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Coupling Efficacy and Selectivity of the Human μ-Opioid Receptor Expressed as Receptor–Gα Fusion Proteins in *Escherichia coli*

Laura Stanasila, *William K. Lim, *Richard R. Neubig, and Franc Pattus

Département des Récepteurs et Protéines Membranaires, UPR 9050 CNRS, ESBS, Illkirch, France; and
**Department of Pharmacology, University of Michigan, Ann Arbor, Michigan, U.S.A.*

Abstract: Two constructs encoding the human μ-opioid receptor (hMOR) fused at its C terminus to either one of two Gα subunits, $G\alpha_{o1}$ (hMOR–$G\alpha_{o1}$) and $G\alpha_{i2}$ (hMOR–$G\alpha_{i2}$), were expressed in *Escherichia coli* at levels suitable for pharmacological studies (0.4–0.5 pmol/mg). Receptors fused to $G\alpha_{o1}$ or to $G\alpha_{i2}$ maintained high-affinity binding of the antagonist diprenorphine. Affinities of the μ-selective agonists morphine, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO), and endomorphins as well as their potencies and intrinsic activities in stimulating guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate) ([^{35}S]GTPγS) binding were assessed in the presence of added purified Gβγ subunits. Both fusion proteins displayed high-affinity agonist binding and agonist-stimulated [^{35}S]GTPγS binding. In the presence of Gβγ dimers, the affinities of DAMGO and endomorphin-1 and -2 were higher at hMOR-$G\alpha_{i2}$ than at hMOR–$G\alpha_{o1}$, whereas morphine displayed similar affinities at the two chimeras. Potencies of the four agonists in stimulating [^{35}S]GTPγS binding at hMOR–$G\alpha_{o1}$ were similar, whereas at hMOR-$G\alpha_{i2}$, endomorphin-1 and morphine were more potent than DAMGO and endomorphin-2. The intrinsic activities of the four agonists at the two fusion constructs were similar. The results confirm hMOR coupling to $G\alpha_{o1}$ and $G\alpha_{i2}$ and support the hypothesis of the existence of multiple receptor conformational states, depending on the nature of the G protein to which it is coupled. **Key Words:** G protein-coupled receptors—Receptor–G protein fusion—μ-Opioid receptor—*Escherichia coli*—Pharmacology.
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Opioid receptors and their endogenous ligands form a neuromodulatory system that is involved in stress-induced analgesia, regulation of emotional responses and self-reward, and controls of neuroendocrine physiology and autonomic functions such as respiration, blood pressure, and gastrointestinal motility. According to their pharmacological properties, opioid receptors are classified into three subtypes: δ, μ, and κ, each possessing distinct ligand selectivity profiles. The μ subtype in particular is the target of morphine, an alkaloid with analgesic and psychotropic effects.

As members of the large family of G protein-coupled receptors (GPCRs), opioid receptors exert their actions through heterotrimeric G proteins (Childers, 1993). The inhibitory effect of opioid ligands on adenylyl cyclase and their activating effect on inward rectifying K^+ channels are mediated by a pertussis toxin-sensitive mechanism, which suggests coupling of the receptors to $G_{i/o}$ proteins (North, 1993; Kieffer, 1995; Satoh and Minami, 1995). For a more precise identification of the Gα protein subtypes activated on binding of opioid agonists, one approach combined azidoanilido [^{32}P]GTP labeling of activated G proteins with their subsequent immunoprecipitation with subtype-specific antibodies (Laugwitz et al., 1993; Prather et al., 1994, 1995; Chakrabarti et al., 1995; Law and Reisine, 1997). Other methods were based on coexpression or in vitro reconstitution of the receptor and distinct Gα subunits (Chan et al., 1995) or on specific blocking of one Gα subtype using antibodies directed against it (Murthy and Makhlouf, 1996).

These different approaches led to the conclusion that opioid receptors do not distinguish among the different $G\alpha_{i/o}$ subtypes. However, in vivo down-regulation of individual Gα subtypes using antisense oligodeoxynucleotides provided evidence that μ- and δ-opioid receptors preferentially signal through $G\alpha_{i2}$ and $G\alpha_z$ (Sanchez-Blazquez et al., 1999).

To investigate the G protein-coupling properties of the μ-opioid receptor and the impact of the different G protein subtypes on the pharmacological properties of the receptor, we undertook expression in *Escherichia*

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Address correspondence and reprint requests to Dr. L. Stanasila at her present address: Institut de Pharmacologie et Toxicologie, Rue du Bugnon 27, 1005, Lausanne, Switzerland. E-mail: Laura.Stanasila@ipharm.unil.ch

Abbreviations used: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin; GPCR, G protein-coupled receptor; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); hMOR, human μ-opioid receptor; MBP, maltose binding protein.

coli of the human μ-opioid receptor (hMOR) as a fusion protein to either one of two putative cognate Gα subunits, $G\alpha_{o1}$ and $G\alpha_{i2}$. Receptor–Gα chimeras were developed as tools for studying interactions between a large array of receptors and G proteins (review by Seifert et al., 1999*a*). The present report is the first application of the receptor–Gα chimera strategy to the study of the μ-opioid receptor. This strategy takes advantage of the main features of the receptor–Gα fusion proteins, namely, the opportunity to study a defined receptor–G protein couple under a defined 1:1 receptor:G protein stoichiometry. This is particularly true in a prokaryotic environment, which is devoid of endogenous GPCR or G protein homologues. The present study is also the first report of the functional expression of receptor–Gα subunit chimeras in bacteria.

EXPERIMENTAL PROCEDURES

Materials

Plasmid pMalp2 was from New England Biolabs. Rat $G\alpha_{o1}$ and $G\alpha_{i2}$ cDNAs were kindly provided by Dr. Randall Reed (Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.) (Jones and Reed, 1987). hMOR cDNA was a gift of Dr. Lei Yu (Indiana State University, Terre Haute, IN, U.S.A.). Restriction endonucleases, DNA modification enzymes, and polyclonal anti-maltose binding protein (anti-MBP) antibody were from New England Biolabs. Anti-$G\alpha_o$ and -$G\alpha_{i2}$ monoclonal antibodies were from Neomarkers. Anti-rabbit peroxidase-conjugated secondary antibody and the ECL Plus detection kit were purchased from Amersham. [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]Enkephalin (DAMGO), naloxone, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), and GDP were from Sigma. Endomorphin-1 and -2 were synthesized at IGBMC (Strasbourg, France). [^{35}S]GTPγS was from Amersham. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was from Fluka. All other analytical-grade chemicals were from Sigma.

