The Local Environment at the Cytoplasmic End of TM6 of the μ Opioid Receptor Differs from Those of Rhodopsin and Monoamine Receptors: Introduction of an Ionic Lock between the Cytoplasmic Ends of Helices 3 and 6 by a L6.30(275)E Mutation Inactivates the μ Opioid Receptor and Reduces the Constitutive Activity of Its T6.34(279)K Mutant†

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Received May 2, 2002; Revised Manuscript Received August 11, 2002

ABSTRACT: Activation of rhodopsin and monoamine G protein-coupled receptors (GPCRs) has been proposed to involve in part the disruption of a conserved E6.30−R3.50 ionic interaction between transmembrane segments (TMs) 3 and 6. However, this interaction does not occur in the opioid receptors, which have L275 at 6.30. On the basis of our findings that mutations of T6.34(279) to K and D produced, respectively, a constitutively active and an inactive form of the μ opioid receptor, we previously suggested that the functional role of the 6.30(275) residue could be assumed by T6.34(279), but the interplay between residues at positions 6.30 and 6.34 remained unresolved. In this study, we examined the effects of introducing an E in position 6.30(275) of the wild type (WT) and of the T6.34(279) mutants of the μ opioid receptor to compare the participation of the 6.30 locus in molecular events during activation in this receptor with its role in other GPCRs. The L6.30(275)E and the L6.30(275)E/T6.34(279)D mutants displayed no constitutive activity and could not be activated by the agonist DAMGO or morphine. The L6.30(275)E/T6.34(279)K mutant had some constitutive activity, but much less than the T6.34(279)K mutant, and could be activated by both agonists. The rank order of affinity for the agonist DAMGO is as follows: T6.34(279)K > WT ≅ L6.30(275)E/T6.34(279)K > L6.30(275)E ≅ T6.34(279)D > L6.30(275)E/T6.34(279)D; however, all constructs have a similar affinity for the antagonist [^{3}H]diprenorphine. These data are interpreted in the context of interactions with the conserved R3.50(165) in TM3. When L6.30(275) is mutated to E, the favorable E6.30(275)−R3.50(165) interaction stabilizes an inactive state, as in rhodopsin, and hence reduces the activities of T6.34(279) mutants. Thus, the μ opioid receptor is shown to be different from rhodopsin and monoamine GPCRs, of which the WTs with native E6.30 can be activated, and the 6.34D or 6.34K mutants display enhanced constitutive activities. Our molecular modeling results suggest that some specific differences in local geometry at the cytoplasmic ends of TM5 and TM6 may account in part for the observed differences in the molecular mechanisms of receptor activation.

Multiple types of opioid receptors (at least μ, δ, and κ) have been demonstrated, and each type mediates distinct physiological functions and pharmacological effects (*1*). The μ opioid receptors are most closely associated with analgesic and euphoric actions elicited by opiates and opioids (*1*). Opioid receptors are coupled to pertussis toxin-sensitive G_i/G_o proteins, and their effectors include adenylate cyclase, potassium channels, calcium channels, and mitogen-activated protein kinase pathways (for a review, see ref *2*). μ, δ, and κ opioid receptors have been cloned (for reviews, see refs *3* and *4* and references therein], and they belong to the rhodopsin subfamily of G protein-coupled receptors (GPCRs)[1] (see refs *5* and *6* for a classification scheme), characterized by the presence of highly conserved "fingerprint" residues (*6*), 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 TMs5−7 (see Experimental Procedures for a definition of the generic residue numbering system).

According to the various models of GPCR function (*7*−*12*), receptors undergo conformational changes in an equilibrium between inactive states that are structurally constrained and unable to couple to the GDP-liganded Gαβγ

† This work was supported by National Institutes of Health Grants DA04745 and DA11263 (to L.-Y.L.-C.) and Grants DA12923 and DA00060 (to H.W.).

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[1] Abbreviations: AR, adrenergic receptor; CHO, Chinese hamster ovary; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol]Enkephalin [Try-D-Ala-Gly-(Me)Phe-Gly-ol]; EDTA, ethylenediaminetetraacetic acid; GPCRs, G protein-coupled receptors; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SEM, standard error of the mean; 5-HT, 5-hydroxytryptamine; TM, transmembrane domain; WT, wild type.

10.1021/bi026067b CCC: $22.00
Published on Web 09/11/2002

heterotrimer, and active states that can interact productively with the heterotrimer to catalyze 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 fully elucidated experimentally. Specific movements of TMs2, -3, and -5−7 have been shown to be important for receptor activation (*10*, *13*−*16*). It has been shown that disruption of an ionic interaction between a negatively charged residue in TM3 and a positively charged residue in TM7 in rhodopsin (*17*) and α_{1b}-adrenergic (*18*, *19*) and δ opioid (*20*) receptors leads to activation of the receptors. Interactions between TM5 and TM6 were suggested to be important for the activation of tachykinin NK-1 receptors (*16*). In addition, hydrogen bonding interactions between TM2 and TM7 were suggested to be important for the activation of 5-hydroxytryptamine$_{2A}$ (5-HT$_{2A}$), gonadotropin-releasing hormone, and μ opioid receptors (*21*−*23*), and this mode of interaction was proposed to be generally important for GPCR activation (*22*, *24*). Moreover, rotations of TM3 and TM6 were proposed to be involved in the activation of rhodopsin and β_2-adrenergic (*25*) and muscarinic m5 receptors (*15*, *26*, *27*).

The high-resolution crystal structure of the inactive form of rhodopsin (*28*) provided information about possible constraints that keep rhodopsin in inactive states. A set of residues was suggested to mediate interactions among the transmembrane helices and among the cytoplasmic surfaces to maintain receptor structure. Most of these interhelical hydrogen bonds, hydrophobic interactions, and salt bridges are formed by highly conserved residues in GPCRs. Arg3.50 in the (D/E)RY motif within TM3, one of the most conserved residues, has been shown to play a critical role in G protein activation (*24*, *29*). The interactions between R3.50(135) and the preceding E3.49(134) as well as with E6.30(247) and T6.34(251) in TM6 are evident in the crystal structure of rhodopsin. These strong ionic interactions were thought to stabilize the inactive form of the receptor, and disruption of the interactions between these residues in TM3 and TM6 was envisioned to be involved in receptor activation (*30*, *31*). As one of the residues that exhibit a high degree of conservation in rhodopsin-like GPCRs, E6.30 is at the X_1 locus within the $X_1BBX_2X_3B$ motif (B being a basic amino acid and X a nonbasic amino acid) in the third intracellular (i3) loop−TM6 junction region (Figure 1). Consonant with structural data for rhodopsin, the E6.30(X_1)−R3.50 salt bridge has been proposed to occur in the 5-hydroxytryptamine$_{2A}$ (5-HT$_{2A}$), m1 muscarinic, and β_2-adrenergic receptors based on computational modeling and supported by mutagenesis studies (*10*, *30*−*33*). The E6.30(X_1)−R3.50 interaction is considered to have a critical role in constraining these receptors in their inactive states, and detailed molecular mechanisms by which this constraint is removed in the process of receptor activation have been proposed recently (*30*, *31*, *34*).

