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Functional role of the spatial proximity of Asp114(2.50) in TMH 2 and Asn332(7.49) in TMH 7 of the μ opioid receptor

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Abstract We examined whether a proposed spatial proximity between Asp114(2.50) and Asn332(7.49) affected the functional properties of the μ opioid receptor. The D114(2.50)N mutant had reduced binding affinities for morphine, DAMGO and CTAP, but not for naloxone and [^{3}H]diprenorphine; this mutation also abolished agonist-induced increase in [^{35}S]GTPγS binding. The N332(7.49)D mutation eliminated detectable binding of either [^{3}H]diprenorphine or [^{3}H]DAMGO. The combined D114(2.50)N-N332(7.49)D mutation restored high affinity binding for [^{3}H]diprenorphine, CTAP and naloxone, and restored partially the binding affinities, potencies and efficacies of morphine and DAMGO. Thus, reciprocal mutations of Asp114(2.50) and Asn332(7.49) compensate for the detrimental effects of the single mutations, indicating that the residues are adjacent in space and that their chemical functionalities are important for ligand binding and receptor activation.

Key words: μ Opioid receptor; Structure-activity relationship; Site-directed mutagenesis

1. Introduction

Opiate and opioid compounds act on opioid receptors to produce their pharmacological actions. Multiple opioid receptors (μ, δ, κ, ε) have been demonstrated from pharmacological, binding, anatomical and molecular data (for reviews, [1–3]). These opioid receptors are coupled through G proteins to affect a variety of effectors, which include adenylate cyclase, potassium channels, calcium channels (for a review, [4]) and a mitogen-activated protein kinase pathway (for example, [5]). The μ opioid receptors are closely associated with analgesic and euphoric actions of opiate and opioid compounds [1]. Two splice variants of μ opioid receptors have been cloned [6–9]. Deduced amino acid sequences of these clones display the motif of putative seven α-helical transmembrane helices (TMHs) connected by alternating intracellular and extracellular hydrophilic loops, that is characteristic of G protein-coupled receptors (GPCRs). The splice variants of the μ opioid receptor share the same putative TMHs with sequence variations in the C-terminal domain.

Our current understanding of the molecular mechanisms of GPCR functions draws on the growing information about structural details of the receptor molecules and the relationship between these structural elements and the functional properties (for reviews, [10–15]). Although high-resolution structures of GPCRs are not yet available, local structural data obtained by site-directed mutagenesis and chimeric receptors have provided some insight into the mechanisms of receptor binding and activation and the potential functional roles of specific residues and domains of receptors (for reviews, [10,13–15]). A feature common to GPCRs is a network of side-chain interactions among TMHs that maintain the structural integrity and underlie receptor activation. Residues that are highly conserved among GPCRs are likely to be essential structural determinants of receptors and to play a role in their functions. An Asp in TMH 2 (Asp2.50 in the generic nomenclature, see Section 2) and an Asn in TMH 7 (Asn7.49 in the generic nomenclature) are conserved in the same positions of the putative TMHs in over 95% of GPCRs [16]. The role of the conserved Asp2.50 has been examined in many GPCRs. Mutation of this Asp has been shown to affect agonist affinity, G protein coupling, sensitivity to Na^+ or guanine nucleotides (for a review, [10]). In the 5-HT_{2A} receptor, mutation of this Asp in TMH 2 to Asn abolished serotonin-induced increase in IP_3 generation. However, an additional mutation of the Asn in TMH 7 to Asp (N7.49D) partially restored the receptor function, indicating that Asp2.50 and Asn7.49 are in close proximity and likely to interact by hydrogen bonding [17]. The corresponding residues are reversed in the wild-type gonadotropin-releasing hormone (GnRH) receptor, which has an Asn in TMH 2 and an Asp in TMH 7. Nevertheless, the same restoration of function was observed from reciprocal mutations of these residues, indicating a spatial proximity and potential for interactions similar to that demonstrated for the 5-HT_{2A} serotonin receptor [18].