Strains

Strain XL1Blue was used for cloning. Strain JM101 was used as the recipient of expression vectors.

Construction of expression vectors

The gene encoding the hMOR was isolated from human brain (Mestek et al., 1995). The MBP–hMOR construct was described elsewhere (Stanasila et al., 1999). The C-terminal fragment of hMOR cDNA starting at the *Bam*HI site (600 bp) was PCR-amplified and subcloned as a *Bam*HI–*Xba*I fragment into the high-copy number pMalp2–hMOR expression vector (Stanasila et al., 1999) to suppress the UAA stop codon. $G\alpha_{o1}$ and $G\alpha_{i2}$ cDNAs were PCR-amplified and subcloned into the *Xba*I site of the newly generated pMalp2–hMOR vector. A six-amino acid spacer was introduced between the hMOR and the Gα subunit fused downstream of the receptor, to allow for a flexible junction between the two proteins. The sequence of the junction between hMOR and Gα cDNAs in the resulting fusion proteins was as follows: hMOR TCT AGA GGT GCA CAT ATG $G\alpha_{o1}$/$G\alpha_{i2}$, i.e., hMOR SRGAHM $G\alpha_{o1}$/$G\alpha_{i2}$. Cloning was done according to standard techniques. All constructs were sequenced to check for the absence of PCR-introduced mutations. Fusion proteins were expressed under the control of the *tac* promoter. Liquid cultures were inoculated at a 1:100 dilution with an overnight preculture issued from a single colony on an agar plate. Cultures were grown at 20°C in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) supplemented with 100 mg/L ampicillin. Expression of the fusion proteins was induced by addition of 0.5 mg/L isopropyl thiogalactoside when the culture had reached an OD_{600} of 0.5.

Ligand binding assays

Bacteria were harvested 5 h after induction and pelleted at 3,400 *g* for 15 min at 4°C. Bacterial membrane preparation was done as described (Stanasila et al., 1999). Protein concentration was estimated using the assay of Bradford (1976).

For saturation binding assays, aliquots of 2×10^9 intact cells or of 50 μg of membrane proteins were incubated for 1 h at 20°C in the presence of various concentrations of [^{3}H]diprenorphine in a final volume of 0.5 ml of TE buffer [50 m*M* Tris (pH 7.4) and 1 m*M* EDTA]. Competition binding was performed on 50-μg aliquots of membrane proteins, incubated at 20°C for 1 h in the presence of 2 n*M* [^{3}H]diprenorphine and various concentrations of agonists in a final volume of 125 μl of TEM [50 m*M* Tris-HCl (pH 7.4), 1 m*M* EDTA, and 10 m*M* $MgCl_2$]. Nonspecific binding was determined in the presence of 10^{-5} *M* naloxone. Reversal of high-affinity agonist binding was achieved in the presence of 100 μ*M* GTPγS. Separation of bound from free ligand was achieved by rapid filtration through GF/B filters pretreated with 0.1% polyethylenimine, followed by three washes with ice-cold 50 m*M* Tris-HCl (pH 7.4) on a Brandel cell harvester. Bound radioactivity was determined by scintillation counting.

Purification of bovine brain Gβγ subunits

Bovine brain Gβγ subunits were purified as described (Taylor et al., 1996) and stored at 1.6 mg/ml in a buffer containing 50 m*M* Tris, 5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* dithiothreitol, 100 m*M* NaCl, and 0.5% cholate, pH 7.6.

Reconstitution with purified Gβγ subunits

Membrane proteins from bacteria expressing hMOR–Gα fusion proteins were incubated at a concentration of 2 μg/μl in TEMC [50 m*M* Tris-HCl (pH 7.4), 1 m*M* EDTA, 10 m*M* $MgCl_2$, and 5 m*M* CHAPS] in the presence or absence of purified Gβγ subunits at a concentration of 200 n*M*. After vigorous vortex-mixing, the incubation was left to proceed for 1 h on ice. Aliquots of membrane proteins were then diluted five times with TEM and used for ligand competition binding or [^{35}S]GTPγS binding assays.

[^{35}S]GTPγS binding assays

Aliquots of bacterial membrane proteins containing the equivalent of 25 fmol of receptor sites (50 and 65 μg of membrane proteins for bacteria expressing MBP–hMOR–$G\alpha_{o1}$ and MBP–hMOR–$G\alpha_{i2}$, respectively) preincubated in TEMC in the presence or absence of purified Gβγ subunits were incubated for 30 min at 20°C in binding buffer (TEM, 10 μ*M* GDP, and 1 n*M* [^{35}S]GTPγS) with various concentrations of agonists in a final volume of 125 μl. Reversal of agonist-induced binding was achieved in the presence of 5×10^{-5} *M* naloxone. Separation of bound from free [^{35}S]GTPγS was performed by rapid filtration through GF/B filters and three washes with ice-cold 50 m*M* Tris-HCl, pH 7.4. Bound radioactivity was determined by scintillation counting.

Immunodetection of the expressed proteins

One-milliliter samples of induced bacterial cultures were spun down, and the bacterial pellets were used for immunodetection of expression. Fusion proteins were detected after so-

dium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% acrylamide gels and transferred onto Immobilon membranes (Millipore) by means of anti-MBP polyclonal antibodies or anti-Gα_o and anti-Gα_{i2} monoclonal antibodies, at a dilution of 1:10,000 and 1:200, respectively. Western blots were developed using peroxidase-conjugated anti-rabbit and anti-mouse IgG, respectively, and the chemiluminescence ECL Plus detection kit.

Data treatment

Binding data were analyzed using PRISM software (GraphPad, San Diego, CA, U.S.A.). Statistical significance of the data was assessed with the paired *t* test.

RESULTS

Expression of receptor–Gα fusion proteins

For expression of hMOR–Gα fusion proteins we used the conditions established as optimal for the expression of human opioid receptors in bacteria (Stanasila et al., 1999). The hMOR was fused at its N terminus to the soluble periplasmic MBP, and *E. coli* strain JM101 was chosen for expression of the recombinant proteins. Immunodetection using the anti-MBP antiserum (Fig. 1a) revealed the presence of protein bands compatible with the expected molecular mass of the MBP–hMOR–Gα fusion proteins (~127 kDa). Several lower-molecular-mass bands were also present and probably represented degradation products. Monoclonal antibodies directed against Gα_o and Gα_{i2} (Fig. 1b) identified a protein band of apparent molecular mass of 120 kDa, which was lacking in the control (bacteria expressing MBP–hMOR). Nonspecific detection of several protein bands was equally present. The bands revealed by anti-MBP and anti-Gα antibodies coincided, thus confirming expression of full-length MBP–hMOR–Gα fusion proteins.

