Functional Role of a Conserved Motif in TM6 of the Rat μ Opioid Receptor: Constitutively Active and Inactive Receptors Result from Substitutions of Thr6.34(279) with Lys and Asp[†]

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ABSTRACT: Mutations within the "$X_1BBX_2X_3B$" motif or its variants in the junction of the third intracellular (i3) loop and the sixth transmembrane domain (TM6) have been shown to lead to constitutive activation of several G protein-coupled receptors (GPCRs). In this study, T6.34(279) at the X_3 locus of the rat μ opioid receptor was mutated to Lys and Asp, and the mutants were examined for binding and signaling properties. The T6.34(279)K mutant was poorly expressed, and pretreatment with naloxone greatly enhanced its expression. This construct exhibited properties identified previously with constitutive activation: (1) compared with the wild type, it produced much higher agonist-independent [^{35}S]GTPγS binding, which was abolished by pertussis toxin treatment; (2) it displayed an enhanced affinity for the agonist DAMGO similar to that of the high-affinity state of the wild type, which was not altered by GTPγS, while having unchanged affinity for the antagonist diprenorphine. The T6.34(279)K mutant displayed a higher intracellular receptor pool than the wild type. Naloxone inhibited the basal [^{35}S]GTPγS binding of the T6.34(279)K mutant, demonstrating inverse agonist activity at this mutant receptor. In contrast, the T6.34(279)D substitution did not increase basal [^{35}S]GTPγS binding, greatly reduced agonist-promoted [^{35}S]GTPγS binding, and markedly decreased affinity for DAMGO. Thus, the T6.34(279)D mutant adopts conformations corresponding to inactive states of the receptor. The results were interpreted in the structural context of a model for the μ opioid receptor that incorporates the information from the crystal structure of rhodopsin. The interaction of T6.34(279) with R3.50(165) in the μ opioid receptor is considered to stabilize the inactive conformations. The T6.34(279)K substitution would then disrupt this interaction and support agonist-free activation, while T6.34(279)D mutation should strengthen this interaction which keeps the receptor in inactive states. T6.34(279) may, in addition, interact with the neighboring R6.35(280) to help constrain the receptor in inactive states, and T6.34(279)K and T6.34(279)D mutations would affect this interaction by disrupting or strengthening it, respectively. To the best of our knowledge, the results presented here represent the first structurally rationalized demonstration that mutations of this locus can lead to dramatically different properties of a GPCR.

Opiate and opioid drugs, acting on membrane-bound opioid receptors, have long been used as analgesics (*1, 2*). These drugs, however, also produce side effects such as respiratory depression, decreased gastrointestinal motility, sedation, mood changes, tolerance, and dependence (*1, 2*). Heroin and morphine are among the most widely abused drugs, causing serious social problems. The site of action of the opioid drugs includes at least three types (μ, δ, and κ) of opioid receptors present in the nervous system (*1*). Opioid receptors are coupled via pertussis toxin (PTX)[1]-sensitive G_i/G_o proteins to a variety of effectors that include adenylate cyclase, potassium channels, calcium channels, and a mitogen-activated protein kinase pathway [for a review, see (*3*)]. Following the cloning of the δ opioid receptor, μ and κ receptors were cloned [for reviews, see (*4, 5*) and references cited therein]. The cloned μ, δ, and κ receptors have seven transmembrane domains (TMs), a characteristic of G protein-coupled receptors (GPCRs) (*6*). The opioid receptors belong to the rhodopsin subfamily [see (*6, 7*) for a classification scheme], characterized by the presence of highly conserved "fingerprint" residues including the DRY motif in TM3, N1.50 in TM1, D2.50 in TM2, W4.50 in TM4, and P5.50, P6.50, and P7.50 in TMs 5−7 (*7*).

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[1] Abbreviations: AR, adrenergic receptor; CAM, constitutively active mutant; CHO cells, Chinese hamster ovary cells; DAMGO, Tyr-D-Ala-Gly-(Me)Phe-Gly-ol; EDTA, ethylenediaminetetraacetic acid; GPCRs, G protein-coupled receptors; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); 5-HT, 5-hydroxytryptamine; i3 loop, the third intracellular loop; PTX, pertussis toxin; TM, transmembrane domain; WT, wild type.

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According to the various models of GPCR function (*8, 9*), receptors undergo conformational equilibria between inactive states that are structurally constrained and unable to couple to GDP-liganded G$\alpha\beta\gamma$ heterotrimer, and active states that can interact productively with the heterotrimer by catalyzing GDP/GTP exchange. GTP-bound G_α and $G_{\beta\gamma}$ are then dissociated to relay signals downstream to a number of intracellular pathways.

The detailed mechanisms underlying the conformational changes from inactive states to activated states have not been elucidated experimentally in their entirety. Movements of TMs 3, 5, and 6 have been shown to be important for activation of GPCRs (*10*−*13*). In rhodopsin, disulfide cross-linking of cytoplasmic ends of TMs 3 and 6 or zinc binding to a metal binding site formed by engineered histidines in intracellular sides of TMs 3 and 6 prevented activation of transducin (*10, 11*). Gether et al. (*12*) showed that agonist binding to the β_2-adrenergic receptor (AR) 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 in activation of the receptor. Activation of the NK-1 receptor was inhibited by zinc binding to a metal binding site formed by engineered histidines in TMs 5 and 6 (*13*). Nevertheless, while the possible mechanistic implication of these observations can be understood in the currently known structural context for GPCRs [for a review, see (*14*)], the detailed molecular events underlying the movement of these transmembrane helices during receptor activation, and thereby determining the conversion from inactive to activated states, are still missing.

Some of these details are being revealed from mutagenesis studies. Mutation in a GPCR resulting in G protein activation in the absence of an agonist was first demonstrated for α_{1B}-AR (*15, 16*) and subsequently in several other receptors [for reviews, see (*17*−*19*)]. Constitutive activity mostly arises from mutations clustered in the cytoplasmic extensions of TMs 3 and 6 as well as within TMs 2, 3, 6, and 7 [for a review, see (*19*)]. Because these mutants likely represent a spectrum of activated states, they can be used to shed light on the conformational changes underlying GPCR activation.