We demonstrated recently that mutation of D3.49(164) of the DRY motif to H, Q, Y, or M, but not to E, led to agonist-independent activation of the μ opioid receptor (*35*). In addition, we have shown that substitutions of T6.34(279) (at the X_3 locus) with Lys and Asp result in constitutively active and inactive forms of the μ opioid receptor, respectively (*36*). On the basis of inferences from our rhodopsin-based model of the μ opioid receptor, we concluded that D3.49(164) and T6.34(279) interact with R3.50(165) within the DRY motif to constrain the μ receptor in inactive states (*35*, *36*).

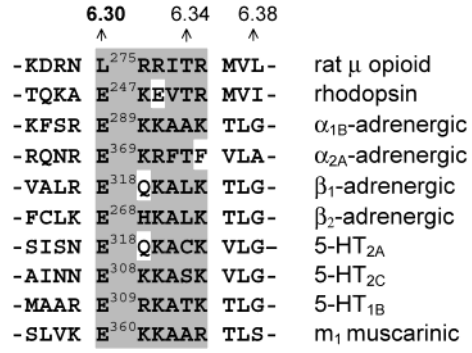

FIGURE 1: Amino acid sequence at the cytoplasmic end of TM6 of the rat μ opioid receptor compared to those of several other GPCRs. The "$X_1BBX_2X_3B$" motifs and variants are highlighted (B being a basic amino acid and X a nonbasic amino acid). The numbering indicates the amino acid numbers in the sequences of the receptors.

While rhodopsin and receptors for the monoamine neurotransmitters have a polar Glu at the 6.30 locus, the opioid receptors have a nonpolar residue, Leu6.30(275), at this locus. Thus, 6.30(X_1)−R3.50 interactions are not likely to have the same role in the opioid receptors as in the receptors with a conserved Glu at this locus. This inference is supported by the finding that while mutation of E6.30 to neutral residues led to constitutive activation of the 5-HT$_{2A}$, m1 muscarinic, and β_2-adrenergic receptors (*10*, *30*−*33*), the wild-type (WT) μ opioid receptor lacking E6.30 does not have high constitutive activity (*35*, *36*). To understand the involvement of the 6.30 and 6.34 loci in the molecular mechanisms of activation of the μ opioid receptor, and contrast them with those in rhodopsin and monoamine GPCRs, we examined the effects of the incorporation of the ubiquitous E6.30 into the μ opioid receptor by the L6.30(275)E mutation and the effect it has on the T6.34(279) mutants of this receptor. We found that the L6.30(275)E mutant could not be activated by DAMGO, a selective μ agonist, and that the L6.30(275)E mutation reduced the constitutive activity of the T6.34(279)K mutant and further inactivated the T6.34(279)D mutant. Examined in the context of a structural model, these results shed new light on the specific interactions that constrain the μ opioid receptor in an inactive state, on the role of structural differences between this receptor and other GPCRs in the rhodopsin-like family, and on the specific nature of the changes that make possible the conformational rearrangements that produce the activated form of the receptor.

EXPERIMENTAL PROCEDURES

Materials. [^{35}S]GTPγS (~1250 Ci/mmol) and [^{3}H]diprenorphine (58 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA). GDP and GTPγS were obtained from Sigma Co. (St. Louis, MO). Naloxone hydrochloride was a gift from DuPont/Merck Co. (Wilmington, DE). [D-Ala^2,*N*-Me-Phe^4,Gly-ol]Enkephalin (DAMGO) was 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), Beohringer-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 scheme used throughout identifies 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 (*37*) and recently applied to opioid receptors (*23*, *35*, *36*, *38*). This combined scheme is used to relate the results obtained for opioid receptors to corresponding positions in other GPCRs (*10*). According to the generic numbering scheme, amino acid residues in TMs are assigned two numbers (N1.N2). N1 refers to the TM number. For N2, the numbering is relative to the most conserved residue in each TM, which is assigned the number 50, and 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 named P6.50(295), and Leu275 is named L6.30(275).

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. (*39*). HA-tagged WT and mutant rat μ receptors were subcloned into *Hin*dIII and *Xba*I sites of the mammalian expression vector pcDNA3 (*38*). The cDNA sequences were determined with the method of Sanger et al. (*40*) to confirm the presence of desired mutations and the absence of unwanted mutations.

Stable Expression of the WT and Mutant Rat μ Opioid Receptors in CHO Cells. Transfection of CHO cells with the cDNA clones of the WT or a mutant of the rat μ opioid receptor in pcDNA3 was performed with Lipofectamine according to the manufacturer's instructions, and cells were grown under the selection pressure of Geneticin (1 mg/mL). CHO cell clones stably expressing the WT or a mutant of the rat μ opioid receptor were established as described previously (*41*). 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 achieve a higher expression density. Cells were harvested for experiments by using a Versene solution (0.54 mM EDTA, 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, and 1 mM glucose) in the presence of 20 μM naloxone.

Membrane Preparations. Membranes were prepared according to the method of Huang et al. (*42*). Protein concentrations were determined by the bicinchoninic acid method of Smith et al. (*43*) 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. Saturation binding of [^{3}H]-diprenorphine to the WT 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 WT 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. In some experiments, 20 μM GTPγS was included in the binding buffer. Binding data were analyzed with the EBDA program (*44*).