Not much is known about the relative proximity of these two loci in opioid receptors. Surratt et al. [19] demonstrated that Asp114(2.50) of the μ opioid receptor was important for high affinity agonist binding and receptor activation. In the present study, we examined by site-directed mutagenesis studies in the rat μ opioid receptors the possible interactions between Asp114(2.50) and Asn332(7.49) and their functional consequences. Binding affinities of morphine (non-peptide agonist), DAMGO (peptide agonist), naloxone (non-peptide an-

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Abbreviations: CHO cells, Chinese hamster ovary cells; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; DAMGO, Try-D-Ala-Gly-N-Me-Phe-Gly-ol; GDP, guanosine diphosphate; GnRH receptor, gonadotropin-releasing hormone receptor; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; HA, hemagglutinin; 5-HT receptor, 5-hydroxytrytamine receptor; TEL buffer, 50 mM Tris-HCl buffer containing 1 mM EGTA and 4 μM leupeptin (pH 7.4); TMH, transmembrane helix

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tagonist) and CTAP (peptide antagonist) were determined by competitive inhibition of [^{3}H]diprenorphine binding. Morphine- and DAMGO-induced increase in [^{35}S]GTPγS binding was used as the functional measure of receptor activation for the various constructs.

2. Materials and methods

2.1. Materials

[^{3}H]diprenophine (58 Ci/mmole), [^{3}H]DAMGO (54 Ci/mmole) and [^{35}S]GTPγS (1000–1200 Ci/mmole) were purchased from NEN Life Science (Boston, MA, USA). Morphine was provided by the National Institute on Drug Abuse. Naloxone was a gift from DuPont/Merck Co. (Wilmington, DE, USA). Muta-Gene kit was purchased from Bio-Rad Co. (Hercules, CA, USA); DAMGO and CTAP from Phoenix Pharmaceuticals, Inc. (Mountain View, CA, USA); Lipofectamine from GIBCO-BRL (Gaitherburg, MD, USA); Vectastain ABC kit from Vector Laboratories (Burlingame, CA, USA); the mouse monoclonal antibody (clone 12CA5) against hemagglutinin (HA) and HA peptide from Boehringer Mannheim Co. (Indianapolis, IN, USA); GDP, GTPγS, geneticin, 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide were from Sigma.

2.2. Numbering schemes for amino acid residues in the μ opioid receptor

Two numbering schemes were used. Amino acid residues in the μ opioid receptor were identified by their sequence numbers. In addition, the generic numbering scheme of amino acid residues in GPCRs proposed by Ballesteros and Weinstein [15] was used. According to this nomenclature, amino acid residues in putative TMHs are assigned two numbers (N1, N2). N1 refers to the TMH number. For N2, the most conserved residue in each TMH is assigned 50, and the other residues are numbered in relation to this conserved residue, with numbers decreasing towards the N-terminus and increasing toward the C-terminus. In TMH 2 of the μ opioid receptor, Asp114 corresponds to the most conserved residue and hence is assigned the index 2.50. The residue is referred to as Asp114(2.50) to enable a structural comparison to the equivalent positions in other GPCRs. The Asn332 of this receptor precedes the most conserved residues in TMH 7, which is Pro333, and hence is identified as Asn332(7.49). The generic numbering allows for cross-reference to the published literature on other GPCRs.

2.3. Oligodeoxynucleotide-directed mutagenesis

Mutations were introduced into the rat μ receptor [6] with the uracil replacement method of Kunkel [20] using the Muta-Gene kit. Mutants were selected by DNA sequence determination with the method of Sanger et al. [21]. Mutant μ receptors were subcloned into the *Hin*dIII site of the mammalian expression vector pcDNA3. Correctly oriented clones were used for subsequent studies.

2.4. Transient expression of the wild-type and mutant μ receptors in CHO cells

CHO cells were transfected with the rat wild-type or mutant μ receptor cDNA with Lipofectamine [22] according to the manufacturer's instructions.

2.5. Stable expression of the wild-type and mutant μ receptors in CHO cells

CHO cell lines stably expressing the cloned rat μ receptor or its mutant were established as described previously [23]. CHO cells expressing similar levels of the wild-type, D114(2.50)N and D114(2.50)N-N332(7.49)D μ receptors (∼0.8 pmole/mg membrane protein) were used in the present study.

