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Molecular Basis of Differences in (−)(*trans*)-3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide-Induced Desensitization and Phosphorylation between Human and Rat κ-Opioid Receptors Expressed in Chinese Hamster Ovary Cells

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ABSTRACT

The agonist (−)(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide [(−)U50,488H] caused desensitization of the human κ-opioid receptor (hkor) and Flag-tagged hkor (Flag-hkor) but not the rat κ-opioid receptor (rkor) and Flag-tagged rkor (Flag-rkor) stably expressed in CHO cells as assessed by guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate) binding. In addition, (−)U50,488H stimulation enhanced phosphorylation of the Flag-hkor, but not Flag-rkor. (−)U50,488H-induced phosphorylation of the Flag-hkor was reduced by expression of the dominant negative mutant GRK2-K220R, demonstrating the involvement of G protein-coupled receptor kinases (GRKs). However, expression of GRK2 and arrestin-2 or GRK3 and arrestin-3 did not result in desensitization or phosphorylation of the Flag-rkor after (−)U50,488H pretreatment. To understand the molecular basis of the species differences, we constructed two Flag-tagged chimeric receptors, Flag-h/rkor and Flag-r/hkor, in which the C-terminal domains of Flag-hkor and Flag-rkor were switched. When stably expressed in CHO cells, Flag-r/hkor, but not Flag-h/rkor, was desensitized and phosphorylated after exposure to (−)U50,488H, indicating that the C-terminal domain plays a critical role in the differences. We then generated a Flag-hkor mutant, in which S358 was mutated to N (Flag-hkorS358N) and a Flag-rkor mutant, in which N358 was substituted with S (Flag-rkorN358S). Although Flag-hkorS358N was not phosphorylated or desensitized by (−)U50,488H stimulation, Flag-rkorN358S underwent (−)U50,488H-induced desensitization with slightly increased phosphorylation. These results indicate that there are differences in (−)U50,488H-induced desensitization and phosphorylation between the hkor and the rkor. In addition, the C-terminal domain plays a crucial role in these differences and the 358 locus contributes to the differences. Our findings suggest caution in extrapolating studies on κ-opioid receptor regulation from rats to humans.

Most G protein-coupled receptors (GPCRs) show attenuated responsiveness to agonists after prolonged or repeated activation. Three temporally distinct processes that occur over a time scale of seconds to days have been demonstrated: desensitization (seconds to hours), internalization (minutes to hours), and down-regulation (hours to days) (for reviews, see Krupnick and Benovic, 1998; Law et al., 2000). Binding of an agonist to a GPCR, in addition to activating downstream effectors, also initiates adaptive responses by enhancing phosphorylation of the receptor. Phosphorylation in most cases occur in the C-terminal domain and/or the third intracellular loop, which is catalyzed by GPCR kinases (GRKs) and, in some cases, protein kinases activated by second messengers. Phosphorylation of the GPCR facilitates the binding of arrestins, leading to the uncoupling of the GPCR from G proteins and, hence, reduced responsiveness to the cognate agonists. Binding of arrestins also results in internalization of the receptor, which is a rapid agonist-induced movement of the receptor into intracellular compartments from the

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; hkor, human κ-opioid receptor; rkor, rat κ-opioid receptor; (−)U50,488H, (−)(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide; U69,593, (5α,7α,8β)-(+)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)benzeneacetamide; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); CHO, Chinese hamster ovary; Flag-hkor, Flag-tagged human κ-opioid receptor; Flag-h/rkor, Flag-tagged chimera of human κ-opioid receptor 1–338/rat κ-opioid receptor 339–380; Flag-hkorS358N, S358N mutant of the Flag-tagged human κ-opioid receptor; Flag-rkor, Flag-tagged rat κ-opioid receptor; Flag-r/hkor, Flag-tagged chimera of rat κ-opioid receptor 1–338/human κ-opioid receptor 339–380; Flag-rkorN358S, N358S mutant of the Flag-tagged rat κ-opioid receptor; rmor, the rat μ-opioid receptor; PCR, polymerase chain reaction.

plasma membrane, where it is unavailable for signal transduction. Down-regulation involves a reduction in the number of the receptor.

Opioid receptors belong to the rhodopsin subfamily of the GPCR family and can be classified into at least three types (μ, δ, and κ) based on pharmacological (for review, see Pasternak, 1988), anatomical (for review, see Mansour et al., 1988), and molecular (for review, see Knapp et al., 1995) analyses. Activation of κ-opioid receptors produces many effects, including analgesia (von Voigtlander et al., 1983; Dykstra et al., 1987), dysphoria (Pfeiffer et al., 1986; Dykstra et al., 1987) and water diuresis (von Voigtlander et al., 1983; Dykstra et al., 1987), hypothermia (Adler and Geller, 1993), and modulation of immune responses (Taub et al., 1991). Activation of κ-opioid receptors is coupled via pertussis toxin-sensitive G proteins to affect a variety of effectors, which include adenylate cyclase, potassium channels, and calcium channels and the p42/p44 mitogen-activated protein kinase pathway (for review, see Law et al., 2000).

Chronic use of κ-opioid agonists causes tolerance (Murray and Cowan, 1988; Bhargava et al., 1989) that can be partially accounted for at the receptor level (von Voigtlander et al., 1983; Bhargava et al., 1989; Morris and Herz, 1989). Agonist-induced desensitization of the κ-opioid receptor was examined in cell systems with different results. Incubation with (*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide (U50,488H) or U69,593 was found to cause desensitization of the human κ-opioid receptor (hkor) stably transfected into Chinese hamster ovary (CHO) cells (Ling et al., 1998; Zhu et al., 1998) or HEK-293 cells (Blake et al., 1997) and the mouse κ-opioid receptor expressed in AtT-20 cells (Tallent et al., 1998). Appleyard et al. (1997) demonstrated that the κ-receptor in guinea pig hippocampal slices was desensitized and phosphorylated in an agonist-dependent manner. In contrast, treatment with U50,488H or U69,593 did not cause desensitization of the mouse κ-receptor in R1.1 thymoma cells (Joseph and Bidlack, 1995) or the rat κ-opioid receptor (rkor) stably expressed in CHO cells (Avidor-Reiss et al., 1995). Inhibition of forskolin-stimulated adenylate cyclase (Avidor-Reiss et al., 1995; Joseph and Bidlack, 1995; Blake et al., 1997; Tallent et al., 1998; Zhu et al., 1998), enhancement of [^{35}S]GTPγS binding (Zhu et al., 1998), increase in K^+ current (Appleyard et al., 1997; Tallent et al., 1998) and extracellular acidification response (Ling et al., 1998) were employed as functional endpoints. In *Xenopus laevis* oocytes expressing the rkor, U69,593 caused a slight desensitization and expression of GRK3 or GRK5 and arrestin-3 greatly enhanced the extent of desensitization with K^+ current as the functional endpoint (Henry et al., 1995; Appleyard et al., 1999). The discrepancy in these studies may be attributed to differences in cell system, functional endpoint, and species of origin of the κ-receptor clones.

We previously observed that after exposure to (−)U50,488H, the hkor expressed in CHO cells underwent desensitization, internalization, and down-regulation (Zhu et al., 1998; Li et al., 1999, 2000) and internalization and down-regulation occurred via GRK-, arrestin-, and dynamin-dependent pathways (Li et al., 1999, 2000). In contrast, the rkor stably expressed in CHO cells did not undergo internalization and down-regulation when activated by (−)U50,488H (Li et al., 1999, 2000; Jordan et al., 2000). However, no comparison between the hkor and the rkor in the same system in agonist-induced desensitization and phosphorylation has been reported. In this study, we first assessed whether there was a difference in (−)U50,488H-induced desensitization of the hkor and the rkor stably expressed in CHO cells. We found that the hkor was desensitized, but the rkor was not. The differences between the rkor and the hkor receptors in CHO cells provided a unique opportunity to delineate the mechanisms underlying agonist-induced regulation of the hkor. The amino acid sequences of the hkor and the rkor are 94.2% identical (Zhu et al., 1995). To understand the molecular basis of the hkor-rkor difference in (−)U50,488H-induced desensitization, we epitope-tagged the hkor and the rkor and generated chimeric and mutant receptors. Whether the chimeras and mutants were desensitized and phosphorylated by (−)U50,488H treatment was investigated. [^{35}S]GTPγS binding, which has been established as a functional measure of the κ-opioid receptor activation (Zhu et al., 1997), was used as the endpoint.

Experimental Procedures

Materials. [^{35}S]GTPγS (~1,250 Ci/mmol), [^{3}H]diprenorphine (58 Ci/mmol), and [^{32}P]orthophosphate (8,500–9,100 Ci/mmol) were purchased from PerkinElmer Life Science (Boston, MA). (−)U50,488H was provided by Upjohn Co. (Kalamazoo, MI). Naloxone was a gift from DuPont/Merck Co. (Wilmington, DE). Rabbit polyclonal antibody against the Flag epitope, GDP, GTPγS, sodium fluouride, calyculin A, and tetrasodium pyrophosphate were obtained from Sigma Co. (St. Louis, MO). Pansorbin was obtained from Calbiochem (La, Jolla, CA). Enhanced chemiluminesence Western blotting detection reagents were purchased from Amersham Biosciences (Piscataway, NJ). Horseradish peroxidase-linked goat polyclonal anti-rabbit IgG was produced by New England Biolabs (Beverly, MA). Geneticin was purchased from Mediatech Co. (Herndon, VA). Protease inhibitor cocktail was obtained from Roche Molecular Biochemicals (Indianapolis, IN). LipofectAMINE and enzymes and chemicals used in molecular biology and mutagenesis experiments were purchased from Invitrogen (Carlsbad, CA), Promega (Madison, WI), Roche Molecular Biochemicals, and QIAGEN Co. (Valencia, CA). Expression constructs of GRK2, GRK2-K220R, GRK3, arrestin-2, arrestin-3, and Flag-tagged β_2-adrenergic receptor were gifts from Dr. Jeffrey L. Benovic of Thomas Jefferson University School of Medicine.

