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# Key Residues Defining the μ-Opioid Receptor Binding Pocket: A Site-Directed Mutagenesis Study

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**Abstract:** Structural elements of the rat μ-opioid receptor important in ligand receptor binding and selectivity were examined using a site-directed mutagenesis approach. Five single amino acid mutations were made, three that altered conserved residues in the μ, δ, and κ receptors (Asn$^{150}$ to Ala, His$^{297}$ to Ala, and Tyr$^{326}$ to Phe) and two designed to test for μ/δ selectivity (Ile$^{198}$ to Val and Val$^{202}$ to Ile). Mutation of His$^{297}$ in transmembrane domain 6 (TM6) resulted in no detectable binding with [$^3$H]DAMGO ($^3$H-labeled D-Ala$^2$,*N*-Me-Phe$^4$,Gly-ol$^5$-enkephalin), [$^3$H]bremazocine, or [$^3$H]ethylketocyclazocine. Mutation of Asn$^{150}$ in TM3 produces a three- to 20-fold increase in affinity for the opioid agonists morphine, DAMGO, fentanyl, β-endorphin$_{1-31}$, JOM-13, deltorphin II, dynorphin$_{1-13}$, and U50,488, with no change in the binding of antagonists such as naloxone, naltrexone, naltrindole, and nor-binaltorphamine. In contrast, the Tyr$^{326}$ mutation in TM7 resulted in a decreased affinity for a wide spectrum of μ, δ, and κ agonists and antagonists. Altering Val$^{202}$ to Ile in TM4 produced no change on ligand affinity, but Ile$^{198}$ to Val resulted in a four- to fivefold decreased affinity for the μ agonists morphine and DAMGO, with no change in the binding affinities of κ and δ ligands. **Key Words:** Endorphins—G protein–coupled receptors—Mutagenesis—Morphine—Opiate—Opioid receptor.
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Several lines of evidence have demonstrated the presence of three types of opioid receptors, referred to as μ, δ, and κ (Wood, 1982; Fowler and Fraser, 1994). These receptors are differentially distributed in the CNS and periphery and have unique pharmacological and receptor binding profiles (Gillan and Kosterlitz, 1982; Goldstein and Naidu, 1989; Mansour et al., 1995*b*). The opioid receptors mediate a host of behavioral effects including antinociception, reward, and reinforcement, as well as a number of neuroendocrine responses. Paralleling the multiple opioid receptors, three opioid peptide families have been described, the proopiomelanocortin, proenkephalin, and prodynorphin peptide families from which the endogenous ligands are derived (Day et al., 1993; Rossier, 1993; Young et al., 1993). The opioid peptides share a common core sequence, Tyr-Gly-Gly-Phe-Leu (or Met), and can bind with varying affinities to all three opioid receptors (Hughes et al., 1973; Magnan et al., 1982; Mansour et al., 1995*c*). The μ-opioid receptor, the main focus of this study, is of particular clinical importance as it is the site at which morphine-like drugs interact to produce their profound analgesic effects. Therefore, a better understanding of the structural features and binding requirements of the μ receptor may lead to the development of more selective and efficacious drugs.

The recent cloning of the μ-, δ-, and κ-opioid receptors suggests that they are members of the G protein family of seven transmembrane domain (TM) receptors, which are negatively linked to adenylyl cyclase (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Meng et al., 1993; Thompson et al., 1993; Wang et al., 1993; Yasuda et al., 1993). The opioid receptors are highly homologous to one another on an amino acid level with an overall amino acid identity of ~60%. Amino acid homology is highest within the transmembrane domains and the connecting intracellular loops, with greatest divergence in the extracellular N-terminal domain, the extracellular loops, and C-terminal intracellular domain. TM4 differs from the other transmembrane domains, as it has comparatively low level amino acid homology when examined across the opioid-receptor types. In addition to the three opioid receptors, a novel receptor has recently been cloned (Bunzow et al., 1994; Fukuda et al., 1994; Mollereau et al., 1994;

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*Abbreviations used:* BSA, bovine serum albumin; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr; DAMGO, D-Ala$^2$,*N*-Me-Phe$^4$, Gly-ol$^5$-enkephalin; deltorphin II, Tyr-D-Ala-Glu-Val-Val-Gly-NH$_2$; DPDPE, D-Pen$^2$-D-Pen$^5$-enkephalin; DSLET, D-Ser$^2$, Leu$^5$-enkephalin; EKC, ethylketocyclazocine; JOM-13, Tyr-c(D-Cys-Phe-D-Pen)OH; KPBS, potassium phosphate–buffered saline; nor-BNI, nor-binaltorphamine; TIPP, H-Tyr-Tic-Phe-Phe-OH; TM, transmembrane domain; U50,488, *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide.

Wick et al., 1994; Lachowicz et al., 1995) that has high amino acid homology and shares many of the structural features of the opioid receptors but does not specifically bind any opiate peptide or alkaloid. The function of this closely related orphan receptor is presently unclear, but it may provide clues as to the structural requirements of the opioid receptors, as well as their common evolution.

The cloning of the opioid receptors provides a unique opportunity to examine issues of opioid-receptor structure and ligand specificity. Previous studies examining structure–function relationships have been limited to systematically manipulating the ligand and examining receptor binding or a pharmacological response. Although these studies have been invaluable in producing highly potent and selective opioid agonists and antagonists, they provide only indirect information concerning receptor structure. With the cloning of the opioid receptors, the receptors may be directly manipulated to examine issues of selectivity and affinity. In general, two approaches have been developed to examine receptor structure. The first is an empirical approach using receptor chimeras, where individual domains of the opioid receptors are exchanged to examine receptor selectivity (Kong et al., 1994; Wang et al., 1994; Xue et al., 1994, 1995; Fukuda et al., 1995; Hjorth et al., 1995; Meng et al., 1995; Minami et al., 1995; Onogi et al., 1995). The second approach involves three-dimensional computer modeling. Using this approach, specific amino acid residues critical to binding are predicted and then tested with site-directed mutagenesis techniques (Kong et al., 1993; Surratt et al., 1994). Both approaches have been useful in delineating specific domains and amino acids that may be critical for receptor binding and selectivity, but caution must be used in interpreting these results, as the precise three-dimensional geometry of the receptor pocket may be altered by either of these structural manipulations.