The fused receptor was able to bind the nonselective opioid antagonist [^{3}H]diprenorphine with high affinity: K_D values were 0.48, 0.55, and 0.66 n*M* for the MBP–hMOR–Gα_{o1}, MBP–hMOR–Gα_{i2}, and MBP–hMOR constructs, respectively. The number of MBP–hMOR–Gα_{o1} and MBP–hMOR–Gα_{i2} binding sites estimated by saturation [^{3}H]diprenorphine binding was 30 (0.53 pmol/mg of membrane protein) and 23 receptors per cell (0.4 pmol/mg of membrane protein), respectively. These values were comparable to the level obtained for MBP–hMOR (Stanasila et al., 1999). Three independent experiments performed in duplicate gave consistent results (data not shown).

MBP–hMOR–Gα_{o1}, MBP–hMOR–Gα_{i2}, and MBP–hMOR fusion proteins will be hereafter referred to as hMOR–Gα_{o1}, hMOR–Gα_{i2}, and hMOR, respectively.

Reconstitution of high-affinity agonist binding

We investigated coupling between the hMOR and the two selected Gα subtypes, Gα_{o1} and Gα_{i2}, within the receptor–Gα fusion protein and the role played by added G$\beta\gamma$ subunits in this interaction. Competition binding experiments were performed to study the ability of various μ-selective agonists to displace [^{3}H]diprenorphine binding to hMOR fused to Gα_{o1} and Gα_{i2}. We chose four

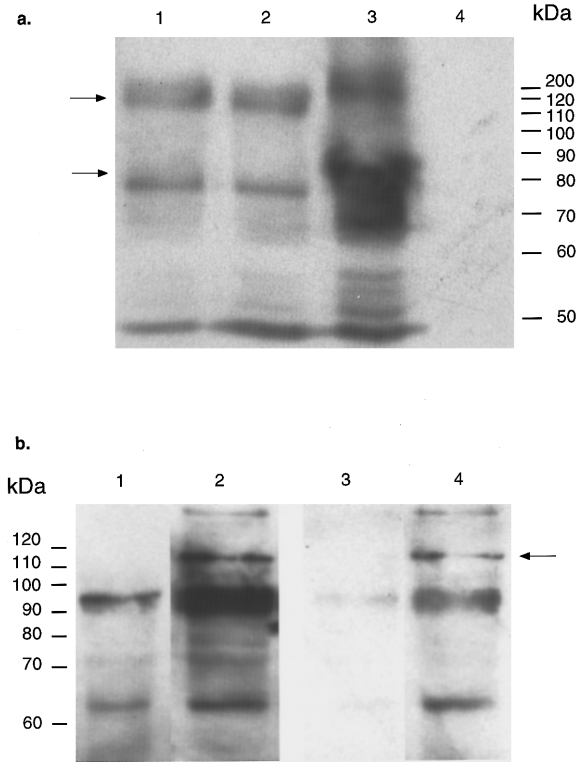

FIG. 1. a: Immunodetection of *E. coli*-expressed hMOR constructs using anti-MBP polyclonal antibodies. Samples were taken from isopropyl thiogalactoside-induced cultures of JM101 bacteria, wild-type (lane 4) or harboring plasmids that encoded MBP–hMOR–Gα_{i2} (lane 1), MBP–hMOR–Gα_{o1} (lane 2), or MBP–hMOR (lane 3). Arrows indicate the position of the MBP–hMOR–Gα fusion proteins (~127 kDa) and of MBP–hMOR (~87 kDa). The higher-molecular-weight band detected in lane 3 possibly represents MBP–hMOR dimers (Stanasila et al., 1999). **b:** Immunodetection of *E. coli*-expressed, hMOR–Gα fusion proteins using anti-Gα monoclonal antibodies. Lanes 1 and 2, anti-Gα_o antibody; lanes 3 and 4, anti-Gα_{i2} antibody. Samples were taken from isopropyl thiogalactoside-induced cultures of JM101 bacteria harboring plasmids that encoded MBP–hMOR–Gα_{i2} (lane 2), MBP–hMOR–Gα_{o1} (lane 4), and MBP–hMOR (lanes 1 and 3). The arrow indicates the position of the receptor–Gα fusion proteins (~127 kDa). The upper bands distinguishable in lanes 2 and 4 might represent MBP–hMOR–Gα protein oligomers (Stanasila et al., 1999).

μ-selective agonists, including morphine, DAMGO, and the recently isolated endogenous μ-agonists endomorphin-1 and endomorphin-2 (Zadina et al., 1997). The fusion proteins were reconstituted in CHAPS with purified bovine brain G$\beta\gamma$ subunits in large molar excess (the receptor–Gα:G$\beta\gamma$ molar ratio was set at 1:250) or were subjected to mock reconstitution in CHAPS only. Competition curves were fitted to a one- or two-site competition model by nonlinear regression, and K_i values were calculated for the low- and high-affinity binding sites. Control competition binding experiments were performed on *E. coli*-expressed hMOR. To check for the presence of contaminating Gα subunits in the G$\beta\gamma$ dimer preparation, achievement of high-affinity DAMGO binding was followed after reconstitution of C6 glioma cell