[^{35}S]GTPγS Binding Assay. Determination of the level of [^{35}S]GTPγS binding to G proteins was carried out as described previously (*42*) with 15 μM GDP and 0.2 nM [^{35}S]-GTPγS in reaction buffer (50 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 0.1% BSA) in a final volume of 0.5 mL. The level of nonspecific binding was determined in the presence of 10 μM GTPγS. After incubation for 60 min at 30 °C, bound and free [^{35}S]GTPγS were separated by filtration with GF/B filters under reduced pressure. The amount of radioactivity was determined by liquid scintillation counting with a counting efficiency of ~95%.

Molecular Modeling of the μ Opioid Receptor Constructs. The construction of molecular models for the WT and mutant receptors has been described in detail in a recent publication (*36*). Briefly, the model was constructed with the use of the homology modeling approach incorporated in the program MODELLER (*45*), with the 2.8 Å resolution crystal structure of rhodopsin (*28*) as a structural template. The sequence alignment 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 (*10*, *37*). 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" (*46*, *47*) and the "arginine cage" (*24*, *32*). As described in detail elsewhere (*10*), 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 microdomains. As described recently (*36*), the mutant constructs were modeled by introducing the appropriate residues in the WT receptor models. Unlike the previous procedure, however, energy minimization was used here to eliminate offending steric contacts and to optimize hydrogen bonding interactions.

RESULTS

K_d and B_{max} Values of the Antagonist Diprenorphine for the WT and Mutants of the μ Opioid Receptor Stably Expressed in CHO Cells. The WT and mutant receptors were transfected into CHO cells and screened by [^{3}H]-diprenorphine binding to intact cells. Multiple clonal cell lines expressing each receptor were established. Saturation binding of [^{3}H]diprenorphine to membrane preparations was performed for one clone for each receptor, and K_d and B_{max} values were determined (Table 1). The affinities of L6.30(275)E, T6.34(279)K, L6.30(275)E/T6.34(279)K, T6.34(279)D, and L6.30(275)E/T6.34(279)D mutant receptors for the antagonist [^{3}H]diprenorphine were similar to those of the WT, suggesting that the mutant receptors retain similar overall structures relative to the WT.

Table 1: K_d and B_{max} Values of [^{3}H]Diprenorphine Binding and Apparent K_i Values of DAMGO Binding to the WT and the L6.30(275)E, T6.34(279)K, L6.30(275)E/T6.34(279)K, T6.34(279)D, and L6.30(275)E/T6.34(279)D Mutants of the Rat μ Opioid Receptor Stably Transfected in CHO Cells[a]

	[^{3}H]diprenorphine		DAMGO		
			K_i (nM) (n_H)		K_i ratio
	K_d (nM)	B_{max} (pmol/mg of protein)	no GTPγS	with GTPγS	with GTPγS/no GTPγS
WT	0.17 ± 0.05	0.46 ± 0.11	7.14 ± 1.28 (0.64)	21.0 ± 2.3 (0.74)[d]	2.94
L6.30(275)E	0.18 ± 0.03	0.49 ± 0.07	667 ± 171 (0.81)	1688 ± 1242 (0.78)[d]	2.53
T6.34(279)K[b]	0.22 ± 0.04	0.26 ± 0.06[c]	0.34 ± 0.06 (0.90)	0.56 ± 0.08 (0.92)[d]	1.65
L6.30(275)E/T6.34(279)K	0.21 ± 0.07	0.45 ± 0.04	6.98 ± 0.46 (0.70)	23.8 ± 1.5 (0.79)[d]	3.41
T6.34(279)D[b]	0.27 ± 0.02	1.63 ± 0.12	684 ± 318 (0.72)	1792 ± 1481 (0.74)[d]	2.62
L6.30(275)E/T6.34(279)D	0.20 ± 0.02	2.04 ± 0.18	3189 ± 989 (1.04)	3007 ± 1645 (0.97)[d]	0.94

[a] Membranes were prepared from each of the CHO clonal cell lines. Saturation binding of [^{3}H]diprenorphine was performed, and K_d and B_{max} values were determined. Competition inhibition of [^{3}H]diprenorphine binding was carried out in the absence (see Figure 3) or presence of 20 μM GTPγS; IC_{50} values and Hill slopes were determined, and apparent K_i values were calculated. Each value represents the mean ± SEM of at least three independent experiments performed in duplicate. [b] Data from ref *36*. [c] CHO clonal cells stably expressing the T6.34(279)K mutant were pretreated with 20 μM naloxone for at least 96 h. [d] Data were derived from the competition binding experiments in the presence of 20 μM GTPγS.

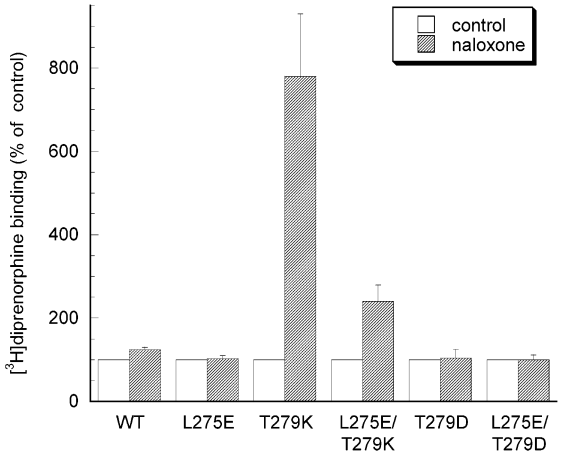

FIGURE 2: Effect of naloxone pretreatment on expression of [^{3}H]-diprenorphine binding of the WT and mutants of the rat μ opioid receptor. CHO cells stably transfected with the WT or a mutant receptor were cultured in the absence or presence of 20 μM naloxone for at least 96 h. Cells were washed and harvested, and membranes were prepared. [^{3}H]Diprenorphine (1 nM) binding to cell membranes was performed. Data are expressed as a percentage of binding without naloxone pretreatment for each receptor. Each value represents the mean ± SEM of data from four to six clonal cell lines for each receptor.