Deletion of the N-terminal domain (amino acids 1–66) or the C-terminal 33 amino acids of the μ receptor produces little change in receptor binding affinities and selectivities, suggesting that the region critical for binding lies within the transmembrane domains and the extracellular loops (Surratt et al., 1994). Studies with μ/κ- and δ/κ-receptor chimeras suggest that extracellular loop 2 is particularly important for the binding of prodynorphin peptides (Wang et al., 1994; Xue et al., 1994; Meng et al., 1995). This may be due to the negatively charged amino acids in the second extracellular loop of the κ receptor that could form "salt bridges" with the positively charged amino acids of the prodynorphin peptides to result in high-affinity binding. In contrast to the effects of large peptides, replacement of extracellular loop 2 has little effect on comparatively small ligands, such as U50,488, a selective κ agonist whose binding may depend on interaction deep in the receptor pocket that is formed by the transmembrane domains.

Several amino acids within the transmembrane domains of the μ have also been shown to be critical for opioid-receptor binding (Surratt et al., 1994). Mutation of aspartic acid residue (114) in TM2 produces a dramatic loss of the receptor binding affinity of agonists such as D-Ala$^2$,*N*-Me-Phe$^4$,Gly-ol$^5$-enkephalin (DAMGO) and morphine, with little change in the binding of the μ antagonist naloxone. A second aspartate residue (147) in TM3 produces smaller changes (fivefold) in both μ agonists and antagonists and may provide the negative counterion for the positively charged nitrogen found in many opiate ligands. In addition to these negatively charged amino acids, a conserved histidine (297) in TM6 of the opioid-receptor family has been identified to be critical in both μ-agonist and antagonist binding.

Notwithstanding the results from chimeric receptors and site-directed mutagenesis studies, comparatively little is known concerning the opioid-receptor structure and how ligands achieve selectivity. The results of studies with chimeric receptors have been somewhat inconsistent, with some studies suggesting that DAMGO may bind to a different region of the μ receptor than morphine and codeine (Onogi et al., 1995), and others argue for a common site of interaction (Xue et al., 1995). Further, studies using μ/δ chimeras suggest that intracellular loop 1 to TM3 is important for DAMGO binding (Fukuda et al., 1995), whereas others using μ/κ chimeras argue that intracellular loop 3 to the carboxyl terminus is important for DAMGO binding (Xue et al., 1995). These disparate findings emphasize the importance of considering the particular receptor background in which the chimeric fragment is inserted when drawing conclusions regarding a ligand's binding domain. The binding of the selective μ agonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr (CTAP) has been difficult to ascribe to any particular region of the μ receptor and may depend on multiple domains (Xue et al., 1995).

The present study, therefore, examines the structural elements of the rat μ receptor that may be important for ligand–receptor binding and selectivity using a site-directed mutagenesis approach. Assuming that His$^{297}$ and Asp$^{147}$ are important anchor points based on the previously noted studies, we aligned several rigid opioid ligands [morphine, bremazocine, ethylketocyclazocine (EKC), naloxone, naltrexone, and naltrindole] such that the phenol of the alkaloid was in the vicinity of the His$^{297}$ and the quaternary nitrogen was near the aspartate on TM3. Based on these computer modeling manipulations, we hypothesized that Tyr$^{326}$ in TM7 and Asn$^{150}$ in TM3 would lie in or near the volume occupied by the opioid ligands. For example, with morphine placed as described, the aliphatic alcohol of morphine would lie adjacent to Tyr$^{326}$. Thus, the change of Tyr$^{326}$ to Phe (removing the hydrogen bonding site) would decrease morphine affinity. In a similar manner, it was noted that with morphine aligned as described, a portion of the aliphatic ring of the ligand was near

Asn[150]. Thus, removal of the polar Asn residue to Ala would enhance binding. To examine the structural basis of $\mu/\delta$ selectivity, two amino acids (Ile[198] and Val[202]) in TM4, the least conserved of the transmembrane domains, were mutated to the corresponding amino acids in the rat $\delta$ receptor. To evaluate whether the changes reflect differences in receptor selectivity, differences between opioid peptides and alkaloids, and agonists vs. antagonists, a series of opioid ligands were characterized. Also examined was whether pharmacologically similar compounds were equally affected by the same mutation.

## MATERIALS AND METHODS

### Molecular modeling

Molecular modeling was done on a Silicon Graphics Indigo Elan workstation using SYBYL 6.0. Only the transmembrane segments were assembled. The transmembrane segments were assumed to be $\alpha$ helixes, with the last three to four residues in a 3–10 helix conformation. The helixes were arranged in a counterclockwise fashion, similar to that described for rhodopsin. It was assumed that small opioid ligands would bind within the cavity of the transmembrane segments, so the model was built by manually placing a variety of opioid ligands (with morphine and bremazocine as primary targets) within the cavity and looking for complementary interaction sites within the receptor helixes. The high degree of homology shown by the opioid receptors in TM6 and 7 suggested this region was important for binding, and emphasis was placed on "docking" ligands to this region. The model was assembled primarily as a tool to predict mutation sites, as opposed to an attempt to rigorously define atomic coordinates for each residue.

### Mutagenesis and expression

Single amino acid mutations of the rat $\mu$ receptor (Thompson et al., 1993) were prepared with the oligonucleotide-directed mutagenesis system provided by Amersham. Oligonucleotides (18–24 bases) were synthesized, purified by HPLC, and annealed to an M13 single-stranded bacteriophage that contained the entire protein coding region of the rat $\mu$ receptor. In brief, the method involved extending the oligonucleotide with Klenow polymerase in the presence of T4 DNA ligase to generate a mutant heteroduplex. The nonmutant strands were then selectively removed with exonuclease digestion and filtration, leaving the mutant strand to regenerate the replicative DNA form that was then subcloned in a pCMV expression vector for transfection into COS-1 cells. A total of five single amino acid mutations were made, three that examined the $\mu$-receptor binding pocket (Asn[150] to Ala, His[297] to Ala, and Tyr[326] to Phe) and two that examined $\mu/\delta$ selectivity (Ile[198] to Val and Val[202] to Ile). All mutations were verified by cDNA sequencing and restriction enzyme mapping.

