G_{i1} and G_{oA} differentially determine kinetic efficacies of agonists for κ-opioid receptor

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Abstract We examined the diversity of single receptor function by measuring receptor–G protein coupling in the baculovirus–Sf21 expression system. In comparative studies using Sf21 cell membranes expressing κ-opioid receptor (KOR) plus $G\alpha_{i1}\beta_1\gamma_2$ or KOR plus $G\alpha_{oA}\beta_1\gamma_2$, there was no significant difference between both preparations in the K_i values of various κ-opioid ligands for the displacement of [^{3}H]U69593 binding. However, a marked difference in the rank order of agonists to stimulate [^{35}S]GTPγS binding was observed between both preparations. These findings suggest that agonist efficacy is dependent on the population of different G proteins expressed in various tissues.

Key words: κ-Opioid receptor; G protein; Reconstitution

1. Introduction

From recent advances in G protein-coupled receptor (GPCR) studies, more complex coupling between GPCR and G protein than previously expected in reconstitution experiments using purified proteins [1,2] has been reported [3,4]. The facts adding the diversity in such functional couplings may include the basal activity of GPCR without agonist stimulation [3] and the existence of alternative splicing variants showing different kinetics of internalization [4]. Opioid receptors are the most representative GPCRs whose diverse functions have been extensively characterized [2]. They were originally classified into μ, δ and κ types, and they were all known to be coupled with pertussis toxin-sensitive G protein, G_i or G_o [2,5], and these functional couplings have been confirmed by reconstitution experiments using cloned receptors [6,7]. Although more than 20 opioid peptides including endorphins have been discovered in mammals [8,9], only three opioid receptor genes have been discovered even after extensive searches [10]. From this reason it has been speculated that each opioid receptor should have diverse sensitivities to different opioid peptides in view of its functional efficacy or kinetics as well as binding affinity. Although some diverse mechanisms might be related to several alternatively splicing variants of opioid receptors, it remains to be determined whether each variant corresponds to each opioid peptide. Our strategy to see this diverse functional sensitivity of opioid receptor to agonists is the determination of agonist efficacy in opioid receptor coupling to specific G protein molecules. For this purpose, analysis in reconstitution experiments using purified recombinant receptor and G protein seems to be ideal. However, because of the instability of these proteins, we have often met a big limitation in detailed analyses for functional coupling. Recently a baculovirus expression system has been introduced into the signal transduction study of receptors. As abundant recombinant proteins are generally expressed, and multiple infections are easily performed in this expression system, protein–protein interaction could be analyzed in detail using membrane preparations, under the minimized consideration of naturally occurring proteins. Here we report the diverse sensitivity of κ-opioid receptor (KOR) to various agonists under different quasi-reconstitution conditions with different G proteins.

2. Materials and methods

2.1. Materials

(5a,7a,8b)-(+)-*N*-Methyl-*N*-(7-(pyrrolidinyl-1-oxaspiro-(4,5)dec-8-(+)-yl) benzeneacetamide (U69593) was kindly provided by Upjohn Japan, norbinaltorphimine (nor-BNI) by Dr. H. Nagase (Toray Industries), rat KOR cDNA by Dr. K. Fukuda (Kyoto University). [^{3}H]U69593 (50.6 Ci/mmol) and [^{35}S]GTPγS (1156 Ci/mmol) were purchased from DuPont/NEN, EX-CELL 400 medium from JRH Bioscience, TNM-FH medium from Sigma, wild-type baculovirus (*Autographa californica* nuclear polyhedrosis virus, AcNPV) DNA from Pharmingen, fetal bovine serum from PAA Laboratories, nitrocellulose transfer membrane from Schleicher and Schuell, and GF/B glass fiber filters from Whatman. All other chemicals were purchased from Sigma.