2.6. Opioid receptor binding

Membranes were prepared from CHO cells and opioid receptor binding was performed with [^{3}H]diprenorphine according to our published procedure [24]. Binding was carried out in 50 mM Tris-HCl buffer containing 1 mM EGTA and 4 μM leupeptin (pH 7.4) (TEL buffer), unless indicated otherwise, at room temperature for 1 h. [^{3}H]diprenorphine saturation binding was performed for determination of K_d and B_{max} values. Competitive inhibition of [^{3}H]diprenorphine binding by drugs was performed with [^{3}H]diprenorphine at a concentration close to its K_d value. Naloxone (1 μM) was used to define non-specific binding. Binding data were analyzed with the EBDA program [25].

2.7. Agonist-induced increase in [^{35}S]GTPγS binding

Determination of [^{35}S]GTPγS binding to G proteins was carried out with our published procedure [26]. EC_{50} and maximal response values were calculated by use of the equation $y = E_{max}/[1+(x/EC_{50})^s]$+ background, where y is the response at the dose x, E_{max} is the maximal response and s is a slope factor. The K_B value of an antagonist was calculated by use of the equation $[A']/[A] = [B]/K_B+1$, where $[A']$ and $[A]$ were the concentration of an agonist which produced an equal response in the presence and absence of the concentration of the antagonist $[B]$ [27].

2.8. Determination of protein content

Protein contents of membranes were determined by the bicinchoninic acid method of Smith et al. [28] with bovine serum albumin as the standard.

2.9. Epitope tagging of the wild-type and N332(7.49)D rat μ opioid receptor with HA

HA sequence (YPYDVPDYA) was inserted into the receptor immediately after the first methionine residue by polymerase chain reaction [29]. HA-tagged receptors were cloned into the *Hin*dIII site of the vector pcDNA3. DNA sequence was determined with the method of Sanger et al. [21] to confirm the correct insertion of the HA sequence and the absence of unwanted mutations.

2.10. Immunocytochemical studies

CHO cells were transfected with the HA-tagged rat wild-type or N332(7.49)D mutant μ receptor cDNAs or empty vector with Lipofectamine. Cells were plated on the Lab-Tek chamber slide 36–48 h after transfection, grown for one day, gently washed, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) at 4°C for 1 h, and washed again to remove the fixative. Immunohistochemistry was performed with the avidin-biotin-peroxidase method according to a published procedure [30]. Fixed cells were incubated with the mouse monoclonal antibody against HA (clone 12CA5) diluted 1:5000 at 4°C for 16–24 h followed by incubation with biotinylated horse

Table 1
K_d and B_{max} values of [^{3}H]diprenorphine binding to the wild-type, D114(2.50)N, N332(7.49)D and D114(2.50)N-N332(7.49)D μ opioid receptor constructs expressed in CHO cells

μ Opioid receptor construct	Transmembrane helix		[^{3}H]diprenorphine binding	
	2	7	K_d (nM)	B_{max} (pmol/mg protein)
Wild-type	D	N	0.03 ± 0.002	0.87 ± 0.08
D114(2.50)N	N	N	0.12 ± 0.02	0.99 ± 0.06
N332(7.49)D	D	D	ud	ud
D114(2.50)N-N332(7.49)D	N	D	0.05 ± 0.009	0.78 ± 0.08

The wild-type and the mutant μ opioid receptors were transfected into CHO cells and clonal cell lines stably expressing each receptor were established. Saturation binding of [^{3}H]diprenorphine to the wild-type, D114(2.50)N, N332(7.49)D and D114(2.50)N-N332(7.49)D μ opioid receptors were performed and K_d and B_{max} values were calculated as described in Section 2. Each value represents the mean ± S.E.M. of three to five independent experiments in duplicate.
Ud, binding was undetectable in CHO cells transiently transfected with the N332(7.49)D mutant μ receptor.

