Constitutive Activation of the μ Opioid Receptor by Mutation of D3.49(164), but Not D3.32(147): D3.49(164) Is Critical for Stabilization of the Inactive Form of the Receptor and for Its Expression†

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ABSTRACT: The roles of conserved aspartates in the third transmembrane domain of the rat μ opioid receptor (RMOR) were explored with mutations of D3.32(147) and D3.49(164). D3.49(164) in the highly conserved DRY motif was mutated to 13 amino acids. Except for the D3.49(164)E mutant, each mutant displayed little or no detectable [^{3}H]diprenorphine binding, and pretreatment with naloxone greatly enhanced binding. D3.49(164)H, -Q, -Y, -M, and -E mutants were further studied. D3.32(147) was substituted with A or N. All seven mutants exhibited similar binding affinities for the antagonist [^{3}H]diprenorphine as the wild-type. The D3.49(164)H, -Q, -Y, and -M mutants, but not the D3.49(164)E and D3.32(147) mutants, exhibited enhanced basal [^{35}S]GTPγS binding which was comparable to the maximally activated level of the wild-type and was related to expression levels. Naloxone, naltrexone, and naloxone methiodide significantly inhibited the basal [^{35}S]GTPγS binding of the D3.49(164) mutants, indicating inverse agonist activities. Treatment of the D3.49(164)Y mutant with pertussis toxin greatly reduced the basal [^{35}S]GTPγS binding, demonstrating constitutive activation of $G\alpha_i/G\alpha_o$. The D3.49(164)H, -Y, -M, and -Q mutants had higher affinities for DAMGO than the wild-type, which were not significantly lowered by GTPγS. Thus, mutation of D3.49(164) to H, Y, M, or Q in RMOR resulted in receptor assuming activated conformations. In contrast, the D3.49(164)E mutant displayed significantly lower basal [^{35}S]GTPγS binding and reduced affinity for DAMGO. Upon incubation of membranes at 37 °C, the constitutively active D3.49(164)Y mutant was structurally less stable, whereas the inactivated D3.49(164)E mutant was more stable, than the wild-type. Computational simulations showed that the E3.49 side chain interacted strongly with the conserved R3.50 in the DRY motif and stabilized the inactive form of the receptor. Taken together, these results indicate that D3.49 plays an important role in constraining the receptor in inactive conformations.

Opiate and opioid compounds act on opioid receptors to produce characteristic pharmacological and physiological effects, most notably analgesia. Multiple opioid receptors (μ, δ, κ) have been demonstrated, and they are coupled through pertussis toxin (PTX)[1]-sensitive G-proteins to a variety of effectors that include adenylate cyclase, potassium channels, calcium channels [for a review, see (*1*)], and a mitogen-activated protein kinase pathway [for example, see (*2*)]. Following the cloning of the δ opioid receptor, μ and κ opioid receptors were cloned [for a review, see (*3*) and references cited therein]. Deduced amino acid sequences of these clones display the motif of seven transmembrane domains (TMs) connected by alternating intracellular and extracellular hydrophilic loops, that is characteristics of G protein-coupled receptors (GPCRs) (*4*). Opioid receptors belong to the rhodopsin subfamily of GPCRs (*5*).

Conformational changes are thought to underlie the activation of GPCRs [for reviews, see (*4*, *6*)]. The importance of specific TMs in the mechanism of activation of opioid receptors may be inferred from information on rhodopsin, β_2-adrenergic, and neurokinin-1 receptors. Upon light activation of rhodopsin, there is a movement of helices relative to

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[1] Abbreviations: CAM, constitutively active mutant; CHO cells, Chinese hamster ovary cells; CHO-D3.49(164)*X* cells, CHO cells stably transfected with D3.49(164) mutants of rat μ opioid receptor; CHO-RMOR cells, CHO cells stably transfected with rat μ opioid receptor; DAMGO, [D-Ala^2, *N*-Me-Phe^4, Gly-ol]-enkephalin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GDP, guanosine 5′-diphosphate; GPCR, G protein-coupled receptor; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HA, hemagglutinin peptide (YPYDVPDYA); HEK293 cell, human embryonic kidney cell; MOR, μ opioid receptor; PTX, pertussis toxin; RMOR, rat μ opioid receptor; TMs, transmembrane domains.

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one another. Disulfide cross-linking of the TMs 3 and 6 prevented activation of transducin, which suggests the importance of this movement for activation of rhodopsin (*7*). Gether et al. (*8*) showed that agonist binding to the β_2-adrenergic receptor caused C3.44(125) in TM3 and C6.47(285) in TM6 to be exposed to a more polar environment, indicating that movements of TMs 3 and 6 are involved as well in activation of this receptor. Zinc binding to an engineered binding site formed by histidines introduced in TMs 5 and 6 in the neurokinin-1 receptor (*9*) and in TMs 3 and 6 in rhodopsin (*10*) prevented receptor activation, indicating the importance of relative movements of TMs 3, 5, and 6. However, the molecular mechanisms of the movement of these transmembrane helices during receptor activation, which thereby determine the conversion from an inactive to an activated state of the receptor, are not well understood. In recent years, some insights about these mechanisms came from the nature and properties of constitutively active mutants (CAMs) that have been generated by point mutation or chimeric receptor approaches of many GPCRs or found naturally in disease states [for reviews, see (*11*−*13*)]. Mutation in a GPCR resulting in G protein activation in the absence of an agonist was first demonstrated for α_{1B}-adrenergic receptor (*14, 15*) and subsequently in several other receptors [for reviews, see (*11*−*13*)]. Substitutions at several loci have been shown to result in enhanced constitutive activation of GPCRs, including those in the cytoplasmic extensions of TMs 3 and 6 as well as TMs 2, 3, 6, and 7 [for a review, see (*13*)]. Because these mutants likely represent a spectrum of activated states, they can be used to shed light on the conformational changes underlying GPCR activation.

The specific mechanistic role of TM3 in the activation of the μ opioid receptor (MOR) is not known. Replacement of D/E3.49 in the highly conserved DRY motif at the interface of the TM3 and second intracellular loop has been shown to result in constitutive activation of several GPCRs, including rhodopsin (*16*) and the α_{1B}- (*17, 18*) and β_2-adrenergic (*19*) receptors. Mutations at the D3.32 locus enhanced agonist-independent activities of α_{1B}-adrenergic and δ opioid receptors (*20, 21*).

Here we show that mutations of D3.49(164), but not D3.32(147), result in agonist-independent activation of the rat μ opioid receptor (RMOR) as determined by [^{35}S]GTPγS binding assay. We also demonstrate that the D3.49(164)E mutation stabilizes the inactive form of the RMOR. In characterizing the binding and coupling properties of the D3.49(164) CAMs, we found that pretreatment with naloxone was necessary for detection of mutant receptor binding and receptor protein by western blot, and we demonstrate that naloxone and some of its congeners have inverse agonist activities. A mechanistic hypothesis of the observed effects of the mutations at the D3.49 (164) locus is provided in the context of a molecular model of the conserved DRY motif in TM3.

MATERIALS AND METHODS

Materials. [^{35}S]GTPγS (~1250 Ci/mmol) and [^{3}H]diprenorphine (58 Ci/mmol) were purchased from NEN Life Sciences (Boston, MA). Guanosine 5′-diphosphate (GDP) and guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) were obtained from Sigma Chemical Co. (St. Louis, MO). Naloxone was a gift from DuPont/Merck Co. (Wilmington, DE). [D-Ala^2, *N*-Me-Phe^4, Gly-ol]-enkephalin (DAMGO) and PTX were purchased from Research Biochemicals International, Inc. (Natick, MA). Mouse monoclonal antibodies against hemagglutinin peptide (HA) and ECL western blotting detection reagents were obtained from BABCO (Berkeley, CA) and Amersham Pharmacia Biotech Co. (Piscataway, NJ). Enzymes and chemicals used in molecular biology and mutagenesis experiments were purchased from Life Technologies Co. (Gaithersburg, MD), Promega (Madison, WI), Boehringer-Mannheim Co. (Indianapolis, IN), and Qiagen Co. (Valencia, CA).

Numbering Schemes for Amino Acid Residues in RMOR and Other GPCRs. The numbering scheme used identifies amino acid residues in RMOR and other GPCRs both by their sequence numbers and by the generic numbering scheme proposed by Ballesteros and Weinstein (*22*) and recently applied to opioid receptors (*23, 24*). This combined scheme is used in order to relate the results obtained for opioid receptors to equivalent positions in other GPCRs. According to the generic numbering scheme, amino acid residues in TMs are assigned two numbers (*N*1, *N*2). *N*1 refers to the TM number. For *N*2, the numbering is relative to the most conserved residue in each TM, which is assigned 50; the other residues in the TM are numbered in relation to this conserved residue, with numbers decreasing toward the N-terminus and increasing toward the C-terminus. The most conserved residue in the TM3 of the RMOR is R165, which is referred to as R3.50(165).