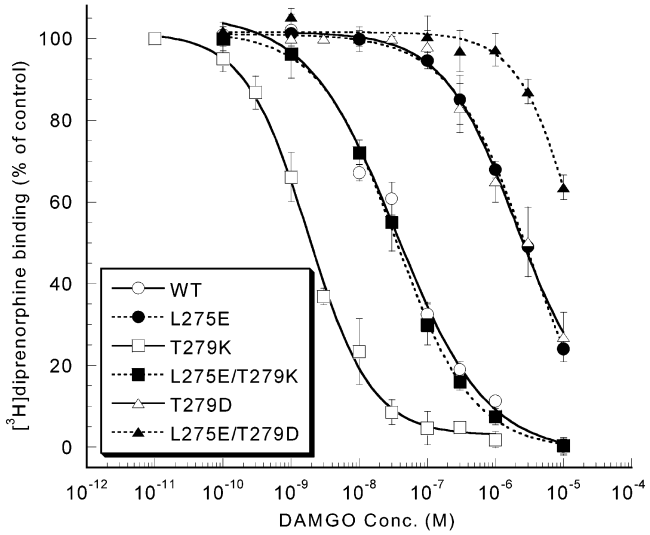

FIGURE 3: Competitive inhibition by DAMGO of [^{3}H]diprenorphine binding to the WT and mutants of the rat μ opioid receptor. Competitive inhibition of [^{3}H]diprenorphine binding by DAMGO was performed with 0.8 nM [^{3}H]diprenorphine for 60 min at room temperature in the presence of various concentrations (10^{-11}−10^{-5} M) of DAMGO. Data are expressed as a percentage of specific binding. Each value represents the mean ± SEM from at least three independent experiments in duplicate. K_i values of DAMGO derived from the curves are shown in Table 1.

Effect of Naloxone Pretreatment on Expression of the Mutant Receptors. We previously demonstrated that preincubation of cells with naloxone greatly increased the expression level of the constitutively active T6.34(279)K and D3.49(164) mutants and had a modest effect on the WT receptor, but had no effect on the inactive T6.34(279)D mutant (*35*, *36*). Pretreatment of cells with 20 μM naloxone for at least 96 h did not upregulate the L6.30(275)E or the L6.30(275)E/T6.34(279)D mutant as determined by assessing [^{3}H]diprenorphine (1 nM) binding to membranes (Figure 2). In contrast, the same treatment increased the level of expression of the L6.30(275)E/T6.34(279)K mutant, but the magnitude of the increase was much smaller than that of the T6.34(279)K mutant (Figure 2). The magnitudes of naloxone upregulation (percentage of the control without naloxone pretreatment for each receptor) were in the following order (high to low): T6.34(279)K (780%) > L6.30(275)E/T6.34(279)K (245%) > WT (124%) > L6.30(275)E, T6.34(279)D, and L6.30(275)E/T6.34(279)D (no upregulation). The size of the increase in the level of WT expression determined by binding using 1 nM [^{3}H]-diprenorphine was similar to what we observed previously by determining B_{max} from saturation binding (*35*). Thus, the L6.30(275)E mutation greatly attenuated the upregulation effect of naloxone on the T6.34(279)K mutant and abolished its modest effect on the WT μ opioid receptor.

The L6.30(275)E Mutation in the WT, T6.34(279)K, and T6.34(279)D Receptors Greatly Decreases Their Affinities for the Agonist DAMGO. The affinities of DAMGO for the WT and mutants were determined (Table 1 and Figure 3). The following rank order of affinity of these mutants for DAMGO was found: T6.34(279)K (K_i = 0.34 nM) > L6.30(275)E/T6.34(279)K (K_i = 6.98 nM) ≅ WT (K_i = 7.14 nM) > L6.30(275)E (K_i = 667 nM) ≅ T6.34(279)D (K_i = 684 nM) > L6.30(279)E/T6.34(275)D (K_i = 3.2 μM). Thus, the L6.30(275)E mutant displayed ~95-fold lower affinity for DAMGO than the WT, but had an affinity similar to that of the T6.34(279)D mutant (Table 1 and Figure 3). In addition, the double mutant L6.30(275)E/T6.34(279)K had a greatly reduced affinity for DAMGO compared to that of

the T6.34(279)K mutant; however, its affinity was similar to that of the WT. The L6.30(275)E/T6.34(279)D double mutant had an even lower affinity for DAMGO than the T6.34(279)D mutant.

Since the $X_1BBX_2X_3B$ motif has been implicated in receptor coupling to G proteins, the effects of GTPγS (20 μM) on the affinities of the WT and mutant receptors for DAMGO were examined to determine whether the mutants are coupled to G proteins. GTPγS lowered the affinities of both the L6.30(275)E and L6.30(275)E/T6.34(279)K mutants for DAMGO by ~3-fold (Table 1), similar to that of the WT. We previously showed that GTPγS lowered the affinity of the T6.34(279)D mutant for DAMGO ~3-fold (*36*). Taken together, these results indicate that the mutants, like the WT, exist in part coupled to G proteins, suggesting that L6.30(275)E, T6.34(279)D, and L6.30(275)E/T6.34(279)K mutants are able to couple to G proteins and that mutations at the 6.30(275) and 6.34(279) loci of μ opioid receptors do not preclude G protein coupling. The L6.30(275)E/T6.34(279)D mutant has a very low affinity for DAMGO, and GTPγS appeared not to have any effect (Table 1), indicating that the reduced affinity of the mutant for DAMGO may be due to a change in receptor conformation by the mutation, but unrelated to G protein coupling, or that the mutant receptor is uncoupled from G proteins.

The L6.30(275)E Mutation Decreases the Activity of the Receptor both in the WT and in the Constitutively Active Mutant T6.34(279)K. Membranes of CHO cell clones stably expressing the WT and mutant receptors were examined for basal and DAMGO-promoted [^{35}S]GTPγS binding.

(A) The L6.30(275)E Mutant Could Not Be Activated by Agonists. No significant difference was observed in the basal [^{35}S]GTPγS binding to membranes of the WT and L6.30(275)E mutant (Figures 4 and 5). However, when the DAMGO dose−response relationship (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 3×10^{-5}, and 10^{-4} M) was examined, the L6.30(275)E mutant could not be activated even at 10^{-4} M. Similarly, morphine at 10^{-5} M failed to stimulate [^{35}S]GTPγS binding to L6.30(275)E membranes. In contrast, 10^{-5} M DAMGO or morphine elevated the level of [^{35}S]GTPγS binding to WT membranes to 118 or 78% above the basal level, respectively (Figure 4).