Establishment of CHO Cell Lines and Cell Culture. Clonal CHO cell lines stably expressing the hkor, rkor, Flag-hkor, Flag-rkor, Flag-r/hkor, Flag-h/rkor, Flag-hkorS358N, and Flag-rkorN358S receptors were established as described previously (Chen et al., 1995). Cells were cultured in 100-mm culture dishes in Dulbecco's modified Eagle's medium F12 HAM supplemented with 10% fetal calf serum, 0.2 mg/ml geneticin, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37°C.

Pretreatment with the κ-Agonist (−)U50,488H. At ~90% confluence, cells were washed once with 100 mM phosphate-buffered saline and treated without (control) or with the κ-opioid agonist (−)U50,488H (1 μM) in the medium for an indicated period. Cells were washed four times with cold Kreb's solution on ice to remove (−)U50,488H.

Membrane Preparation. Membranes were prepared according to Li et al. (2001) with some modifications. Briefly, the CHO cells were pelleted, frozen at −80°C for at least 30 min, thawed in cold lysis buffer (5 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSF, 10 μM leupeptin, 10 mM sodium fluouride, and 10 mM tetrasodium pyrophosphate, pH 7.4) and vortexed. Cell suspension was passed through a 1-ml 29_G3/8 syringe needle five times and centrifuged. Pellets were resuspended in 50 mM Tris-HCl buffer (pH

7.0), passed through the syringe needle, and centrifuged, and the processes were repeated. Membranes were suspended in 50 mM Tris-HCl buffer (pH 7.4), and protein concentration was determined by the bicinchoninic acid method of Smith et al. (1985).

Saturation Binding of [^{3}H]Diprenorphine. Saturation binding of [^{33}H]diprenorphine to the wild-type, chimeric, and mutant κ-opioid receptors was performed with at least six concentrations of [^{33}H]diprenorphine (ranging from 25 pM to 1–2 nM) and K_d and B_{max} values were determined. Binding was carried out in 50 mM Tris-HCl buffer containing 1 mM EGTA (pH 7.4) at room temperature for 1 h in duplicate in a final volume of 1 ml with 10 to 20 μg of membrane protein. Naloxone (10 μM) was used to define nonspecific binding. Binding data were analyzed with the EBDA program (McPherson, 1983).

[^{35}S]GTPγS Binding Assay. Determination of [^{35}S]GTPγS binding to G proteins was carried out with a procedure modified from that of Li et al. (2001). For each experiment, 10 μg of membrane protein was incubated with 15 μM GDP and 0.2 nM [^{35}S]GTPγS in reaction buffer (50 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 0.1% bovine serum albumin, pH 7.4) in a final volume of 0.5 ml. Nonspecific binding was determined in the presence of 10 μM GTPγS. Seven concentrations (10 pM–10 μM) of (−)U50,488H were used to generate dose-response curves. After 60 min of incubation at 30°C, bound and free [^{35}S]GTPγS were separated by filtration with GF/B filters. Radioactivity on filters was determined by liquid scintillation counting. The results were expressed as (−)U50,488H-stimulated [^{35}S]GTPγS binding in femtomoles per milligram of protein with the basal binding subtracted. EC_{50} values and maximal responses (E_{max}) of the drug were determined by curve fitting to the equation for a sigmoidal curve $E = (E_{max} / [1 + ([D] / EC_{50})^n]) +$ basal level, where E is effect produced by a certain concentration of the drug, $[D]$, E_{max} is the maximal response elicited by the drug, and n is a fitting parameter.

Transient Expression of GRKs and Arrestins in CHO-Flag-rkor Cells and GRK2-K220R in CHO-Flag-hkor Cells. CHO-Flag-rkor cells were transiently transfected with 4 μg of GRK2/100-mm dish in pcDNA3.1 Zeo(+) plus 4 μg of arrestin-2/100-mm dish in pcDNA 3.1 Zeo(+), 4 μg of GRK3/100-mm dish in pcDNA 3 plus 4 μg of arrestin-3/100-mm dish in pcDNA 3, or 4 μg/100-mm dish each of GRK2 and GRK3 (Sterne-Marr and Benovic, 1995, and references therein) or the vectors by using LipofectAMINE (50 μl) following the manufacturer's instructions. CHO-Flag-hkor cells were similarly transfected with 8 μg of GRK2-K220R/100-mm dish (Kong et al., 1994) in pcDNA3.1 Zeo(+). Forty-eight to 72 h after transfection, cells were used for desensitization or phosphorylation experiments.

Phosphorylation of the Wild-Type, Chimeric, and Mutant κ-Opioid Receptors. Phosphorylation was conducted according to a procedure we described previously (Carman et al., 2000). CHO cells stably expressing each construct were transferred from 100-mm dishes into 6-well plates and cultured overnight to confluence. Cells were then grown in 1 ml/well phosphate-free medium and incubated at 37°C for 2 h. [^{32}P]orthophosphate (0.25 mCi/well) was added and incubated for another 2 h and medium was aspirated. Cells were incubated without or with 1 μM (−)U50,488H for an indicated period of time at 37°C, cooled on ice, and washed three times with ice-cold phosphate-buffered saline. All subsequent steps were carried out at 4°C. Cells were solubilized for at least 1 h with solubilization buffer [2% digitonin, 0.5% sodium deoxycholate, 2 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 20 nM calyculin A, and a protease inhibitor cocktail (5 μg/ml antipain dihydrochloride, 0.2 μg/ml aprotinin, 4 μg/ml bestatin, 0.6 μg/ml chymostatin, 0.05 μg/ml E-64, 0.02 mg/ml EDTA, 0.01 mg/ml Pefabloc SC, 0.07 μg/ml pepstatin, 1 μg/ml phosphoramidon, and 0.05 μg/ml leupeptin)] and centrifuged at 100,000*g* for 1 h. Immunoprecipitation of the wild-type and mutant rkor and hkor was performed with a specific polyclonal antibody against Flag followed by Pansorbin (final 1/200, 4°C, 1 h) according to a modified procedure of Luthin et al. (1988). The mixture was centrifuged and the pellets were washed three times by centrifugation and resuspension. Immunoprecipitated materials were dissolved in 2× Lammeli sample buffer and subjected to 7 or 8% SDS-polyacrylamide gel electrophoresis (Chen et al., 1995) and autoradiography.

Western Blot. Western blot was performed to examine the expression of the Flag-tagged wild-type and mutant rkor and hkor proteins as described previously (Li et al., 2001). Briefly, stably transfected CHO cells or membranes were treated as indicated, solubilized with Laemmli sample buffer, subjected to SDS-PAGE, and transferred onto nitrocellulose membranes. Nitrocellulose membranes were treated with blocking solution, incubated with a rabbit polyclonal antibody against Flag and then goat anti-rabbit polyclonal IgG conjugated with horseradish peroxidase, reacted with enhanced chemiluminescence Western blotting detection reagents, and exposed to X-ray films.

Construction of Flag-Tagged Wild-Type, Chimeric, and Mutant Receptors. Correct generations of all constructs were confirmed by DNA sequence determination.

Flag Tag Fragment. An ~130-bp fragment containing a signal peptide and the Flag tag sequence was excised with *Hin*dIII and *Nco*I from a construct of Flag-tagged β_2-adrenergic receptor in pcDNA3, with Flag-tagged 5′ to the initiation codon (Guan et al., 1992).

Flag-Tagged hkor (Flag-hkor). The Flag tag fragment was inserted 5′ to the Met start codon, and Flag-hkor inserted in *Hin*dIII and *Xba*I sites of vector pcDNA3 was generated previously (Xu et al., 2000).

Flag-Tagged rkor (Flag-rkor). The fragment containing the coding region and a short 3′-noncoding region of the rkor was generated by polymerase chain reaction (PCR) using the *Nco*I-RK (ATC ACC ATG GAG TCC CCC ATC) and RK-*Not*I (TAT GCG GCC GCA CCA AGA TCA TTG AAC TC) as primers and the rkor in pcDNA3 (Li et al., 1993) as the template. The resulting PCR product was treated with *Nco*I and *Not*I. The *Hin*dIII/*Nco*I-generated ~130-bp Flag fragment and the *Nco*I/*Not*I-treated PCR product (1.3 kilobases) were ligated into *Hin*dIII and *Not*I sites of the vector pcDNA3 to generate the Flag-rkor.

