTP analog [$^{35}$S]GTPγS to Gα in the presence of various concentrations of GDP. Using the 8.5 kDa chemokine domain of fractalkine (sFKN) as a $CX_3CR1$-agonist, we observed agonist-dependent [$^{35}$S]GTPγS binding to Sf9 cell membranes expressing $CX_3CR1$-$G\alpha_{i2}$, indicating that the fused $CX_3CR1$ and $G\alpha_{i2}$ are functionally coupled (Fig. 1). The $EC_{50}$ was estimated to be $>2 \times 10^{-7}$ M. In contrast, sFKN did not affect [$^{35}$S]GTPγS binding to membranes expressing either $CX_3CR1$-$G\alpha_{16}$ or $CX_3CR1$ alone. Coexpression of $G\beta_1\gamma_2$ subunits did not affect the sFKN-induced [$^{35}$S]GTPγS binding to either $CX_3CR1$-$G\alpha_{i2}$ or $CX_3CR1$-$G\alpha_{16}$.

To determine if these fusion proteins can activate downstream signals, $CX_3CR1$-$G\alpha_{i2}$ and $CX_3CR1$-$G\alpha_{16}$ as well as $CX_3CR1$ or $CX_3CR1$ plus $G\alpha_{16}$ were stably expressed in CHO cells, and the expression of $CX_3CR1$ and $G\alpha_{16}$ was confirmed by semi-quantitative RT-PCR. In

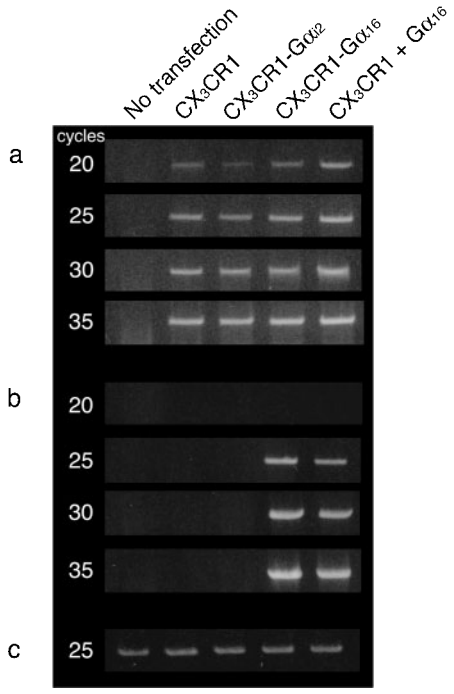


Fig. 2. **Expression of $CX_3CR1$ and $G\alpha_{16}$ mRNA in stably transfected CHO cells.** Cells were stably transfected with a plasmid encoding $CX_3CR1$, $CX_3CR1$-$G\alpha_{i2}$, $CX_3CR1$-$G\alpha_{16}$ or $CX_3CR1$ plus $G\alpha_{16}$, and then mRNA levels were determined by semi-quantitative RT-PCR using gene-specific primers for $CX_3CR1$ (a), $G\alpha_{16}$ (b), and glyceraldehyde-3-phosphate dehydrogenase (c). The number of PCR cycles is indicated on the left of each gel. Representative gels of three experiments with similar results are shown.