(B) The L6.30(275)E Mutation Reduced the Constitutive Activity of the T6.34(279)K Mutant μ Opioid Receptor but Had Little Effect on Its Agonist-Induced [^{35}S]GTPγS Binding. The double mutant L6.30(275)E/T6.34(279)K exhibited an elevated basal level of [^{35}S]GTPγS binding, which was 124% of that of the WT at a comparable expression level. In contrast, the T6.34(279)K mutant had a much higher basal level of [^{35}S]GTPγS binding, 210% of that of the WT and similar to the DAMGO (10^{-5} M)- or morphine (10^{-5} M)-stimulated WT level (Figure 4), which is consistent with our previous findings (*36*). DAMGO further stimulated [^{35}S]-GTPγS binding to similar extents (percentage of the WT basal) in both the L6.30(275)E/T6.34(279)K (318%) and T6.34(279)K mutants (382%). However, morphine stimulated [^{35}S]GTPγS binding to a much smaller extent in the L6.30(275)E/T6.34(279)K mutant (208% of the WT basal level) than in the T6.34(279)K mutant (380% of the WT basal level) (Figure 4).

The relation between basal levels of [^{35}S]GTPγS binding and the level of receptor expression was determined for several clones of varying expression levels for each receptor. A linear relationship was observed for both L6.30(275)E/T6.34(279)K and T6.34(279)K mutant receptors (Figure 5). However, the line for the T6.34(279)K mutant had a much larger slope than that for the L6.30(275)E/T6.34(279)K mutant (2.69 vs 0.51), indicating that the L6.30(275)E/T6.34(279)K mutant had lower constitutive activity per receptor than the T6.34(279)K mutant.

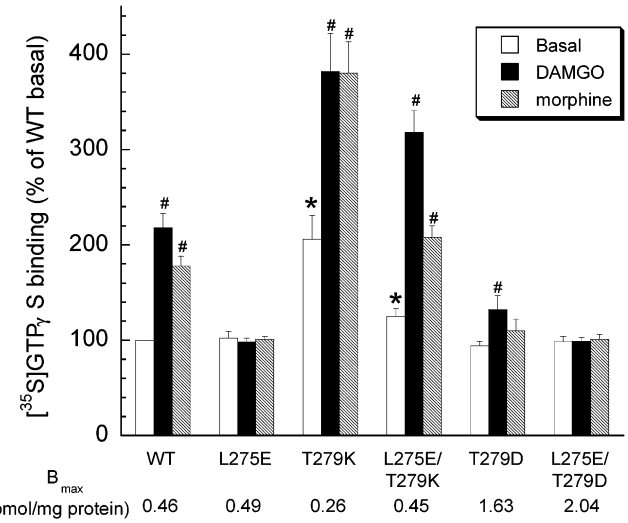

FIGURE 4: [^{35}S]GTPγS binding mediated by the WT and the L6.30(275)E, T6.34(279)K, L6.30(275)E/T6.34(279)K, T6.34(279)D, and L6.30(275)E/T6.34(279)D mutants of the rat μ opioid receptor. Membranes were prepared from CHO cells stably transfected with the WT or a mutant receptor (see Table 1 for K_d 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 morphine. The level of nonspecific binding, determined in the presence of 10 μM GTPγS, was ~500 cpm and was subtracted from each value. Data were normalized as a percentage of the basal level of [^{35}S]GTPγS binding of the WT (~3500 cpm). Each value represents the mean ± SEM of at least three independent experiments performed in duplicate. Asterisks denote p values of <0.01, compared with the WT basal level of [^{35}S]GTPγS binding. Pound signs (#) denote p values of <0.01, compared with its own basal level of [^{35}S]GTPγS binding by one-way ANOVA followed by a Dunnett multiple-comparisons test.

(C) The L6.30(275)E/T6.34(279)D Mutant Displayed No Constitutive Activity and Could Not Be Activated by Agonists. We showed previously that the T6.34(279)D mutant receptor did not have enhanced constitutive activity and DAMGO stimulated [^{35}S]GTPγS binding only slightly (*36*). The L6.30(275)E/T6.34(279)D double mutant had a basal level of [^{35}S]GTPγS binding similar to those of the T6.34(279)D and L6.30(275)E mutants and the WT receptor (Figure 5). The DAMGO dose−response relationship for the T279D and the L275E/T279D mutants (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 3×10^{-5}, and 10^{-4} M) showed that the L275E/T279D mutant could not be activated even at 10^{-4} M, whereas the T279D mutant could be activated by DAMGO at only 10^{-5}, 3×10^{-5}, and 10^{-4} M with increases in the level of [^{35}S]GTPγS binding of 32, 44, and 67% above the basal level, respectively. Morphine at 10^{-5} M could not activate either the L6.30(275)E/T6.34(279)D or T6.34(279)D mutant. Figure 4 shows data obtained at 10^{-5} M morphine and DAMGO, with both mutants at higher expression levels than the WT. Thus, compared with the T6.34(279)D mutant, the

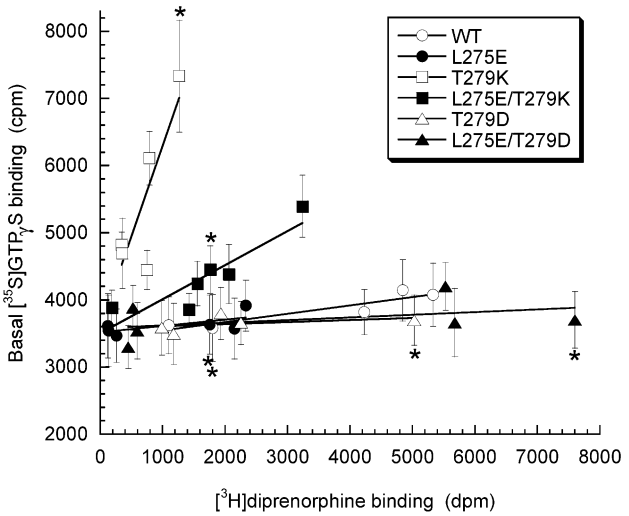

FIGURE 5: Relationship between the basal level of [^{35}S]GTPγS binding and the receptor level of the WT and mutant receptors. Membranes were prepared from CHO cells stably expressing different levels of the WT and mutant receptors. T6.34(279)K clonal cells were treated with 20 μM naloxone for at least 96 h prior to membrane preparation. 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 (10 μg) was conducted and was used to indicate receptor levels. The basal level of [^{35}S]GTPγS binding was plotted against the level of [^{3}H]-diprenorphine binding. Clonal cells shown in Table 1 and Figure 4 are denoted with asterisks. Data are expressed as means ± sem of three or four independent experiments performed in duplicate.