Flag-Tagged hkor1-338/rkor339-380 (Flag-h/rkor). This chimera combined the fragment of the N terminus to the end of TM7 of the hkor with the C-terminal domain of the rkor (see Fig. 5) with the overlap PCR method (Higuchi et al., 1988). The hkor and rkor have the same nucleotide sequence (GCC TTT CTT GAT GAA AAC TTC AAG) corresponding to ^{331}AFLDENFK^{338} at the end of TM7 and the beginning of C-terminal domain. Sense and antisense oligodeoxynucleotides corresponding to the amino acids 331–338 were synthesized for use as primers. The fragment of the hkor(N terminus-TM7) was generated by PCR using the *Nco*I-HK (ATC ACC ATG GAC TCC CCG ATC) and the antisense oligodeoxynucleotide for amino acids 331–338 as primers and the hkor in pBK/CMV (Stratagene, La Jolla, CA; Zhu et al., 1995) as the template. The rkor(C-terminal domain) was produced by PCR using the sense oligodeoxynucleotide (for amino acids 331–338) and RK-*Not*I (TAT GCG GCC GCA CCA AGA TCA TTG AAC TC) as primers and the rkor in pcDNA3 (Li et al., 1993) as the template. Overlap PCR was performed with the hkor(N terminus-TM7) and the rkor(C-terminal domain) as the templates and *Nco*I-HK and RK-*Not*I as primers. The resulting PCR product was treated with *Nco*I and *Not*I. The *Hin*dIII/*Nco*I-generated ~130-bp Flag tag fragment and the *Nco*I/*Not*I-treated PCR product [hkor(N terminus-TM7)/rkor(C-terminal domain)] were ligated into *Hin*dIII and *Not*I sites of the vector pcDNA3 to generate the chimera construct.

Flag-Tagged rkor1-338/hkor339-380 (Flag-r/hkor). This chimera contains the fragments of the rkor(N terminus-TM7) and the hkor(C-terminal domain) (see Fig. 5). The rkor(N terminus-TM7) fragment was generated by PCR using the NcoI-RK primer (ATC ACC ATG GAG TCC CCC ATC) and the antisense oligodeoxynucleotide (for amino acids 331–338) as primers and the rkor in pcDNA3

(Li et al., 1993) as the template. The hkor(C-terminal domain) was produced by PCR using the sense oligodeoxynucleotide (for amino acids 331–338) and HK-*Not*I primer (TAT GCGGCC GCA GTG ATC TGA GTT AAA CC) as primers and the hkor in pBK/CMV (Zhu et al., 1995) as the template. Overlap PCR was performed with the rkor(N terminus-TM7) and hkor(C-terminal domain) as the templates and *Nco*I-RK and *Not*I-HK as primers. The resulting PCR product was treated with *Nco*I and *Not*I. The *Hin*dIII/*Nco*I-generated ~130-bp Flag tag fragment and the *Nco*I/*Not*I-treated PCR product [rkor(N terminus-TM7)/hkor(C-terminal domain)] were ligated into *Hin*dIII and *Not*I sites of the vector pcDNA3 to generate the chimera construct.

S358N Mutant of the Flag-hkor (Flag-hkorS358N). S358N mutation was introduced into the Flag-hkor (see Fig. 5) with the overlap PCR method (Higuchi et al., 1988) and primers with point mutations. The fragment of hkor(N terminus to 358N) was generated by PCR using the *Nco*I-HK and the antisense of AGA GCA CTA ACA GAG TCC CG (the point mutation is underlined) as primers and the hkor in pBK/CMV (Zhu et al., 1995) as the template. The fragment of 358N to the 3′-noncoding region was generated by PCR using the sense sequence of AGA GCA CTA ACA GAG TCC CG and HK-*Not*I as primers and the hkor in pBK/CMV (Zhu et al., 1995) as the template. Overlap PCR was performed with the *Nco*I-HK and the HK-*Not*I as primers and two fragments as the template. The resulting PCR product (hkor-S358N) was treated with *Nco*I and *Not*I. The *Hin*dIII/*Nco*I-generated ~130-bp Flag tag fragment and the *Nco*I/*Not*I-treated PCR product (hkor-S358N) were ligated into *Hin*dIII and *Not*I sites of the vector pcDNA3 to generate the Flag-hkorS358N.

N358S Mutant of the Flag-rkor (Flag-rkorN358S). N358S mutation was introduced into the Flag-rkor (see Fig. 5). The fragments of Flag-rkor (5′-noncoding region to 358S) was generated by PCR using CTG GCT AAC TAG AGA ACC (the pcDNA3 5′ primer) and antisense of CAG AGC ACA AGC AGA GTT AGA (the point mutation is underlined) as primers and the Flag-rkor in pcDNA3 as the template. The fragment of 358S to the 3′-noncoding region was generated by PCR using the sense sequence of CAG AGC ACA AGC AGA GTT AGA and GGC AAA CAA CAG ATG GCT GGC AAC TA (the pcDNA3 3′ primer) as primers and the Flag-rkor as the template. Overlap PCR was performed with the 5′ primer and the 3′ primer and two PCR fragments as the template. The resulting PCR product was treated with *Eco*RI and *Not*I. The *Hin*dIII/*Eco*RI-generated fragment containing the Flag fragment and part of the rkor (~500 bp) from Flag-rkor in pcDNA3 and the *Eco*RI/*Not*I-treated PCR product were ligated into *Hin*dIII and *Not*I sites of the vector pcDNA3 to generate the Flag-rkorN358S.

Results

Determination of K_d and B_{max} Values of [^{3}H]Diprenorphine Binding to the Wild-Type and Flag-Tagged Wild-Type, Chimeric, and Mutant κ-Opioid Receptors. Each of the constructs hkor, rkor, Flag-hkor, Flag-rkor, Flag-h/rkor, Flag-r/hkor, Flag-hkorS358N, and Flag-rkorN358S was stably expressed in CHO cells. Clonal cell lines for each construct were established. Cell lines that expressed 1 to 3 pmol of receptor/mg of protein were selected. Saturation binding of [^{3}H]diprenorphine to one clonal cell line of each receptor in membranes was performed and K_d and B_{max} values were determined. All chimeras and mutants displayed similar affinities for the antagonist [^{3}H]diprenorphine as the wild types, with K_d values ranging from 0.08 to 0.13 nM (Table 1), indicating that binding pockets of the chimeras and mutants do not differ significantly from those of the wild types. The B_{max} values ranged from 1.00 to 2.74 pmol/mg of protein (Table 1).

Differences in (−)U50,488H-Induced Desensitization between the hkor and the rkor. Pretreatment of CHO-hkor cells with 1 μM (−)U50,488H for 60 min significantly reduced (−)U50,488H-stimulated [^{35}S]GTPγS binding (Fig. 1A; Table 2). Dose-response curve of (−)U50,488H was shifted downward and rightward. The EC_{50} of (−)U50,488H was significantly increased and maximal [^{35}S]GTPγS binding was decreased, compared with the control. In contrast, the same pretreatment did not reduce responsiveness of the rkor (Fig. 1B; Table 2). Neither the EC_{50} nor the maximal [^{35}S]GTPγS binding of (−)U50,488H was changed significantly. There were no significant differences in basal [^{35}S]GTPγS binding between the control and (−)U50,488H-treated CHO-hkor or CHO-rkor cells. These results indicate that there is a difference in agonist-induced desensitization between the hkor and the rkor expressed in CHO cells.

To facilitate biochemical studies, we epitope-tagged both receptors with the Flag sequence. Similar differences in (−)U50–488H-induced desensitization were observed between the Flag-hkor and the Flag-rkor stably transfected into CHO cells (Fig. 1, C and D; Table 2), indicating that the presence of the Flag sequence did not influence desensitization properties of the hkor and the rkor after exposure to (−)U50,488H. The Flag tag did not significantly affect the EC_{50} and E_{max} values of (−)U50,488H in stimulating [^{35}S]GTPγS binding. There were no significant differences in basal [^{35}S]GTPγS binding between the control and (−)U50,488H-treated CHO-Flag-hkor or CHO-Flag-rkor cells. We examined three clone cell lines each for the Flag-hkor and the Flag-rkor, and they yielded similar results.

Differences in (−)U50,488H-Promoted Phosphorylation between the Flag-hkor and the Flag-rkor. The difference between the hkor and the rkor in (−)U50,488H-induced desensitization may be due to differential (−)U50,488H-promoted phosphorylation of the receptors. We thus examined receptor phosphorylation after exposure to (−)U50,488H. Treatment with (−)U50,488H for 15 min enhanced phosphorylation of the Flag-hkor, but not the Flag-rkor (Fig. 2A), although both receptors were expressed well as determined by Western blot (Fig. 2B). Increasing pretreatment time to 30 or 60 min neither affected the degree of phosphorylation of the Flag-hkor appreciably nor enhanced phosphorylation of the Flag-rkor (Fig. 2A). Two clone cell lines each of the Flag-hkor and the Flag-rkor were examined and they yielded similar results. Therefore, we assessed (−)U50,488H-induced phosphorylation at 15 min in subse-

TABLE 1

K_d and B_{max} values of [^{3}H]diprenorphine binding to the wild-type, chimeric, and mutant κ opioid receptors stably expressed in CHO cells

Membranes were prepared from CHO cells stably expressing each receptor construct. Saturation binding of [^{3}H]diprenorphine was performed and K_d and B_{max} values were determined. Results are expressed as mean ± S.E.M. n = number of independent experiments in duplicate.

Receptor Construct	K_d	B_{max}	n
	nM	*pmol/mg of protein*	
hkor	0.16 ± 0.02	1.22 ± 0.05	3
rkor	0.20 ± 0.20	1.00 ± 0.14	3
Flag-hkor	0.10 ± 0.02	1.33 ± 0.15	6
Flag-rkor	0.26 ± 0.10	2.09 ± 0.31	6
Flag-r/hkor	0.12 ± 0.01	2.74 ± 0.14	4
Flag-h/rkor	0.10 ± 0.01	2.34 ± 0.13	3
Flag-rkorN358S	0.13 ± 0.02	2.25 ± 0.03	3
Flag-hkorS358N	0.08 ± 0.01	1.27 ± 0.15	6

quent experiments unless indicated otherwise. Phosphorylated Flag-hkor appeared as a broad and diffuse band with a molecular mass range of 52 to 63 kDa and the median of 58 kDa (Fig. 2A), indicating that it is a glycoprotein.