2.2. Recombinant baculovirus of the κ-opioid receptor

The cDNA of rat KOR was amplified using two synthetic oligo primers complementary to the 5′ and 3′ ends of the coding region and carrying a recognition site for *Xba*I on each end. The *Xba*I–*Xba*I fragment of the PCR product was inserted into an *Nhe*I site downstream of the polyhedrin promoter in the transfer vector pJVP10Z using cohesive end ligation [11]. The recombinant plasmid, pJVP10Z/KOR, carrying the insert in the correct orientation was identified by sequencing. The baculovirus transfer vector pJVP10Z used in this study is based on pJV(*Nhe*I) [12] containing a β-galactosidase gene, which allows blue/white screening via production of β-galactosidase. The recombinant plasmid pJVP10Z/KOR was transfected into Sf21 cells together with wild-type baculovirus (AcNPV) DNA using a lipofectin-mediated co-transfection method [13]. A recombinant virus was plaque-purified by four rounds of color selection using X-gal, and amplified four rounds.

2.3. Co-expression of the KOR and G proteins

Spodoptera frugiperda Sf21 cells were maintained in suspension culture in EX-CELL 400 medium containing 25 μg/ml gentamicin at 27°C, and for infection Sf21 cells were grown in TNM-FH medium containing 10% fetal bovine serum and 25 μg/ml gentamicin at 27°C [14]. Insect cells and baculovirus (AcNPV) were manipulated essentially as described [15]. The Sf21 cells were seeded in 75 cm^2 flasks (Nunc) in TNM-FH insect medium (Sigma) containing 10% fetal bo-

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vine serum and infected with recombinant viruses at a multiplicity of infection (m.o.i.) of 10 for KOR, 3 for Gα subunit and 1 for $G\beta_1\gamma_2$ subunits [16] unless otherwise stated. $G\beta_1$ and $G\gamma_2$ subunits were placed downstream of two polyhedrin promoters arranged back to back in the recombinant virus coding both subunits. Cells were harvested 2–3 days after infection.

2.4. [^{35}S]GTPγS binding assay

Cells were harvested, washed with phosphate-buffered saline and homogenized in 20 mM Tris–HCl buffer, pH 7.5 containing 1 mM EDTA, 1 mM dithiothreitol with a Potter-type homogenizer. The homogenate was centrifuged at $1000\times g$ for 10 min and the supernatant was further centrifuged at $20\,000\times g$ for 20 min. The resulting pellet was resuspended in buffer A (20 mM HEPES, 1 mM $MgCl_2$, 100 mM NaCl and 1 mM dithiothreitol, pH 7.5) to adjust at 0.3–1 mg/ml protein. In some studies $MgCl_2$ was omitted to assess its role. [^{35}S]GTPγS binding assays were carried out as described [17]. The membrane fractions from Sf21 cells were incubated with [^{35}S]GTPγS for 60 min or indicated periods at 30°C.

2.5. [^{3}H]U69593 binding

Membrane proteins (50–150 μg) were mixed with 50 mM Tris–HCl buffer, pH 7.5 with varying concentrations (0.5–5 nM) of the κ-opioid agonist [^{3}H]U69593 in a total of 1 ml. The incubation was started by addition of the membrane suspension and was carried out for 40 min at 25°C. The reaction was terminated by a rapid filtration through GF/B filters presoaked in 0.1% polyethyleneimine, under vacuum. The filters were washed six times with 3 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.5. Filters were transferred to scintillation vials containing 7 ml of Clearsol I scintillation cocktail (Nacalai Tesque) and bound radioactivity was quantitated by liquid scintillation counting. In the displacement experiments, 1 nM [^{3}H]U69593 was used. In all experiments, the non-specific binding was represented as the binding activity in the presence of 1 μM nor-BNI.