Similarly, the junction region between the i3 loop to TM6 has been implicated in these mechanisms. Mutations in the "$X_1BBX_2X_3B$" motif (B, basic amino acid; X, nonbasic amino acid) or its variants within this region have been shown to lead to constitutive activation of several GPCRs, including α_{1B}-adrenergic (*15, 16*), β_2-adrenergic (*9*), α_{2A}-adrenergic (*20*), β_1-adrenergic (*21*), m1 muscarinic (*22*), 5-hydroxytryptamine 2A (5-HT_{2A}) (*23*), 5-HT_{2C} (*24*), 5-HT_{1B} (*25*), and cannabinoid CB1 (*26*) receptors (Figure 1). This region, thus, has been suggested to be involved in constraining GPCRs in inactive conformations, and mutations abolishing the constraints result in constitutive activation of the receptors. We tested a mechanistic hypothesis for the role of this region in the equilibrium between inactive and active forms of the μ opioid receptor. To this end, we mutated T6.34(279) at the X_3 site within the "$X_1BBX_2X_3B$" motif of the rat μ opioid receptor to positively and negatively charged amino acids, Lys and Asp, respectively, and characterized the binding and signaling properties of these mutants. [^{35}S]-GTPγS binding was used as the functional measure of receptor activation. The two mutant constructs are shown to adopt different functional forms of the receptor. The results

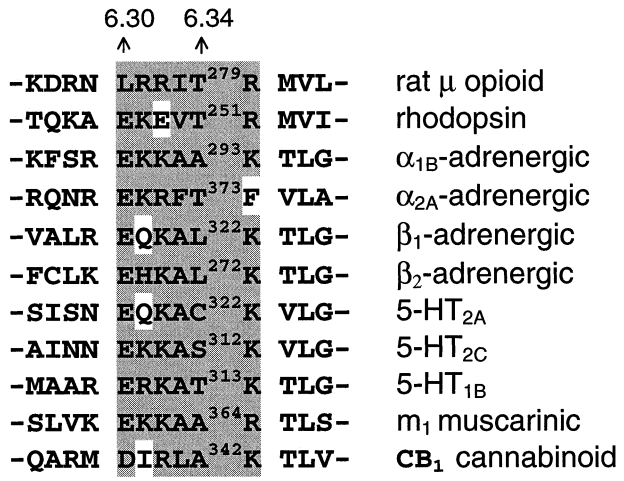

FIGURE 1: Amino acid sequence at the i3 loop−TM6 junction region of the rat μ opioid receptor and comparison with those of several other GPCRs. The "$X_1BBX_2X_3B$" motifs and variants are highlighted (B, basic amino acid; X, nonbasic amino acid). The numbers indicate amino acid numbers in the sequences of the receptors.

are analyzed in a structural context that supports the mechanistic role of the arginine cage region (*27*) in the function of GPCRs.

MATERIALS AND METHODS

Materials. [^{35}S]GTPγS (~1250 Ci/mmol) and [^{3}H]diprenorphine (58 Ci/mmol) were purchased from NEN Life Science (Boston, MA). GDP and GTPγS were obtained from Sigma Chemical Co. (St. Louis, MO). Naloxone was a gift from DuPont/Merck Co. (Wilmington, DE). Tyr-D-Ala-Gly-(Me)Phe-Gly-ol (DAMGO) and pertussis toxin (PTX) were purchased from Research Biochemicals International, Inc. (Natick, MA). 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 the μ Opioid Receptor and Other GPCRs. The numbering schemes used throughout identify amino acid residues in opioid receptors and other GPCRs both by their sequence numbers and by the generic numbering scheme proposed by Ballesteros and Weinstein (*28*) and recently applied to opioid receptors (*29, 30*). This combined scheme is used in order to relate the results obtained for opioid receptors to corresponding 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 TM6 of the rat μ opioid receptor is Pro295, which is referred to as P6.50(295). T279 is referred to as T6.34(279).

Oligodeoxynucleotide-Directed Mutagenesis. Site-directed mutagenesis was performed on the rat μ receptor with the overlap polymerase chain reaction method described by Higuchi et al. (*31*). HA-tagged wild type and mutant rat μ

receptors were subcloned into *Hin*dIII and *Xba*I sites of the mammalian expression vector pcDNA3 (*29*). The cDNA sequences were determined with the method of Sanger et al. (*32*) to confirm the presence of desired mutations and the absence of unwanted mutations.

Stable Expression of the Wild Type and Mutant Rat μ Opioid Receptors in CHO Cells. Transfection of CHO cells with the cDNA clones of the wild type or a mutant of the rat μ opioid receptor in 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 the wild type or a mutant of the rat μ opioid receptor were established as described previously (*33*). Clonal cell lines with different expression levels were obtained. In some experiments, clonal cells transfected with the T6.34(279)K mutant receptor were pretreated with naloxone (20 μM) for at least 96 h to reach higher expression density. Cells were harvested for experiments by use of Versene solution (EDTA 0.54 mM, NaCl 140 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.46 mM, and glucose 1 mM) in the presence of 20 μM naloxone.

Opioid Receptor Binding in Intact Cells. CHO cells stably transfected with the wild type or a mutant receptor were cultured in the absence or presence of 20 μM naloxone for at least 96 h and washed to remove naloxone. [^{3}H]-Diprenorphine (1 nM) binding to the receptor in intact cells was performed in Kreb's buffer (NaCl 130 mM, KCl 4.8 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 1.3 mM, $MgSO_4$ 1.2 mM, glucose 10 mM, and HEPES 25 mM, pH 7.4) at room temperature for 60 min. Naloxone (10 μM) was used to define nonspecific binding. Bound and free radioactivity was separated by filtration.

Determination of Intracellular Receptors. Intracellular receptors were assessed according to a modification of a previously described procedure (*34*). CHO cells stably transfected with the wild type or the T6.34(279)K mutant were cultured with or without naloxone, washed, and harvested. Binding was performed on intact cells in suspension in Kreb's buffer solution. Total receptor levels were assessed by binding with 1 nM [^{3}H]diprenorphine in the presence or absence of 10 μM naloxone, while surface receptors were measured by binding with 1 nM [^{3}H]-diprenorphine in the presence or absence of 10 μM DAMGO. Binding was performed at room temperature for 60 min, and bound and free radioactivity was separated by filtration.