(−)U50,488H-promoted phosphorylation of the Flag-hkor was blocked by the antagonist naloxone (Fig. 2A), indicating that receptor activation is required. Expression of the dominant negative mutant GRK2-K220R reduced U50,488H-induced phosphorylation of the Flag-hkor, demonstrating that the phosphorylation is GRK-mediated (Fig. 2C).

Effects of Expression of GRK2 and Arrestin-2 or GRK3 and Arrestin-3 on Response of the Flag-rkor to

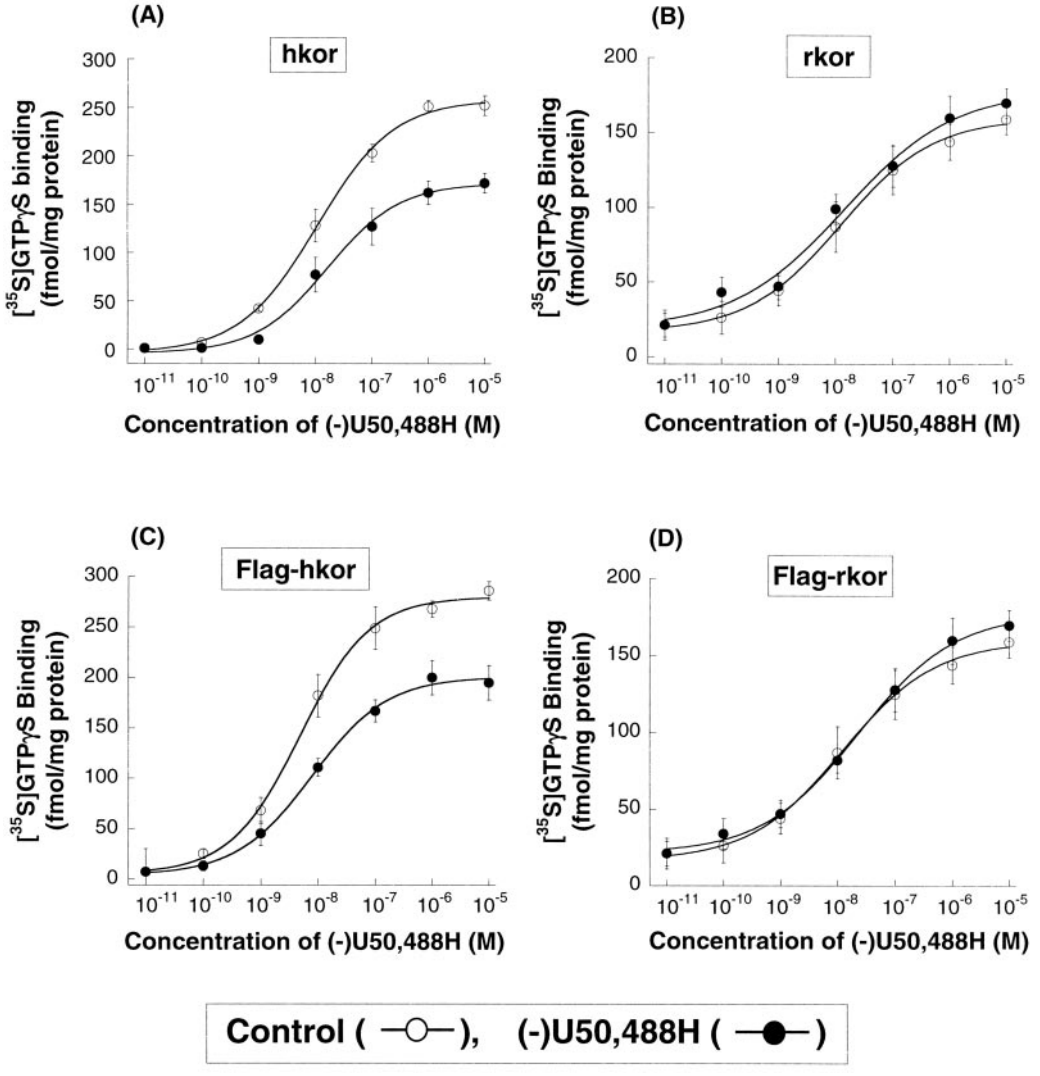

Fig. 1. Effect of (−)U50,488H pretreatment of CHO-hkor cells (A), CHO-rkor cells (B), CHO-Flag-hkor cells (C), and CHO-Flag-rkor cells (D) on (−)U50,488H-stimulated [^{35}S]GTPγS binding. Cells were pretreated without (control) or with 1 μM (−)U50,488H at 37°C for 60 min and washed to remove the agonist. Membranes were prepared and [^{35}S]GTPγS binding in response to (−)U50,488H was performed. [^{35}S]GTPγS binding data were normalized to femtomoles per milligram of protein. Basal binding, about 150 fmol/mg of protein, was subtracted from each value, and data are expressed as (−)U50,488H-stimulated [^{35}S]GTPγS binding. Each point represents the mean ± S.E.M. of 4 (A), 3 (B), 7 (C), and 9 (D) independent experiments in duplicate. EC_{50} values and maximal [^{35}S]GTPγS binding are shown in Table 2.

TABLE 2

Effect of (−)U50,488H pretreatment of the wild-type, chimeric, and mutant κ opioid receptors on [^{35}S]GTPγS binding stimulated by the subsequent application of (−)U50,488H: EC_{50} values and maximal responses (E_{max}) of (−)U50,488H

Clonal CHO cells stably transfected with each of the receptor constructs were treated without (control) or with 1 μM (−)U50,488H at 37°C for 60 min and washed. Membranes were prepared and [^{35}S]GTPγS binding was performed. Results are expressed as mean ± S.E.M. n = number of independent experiments in duplicate.

Receptor Construct	Control		(−)U50,488H-Treated		n
	EC_{50}	E_{max}	EC_{50}	E_{max}	
	nM	*fmol/mg of protein*	*nM*	*fmol/mg of protein*	
hkor	6.32 ± 0.97	258.0 ± 10.0	13.79 ± 3.07[a]	184.5 ± 9.6[b]	4
rkor	5.16 ± 0.38	144.3 ± 20.7	8.27 ± 0.64	141.3 ± 14.6	3
Flag-hkor	4.69 ± 0.91	287.7 ± 8.5	15.35 ± 4.17[a]	200.9 ± 18.5[b]	7
Flag-rkor	2.11 ± 0.25	143.4 ± 7.0	4.97 ± 0.53	156.6 ± 7.03	9
Flag-h/rkor	4.13 ± 0.82	175.1 ± 7.8	7.32 ± 1.61	190.8 ± 9.54	8
Flag-r/hkor	2.72 ± 0.59	332.8 ± 7.0	18.67 ± 3.96[b]	237.6 ± 28.4[b]	5
Flag-rkorN358S	3.64 ± 1.35	336.3 ± 29.2	1.25 ± 0.26	236.4 ± 13.5[b]	5
Flag-hkorS358N	3.30 ± 1.08	253.1 ± 27.6	1.84 ± 1.01	283.0 ± 36.6	7

[a] $p < 0.05$.
[b] $p < 0.01$ compared with the control by Student's t test.

(−)U50,488H Pretreatment. The inability of the Flag-rkor to undergo desensitization and phosphorylation may be due to insufficient amounts of GRKs and arrestins present in cells. We thus examined whether expression of GRK2 and arrestin-2 or GRK3 and arrestin-3 had any effects. Transfection efficiency was about 60% as determined by transfection with a green fluorescent protein construct. There was no (−)U50,488H-induced desensitization of the Flag-rkor in cells transfected with GRK2 and arrestin-2 or GRK3 and arrestin-3, similar to cells transfected with the vector pcDNA3 or pcDNA3.1Zeo(+) (Fig. 3). In addition, expression of GRK2 and GRK3 did not increase the extent of phosphorylation of the Flag-rkor after (−)U50,488H treatment (Fig. 4A), nor did expression of GRK2 or GRK3 alone (data not shown). As a positive control, although morphine only slightly enhanced phosphorylation of the rat μ-opioid receptor (rmor) in CHO cells, expression of GRK2 and GRK3 greatly enhanced morphine-promoted phosphorylation (Fig. 4B), similar to what Zhang et al. (1998) and we (Carman et al., 2000) reported. There results indicate that the failure of the Flag-rkor to undergo (−)U50,488H-induced desensitization and phosphorylation is not due to low levels of GRKs and arrestins.