2.6. Immunoblot analysis

Membrane proteins (10 μg), prepared from uninfected or infected Sf21 cells, were heated in Laemmli sample buffer for 5 min at 95°C and subjected to SDS–polyacrylamide gel electrophoresis on a 4% stacking/10% running gel [18]. SeeBlue Pre-Stained Standards (Novex) were run in parallel. After electrophoresis, the resolved proteins were electro-transferred to Immobilon transfer membranes (Millipore). The membranes were sequentially incubated with antibodies recognizing $G\alpha_{i1}$ (AS/7; rabbit antiserum, against C-terminal, 1:1000 dilution), $G\alpha_{11}$ (rabbit antiserum, against C-terminal, 1:3000 dilution, [16]), $G\alpha_s$ (rabbit antiserum, against N-terminal, 1:3000 dilution), $G\alpha_o$ (rabbit antiserum, against N-terminal, 1:500 dilution), or $G\beta_1$ (mouse IgG, against N-terminal, 1:1500 dilution) for 3 h at room temperature. Secondary incubations were performed for 2 h at room temperature using goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase and bound enzyme was visualized with H_2O_2 and 4-chloro-1-naphthol.

3. Results

3.1. Opioid agonist-stimulated [^{35}S]GTPγS binding

Agonist stimulation of [^{35}S]GTPγS binding is known to be critical for the concentration of GDP to inhibit non-specific binding of [^{35}S]GTPγS to the preparation. In preliminary studies we observed that there was no significant stimulation by U69593 in its absence or in the presence of a high concentration (100 μM) of GDP in membrane preparations from Sf21 cells infected with $G\alpha_{i1}$ and $G\beta_1\gamma_2$ viruses together with KOR virus. At concentrations of GDP between 0.3 and 10 μM, however, basal levels were decreased, giving rise to significant agonist-induced stimulation (Fig. 1A). When the influence of Mg^{2+} on the binding of [^{35}S]GTPγS was investigated in the absence and presence of 1 μM U69593, the condition in the presence of 1 mM Mg^{2+} gave the best result (Fig. 1B). Here we decided to use 3 μM GDP and 1 mM Mg^{2+} for standard assay conditions unless otherwise stated. The κ-opioid agonist U69593 stimulated the [^{35}S]GTPγS binding in a concentration-dependent manner (Fig. 1C). The median effective concentration (EC_{50}) was 54.7 ± 11.1 nM ($n = 3$). No significant stimulation of the binding was observed by [D-Ala^2,MePhe^4,Gly^5-ol]-enkephalin or [D-Ser^2,Leu^5]-enkephalin-Thr^6, specific μ- or δ-opioid agonists [8], respectively (data not shown). This stimulation by U69593 was completely antagonized by the κ-opioid antagonist nor-BNI (1 μM), indicating the involvement of specific κ-opioid receptor in the U69593-stimulated [^{35}S]GTPγS binding.

3.2. Selective activation of G_{i1} or G_{oA} by KOR in the reconstitution membrane

In membrane preparations of Sf21 cells infected with KOR alone, 1 μM U69593 did not show any significant change in the [^{35}S]GTPγS binding for 3 h, as shown in Fig. 1D. When Sf21 cells were infected with $G\beta_1\gamma_2$ and $G\alpha_{i1}$ or $G\alpha_{oA}$ viruses together with KOR virus, significant stimulation was observed by 1 μM U69593 treatment (Fig. 1E,F). However, there was no significant stimulation of [^{35}S]GTPγS binding in preparations with $G\alpha_s$ or $G\alpha_{11}$ virus (Fig. 1G,H), though protein expression of four Gα subunits and $G\beta_1$ subunit was detected in cell membranes by immunoblot analysis (Fig. 1I).