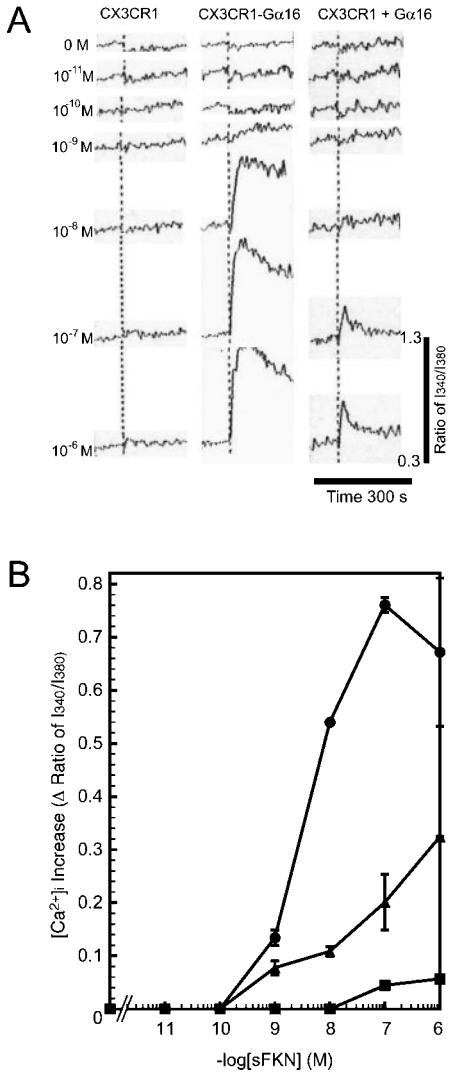


Fig. 3. **$Ca^{2+}$ mobilization in stably transfected CHO cells.** Cells stably transfected with a plasmid encoding $CX_3CR1$, $CX_3CR1$-$G\alpha_{16}$ or $CX_3CR1$ plus $G\alpha_{16}$ were loaded with 5 μM fura-2, and then sFKN-induced changes in intracellular $Ca^{2+}$ were measured using FDSS. (A) Dose-dependent intracellular $Ca^{2+}$ responses in cells expressing $CX_3CR1$ alone, $CX_3CR1$-$G\alpha_{16}$ fusion protein or $CX_3CR1$ plus $G\alpha_{16}$. sFKN concentrations are shown on the left. Changes in intracellular $Ca^{2+}$ are expressed as $I_{340}/I_{380}$. Vertical dotted lines indicate the time of addition of sFKN. Each tracing is representative of three independent experiments performed in triplicate. (B) Dose-response curves of intracellular $Ca^{2+}$ mobilization. Cells expressing $CX_3CR1$ (closed squares), $CX_3CR1$-$G\alpha_{16}$ (closed circles), or $CX_3CR1$ plus $G\alpha_{16}$ (closed triangles) were incubated with sFKN, and then intracellular $Ca^{2+}$ increases were measured as $\Delta I_{340}/I_{380}$, defined as the difference of $I_{340}/I_{380}$ in the presence and absence of the ligand. No intracellular $Ca^{2+}$ increase was induced by the addition of sFKN to the parental CHO cells. Each point represents the mean ± SEM of three determinations (n = 3), and is representative of at least three independent experiments.

CHO cells transfected with a plasmid encoding $CX_3CR1$, $CX_3CR1$-$G\alpha_{i2}$, $CX_3CR1$-$G\alpha_{16}$ or $CX_3CR1$ plus $G\alpha_{16}$, the level of $CX_3CR1$-specific mRNA was essentially the same (Fig. 2a). The amounts of expressed $CX_3CR1$ assessed as [$^{125}$I]sFKN binding sites were 0.41, 1.0, 0.61, and 0.54 pmol/mg total protein for cells transfected with $CX_3CR1$ alone, $CX_3CR1$-$G\alpha_{i2}$, $CX_3CR1$-$G\alpha_{16}$, and $CX_3CR1$ plus $G\alpha_{16}$, respectively. The expression level of $G\alpha_{16}$ mRNA was similar for cells transfected with $CX_3CR1$-$G\alpha_{16}$ and ones transfected with $CX_3CR1$ plus $G\alpha_{16}$ (Fig. 2b). This result was confirmed by immunostaining with antiserum directed against the C-terminus of $G\alpha_{16}$ (data not shown).

We examined whether or not sFKN can cause an increase in the intracellular $Ca^{2+}$ concentration in these cells. While sFKN had no effect on the $Ca^{2+}$ concentration in the parental CHO cells, it induced a slight increase in intracellular $Ca^{2+}$ followed by a decrease to the basal level within ~3 min in cells transfected with $CX_3CR1$ (Fig. 3A) or $CX_3CR1$-$G\alpha_{i2}$ (data not shown). In contrast, the addition of sFKN to cells expressing $CX_3CR1$-$G\alpha_{16}$ induced a robust, sustained increase in intracellular $Ca^{2+}$, with an $EC_{50}$ of approximately $3 \times 10^{-9}$ M (Fig. 3, A and B). In cells coexpressing $CX_3CR1$ and $G\alpha_{16}$, however, the sFKN-stimulated $Ca^{2+}$ increase was smaller and more transient than in ones expressing $CX_3CR1$-$G\alpha_{16}$ (Fig. 3, A and B). Moreover, most clones (15 out of 16) transfected with $CX_3CR1$-$G\alpha_{16}$ exhibited an agonist-induced $Ca^{2+}$ increase, while only a few clones (3 out of 12) cotransfected with $CX_3CR1$ plus $G\alpha_{16}$ exhibited an agonist-induced $Ca^{2+}$ increase. The reason for the low frequency of functional expression by the latter is not known, but it is unlikely to be due to a low expression level because 10 out of 12 clones were found to be positive for both $CX_3CR1$ and $G\alpha_{16}$ on RT-PCR. Various clones expressing $CX_3CR1$-$G\alpha_{16}$ showed similar $Ca^{2+}$ increases and $EC_{50}$ values, whereas clones coexpressing $CX_3CR1$