Oligodeoxynucleotide-Directed Mutagenesis. Site-directed mutagenesis was performed on the RMOR with the overlap polymerase chain reaction method described by Higuchi et al. (*25*). HA-tagged wild-type and mutants of RMOR were subcloned into *Hin*dIII and *Xba*I sites of the mammalian expression vector pcDNA3 (*23*). The cDNA sequences were determined with the method of Sanger et al. (*26*) to confirm the presence of desired mutations and the absence of unwanted mutations.

Transient Expression of the Wild-Type and Mutant RMOR in HEK293 Cells. Human embryonic kidney cells (HEK293 cells) were grown in 100 mm culture dishes in minimum essential medium supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37 °C. The cells were transfected with the wild-type or a RMOR mutant cDNA (5 μg/dish plus 15 μg of vector) using the calcium phosphate method (*27*). Twenty-four hours after transfection, cells were placed in fresh medium containing 20 μM naloxone. Twenty-four to fourty-eight hours later, cells were harvested for experiments by use of Versene solution [0.54 mM ethylenediaminetetraacetic acid (EDTA), 0.14 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, 1 mM glucose] in the presence of 20 μM naloxone.

Stable Expression of the Wild-Type and Mutant RMOR in CHO Cells. Transfection of Chinese hamster ovary (CHO) cells with the wild-type or a mutant RMOR in the vector pcDNA3 was performed with Lipofectamine according to the manufacturers' instructions, and cells were grown under the selection pressure of Geneticin (1 mg/mL). CHO cell clones stably expressing different levels of the wild-type or a mutant of RMOR were established as described previously

(*28*), except that naloxone (20 μM) was kept in the medium during the entire process. These CHO cells were cultured in Dulbecco's modified Eagle's medium F_{12} HAM supplemented with 10% fetal calf serum, 0.5 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. Prior to experiments, naloxone (20 μM) was added to the medium for at least 96 h. Cells were harvested for experiments by use of Versene solution in the presence of 20 μM naloxone. Cells were washed 3 times with 15 mL/100 mm dish of phosphate-buffered saline by resuspension and centrifugation. Additional washes were accomplished during membrane preparation. We found this procedure to be adequate for removal of naloxone.

Western Blot. Western blot was performed to examine the expression of the HA-tagged wild-type and mutant RMOR according to Harlow and Lane (*29*). CHO cells stably transfected with the wild-type or D3.49(164)Q mutant were treated with or without 20 μM naloxone for 96 h and solubilized with Laemmli sample buffer and subjected to SDS−PAGE as we described previously (*30*). Protein bands thus formed were electrophorectically transferred onto nitrocellulose membranes. Membranes were treated with blocking solution [5% nonfat dry milk in 0.1% Tween 20/0.15 M NaCl/25 mM Tris-HCl (TBS), pH 7.5], incubated with monoclonal antibodies against HA in the blocking solution for 18 h at 4 °C on a shaker, and then washed 3 times with TBS. Membranes were incubated with goat anti-mouse IgG conjugated with horseradish peroxidase in the blocking solution for 1 h at room temperature followed by washing 3 times with TBS. The target proteins were located by chemiluminescence using ECL western blotting detection reagents followed by exposure to X-ray films.

Membrane Preparation. Membranes were prepared according to Zhu et al. (*31*) with some modifications. HEK293 cells or CHO cells were pelleted by spinning at 500*g* for 10 min at 4 °C and frozen at −80 °C for at least 30 min. Frozen cell pellets were thawed in 1 mL/dish cold lysis buffer [5 mM Tris-HCl, 5 mM EDTA, 5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4] for 30 min and vortexed. Cell suspension was passed through a 29_G3/8 syringe needle at least 5 times, added to 15 mL/dish of lysis buffer, and centrifuged at 48000*g* for 35 min at 4 °C. The pellet resuspended in 50 mM Tris-HCl buffer (pH 7.0) was passed through the syringe needle 5 times and centrifuged again. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.0) and centrifuged again. Membranes were suspended in 50 mM Tris-HCl buffer in the presence of 1 mM EGTA (pH 7.4). Protein concentration was determined by the bicinchoninic acid method of Smith et al. (*32*) with bovine serum albumin as the standard. Membranes were then aliquoted at 0.2 mg/mL and stored at −80 °C.

Opioid Receptor Binding. Saturation binding of [^{3}H]diprenorphine to the wild-type and mutant RMOR was performed with at least six concentrations of [^{3}H]diprenorphine (ranging from 25 pM to 1−2 nM), and K_d and B_{max} values were determined. Competition inhibition by DAMGO of [^{3}H]diprenorphine binding to the wild-type and mutant RMOR receptors was performed with 0.4 nM [^{3}H]diprenorphine in the absence or presence of different concentrations of DAMGO, and K_i values of DAMGO 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 10−20 μg of membrane protein. Naloxone (10 μM) was used to define nonspecific binding. Binding data were analyzed with the EBDA program (*33*).

[^{35}S]GTPγS Binding Assay. Determination of [^{35}S]GTPγS binding to G proteins was carried out with a modified procedure of Zhu et al. (*31*). [^{35}S]GTPγS stock was diluted 100-fold in 10 mM Tricine, pH 7.4, 10 mM DL-dithiothreitol, aliquoted, and stored at −80 °C. For each experiment, 10 μg of membrane protein was incubated with 15 μM GDP and 0.2 nM [^{35}S]GTPγS in the presence or absence of a drug in reaction buffer (50 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 0.1% BSA) in a final volume of 0.5 mL. Nonspecific binding was determined in the presence of 10 μM GTPγS. After 60 min incubation at 30 °C, bound and free [^{35}S]GTPγS were separated by filtration with GF/B filters under reduced pressure with a Brandel cell harvester, and the filter was washed 3 times with cold washing buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, and 50 mM NaCl, pH 7.4). Radioactivity in filters was determined by liquid scintillation counting.

Effect of PTX on [^{35}S]GTPγS binding. CHO cells stably transfected with the wild-type or D3.49(164)Y mutant receptor, which had been cultured for over 96 h in the presence of 20 μM naloxone, were pretreated with or without 100 ng/mL PTX for 24 h, and membranes were prepared. [^{35}S]GTPγS binding in the absence and presence of 10 μM DAMGO was determined.

Incubation of Membranes of CHO-RMOR, CHO-D3.49(164)Y, and CHO-D3.49(164)E Cells at 37 or 4 °C. Membranes were prepared from CHO-RMOR and CHO-D3.49(164)E cells. CHO-D3.49(164)Y cells were pretreated with 20 μM naloxone for at least 96 h, and membranes were prepared. Membranes were incubated at 4 or 37 °C in the presence of protease inhibitors (antipain dihydrochloride, 5 μg/mL; aprotinin, 0.2 μg/mL; bestatin, 4 μg/mL; chymostatin, 0.6 μg/mL; E-64, 0.05 μg/mL; EDTA, 0.02 mg/mL; prefabloc SC, 0.01 mg/mL; pepstatin, 0.07 μg/mL; phosphoramidon, 1 μg/mL; leupeptine, 0.05 μg/mL) for an indicated period of time. [^{3}H]Diprenorphine binding and western blot were performed.

Modeling and Simulation of Hydrogen Bonding in the DRY Motif. The effect of the D3.49E mutation on the local structure of the "arginine cage" in TM3 (*34*) was investigated in the context of the crystal structure of rhodopsin (*35*). Two constructs were considered: one with glutamate at position 3.49 (as in wild-type rhodopsin), and another with aspartate at that position (as in the wild-type μ opioid receptor). Computational simulations were carried out to calculate the average bond length and interaction energy between the conserved arginine R3.50(135) and the residue at position 3.49(134) (either E or D). Monte Carlo simulations were carried out with the CHARMM27 program package (*36*). The degrees of freedom included all torsional angles of the side chains surrounding the arginine cage. This neighborhood was defined by any residue with atoms within 5 Å of any atom of E3.49 or R3.50 in the rhodopsin crystal structure, and was found to include residues in TM2 [L2.39(72)]; TM3 [L3.46(131); V3.53(138); V3.54(139)], and TM6 [E6.30(247); V6.33(250); T6.34(251); V6.37(254)]. The Monte Carlo

Table 1: K_d and B_{max} Values of [^{3}H]Diprenorphine Binding to the Wild-Type (WT) and D3.49(164) Mutants of the RMOR[a]