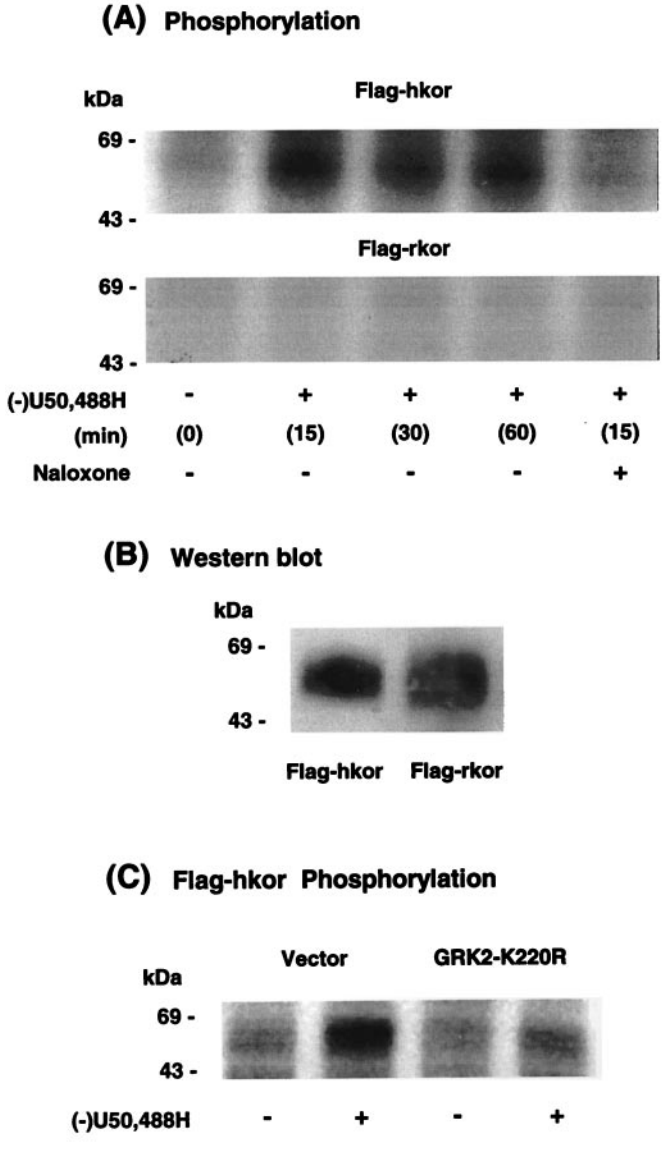

Fig. 2. Effect of (−)U50,488H treatment on phosphorylation of the Flag-hkor and the Flag-rkor (A) and Western blot of Flag-hkor and Flag-rkor (B). C, effect of expression of the dominate negative mutant GRK2-K220R on phosphorylation of Flag-hkor stably expressed in CHO cells. A, CHO-Flag-hkor cells and CHO-Flag-rkor cells were grown for 24 h in 6-well plates. Cells were metabolically labeled with [^{32}P]orthophosphate, incubated without or with 1 μM (−)U50,488H and 10 μM naloxone at 37°C for indicated periods, and then solubilized. Flag-hkor and Flag-rkor were immunoprecipitated with a rabbit polyclonal antibody against Flag and immunoprecipitated mixtures were subjected to SDS-PAGE followed by autoradiography as described under *Experimental Procedures*. This figure represents one of the three experiments with similar results. B, Western blot of Flag-hkor and Flag-rkor was carried out with a rabbit polyclonal antibody against Flag to determine the relative molecular mass and assess the expression of the receptor proteins. The figure represents one of the two experiments performed with similar results. C, CHO-Flag-hkor cells were grown in 6-well plates and then transfected with 1.5 μg/well of the vector pcDNA3.1 Zeo(+) or the dominate negative mutant GRK2-K220R in pcDNA3.1 Zeo(+). Forty-eight to 72 h later, cells were metabolically labeled with [^{32}P]orthophosphate, incubated without or with 1 μM (−)U50,488H at 37°C for 15 min and processed for receptor phosphorylation as in A. This figure represents one of the two experiments with similar results.

Role of the C-Terminal Domain of the κ-Opioid Receptor in (−)U50,488H-Induced Desensitization and Phosphorylation. The amino acid sequences of intracellular regions of the rkor and the hkor are highly homologous with only some differences in the C-terminal domain (Fig. 5). To understand the molecular basis of the differences in (−)U50,488H-induced desensitization and phosphorylation between the hkor and the rkor, we constructed two Flag-tagged chimeric receptors, Flag-h/rkor [Flag-hkor(1–338)/rkor(339–380)] and Flag-r/hkor [Flag-rkor(1–338)/hkor(339–380)], in which the C-terminal domains were swapped. Unlike the rkor or the Flag-rkor, the Flag-r/hkor expressed stably in CHO cells underwent (−)U50,488H-promoted desensitization (Fig. 6A; Table 2). The dose-response curve of (−)U50,488H was shifted downward and rightward with its EC_{50} value increased by ~7-fold, and the maximal [^{35}S]GTPγS binding decreased by ~30%, compared with the control. In contrast to the hkor and the Flag-hkor, the Flag-h/rkor pretreated with (−)U50,488H did not exhibit significant desensitization and neither the E_{max} value nor the EC_{50} was changed significantly compared with those of the control (Fig. 6A; Table 2). There were no significant differences in basal [^{35}S]GTPγS binding between the control and (−)U50,488H-treated CHO-Flag-h/rkor or CHO-Flag-r/hkor cells. In addition, (−)U50,488H enhanced phosphorylation of the Flag-r/hkor but not the Flag-h/rkor (Fig. 6B), although both constructs were expressed to apparent similar extents (Fig. 6B). Experiments were performed on two and three clonal cell lines of the Flag-r/hkor and the Flag-h/rkor, respectively, with similar results. These results demonstrate that the C-terminal domain plays a crucial role in the observed species differences.

Role of the 358 Residues in the C-Terminal Domains of the hkor and the rkor in (−)U50,488H-Induced Desensitization and Phosphorylation. Comparison of the C-terminal domain amino acid sequences between the hkor and the rkor reveals that only seven residues are different (Fig. 5). One notable difference is the locus 358, where it is Ser in the hkor, but Asn in the rkor. Because GRKs, which are Ser/Thr kinases, have been implicated in (−)U50,488H-induced phosphorylation of the Flag-hkor, we constructed two mutants, Flag-hkor-S358N and Flag-rkor-N358S, to fur-

ther delineate the molecular basis of the observed species differences.

Pretreatment of CHO-Flag-hkorS358N cells with (−)U50,488H did not induce desensitization of the mutant (Fig. 7A; Table 2). In contrast, preincubation of CHO-Flag-rkorN358S cells with (−)U50,488H caused profound desensitization (Fig. 7A; Table 2). The E_{max} was reduced by ~30% with no significant change in EC_{50}. There were no significant differences in basal [^{35}S]GTPγS binding between the control and (−)U50,488H-treated Flag-hkorS358N or Flag-rkorN358S cells. In addition, although S358N mutation in the Flag-hkor completely abolished (−)U50,488H-induced phosphorylation, (−)U50,488H only slightly increased phosphorylation of the N358S mutant of the Flag-rkor (Fig. 7B). (−)U50,488H treatment did not seem to affect the amount of either receptor protein, as detected by Western blot. Extending the U50,488H treatment period from 15 min to 30 or 60 min did not increase the extent of phosphorylation of the Flag-rkorN358S (data not shown). Both receptor constructs were expressed well (Fig. 7B). Experiments were performed on two clonal cell lines each of the Flag-hkorS358N and the Flag-rkorN358S with similar results. These results indicate that the S358 in C-terminal domain of the hkor plays a key role in conferring the ability to undergo (−)U50,488H-induced desensitization and phosphorylation.

Discussion

In the present study, we have shown that after exposure to (−)U50,488H, the hkor and the Flag-hkor were desensitized and the Flag-hkor was phosphorylated. In contrast, the rkor and the Flag-rkor were not desensitized and the Flag-rkor was not phosphorylated. The C-terminal domains contribute to, and the 358 locus plays a nonexclusive role in, the differences in desensitization and phosphorylation between the hkor and the rkor. To the best of our knowledge, this study represents the first demonstration of such species difference in the regulation of GPCRs. In addition, this study provides first evidence for the importance of Ser-358 in (−)U50,488H-induced phosphorylation and desensitization of the hkor.

Desensitization and Phosphorylation of the hkor and the Flag-hkor. The hkor and the Flag-hkor were readily desensitized by (−)U50,488H pretreatment. This finding is consistent with our previous report (Zhu et al., 1998) and those of Blake et al. (1997) and Ling et al. (1998).

A 15-min incubation with 1 μM (−)U50,488H enhanced phosphorylation of the Flag-hkor, which was blocked by the antagonist naloxone, indicating that receptor activation is required for phosphorylation. A longer incubation time (30 min or 60 min) did not seem to increase the degree of phosphorylation of the Flag-hkor. We have previously shown that significant desensitization of the hkor is observed after a 15-min incubation with 1 μM (−)U50,488H (Zhu et al., 1998). One-hour pretreatment with 1 μM (−)U50,488H led to a slightly higher extent of desensitization of the hkor with no change in receptor number (Zhu et al., 1998). Thus, there is a correlation between desensitization and phosphorylation of the hkor.

Phosphorylated Flag-hkor seems to be a glycoprotein with a molecular mass range of 52 to 65 kDa (median 58 kDa). The observed molecular mass range in this study is similar to that reported by Appleyard et al. (1997), who showed that the phosphorylated guinea pig κ-opioid receptor had a molecular mass of 53 kDa. The hkor used in this study was epitope-tagged with Flag, which added about 1 kDa to the molecular mass.

Expression of the dominant negative mutant GRK2-K220R reduced (−)U50,488H-induced phosphorylation of the Flag-hkor, indicating that phosphorylation is mediated by GRKs. These results are in accord with our previous reports. We have shown previously that expression of dominant negative mutants of GRK2 and arrestin-2 reduces (−)U50,488H-promoted internalization and down-regulation of the hkor (Li et al., 1999, 2000), demonstrating the involvement of GRKs in the regulation of the hkor.