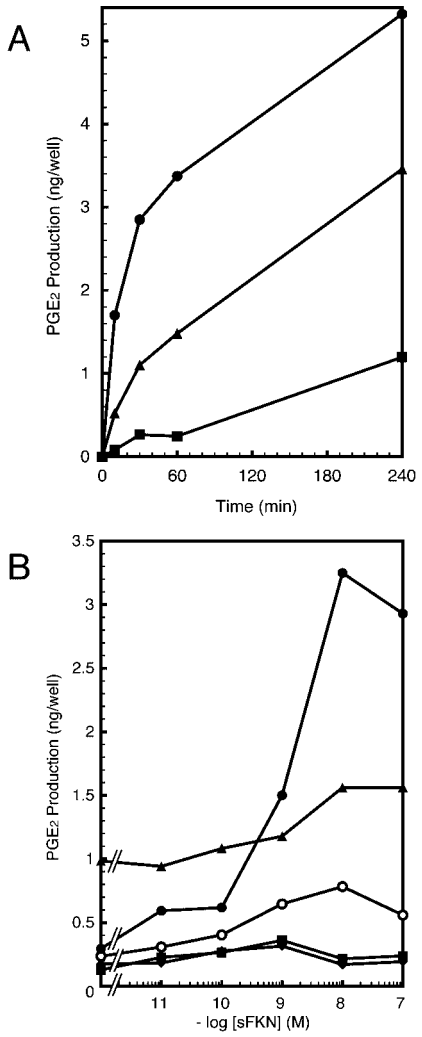


Fig. 4. **PGE$_2$ generation in stably transfected CHO cells.** (A) Time course of PGE$_2$ generation. Cells expressing CX$_3$CR1 (closed squares), CX$_3$CR1-Gα$_{16}$ (closed circles), or CX$_3$CR1 plus Gα$_{16}$ (closed triangles) were stimulated with $10^{-8}$ M sFKN for the times indicated, and then PGE$_2$ released into the culture supernatant was measured using a PGE$_2$-enzyme immunoassay kit. A representative result of three experiments is shown. (B) Dose-response curves of PGE$_2$ generation. Cells expressing CX$_3$CR1 (closed squares), CX$_3$CR1-Gα$_{i2}$ (open circles), CX$_3$CR1-Gα$_{16}$ (closed circles), or CX$_3$CR1 plus Gα$_{16}$ (closed triangles), and parental CHO cells (closed diamonds) were stimulated with various concentrations of sFKN for 4 h, and then PGE$_2$ released into the culture supernatant was measured. A representative result of three experiments is shown.