		HEK293			CHO cells		
	naloxone	K_d (nM)	B_{max} (pmol/mg of protein)	n	K_d (nM)	B_{max} (pmol/mg of protein)	n
WT	−	0.18 ± 0.05	1.8 ± 0.2	5			
WT	+	0.14 ± 0.02	2.1 ± 0.2	6			
WT II	−				0.21 ± 0.02	7.3 ± 0.2	8
WT II	+				0.20 ± 0.01	9.9 ± 0.3	4
WT III	−				0.20 ± 0.02	1.8 ± 0.1	8
WT III	+				0.27 ± 0.03	2.4 ± 0.1	4
D3.49(164)E	−	0.25 ± 0.09	1.0 ± 0.1	3	0.16 ± 0.01	5.0 ± 0.2	4
D3.49(164)E	+	0.18 ± 0.07	2.0 ± 0.1	3	0.16 ± 0.01	8.2 ± 0.1	4
D3.49(164)Q	−	NB	NB	3	NB	NB	4
D3.49(164)Q	+	0.28 ± 0.11	1.4 ± 0.1	4	0.22 ± 0.01	5.9 ± 0.2	4
D3.49(164)H	−	NB	NB	3			
D3.49(164)H	+	0.27 ± 0.08	1.3 ± 0.1	6	0.49 ± 0.03	0.6 ± 0.1	3
D3.49(164)M	−	NB	NB	3			
D3.49(164)M	+	0.35 ± 0.13	1.2 ± 0.1	5	0.37 ± 0.04	7.2 ± 0.7	6
D3.49(164)Y	−	NB	NB	3			
D3.49(164)Y	+	0.46 ± 0.02	1.1 ± 0.1	6	0.45 ± 0.01	3.8 ± 0.1	6

[a] The WT or mutant receptor was expressed transiently in HEK293 cells or stably in CHO cells and treated with naloxone for at least over 96 h as described under Materials and Methods. Saturation binding of [^{3}H]diprenorphine to membrane preparations was performed, and K_d and B_{max} values were determined. Each value was expressed as means ± SEM. n = number of independent experiments in duplicate. NB: displayed no detectable [^{3}H]diprenorphine binding.

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                    3.32              3.49
RMOR        138 ILCKIVISIDYYNMFTSIFTLCTMSVDRYIAV 169
HMOR        140 ILCKIVISIDYYNMFTSIFTLCTMSVDRYIAV 171
HDOR        119 LLCKAVLSIDYYNMFTSIFTLTMMSVDRYIAV 150
HKOR        129 VLCKIVISIDYYNMFTSIFTLTMMSVDRYIAV 160
β2-AR       104 FWCEFWTSIDVLCVTASIETLCVIAVDRYFAI 135
α1B-AR      116 IFCDIWAAVDVLCCTASILSLCAISIDRYIGV 147
rhodopsin   108 TGCNLEGFFATLGGEIALWSLVVLAIERYVVV 139
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FIGURE 1: Amino acid sequence of the TM3 of the RMOR and comparison to those of the human μ opioid (HMOR), human δ opioid (HDOR), human κ opioid (HKOR), human β_2-adrenergic (β_2-AR), and human α_{1B}-adrenergic (α_{1B}-AR) receptors and bovine rhodopsin. D3.32(147) and D3.49(164) of the RMOR were mutated to other amino acid residues in this study. D3.32 and D/E3.49 of each receptor are bold. The numbers refer to the positions of the residues within the protein sequences.

simulations were carried out for each construct for 3 million steps, saving a structure every 5000 steps for a total of 600 representative structures. The interaction energy between the residues at positions 3.49 (E or D) and 3.50 (R) was calculated for each representative structure.

RESULTS

Effect of D3.49(164) Mutations on [^{3}H]Diprenorphine Binding. The D3.49(164) residue of the highly conserved DRY motif at the cytoplasmic end of the TM3 of the RMOR was mutated to Q, S, M, H, I, V, L, A, T, N, E, W, and Y (Figure 1). When transiently transfected into HEK293 cells, the mutant receptors exhibited little or no [^{3}H]diprenorphine binding except for the D3.49(164)E mutant, which displayed [^{3}H]diprenorphine binding comparable to the wild-type (Table 1). Pretreatment with 20 μM naloxone of HEK293 cells transiently transfected with the mutant receptors enhanced [^{3}H]diprenorphine binding greatly (Figure 2, Table 1). Among the 12 mutants [not including D3.49(164)E], we selected 4 [D3.49(164)Q, D3.49(164)M, D3.49(164)H, and D3.49(164)Y] for further studies, because of their relatively high [^{3}H]diprenorphine (0.4 nM) binding after naloxone pretreatment. Saturation [^{3}H]diprenorphine binding to the five mutants and the wild-type was performed, and K_d and B_{max} values were determined (Figure 2, Table 1). For naloxone pretreatment, HEK293 cells were pretreated with the drug

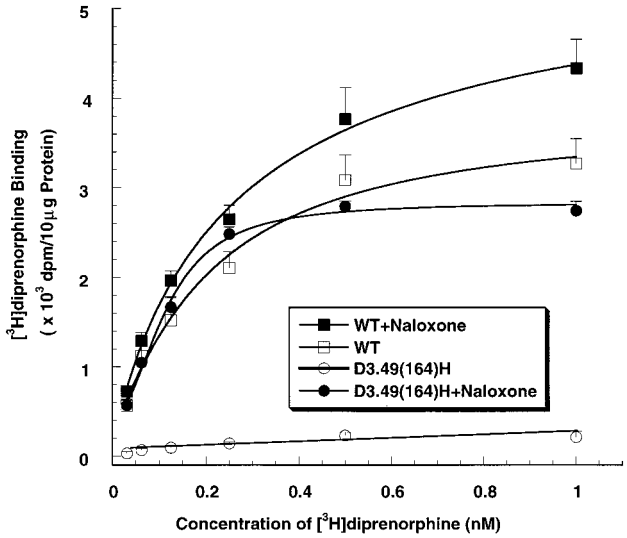

FIGURE 2: Saturation [^{3}H]diprenorphine binding to the wild-type and the D3.49(164)H mutant of the RMOR transiently expressed in HEK293 cells grown in the absence or presence of naloxone. HEK293 cells were transiently transfected with the wild-type or the D3.49(164)H mutant of RMOR. Twenty-four hours later, cells were placed in medium with or without 20 μM naloxone and grown for another 36−48 h. Cells were harvested and membranes prepared. For naloxone-pretreated cells, naloxone was kept until lysis buffer was added. Saturation [^{3}H]diprenorphine binding to membranes was performed. The data are expressed as the mean ± SEM from 3−6 independent experiments in duplicate. K_d and B_{max} values of the wild-type and mutant receptors are shown in Table 1.

(20 μM) for 24−48 h, and during membrane preparation, naloxone was included in the buffered solutions until cell lysis. Naloxone pretreatment of the transfected cells dramatically increased [^{3}H]diprenorphine binding to the D3.49(164)H, -Y, -M, and -Q mutants, whereas the same treatment increased the B_{max} values of the wild-type by only 16% (Figure 2, Table 1). Naloxone pretreatment also doubled the expression of the D3.49(164)E mutant (Table 1). The K_d values of these mutants ranged from ∼0.7- to ∼3-fold of that of the wild-type, indicating that these mutants retain similar affinities for [^{3}H]diprenorphine.

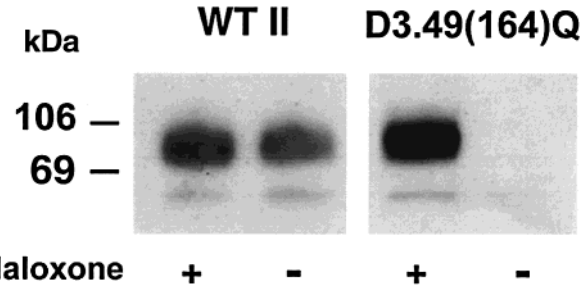

FIGURE 3: Western blot of the HA-tagged wild-type and D3.49(164)Q mutant of the RMOR stably expressed in CHO cells. Cells were treated with or without naloxone for 96 h and subjected to SDS−PAGE, and western blot was performed with a monoclonal antibody against the HA epitope as described under Materials and Methods. The amount of proteins loaded in each lane was 5 μg for the wild-type and 20 μg for the D3.49(164)Q mutant. The blot represents one of the two experiments performed.

Because of difficulties in obtaining consistent basal and DAMGO-stimulated [^{35}S]GTPγS binding data using cells transiently transfected with the wild-type and mutant receptors, clonal CHO cell lines stably transfected with each of the D3.49(164)E, D3.49(164)Q, D3.49(164)M, D3.49(164)H, and D3.49(164)Y mutants were established. The presence of naloxone in the culture medium of these CHO cell lines was again found to be essential for detection of [^{3}H]diprenorphine binding to four of the D3.49(164) mutants (Q, M, H, and Y). CHO cells were grown in the presence of naloxone for at least 96 h until cell lysis. K_d and B_{max} values of [^{3}H]diprenorphine binding to membranes of these stable cell lines were determined (Table 1). K_d values of [^{3}H]diprenorphine binding to the wild-type and mutants were similar to those obtained from the receptors transiently expressed in HEK293 cells (Table 1). For each mutant, we have selected a number of cell lines, which display different levels of [^{3}H]diprenorphine binding (see Figure 6).