Table 2
K_i values (nM) of DAMGO, morphine, CTAP and naloxone in inhibiting [^{3}H]diprenorphine binding to the wild-type, D114(2.50)N and D114(2.50)N-N332(7.49)D μ receptors

Ligand	Wild-type	D114(2.50)N	Fold change [D114(2.50)N/wt]	D114(2.50)N-N332(7.49)D	Fold change [D114(2.50)N-N332(7.49)D/wt]
DAMGO	1.4 ± 0.3	296 ± 52	(212)	61.1 ± 23.2	(44)
Morphine	5.1 ± 1.5	684 ± 115	(134)	192 ± 54	(38)
CTAP	0.0057 ± 0.0006	3.9 ± 1.8	(684)	0.0066 ± 0.0029	(1.2)
Naloxone	0.7 ± 0.1	1.1 ± 0.1	(1.6)	0.9 ± 0.2	(1.3)

Inhibition of [^{3}H]diprenorphine binding by each ligand to the wild-type and the mutant μ opioid receptors was performed and K_i values were determined as described in Section 2. Each value is the mean ± S.E.M. of three to five independent experiments in duplicate. Wt, wild-type.

anti-mouse Ig G (1:200) at room temperature for 45 min. Subsequently cells were incubated with avidin-biotin-peroxidase complex for 45 min and reacted with 3,3′-diaminobenzidine tetrahydrochloride (0.006%) and hydrogen peroxide (0.0018%) for 3–6 min. Between incubations, cells were washed three times with 10 mM phosphate buffer (pH 7.4), 1% normal horse serum and 0.1% NP-40 at room temperature. Two types of controls were performed: incubation with antibody preincubated with HA peptide (10 μg/ml) and cells transfected with the vector alone. Cells were dehydrated through 70, 95, and 100% ethanol, cleared with xylene and coverslipped with Permount. The cells were then examined using bright-field and phase-contrast microscopy. The deep brown reaction product indicates the presence of HA-tagged receptor.

3. Results

3.1. Effects of D114(2.50)N, N332(7.49)D and D114(2.50)N-N332(7.49)D mutations on ligand binding affinities

The wild-type and D114(2.50)N, N332(7.49)D and D114(2.50)N-N332(7.49)D mutant μ opioid receptors were first transiently transfected into CHO cells and [^{3}H]diprenorphine binding was examined. While the wild-type and D114(2.50)N and D114(2.50)N-N332(7.49)D mutants had robust binding, the N332(7.49)D mutant did not. The N332(7.49)D mutant also did not exhibit binding for [^{3}H]DAMGO.

The wild-type and D114(2.50)N and D114(2.50)N-N332(7.49)D mutants were stably transfected into CHO cells and clonal cell lines established. Saturation [^{3}H]diprenorphine binding was performed and cell lines with similar receptor expression levels (~0.8 pmole/mg protein) were used for the study. Table 1 shows K_d and B_{max} values of [^{3}H]diprenorphine binding to the wild-type, D114(2.50)N and D114(2.50)N-N332(7.49)D μ receptors. Both D114(2.50)N and D114(2.50)N-N332(7.49)D mutants retained high affinity [^{3}H]diprenorphine binding.

We then determined whether the lack of binding to the N332(7.49)D mutant was due to poor expression. Both the wild-type and N332(7.49)D μ receptors were tagged with HA. When transiently expressed in CHO cells, the HA-tagged wild-type, but not the HA-tagged N332(7.49)D mutant, exhibited high levels of [^{3}H]diprenorphine binding. Immunohistochemistry of CHO cells transiently transfected with HA-tagged N332(7.49)D mutant using anti-HA monoclonal antibody showed intense staining, similar to the HA-tagged wild-type receptor (Fig. 1), indicating that the N332(7.49)D mutant is expressed. At light microscopy level, it is not possible to discriminate between intracellular and membrane expression of the receptor. Thus, the lack of binding to the N332(7.49)D mutant is due to disruption of the binding pocket by the N332(7.49)D mutation or to improper processing of the mutant receptor protein.

K_i values of four ligands (DAMGO, morphine, CTAP and naloxone) in inhibiting [^{3}H]diprenorphine binding are shown in Table 2. D114(2.50)N mutation greatly reduced the affinities of DAMGO, morphine and CTAP with the K_i values increased by 212-, 134- and 684-fold, respectively, compared with those of the wild-type. However, the same mutation affected the affinity of naloxone only slightly. Remarkably, the combined D114(2.50)N-N332(7.49)D mutant retained high affinity binding for CTAP, with the K_i value similar to that of the wild-type μ receptor. In addition, binding affinities of morphine and DAMGO, which were lowered dramatically by the D114(2.50)N mutation, were restored partially by the combined D114(2.50)N-N332(7.49)D mutation.