3.3. No change in the affinities of various κ-opioid ligands to [^{3}H]U69593 binding between preparations expressing G_{i1} or G_{oA}

From Scatchard plot analysis, K_d and B_{max} were calculated to be 2.7 ± 0.5 nM and 139.5 ± 38.7 fmol/mg protein ($n = 3$) in the preparation with KOR plus $G\alpha_{i1}\beta_1\gamma_2$, respectively, while these values were 2.5 ± 0.5 nM and 97.4 ± 28.5 fmol/mg protein ($n = 3$) in the preparation with KOR plus $G\alpha_{oA}\beta_1\gamma_2$, respectively. There was no significant difference in K_d value between both preparations, though some differences in B_{max} were observed. The latter difference may be attributed to different expression of either KOR or G protein. In the displacement experiments the inhibitory effects of various κ-opioid ligands on [^{3}H]U69593 (1 nM) binding in both preparations expressing G_{i1} or G_{oA} were examined. As shown in Fig. 2, all these compounds markedly inhibited the binding in the range of 1 nM to 1 μM in both preparations. However, there were no significant differences in the displacement curves by any opioid ligands between the preparations. Detailed analysis also confirmed this conclusion in view of K_i values (Table 1).

3.4. Different kinetic potencies of various κ-opioid agonists in different preparations expressing G_{i1} or G_{oA}

We characterized the kinetic potencies of κ-opioid agonists

Table 1
Inhibition of specific [^{3}H]U69593 binding to membranes of KOR/$\alpha_{i1}\beta_1\gamma_2$ and KOR/$\alpha_{oA}\beta_1\gamma_2$ virus-infected Sf21 cells by κ agonists and antagonist

Compound	K_i (nM)	
	G_{i1}	G_{oA}
U69593	2.0 ± 0.3	2.2 ± 0.3
U50488H	1.8 ± 0.1	1.6 ± 0.3
DynA(1–17)	7.8 ± 3.6	4.3 ± 3.1
DynB	32.3 ± 14.5	25.9 ± 11.3
nor-BNI	13.6 ± 4.5	16.0 ± 5.6

Results represent the mean ± S.E.M. from three separate experiments performed in duplicate.

