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Studies on μ and δ opioid receptor selectivity utilizing chimeric and site-mutagenized receptors

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ABSTRACT **Opioid receptors are members of the guanine nucleotide binding protein (G protein)-coupled receptor family. Three types of opioid receptors have been cloned and characterized and are referred to as the δ, κ, and μ types. Analysis of receptor chimeras and site-directed mutant receptors has provided a great deal of information about functionally important amino acid side chains that constitute the ligand-binding domains and G-protein-coupling domains of G-protein-coupled receptors. We have constructed δ/μ opioid receptor chimeras that were expressed in human embryonic kidney 293 cells in order to define receptor domains that are responsible for receptor type selectivity. All chimeric receptors and wild-type δ and μ opioid receptors displayed high-affinity binding of etorphine (an agonist), naloxone (an antagonist), and bremazocine (a mixed agonist/antagonist). In contrast, chimeras that lacked the putative first extracellular loop of the μ receptor did not bind the μ-selective peptide [D-Ala^2,MePhe^4,Gly^5-ol]enkephalin (DAMGO). Chimeras that lacked the putative third extracellular loop of the δ receptor did not bind the δ-selective peptide, [D-Ser^2,D-Leu^5]enkephalin-Thr (DSLET). Point mutations in the putative third extracellular loop of the wild-type δ receptor that converted vicinal arginine residues to glutamine abolished DSLET binding while not affecting bremazocine, etorphine, and naltrindole binding. We conclude that amino acids in the putative first extracellular loop of the μ receptor are critical for high-affinity DAMGO binding and that arginine residues in the putative third extracellular loop of the δ receptor are important for high-affinity DSLET binding.**

Morphine, related opioid drugs, and the endogenous opioid peptides activate signal transduction pathways by binding to opioid receptors, which are members of the guanine nucleotide binding protein (G protein)-coupled receptor family (1–3). Three major types of opioid receptor, δ, κ, and μ, have been cloned and characterized extensively (4–12). The opioid receptor types share ≈60% amino acid sequence identity. The δ, κ, and μ opioid receptors have unique ligand specificities, anatomical distribution, and physiological functions (13). Several studies have indicated that opioid receptor types interact with multiple G proteins (14–16) to regulate adenylyl cyclase, Ca^{2+} channels, and K^+ channels (17, 18).

The aim of this investigation was to determine the domains in the δ and μ opioid receptors that are responsible for type selectivity. The strategy was to construct a series of receptor chimeras and characterize the ability to bind peptide and nonpeptide ligands, including agonists and antagonists, that differed in receptor specificity. It was found that δ and μ opioid receptor selectivity toward [D-Ser^2,D-Leu^5]enkephalin-Thr (DSLET) and [D-Ala^2,MePhe^4,Gly^5-ol]enkephalin (DAMGO), respectively, was mediated by distinct domains located in extracellular loops. Information gained from analysis of receptor chimeras was used to focus site-directed mutagenesis to the third extracellular loop of the δ opioid receptor.