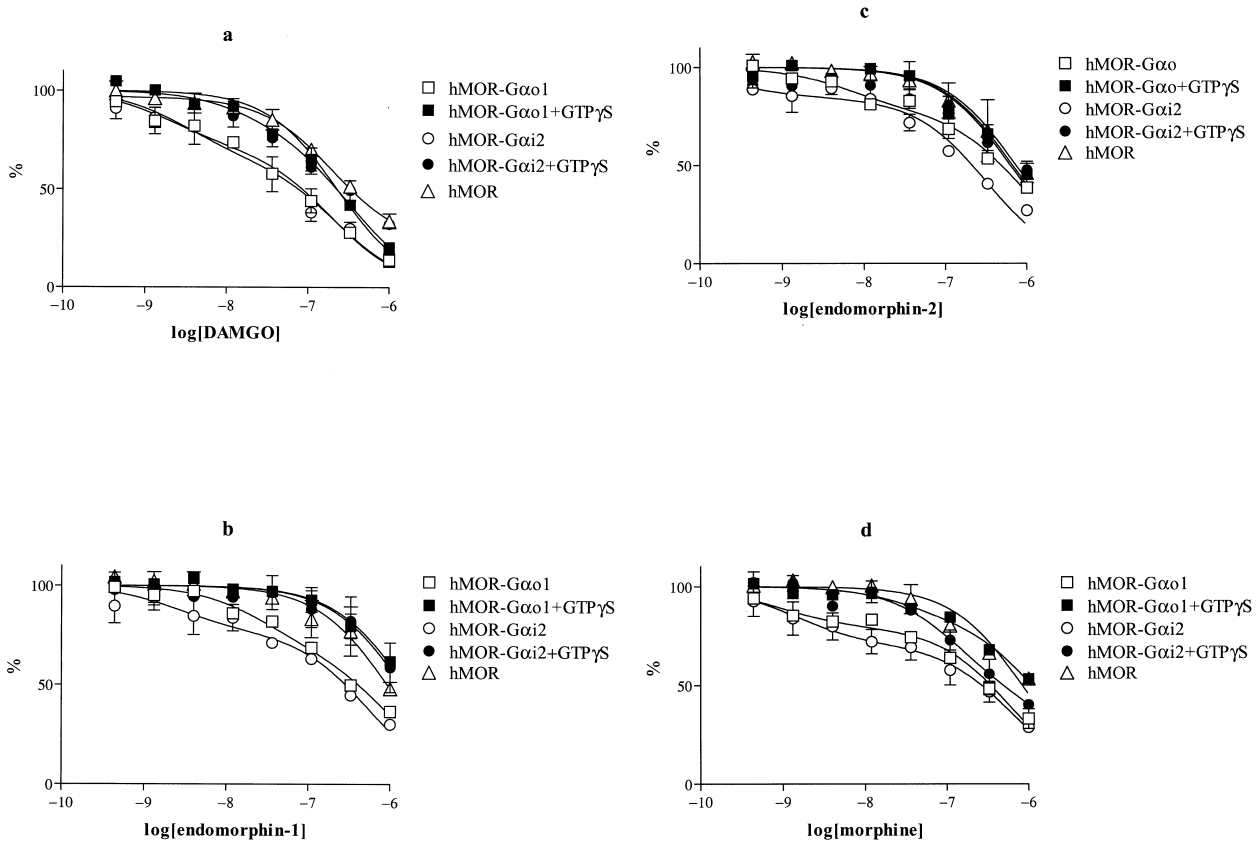

FIG. 2. Competition binding experiments on receptor–Gα fusion proteins reconstituted with G$\beta\gamma$ subunits. Curves represent [^{3}H]diprenorphine competition by (**a**) DAMGO, (**b**) endomorphin-1, (**c**) endomorphin-2, and (**d**) morphine on bacterial membranes preincubated with 200 n*M* G$\beta\gamma$. The curves were fitted to a two- or one-site competition model using the program PRISM. Reversal of high-affinity agonist binding was performed in the presence of 100 μM GTPγS. Nonspecific binding was determined in the presence of 10^{-5} *M* naloxone. Data are mean ± SEM (bars) values of three independent experiments performed in duplicate.

membranes constitutively expressing the μ-opioid receptor with G$\beta\gamma$ dimers and/or purified Gα_{i1} subunits (W. K. Lim and R. R. Neubig, manuscript submitted). No significant increase in [^{3}H]DAMGO binding was observed with either Gα or G$\beta\gamma$ subunits alone, whereas high-affinity binding could be restored by reconstituting the receptor with both Gα and G$\beta\gamma$. This showed that the Gα contamination of the G$\beta\gamma$ preparation, if present at all, was negligible.

As described previously (Stanasila et al., 1999), competition binding of μ-opioid agonists at *E. coli*-expressed hMOR showed only the presence of a low-affinity site. The binding properties of these agonists at the hMOR–Gα fusion proteins were significantly different. In the absence of G$\beta\gamma$ subunits, competition binding experiments with all four agonists on receptor–Gα_{i2} and with DAMGO and morphine on receptor–Gα_{o1} showed that two populations of binding sites, of high and low affinity, were present. However, binding of endomorphin-1 and -2 to hMOR-Gα_{o1} fitted best a one-site competition model (data not shown). Figure 2 displays the competition curves obtained with each agonist on the two receptor–Gα fusion proteins after reconstitution with purified G$\beta\gamma$ subunits, as compared with hMOR alone. High-affinity binding could be reversed by addition of excess GTPγS, confirming that the increase in agonist affinity was due to receptor coupling to the fused Gα subunit. As a rule, data from experiments performed on receptor–Gα fusion proteins in the absence of GTPγS fitted better a two-site competition model, whereas data from control experiments in the presence of excess GTPγS matched a one-site competition model.

Table 1 shows K_i values calculated for the high- and low-affinity binding sites of each of the four agonists tested on the two receptor–Gα fusion proteins, in the absence of G$\beta\gamma$ or reconstituted with purified G$\beta\gamma$ subunits. Differences could be detected in the behavior of the two receptor fusion proteins toward agonists in the absence of G$\beta\gamma$ subunits. DAMGO and morphine detected the presence of two receptor populations at both hMOR–Gα_{o1} and hMOR–Gα_{i2}; their binding at the high-affinity as well as the low-affinity sites at the two receptor–Gα chimeras was characterized by similar K_i values, irrespective of the nature of the fused Gα subunit. Binding of endomorphin-1 and -2 at hMOR–Gα_{o1} was best fitted to a one-site competition model representing low-affinity binding. Yet, binding of endomorphins at hMOR–Gα_{i2} could be fitted to a two-site competition

TABLE 1. *Competition binding in the absence and in the presence of Gβγ subunits*

	DAMGO		Endomorphin-1		Endomorphin-2		Morphine	
	− Gβγ	+ Gβγ	− Gβγ	+ Gβγ	− Gβγ	+ Gβγ	− Gβγ	+ Gβγ
hMOR–$G\alpha_{o1}$								
High-affinity	2 ± 1	1 ± 0.2	—	5 ± 0.4	—	1 ± 0.2	0.8 ± 0.4	0.2 ± 0.04
Low-affinity	110 ± 50	40 ± 8	30 ± 8[a]	200 ± 40	90 ± 10[a]	170 ± 10	180 ± 40	100 ± 10
hMOR–$G\alpha_{i2}$								
High-affinity	7 ± 3	0.1 ± 0.04[b,c]	11 ± 5	0.3 ± 0.2[b,c]	18 ± 9	0.06 ± 0.002[b,c]	0.4 ± 0.2	0.2 ± 0.06
Low-affinity	230 ± 120	33 ± 2	250 ± 140	120 ± 10	410 ± 160	70 ± 10	170 ± 50	180 ± 20
hMOR[a]	50 ± 20		170 ± 90		100 ± 20		200 ± 30	

Data are mean ± SEM K_i values (in n*M*) of [^{3}H]diprenorphine displacement by agonists on bacterial membranes in the absence of Gβγ or reconstituted with 200 n*M* purified Gβγ subunits from three independent experiments performed in duplicate.