3.2. Effects of D114(2.50)N and D114(2.50)N-N332(7.49)D mutations on μ receptor activation by morphine and DAMGO

The D114(2.50)N mutation essentially abolished agonist activities of morphine and DAMGO in increasing [^{35}S]GTPγS

Table 3
Agonist-stimulated [^{35}S]GTPγS binding to membranes: EC_{50} values and maximal effects of morphine and DAMGO in activating the wild-type and D114(2.50)N, N332(7.49)D and D114(2.50)N-N332(7.49)D mutant μ opioid receptors

Agonist	μ Opioid receptor construct	EC_{50} (nM)	Maximal stimulated binding	
			(fmol/mg protein)	(% of wild-type)
Morphine	Wild-type	81.0 ± 4.6	109 ± 10	100
	D114(2.50)N	nd	2.3 ± 0.4	2.1
	D114(2.50)N-N332(7.49)D	328 ± 19	60.7 ± 4.0	55.8
DAMGO	Wild-type	59.5 ± 10.8	152 ± 20	100
	D114(2.50)N	nd	8.9 ± 2.2	5.9
	D114(2.50)N-N332(7.49)D	197 ± 10	86.5 ± 1.2	57.0

Activation of the wild-type, D114(2.50)N, N332(7.49)D and D114(2.50)N-N332(7.49)D mutant μ opioid receptors by morphine or DAMGO to increase [^{35}S]GTPγS binding was determined as described in Section 2. Each value is the mean ± S.E.M. of three independent experiments in duplicate.
Nd, can not be determined.

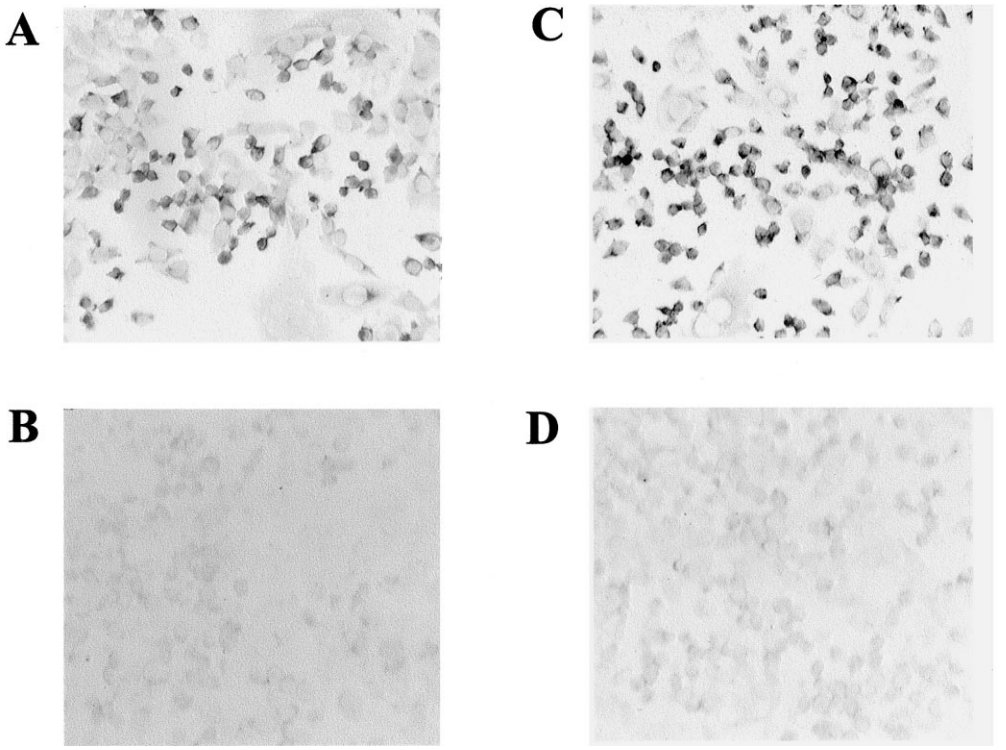

Fig. 1. Immunohistochemical staining of HA-tagged wild-type (A) and N332(7.49)D (C) μ opioid receptors transiently expressed in CHO cells with the 12CA5 monoclonal antibody against HA. Controls were performed with CHO cells transfected with the expression vector or antibody pre-absorbed with the HA peptide (B, wild-type; D, N332(7.49)D). Magnification is 800× for each. The experiments were conducted three times with the same results.

binding (Table 3). In contrast, the potencies and efficacies of morphine and DAMGO in enhancing [^{35}S]GTPγS binding were partially retained in the D114(2.50)N-N332(7.49)D mutant (Table 3). EC_{50} values of morphine and DAMGO were only 4- and 3.3-fold higher than in the wild-type and the maximal responses were 56% and 57% of those of the wild-type, respectively.