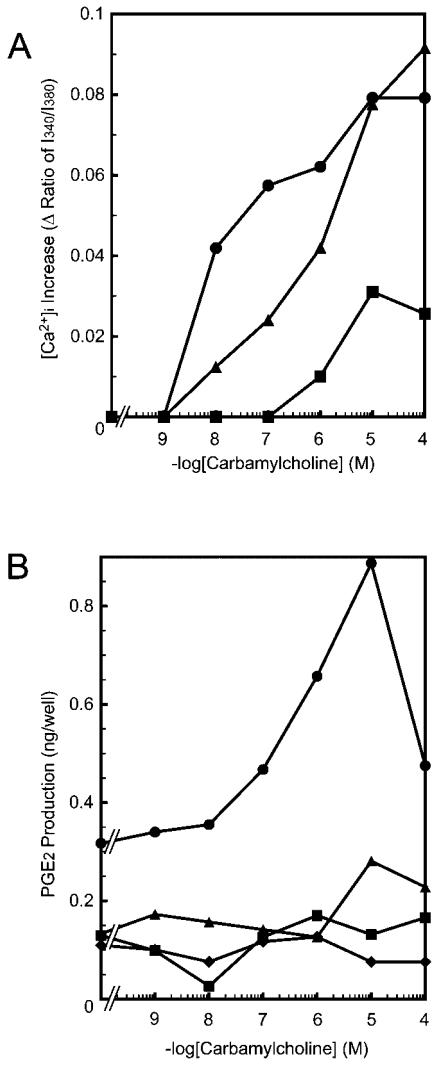


Fig. 5. **Ca$^{2+}$ mobilization and PGE$_2$ generation in CHO cells transiently transfected with a plasmid encoding M$_2$, M$_2$-Gα$_{16}$ or M$_2$ plus Gα$_{16}$.** (A) Dose-response curves of intracellular Ca$^{2+}$ mobilization. Cells expressing M$_2$ (closed squares), M$_2$-Gα$_{16}$ (closed circles), or M$_2$ plus Gα$_{16}$ (closed triangles) were loaded with 5 μM fura-2, and then carbamylcholine-induced changes in intracellular Ca$^{2+}$ were determined as Δ $I_{340}/I_{380}$. No intracellular Ca$^{2+}$ increase was induced by the addition of carbamylcholine to the parental CHO cells. A representative result of three experiments is shown. (B) Dose-response curves of PGE$_2$ generation. Cells expressing M$_2$ (closed squares), M$_2$-Gα$_{16}$ (closed circles), or M$_2$ plus Gα$_{16}$ (closed triangles), and parental CHO cells (closed diamonds) were stimulated with various concentrations of carbamylcholine for 4 h, and then PGE$_2$ released into the culture supernatant was measured. A representative result of three experiments is shown.

plus Gα$_{16}$ showed various Ca$^{2+}$ increases. We chose clones exhibiting the highest Ca$^{2+}$ increases in both cases and used them in the present studies.

We also examined agonist-induced PGE$_2$ generation in these clones. sFKN-induced PGE$_2$ generation was detectable within 10 min, and increased gradually over 4 h (Fig. 4A). In both parental CHO cells and cells expressing CX$_3$CR1, sFKN had no effect on PGE$_2$ generation, while agonist-induced PGE$_2$ generation was somewhat higher in cells expressing CX$_3$CR1-Gα$_{i2}$ (Fig. 4B). In cells expressing CX$_3$CR1-Gα$_{16}$ (Fig. 4B), however, sFKN induced marked PGE$_2$ generation. The $EC_{50}$ was estimated to be approximately $1 \times 10^{-9}$ M. The ability of the agonist to induce PGE$_2$ generation was lower in cells coexpressing CX$_3$CR1 plus Gα$_{16}$ (Fig. 4B) than in ones expressing CX$_3$CR1-Gα$_{16}$. In cells expressing CX$_3$CR1-Gα$_{16}$, PGE$_2$ generation was approximately 11-fold higher in the presence of $10^{-8}$ M sFKN than in its absence, whereas in cells coexpressing CX$_3$CR1 plus Gα$_{16}$ and ones expressing CX$_3$CR1-Gα$_{i2}$, 1.6- and 3.3-fold increases, respectively, were observed.

*M$_2$-Gα$_{16}$ Fusion Protein*—We transiently expressed M$_2$, M$_2$-Gα$_{16}$ or M$_2$ plus Gα$_{16}$ in CHO cells, and then examined