[a]According to a one-site competition model.

[b]Significantly different from the K_i value for high-affinity binding obtained in the absence of Gβγ ($p < 0.001$).

[c]Significantly different from the corresponding value at hMOR–$G\alpha_{o1}$ ($p < 0.001$).

model, corresponding to a mixed population of high- and low-affinity binding sites. K_i values of all four agonists at the low-affinity site were in agreement with values obtained on *E. coli*-expressed hMOR.

After addition of Gβγ subunits, the binding of the two endomorphins on hMOR–$G\alpha_{o1}$ could detect the presence of a second population, composed of high-affinity binding sites. K_i values for the high-affinity binding of DAMGO and endomorphins at hMOR–$G\alpha_{i2}$, but not at hMOR–$G\alpha_{o1}$, were significantly decreased. Morphine constituted an exception as its affinity for hMOR–$G\alpha_{i2}$ remained unaffected by addition of Gβγ. As expected, addition of Gβγ did not significantly affect K_i values corresponding to binding at the low-affinity sites at any one receptor–Gα fusion.

Reconstitution of the receptor–Gα fusion proteins with purified Gβγ dimers underscored differences in the pharmacological properties conferred on the receptor by the presence of the different Gα subunits. A comparison of K_i values for high-affinity binding on hMOR–$G\alpha_{o1}$ and hMOR–$G\alpha_{i2}$ in the presence of Gβγ revealed differences in the behavior of the four agonists. Whereas morphine displayed similar affinities for the two receptor–Gα fusion proteins, the other three agonists had significantly higher affinities for hMOR–$G\alpha_{i2}$ than for hMOR-$G\alpha_{o1}$. The selectivity factor, defined as the ratio between the two K_i values corresponding to binding at the high-affinity site of hMOR–$G\alpha_{o1}$ and hMOR–$G\alpha_{i2}$, was 10, 17, 17, and 1 for DAMGO, endomorphin-1, endomorphin-2, and morphine, respectively.

[^{35}S]GTPγS binding activity

To assess further the functionality of the receptor–Gα protein fusion proteins, we studied agonist stimulation of [^{35}S]GTPγS binding on the fused Gα subunit. The time course of [^{35}S]GTPγS binding on the two receptor–Gα chimeras in the absence of agonist and Gβγ subunits showed significant ($p < 0.01$) basal binding activity of the hMOR–$G\alpha_{o1}$, but not hMOR–$G\alpha_{i2}$, fusion protein as compared with the hMOR control (Fig. 3). Reconstitution with purified Gβγ subunits before [^{35}S]GTPγS binding assay generated a stronger signal at both receptor–Gα fusion proteins. After addition of Gβγ subunits, the basal [^{35}S]GTPγS binding activity at hMOR–$G\alpha_{i2}$ was also increased to values significantly above the hMOR control ($p < 0.05$). Lack of known inverse agonists for the μ-opioid receptor subtype prevented further investigation of whether the presence of the receptor was

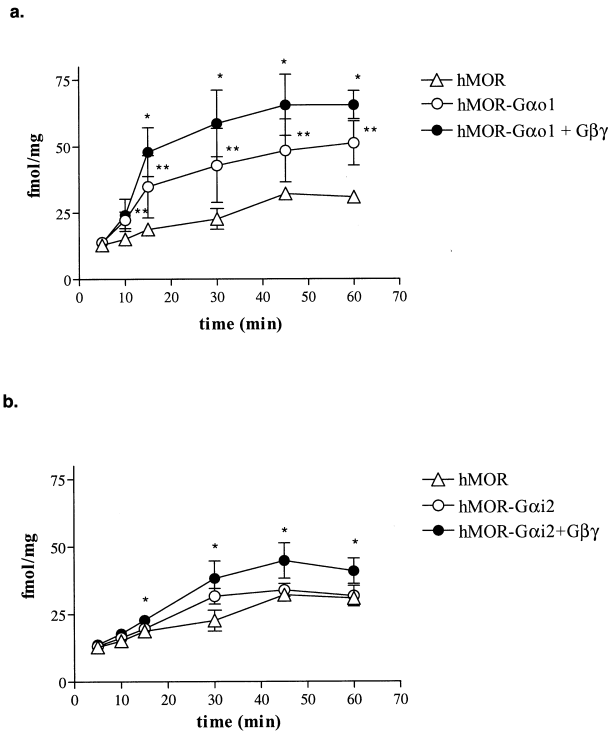

FIG. 3. Time course of [^{35}S]GTPγS binding in the absence of agonist. The concentration of receptor sites in each aliquot was 0.2 n*M*. Data are mean ± SEM (bars) values, in fmol of [^{35}S]GTPγS bound/mg of membrane protein, of three independent experiments performed in duplicate. **a:** [^{35}S]GTPγS binding at hMOR–$G\alpha_{o1}$. *$p < 0.05$, significantly different from values obtained in the absence of Gβγ subunits; **$p < 0.01$, significantly different from hMOR control values. **b:** [^{35}S]GTPγS at hMOR–$G\alpha_{i2}$. *$p < 0.05$, significantly different from hMOR control values.