MATERIALS AND METHODS

Construction of Receptor Chimeras. Computer-aided alignment of the nucleotide sequences of the δ and μ opioid receptor cDNAs revealed six domains that have at least 15 identical contiguous nucleotides. These domains are located at the junction of the first intracellular loop and transmembrane domain 2 (TM2), at the junction of TM3 and the second intracellular loop, and in TM3, -5, -6, and -7. Junction sites used in the present studies are shown in Fig. 1. Pairs of complementary oligodeoxyribonucleotides were synthesized (Operon Technologies, Alameda, CA) corresponding to these homologous domains and had the following sequences (written in the 5′ to 3′ direction): TM2+, GCCACCAACATCTACAT; TM2−, ATGTAGATGTTGGTGGC; TM3+, TACTACAACATGTTCAC; TM3−, GTGAACATGTTGTAGTA; TM5+, TGCCGATCCTCATCATCAC; TM5−, GTGATGATGAGGATCGGCA; TM6+, ATGGTGCTGGTGGTC/GGTG; TM6−, CACG/CACCACCAGCACCAT; TM7+, GTTCTTTACGCCTTCCTGG; TM7−, CCAGGCAGGCGTAAAGAAC. Expression plasmids encoding receptor chimeras were constructed by using a two-step recombinant PCR protocol (Fig. 2). Template DNAs were the δ receptor cDNA (4) that we had subcloned into the vector pCR3 (Invitrogen) and the μ receptor cDNA (7) that we subcloned into the pRc/CMV vector (Invitrogen). The pCR3 and pRc/CMV vectors contain T7 and Sp6 RNA polymerase promoters upstream and downstream of the receptor cDNA inserts, respectively, and oligodeoxyribonucleotides corresponding to these promoters were used in combination with the TM+/− receptor oligodeoxyribonucleotides to generate the primary PCR fragments. An aliquot from each primary PCR was analyzed by agarose gel electrophoresis to estimate yield and specificity, and then aliquots were annealed and subjected to secondary PCR by using the T7 and Sp6 promoter oligodeoxyribonucleotides as primers. The secondary PCR product encoding a chimeric opioid receptor was directly ligated without further purification to the mammalian expression vector pCR3 (Invitrogen). All chimeric receptor constructs were fully sequenced as described (19) to verify the location of the junction site and to ensure that no mutations were introduced during synthesis.

Site-Directed Mutagenesis. A double mutation was introduced into the wild-type δ receptor that converted both arginine residues 291 and 292 in the putative third extracellular loop to glutamine (Fig. 1), using a two-step PCR protocol similar to that described above. The sequences of the complementary primers bearing the R291Q/R292Q mutation were

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Abbreviations: DAMGO, [D-Ala^2,MePhe^4,Gly^5-ol]enkephalin; DSLET, [D-Ser^2,D-Leu^5]enkephalin-Thr; G protein, guanine nucleotide binding protein; TM, transmembrane domain(s).

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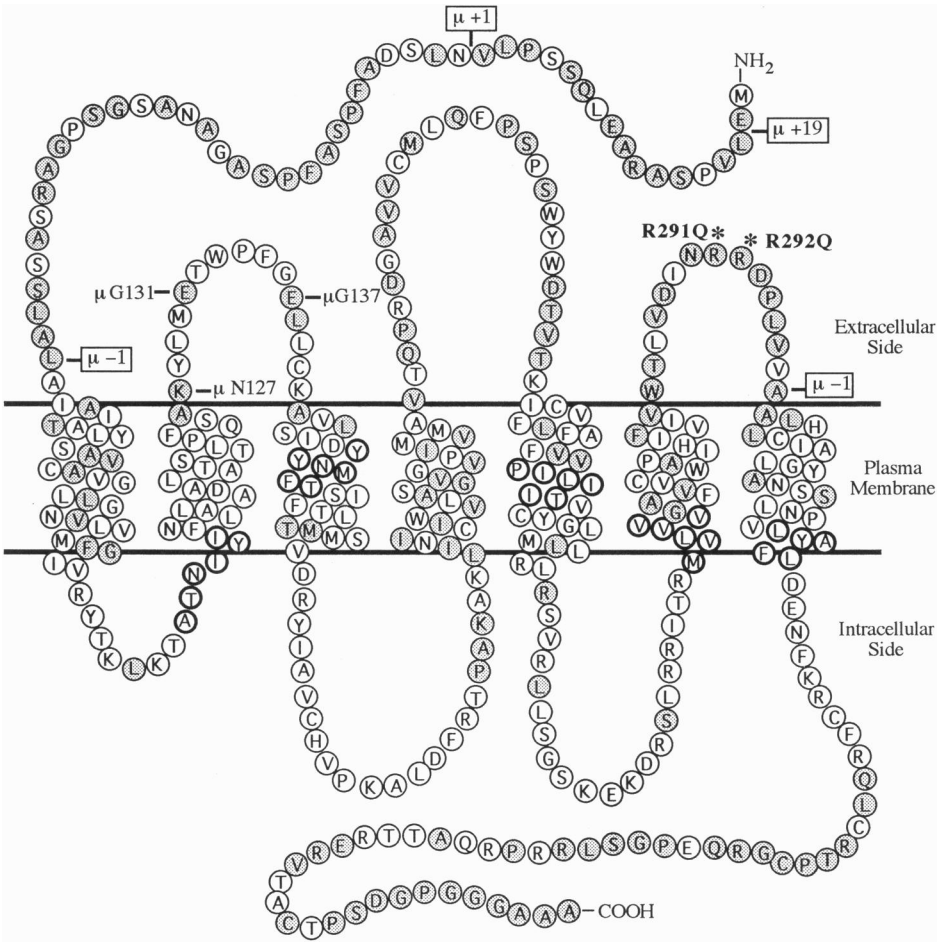