### Transfection

COS-1 cells were grown in Dulbecco's modified Eagle medium with 10% fetal calf serum and subcultured in 100-mm tissue-culture plates ($1.0–1.5 \times 10^6$ cells) 24 h before transient transfection using a calcium phosphate precipitation procedure (Chen and Okayama, 1987). Each 100-mm plate of cells was transfected with 20 $\mu$g of pCMV-$\mu$ wild-type or pCMV-$\mu$ mutant DNA. Plasmid DNAs were added to 0.25 *M* $CaCl_2$ and 2× BBS [50 m*M* *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 m*M* NaCl, 1.5 m*M* sodium phosphate, pH 6.95] to result in a final volume of 1 ml. This mixture was allowed to remain at 22°C for 10–20 min, then slowly dripped onto one 100-mm plate of cells. The cells were then grown overnight at 37°C, 3% $CO_2$, washed twice in Versine and once in medium, and allowed to grow for an additional 24 h (37°C, 5% $CO_2$) before harvesting.

### Radioligand binding assays

At the time of cell harvesting, the culture medium was removed and each plate of cells was washed in 50 m*M* Tris (10 ml, pH 7.4, 22°C), scraped in 5 ml of fresh 50 m*M* Tris buffer, and pelleted (5,000 rpm, 5 min). Cellular pellets of COS-1 cells expressing the wild-type $\mu$ or mutant receptor were homogenized (Kinematic Polytron) in 50 m*M* Tris buffer and aliquots of 200 $\mu$l (50–100 $\mu$g of protein) were added to each incubation tube. The $\mu$-opioid receptors were labeled with [$^3$H]DAMGO (50.5 Ci/mmol), [$^3$H]bremazocine (31.1 Ci/mmol), or [$^3$H]EKC (20.2 Ci/mmol); all were purchased from New England Nuclear (Wilmington, DE, U.S.A.). All three ligands were used to determine ligand affinity constants ($K_D$), with [$^3$H]bremazocine used in subsequent competition studies with unlabeled ligands. Nonspecific binding for all three ligands was defined by 1 $\mu M$ naltrexone. COS-1 cell homogenates were added to each incubation tube, bringing the total membrane homogenate–ligand volume to 250 $\mu$l/tube. All determinations were performed in duplicate. After a 60-min incubation (22°C), membranes were filtered under vacuum through glass filters (Schleicher and Schuell, no. 32) using a Brandel cell harvester. The filters were washed twice in 4 ml of 50 m*M* Tris (pH 7.0, 0°C) and counted by liquid scintillation spectrophotometry.

Saturation studies to determine the receptor affinities and binding capacities of the wild-type $\mu$ and $\mu$-mutant receptors were performed with a minimum of six concentrations of tritiated ligand. Competition studies used a minimum of 12 concentrations of opioid peptides or alkaloids ranging from 0.001 n*M* to 10 $\mu M$. The labeling concentration of [$^3$H]-bremazocine used in the competition studies was 1 n*M*, which is approximately its $K_D$ value for the $\mu$ wild-type and mutant receptors. The competition studies were performed in parallel with the same tritiated ligand and unlabeled ligand dilution curve used across the wild-type $\mu$ and mutant receptors. In competition studies with Leu-enkephalin, dynorphin$_{1-13}$, and $\beta$-endorphin$_{1-31}$, incubations were performed at 0°C (1 h) and 0.3% bovine serum albumin (BSA) was added to the 50 m*M* Tris (pH 7.0) incubation buffer. All three peptides were initially resuspended in methanol/0.1 *M* HCl (1:1) and diluted in 50 m*M* Tris buffer (pH 7.0, 0°C) containing 0.3% BSA immediately before the competition binding assay. Other compounds used in this study include CTAP; DAMGO; D-Pen$^2$-D-Pen$^5$-enkephalin (DPDPE), Tyr-D-Ala-Glu-Val-Val-Gly-$NH_2$ (deltorphin II), Ser$^2$,-Leu$^5$-enkephalin (DSLET), fentanyl, Tyr-c(D-Cys-Phe-D-Pen)OH (JOM-13), morphine, naloxone, naltrexone, naltrindole, nor-binaltorphamine (nor-BNI), H-Tyr-Tic-Phe-Phe-OH) (TIPP), and *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (U50,488), kindly provided by James Woods of the University of Michigan, and were dissolved in sterile distilled water. All receptor binding data were analyzed by the LIGAND program developed by Munson and Rodbard (1980), and

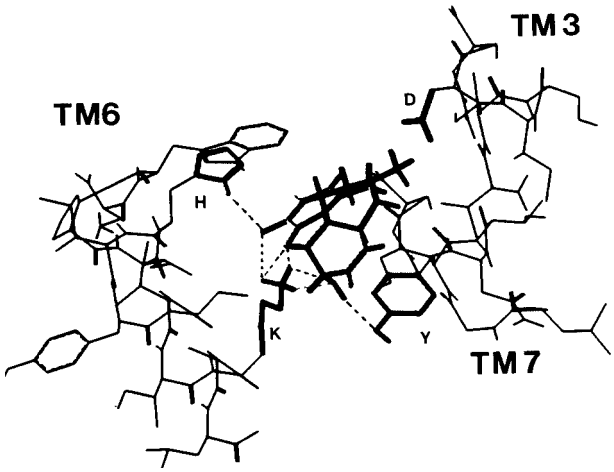


**FIG. 1.** This figure shows morphine within the cavity defined by the seven transmembrane domains. Morphine and the residues believed to be important contact points are highlighted as rods; the amino acid residues are labeled with single-letter designations. The receptor has been rotated within the plane vertical to the membrane and portions of the molecule removed from view to maximize visualization of interactions. Potential hydrogen bonding interactions are shown as dotted lines. In this interpretation, the phenol hydroxyl of morphine lies near the opioid invariant His(H) on TM6. The quaternary nitrogen of morphine is adjacent to the invariant Asp(D) on TM3. The tyrosine (Y) on TM7 is speculated to hydrogen-bond with the aliphatic hydroxyl of morphine.

cellular proteins were quantified by a Bio-Rad protein assay system (Richmond, CA, U.S.A.).