3.3. Potencies of the antagonists naloxone and CTAP at the wild-type and D114(2.50)N-N332(7.49)D mutant μ receptors

K_B values were determined for naloxone and CTAP in antagonizing DAMGO activation of the wild-type and D114(2.50)N-N332(7.49)D mutant μ receptors (Table 4). The D114(2.50)N-N332(7.49)D mutation did not affect the potency of either antagonist.

3.4. GTP shift of DAMGO binding affinities to the wild-type, D114(2.50)N and D114(2.50)N-N332(7.49)D μ opioid receptors

Multiple agonist affinity states of opioid receptors and other GPCRs were reported to represent various G protein-associated and dissociated states [31]. While high affinity states represent G protein-coupled states, low affinity states represent conformations that are uncoupled from G proteins or associated with G proteins with less efficiency. Competitive inhibition of [^{3}H]diprenorphine binding by DAMGO was conducted in the presence of 100 mM NaCl, 5 mM $MgCl_2$ and 30 μM GTPγS, which has been shown to uncouple receptors from G proteins and convert all receptors to a low affinity state for agonists [32,33]. The presence of NaCl, $MgCl_2$ and GTPγS decreased the affinity of DAMGO for the wild-type by 46-fold; however, it had no effect on the K_i value of DAMGO for the D114(2.50)N mutant and increased its K_i value for the D114(2.50)N-N332(7.49)D mutant only by 1.9-fold (Table 5). These results indicate that while the wild-type receptor is tightly pre-coupled to G proteins, the D114(2.50)N mutant is uncoupled from G protein and the revertant D114(2.50)N-N332(7.49)D mutant is weakly pre-coupled to G protein.

4. Discussion

If two residues play independent roles in a certain property of the receptor, their simultaneous mutation is expected to have additive effects. This is not the case for residues

Table 4
Potencies of naloxone and CTAP in antagonizing activation of the wild-type and D114(2.50)N-N332(7.49)D mutant μ opioid receptors by DAMGO

Antagonist	μ Opioid receptor construct	K_B (nM)
Naloxone	Wild-type	0.94 ± 0.12
	D114(2.50)N-N332(7.49)D	1.2 ± 0.18
CTAP	Wild-type	0.009 ± 0.002
	D114(2.50)N-N332(7.49)D	0.014 ± 0.004

Dose-response of DAMGO-induced increase in [^{35}S]GTPγS binding to membranes of CHO cells stably transfected with the wild-type or D114(2.50)N-N332(7.49)D mutant μ opioid receptor was performed in the presence or absence of 10 nM naloxone or 0.1 nM CTAP and K_B values were determined as described in Section 2. Each value represents the mean ± S.E.M. of three to four independent determinations in duplicate.

Table 5
K_i values (nM) of DAMGO binding to the wild-type, D114(2.50)N and D114(2.50)N-N332(7.49)D μ receptors in the presence and absence of GTPγS

μ Opioid receptor construct	K_i in TEL buffer	K_i in GTPγS buffer	K_i (GTPγS buffer)/K_i (TEL buffer)
Wild-type	1.5 ± 0.3	69 ± 8.9	46
D114(2.50)N	279 ± 55	308 ± 52	1.1
D114(2.50)N-N332(7.49)D	59 ± 20	111 ± 13	1.9

Competitive inhibition of [^{3}H]diprenorphine binding by DAMGO to the wild-type and the mutant μ opioid receptors was performed in TEL buffer or in TEL buffer plus 100 mM NaCl, 5 mM $MgCl_2$ and 30 μM GTPγS (GTPγS buffer) as described in Section 2. Each value is the mean ± S.E.M. of three independent experiments in duplicate.