FIG. 1. Proposed transmembrane topology of the mouse δ opioid receptor. Differences in amino acid number resulting from alignment of the μ receptor are shown in boxes at the site of the change (+, insertion; −, deletion of *n* amino acids). Amino acid differences in the putative first extracellular loop that alter the local electrostatic charge of the μ receptor relative to the δ receptor are indicated. Junction sites used to construct δ/μ receptor chimeras are indicated with boldface circles. Other identical amino acids shared by the μ and δ receptors are shown as open circles; amino acids that differ are shaded. Arg-291 and -292 in the putative third extracellular loop of the δ receptor that were altered to glutamine are indicated with asterisks.

5′-GGACATCAATCAGCAGGACCCACTTGT-3′ and 5′-ACAAGTGGGTCCTGCTGATTGATGTCC-3′.

Transfection and Radioligand Binding Assays. Human embryonic kidney 293 cells (American Type Culture Collection CRL 1573) were transfected by the calcium phosphate method as described (20). Cells stably expressing opioid receptors were selected in medium containing G418 (0.5 mg/ml) (GIBCO/BRL). Opioid receptor binding assays (21) were conducted in duplicate on membrane preparations resuspended in 50 mM Tris·HCl/1 mM Na_4EDTA, pH 7.4, buffer using [^{3}H]bremazocine (New England Nuclear; specific activity, 20–30 Ci/mmol; 1 Ci = 37 GBq) and 10 μM naloxone to define nonspecific binding. Competition analysis was performed with [^{3}H]bremazocine at concentrations equal to the dissociation constant and nine different concentrations of competing ligands. After a 1-h incubation at 22°C, binding assays were terminated by filtration through Whatman GF/B filters that had been presoaked in 0.1% bovine serum albumin. Filters were soaked in BCS liquid scintillation mixture (Amersham) prior to determination of filter-bound radioactivity with a Beckman LS 1701 scintillation counter. Receptor binding data were analyzed by nonlinear regression using the PRISM program (GraphPad Software, San Diego).

RESULTS

A series of δ/μ opioid receptor chimeras were constructed to determine which receptor domains are responsible for mediating receptor-type selectivity (Fig. 3). The ability of the chimeras to bind peptide and nonpeptide ligands, including agonists and antagonists with varying receptor selectivity, was then assessed.

Bremazocine is a universal ligand of the benzomorphan series with high affinity for δ, μ, and κ opioid receptors (22). Nonlinear regression analysis of saturation curves revealed that bremazocine had a slightly greater affinity for μ compared to δ receptors (K_D = 0.8 and 2.8 nM, respectively). Similar values for the cloned μ and δ receptors have been reported (23). The dissociation constants of [^{3}H]bremazocine for the receptor chimeras ranged from 0.5 to 5.3 nM (Table 1), indicating that high-affinity binding sites for bremazocine were maintained in each of the chimeras.