### Immunohistochemical studies

To examine whether the wild-type μ and the μ-mutant receptor proteins were expressed, immunohistochemical studies were performed on transiently transfected COS-1 cells. COS-1 cells were subcultured into six-well tissue-culture plates and transfected as described above with either the wild-type μ receptor or one of the mutant receptor DNAs (2 μg of plasmid/well). The cells were grown overnight at 37°C, 3% $CO_2$, washed twice in Versine and once in Dulbecco's modified Eagle medium with 10% fetal calf serum, and allowed to grow for an additional 24 h (37°C, 5% $CO_2$) before immunohistochemical staining.

Transfected COS-1 cells were postfixed in Zamboni fixative (2 h), washed in 50 m*M* potassium phosphate–buffered saline (KPBS), treated with 0.3% $H_2O_2$, and incubated with a μ-receptor antiserum generated to the C-terminal 63 amino acids of the cloned rat μ receptor (Mansour et al., 1995*a*) for 24 h (1:1,000 dilution in 50 m*M* KPBS, 0.4% Triton, 1% BSA, 1% normal goat serum) at 4°C. Transfected cells were then incubated with biotinylated goat antirabbit IgG (1:200, 1 h, 22°C), followed by an avidin-biotin complex–coupled horseradish peroxidase (1:200, 1 h, 22°C, Vector Elite, Burlingame, CA, U.S.A.). The reaction product was visualized with 0.04% 3,3′-diaminobenzidine tetrahydrochloride, 2.5% nickel chloride, and 0.01% $H_2O_2$, dissolved in 0.1 *M* sodium acetate. The reaction was terminated by two consecutive 0.9% NaCl washes, and the cells were then treated with graded alcohols, xylene, coverslipped with Permount, and viewed with a Zeiss Axiophot microscope. Immunohistochemical controls included the preabsorption and coincubation of the μ-receptor primary antibody with the μ-receptor fusion protein (4 μ*M*) to which the antibody was raised.

## RESULTS

A working qualitative model of the μ receptor was assembled using Sybyl software running on a Silicon Graphics Indigo Elan Workstation. This model's purpose was to assist in predicting site-directed mutagenesis, as opposed to defining a rigorous three-dimensional entity. In brief, the sequences of the opioid receptors were aligned. Transmembrane segments were selected by examination of hydrophobicity plots, with emphasis on including residues conserved in all the opioids. The transmembrane segments were assumed to be α-helical. Whenever possible, the helix was started at a Pro residue. Also, if possible, the cytosolic end was chosen as Arg or Lys. The seven transmembrane segments were manually assembled in a counterclockwise, contiguous arrangement, such that the most hydrophilic areas were facing into the cavity defined by these segments. It was assumed that the opioid invariant His in TM6 and the invariant Asp in TM3 were essential elements of the opioid-receptor structure. Accordingly, morphine was placed within the receptor cavity such that its phenol functional group was in the vicinity of the His of TM6 and the quaternary nitrogen of morphine was placed near the Asp on TM3 (Fig. 1). Using this as a "core template," the rest of the helix segments were manually placed around the mor-

**TABLE 1.** *Ligand affinities for the μ-receptor mutations and the wild-type receptor ($K_D$, n*M *± SEM)*

| | $Ile^{198}$–Val | $Val^{202}$–Ile | $Asn^{150}$–Ala | $His^{297}$–Ala | $Tyr^{326}$–Phe | Wild type |
|---|---|---|---|---|---|---|
| [$^3$H]Bremazocine | 1.90 | 2.66 | 1.09 | NSB | 0.81 | 1.58 |
| | (0.40) | (0.04) | (0.01) | | (0.03) | (0.01) |
| [$^3$H]DAMGO | 1.23 | 1.38 | 0.48 | NSB | NSB | 1.17 |
| | (0.09) | (0.05) | (0.05) | | | (0.10) |
| [$^3$H]EKC | 1.28 | 1.05 | 0.46 | NSB | 1.37 | 0.96 |
| | (0.21) | (0.08) | (0.04) | | (0.11) | (0.02) |

The μ-receptor mutants and wild-type receptor were labeled by either [$^3$H]bremazocine, [$^3$H]DAMGO, or [$^3$H]EKC. NSB, no specific binding.

**TABLE 2.** *Pharmacological profile of the μ-receptor mutations and wild-type receptor ($K_i$, nM ± SEM)*

| | Ile$^{198}$–Val | Val$^{202}$–Ile | Asn$^{150}$–Ala | Tyr$^{326}$–Phe | Wild-type μ |
|---|---|---|---|---|---|
| μ selective | | | | | |
| Morphine | 79.05 (15.64) | 24.25 (3.32) | 2.93 (0.41) | 118.89 (22.95) | 19.95 (3.51) |
| DAMGO | 51.75 (12.42) | 10.56 (1.33) | 1.19 (0.22) | 227.25 (28.96) | 9.80 (0.15) |
| Fentanyl | 61.01 (16.92) | 40.34 (7.50) | 2.04 (0.14) | >3,000.00 | 41.63 (2.65) |
| CTAP | 6.64 (0.70) | 4.66 (0.16) | 7.48 (0.79) | 25.56 (1.39) | 4.82 (0.28) |
| Naloxone | 9.76 (1.63) | 5.93 (0.48) | 4.38 (0.22) | 47.25 (5.79) | 4.35 (0.11) |
| Naltrexone | 1.46 (0.21) | 2.30 (0.18) | 1.27 (0.12) | 12.32 (1.20) | 1.93 (0.04) |
| μ/δ selective | | | | | |
| β-Endorphin | 46.91 (0.20) | 33.32 (1.71) | 16.25 (1.41) | 352.80 (65.53) | 46.65 (0.80) |
| DSLET | 393.50 (27.22) | 264.50 (25.24) | 32.00 (5.74) | >3,000.00 | 218.00 (73.25) |
| δ selective | | | | | |
| DPDPE | >3,000.00 | >3,000.00 | >3,000.00 | >3,000.00 | >3,000.00 |
| JOM-13 | >3,000.00 | >3,000.00 | 145.15 (40.44) | >3,000.00 | 1,249.39 (438.76) |
| Deltorphin II | >3,000.00 | >3,000.00 | 1,001.17 (764.86) | >3,000.00 | >3,000.00 |
| TIPP | >3,000.00 | >3,000.00 | >3,000.00 | >3,000.00 | >3,000.00 |
| Naltrindole | 18.95 (0.55) | 29.00 (0.79) | 28.85 (2.42) | 148.00 (12.87) | 23.65 (2.72) |
| κ selective | | | | | |
| Dynorphin$_{1-13}$ | 107.05 (33.32) | 65.51 (14.87) | 4.39 (0.38) | 746.38 (321.35) | 95.50 (8.53) |
| U50,488 | >3,000.00 | >3,000.00 | 283.30 (49.79) | >3,000.00 | 1,322.15 (150.62) |
| nor-BNI | 65.05 (14.12) | 64.07 (9.97) | 44.60 (1.44) | 152.50 (0.49) | 62.83 (3.04) |

phine. Residues in the vicinity of functional groups on morphine and other alkaloids were chosen as mutation targets.