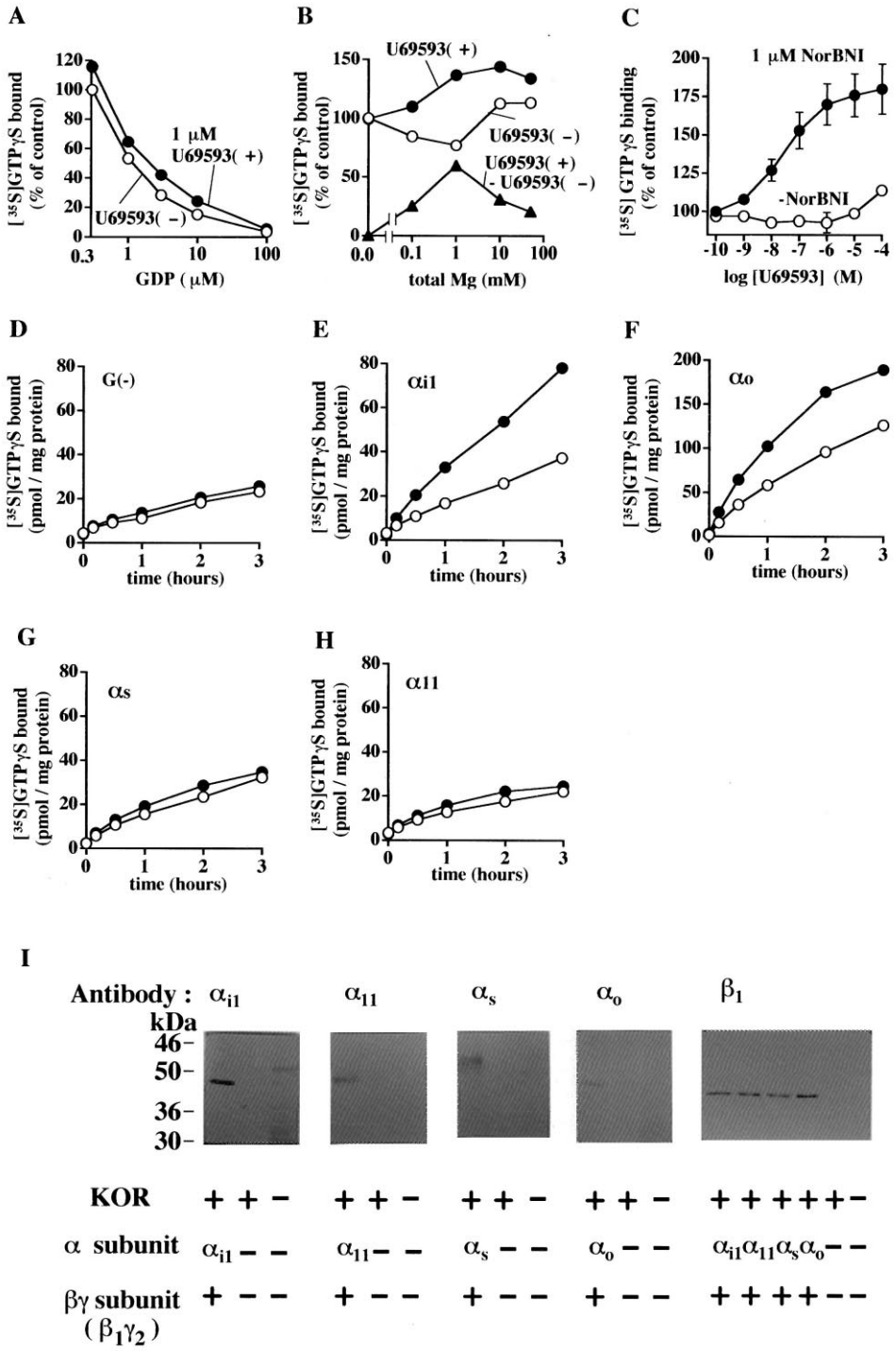

Fig. 1. Characterization of KOR-mediated [^{35}S]GTPγS binding. In the experiments, monolayer cultures of Sf21 cells were infected with recombinant viruses at a m.o.i. of 10 for KOR, 3 for $G\alpha_{i1}$ subunit and 1 for $G\beta_1\gamma_2$ subunits. Membrane fractions were prepared from the infected cells after 72 h, and subjected to [^{35}S]GTPγS binding assay. A, B: Effects of GDP (A) and Mg^{2+} (B) on the binding of [^{35}S]GTPγS to membranes of Sf21 cells infected with KOR, $G\alpha_{i1}$ and $G\beta_1\gamma_2$ subunit viruses, and stimulation by 1 μM U69593. All results are from a single experiment performed in duplicate. C: Antagonism of [^{35}S]GTPγS binding stimulated by various concentrations of U69593 by nor-BNI (1 μM) in $G\alpha_{i1}\beta_1\gamma_2$ preparations. Results represent means ± S.E.M. from three separate experiments performed in duplicate. Basal [^{35}S]GTPγS binding was 39.4 ± 6.3 pmol/mg of protein in the absence of U69593 ($n = 3$). D–H: U69593-induced stimulation of [^{35}S]GTPγS binding to membranes of Sf21 cells infected with KOR and $G\beta_1\gamma_2$ alone (D), plus $G\alpha_{i1}$ (E), $G\alpha_{oA}$ (F), $G\alpha_s$ (G) or $G\alpha_{11}$ viruses (H). Open and closed circles represent the absence and presence of 1 μM U69593, respectively. I: Immunoblot analysis of Gα subunits and Gβ subunit in Sf21 cells infected with KOR and $G\beta_1\gamma_2$ with and without $G\alpha_{i1}$, $G\alpha_{11}$, $G\alpha_s$ or $G\alpha_o$ viruses.