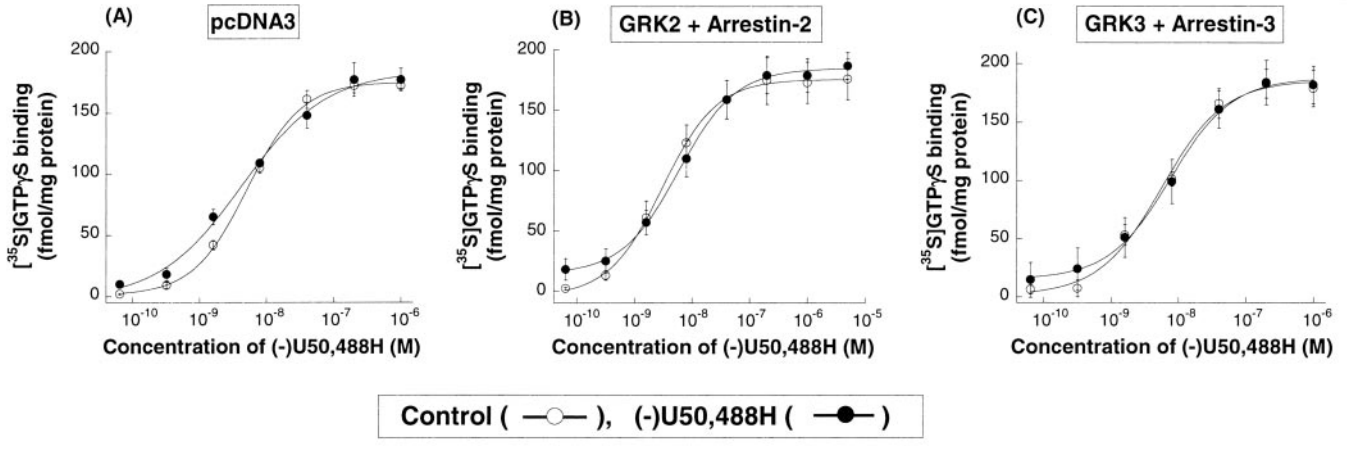

Fig. 3. Effects of transfection of the vector pcDNA3.1 Zeo(+) (A), GRK2 and arrestin-2 (B), and GRK3 and arrestin-3 (C) on response of Flag-rkor to (−)U50,488H pretreatment. CHO-Flag-rkor cells were transiently transfected with 8 μg/100-mm dish of the vector pcDNA3.1 Zeo(+), 4 μg/100-mm dish each of bovine GRK2 and arrestin-2 or 4 μg/100-mm dish each of bovine GRK3 and arrestin-3. Forty-eight to 72 h after transfection, cells were pretreated without (control) or with 1 μM (−)U50,488H at 37°C for 60 min and washed to remove (−)U50,488H. Membranes were prepared and [^{35}S]GTPγS binding in response to (−)U50,488H was performed. [^{35}S]GTPγS binding data were normalized to femtomoles per milligram of protein. Basal binding, about 160 fmol/mg of protein, was subtracted from each value and data are expressed as (−)U50,488H-stimulated [^{35}S]GTPγS binding. Each point represents the mean ± S.E.M. of eight independent experiments in duplicate. Transfection with pcDNA3 yielded similar results as pcDNA3.1 Zeo(+).

Lack of Desensitization and Phosphorylation of the rkor and the Flag-rkor. A 1-h incubation with 1 μM (−)U50,488H did not cause desensitization of the rkor and the Flag-rkor (see Fig. 1), and treatment of the Flag-rkor with 1 μM (−)U50,488H for 15 min, 30 min, or 1 h did not enhance phosphorylation (see Fig. 2). Our results that the rkor and Flag-rkor were not desensitized by (−)U50,488H are similar to reports by Joseph and Bidlack (1995) and Avidor-Reiss et al. (1995), who showed that treatment with (−)U50,488H or U69,593 did not cause desensitization of the mouse κ-receptor in R1.1 thymoma cells or the rkor stably expressed in CHO cells. There findings are also in accord with our previous observations that the rkor was not internalized or down-regulated by (−)U50,488H pretreatment (Li et al., 1999, 2000). In contrast, Tallent et al. (1998) reported that (−)U50,488H pretreatment caused desensitization of mouse κ-opioid receptor expressed in AtT-20 cells, with inhibition of adenylate cyclase and increase in K^+ current as functional measures. The discrepancy among these results may be due to different cell systems and functional endpoints used.

One may argue that the lack of (−)U50,488H-induced phosphorylation of the Flag-rkor may be due to inability of the Flag antibody to immunoprecipitate the Flag-rkor because of glycosylation in the N-terminal domain. This seems not to be the case, because the Flag antibody could immunoprecipitate the Flag-r/hkor (see Fig. 6B), which has the identical N-terminal domain and a similar molecular mass range, indicating similar degree of glycosylation as the Flag-rkor. In addition, the lack of (−)U50,488H-induced phosphorylation was not because the Flag-rkor was not expressed on the cell surface, because about 85% of total receptors are extracellular (Li et al., 1999; our manuscript in preparation).

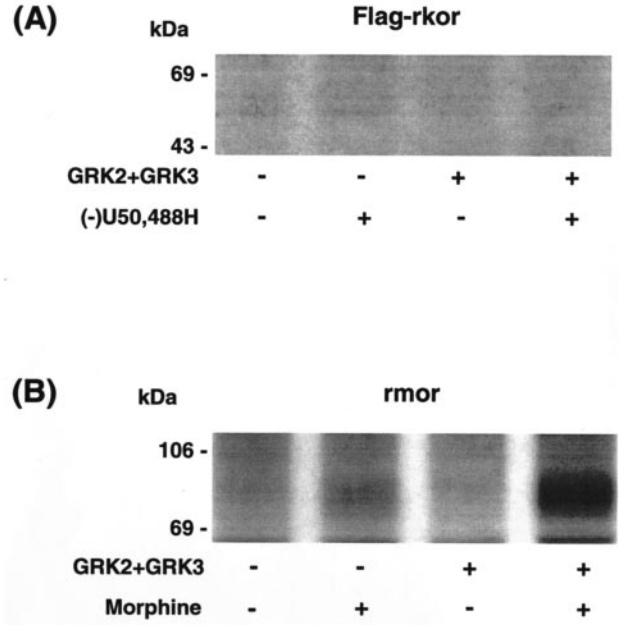

Fig. 4. Effect of expression of GRK2 and GRK3 on phosphorylation of the Flag-rkor after exposure to (−)U50,488H (A) and the rat μ-opioid receptor (rmor) after morphine treatment (B). CHO cells stably expressing the rmor or the Flag-rkor were grown in 6-well plates and then transfected transiently with 1.5 μg/well of the vector or GRK2 and GRK3. Forty-eight hours after transfection, cells were metabolically labeled with [^{32}P]orthophosphate and incubated without or with 1 μM U50,488H or 10 μM morphine at 37°C for 15 min and solubilized. rmor was immunoprecipitated with antiserum against a C-terminal domain peptide (Carman et al., 2000) and the Flag-rkor with a rabbit polyclonal antibody against Flag. The immunoprecipitated mixtures were subjected to SDS-PAGE followed by autoradiography as described under *Experimental Procedures*. This figure represents one of the three experiments with similar results.

The rkor clone used in the present study has Tyr at the 345 position (Li et al., 1993), whereas others have Cys at this position (for review, see Knapp et al., 1995), which was postulated to be the putative palmitoylation site. We generated a Flag-rkorY345C mutant and examined whether this mutation affected the phosphorylation of the Flag-rkor. After (−)U50,488H stimulation for 15 or 60 min, the Flag-rkorY345C mutant was not phosphorylated (data not shown), similar to the Flag-rkor, indicating that the difference in the 345 position did not have effect on lack of (−)U50,488-induced phosphorylation of Flag-rkor.

The lack of desensitization and phosphorylation of the Flag-rkor by (−)U50,488H may be due to insufficient levels of GRKs and arrestins for the Flag-rkor to undergo these processes, even though the levels seem to be sufficient for the Flag-hkor. Chavkin and colleagues (Appleyard et al., 1999) reported that the rkor expressed in *X. laevis* oocytes exhibited only slight desensitization in response to (−)U50,488H, using K^+ currents as the endpoint, but expression of GRK3 or GRK5 and arrestin-3 greatly increased the extent of desensitization. Zhang et al. (1998) and we (Carman et al., 2000) showed that morphine did not increase phosphorylation or cause desensitization of the μ-opioid receptor and expression of GRK2 enabled morphine to cause phosphorylation and desensitization of the μ-opioid receptor. We reported previously that etorphine, unlike (−)U50,488H, did not promote internalization of the hkor and expression of GRK2 and arrestin-2 permitted etorphine to induce internalization (Li et al., 1999). We thus examined whether expression of GRK and arrestin would allow the Flag-rkor to undergo desensitization and phosphorylation. To our surprise, even with expression of GRK2 and arrestin-2 or GRK3 and arrestin-3 using a protocol described previously (Li et al., 1999), the Flag-rkor was not desensitized or phosphorylated after (−)U50,488H exposure, indicating that the lack of desensitization and phosphorylation of the Flag-rkor is not the result of insufficient GRKs and arrestins. The differences between our results and those of Appleyard et al. (1999) are most probably due to different systems and possibly different functional endpoints used. Whereas we used CHO cells and [^{35}S]GTPγS binding, these researchers used *X. laevis* oocytes and K^+ currents. Such a lack of effect of GRK2 is not without precedent. Morphine did not elicit phosphorylation or internalization of the δ-opioid receptor or stimulate β-arrestin translocation and expression of GRK2 did not have any effect (Zhang et al., 1999). Whether the rkor is desensitized in vivo requires further investigation.

Arrestin-2 and arrestin-3 have been shown to bind to phosphorylated and unphosphorylated GPCRs, with higher affinities for phosphorylated receptors (Krupnick and Benovic, 1998). Cen et al. (2001) have shown recently that arrestin-2 and arrestin-3 bind to the unphosphorylated C-terminal domain of the hkor and mutation of the four Ser/Thr residues in the C-terminal domain abolished the binding. We found that overexpression of arrestin-2 or arrestin-3 did not attenuate the G proteins-coupling of the Flag-rkor nor did it cause the Flag-rkor to undergo desensitization in response to

U50,488H (see Fig. 3). This may be due to lack of binding or low-affinity binding of arrestin-2 and arrestin-3 to the C-terminal region of the rkor.