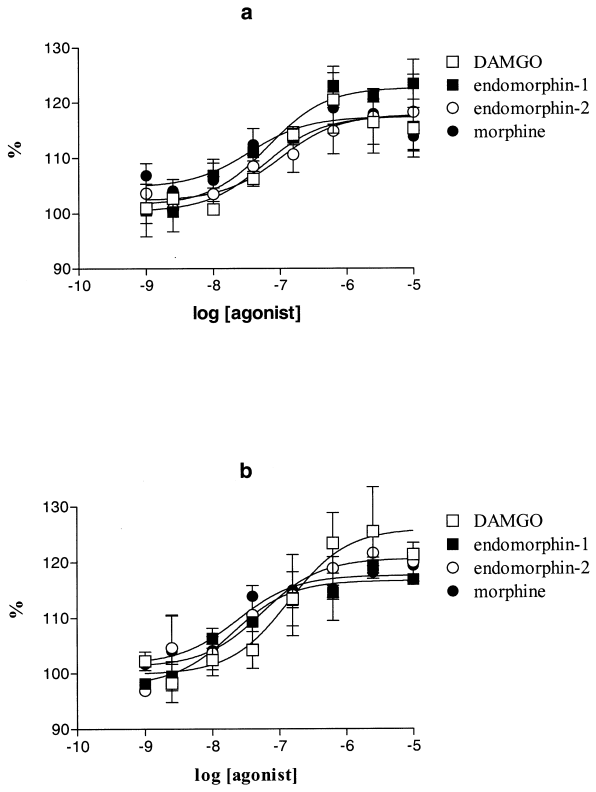

FIG. 4. Concentration-dependent stimulation of [^{35}S]GTPγS binding by μ-opioid agonists: (**a**) hMOR-Gα_{o1} and (**b**) hMOR–Gα_{i2}. The concentration of receptor sites in each aliquot was 0.2 n*M*. Data are mean ± SEM (bars) values, in percentages of bound [^{35}S]GTPγS in the absence of agonist on bacterial membranes pretreated with 200 n*M* purified Gβγ subunits, of three independent experiments performed in duplicate.

in any way responsible for this apparent constitutive activity or whether it was solely due to basal [^{35}S]GTPγS binding at the Gα subunits.

Figure 4 displays dose–response curves of hMOR–Gα_{o1} and hMOR–Gα_{i2} in the presence of Gβγ subunits, at various concentrations of agonist, ranging from 10^{-9} to 10^{-5} *M*. All four agonists tested were able to stimulate [^{35}S]GTPγS binding on both receptor–Gα fusion proteins. DAMGO, endomorphins, and morphine displayed comparable potencies in stimulating [^{35}S]GTPγS binding at hMOR–Gα_{o1}. In contrast, at hMOR–Gα_{i2}, endomorphin-1 and morphine were significantly more potent than DAMGO and endomorphin-2 in stimulating [^{35}S]GTPγS binding ($p < 0.05$; Table 2).

Figure 5 shows maximal levels of stimulation of [^{35}S]GTPγS binding reached with the four agonists on the two receptor–Gα fusion proteins after reconstitution with purified Gβγ dimers. Intrinsic activities of the four agonists tested were similar at any one receptor–Gα fusion protein. Agonist-induced [^{35}S]GTPγS binding could in each case be displaced by an excess of the nonselective opioid antagonist naloxone. Reversal of agonist-induced [^{35}S]GTPγS binding in the presence of naloxone was not complete, probably owing to the fact

TABLE 2. *Agonist potencies in stimulating [^{35}S]GTPγS binding, measured as EC_{50} values*

	EC_{50} (n*M*)			
	DAMGO	Endomorphin-1	Endomorphin-2	Morphine
hMOR–Gα_{o1}	81 ± 25	46 ± 15	61 ± 6	53 ± 7
hMOR–Gα_{i2}	110 ± 38	28 ± 3	113 ± 39	18 ± 4

[^{35}S]GTPγS was used at a fixed concentration of 1 n*M*. Agonists were added at various concentrations ranging from 9×10^{-10} to 10^{-5} *M*, in the presence of 200 n*M* Gβγ subunits. Data are mean ± SEM values of three independent experiments performed in duplicate.

that in our experimental conditions the excess of naloxone over the agonist was only fivefold. The magnitude of the [^{35}S]GTPγS binding signal was low: The fraction of the hMOR–Gα population that bound [^{35}S]GTPγS in the presence of 10^{-5} *M* agonist amounted to 14–15% and 11–12% of the total receptor sites in the case of hMOR–Gα_{o1} and hMOR–Gα_{i2}, respectively. This suggests that all the receptor–Gα molecules that were able to bind ligands were not equally functional in terms of Gα activation. This could be due to the partial degradation of the fusion proteins, as suggested by the presence of a band of ~80 kDa detected by the anti-MBP antiserum that could correspond to an MBP–hMOR product after cleavage of the Gα subunit (Fig. 1). This supposition is reinforced by the fact that the 80-kDa band was not detected by anti-Gα antibodies.

DISCUSSION

We have previously shown that human opioid receptors expressed in *E. coli* are able to bind opioid ligands and that high-affinity binding of agonists can be reconstituted by addition of purified trimeric G proteins (Stanasila et al., 1999). Here we report expression in *E. coli*

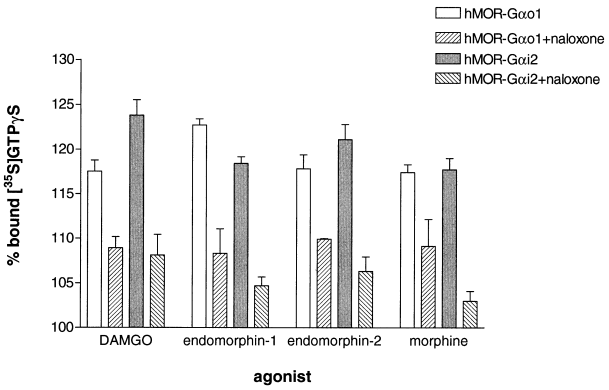

FIG. 5. Maximal stimulation of [^{35}S]GTPγS binding. Data are mean ± SEM (bars) values, in percentages of bound [^{35}S]GTPγS in the presence of 10^{-5} *M* agonist on bacterial membranes pretreated with 200 n*M* purified Gβγ subunits, of two or three independent experiments performed in duplicate. Reversal of agonist-stimulated [^{35}S]GTPγS binding was performed in the presence of 5×10^{-5} *M* naloxone.

of the hMOR fused at its C terminus to either one of two Gα subunits, Gα_{o1} and Gα_{i2}, previously identified as possible coupling partners (Laugwitz et al., 1993; Chan et al., 1995; Murthy and Makhlouf, 1996). The *E. coli*-expressed receptor–Gα chimeras were functional, as shown by the facts that the presence of the fused Gα subunit restored high-affinity agonist binding and that agonists were able to stimulate [^{35}S]GTPγS binding at the Gα subunit fused to the receptor C terminus. Reconstitution of coupling between the fused receptor and the Gα_{o1} and Gα_{i2} subunits confirmed that these two subtypes of Gα subunits are involved in signaling through the hMOR. It is interesting that the pharmacological characterization of the two individual receptor–Gα couples provided evidence that the affinity of the receptor for the different agonists depended on the nature of the Gα subunit to which it is coupled. DAMGO and endomorphin-1 and -2 showed a higher affinity for the hMOR–Gα_{i2} fusion protein, suggesting that these agonists stabilize the receptor population that is coupled to Gα_{i2} better than the population that coupled to Gα_{o1}. Morphine showed no such preference, indicating that its binding to the hMOR has different requirements than that of the other three agonists. The observed differences in agonist recognition by the receptor fused to one or to the other Gα subunit, in the presence or in the absence of G$\beta\gamma$ dimers, may serve as a means to characterize the multiple states the receptor is able to adopt as a function of its interaction partners.