Table 1 illustrates the binding affinities of several tritiated ligands for the μ-receptor mutants and the wild-type receptor. Mutation of His$^{297}$ in TM6 markedly affected opioid-receptor binding, with no specific binding detected with the selective μ ligand [$^3$H]-DAMGO or the nonselective benzomorphans [$^3$H]-bremazocine and [$^3$H]EKC. In a similar manner, the Tyr$^{326}$ mutation in TM7 is important for [$^3$H]DAMGO binding, with mutation to Phe resulting in a total loss of [$^3$H]DAMGO binding. In contrast, the Tyr$^{326}$ mutation has little effect on the binding affinities of the smaller benzomorphans [$^3$H]bremazocine and [$^3$H]EKC.

Twofold increases in [$^3$H]DAMGO and [$^3$H]EKC affinities are seen with the Asn$^{150}$ to Ala mutation, with more modest changes in affinity detected with [$^3$H]bremazocine. As the affinity of [$^3$H]bremazocine is only minimally altered by the Ile$^{198}$, Val$^{202}$, Asn$^{150}$, and Tyr$^{326}$ mutations, it was used as the labeling ligand in competition studies. Given that the His$^{297}$ mutation showed no specific binding with any of the ligands tested, it was not characterized further in binding studies.

Competition studies suggest that Asn$^{150}$ and Tyr$^{326}$ are critical amino acids in determining the affinity of a wide range of opioid peptides and alkaloids and may be important in defining the opioid binding pocket (Table 2; Figs. 2–4). Mutation of Asn$^{150}$ to Ala results in a three- to 20-fold increase in affinity for opioid agonists, with little change in the antagonist binding affinities of CTAP, naloxone, naltrexone, naltrindole, or nor-BNI (Figs. 2 and 3). The smallest increase in affinity with the Asn$^{150}$ mutation is seen with β-endorphin$_{1-31}$, with the largest differences in affinity observed with dynorphin$_{1-13}$ and fentanyl. Further, the Asn$^{150}$ mutation results in increased affinity for μ

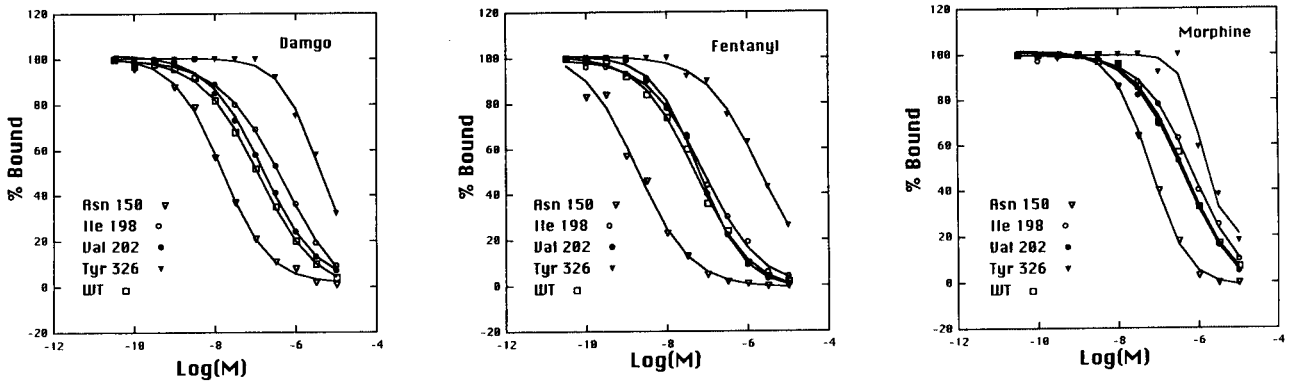


**FIG. 2.** Competition curves of the μ-receptor mutants and wild-type (WT) receptor for the μ agonists DAMGO, fentanyl, and morphine.

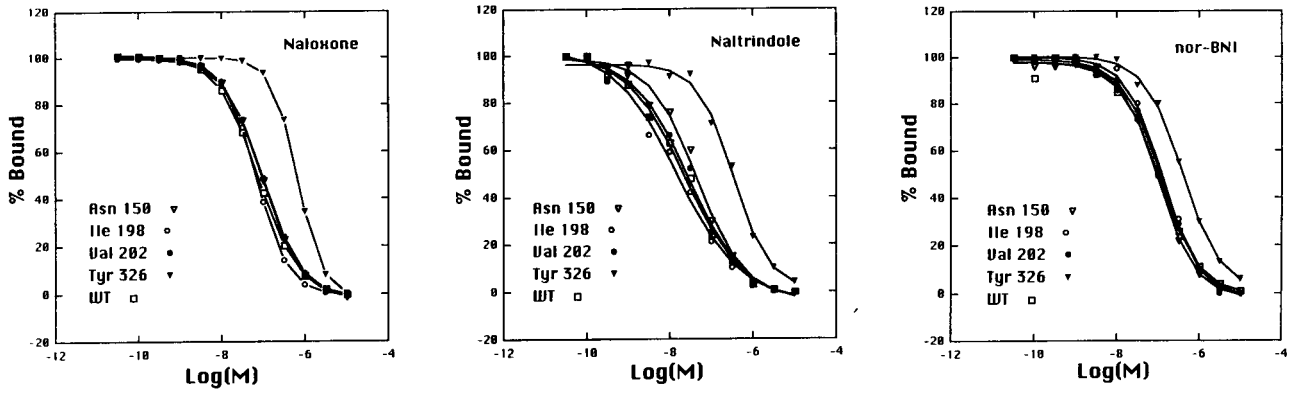


**FIG. 3.** Competition curves of the μ-receptor mutants and wild-type (WT) receptor for the opioid antagonists naloxone, naltrindole, and nor-BNI.