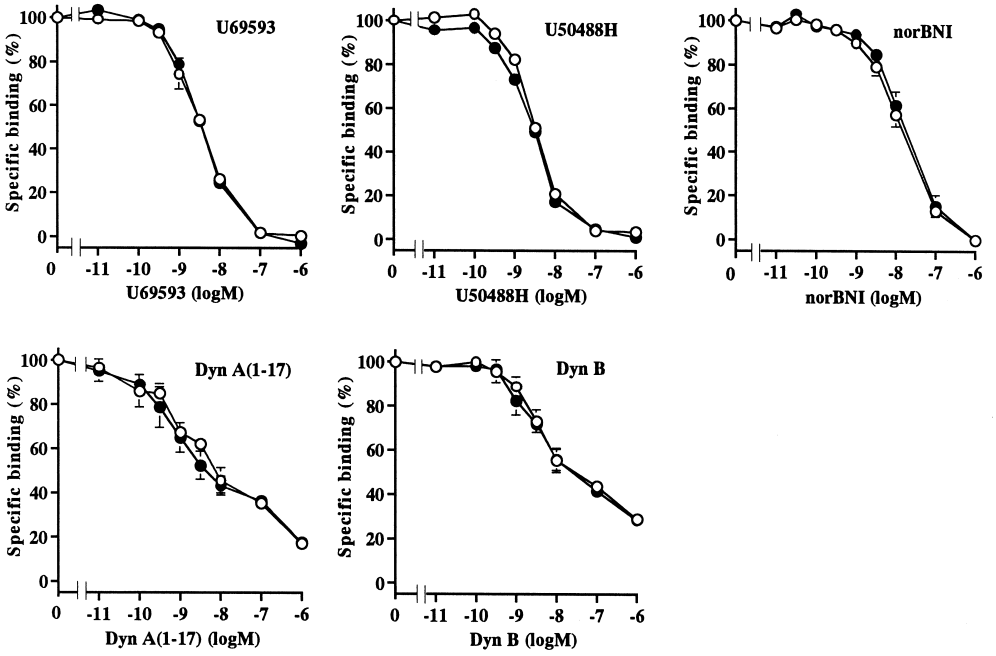

Fig. 2. Displacement of [^{3}H]U69593 binding by various κ-opioid ligands. Open and closed circles represent the results with $G\alpha_{i1}\beta_1\gamma_2$ and $G\alpha_{oA}\beta_1\gamma_2$ preparations, respectively. Each data point represents the mean ± S.E.M. from three separate experiments.

in the membrane from Sf21 cells expressing G_{i1} or G_{oA}. Because multiple-virus infection was needed to reconstitute the expression system, the absolute value of [^{35}S]GTPγS binding was irregular in each experiment. To normalize the agonist stimulation of the [^{35}S]GTPγS binding from preparation to preparation, each agonist-stimulated activity was evaluated as percent of maximal stimulation caused by 100 μM U69593. As shown in Fig. 3, no significant difference in the agonist stimulation of [^{35}S]GTPγS binding between G_{i1} and G_{oA} preparations was observed with U69593 and dynorphin A (DynA) 1–17. However, there were significant differences in the profiles of agonist stimulation of [^{35}S]GTPγS binding between both preparations with U50488H and DynB. The difference includes changes in the EC_{50} or maximal activity. In the case of U50488H, the maximal stimulation of [^{35}S]GTPγS binding was lower in the preparation expressing G_{i1} than in the preparation expressing G_{oA}. In the case of DynB, however, a higher sensitivity of agonist stimulation of [^{35}S]GTPγS binding was observed in the G_{i1} preparation than in the G_{oA} preparation, where there was a marked parallel shift, but no significant difference in maximal effects between preparations. The EC_{20}, EC_{50}, and EC_{80} of G_{oA} stimulation increased 10.0-, 50.3-, and 18.6-fold compared to those of G_{i1} stimulation.