To determine whether lack of [^{3}H]diprenorphine binding of the D3.49(164) mutants was due to the absence of receptor protein expression or conformation changes, we performed western blot on CHO cells stably expressing D3.49(164)Q mutant using monoclonal antibodies against HA. As shown in Figure 3, without naloxone pretreatment, no HA-tagged D3.49(164)Q mutant receptor protein was detected; naloxone pretreatment for 96 h greatly enhanced its expression. In contrast, naloxone pretreatment only slightly increased the expression of the wild-type (Figure 3). These results were consistent with those obtained from the receptor binding assay.

Effect of Mutations of D3.32(147) on [^{3}H]Diprenorphine Binding. When transiently transfected into HEK293 cells, the D3.32(147)A or D3.32(147)N mutant showed decreased [^{3}H]diprenorphine binding compared with wild-type (data not shown). Clonal CHO cells stably expressing each mutant were established, and clones expressing 1−2 pmol of receptor/mg of protein were chosen. K_d and B_{max} values of [^{3}H]diprenorphine binding to membranes of these stable cell lines were determined, and clones with similar B_{max} values as wild-type clones were selected for further studies (Table 2). These mutants have similar binding affinities for [^{3}H]diprenorphine as the wild-type.

Effect of D3.49(164) Mutations on Basal and DAMGO-Stimulated [^{35}S]GTPγS Binding. [^{35}S]GTPγS binding has been established as a functional assay of MOR activation

Table 2: K_d and B_{max} Values of [^{3}H]Diprenorphine Binding to the Wild-Type (WT) and D3.32(147) Mutants of the RMOR Stably Expressed in CHO Cells[a]

	K_d (nM)	B_{max} (pmol/mg of protein)	n
WT III[b]	0.20 ± 0.02	1.8 ± 0.1	8
D3.32(147)A	0.14 ± 0.18	1.2 ± 0.1	3
D3.32(147)N	0.24 ± 0.16	1.6 ± 0.1	3

[a] Saturation binding of [^{3}H]diprenorphine to membrane preparations was performed, and K_d and B_{max} values were determined. Data were expressed as means ± SEM. n represents the number of independent experiments in duplicate. [b] From Table 1.

(*37*, *38*). Both basal and DAMGO-stimulated [^{35}S]GTPγS binding was examined in membranes of CHO cells stably transfected with the wild-type (CHO-RMOR cells) and the D3.49(164) mutants [CHO-D3.49(164)*X* cells] of the RMOR. With the CHO cell clones shown in Table 1, four of the D3.49(164) mutants (i.e., to H, Y, Q, and M) exhibited significantly higher basal [^{35}S]GTPγS binding than the wild-type (Figure 4A), despite lower receptor expression levels than the wild-type (see Table 1). The selective MOR agonist DAMGO (10 μM) increased [^{35}S]GTPγS binding of the wild-type preparation to 206 ± 13% (n = 6, mean ± SEM) of its basal level. The basal [^{35}S]GTPγS binding of the four D3.49(164) mutants was similar to or even higher than the maximal DAMGO-stimulated activities of the wild-type receptor (Figure 4A). These results indicate that D3.49(164)H, D3.49(164)Y, D3.49(164)Q, and D3.49(164)M mutations result in agonist-independent activation of the RMOR. Unlike the wild-type, these four mutants displayed no or small increases in [^{35}S]GTPγS binding upon DAMGO (10 μM) stimulation.

In contrast to the four D3.49(164) mutants, the D3.49(164)E mutant displayed significantly lower basal [^{35}S]GTPγS binding compared with the wild-type. However, DAMGO stimulated [^{35}S]GTPγS binding in the D3.49(164)E mutant preparations to a level similar to that achieved in the wild-type (Figure 4A).

Effect of Mutations of D3.32(147) on Basal and DAMGO-Stimulated [^{35}S]GTPγS Binding. Replacement of D3.32(147) in the TM3 with A or N did not affect basal [^{35}S]GTPγS binding, but both mutations abolished 10 μM DAMGO-stimulated [^{35}S]GTPγS binding (Figure 4B). These results indicate that unlike the D3.49(164) mutants, the D3.32(147) mutants do not have enhanced constitutive activity and cannot be activated by DAMGO.

Effect of Naloxone, Naltrexone, Naloxone Methiodide, Diprenorphine, and CTAP on the Basal [^{35}S]GTPγS Binding of the D3.49(164) Mutants. Inverse agonists have been shown to inhibit the enhanced activities of CAMs of GPCRs (*39*). As shown in Figure 4A, naloxone significantly reduced basal [^{35}S]GTPγS binding induced by mutations of D3.49(164) to H, Y, Q, and M to levels similar to the basal [^{35}S]GTPγS binding of the WT II. Thus, naloxone acts as an inverse agonist at these mutant receptors. Although naloxone appeared to reduce basal [^{35}S]GTPγS binding of the wild-type preparation, the changes did not reach statistical significance. Interestingly, naloxone increased the basal binding of the D3.49(164)E mutant to a level similar to that of the wild-type (Figure 4A).

Other opioid receptor antagonists were then examined for their inverse agonist activities on the D3.49(164)Q mutant

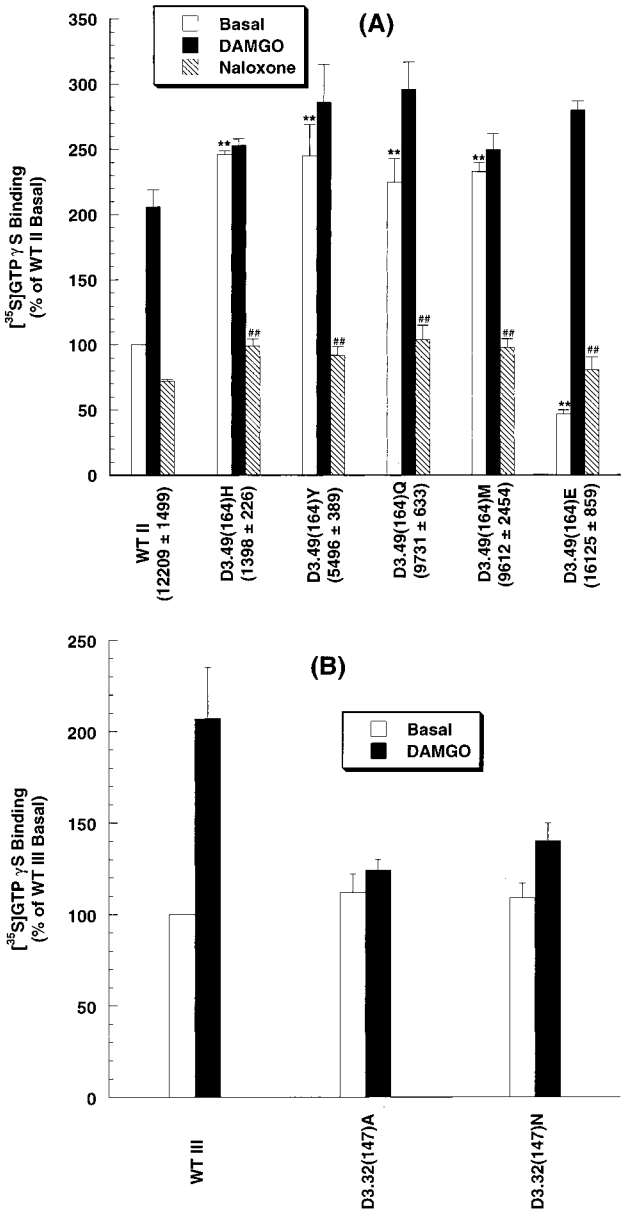

FIGURE 4: [^{35}S]GTPγS binding mediated by the wild-type and mutant RMOR stably expressed in CHO cells: basal, +DAMGO, or +naloxone. Data in panels A and B were derived from cell clones shown in Table 1 and Table 2, respectively. (A) D3.49(164) mutants. CHO cells stably transfected with the wild-type or the D3.49(164)H, D3.49(164)Y, D3.49(164)Q, D3.49(164)M, or D3.49(164)E mutant were treated with 20 μM naloxone for at least 96 h, and membranes were prepared. (B) D3.32(147) mutants. Membranes were prepared from CHO cells stably transfected with the D3.32(147)A or D3.32(147)N mutant of RMOR without naloxone pretreatment. [^{35}S]GTPγS binding was performed with 10 μg of membrane proteins, 0.2 nM [^{35}S]GTPγS, and 15 μM GDP for 60 min at 30 °C in the absence (basal) or presence of 10 μM DAMGO or 10 μM naloxone. [^{3}H]Diprenorphine (1 nM) binding to the same batch of membranes was conducted. The numbers in parentheses indicate [^{3}H]diprenorphine binding (dpm/1 nM [^{3}H]diprenorphine/10 μg of protein). Data were normalized against the basal [^{35}S]GTPγS binding of the wild-type. The results are expressed as the mean ± SEM of at least 3 independent experiments in duplicate. $^{**}p < 0.01$, compared with the WT II basal; $^{\#\#}p < 0.01$ compared with its own basal by one-way ANOVA followed by Dunnett Multiple Comparisons Test.