Desensitization and Phosphorylation of Chimeric and Mutant Receptors. Pretreatment of CHO-Flag-r/hkor cells with (−)U50,488H resulted in desensitization and enhanced phosphorylation. In contrast, Flag-h/rkor did not undergo desensitization and phosphorylation after exposure to (−)U50,488H. The results demonstrate that differences in the C-terminal domain contribute to the difference in (−)U50,488H-induced desensitization and phosphorylation between the hkor and the rkor.

GRKs are Ser/Thr kinases. The C-terminal domains of both the hkor and rkor have two Ser and two Thr residues: S356, T357, S358, and T363 in the hkor and S356, T357, T363, and S369 in the rkor (see Fig. 5). One Ser/Thr present in the hkor, but not in the rkor, is S358. S358N mutation in the Flag-hkor abolished (−)U50,488H-induced desensitization and phosphorylation. In contrast, the N358S substitution in the rkor enabled the rkor to be desensitized by (−)U50,488H, but the agonist only slightly increased phosphorylation. These results indicate the role of S358 of the hkor in these processes and its contribution to the difference between the hkor and the rkor.

Our results that S358N mutation of the hkor abolished U50,488H-induced desensitization and phosphorylation are consistent with those of Cheng et al. (1998). These researchers reported that expression of arrestin-2 reduced hkor-mediated enhancement of [^{35}S]GTPγS binding and inhibition of forskolin-stimulated adenylate cyclase activity. S356A/T357G/S358G or S356A/T357G/S358G/T363A mutation abolished the modulatory effect of arrestin-2, but T363A mutation had no effect, indicating that S356/T357/S358 play an important role in the arrestin-2 effect. However, no phosphorylation was performed in their study.

Two interpretations are likely for the observation that S358N mutation in the Flag-hkor abolished (−)U50,488H-promoted phosphorylation and desensitization. One possibility is that Ser-358 is the phosphorylation site. The other is that S358N substitution changes the conformation of the C-terminal domain, thus abolishing phosphorylation. Further studies are required to distinguish the two possibilities. The 358 locus in the guinea pig κ-opioid receptor is Ser (Xie et al., 1994), similar to the hkor, and the κ-opioid receptor in the guinea pig hippocampal slices was desensitized and phosphorylated (Appleyard et al., 1997).

Differences in (−)U50,488H-Promoted Phosphorylation and Desensitization between the Flag-r/hkor and the Flag-rkorN358S. (−)U50,488H enhanced phosphorylation of the Flag-r/hkor to a much greater extent than the Flag-rkorN358S mutant. In addition, during (−)U50,488H-induced desensitization of the Flag-r/hkor or the Flag-hkor, there were

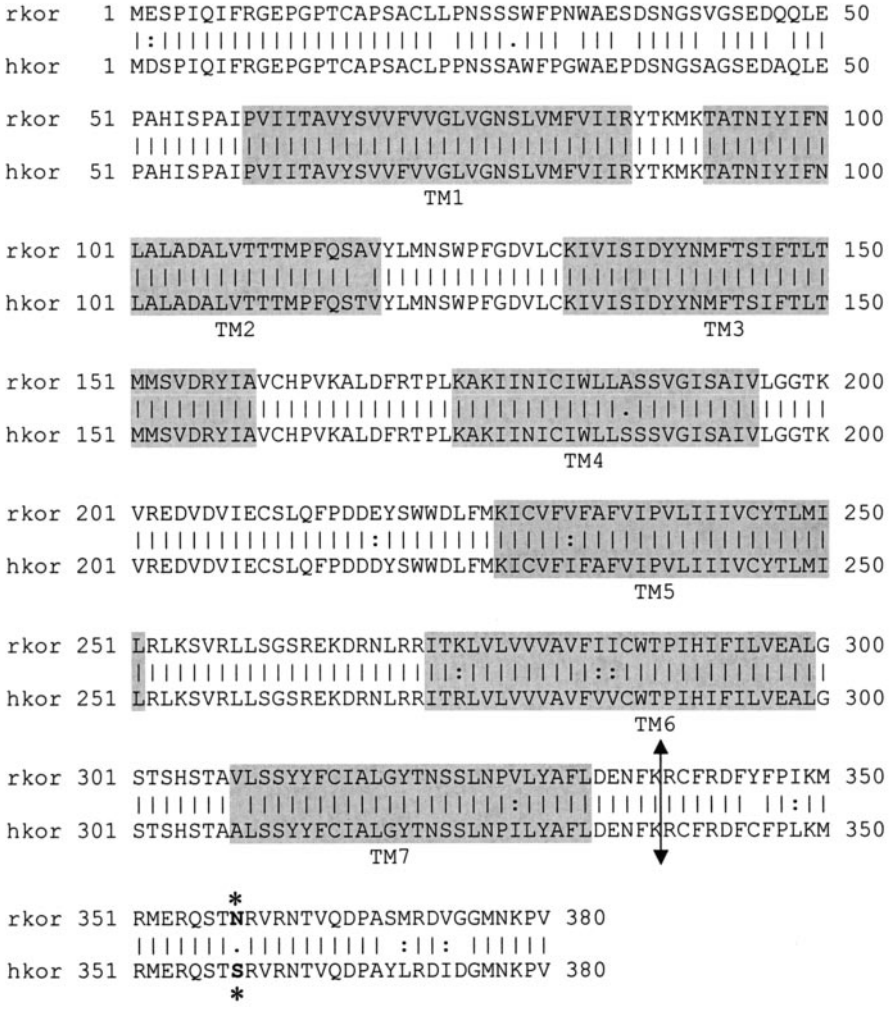

Fig. 5. Amino acid sequence comparison between the hkor (Zhu et al., 1995) and the rkor (Li et al., 1993), points of exchange for generation of chimeric receptors and amino acid residues mutated in the hkor-S358N and rkor-N358S mutants. Amino acid residue numbers are indicated on both sides. Seven putative transmembrane domains (TMs) are shaded. Chimeras Flag-r/hkor (rkor1-338/hkor339-380) and Flag-h/rkor (hkor1–338/rkor339-380) were generated by an exchange of the C-terminal domain fragments 338–380 as indicated by an arrow. *, amino acid residues 358. Ser-358 in the hkor was mutated to Asn and Asn-358 in the rkor was substituted with Ser.

an increase in EC_{50} and a decrease in E_{max}, whereas desensitization of the Flag-rkorN358S involved a decrease in E_{max} with no change in EC_{50}. Thus, sequence differences in the C-terminal domain, besides the S versus N at the 358 locus, also contribute to the differences between the hkor and the rkor. The dissimilarity between the sequences may lead to conformational differences of the C-terminal domain between the hkor and the rkor, which in turn lead to differential interactions of GRKs with this region.

Correlation between Phosphorylation and Desensitization. The Flag-hkor and Flag-r/hkor underwent (−)U50,488H-promoted phosphorylation and desensitization, but the Flag-rkor, Flag-h/rkor, and Flag-hkorS358N did not. Thus, there seems to be a correlation between phosphorylation and desensitization, which is consistent with studies on other GPCRs (for review, see Krupnick and Benovic, 1998). However, the N358S mutant of the Flag-rkor gained the ability to desensitize in response to (−)U50,488H, yet the agonist only slightly increased phosphorylation of this mutant. It has been demonstrated that arrestin-2 and arrestin-3 can bind to both phosphorylated and nonphosphorylated GPCRs, but with higher affinities for phosphorylated states of GPCRs (for review, see Krupnick and Benovic, 1998). Unphosphorylated mutants of GPCRs have been shown to undergo agonist-induced desensitization and internalization. For instance, a δ-opioid receptor mutant truncated at 344, which lacks all Ser and Thr residues in the C-terminal domain, was not phosphorylated, but was able to undergo dynamin- and arrestin-2-dependent internalization in HEK293 cells (Murray et al., 1998).