L6.30(275)E/T6.34(279)D mutant completely lost the ability to respond to DAMGO.

DISCUSSION

The 6.30 locus at the X_1 site within the $X_1BBX_2X_3B$ motif at the junction region of the i3 loop and TM6 is Glu(E) in rhodopsin and monoamine receptors (Figure 1). An ionic interaction between E6.30 and the conserved R3.50 in TM3 has been identified in many of these receptors, and this interaction is important for stabilization of the inactive states of the receptors (*10*, *30*−*33*). We have shown previously (*36*) that this functional role of residues at the 6.30(275) locus can be replaced in the μ opioid receptor by the T6.34(279) residue in the X_3 position of the motif. However, the question regarding the possible participation of the 6.30 locus in mechanisms that modulate the activation of opioid receptors remained open because in opioid receptors the residue in this locus is L6.30(275). In this study, we have found that the L6.30(275)E mutation inactivates the μ opioid receptor, attenuates the constitutive activity of the T6.34(279)K mutant, and further inactivates the T6.34(279)D mutant. It is evident from these findings that the proximity of the 6.30(275) locus to the DRY motif in TM3 endows the residues at this locus with direct effects on the functional properties of the opioid receptors. In addition, the results show that there must be local structural differences between rhodopsin and monoamine receptors and opioid receptors affecting the constraints that keep the receptors in inactive states.

L6.30(275)E Mutant. The L6.30(275)E mutant could not be activated by agonists and displayed a lower affinity for the agonist DAMGO, indicating that in this mutant the inactive state is strongly stabilized. According to our rhodopsin-based molecular model of the μ opioid receptor, the loss of function of the L6.30(275)E mutant is due to the additional interaction of E6.30(275) with R3.50(165) introduced by the L6.30(275)E mutation (Figure 6B), compared with the WT receptor (Figure 6A). This interaction, combined with all the original constraints that include T6.34(279)−R3.50(165) bonding, would keep the μ opioid receptor in an overly restrained inactive conformation that cannot be transformed into active conformations upon agonist binding (Figure 6B). Calculations of the electrostatic interaction between TM3 and TM6 in our model of the μ opioid receptor (see Experimental Procedures) showed that the interaction energy in the L6.30E mutant (−92 kcal/mol) calculated with the CHARMM program (*48*) and using a distance-dependent dielectric constant ($\epsilon = r$) is much more favorable than that in the WT receptor (−14 kcal/mol). The additional stabilization of the inactive form in the L6.30E mutant is consistent with the experimental findings.

L6.30(275)E/T6.34(279)K Mutant. We have previously shown that the T6.34(279)K mutation in the μ opioid receptor results in markedly high constitutive activity and enhanced agonist affinity (*36*). In contrast, compared with the WT, the L6.30(275)E/T6.34(279)K mutant exhibited an only slightly increased agonist-independent activity and a similar affinity for DAMGO. Moreover, this mutant could be stimulated more effectively by the full agonist DAMGO than the partial agonist morphine. Thus, the L6.30(275)E/T6.34(279)K mutant exhibited properties that are intermediate between those of the L6.30(275)E mutant and the T6.34(279)K mutant.

Calculations of the electrostatic interaction between TM3 and TM6 showed that in the T6.34K mutant an unstable state was created by the repulsion (11.1 kcal/mol) between the positively charged R3.50 and the similarly charged K6.34 (Figure 6Ca), and this unstable state is avoided when the cytoplasmic end of TM6 distances itself from TM3. Such a rearrangement has been shown to be associated with activation of GPCRs (*10*, *14*, *49*, *50*) and to involve the conserved proline P6.50 (*51*). Calculation for such an activated state of the μ opioid receptor (*10*, *34*) indicates that the repulsion is essentially eliminated by the change in the TM3−TM6 distance (Figure 6Ca,b) from ∼5.6 to ∼14.7 Å. This change corresponds to the increase in distance inferred from a variety of experiments (*10*, *14*, *49*, *50*). Thus, the avoidance of repulsion in the inactive form of the T6.34K mutant receptor is likely to be the major driving force for the transition of the structure to the activated-like form expressed in the constitutive activation.

Similar calculations in the model of the inactive receptor form of the double mutant T6.43K/L6.30E show that the TM3−TM6 interaction becomes stabilized by the E6.30−R3.50 ionic interaction (Figure 6D) to a value (−17 kcal/mol) that is comparable to that of the WT (−14 kcal/mol) (Figure 6A). This result agrees well with our finding that the added L6.30E mutation diminishes significantly the constitutive activity compared to the single T6.34K mutant. These calculations show how the simultaneous L6.30E mutation, which introduces a negatively charged residue into the structural environment of the TM3−TM6 interaction (Figure 6D), adds a restraining force that diminishes the likelihood of TM6 moving away from TM3 to conform with the activated state the receptor.