at a concentration at least 1000-fold that of their K_i values at the RMOR. Naltrexone and naloxone methiodide significantly inhibited basal [^{35}S]GTPγS binding (Figure 5),

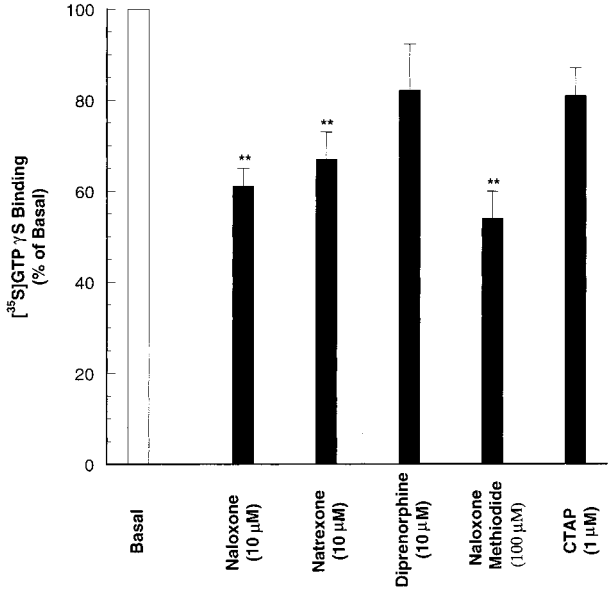

FIGURE 5: Effect of opioid drugs on basal [^{35}S]GTPγS binding in membranes of the CHO cells stably expressing the D3.49(164)Q mutant. CHO cells stably expressing the D3.49(164)Q mutant receptor were treated with 20 μM naloxone for at least 96 h. Naloxone was removed, and membranes were prepared. [^{35}S]GTPγS binding was performed in the absence (basal) or presence of naloxone, naltrexone, diprenorphine, naloxone methiodide, and CTAP at the indicated concentrations. Results were normalized as percent of the basal binding and are expressed as mean ± SEM of 5 independent experiments in duplicate. $^{**}p < 0.01$, compared with the basal [^{35}S]GTPγS binding by one-way ANOVA followed by Dunnett Multiple Comparisons Test.

indicating that they too act as inverse agonists. In contrast, diprenorphine and CTAP appeared to reduce basal [^{35}S]GTPγS binding only slightly, and the decreases were not statistically significant.

Relationship between Constitutive Activities of D3.49(164) Mutants and Receptor Levels. To further characterize these D3.49(164) mutants, we investigated whether the enhanced basal [^{35}S]GTPγS binding was related to receptor levels. For the wild-type and each mutant, 4−6 CHO cell clones stably expressing different levels of the mutant receptor were examined. [^{35}S]GTPγS binding to G proteins and [^{3}H]diprenorphine binding to receptors were performed on the same batch of membranes. Since the K_d values of the mutant receptors were similar to those of the wild-type, the relative expression levels of the wild-type and mutant receptors were compared from the binding of 1 nM [^{3}H]diprenorphine and expressed in dpm/10 μg of protein. Results in Figure 6 show that the basal levels of [^{35}S]GTPγS binding increased as [^{3}H]diprenorphine binding increased. There is a linear portion in which the regression lines of [^{35}S]GTPγS binding vs receptor binding for the D3.49(164) to H, Y, Q, and M mutants were shifted upward and had larger slopes than that of the wild-type. The greater slopes of the lines for the mutants indicate a greater increase in [^{35}S]GTPγS binding per receptor for the mutants than for the wild-type. This is consistent with the constitutive activity of each of the four mutants. When compared with the wild-type of similar expression levels, the order of agonist-independent activity is D3.49(164)H > D3.49(164)Y > D3.49(164)Q = D3.49(164)M (Figure 6). The observation that multiple clonal cell lines of each mutant receptor displayed enhanced

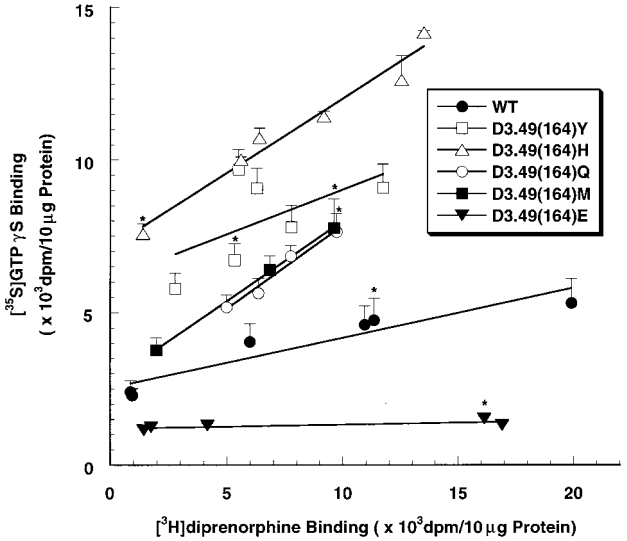

FIGURE 6: Relationship between receptor level and basal [^{35}S]GTPγS binding mediated by the wild-type and mutant RMOR. CHO cells stably expressing different levels of the wild-type or the D3.49(164)H, D3.49(164)Y, D3.49(164)Q, D3.49(164)M, or D3.49(164)E mutant were treated with 20 μM naloxone for at least 96 h, and membranes were prepared. [^{35}S]GTPγS binding was performed in the absence of agonist. [^{3}H]Diprenorphine (1 nM) binding to the same batch of membranes was conducted. Data are expressed as the mean ± SEM of at least 3 independent experiments in duplicate. An asterisk indicates the clones shown in Table 1 and Figure 4A.

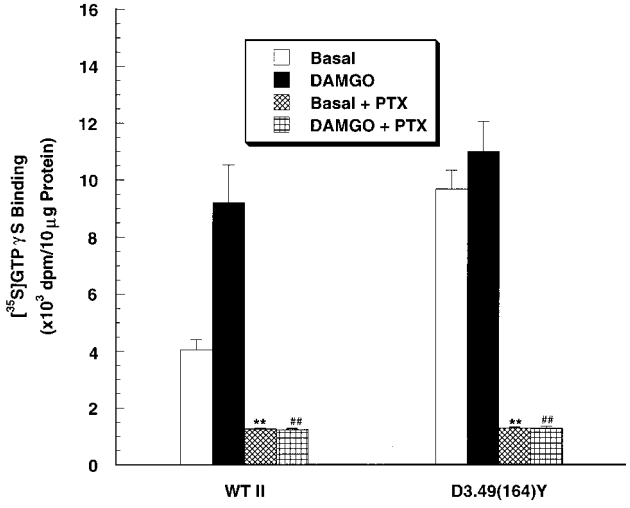

FIGURE 7: Effect of PTX pretreatment on basal and DAMGO-stimulated [^{35}S]GTPγS binding mediated by the wild-type and D3.49(164)Y mutant RMOR. CHO cells stably transfected with the wild-type or the D3.49(164)Y mutant were grown in the presence of 20 μM naloxone for at least 96 h and treated with PTX (100 ng/mL) for 24 h, and membranes were prepared. [^{35}S]GTPγS binding was performed in the absence (basal) or presence of 10 μM DAMGO. Data are expressed as the mean ± SEM of at least 3 independent experiments in duplicate. $^{**}p < 0.01$, compared with basal without PTX; $^{\#\#}p < 0.01$ compared with DAMGO-stimulated binding without PTX by Student's *t*-test.

constitutive activity further indicates that these mutations led to agonist-independent activation of the receptor.

In contrast to the constitutively active D3.49(164) mutants, the D3.49(164)E mutant exhibited significantly lower basal [^{35}S]GTPγS binding than the wild-type, and the slope of the regression line was smaller than that of wild-type, indicating a reduced activity of the mutant receptor. Notably, the basal [^{35}S]GTPγS binding exhibited by the wild-type preparations also increased linearly as the receptor level increased, indicating that there is a constant, albeit low, level of constitutive activation of G proteins by the wild-type receptor.

Effect of PTX Treatment on [^{35}S]GTPγS Binding Mediated by the Wild-Type and D3.49(164)Y Mutant. Pretreatment of CHO-RMOR and CHO-D3.49(164)Y cells with 100 ng/mL PTX for 24 h greatly decreased the basal level of [^{35}S]GTPγS binding of both the wild-type and D3.49(164)Y mutant and completely abolished DAMGO-stimulated [^{35}S]GTPγS binding (Figure 7). Thus, the agonist-independent activities of the D3.49(164)Y mutant receptor, and likely D3.49(164)H, D3.49(164)M, and D3.49(164)Q mutants, were due to constitutive activation of PTX-sensitive G_i and/or G_o proteins.