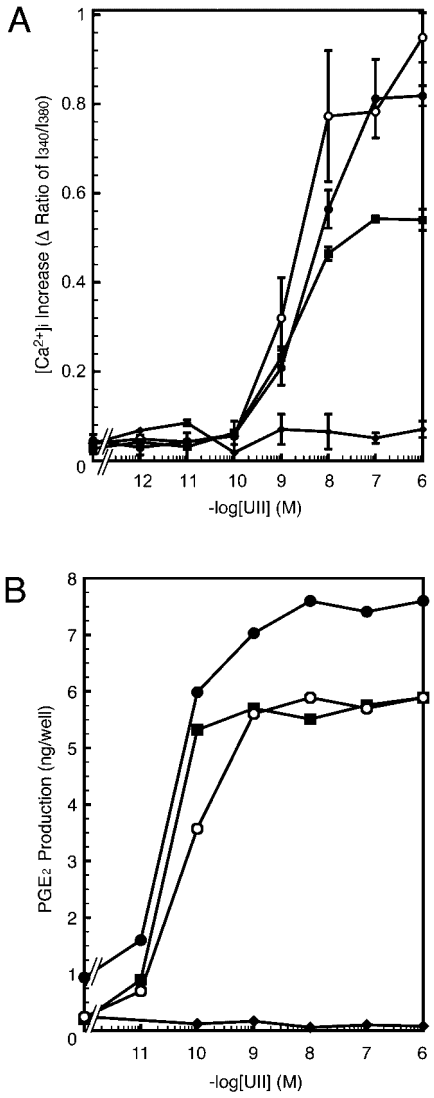


Fig. 6. **$Ca^{2+}$ mobilization and $PGE_2$ generation in CHO cells stably expressing urotensin II receptor, urotensin II receptor-$G\alpha_{i2}$ or urotensin II receptor-$G\alpha_{16}$.** (A) Dose-response curves of intracellular $Ca^{2+}$ mobilization. Cells expressing urotensin II receptor (closed squares), urotensin II receptor-$G\alpha_{i2}$ (open circles), or urotensin II receptor-$G\alpha_{16}$ (closed circles), and parental CHO cells (closed diamonds) were loaded with 5 μM fura-2, and then urotensin II-induced changes in intracellular $Ca^{2+}$, measured as $\Delta I_{340}/I_{380}$, were determined. Each point represents the mean ± SEM of three determinations ($n$ = 3), and is representative of at least three independent experiments. (B) Dose-response curves of $PGE_2$ generation. Cells expressing urotensin II receptor (closed squares), urotensin II receptor-$G\alpha_{i2}$ (open circles), or urotensin II receptor-$G\alpha_{16}$ (closed circles), and parental CHO cells (closed diamonds) were stimulated with various concentrations of urotensin II for 4 h, and then $PGE_2$ released into the culture supernatant was measured. A representative result of three experiments is shown.

the effects of an agonist, carbamylcholine, on the intracellular $Ca^{2+}$ increase and $PGE_2$ generation. Carbamylcholine had no effect on the intracellular $Ca^{2+}$ concentration in the parental CHO cells, and induced a small, transient increase in cells expressing $M_2$. In contrast, we observed robust, prolonged agonist-induced increases in intracellular $Ca^{2+}$ in cells transfected with $M_2$-$G\alpha_{16}$ and ones cotransfected with $M_2$ plus $G\alpha_{16}$ (Fig. 5A). When we

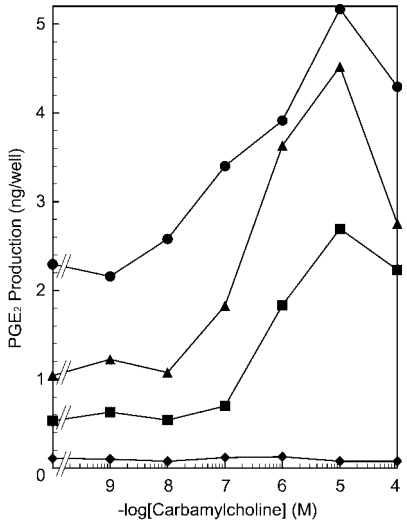


Fig. 7. **$PGE_2$ generation in CHO cells transiently transfected with a plasmid encoding $M_1$, $M_1$-$G\alpha_{16}$ or $M_1$ plus $G\alpha_{16}$.** Cells expressing $M_1$ (closed squares), $M_1$-$G\alpha_{16}$ (closed circles), or $M_1$ plus $G\alpha_{16}$ (closed triangles), and parental CHO cells (closed diamonds) were stimulated with various concentrations of carbamylcholine for 4 h, and then $PGE_2$ released into the culture supernatant was measured. A representative result of three experiments is shown.

assayed carbamylcholine-induced $PGE_2$ generation in these cells, we observed robust stimulation in cells transfected with $M_2$-$G\alpha_{16}$, but not in ones transfected with $M_2$ or $M_2$ plus $G\alpha_{16}$ (Fig. 5B).