Membrane Preparations. Membranes were prepared according to Huang et al. (*35*). Protein concentration was determined by the bicinchoninic acid method of Smith et al. (*36*) with bovine serum albumin as the standard. Membranes were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose at ~0.5 mg/mL and then aliquoted and stored at −80 °C.

Opioid Receptor Binding in Membrane Preparations. Saturation binding of [^{3}H]diprenorphine to the wild type and mutant μ opioid receptors 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 rat μ opioid receptors was performed with 0.8 nM [^{3}H]diprenorphine in the absence or presence of increased concentrations of DAMGO, and the K_i value of DAMGO was 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. Bound and free radioactivity was separated by filtration. In some experiments, 20 μM GTPγS was included in the binding buffer. Binding data were analyzed with the nonlinear regression analysis in the GraphPad Prism program. In some cases, two-site competitive binding fit was performed.

Western Blot. Western blot was performed to examine the expression of the HA-tagged wild type and mutant μ opioid receptors. CHO cells stably transfected with the wild type, T6.34(279)K, or T6.34(279)D receptor 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 (*37*). 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 three washes with TBS. The target proteins were located by chemiluminescence using ECL western blotting detection reagents followed by exposure to X-ray films.

[^{35}S]GTPγS Binding Assay. Determination of [^{35}S]GTPγS binding to G proteins was carried out as described previously (*35*) 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 EGTA, 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. Radioactivity was determined by liquid scintillation counting.

Effect of PTX on [^{35}S]GTPγS Binding. CHO cells stably transfected with the wild type or a mutant receptor were pretreated with or without 200 ng/mL PTX overnight, and membranes were prepared. [^{35}S]GTPγS binding in the absence and presence of 10 μM DAMGO was determined.

A Molecular Model of the μ Opioid Receptor. The model was constructed with the use of the homology modeling approach incorporated in the program MODELLER (*38*); the 2.8 Å resolution crystal structure of rhodopsin (*39*) was used as a structural template. The sequence alignments between the μ opioid receptor and rhodopsin-like GPCRs and the criteria and procedures for further refinements of the model were performed as described in detail in recent reviews (*14*, *28*). Special attention was given to the relative positions and interactions in structural motifs, identified in TM6 and TM3, that have been shown to correspond to functional microdomains such as the "aromatic cluster" (*40*, *41*) and the "arginine cage" (*27*, *42*). As described in detail elsewhere (*14*), the receptor sequence is parsed into groups of residues that correspond to such "microdomains" exhibiting a very high degree of conservation. The (E/D)RY motif and the $X_1BBX_2X_3B$ motif studied here are among such micro-

Table 1: K_d and B_{max} Values of [^{3}H]Diprenorphine Binding to the WT and T279K and T279D Mutants of the Rat μ Opioid Receptor Stably Transfected in CHO Cells[a]

	K_d (nM)	B_{max} (pmol/mg of protein)
WT I	0.12 ± 0.06	0.48 ± 0.15
WT II	0.20 ± 0.01	1.8 ± 0.1
WT III	0.16 ± 0.01	8.5 ± 0.5
T279K I	0.18 ± 0.04	0.19 ± 0.04*
T279K II	0.22 ± 0.04	0.26 ± 0.06*
T279D I	0.24 ± 0.05	0.92 ± 0.25
T279D II	0.27 ± 0.02	1.6 ± 0.1

[a] Membranes were prepared from CHO clonal cell lines, three for the wild type and two for the T6.34(279)D mutant. Two CHO clonal cell lines stably expressing the T6.34(279)K mutant were pretreated with 20 μM naloxone for at least 96 h, and membranes were prepared (*). Saturation binding of [^{3}H]diprenorphine to membrane preparations was performed, and K_d and B_{max} values were determined. Each value represents the mean ± SEM of at least three independent experiments performed in duplicate.

domains. They received special attention in the refinement of the model based on the structure of rhodopsin, the functional inferences about the "arginine cage" (*27*), and the interaction between residues at positions 3.50 and 6.30 (*14*). The mutant constructs T6.34(279)K and T6.34(279)D were modeled by introducing the appropriate residues and using a combination of manual reorientation and calculations with the program CHARMM (*43*) to eliminate unfavorable steric contacts.

RESULTS

[^{3}H]Diprenorphine Binding to the Wild Type (WT) and Mutant Receptors. (A) Increased Expression of the T6.34-(279)K Mutant Receptor by Naloxone Pretreatment. The WT and mutant receptors were transfected into CHO cells, and multiple clonal cell lines expressing each receptor were established. Saturation binding of [^{3}H]diprenorphine to membrane preparations was performed, and K_d and B_{max} values of three WT, two T6.34(279)K, and two T6.34(279)D cell clones were determined (Table 1).

The T6.34(279)D mutant receptor bound [^{3}H]diprenorphine with similar K_d values as the WT (Table 1), suggesting that the mutant receptor retains a similar overall structure as the WT. Two T6.34(279)D clonal cell lines have B_{max} values of 0.92 and 1.6 pmol/mg of protein, respectively.

In contrast to the T6.34(279)D mutant, clonal cells for the T6.34(279)K mutant displayed low [^{3}H]diprenorphine binding. We recently demonstrated that preincubation of cells with naloxone greatly increased the expression levels of the constitutively active D3.49(164) mutants of the μ opioid receptor (*61*). Whether naloxone pretreatment increased expression of the T6.34(279)K mutant was therefore examined. Pretreatment of CHO cells stably expressing the T6.34-(279)K mutant receptor with 20 μM naloxone for at least 96 h greatly increased [^{3}H]diprenorphine (1 nM) binding to the receptor in intact cells following removal of the drug by washing (Figure 2A). Since diprenorphine and naloxone are hydrophobic ligands, the binding paradigm detects cell-surface and intracellular receptors.