Receptor–Gα fusion proteins have become an increasingly popular tool for the study of GPCR–G protein signaling pathway. The β_2-adrenergic receptor fused to its coupling partner Gα_s was able to restore adenylyl cyclase activation in a cell line defective in endogenous Gα_s (Bertin et al., 1994). This first report of receptor–Gα fusion functionality has been followed by several studies of receptor–G protein interactions where the receptor and the Gα were expressed from a chimeric open reading frame (for review, see Seifert et al., 1999*a*; Milligan, 2000).

One major inconvenience of eukaryotic expression systems, commonly used for pharmacological studies, is the simultaneous presence within the cell of endogenous G protein subtypes, resulting in a potentially high background. This issue remains relevant for studies of receptor–Gα chimeras. In the case of the α_2-adrenergic receptor–Gα_{i1} fusion protein, it has been shown that receptor interactions are not restricted to the tethered Gα subunit, but they can also occur with endogenous G proteins (Burt et al., 1998). Different means were used so far to lower the background signal. Receptor–Gα chimeras were expressed in insect cells where the receptor was known not to couple effectively to the endogenous G proteins (Seifert et al., 1998). Also, a mutant Gα subunit resistant to pertussis toxin treatment was used to allow for selective inactivation of the endogenous $G_{i/o}$ (Wise and Milligan, 1997). The first strategy is not always applicable as coupling to insect G proteins has been shown to occur with many GPCRs expressed in insect cells (Van den Broeck, 1996). Also, Gα mutants resistant to toxin treatment are not always available, and an accurate assessment of G protein behavior may require the use of native proteins. Bacteria lack a G protein-mediated signal transduction pathway; thus, the coupling specificity of an *E. coli*-expressed GPCR may be studied in an environment devoid of interfering endogenous G proteins or regulatory elements such as regulators of G protein signaling, GPCR kinases, and arrestins.

hMOR–Gα fusion proteins were expressed in *E. coli* at levels compatible with pharmacological studies, although low (~30 receptor sites per cell, or 0.5 pmol/mg of membrane protein). The hMOR fused at its C terminus to either Gα_{o1} or Gα_{i2} bound the antagonist diprenorphine with high affinity, indicating, as expected, that the presence of the Gα subunit had no bearing on its antagonist binding characteristics.

Absolute values drawn from experiments with receptor–Gα fusion proteins are probably biased as a consequence of the constrained proximity between the receptor and the Gα subunit. Thus, they do not necessarily accurately describe the behavior of receptors and G proteins in vivo. Further experiments, involving a comparison between receptor–Gα constructs and receptors reconstituted with purified Gα subunits, are necessary to ascertain to what extent tethering of the Gα subunit bears on its receptor-coupling properties. Previous reports of reconstitution studies of the μ-opioid receptor with purified Gα subunits (Ueda et al., 1988; Fan et al., 1995; Gaibelet et al., 1999) do not determine pharmacological profiles of the receptor in the presence of the different Gα subunits but merely assess the general capacity of this receptor to couple to the members of G$\alpha_{i/o}$ family of G proteins. Yet, so far experimental data have validated the use of receptor–Gα fusion constructs as models for the functional studies of GPCRs (Milligan, 2000). Moreover, our conclusions are based on a comparative analysis of results using two different, similarly constructed, hMOR–Gα fusion proteins; this approach can be expected to minimize the impact of the physical constraint of the two partners on the pharmacological properties of the receptor.

It can be estimated (Fig. 5; see Results) that only a fraction of the total ligand binding sites also represents functional [^{35}S]GTPγS binding sites. This could be attributed to proteolytic degradation of the MBP–hMOR–Gα fusion proteins, with the Gα subunit being probably cleaved off. A different proteolytic rate between hMOR–Gα_{o1} and hMOR–Gα_{i2} could bias the results of ligand affinity and efficacy determination at the two fusion proteins. However, the fraction of [^{35}S]GTPγS binding sites of the total number of ligand binding sites is not significantly different between the two receptor–Gα fusion proteins. Moreover, data concerning agonist stimulation of [^{35}S]GTPγS binding are represented as percentages of the background [^{35}S]GTPγS binding at each hMOR–Gα fusion protein. Thus, results are independent of the expression level of each fusion protein.

According to the ternary complex model describing receptor–G protein interactions (Leff et al., 1997), efficient coupling of the receptor to its cognate G protein manifests itself as high-affinity agonist binding and as enhanced GDP–GTP exchange at the Gα subunit. This latter may be estimated in terms of [^{35}S]GTPγS binding on Gα (Befort et al., 1996). We checked whether the presence of $G\alpha_{o1}$ and $G\alpha_{i2}$ fused downstream of the hMOR was able to induce high-affinity binding of several μ-selective agonists. In the presence of purified Gβγ subunits, high-affinity binding of all four agonists tested was achieved with both fusion constructs. Both fused Gα subunits kept their capacity of [^{35}S]GTPγS binding, further stimulated by agonists, as shown by the dose–response curves in the presence of increasing concentrations of the ligand. These results confirm that the hMOR is capable of effective coupling to both $G\alpha_{o1}$ and $G\alpha_{i2}$, as previously suggested by studies performed on heterologously expressed hMORs in mammalian cells (Laugwitz et al., 1993; Chan et al., 1995; Murthy and Makhlouf, 1996).