*Urotensin II Receptor-$G\alpha_{16}$ Fusion Protein*—When we measured agonist-stimulated [$^{35}$S]GTPγS binding to Sf9 cell membranes expressing urotensin II receptor-$G\alpha_{i2}$ or urotensin II receptor-$G\alpha_{16}$, we observed a reduced effect compared with in the case of cell membranes expressing $CX_3CR1$-$G\alpha_{i2}$ (data not shown). Urotensin II-stimulated [$^{35}$S]GTPγS binding was only 1.5-fold higher in the presence of excess urotensin II than in its absence. The $EC_{50}$ values for urotensin II receptor-$G\alpha_{i2}$ and urotensin II receptor-$G\alpha_{16}$ were approximately $9 \times 10^{-9}$ M and $4 \times 10^{-8}$ M, respectively. Coexpression of $G\beta_1\gamma_2$ did not increase the effect of the agonist. Urotensin II did not affect [$^{35}$S]GTPγS binding to membranes expressing urotensin II receptor alone.

We stably expressed urotensin II receptor-$G\alpha_{i2}$, urotensin II receptor-$G\alpha_{16}$ or urotensin II receptor alone in CHO cells, and then examined the effects of the agonist on the intracellular $Ca^{2+}$ increase and $PGE_2$ generation. The addition of urotensin II caused a robust, sustained increase in intracellular $Ca^{2+}$ with $EC_{50}$ values of 1.5–3.5 $\times 10^{-9}$ M for all of these cells (Fig. 6A). Furthermore, each of these constructs showed marked $PGE_2$ generation in response to urotensin II stimulation with $EC_{50}$ values of $<10^{-10}$ M (Fig. 6B).

*$M_1$-$G\alpha_{16}$ Fusion Protein*—To determine if the above observation could be applied to other $G_q$-coupled receptors, we also examined cells transiently transfected with $M_1$, $M_1$-$G\alpha_{16}$ or $M_1$ plus $G\alpha_{16}$. In response to agonist carbamylcholine, each showed marked $PGE_2$ generation (Fig. 7), as well as a robust, sustained increase in intracellular $Ca^{2+}$ (not shown).

## DISCUSSION

It has been reported that GPCRs and Gα interact more efficiently when they are fused than when they are expressed as independent entities (*17–21*). It has also been shown that G$\alpha_{16}$ interacts with different kinds of GPCRs, which are coupled to $G_s$, $G_i$ or $G_q$ family G proteins (*9*, *10*). These findings suggest that fusion proteins of GPCRs with G$\alpha_{16}$ may be useful for the screening of endogenous and surrogate ligands of GPCRs, and this screening should be particularly useful for $G_i$-coupled receptors and orphan GPCRs, because efficient screening systems are not available for $G_i$-coupled receptors, and coupled G proteins are usually unknown for orphan GPCRs. We therefore expressed fusion proteins of G$\alpha_{16}$ and four GPCRs—$CX_3CR1$, $M_2$ receptor, urotensin II receptor, and $M_1$ receptor—in CHO cells as model systems, and examined whether or not GPCR-G$\alpha_{16}$ fusion proteins could activate downstream signals and be used as ligand screening systems.

We found that cells expressing $CX_3CR1$-G$\alpha_{16}$ fusion protein showed greater responses as to an agonist-induced intracellular $Ca^{2+}$ increase and $PGE_2$ generation than ones expressing $CX_3CR1$ and G$\alpha_{16}$ together but as independent entities. While previous reports suggested that the coexpression of $G_i$-coupled receptors with G$\alpha_{16}$ may be utilized to screen for ligands (*12*, *13*), our results for both $CX_3CR1$-G$\alpha_{16}$ and $M_2$-G$\alpha_{16}$ indicate that a strategy involving fusion proteins of $G_i$-coupled receptors with G$\alpha_{16}$ would be more effective and sensitive than the coexpression method. Furthermore, it should be noted that more than 90% of $CX_3CR1$-G$\alpha_{16}$ transfected clones showed a robust agonist-induced $Ca^{2+}$ increase, whereas only 25% of clones cotransfected with $CX_3CR1$ plus G$\alpha_{16}$ showed an agonist-induced $Ca^{2+}$ increase. This is particularly important when ligands for orphan GPCRs are screened, because positive control experiments are not possible in the initial stage.