Saturation binding of [^{3}H]diprenorphine was performed on crude membranes of cells pretreated with naloxone (including plasma membranes and membranes of intracellular

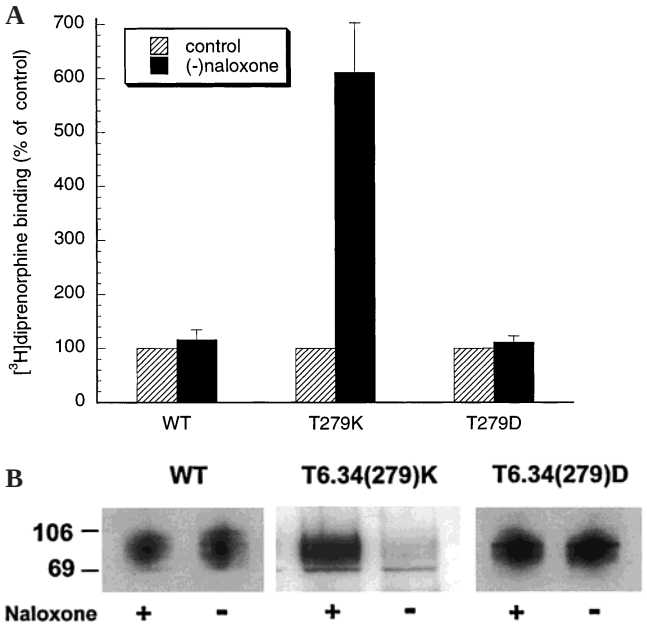

FIGURE 2: (A) Effect of naloxone pretreatment on expression of [^{3}H]diprenorphine binding of the T6.34(279)K and T6.34(279)D mutants of the rat μ opioid receptor. CHO cells stably transfected with the wild type or the T6.34(279)K or T6.34(279)D mutant receptor were cultured in the absence or presence of 20 μM naloxone for at least 96 h. Cells were washed, and [^{3}H]diprenorphine (1 nM) binding to the receptor in intact cells was performed. Data are expressed as percent of binding without naloxone pretreatment for each receptor. Each value represents the mean ± SEM of at least three independent experiments performed in duplicate. (B) Western blot of the HA-tagged wild type and T6.34-(279)K mutant of the rat μ opioid receptor 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 blot represents one of the two experiments performed.

organelles). Two clonal cell lines bound [^{3}H]diprenorphine with similar K_d values as the WT receptor and had B_{max} values of 0.19 and 0.26 pmol/mg of protein, respectively (Table 1). In contrast, naloxone pretreatment did not significantly enhance the expression levels of the WT and T6.34(279)D mutant receptors (Figure 2A).

To determine whether the low level of [^{3}H]diprenorphine binding of the T6.34(279)K mutant was due to the absence of receptor protein expression or receptor conformational changes, we performed western blot on CHO cells stably expressing the mutant using monoclonal antibodies against HA. As shown in Figure 2B, without naloxone pretreatment, only a faint band of the 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 2B). Thus, the low level of binding is due to the low expression of the T6.34(279)K construct in the absence of naloxone pretreatment.

(B) The T6.34(279)K Mutant Receptor Has a Higher Internal Receptor Pool than the Wild Type. The percent of receptors that are intracellular for the wild type and the T6.34(279)K mutant was determined to be 14.7% ± 3.0% (mean ± SEM, $n = 4$) and 33.0% ± 4.0% ($n = 6$), respectively. Pretreatment of cells with naloxone did not significantly change the proportion of the receptors that are

intracellular. Thus, the mutant has higher internal pool of receptors as percent of total receptors.

T6.34(279)K Substitution Results in Constitutive Activation of the Receptor. (A) Basal [^{35}S]GTPγS Binding Is Elevated by the T6.34(279)K Mutant. Membranes of CHO cell clones stably expressing the WT and T6.34(279)K mutant receptors were examined for basal and DAMGO-promoted [^{35}S]-GTPγS binding. The T6.34(279)K mutant at 0.26 pmol of receptor/mg of protein exhibited much higher basal [^{35}S]-GTPγS binding than the WT I at 0.48 pmol of receptor/mg of protein (Figure 3A). DAMGO (10^{-6} M) further enhanced [^{35}S]GTPγS binding of the T6.34(279)K mutant membranes to a significantly higher level over its basal level (Figure 3A).

(B) Constitutive Activation of PTX-Sensitive G Proteins by the T6.34(279)K Mutant. Overnight pretreatment of cells with PTX greatly reduced the basal [^{35}S]GTPγS binding of the T6.34(279)K mutant membranes, indicating the agonist-independent activation of PTX-sensitive G proteins by the T6.34(279)K mutant (Figure 3A). PTX treatment also abolished the DAMGO-stimulated increase in [^{35}S]GTPγS binding of the WT and the T6.34(279)K mutant.

(C) Naloxone Is an Inverse Agonist at the T6.34(279)K Mutant. Inverse agonists have been shown to inhibit the agonist-independent activity of constitutively active mutants (CAMs) of GPCRs (*44*). Naloxone at 10^{-6} M decreased the basal [^{35}S]GTPγS binding with the T6.34(279)K mutant to a level similar to that with the WT (Figure 3A), indicating that naloxone is an inverse agonist at this CAM.

(D) Constitutive Activity of the T6.34(279)K Mutant Is Related to Receptor Level. Multiple T6.34(279)K clonal cell lines were investigated to determine whether the elevated basal [^{35}S]GTPγS binding correlates with the level of receptor expression. As shown in Figure 4, among the cell clones obtained, basal [^{35}S]GTPγS binding with the T6.34-(279)K mutant increased with the receptor expression level. The WT also showed enhanced [^{35}S]GTPγS binding with increasing receptor density. However, the slope of the binding curve with the T6.34(279)K mutant was much larger than that with the wild type. The observation that multiple T6.34-(279)K clonal cell lines showed enhanced agonist-independent [^{35}S]GTPγS binding provides further evidence that the T6.34(279)K mutant is constitutively active.

T6.34(279)D Substitution Did Not Result in Agonist-Independent Activation of the Receptor and Greatly Reduced Agonist-Promoted [^{35}S]GTPγS Binding. No significant difference was observed in the basal [^{35}S]GTPγS binding to membranes of the T6.34(279)D mutant and WT receptors (Figures 3B and 4). In clonal cells expressing 0.92 and 1.6 pmol of receptor/mg of protein, DAMGO at 10^{-5} M stimulated [^{35}S]GTPγS binding to T6.34(279)D membranes by 15% and 40% above the basal level, respectively (Figure 3B). Morphine (10^{-5} M) enhanced [^{35}S]GTPγS binding only slightly in both T6.34(279)D clonal cells. In contrast, the WT receptor had much higher responses to DAMGO or morphine. DAMGO or morphine at 10^{-5} M elevated [^{35}S]-GTPγS binding of WT membranes by ~100% or 70% at a receptor level of 0.48 pmol/mg of protein (Figure 3B, WT I) and by 190% or 130% at 1.8 pmol/mg of protein, respectively (Figure 3B, WT II).