It is known that the presence of Gβγ dimers promotes coupling between the receptor and the catalytic Gα subunit. Previous reports showed that Gβγ subunits were needed for coupling to occur in vitro in reconstituted systems, for instance, between *E. coli*-expressed A_1 adenosine receptor (Jockers et al., 1994). Our results tend to support the favorable effect of Gβγ dimers on receptor–Gα coupling. After reconstitution with Gβγ, high-affinity binding for endomorphins was restored at hMOR–$G\alpha_{o1}$, and K_i values of high-affinity binding sites for DAMGO and endomorphins were decreased at hMOR–$G\alpha_{i2}$ (Table 1). Yet, coupling to Gα in the absence of Gβγ dimers was surprisingly effective as high-affinity agonist binding was apparent in all cases except for the binding of endomorphins at hMOR–$G\alpha_{o1}$ (Table 1). This supports the notion that the high-affinity state of the receptor can also be stabilized through interaction with the Gα subunit alone. Presumably the covalent link engineered between the receptor and Gα reduces the degrees of freedom in the mobility of the Gα subunit and thus increases coupling efficacy, accounting in part for these results.

Yet, in some cases tethering of the Gα subunit to the receptor does not compensate for the absence of Gβγ: Addition of Gβγ dimers to Gβγ-depleted membranes containing the A_1-adenosine receptor fused to $G\alpha_{i1}$ promoted high-affinity agonist binding (Waldhoer et al., 1999). It is likely that some variability exists, related to the specific ligand–receptor–Gα triad under study. Our results support this hypothesis, providing evidence that the affinities of the different agonists tested were differently sensitive to the presence of Gβγ. For instance, on addition of Gβγ, the affinity of morphine at hMOR–$G\alpha_{i2}$ remained virtually unchanged, whereas the affinities of DAMGO and endomorphins were significantly increased (Table 1).

It has been already shown that different Gα subunits have different affinities for GDP or for GTP; $G\alpha_{o1}$ has a higher basal rate of nucleotide exchange than $G\alpha_{i2}$ (Ferguson et al., 1986). Thus, we cannot rule out that the apparently higher constitutive activity of the hMOR–$G\alpha_{o1}$ fusion protein (Fig. 3) may be entirely due to different affinity constants for guanine nucleotides. At the same time, this prevents comparison between maximal levels of [^{35}S]GTPγS binding induced by one agonist at the two receptor–Gα fusion proteins. Nevertheless, it is relevant to compare intrinsic activities and efficacies of different agonists at each receptor–Gα chimera. In the presence of Gβγ dimers, all agonists tested were equally potent in stimulating [^{35}S]GTPγS binding at hMOR–$G\alpha_{o1}$. At hMOR-$G\alpha_{i2}$, under the same conditions, endomorphin-1 and morphine were significantly more potent than DAMGO and endomorphin-2 (Table 2). No correlation was found between K_i and EC_{50} values of the different agonists, supporting the view that agonist binding and agonist-induced activation of the G protein are two distinct processes.

Endomorphin-1 and -2 have been reported to be partial agonists of the μ-opioid receptor, in native tissues as well as in transient expression systems (Alt et al., 1998; Sim et al., 1998). In the presence of Gβγ dimers, no evidence of partial agonist behavior was found in our experiments. All agonists tested induced similar maximal [^{35}S]GTPγS binding levels (Fig. 5).

The main outcome of the present study is that activities of different agonists were differently sensitive to the nature of the Gα subunit that was attached to the receptor and to the presence or absence of Gβγ. DAMGO and endomorphins, but not morphine, showed a higher affinity for hMOR–$G\alpha_{i2}$ than for hMOR–$G\alpha_{o1}$. The rank orders of agonist potencies in stimulating [^{35}S]GTPγS binding at hMOR–$G\alpha_{o1}$ and hMOR–$G\alpha_{i2}$ did not coincide. These discrepancies depict a complex picture of the interactions within the triad ligand–receptor–G protein. Our results are in agreement with the hypothesis that the receptor adopts different conformations depending on the nature of the Gα subunit to which it is coupled (Leff et al., 1997). There is increasing evidence in support of the notion that G protein type influences agonist efficacy (Seifert et al., 1999*b*; Yang and Lanier, 1999). In vivo experiments of selective down-regulation of different Gα subunits by antisense oligodeoxynucleotide treatment (Sanchez-Blazquez et al., 1999) showed that agonists activate with different efficacies the G proteins regulated by the μ receptor. In particular, an antisense probe targeting $G\alpha_{i2}$ blocked the analgesic effects of DAMGO, endomorphin-2, and morphine, whereas antisense treatment directed against $G\alpha_{o1}$ had little or no effect on opioid-induced analgesia. Our results, showing a higher affinity of DAMGO and endomorphins for the hMOR–$G\alpha_{i2}$ complex than for hMOR–$G\alpha_{o1}$, suggest that this is due to preferential binding of these agonists to receptors coupled to $G\alpha_{i2}$. A different mechanism probably applies in the case of morphine, which displayed similar affinities for the two receptor–Gα complexes.

The concept of the simultaneous existence of multiple possible receptor conformations, selectively favored by

interaction with different agonists and G proteins, might help explain the phenomenon of differential agonist regulation of the μ-opioid receptor. It has been found that prolonged exposure of the μ-opioid receptor to the opioid agonists methadone and buprenorphine, but not to DAMGO or morphine, abolished the ability of opioids to inhibit adenylyl cyclase (Blake et al., 1997). Distinct differences in protein kinase A-mediated phosphorylation pattern of the μ-opioid receptor were generated by morphine and related alkaloids as compared with DAMGO and other enkephalin analogues (Chakrabarti et al., 1998). A peculiar behavior has been described for morphine, which is unable to induce internalization of the μ-opioid receptor (Sternini et al., 1996). It has been shown that morphine binding at the receptor favors a receptor conformation that interacts ineffectively with β-arrestin and subsequently with clathrin (Whistler and von Zastrow, 1998; Zhang et al., 1998). It is intriguing that in our experiments morphine behaved distinctly from the other agonists tested: Its binding to the fused receptor was little influenced by the nature of the Gα subunit or by the presence or absence of Gβγ dimers.

Our results converge toward the hypothesis of a diversity of possible receptor conformations, differently able to interact productively with ligands, G proteins, or, possibly, regulators of signaling. At the same time, the results prove that *E. coli*-expressed receptor–Gα fusion proteins are functional and apt for reconstitution with purified Gβγ subunits. As the prokaryotic environment allows control over the receptor and G protein subtype composition of the experimental setup, *E. coli*-expressed receptor–Gα chimeras should prove a useful tool in dissecting the interplay of elements involved in G protein-mediated signal transduction.

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