Asp114(2.50) and Asn332(7.49) in the rat μ opioid receptor. We found that the D114(2.50)N mutation reduced binding affinities, potencies and efficacies of DAMGO and morphine as well as the binding affinity of CTAP, while the N332(7.49)D mutation eliminated [^{3}H]diprenorphine and [^{3}H]DAMGO binding. However, the combination of D114(2.50)N and N332(7.49)D mutations was not detrimentally additive, but rather restored binding affinity of CTAP and partially restored the binding affinities, potencies and efficacies of DAMGO and morphine. The non-additivity of their effects indicates that Asp114(2.50) and Asn332(7.49) are not independent of each other, and because they seem to be nearly interchangeable, they are likely to share the same microenvironment. This observation is similar to those on 5-HT_{2A} receptor [17] and GnRH receptor [18]. By pharmacological studies and computational simulations, Zhou et al. [18] and Sealfon et al. [17] showed that TMH 2 and TMH 7 are adjacent in space and likely to form hydrogen bonds between Asp and Asn in GnRH receptor and 5-HT_{2A} receptor. Our results suggest a similar interaction between Asp114(2.50) and Asn332(7.49) in the μ opioid receptor. The fact that reversal of chemical functionalities at these two loci restores activities lost upon single mutations further indicates that they have a joint role in the underlying mechanisms. Such a role has been suggested recently [34].

Notably, the wild-type and D114(2.50)N-N332(7.49)D mutant μ opioid receptors share some pharmacological properties and differ in others. Thus, the antagonists naloxone and CTAP had similarly high binding affinities and were similarly potent as antagonists at both receptors. However, the agonists morphine and DAMGO exhibited lower affinities, potencies and efficacies at the D114(2.50)N-N332(7.49)D mutant than the wild-type. In addition, the presence of Mg^{2+}, Na^+ and GTPγS reduced DAMGO affinity for the wild-type much more profoundly than they did for the D114(2.50)N-N332(7.49)D mutant. These differences in agonist binding and G protein coupling indicate that the Asp114 at position 2.50 in TMH 2 and Asn332 at position 7.49 in TMH 7 are not entirely interchangeable in their functions, suggesting that these two residues are part of a more complex network with other loci. Based on molecular dynamics simulation of constitutively active α_{1B}-adrenergic receptor, Scheer et al. [35,36] identified a conserved polar pocket near the cytosolic interface formed by a network of hydrogen bonds among the conserved residues Asn63(1.50), Asp91(2.50), Asn344(7.49) and Tyr348(7.53). It was postulated [35,36] that these interactions constrain the receptor in an inactive state and that the shift of Arg143(3.50) in the conserved DRY sequence in TMH 3 out of the polar pocket leads to receptor activation. More recently, Ballesteros et al. [34] concluded, from mutagenesis studies and molecular dynamics simulations on the TMH containing the same DRY motif in the GnRH receptor, that the conserved Arg at position 3.50 is constrained by an interaction with the preceding Asp3.49 of DRY in the inactive form of the GPCRs. When the GPCR is activated, the Asp at position 3.49 becomes protonated and Arg3.50 is released to enable its interaction with Asp2.50 and Asn7.49 (in the case of GnRH receptor, Asn2.50 and Asp7.49). This is consistent with the repeated findings including the present results, showing that mutation of Asp2.50 to Ala or Asn eliminates or profoundly reduces receptor activation by agonists in many GPCRs (for reviews, [10–15]).

In the N332(7.49)D μ receptor, which has an Asp in both TMH 2 and TMH 7, ligand binding and/or receptor protein processing are disrupted. The immunohistochemistry results could not differentiate between intracellular retention of the receptor protein and membrane expression of a receptor with improperly packed TMHs. Either possibility suggests a complementary Asp/Asn pair in TMH 2/TMH 7 of this receptor. Notably, the Asn332(7.49) is not likely to be directly involved in ligand binding. This is suggested by two observations. One is the strong conservation of an Asn or Asp at this site in GPCRs with ligands of very diverse structures. The other is that the D114(2.50)N-N332(7.49)D double mutant has binding affinities for naloxone, diprenorphine and CTAP that are indistinguishable from those of the wild-type. The present findings as well as data from other studies in which this locus was mutated [17,18] indicate that Asn332(7.49) is more likely to be involved indirectly in maintaining the integrity of the binding pocket. In particular, the specific structural property of the Asn7.49 Pro7.50/Asp7.49 Pro7.50 motif that has been characterized recently [37] indicates the mode in which this structural role can be expressed.