Compared with WT, Agonist Affinity Is Increased in the T6.34(279)K Mutant and Decreased in the T6.34(279)D Mutant. Competitive inhibition of [^{3}H]diprenorphine binding by DAMGO to the WT and T6.34(279)K and T6.34(279)D mutant receptors was measured (Figure 5, Table 2). The T6.34(279)K mutant showed ~10-fold higher affinity for DAMGO (K_i = 0.34 nM) than the WT (K_i = 3.0 nM),

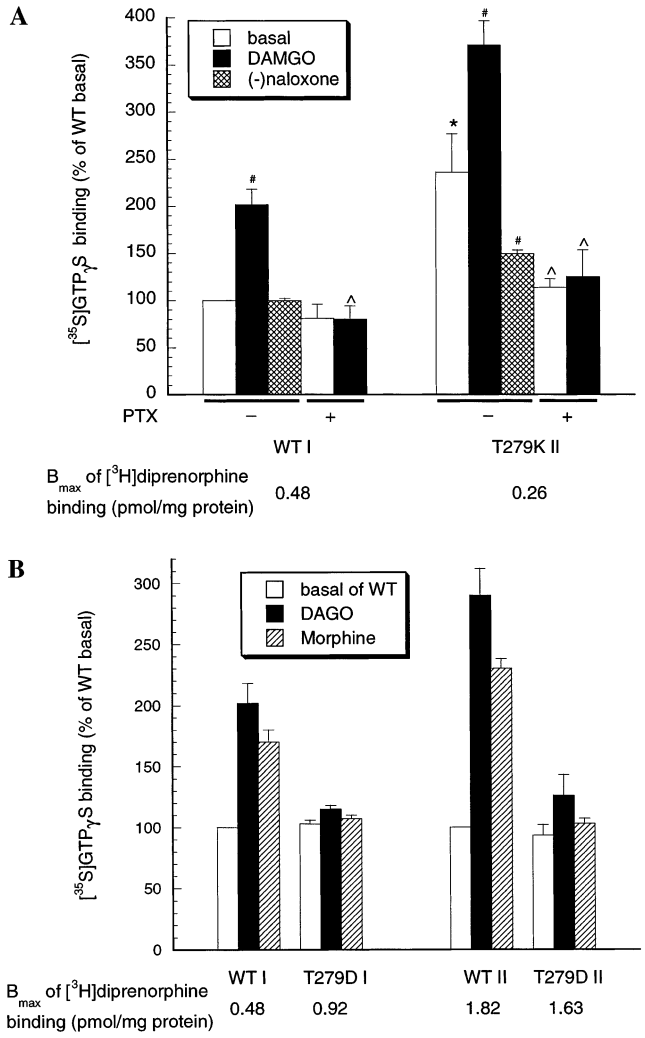

FIGURE 3: [^{35}S]GTPγS binding mediated by the wild type and the T6.34(279)K (A) and T6.34(279)D (B) mutants of the rat μ opioid receptor. (A) CHO cells stably transfected with the wild type (WT I) were treated with or without pertussis toxin (200 ng/mL) for ~24 h. CHO cells stably expressing the T6.34(279)K mutant [T6.34(279)K II] were cultured in the presence of 20 μM naloxone for at least 96 h and similarly treated with or without pertussis toxin for the last ~24 h. Membranes were prepared. (B) Membranes were prepared from CHO cells stably transfected with the WT and T6.34(279)D mutant receptors. Clonal cell lines WT I and WT II or T6.34(279)D I and T6.34(279)D II were used (see Table 1 for K_d and B_{max} values of [^{3}H]diprenorphine binding to each clonal cell line). [^{35}S]GTPγS binding was performed with 10 μg of membrane proteins in the absence (basal) or presence of 10 μM DAMGO or 10 μM naloxone as described under Materials and Methods. Nonspecific binding, determined in the presence of 10 μM GTPγS, was ~500 cpm and was subtracted from each value. Data were normalized as percent of the basal [^{35}S]GTPγS binding of WT I or WT II. Each value represents the mean ± SEM of at least three independent experiments performed in duplicate. $^*p < 0.01$, compared with the WT I basal [^{35}S]GTPγS binding; $^\#p < 0.01$, compared with its own basal [^{35}S]GTPγS binding; and $\wedge p < 0.01$, compared with the groups without pertussis toxin treatment by one-way ANOVA followed by Dunnett Multiple Comparisons Test.

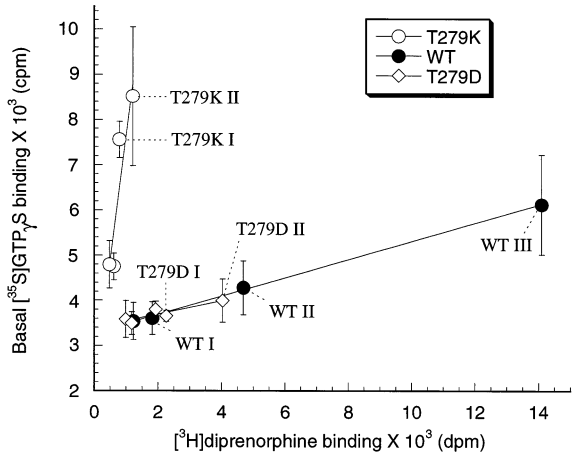

FIGURE 4: Relationship between basal [^{35}S]GTPγS binding (in the absence of agonist) and receptor density of T6.34(279)K and T6.34-(279)D mutant and WT receptors. CHO cells stably expressing different levels of the T6.34(279)K mutant were treated with 20 μM naloxone for at least 96 h, and membranes were prepared. Membranes were also prepared from CHO cells transfected with the wild type or T6.34(279)D mutant without naloxone pretreatment. Basal [^{35}S]GTPγS binding was performed with 10 μg of membrane proteins. [^{3}H]Diprenorphine (1 nM) binding to receptors in the same batches of membranes was conducted and was used to indicate receptor levels. Basal [^{35}S]GTPγS binding was plotted against [^{3}H]-diprenorphine binding. Data are expressed as the mean ± SEM of 3−5 independent experiments performed in duplicate.