CAMs Had Higher Affinities for DAMGO. Many, but not all, constitutively active mutants of GPCRs exhibit higher affinities for agonists than the wild-type [for reviews, see (*11*, *12*)]. We thus examined whether this held true for the binding of the MOR-selective agonist DAMGO to the D3.49(164) CAMs. Competitive inhibition of [^{3}H]diprenorphine binding to the wild-type and the mutants was performed to determine the apparent K_i values of DAMGO (Figure 8, Table 3). The affinities of the D3.49(164)M, D3.49(164)Q, D3.49(164)H, and D3.49(164)Y mutants for DAMGO were 5.4-, 5.0-, 8.6-, and 20.7-fold that of the wild-type. Thus, the constitutively active D3.49(164) mutants had enhanced affinities for DAMGO. In contrast, the affinity of

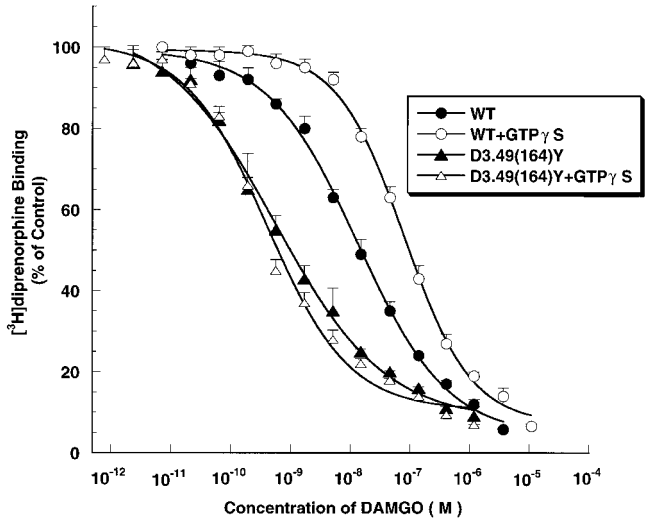

FIGURE 8: Competitive inhibition by DAMGO of [^{3}H]diprenorphine binding to the wild-type and the D3.49(164)Y mutant of the RMOR. The wild-type and the mutant receptors were transiently expressed in HEK293 cells and treated with naloxone, and membranes were prepared. Competitive inhibition of [^{3}H]diprenorphine binding by DAMGO was performed with 0.4 nM [^{3}H]diprenorphine for 60 min at room temperature in the presence of various concentrations of DAMGO with or without 20 μM GTPγS. Data are expressed as means ± SEM from at least 3 independent experiments in duplicate. The K_i values of DAMGO are shown in Table 2.

the D3.49(164)E mutant for DAMGO was significantly lower than that of the wild-type (Table 3). GTPγS, which uncoupled receptors from G proteins, lowered the affinity of the wild-type for DAMGO by about 4-fold (Figure 8, Table 3), but caused no or a smaller reduction in the affinities of the constitutively active D3.49(164) mutants for DAMGO (Figure 8, Table 3). Thus, in the presence of GTPγS, the D3.49(164)M, D3.49(164)Q, D3.49(164)H, and D3.49(164)Y mutants displayed 10−121-fold higher affinities for DAMGO

Table 3: Apparent K_i Values of DAMGO in Inhibiting [^{3}H]Diprenorphine Binding to the Wild-Type (WT) and D3.49(164) Mutants of the RMOR[a]

	in absence of GTPγS			in presence of GTPγS		
	apparent K_i (nM)	WT/ mutant	*n*	apparent K_i (nM)	WT/ mutant	*n*
WT	6.00 ± 0.97	−	8	25.33 ± 4.14	−	4
D3.49(164)M	1.12 ± 0.30	5.4	6	2.63 ± 0.19	9.6	3
D3.49(164)Q	1.19 ± 0.29	5.0	6	1.81 ± 0.06	14.0	3
D3.49(164)H	0.70 ± 0.14	8.6	5	1.25 ± 0.13	20.3	3
D3.49(164)Y	0.29 ± 0.07	20.7	8	0.21 ± 0.04	120.6	3
D3.49(164)E	70.8 ± 22.9	0.08	4			

[a] HEK293 cells were transfected with cDNA clones encoding the WT or a D3.49(164) mutant of RMOR and treated with naloxone as described under Materials and Methods. Competitive inhibition of 0.4 nM [^{3}H]diprenorphine binding to membranes by DAMGO was performed in Tris buffer in the absence or the presence of 20 μM GTPγS. Data are expressed as means ± SEM, and *n* represents the number of independent experiments performed in duplicate.

than the wild-type. These results indicate that the enhanced affinities of the constitutively active D3.49(164) mutants for DAMGO are intrinsic properties of the mutant receptors, unrelated to G protein coupling.

Structural Stability of the Wild-Type and the D3.49(164)Y and D3.49(164)E Mutants in Membranes. Membranes were prepared from CHO-RMOR, CHO-D3.49(164)Y, and CHO-D3.49(164)E cells. Incubation of membranes at 4 °C for 6 h in the presence of various protease inhibitors decreased [^{3}H]diprenorphine binding of the D3.49(164)Y mutant without affecting binding of the wild-type and the D3.49(164)E mutant (Figure 9A). In contrast, incubation of membranes at 37 °C in the presence of various protease inhibitors decreased [^{3}H]diprenorphine binding of the wild-type and the mutant receptors (Figure 9A). Notably, the initial rate of the decrease was higher for the D3.49(164)Y mutant than the wild-type (Figure 9A), with greater decline in the first 2 h, but reaching a similar level of ~20% at 4 and 6 h. In contrast, [^{3}H]diprenorphine binding of the D3.49(164)E mutant decreased to the same extent as that of the wild-type at 1 h; however, the decline was significantly less for the D3.49(164)E mutant at 2, 4, or 6 h. In contrast to the decreased [^{3}H]diprenorphine binding, there was no apparent change in the amount of the wild-type and mutant receptor proteins after incubation at 37 °C for 6 h as detected by western blot (Figure 9B). Thus, the loss of binding activity of the wild-type and the mutants represents denaturation, but not degradation, of the receptor proteins. These results indicate that compared with the wild-type, the constitutively active D3.49(164)Y mutant is structurally less stable, but the D3.49(164)E mutant is more stable.

The D3.49(164)E Mutant Exhibits Stronger Interactions than the Wild-Type with the Conserved R3.50(165). The length of the hydrogen bond between the residues at positions 3.49 and 3.50 in the 600 structures collected from the Monte Carlo simulations is shown in Figure 10. Both the length of the hydrogen bond and the fluctuations around the average are smaller for the E3.49 mutant than for the wild-type, indicating a stronger interaction with the conserved R3.50. The difference is significant, as the average length for E3.49−R3.50, calculated from the 600 structures (Figure 10), is 2.59 ± 0.08 Å [in excellent agreement with the value of 2.63 Å in the crystal structure of rhodopsin (*35*)], while in

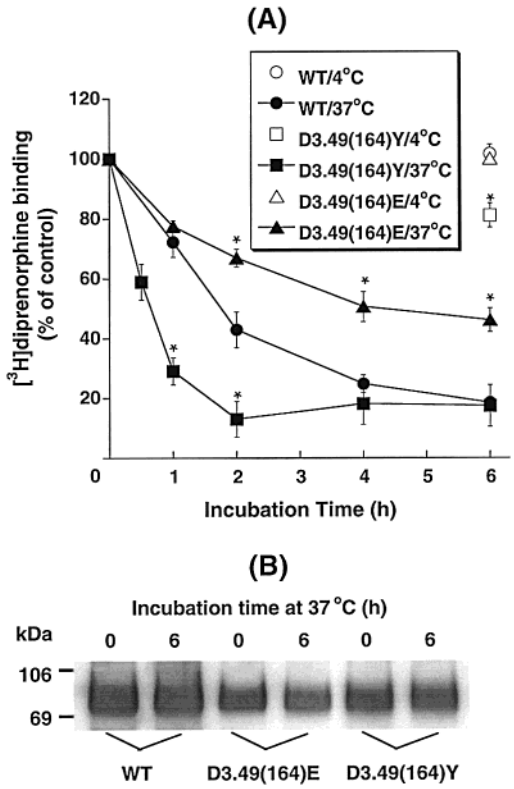

FIGURE 9: Stability of the wild-type and the D3.49(164)Y and D3.4(164)E mutants of the RMOR in membranes. Membranes were prepared from CHO-RMOR and CHO-D3.49(164)E cells. CHO-D3.49(164)Y cells were pretreated with 20 μM naloxone for at least 96 h, and membranes were prepared. Membranes were incubated at 4 or 37 °C for the indicated periods of time in the presence of protease inhibitors. (A) [^{3}H]Diprenorphine binding to the wild-type and mutant receptors in membranes was performed. Results were normalized to dpm of [^{3}H]diprenorphine binding/10 μg of protein and expressed as percent of binding without incubation. Each value represents the mean ± SEM of three independent experiments in duplicate. *p < 0.01, compared with the wild-type by Student's *t*-test. (B) Western blot was performed using monoclonal antibody against the HA epitope. The figure represents one of the two experiments performed with similar results.

the construct corresponding to the wild-type D3.49−R3.50 interaction of the MOR, the average bond length is 2.93 ± 0.16 Å. The corresponding interaction energies were calculated to be −30.2 ± −1.0 and −23.3 ± 3.6 kcal/mol, respectively. Since the interaction between the side chains of D3.49 and R3.50 was shown to be the main factor in the "caging" of the conserved R3.50, in the conformation corresponding to the inactive form of the receptor (*4*, *34*), these results indicate that the E3.49 construct is likely to stabilize the inactive form of the receptor through the stronger interaction with R3.50.