(morphine, DAMGO, and fentanyl), δ (deltorphin II and JOM-13), and κ (dynorphin-13 and U50,488) ligands and may be a critical residue in all three opioid receptors in modulating the affinity of a wide variety of ligands.

In contrast to the $Asn^{150}$ mutation, changing $Tyr^{326}$ to Phe results in a decreased affinity for a wide spectrum of ligands (Table 2; Figs. 2–4). The $Tyr^{326}$ mutation results in a decreased affinity for the μ agonists morphine, DAMGO, and fentanyl, as well as the antagonists CTAP, naloxone, and naltrexone (Table 2; Figs. 2 and 3). In addition, a reduced affinity is seen with δ-selective (DSLET and naltrindole) and κ-selective ($dynorphin_{1-13}$, U50,488, and nor-BNI) ligands with the $Tyr^{326}$ mutation (Fig. 4). Both opioid peptides and alkaloids are affected by this mutation, with fentanyl showing the largest decrease in binding affinity. Only the affinities of the benzomorphans bremazocine and EKC are unaltered by the $Tyr^{326}$ mutation (Table 1).

Of the mutations examining μ/δ selectivity, $Val^{202}$ to Ile produced little or no effect on ligand affinity for μ, δ, or κ agonists or antagonists (Table 2; Figs. 2–4). Only small, but difficult to interpret, changes in affinity for JOM-13 and U50,488 are seen with the $Val^{202}$ mutation. Mutating $Ile^{198}$ to the Val residue that is found in the δ receptor, however, resulted in a four- to fivefold decreased affinity for the μ agonists morphine and DAMGO. The $Ile^{198}$ mutation produced comparatively little change in binding affinity for the μ agonist fentanyl, however, or the antagonists CTAP and naltrexone. Naloxone is the only antagonist tested that shows a decreased binding affinity with the $Ile^{198}$ mutation. The changes observed with the $Ile^{198}$ mutation are selective for μ ligands, with no detectable changes observed in the binding affinities of δ or κ agonists or antagonists. Of the opioid peptides tested, the binding affinities of large peptides, such as β-endorphin$_{1-31}$ and dynorphin$_{1-13}$, are unchanged by the $Ile^{198}$ mutation, but smaller enkephalin analogues, such as DSLET and DAMGO, show a two- to fivefold decrease in binding affinities.

Immunohistochemical studies with an antibody directed to the C-terminal 63 amino acids of the μ receptor demonstrate that all the mutant-receptor proteins and the wild-type receptor are expressed with transient transfection (Fig. 5). Ten to 40% of COS-1 cells transiently transfected with the $Ile^{198}$, $Val^{202}$, $Asn^{150}$, $His^{297}$, $Tyr^{326}$, or wild-type μ receptor were stained with the μ-receptor antibody, suggesting that the mutations did not markedly alter the receptor proteins. The

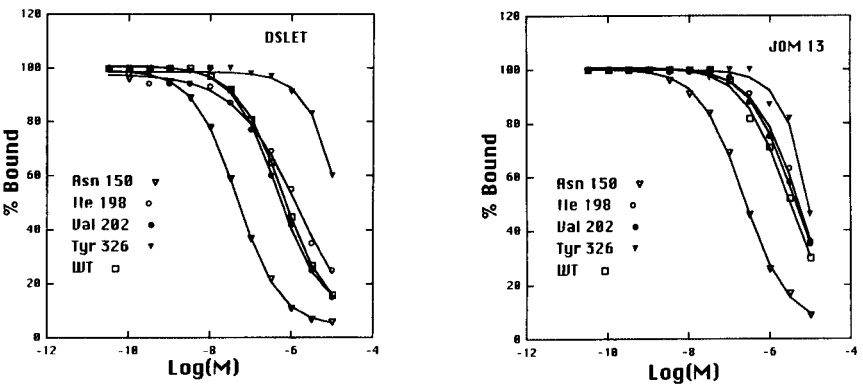


**FIG. 4.** Competition curves of the μ-receptor mutants and wild-type (WT) receptor for the δ agonists DSLET and JOM-13.

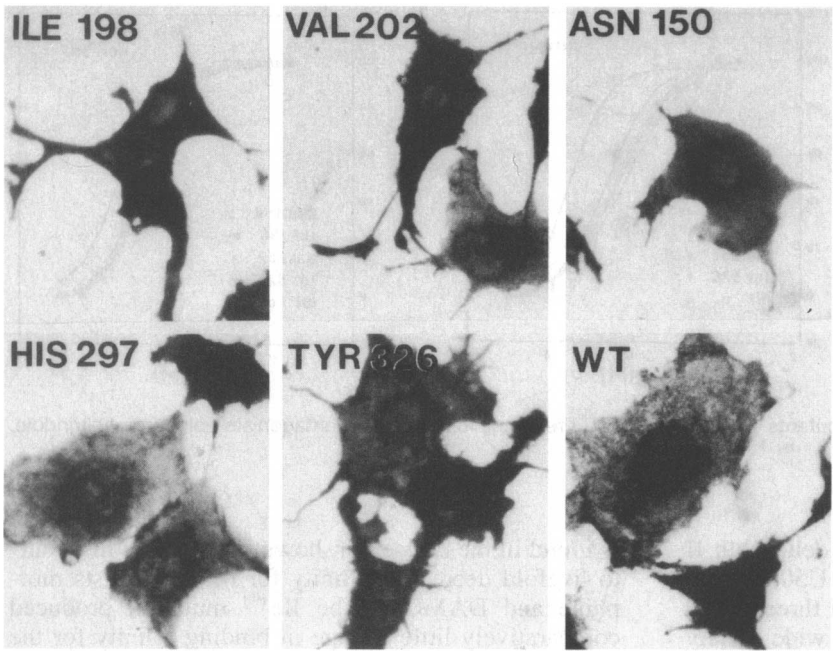


**FIG. 5.** Immunohistochemical $\mu$-receptor staining of COS-1 cells transiently transfected with either the $\mu$-receptor mutants or wild-type (WT) receptor. All staining was blocked by preabsorption of the antibody with the fusion protein to which the antibody was generated (data not shown). These results suggest that the immunohistochemical staining is specific and that all the mutant receptors and wild-type $\mu$ receptors were expressed.

immunohistochemical staining is specific and can be completely blocked by preabsorption of the antibody with the peptide to which the antibody was raised.