In contrast to the N332(7.49)D mutant, the D114(2.50)N mutant with Asn in both loci was tolerated well. High affinity binding was retained for both the antagonists naloxone and diprenorphine, consistent with the findings of Surratt et al. [19]. It is likely that Asn can act as both a hydrogen-bond acceptor (C = O) and a donor (NH_2) but Asp can only be an acceptor at the ε position [18]. Thus, interactions such as hydrogen bonding in both the D114(2.50)N mutant and D114(2.50)N-N332(7.49)D double mutant receptors may retain receptor conformation to some extent. In this regard, the μ opioid receptor is similar to the GnRH receptor although there are Asn in the TMH 2 and Asp in the TMH 7 in the wild-type GnRH receptor. But the μ receptor is different from the 5-HT_{2A} receptor in that the mutant 5-HT_{2A} receptor retained high affinity ligand binding and G protein coupling when Asp was present at both loci [17]. The specific behaviors of the mutant μ opioid, the GnRH and the 5-HT_{2A} receptors are most likely due to differences in the immediate environment of Asp2.50 and Asn7.49 in these particular receptors,

which differ in the identity of the amino acid side chains of surrounding residues.

Our results showing that the D114(2.50)N mutation greatly reduced the affinities of DAMGO and morphine for the μ receptor and abolished the abilities of DAMGO and morphine to activate the receptor are consistent with a large number of reports indicating that Asp at position 2.50 is important for high affinity agonist binding and receptor-G protein coupling (for a review, [10]). In particular, Surratt et al. [19] demonstrated this for the μ opioid receptor. The differential effects of the D114(2.50)N mutation on naloxone and diprenorphine vs. morphine and DAMGO suggest that these agonists and antagonists may bind to different conformations of the receptor or have different binding domains.

Recent molecular dynamics simulations of 5-HT_{2A} receptor models provided some insights into the role of Asp in TMH 2 in the rearrangement of receptor structure due to agonist, but not antagonist binding. Upon activation by 5-HT, there are significant conformational changes observed in the TMHs 5 and 6 toward the intracellular side [17], consistent with experimental data for other GPCRs [38–40]. In contrast, the TMH 2 Asp to Asn mutant showed a smaller conformational change, in an opposite direction, that may not facilitate coupling to the G proteins [17].

While D114(2.50)N mutation did not affect binding affinity of diprenorphine and naloxone significantly, it dramatically decreased the affinity of CTAP. Notably, the affinity of CTAP was restored to a level similar to that of the wild-type by the combined D114(2.50)N-N332(7.49)D mutation. The distinct structural feature of CTAP may contribute to this difference. Naloxone and diprenorphine have the epoxymorphinan structure shared by many opioid ligands, whereas CTAP is a peptide with the sequence of D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2. One possibility is that CTAP may be more sensitive to the agonist conformation of the receptor although it is an antagonist. Indeed, Hawkins et al. [41] reported that the affinity of CTOP for the μ opioid receptor was reduced by metal ions and GTP in a manner similar to effects on high affinity agonist binding. Since CTOP is an analog of CTAP with ornithine at the fifth position instead of arginine, it is likely that CTAP is similarly affected by GTP and metal ions.

We conclude that the interaction between Asp114 at position 2.50 in TMH 2 and the Asn332 at position 7.49 in TMH 7 is important for structural and functional integrity of the μ opioid receptor. The present findings indicating the reciprocal effects of Asp114(2.50) and Asn332(7.49) of the μ opioid receptor underscore the structural relationship of this receptor to the family of GPCRs. The integrity of the interaction between these loci is found to be crucial for high affinity ligand binding and activation of μ opioid receptors in a manner consistent with current structural models and mechanistic inferences from other GPCRs. This should have significant impact on the ability to characterize the mechanistic details of μ opioid receptor function and ligand interaction in the broader context offered by work on the other GPCR systems.

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