DISCUSSION

Our current results show that mutation of D3.49(164) to H, Y, M, and Q, but not D3.49(164) to E or D3.32(147) to A or N, results in enhanced agonist-independent [^{35}S]GTPγS binding levels compared with that of the wild-type at similar or higher receptor levels. The agonist-independent activities of these mutants were related to receptor level and resulted from constitutive activation of PTX-sensitive G proteins. These mutants displayed higher affinities for the agonist DAMGO, which were intrinsic to the mutants. Thus, these

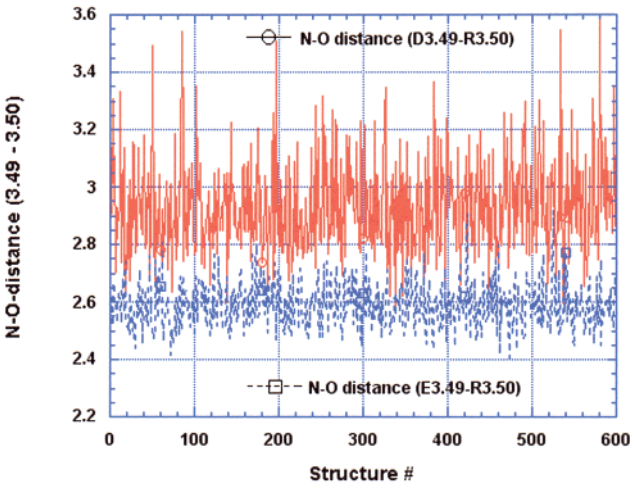

FIGURE 10: Distance between the hydrogen-bonded atoms in R3.50(N) and E3.49(O) calculated from Monte Carlo simulations. The calculations were done for the D/ERY motif in the crystal structure of rhodopsin (*35*). The values shown (in Å) are for the 600 representative structures extracted from the run of 3 million structures as described under Materials and Methods.

mutants appear to assume molecular conformations that resemble those of the activated states of the receptor. To the best of our knowledge, this represents the first report that shows that various mutations of D3.49 in opioid receptors result in constitutive activation of the receptor. In addition, constitutively active MOR allow determination of inverse agonist activities, and this is the first demonstration that naloxone, naltrexone, and naloxone methiodide are inverse agonists.

Mutation of D3.49(164) to H, Y, M, or Q Led to Constitutive Activation of the μ Receptor, but D3.49(164)E Substitution Stabilized the Inactive Form. The D/ERY motif at the interface of the TM3 and second intracellular loop is highly conserved among members of the rhodopsin subfamily of GPCRs (*22, 40*) and has been shown to be important in GPCR activation (*34, 41*). The observations that in the RMOR mutation of D3.49(164) to H, Y, M, or Q led to constitutive activation of the receptor and the D3.49(164)E substitution reduced basal activity indicate that the carboxylate group of D3.49(164) is important for stabilizing the inactive state. Mutation of this locus was shown to result in constitutive activation of several GPCRs, including rhodopsin (*16*), α_{1B}-adrenergic (*17, 18*) and β_2-adrenergic (*19*), V_2 vasopressin (*42*), and H_2 histamine (*43*) receptors, but not some other GPCRs, such as the m1 muscarinic (*44*), luteinizing hormone/chorionic gonadotropin (*45*), and gonadotropin-releasing hormone (*46*) receptors. Substitution of D3.49(142) with E did not result in enhanced basal activity of the α_{1B}-adrenergic receptor (*17*). However, our results represent the first demonstration that D3.49E mutation lowered the basal activity of a GPCR. In fact, our computational results show that the interaction between R3.50 and its neighbor at 3.49 is strengthened by the D3.49E mutation in the RMOR and stabilizes the inactive form of the receptor.

These findings are consistent with the involvement of the DRY motif in GPCR activation proposed by Ballesteros et al. (*34*). Based on mutagenesis studies and molecular dynamics simulations, these researchers have concluded that the conserved R3.50 of the DRY motif is constrained by an ionic interaction with the preceding D3.49 in the inactive form of the GPCRs. The recently published crystal structure of rhodopsin suggests that the carboxylate group of E3.49(134) forms a salt-bridge with the guanidium group of R3.50(135) (*35*). Arnis et al. (*47*) and Fahmy et al. (*48*) have demonstrated that a key event in forming the metarhodopsin II state of rhodopsin is uptake of protons and one proton is most likely taken up by E3.49(134). Thus, when the GPCR is activated, the D/E at position 3.49 is hypothesized to become protonated. The conformational changes that are likely to ensue from this change in protonation state should allow R3.50 to be repositioned to support the interaction with G proteins (*34, 47*). Our findings that mutation of D3.49(164) to H, Y, M, or Q, but not E, results in agonist-independent activation of the RMOR are in accord with this notion as the lack of charged side chain will release the R3.50. In the normal function of the wild-type receptor, protonation of D3.49 could similarly release R3.50 from its local interaction with D3.49. Notably, the stronger interaction of R3.50 with the E3.49 side chain than with D3.49, as demonstrated by the results from our Monte Carlo simulations, reduces the probability of agonist-independent activation of the D3.49E mutant by making the release of R3.50 more difficult energetically.

Once freed from the "arginine cage" (*34*), the arginine side chain can interact with other microdomains in the receptor such as D2.50 and N7.49 (in the case of gonadotropin-releasing hormone receptor, N2.50 and D7.49) (*34*). We have shown that the D2.50(114)N mutant of the MOR could not be activated by agonists and that D2.50(114) and N7.49(332) of RMOR interact with each other as part of the interhelical interaction network (*24*).

The increased agonist-independent [^{35}S]GTPγS binding of the D3.49(164)Y mutant preparations was inhibited by PTX pretreatment, indicating spontaneous receptor coupling to G_i/G_o. These findings, coupled with the observation that the constitutively active D3.49(164) CAMs have higher affinity for the agonist DAMGO, strengthen the idea that CAMs mimic the "active" states of GPCRs, adopting conformations similar to those induced by an agonist. Interestingly, PTX pretreatment also reduced the basal [^{35}S]GTPγS binding of the wild-type preparations. Thus, there is an agonist-independent coupling of the wild-type receptor to PTX-sensitive G proteins as well, similar to the observation of Traynor and Nahorski (*37*).

Among the four constitutively active mutants, the levels of their agonist-independent activities are in the order of H > Y > M = Q (see Figure 6), reflecting either a graded difference in the probability of reaching the activated form of the receptor or a spectrum of active conformations exhibited by these mutants. Replacing D3.49(142) in the α_{1B}-adrenergic receptor with all possible natural amino acids resulted in mutants with various levels of agonist-independent activity (*17*). Among the four substitutions at D3.49(142) of the α_{1B}-adrenergic receptor, the order for the constitutive activity is M = Q ≫ H > Y. These researchers found that the levels of constitutive activity are inversely related to the solvent-accessible area of the residue at D3.49(142) (*17*). This relationship appears not to be applicable to the MOR. This may be due to the different G proteins to which the MOR and α_{1B}-adrenergic receptors are coupled, G_i/G_o and G_q, respectively, and to the possibility that different receptor

conformations may be required for activation of these G proteins.

D3.49(164) CAMs Had Higher Affinities, but D3.49(164)E Had Lower Affinity, for the Agonist DAMGO. According to the original and extended ternary complex models on the interaction of agonists, GPCRs and G proteins, agonists have a higher affinity for the activated states of GPCRs (*49*, *50*). We found that compared with the wild-type, the four D3.49(164) CAMs showed significantly higher affinities for the agonist DAMGO, but had similar affinities for the antagonist [^{3}H]diprenorphine, which is in agreement with the observations reported for CAMs of many GPCRs [for reviews, see (*11*, *12*)]. The enhanced affinities for DAMGO of these mutants were unaffected or affected less by GTPγS, indicating that this is an intrinsic property of these mutants, unrelated to their coupling to G proteins. Thus, among these four D3.49(164) CAMs, enhanced constitutive activities are associated with increased agonist affinities. Notably, this property is not always observed for CAMs of GPCRs. For example, in the δ opioid receptor, the D3.32(128)N, D3.32(128)A, Y3.33(129)A, Y3.33(129)F, and Y7.43(308)F mutants exhibited increased agonist-independent activities (*21*), but had lower agonist affinities (*21*, *51*, *52*). In addition, the D3.49(142)R mutants of the α_{1B}-adrenergic receptor had increased affinity for epinephrine, yet displayed no constitutive activity (*17*).