## DISCUSSION

The results of this study demonstrate the following: First, $His^{297}$ in TM6 is critical for opioid-receptor binding. Mutation of $His^{297}$ to Ala results in a complete loss of opioid-receptor binding. Second, mutation of $Asn^{150}$ in TM3 produces a selective increase in binding affinities for agonists with no change in antagonists. This novel increase in affinity by mutation of the $Asn^{150}$ residue to Ala suggests that it may normally hinder the binding of opioid agonists, and its effects on $\mu$, $\delta$, and $\kappa$ agonists suggest it may have a similar function in the other opioid receptors. Third, an additional residue has been identified in TM7 ($Tyr^{326}$) that may be critical for opioid-receptor binding, affecting $\mu$, $\delta$, and $\kappa$ agonists and antagonists, both opioid peptides and alkaloids.

The $His^{297}$ to Ala mutation produces the most dramatic loss of opioid-receptor binding, resulting in no detectable binding with [$^3$H]DAMGO, [$^3$H]bremazocine, or [$^3$H]EKC. This loss cannot be attributed to a lack of $\mu$ protein expression, as immunohistochemical results demonstrate the presence of the $\mu$-receptor protein in transfected cells. These findings are consistent with a previous study (Surratt et al., 1994) and suggest that $His^{297}$ may be an important residue in determining ligand–receptor interactions. The $His^{297}$ residue is invariant across the $\mu$, $\delta$, and $\kappa$ receptors and lies in a region of high amino acid homology. In TM6, it is surrounded by a large number of aliphatic nonpolar amino acids. This enhances the electronic interactions between the ligand and receptor. By orienting the positively charged nitrogen of the opioid alkaloids near the negatively charged aspartate in TM3, $His^{297}$ may provide the critical hydrogen bonding necessary for opioid-receptor binding. With $Asp^{147}$ in TM3 and $His^{297}$ in TM6 serving as key anchor points, $Asn^{150}$ and $Tyr^{326}$ in TM3 and 7 provide additional sites of hydrogen bonding and further define the opioid-receptor binding pocket.

In contrast to the total loss of receptor binding observed with the $His^{297}$ mutation, changes of $Asn^{150}$ to Ala or of $Tyr^{326}$ to Phe produce markedly different effects on ligand affinities. The enhanced agonist affinities observed with the $Asn^{150}$ mutation may be due to a reduction of the volume near the critical $Asp^{147}$ that lies one helix turn above this Asn. This may allow agonists such as morphine, DAMGO, and fentanyl to lie closer to the negatively charged $Asp^{147}$, increasing the strength of ionic interaction to result in an increased ligand affinity. The increased binding affinity is limited to agonists and is not seen with the opiate antagonists naltrexone, naloxone, CTAP, naltrindole, or nor-BNI. Similar effects have been reported with mutation of the Asp residue in TM2 of the $\mu$ and $\delta$ receptor, where changes in affinity are limited to $\mu$ and $\delta$ agonists and do not extend to antagonists (Kong et al., 1993; Surratt et al., 1994). In contrast, the $Tyr^{326}$ mutation resulted in a reduced affinity for a wide range of opioid alkaloids and peptides, possibly due to a loss of hydrogen bonding.

Another observation with regard to the $Asn^{150}$ mutation that is interesting is the difference in DAMGO affinity when determined directly with [$^3$H]DAMGO

in the form of saturation studies, and in competition studies with [$^3$H]bremazocine as the labeling ligand. This difference in apparent affinity (1.23 vs. 51.75 n*M*) suggests that the high-affinity [$^3$H]DAMGO binding site may be different from those DAMGO displaces when the receptor is labeled by bremazocine.

Some of the differences observed between agonists and antagonists with regard to the Asn$^{150}$ mutation may be related to the structure of the naltrexone-derived compounds. Modeling of these alkaloids suggests that these ligands may reside higher in the binding cavity (above the Asn) than morphine and are unable to interact with Asn$^{150}$ in TM3. Consistent with the differential effects on agonists and antagonists observed with the Asn$^{150}$ mutation, [$^3$H]bremazocine showed the smallest change in affinity, which may be related to the finding that it acts as an antagonist at μ-binding sites. On the other hand, the Asn$^{150}$ mutations may alter the coupling of the μ receptor to G proteins. Whereas the present study cannot differentiate between these alternative hypotheses, future experiments must examine receptor efficacy in stably transfected mutant cell lines.

The magnitude of the changes in receptor binding affinity varies markedly with the specific ligand tested and is likely dependent on the structure of the alkaloid or peptide, the overall topology of the receptor, and the specific mutation that has been introduced. For example, with the Asn$^{150}$ mutation, a 20-fold increase in binding affinity is seen with fentanyl, compared with the 10-fold differences observed with morphine, DAMGO, and DSLET. These effects may be related to specific features of fentanyl that differentiate it from morphine. By placing the amide carbonyl of fentanyl in proximity to the His$^{297}$ in TM6 and the quaternary nitrogen near Asp$^{147}$ of TM3, the phenyl ring of fentanyl lies immediately adjacent to the Asn of TM3. Replacement of this Asn with Ala allows a more favorable steric and electronic interaction between the ligand and receptor and henceforth a larger increase in relative affinity.