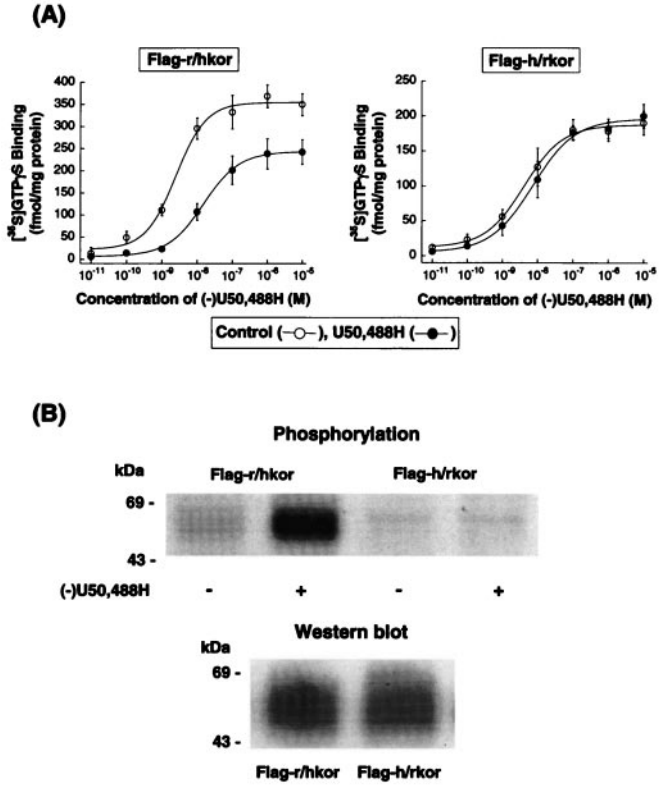

Fig. 6. Effect of (−)U50,488H pretreatment of CHO-Flag-r/hkor cells and CHO-Flag-h/rkor cells on (−)U50,488H-stimulated [^{35}S]GTPγS binding (A) and phosphorylation (B). A, CHO-Flag-r/hkor cells and CHO-Flag-h/rkor cells were pretreated without (control) or with 1 μM (−)U50,488H at 37°C for 60 min and washed thoroughly for removal of (−)U50,488H. Membranes were prepared and [^{35}S]GTPγS binding in response to (−)U50,488H was performed. [^{35}S]GTPγS binding data were normalized to femtomoles per milligram of protein. Basal binding, about 160 fmol/mg protein, was subtracted from each value and data are expressed as (−)U50,488H-stimulated [^{35}S]GTPγS binding. Each point represents the mean ± S.E.M. of five (Flag-r/hkor) and eight (Flag-h/rkor) independent experiments in duplicate. Maximal [^{35}S]GTPγS binding and EC_{50} values are shown in Table 2. B, CHO-Flag-r/hkor and CHO-Flag-h/rkor cells were metabolically labeled with [^{32}P]orthophosphate, incubated without or with 1 μM U50,488H at 37°C for 15 min, solubilized, and immunoprecipitated with a rabbit polyclonal antibody against Flag. The immunoprecipitated mixtures were subjected to SDS-PAGE followed by autoradiography. Western blot was performed on unlabeled receptors with a rabbit polyclonal antibody against Flag to determine the relative molecular mass and to assess the expression of receptor proteins. These figures represent one of the three (phosphorylation) or two (Western blot) experiments with similar results.

Effect of (−)U50,488H Pretreatment on Basal [^{35}S]GTPγS Binding. (−)U50,488H pretreatment followed by extensive washing did not change basal [^{35}S]GTPγS binding of any of the wild-type, chimeric, or mutant κ-opioid receptors. This is different from a recent report by Liu and Prather (2001) showing that agonist treatment of cells expressing the μ-opioid receptor exhibited enhanced basal activity. The reason for the discrepancy is unclear. Perhaps different receptors react differently to chronic agonist treatment.

Role of the C-terminal Domains of the rkor and the hkor in (−)U50,488H-Induced [^{35}S]GTPγS Binding. It is interesting to note that the receptor constructs that contain the C-terminal domain of the rkor, such as rkor, Flag-rkor, and Flag-h/rkor, have lower maximal [^{35}S]GTPγS binding than those that have the C-terminal domain of the hkor, such as hkor, Flag-hkor and Flag-r/hkor, despite comparable or even higher expression levels (see Table 2). Thus, in addition to playing a critical role in the differential (−)U50,488H-promoted desensitization and phosphorylation between the rkor and the hkor, the C-terminal domains may be an important determinant in the relative degree of U50,488H-promoted [^{35}S]GTPγS binding. These two observations may be related in that [^{35}S]GTPγS binding reflects G protein activation, which leads to activation of GRK2 and GRK3 via the βγ-subunits. Therefore, the rkor may be a weaker activator of GRKs than the hkor.

Adaptation to Agonist Exposure. Desensitization, internalization, and down-regulation are processes that cells use to adapt to prolonged activation of GPCRs by agonists. In addition to lack of desensitization, the rkor expressed in CHO cells was not internalized or down-regulated after exposure to (−)U50,488H or etorphine (Li et al., 1999, 2000). In contrast, the hkor underwent internalization and down-regulation. Thus, during prolonged activation, the rkor will be constantly stimulated, which may lead to more profound changes of downstream signal transduction pathways to maintain homeostasis. von Zastrow and colleagues (Whistler et al., 1999) postulated that these downstream adaptive changes may contribute to the development of tolerance and lack of receptor internalization may lead to a higher degree of downstream adaptive changes. Whether this scenario is true needs further investigations.

Conclusions

We have demonstrated that the hkor, but not the rkor, stably expressed in CHO cells undergoes U50,488H-pro-

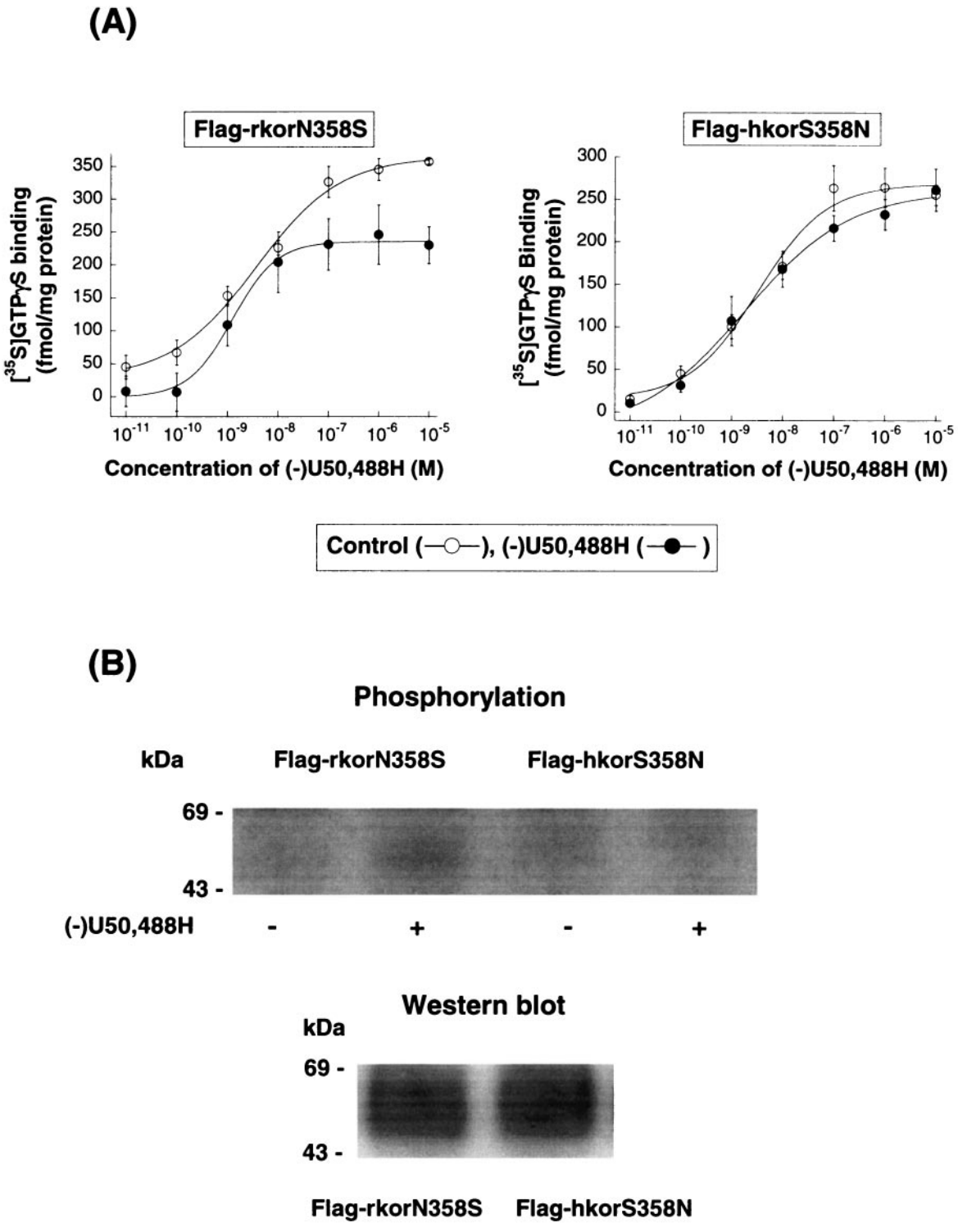

Fig. 7. Effect of U50,488H pretreatment of CHO-Flag-rkorN358S and CHO-Flag-hkorS358N cells on U50,488H-stimulated [^{35}S]GTPγS binding (A) and phosphorylation (B). A, CHO-Flag-rkorN358S cells and CHO-Flag-hkorS358N cells were pretreated without (control) or with 1 μM U50,488H at 37°C for 60 min and washed for removal of U50,488H. Membranes were prepared and [^{35}S]GTPγS binding in response to U50,488H was performed. [^{35}S]GTPγS binding data were normalized to femtomoles per milligram of protein. Basal binding, about 160 fmol/mg protein, was subtracted from each value, and data are expressed as (−)U50,488H-stimulated [^{35}S]GTPγS binding. Each point represents the mean ± S.E.M. of five to eight independent experiments in duplicate. Maximal [^{35}S]GTPγS binding and EC_{50} values are shown in Table 2. B, CHO-Flag-rkorN358S cells and CHO-Flag-hkorS358N cells were metabolically labeled with [^{32}P]orthophosphate, incubated without or with 1 μM U50,488H at 37°C for 15 min, solubilized, and immunoprecipitated with antibody against Flag. The immunoprecipitated mixtures were subjected to SDS-PAGE followed by autoradiography. Western blot of the Flag-rkorN358S and the Flag-hkorS358N was performed with a rabbit polyclonal antibody against the Flag to determine the relative molecular mass and assess the expression of receptor proteins. The figures represent one of the three (phosphorylation) and two (Western blot) experiments performed with similar results.

moted desensitization and phosphorylation and the C-terminal domain contributes to, and the 358 locus has a role in, the differences. Our results suggest that one should exercise caution when extrapolating results on regulation of κ-opioid receptors from the rat to the human.

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