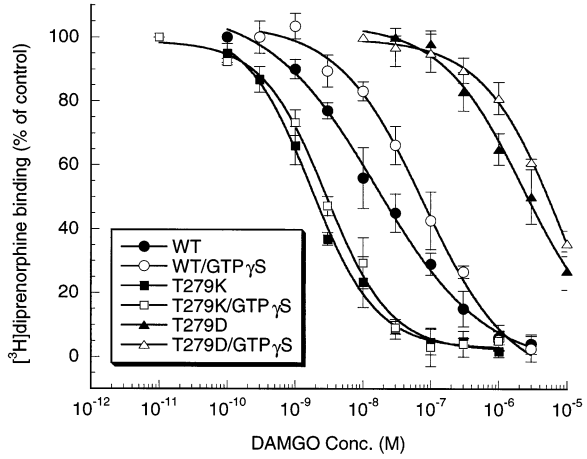

FIGURE 5: Competitive inhibition by DAMGO of [^{3}H]diprenorphine binding to the wild type (circles) and T6.34(279)K (squares) and T6.34(279)D (triangles) mutants of the rat μ opioid receptor. Competitive inhibition of [^{3}H]diprenorphine binding by DAMGO was performed with 0.8 nM [^{3}H]diprenorphine for 60 min at room temperature in the presence of various concentrations (10^{-11}−10^{-5} M) of DAMGO in the absence (close symbols) or presence (open symbols) of 20 μM GTPγS. Data are expressed as percent specific binding. Each value represents the mean ± SEM from at least 3 independent experiments in duplicate. K_i values of DAMGO and Hill slopes derived from the curves are shown in Table 2.

whereas the T6.34(279)D mutant had ~230-fold lower affinity (K_i = 684 nM) than the WT (Table 2).

The effect of GTPγS on the affinity for DAMGO was measured to determine whether the high affinity of the T6.34-(279)K mutant for DAMGO was due to receptor−G protein coupling. GTPγS (20 μM), which uncouples receptors from G proteins, did not significantly increase the K_i value of DAMGO binding to the T6.34(279)K mutant (from 0.34 to 0.56 nM), but it increased the K_i value of DAMGO binding to the WT from 3.0 to 15.6 nM (Figure 5 and Table 2). These results indicate that the enhanced affinity of the T6.34(279)K mutant for DAMGO is likely to be due to a change of receptor conformation by the mutation, but is not related to G protein coupling. Although the T6.34(279)D mutant had a lower affinity for DAMGO, GTPγS further lowered the affinity, demonstrating that the mutant is coupled to G proteins, but the receptor conformation results in a lower affinity for agonist than in the WT.

Table 2: Apparent K_i Values (nM) of DAMGO for WT, T279K, and T279D Mutant Receptors in the Presence and Absence of GTPγS[a]

	K_i(−GTPγS)	n_H(−GTPγS)	K_i(+GTPγS)	n_H(+GTPγS)
WT	3.0 ± 0.6	0.53	15.6 ± 3.6	0.69
T279K	0.34 ± 0.06	0.90	0.56 ± 0.08	0.92
T279D	684 ± 318	0.72	1792 ± 1481	0.74

[a] Apparent K_i values were calculated from the equation: $K_i = IC_{50}/(1 + [L]/K_d)$. IC_{50} values and Hill slopes were derived from the competition curves shown in Figure 5. Each value represents mean ± SEM of three independent experiments performed in duplicate.

While the WT displayed a shallow inhibition curve with a Hill slope of 0.54 in the absence of GTPγS, the T6.34-(279)K mutant had a Hill slope approaching unity both in the presence and in the absence of GTPγS (Table 2). The binding data of the WT were analyzed using two-site competition curve fit. The WT exhibited high and low affinities to DAMGO with K_i(H) of 0.73 ± 0.33 nM and K_i(L) of 26.8 ± 13.3 nM, respectively. Thus, the high-affinity binding to the T6.34(279)K mutant is likely to involve a conformation similar to the high agonist affinity state of the WT.

The Favorable Interaction between TM3 and TM6 in the Receptor Model Is Increased by the T6.34(279)D Mutation, and Decreased by the T6.34(279)K Mutation. In the models of the mutants obtained as described under Materials and Methods, the close proximity of the cytoplasmic ends of TM3 and TM6 near positions 3.50 and 6.34 brings the respective side chains into close contact. In the case of the T6.34(279)D mutant, this contact is stabilizing as a result of the favorable electrostatic interaction between the acidic group of the aspartate and the basic side chain of the conserved arginine at position 3.50 (see Figure 6). The electrostatic energy of interaction between TM3 and TM6 of WT (−4.8 kcal) and T6.34(279)D mutant (−38.29 kcal), calculated with the CHARMM program using a distance-dependent dielectric constant ($\epsilon = r$), indicates that the mutation stabilizes the close proximity between the two helices. In contrast, for the T6.34(279)K mutant, even when the orientation of the side chain of K6.34 is manually modified to minimize van der Waals clashes with its neighbors, the calculated electrostatic energy value remains positive (i.e., repulsive, +11.1 kcal). Thus, the D6.34 side chain causes a stabilization of the inactive form of the receptor in which the two helices are close at their cytoplasmic ends (*14*) [as in the inactive structure of rhodopsin (*39*)]. In contrast, the repulsion introduced by a T6.34(279)K mutation destabilizes the inactive form and would therefore favor the movement of the cytoplasmic part of TM6 away from TM3. Such a movement is associated with receptor activation (*10*−*12*, *14*).