As seen with the Asn$^{150}$ mutation, altering the Tyr$^{326}$ residue to Phe results in a broad range of affinity changes in μ, δ, and κ opioids. Of the antagonists tested, nor-BNI shows the smallest change (2.5-fold) in affinity, with seven- to 10-fold differences observed with naltrexone, naloxone, CTAP, and naltrindole. These results suggest that nor-BNI may interact with different or multiple domains of the receptor to achieve high-affinity binding. Receptor chimera studies suggest that the top of TM6 and the third extracellular loop may be particularly important for nor-BNI binding (Hjorth et al., 1995; Meng et al., 1995; Xue et al., 1995). Differences are also seen between the selective peptides such as DAMGO that show no binding with the Tyr$^{326}$ mutation and the nonselective benzomorphans, bremazocine, and EKC, whose binding affinities are unaltered by this mutation. As mutation of Tyr$^{326}$ to Phe maintains the aromatic configuration of the residue, the effects observed are most likely due to a loss of hydrogen bonding.

In examining the opioid peptides, β-endorphin$_{1-31}$ shows small changes in binding affinity with these single amino acid mutations compared with the enkephalin analogues DAMGO and DSLET. For instance, the Asn$^{150}$ and Tyr$^{326}$ mutations produce only three- or eightfold changes in β-endorphin$_{1-31}$ binding affinity, with no changes observed in the Ile$^{198}$ or Val$^{202}$ mutations when compared with the wild-type receptor. In contrast, a 10–20-fold change in binding affinity is seen with DAMGO and DSLET with the Asn$^{150}$ and Tyr$^{326}$ mutations and the fivefold change in binding affinity of DAMGO found with the Ile$^{198}$ mutation. These differences may be related to the size and flexibility of β-endorphin$_{1-31}$ that allow it to assume multiple conformations and interact with multiple domains of the μ receptor, possibly including the extracellular loops. In contrast, DAMGO and DSLET are smaller, more rigid peptides that may interact at more limited regions of the μ receptor.

That mutations such as Tyr$^{326}$ that are relatively deep within the binding cavity formed by the transmembrane domains can have effects on smaller peptides such as DAMGO, DSLET, and dynorphin$_{1-13}$, as well as on the larger β-endorphin$_{1-31}$, suggests these peptides not only interact with the extracellular loops of the receptor, but likely extend to some extent within the binding cavity itself that is formed by the seven transmembrane helixes. Similar effects have been observed with other peptide receptors, suggesting that peptides not only interact at the extracellular surface, but also extend within the receptor to interact deep within the receptor cavity (Krystek et al., 1994).

Of the mutations designed to examine μ/δ selectivity, only the Ile$^{198}$ mutation to Val resulted in any change in μ- or δ-ligand affinity. Substitution of Val$^{202}$ in TM4 to the corresponding residue in the δ receptor (Ile) resulted in no change in the binding profile when compared with the wild-type μ receptor. Substitution of the Ile$^{198}$ to the corresponding residue of the δ receptor (Val) produced a four- to fivefold reduced affinity for morphine and DAMGO and an approximately twofold decrease in DSLET binding affinity. Few changes are seen in the affinities of δ- and κ-specific ligands to suggest an altered receptor selectivity.

Similar to the results observed with the Asn$^{150}$ mutation, the Ile$^{198}$ mutation resulted in little change in binding affinity for large peptides such as β-endorphin$_{1-31}$ and dynorphin$_{1-13}$, but produced two- to fivefold decreases in affinity for the smaller opioid peptides DSLET and DAMGO. Larger peptides such as β-endorphin$_{1-31}$ and dynorphin$_{1-13}$ likely interact more significantly with extracellular domains of the receptor, so that small changes in a helix may not affect the overall binding affinity of the ligand. Smaller peptides, such as DAMGO, whose phenyl rings can potentially lie within the binding cavity defined by the transmem-

brane domains, may be more dramatically affected given that there are fewer total sites of interaction.

Although TM4 is the least conserved of the transmembrane domains, it may not be integral in conveying receptor selectivity. Recent opioid-receptor chimera studies suggest that TM6 and extracellular loop 3 may be critical in imparting a $\delta$-receptor profile for all $\delta$ ligands, with extracellular loop 2 also necessary for the $\delta$-selective peptides (Meng et al., 1995). The effects observed here with the $Ile^{198}$ mutation in TM4 may, therefore, reflect more subtle interactions of morphine, DAMGO, DSLET, and naloxone within the $\mu$-receptor pocket and not those related to receptor selectivity.

What emerges from studies of chimeric opioid receptors (Kong et al., 1994; Wang et al., 1994; Xue et al., 1994, 1995; Fukuda et al., 1995; Hjorth et al., 1995; Meng et al., 1995; Minami et al., 1995; Onogi et al., 1995) and the mutagenesis results described here is that there may be residues that converge across the opioid receptors that are important in defining the "message" portion of an opioid ligand (Schwyzer, 1977). These residues are likely to be conserved across the opioid receptors, present within the transmembrane domains, and mutation of these amino acids will result in changes in affinity to a broad spectrum of opioid ligands (e.g., $Asn^{150}$, $His^{297}$, and $Tyr^{326}$). The extracellular loops, on the other hand, may define the "address" with specific residues serving to attract particular classes of peptide ligands, with others functioning to reject unfavored ligands. The second and third extracellular loops of the $\mu$ and $\kappa$ receptors are excellent examples of domains that may serve to interpret the address portion of the ligand. In the $\kappa$ receptor, for instance, the negatively charged amino acids attract the larger positively charged prodynorphin peptides and reject the smaller uncharged Met- and Leu-enkephalin peptides (Mansour et al., 1995*c*).

In summary, the results of this study provide a better understanding of the opioid-receptor binding pocket and how opioid peptides and alkaloids interact to initiate the conformational changes that will ultimately result in G protein coupling and signal transduction. $His^{297}$ in TM6 is critical for opioid-receptor binding. $Asn^{150}$ in TM3 and $Tyr^{326}$ in TM7 further define the binding pocket. As these amino acids are invariant across the opioid receptors and mutation of these residues results in changes in a broad spectrum of opioid drugs, the relevance of these residues may not be limited to the $\mu$ receptor and may apply to the $\delta$ and $\kappa$ receptors as well. Further mutagenesis studies focused on the $\delta$ and $\kappa$ receptors will be necessary to determine the generalizability of these results. Amino acid residues such as $Ile^{198}$ in TM4 provide more subtle interactions, modulating the affinity of select opioid agonists and antagonists. These effects are dependent on the precise structure of the ligand, its planar orientation within the receptor pocket, and the local environment formed by the transmembrane domains.

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