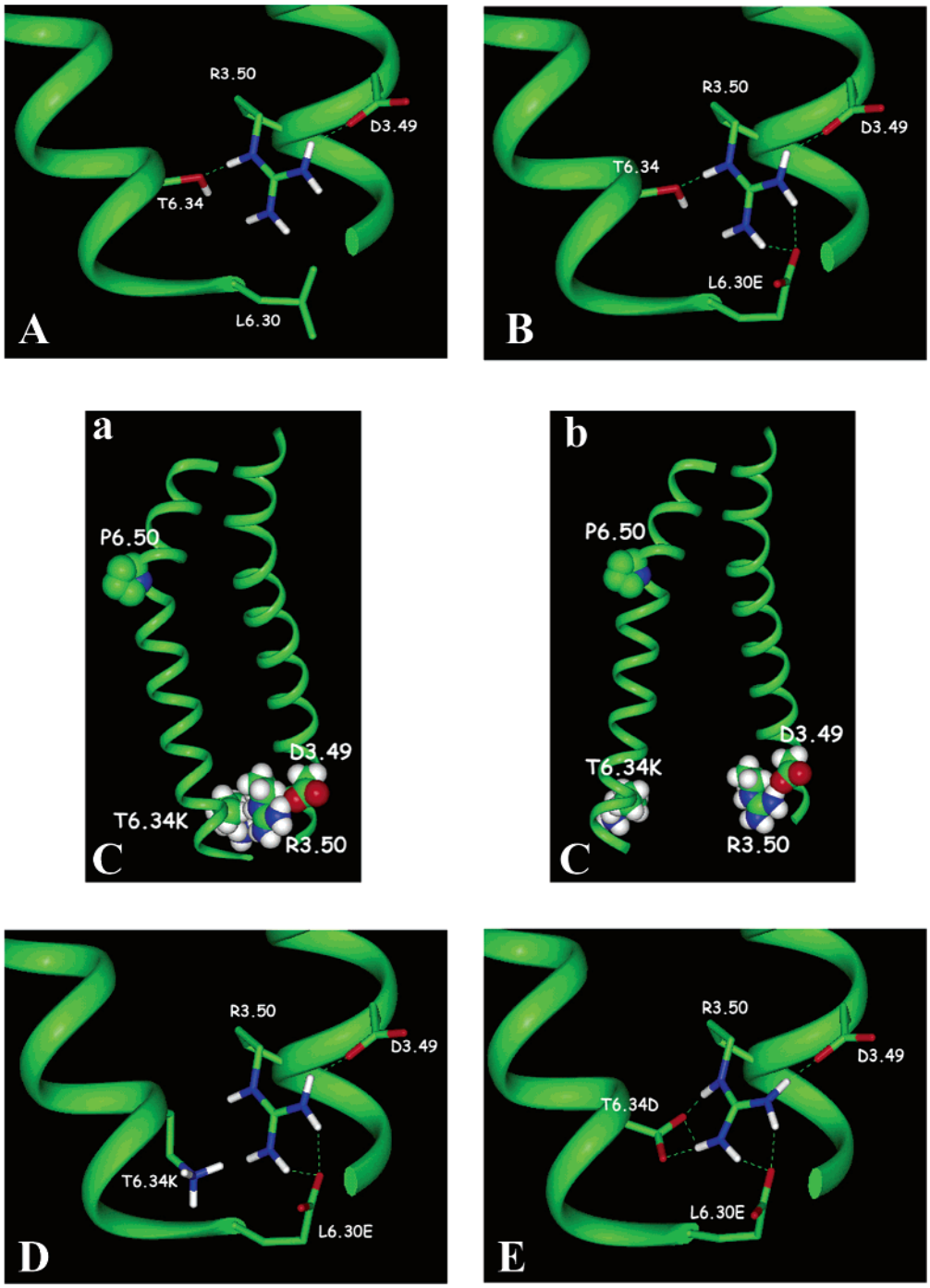

FIGURE 6: Interactions between R3.50(165) and residues at 6.30(275) and 6.34(279) in the WT and mutant μ opioid receptors. (A) In the WT receptor, the T6.34(279)/R3.50(165) interaction, shown here in a model of the μ opioid receptor based on the rhodopsin crystal structure (*28*), enhances the hydrogen bond network around R3.50(165), stabilizing the inactive state of the receptor. (B) In the L6.30(275)E mutant, the stabilizing interaction introduced by the L6.30(275)E mutation reinforces the interaction between TM3 and TM6 supported by the T6.34(279)−R3.50(165) hydrogen bond, to restrain the μ opioid receptor in the inactive conformation. (C) Space filling model of the T6.34(279)K mutant. (a) Steric crowding is created in the region of interaction between TM3 and TM6 in the inactive form of the receptor, by substitution of T6.34(279) for K. The unfavorable interaction is enhanced by the electrostatic repulsion between the positively charged lysine in the T6.34(279)K mutant and R3.50(165). (b) In the model of the activated form of the receptor, the rearrangement around P6.50(295) associated with activation of GPCRs (*10*, *51*) relieves the crowding and practically eliminates the repulsion between T6.34(279)K and R3.50(165) by increasing the distance between the cytoplasmic ends of TM6 and TM3. (D) In the L6.30(275)E/T6.34(279)K mutant, the ionic interaction between the glutamate introduced at position 6.30(275) and the R3.50(165) reduces the degree of repulsion introduced by the T6.34(279)K mutation. (E) In the L6.30(275)E/T6.34(279)D mutant, the ionic lock on R3.50(165) produced by the L6.30(275)E mutation (e.g., see refs *31* and *34*) is further strengthened in the double mutant by the introduction of an aspartate at position 6.34(279), leading to enhanced stabilization of the inactive form of the receptor.

The expression of the L6.30(275)E/T6.34(279)K mutant was enhanced when cells were pretreated with naloxone, but the increase was smaller than that of the T6.34(279)K mutant. We have shown previously that naloxone pretreatment enhances the expression of the constitutively active D3.49(164)Q mutant by two mechanisms: inhibition of constitutive internalization and downregulation and stabilization of the receptor structure (*52*). It is likely that similar mechansims

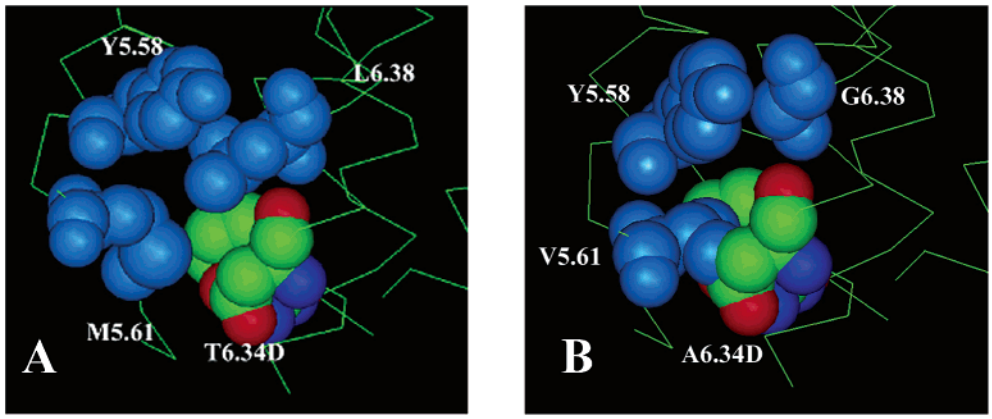

FIGURE 7: Spatial relations among conserved residues in the cytoplasmic ends of TM5 and TM6 in (A) the μ opioid receptor and (B) the α_{1B}-adrenergic receptor as an example of the monoamine receptors. Note that in the μ opioid receptor the spacing defined by the spatially adjacent Y5.58 and L6.38 allows for an ample gap between the residues at positions 6.34 and 5.61 so that mutations placing larger side chains at position 6.34 can be tolerated. In contrast, the spacing between Y5.58 and G6.38 is much smaller in the monoamine receptors so that the residues at positions 5.61 and 6.34 are crowded and mutations to larger side chains at position 6.34 cannot be tolerated. For example, D6.34 shown in panel B clashes with V5.61. The mutation cannot be accommodated without structural rearrangement that must affect the cytoplasmic ends of both TM5 and TM6 in the region that is known to be involved in coupling with G proteins. The nature of the structural rearrangement that would be required to avoid the steric clash shown in panel B will affect intracellular loop 3 and is the same as that proposed to be involved in the activation of the α_{1B}-adrenergic receptor.