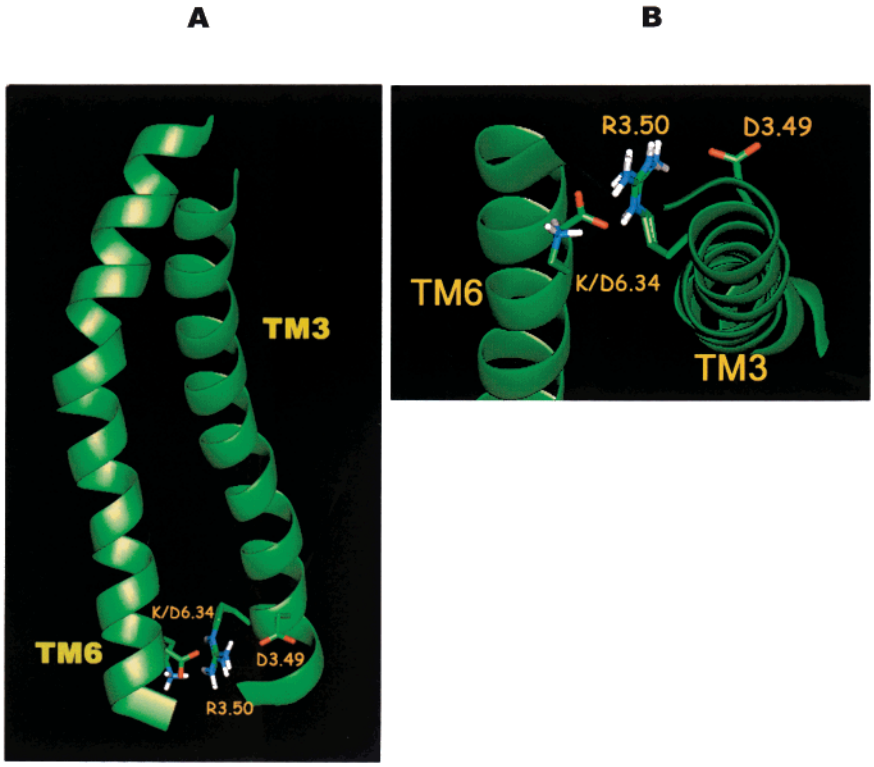

FIGURE 6: Structural organization of residues at the 6.34(279) locus in TM6 and the arginine cage involving residues R3.50(165), D3.49-(164) in TM3 of the μ opioid receptor, shown in a model of the inactive state. The model is based on the crystal structure of rhodopsin (*39*) and a number of specific criteria [see (*14*) and Materials and Methods for details]. Both an Asp and a Lys are depicted at the 6.34 locus to indicate the similarity in orientation and proximity to the conserved Arg at 3.50 achieved by the two residues with opposing mechanistic effects. Panel A shows TMs 3 and 6 in a view parallel to the membrane plane, and panel B focuses in on a detail of the region, viewed perpendicular to the membrane plane from the intracellular side.

DISCUSSION

The T6.34(279)K Mutant Is Constitutively Active, but the T6.34(279)D Mutant Is Inactive. The T6.34(279)K mutant exhibited markedly elevated agonist-independent [^{35}S]GTPγS binding, which resulted from spontaneous coupling to G_i/G_o proteins, and showed enhanced affinity for the agonist DAMGO, which was intrinsic to the mutant and equivalent to the high agonist affinity state of the WT. The increased basal level of receptor activity was inhibited by naloxone, previously shown to have inverse agonist properties at the D3.49(164) CAMs (*61*). These findings indicate that the T6.34(279)K mutant assumes conformations mimicking those of activated states of the WT μ receptor. The T6.34(279)K mutant had a higher intracellular receptor pool than the wild type, perhaps related to its constitutive activity. The increased agonist binding affinity and unaltered antagonist affinity of the T6.34(279)K mutant are in agreement with the observations reported for CAMs of many GPCRs [for reviews, see (*17*, *18*)]. In contrast, T6.34(279)D mutation did not exhibit enhanced basal [^{35}S]GTPγS binding, but had largely reduced levels of agonist-promoted [^{35}S]GTPγS binding. The T6.34-(279)D mutant also displayed much lower affinity for DAMGO than the WT. Thus, the T6.34(279)D mutant appears to adopt conformations corresponding to the inactive states of the receptor.

Our observation that mutation of T6.34(279) (X_3) to Lys in the X_3 position of the $X_1BBX_2X_3B$ motif in the i3 loop−TM6 junction region of the rat μ opioid receptor caused agonist-independent activation of the receptor is consistent with previous reports. Substitutions at this locus have been shown to induce constitutive activation of several GPCRs, e.g., the mutation of A293 in α_{1B}-AR (*16*), of T373 in α_{2A}-AR (*20*), and of L322 in β_1-AR (*21*), and of C322 in the 5-HT_{2A} (*23*), of S312 in the 5-HT_{2C} (*24*), and of T313 in the 5-HT_{1B} (*25*) receptors (see Figure 1). In the α_{1B}-adrenergic receptor, all 19 possible amino acid substitutions at the X_3 locus (Ala293) were generated, and the mutant receptors exhibited varying levels of constitutive activities with the A293K mutant having the highest activity (*16*).

However, our findings show that the effect of a mutation at this locus can be dramatically dependent on the nature of the substitution. In contrast to the T6.34(279)K mutation, the T6.34(279)D mutation in the μ opioid receptor was shown here not to lead to constitutive activation of the receptor, but to reduce greatly the responses to DAMGO and morphine. This result is different from findings on several GPCRs. Substitutions of the corresponding X_3 locus with Asp or Glu still caused agonist-independent activation of several GPCRs, including the A293D/E mutant of the α_{1B}-AR (*16*), the T373E mutant of the α_{2A}-AR (*20*), the L322E mutant of the β_1-AR (*21*), and the C322E mutant of the 5-HT_{2A} receptor (*23*). Notably, however, these receptors have acidic residues in the key 6.30 (X_1) position (see Figure 1) that plays an important role in constraining the interaction between TM6 and TM3 [for a discussion, see (*14*)]. The structural basis for this constraint is evident in the crystal structure of bovine rhodopsin from X-ray diffraction data at 2.8 Å resolution (*39*).