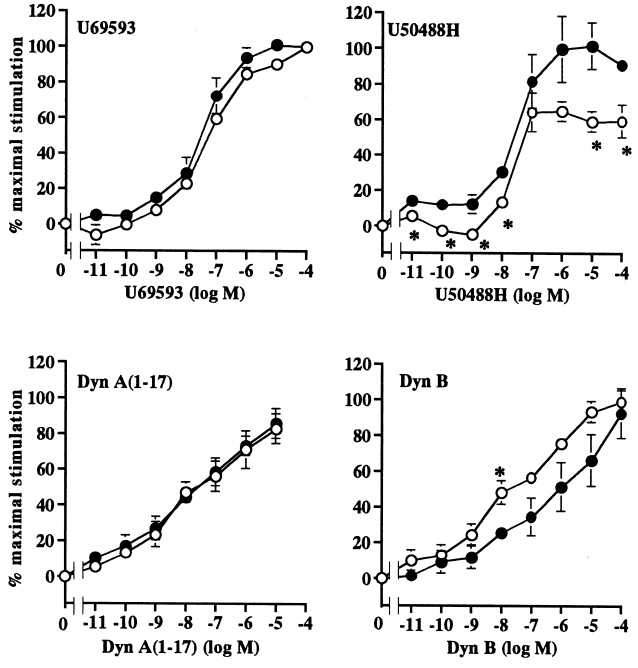

Fig. 3. κ-Opioid agonist-induced stimulation of [^{35}S]GTPγS binding in either $G\alpha_{i1}\beta_1\gamma_2$ (open circles) or $G\alpha_o\beta_1\gamma_2$ preparations (closed circles). Each data point represents the mean ± S.E.M. from three separate experiments. $^*P < 0.05$, compared with the data with $G\alpha_o\beta_1\gamma_2$ preparations at the same concentration of agonist (Student's *t*-test).

4. Discussion

Here we obtained two major findings in KOR–G protein coupling. The first finding is that KOR is coupled to G_{i1} and G_{oA} in baculovirus-infected Sf21 cell membranes, which is consistent with previous pharmacological reports which showed the pertussis toxin-sensitive actions by κ-opioid agonists. In the present study, we confirmed that this receptor did not couple with G_s or G_{11} which can be coupled with D1 dopamine receptor or lysophosphatidic acid receptor respectively [19]. As the stoichiometry of receptor coupling to $G_{q/11}$ was reported to be very low, compared to $G_{i/o}$- or G_s-coupled receptors [20], it is difficult to prove this type of uncoupling unless reconstitution with recombinant $G_{q/11}$ is performed. Nakamura et al. [16] have reported that muscarinic M1 receptor stimulated the [^{35}S]GTPγS binding to G_{11} in membranes of Sf9 cells infected with M1 and G_{11} baculoviruses. Most recently we have found that melittin, an amphiphilic peptide, directly stimulated [^{35}S]GTPγS binding in a prepara-

tion from Sf21 cells infected with the G_{11} baculovirus, but not in a preparation from uninfected cells [19]. Therefore, it is evident that KOR is not functionally coupled to G_{11}. Similarly, as we have found that β2-adrenoceptors or D1 dopamine receptors were coupled with G_s in in vivo reconstitution experiments [17], the uncoupling between KOR and G_s is also evident.

The second finding is that the rank order of potencies of various agonists in stimulation of [^{35}S]GTPγS binding was different between G_{i1} and G_{oA} preparations, while there was no significant kinetic change in [^{3}H]U69593 binding between these preparations. In the displacement experiments of [^{3}H]U69593 binding, the inhibitory kinetics of various κ-opioid ligands were identical between preparations expressing G_{i1} and G_{oA}.

On the other hand, all κ-opioid agonists used here stimulated [^{35}S]GTPγS binding in both preparations expressing G_{i1} or G_{oA}. When the stimulated [^{35}S]GTPγS binding by each agonist was represented as percent of U69593 (100 μM)-stimulated binding in either G_{i1} or G_{oA} preparations, differences in the sensitivity to each agonist between the two preparations were observed (Fig. 3). Although there was no significant difference between preparations in the case of U69593 and DynA(1–17), both U50488H and DynB showed significant differences between preparations. Interestingly, the difference was observed in the kinetics. A marked decrease in the maximum effect was observed with U50488H-stimulated [^{35}S]GTPγS binding in G_{oA} preparations, without a change in ED_{50}, compared to the G_{i1} preparations. In contrast, a lower sensitivity to DynB when evaluated by ED_{50} was observed in G_{i1} preparations than in G_{oA} preparations, without any change in the maximal effect. Thus, all these findings suggest that the rank order and kinetic efficacy of agonists in the stimulation of [^{35}S]GTPγS binding differ in preparations coexpressing different G proteins. Taking into account the fact that G_o is abundant in the brain, but not in the periphery [21], compared with G_{i1}, the physiological significance of DynB in the brain should be less than in the periphery.