Consistent with a stabilization of the inactive form of the receptor in the D3.49(164)E mutant, this construct displayed a lower affinity for DAMGO and lower basal [^{35}S]GTPγS binding compared with the wild-type.

Importance of D3.49(164) for the Expression of the μ Opioid Receptor. Replacement of D3.49(164) of RMOR with Q, S, M, H, I, V, L, A, T, N, W, or Y amino acid residues results in loss of binding activity. Substitution of D3.49(164) with Q, and likely with others, abolished receptor protein expression. In contrast, the D3.49(164)E mutant receptor was expressed to a high level without naloxone pretreatment. These results indicate that the carboxylate moiety of D3.49(164) also plays an important role in the expression of the MOR, which is most likely related to its role in constraining the receptor in inactive conformations. However, D3.49 mutations which result in constitutive activation of GPCRs in most cases reduced, but did not abolish, receptor binding. The lack of expression of the D3.49(164) CAMs of the μ receptor most likely represents an extreme case of what has been observed for D3.49 CAMs of other GPCRs. However, there are also GPCRs in which D3.49 mutations greatly abolished expression of binding activities, but the mutants displayed no constitutive activities, for example, the m1 muscarinic (*44*), luteinizing hormone/chorionic gonadotropin (*45*), and gonadotropin-releasing hormone (*46*) receptors.

D3.49(164)Y Mutant Is Structurally Less Stable, whereas D3.49(164)E Mutant Is More Stable, than the Wild-Type. Upon incubation of membranes at 37 °C, the constitutively active D3.49(164)Y mutant lost binding activity at a greater rate than the wild-type in the first 2 h. In contrast, the D3.49(164)E mutant lost binding activity to a lesser extent than the wild-type. However, there was no appreciable change in the amount of the wild-type and mutant receptor proteins. The difference between the results obtained from western blot and receptor binding indicates that the wild-type and mutants are denatured, but not degraded, and while the D3.49(164)Y mutant is more easily denatured, the D3.49(164)E mutant is less so, than the wild-type. It has been reported that the CAMs of the β_2-adrenergic (*19*, *53*) and the histamine H_2 receptor (*43*) are structurally less stable. The susceptibility to denaturation was considered to relate to the same structural changes that enhance the probability of transitions between the inactive and the activated states of the receptor in the CAM (*53*). Conversely, the higher stability of the D3.49(164)E mutant is most likely due to the stronger interaction of E3.49(164) with R3.50(165) than the wild-type D3.49(164), and consistent with the stabilization of the inactive state of the receptor.

Naloxone Up-Regulates the D3.49(164) CAMs. Naloxone pretreatment of the cells expressing the constitutively active D3.49(164) mutants was essential for [^{3}H]diprenorphine binding and HA-tagged D3.49(164)Q mutant receptor protein to be detected. These observations are similar to previous findings showing that sustained treatment of cells expressing CAMs of several GPCRs with inverse agonists increased expression levels (*43*, *53*−*56*). We have found that naloxone acts by two main mechanisms: stabilization of the unstable mutant receptor structure and blockade of constitutive internalization and down-regulation of these mutants (Li, J., et al., submitted for publication).

Inverse Agonists at the CAMs. Naloxone has been shown to be an inverse agonist at the δ opioid receptor (*57*). Here, we found that naloxone reduced the basal [^{35}S]GTPγS binding of the D3.49(164)H, -Y, -M, and -Q mutants to levels similar to that of the wild-type. In addition, naltrexone and naloxone methiodide were also shown to decrease the basal [^{35}S]GTPγS binding of the D3.49Q mutant, indicating that the drugs act as inverse agonists. Naloxone also reduced the basal [^{35}S]GTPγS binding of the wild-type II clone, which expresses a high level of the receptor. However, this reduction is much smaller than observed in the CAMs. Interestingly, we found that the inhibitory effects of naloxone on the basal [^{35}S]GTPγS binding of the CAMs were observed when naloxone was added to membranes 5−10 min prior to the addition of [^{35}S]GTPγS, but not when naloxone and [^{35}S]GTPγS were added to membranes simultaneously. These observations suggest that interaction of naloxone, and likely other inverse agonists, with the CAM requires some time, perhaps in order to allow the receptor to assume certain conformations. To the best of our knowledge, this is the first demonstration that naloxone, naltrexone, and naloxone methiodide are inverse agonists at the MOR.

An intriguing finding is that while naloxone inhibited the enhanced basal activity of constitutively active D3.49(164) mutants to levels similar to the basal activity of the wild-type, it enhanced the [^{35}S]GTPγS binding of the D3.49(164)E mutant to an equivalent level (see Figure 4A). These results suggest that naloxone binding induces certain conformations in the receptors which yielded [^{35}S]GTPγS binding similar to the basal level of the wild-type, regardless of what conformations the receptors have prior to naloxone binding.

D3.32(147) Mutants Are Not CAMs. Unlike the mutations at the D3.49 site, mutation of D3.32(147) to A or N in the RMOR did not result in constitutive activation of the receptor. Thus, in the MOR, this residue appears not to be involved in receptor activation via a mechanism involving disruption of the TM3−TM7 interaction as described for

rhodopsin and α_{1B}-adrenergic and δ opioid receptors (*20*, *21*, *58*). This observation is similar to that of Kristiansen et al. (*59*), who reported that mutation of the homologous D3.32(155) in the 5-HT2A serotonin receptor did not enhance agonist-independent activity. In contrast, substitution of D3.32 in the δ opioid or α_{1B}-adrenergic receptor or E3.28(113) in rhodopsin, one helical turn extracellular to the 3.32 locus, led to constitutive activation of the receptors (*20*, *21*, *58*). The mechanistic hypothesis presented to explain these results was that E3.28(113) in rhodopsin forms a salt bridge with K7.43(296) (*58*) and so does D3.32(125) in the α_{1B}-adrenergic receptor with K7.36(331) (*20*), and these salt bridges constrain the receptors in inactive states. Mutations that disrupt the salt bridges could therefore lead to agonist-independent activation. Our result supports the notion that D3.32(147) in the MOR plays a different role from the homologous residue D3.32(128) in the δ opioid receptor. While D3.32(147) in the MOR is involved directly in ligand binding, with its carboxylate group forming ion-pairing with the protonated nitrogen of naltrexone and morphine (*60*), D3.32(128) in the δ opioid receptor was demonstrated not to be the anionic counterpart of the cationic opioids (*51*), but to interact with Y7.43(308) as part of a network of interhelical bonds, which contribute to maintaining the δ receptor under an inactive conformation (*21*).

Other Mutants Examined for Constitutive Activity. CAMs have also been reported in other regions of the receptors, including N1.50 (*63*) in TM1 of the α_{1B}-AR (*18*) and L3.43(116) in TM3 of the m1 muscarinic receptor (*61*). We found that in the RMOR, mutation of N1.50(86) in the TM1 to D, L, or H or substitution of L3.43(158) in the TM3 with A abolished [^{3}H]diprenorphine binding (data not shown). The inclusion of naloxone in the culture medium for 48 h did not increase [^{3}H]diprenorphine binding (data not shown). Thus, N1.50(86) and L3.43(158) may have specific important roles in binding and/or expression of the MOR, and it was not possible to determine whether these mutants are constitutively active.

Concluding Remarks. The substitution of D3.49(164) with H, Y, M, or Q results in constitutive activation of the MOR and structural instability. However, unlike the α_{1B}-adrenergic and δ opioid receptors (*20*), mutations of D3.32(147) did not lead to increased constitutive activities of the μ opioid receptor (*21*). The CAMs produced by the substitutions of D3.49(164) allowed the determination of inverse agonist activities, and naloxone, naltrexone, and naloxone methiodide were shown to be inverse agonists. In contrast to the effects of these mutations, the D3.49(164)E substitution was shown to stabilize the inactive state of the receptor. Taken together, these findings indicate that in the wild-type MOR the carboxylate group of the residue at the D3.49(164) position is involved in constraining the receptor in an inactive conformation (*4*, *34*). The constraint is likely to result from the ion-pairing interaction with R3.50(165), as observed in the crystal structure of inactive rhodopsin (*35*). D3.49(164)E had lower basal activity and higher structural stability, most likely because of the stronger interaction of E3.49(164) with R3.50(165), compared to the wild-type D3.49(164), that was shown by computational simulations. The identified importance of D3.49(164) for expression of the MOR is likely related to its role in constraining the receptor in inactive conformations.

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