Proposed Constraint of R3.50(165) by T6.34(279) in the μ Opioid Receptor for Stabilization of Inactive States. Arg3.50, one of the most conserved residues, in the (D/E)-RY motif within the TM3 has been shown to play a critical role in G protein activation (*27, 45*). In the crystal structure of rhodopsin, R3.50 interacts with E6.30(247) (the X_1 locus) and T6.34(251) (the X_3 locus) within the $X_1BBX_2X_3B$ motif (Figure 1), by a salt-bridge and a hydrogen bond, respectively (*39*). These interactions are suggested to be the critical constraints keeping rhodopsin in an inactive conformation. While the D/E3.49−R3.50 salt-bridge within the (D/E)RY motif has been demonstrated to be a common constraint for many GPCRs (*27, 46−48*), the E6.30(X_1)−R3.50 salt-bridge and the T6.34(X_3)−R3.50 hydrogen bond interactions were revealed only recently through the crystal structure of rhodopsin (*39*). The E6.30(X_1)−R3.50 salt-bridge was proposed to occur in the 5-HT_{2A} receptor based on computational modeling and supported by the observation that mutations of E6.30(318) to Asn, Gln, or Leu led to constitutive activation of the receptor (*14, 42*). In addition, substitution of Glu6.30 (the X_1 site) with Ala in the m1 muscarinic or D2 dopamine[2] receptor resulted in elevated constitutive activity (*22*).

Based on inferences from our rhodopsin-based model of the μ opioid receptor, we postulate that T6.34(279) (at the X_3 locus) interacts with R3.50(165) within the DRY motif to constrain the μ receptor in inactive states. Unlike rhodopsin and receptors for the monoamine neurotransmitters, where the residue at the 6.30 locus is polar, the X_1 site of the μ opioid receptor is L6.30(275), thus increasing the importance of the R3.50−T6.34 interaction. We propose, therefore, that replacing T6.34(279)(X_3) by Lys disrupts the T6.34(279)-(X_3)−R3.50(165) interaction due to the electrostatic repulsion between the positively charged side chains, resulting in agonist-independent activity. On the other hand, substitution of T6.34(279)(X_3) with Asp reinforces the interaction, likely by an D6.34(279)(X_3)−R3.50(165) salt-bridge between the oppositely charged side chains. This strong structural constraint stabilizes the T6.34(279)D mutant receptor in an inactive state, consistent with the lower affinity for DAMGO and impaired response to DAMGO and morphine.

It is noteworthy that mutation of the X_3 residue to Asp or Glu causes constitutive activation of several GPCRs, including the α_{1B}-AR (*16*), the α_{2A}-AR (*20*), the β_1-AR (*21*), and the 5-HT_{2A} receptor (*23*). Similar to rhodopsin, these GPCRs have Glu at the X_1 locus, whereas in the μ opioid receptor there is a Leu at this position (see Figure 1). Mutation of the X_3 residue, which is on the same face of the helix as the X_1 residue, to Asp or Glu in the α_{1B}-, α_{2A}-, and β_1-adrenergic and 5-HT_{2A} receptors is likely to cause repulsion of the Glu residue at the X_1 position, which may lead to changes in the distance between the two residues and their orientations in the helical structure and weaken the interactions between Arg3.50 and Glu at the X_1 locus and mutated Glu or Asp at the X_3 site. In these structures, therefore, the X_3 to Asp or Glu mutation would disrupt the original interactions and lead to constitutive activation of the GPCRs. Consequently, the present findings provide a mechanistic explanation for the multifaceted roles of the (D/E)RY and the $X_1BBX_2X_3B$ motif and its variants in the stabilization of either the inactive or the activated forms of the GPCRs in the rhodopsin-like family.

Possible Interaction between T6.34(279) and R6.35(280). The $X_1BBX_2X_3B$ motif and its variants in the i3 loop have been shown to be important for G protein activation [for example, see (*49−52*)]. In particular, Wang (*53*) reported that R6.35(280)L mutation of the rat μ opioid receptor greatly reduced the ability of DAMGO to inhibit forskolin-stimulated cyclic AMP production, whereas combined R6.31(276)L/R6.32(277)M mutation did not have any effect, indicating that R6.35(280) is critical for activation of G proteins. Thus, we can conjecture that T6.34(279) interacts with the neighboring R6.35(280) to strengthen the network of interactions that stabilize the inactive states of the receptor. The proposed structural arrangement would be similar to the network of interactions observed in the rhodopsin crystal structure (*39*) where the interaction of D3.49 with the neighboring R3.50 helps position the latter so as to improve the interaction with E6.30 [see Figure 5D in (*39*)]. Here, the putative T6.34/R6.35 interaction would stabilize the TM3/TM6 interaction. Our T6.34(279)D mutation would sustain this receptor inactivation construct by interacting with both R6.35(280) and R3.50-(165). In contrast, the T6.34(279)K mutation would have the opposite effect. Thus, the interaction between T6.34(279) and R6.35(280) may be in addition to or alternative to the T6.34(279)−R3.50(165) interaction.

Naloxone Pretreatment Enhances Expression of the T6.34-(279)K Mutant. T6.34(279)K substitution greatly reduced receptor expression, and naloxone pretreatment increased the expression of this mutant. This observation is similar to previous findings showing that sustained treatment of cells expressing CAMs of several GPCRs with inverse agonists increased expression levels (*48, 54−59*). We have found that naloxone pretreatment enhances expression of the constitutively active D3.49(164) mutants of the μ opioid receptor by two main mechanisms: stabilization of the unstable mutant receptor structure and blockade of constitutive internalization and down-regulation of these mutants (*60*). Although pretreatment with antagonists or inverse agonists increases expression of many GPCR CAMs, an enhanced expression by such treatments does not necessarily mean that the receptor is constitutively active. For example, pretreatment of R3.50(116)A or R3.50(116)N mutant of the H_2 histamine receptor with an inverse agonist enhanced their expression, but these two mutants do not have enhanced agonist-independent activities (*58*).

Concluding Remarks. We have demonstrated that two substitutions of T6.34(279) in the i3 loop−TM6 junction region of the rat μ opioid receptor produce dramatically different changes in receptor properties. While the T6.34-(279)K mutation results in agonist-independent activation of PTX-sensitive G proteins by the receptor, the T6.34(279)D substitution does not produce such constitutive activity, but greatly reduces responses to agonists. Results from computational modeling based on structural data from the crystallographic analysis of the GPCR rhodopsin (*39*) indicate that the likely conformational changes produced by the different mutations at the T6.34 locus will affect differently the interaction between the cytoplasmic ends of TM3 and TM6 that involves the highly conserved arginine at position 3.50 and possibly arginine at position 6.35.

[2] Javitch, J. A., personal communication.

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