In conclusion, here we demonstrated the different kinetic efficacy of opioid agonists in stimulating G protein in preparations expressing different G proteins. The present study may provide a mechanism to answer the question how a single opioid receptor distinguishes multiple opioid peptides.

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References

[1] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–649.
[2] Ueda, H., Nozaki, M. and Satoh, M. (1991) Comp. Biochem. Physiol. 98c, 157–169.
[3] Milligan, G., MacEwan, D.J., Mercouris, M. and Mullancy, I. (1997) Receptors Channels 5, 209–213.
[4] Koch, T., Schulz, S., Schroeder, H., Wolf, R., Raulf, E. and Hollt, V. (1998) J. Biol. Chem. 273, 13652–13657.
[5] Ueda, H., Harada, H., Nozaki, M., Katada, T., Ui, M., Satoh, M. and Takagi, H. (1988) Proc. Natl. Acad. Sci. USA 85, 7013–7017.
[6] Ueda, H., Miyamae, T., Hayashi, C., Watanabe, S., Fukushima, N., Sasaki, Y., Iwamura, T. and Misu, Y. (1995) J. Neurosci. 15, 7485–7499.
[7] Ueda, H., Miyamae, T., Fukushima, N., Takeshima, H., Fukuda, K., Sasaki, Y. and Misu, Y. (1995) Mol. Brain Res. 32, 166–170.
[8] Corbett, A.D., Paterson, S.J. and Kosterlitz, H.W. (1993) in: Handbook of Experimental Pharmacology: Opioids I (Herz, A., Ed.), pp. 645–679, Springer-Verlag, Berlin.
[9] Zadina, J.E., Hackler, L., Ge, L.J. and Kastin, A.J. (1997) Nature 386, 499–502.
[10] Law, P.Y. and Loh, H.H. (1999) J. Pharmacol. Exp. Ther. 289, 607–624.
[11] Hattori, S., Okuda, K., Hamajima, K., Sakimura, K., Mishina, M. and Kawamoto, S. (1994) Brain Res. 666, 43–52.
[12] Vialard, J., Lalumiere, M., Vernet, T., Briedis, D., Alkhatib, G., Henning, D., Levin, D. and Richardson, C. (1990) J. Virol. 64, 37–50.
[13] Groebe, D.R., Chung, A.E. and Ho, C. (1990) Nucleic Acids Res. 18, 4033.
[14] Kawamoto, S., Onishi, H., Hattori, S., Miyagi, Y., Amaya, Y., Mishina, M. and Okuda, K. (1991) Biochem. Biophys. Res. Commun. 181, 756–763.
[15] Smith, G.E., Summers, M.D. and Fraser, M.J. (1983) Mol. Cell. Biol. 3, 2156–2165.
[16] Nakamura, F., Kato, M., Kameyama, K., Nukada, T., Haga, T., Kato, H., Takenawa, T. and Kikkawa, U. (1995) J. Biol. Chem. 270, 6246–6253.
[17] Fukushima, N., Kohno, M., Kato, T., Kawamoto, S., Okuda, K., Misu, Y. and Ueda, H. (1998) Peptides 19, 811–819.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Yoshida, A. and Ueda, H. (1999) Biochem. Biophys. Res. Commun. 259, 78–84.
[20] Pang, I.-H. and Sternweis, P.C. (1990) J. Biol. Chem. 265, 18707–18712.
[21] Asano, T., Semba, R., Ogasawara, N. and Kato, K. (1987) J. Neurochem. 48, 1617–1623.