Fusion proteins of $G_i$-coupled receptors with G$\alpha_{15}$, a mouse homologue of human G$\alpha_{16}$, have been reported for $\alpha_{2A}$-, $\alpha_{2B}$-, and $\alpha_{2C}$-adrenoceptors, and 5-$HT_{1A}$ receptor (*36–39*). They were expressed in CHO cells, and agonist-induced increases in intracellular $Ca^{2+}$ concentration were detected. The cells expressing a fusion protein of $\alpha_{2B}$-adrenoceptor showed greater responses to an agonist than ones coexpressing $\alpha_{2B}$-adrenoceptor with G$\alpha_{15}$ (*37*), which is consistent with the present results for $CX_3CR1$-G$\alpha_{16}$ and $M_2$-G$\alpha_{16}$. On the other hand, cells expressing a fusion protein of $\alpha_{2A}$-adrenoceptor or 5-$HT_{1A}$ receptor with G$\alpha_{15}$ have been reported to be less sensitive to each agonist than ones coexpressing $\alpha_{2A}$-adrenoceptor or 5-$HT_{1A}$ receptor with G$\alpha_{15}$ (*36*, *39*), and cells expressing a fusion protein of $\alpha_{2C}$-adrenoceptor with G$\alpha_{15}$ showed a weak $Ca^{2+}$ response to an agonist (*38*).

To the best of our knowledge, there have been no reports on agonist-induced stimulation of cells expressing fusion proteins of $G_q$- or $G_s$-coupled receptors with G$\alpha_{16}$. Here we have shown that in cells expressing fusion proteins of G$\alpha_{16}$ with $G_q$-coupled receptors, *i.e.* urotensin II receptor and $M_1$, relevant agonists induced marked responses, although the extent of stimulation was similar to that in cells expressing each receptor alone. In addition, our preliminary findings indicate that $G_s$-coupled $\beta_2$-adrenoceptors coexpressed with G$\alpha_{16}$ in CHO cells are able to mediate agonist-induced generation of $PGE_2$ (data not shown). Taken together, these results suggest that an assay system involving GPCR-G$\alpha_{16}$ fusion proteins might be applied to many, but not all GPCRs.

We utilized three assays to test the effects of agonists on GPCRs–[$^{35}$S]GTPγS binding to Sf9 cell membranes, an intracellular $Ca^{2+}$ increase, and $PGE_2$ generation. Agonist-induced [$^{35}$S]GTPγS binding has been clearly observed for membranes expressing fusion proteins with G$\alpha_i$ of $G_i$-coupled receptors, and suitable for the screening ligands of $G_i$-coupled receptors (*22*, *40*, this study and unpublished data *41*, *42*). The agonist-induced [$^{35}$S]GTPγS binding assay is simple and suitable for large-scale screening, although it is not as sensitive as other assays that involve cultured cells. In the case of fusion proteins with G$\alpha_{16}$ of $G_i$- or $G_q$-coupled receptors, however, the extent of the agonist effect on Sf9 membrane preparations was much less than that for fusion proteins with G$\alpha_i$ of $G_i$-coupled receptors (*22* and this study). An agonist-induced $Ca^{2+}$ increase is used most commonly for the large-scale screening of ligands. As shown in the present report, it is useful for fusion proteins with G$\alpha_{16}$ of $G_i$- or $G_q$-coupled receptors. Furthermore, we showed that agonist-induced $PGE_2$ generation is also useful for such fusion proteins and more sensitive than the $Ca^{2+}$ assay. It might be utilized for the screening of endogenous ligands, since the amounts of available endogenous ligands are usually very limited. The demerit of these screening systems involving whole cells is the false positive responses with endogenous GPCRs. Coexpression of G$\alpha_{16}$ with exogenous GPCRs might also cause increases in the responses by endogenous GPCRs. Fusion proteins of exogenous GPCRs with G$\alpha_{16}$ might increase the responses through exogenous but not endogenous GPCRs. Thus, GPCR-G$\alpha_{16}$ fusion proteins might be effective in reducing false positive responses.

In summary, we have shown that cells expressing fusion proteins with G$\alpha_{16}$ of $G_i$- and $G_q$-coupled receptors are responsive to agonists and activate downstream signaling. We have also found that agonist-induced cellular responses were greater for cells expressing fusion proteins with G$\alpha_{16}$ of $G_i$-coupled receptors than for ones expressing $G_i$-coupled receptor alone or both $G_i$-coupled receptor and G$\alpha_{16}$ separately. These results suggest that GPCR-G$\alpha_{16}$ fusion proteins may provide an effective means of the screening of ligands of $G_i$-coupled receptors and orphan GPCRs.

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