contribute to the upregulatory effects of naloxone on the L6.30(275)E/T6.34(279)K mutant.

L6.30(275)E/T6.34(279)D Mutant. We previously demonstrated that the T6.34(279)D mutation did not to lead to constitutive activation of the μ receptor, and it greatly reduced the magnitudes of the responses to DAMGO and morphine (*36*). Here, we found that the L6.30(275)E/T6.34(279)D mutant displayed basal activities similar to that of the WT, but its response to DAMGO or morphine was completely eliminated. These results suggest that the substitution of T6.34(279) with Asp strengthened the interaction between 6.34(279) and R3.50(165), and that the L6.30(274)E mutation in the T6.34(279)D mutant leads to an additional interaction of E6.30(274) and R3.50(165) (Figure 6E). If no change in ionization states is assumed, the TM3−TM6 interaction energy is more favorable (less than −100 kcal/mol) for the L6.30(275)E/T6.34(279)D double mutant than for the T6.34D construct (−38 kcal/mol) This reinforced electrostatic lock thus further constrains the receptor in the inactive state and explains the complete inability of the agonist to activate this construct.

It is interesting to note that the L6.30(279)E/T6.34(275)D mutant had a lower affinity for DAMGO than the L6.30(275)E and T6.34(279)D mutants, which may be due to differences in the inactive state conformations.

Comparisons between the μ Opioid Receptor and GPCRs of Monoamines. The L6.30E mutant of the μ opioid receptor could not be activated and appeared to assume the conformation of an inactive receptor state. However, E6.30 naturally occurs in rhodopsin and the receptors for monoamines, and the receptors can be activated by agonists (see Figure 1). In addition, the L6.30E/T6.34K mutant of the μ opioid receptor displayed only the low constitutive activity of the receptor. In contrast, substitutions at the 6.34 locus with Lys have been shown to result in high levels of agonist-independent activity in several GPCRs containing E6.30, e.g., the mutation of A293 in the α_{1B}-adrenergic (*53*), of T373 in the α_{2A}-adrenergic (*54*), of L322 in the β_1-adrenergic (*55*), of C322 in the 5-HT_{2A} (*56*), of S312 in the 5-HT_{2C} (*57*), and of T313 in the 5-HT_{1B} (*58*) receptors (see Figure 1). Indeed, in the α_{1B}-adrenergic receptor, all 19 possible amino acid substitutions at this 6.34 locus (Ala293) resulted in varying levels of constitutive activity, with the A293K mutant having the highest activity (*53*). Most dramatically, our finding that the L6.30E/T6.34D mutant of the μ opioid receptor could not be activated by DAMGO, had a very low affinity for DAMGO, and did not show constitutive activity is different from that of GPCRs for monoamines. Substitutions of the 6.34 locus with Asp or Glu in several GPCRs containing the native E6.30 exhibited enhanced agonist-independent activities and higher agonist affinities, including the α_{1B}-adrenergic (*53*), α_{2A}-adrenergic (*54*), β_1-adrenergic (*55*), and 5-HT_{2A} (*56*) receptors. Thus, compared with rhodopsin and receptors of monoamines, the μ receptor may have additional interactions or a different local geometry in the vicinity of the $X_1BBX_2X_3B$ motif to keep it in inactive states.

Indeed, our modeling shows that the local environments are different between the μ opioid receptor and monoamine receptors. Among the monoamine receptors, the most common residue at the 6.38 locus is Gly, followed by Ala and Ser, while the opioid receptors have Leu at this locus. In the μ opioid receptor, the packing between the cytoplasmic ends of TM5 and TM6 is defined by the side chains of the adjacent residues at positions 5.58 and 6.38. In the μ opioid receptor, the bulky side chains of Y5.58 and L6.38 allow for an ample gap between residues at positions 5.61 and 6.34 (Figure 7A) so that mutations placing larger side chains (e.g., an Asp residue) at position 6.34 can be tolerated. In contrast, the spacing between Y5.58 and G6.38 is smaller in the monoamine receptors (for example, in the α_{1B}-adrenergic receptor) so that the residues at positions 5.61 and 6.34 are crowded and mutations to larger side chains at 6.34 cannot be tolerated. For instance, Figure 7B shows that in the α_{1B}-adrenergic receptor mutation of the residue at position 6.34 to a Glu would produce a steric clash with V5.61 in the rhodopsin-like receptor structure. Avoidance of such a clash by conformational rearrangement must affect the local structure at the cytoplasmic ends of both TM5 and TM6 that

are known to be involved in coupling with G proteins (*16*). These differences may account for the observations that E6.30/(E/D)6.34 combinations result in enhanced constitutive activity for the monoamine receptors, but not for the μ opioid receptor, which does not have the steric clash. This local crowding in the monoamine receptors should also explain why the K6.34 mutants, which in these receptors are combined with a native E6.30, have much higher constitutive activities than the E6.30/K6.34 mutant of the μ opioid receptor.

The present analysis shows, therefore, that while receptor activation in the rhodopsin subfamily of GPCRs may be associated with similar conformational changes, different receptors may employ specialized sets of intramolecular interactions to produce these changes. Our findings illustrate how the interactions depend on the residues and local environments at the intracellular ends of TM3, TM5, and TM6 in the family of opioid receptors, but also that sequence differences in this region of other GPCRs in the rhodopsin family are likely to be shown to support locally different forms of the activation mechanism.

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BI026067B