The affinities of etorphine (an alkaloid agonist), naloxone (an alkaloid antagonist), DSLET (a δ receptor-selective peptide agonist), and DAMGO (a μ receptor-selective peptide agonist) for the wild-type and chimeric receptors were determined by competition analysis (Table 2). Etorphine displayed equal affinities toward wild-type μ and δ receptors (K_i = 20 nM) and had slightly lower inhibition constants for most of the receptor chimeras, ranging from 3 to 10 nM. The exception was the D7M variant, for which the K_i of etorphine increased to 45 nM. Naloxone bound to μ receptors with a 20-fold higher affinity than to δ receptors (K_i = 0.5 and 12 nM, respectively), while inhibition constants for all but one of the receptor chimeras were intermediate (2–5 nM). Surprisingly, naloxone

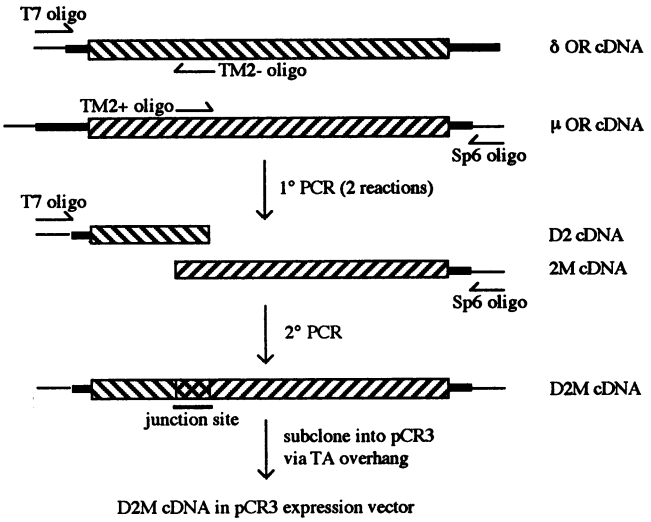

FIG. 2. Strategy for construction of δ/μ receptor chimeras. Oligodeoxyribonucleotides are shown by directional arrows; thin horizontal lines, vector sequences; thick horizontal lines, untranslated regions; hatched areas, receptor open reading frames; cross-hatched section, junction site in TM2. Two separate primary PCRs were carried out to synthesize DNA encoding the D2M chimera. Aliquots of the primary PCRs were mixed and used for a secondary PCR with the T7 and Sp6 polymerase oligodeoxyribonucleotides. Secondary PCR products were directly ligated into the pCR3 expression plasmid.

bound poorly to the M2D variant (K_i = 124 nM). These data indicated again that, with a couple of exceptions, the binding sites for etorphine and naloxone, like those for bremazocine, were similar in the chimeras and wild-type receptors.

In contrast to bremazocine, etorphine, and naloxone, both selective peptide agonists exhibited a marked preference for certain chimeras. DAMGO bound selectively to wild-type μ receptors with high affinity (K_i = 3 nM), as reported (7–10, 23). The affinity for the D2M construct was lowered 10-fold to 30 nM (Table 2). DAMGO did not bind to the reciprocal chimera, M2D, indicating that amino acids in the domain extending from the N terminus to the putative beginning of TM2 in the μ receptor play a role in, but are not sufficient for, formation

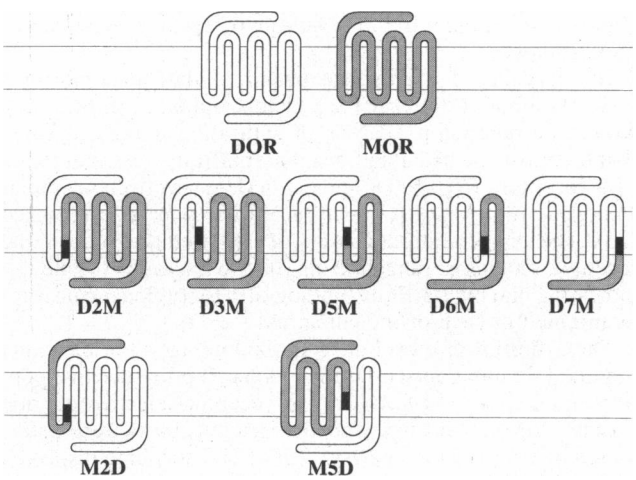

FIG. 3. Schematic representation of structures of wild-type μ and δ opioid receptors and μ/δ receptor chimeras. DOR, wild-type δ opioid receptor; MOR, wild-type μ opioid receptor. Designations for chimeras indicate the origin of the N-terminal domain on the left and the C-terminal domain on the right (μ = M, δ = D), separated by a number that refers to which transmembrane helix is the site of the junction. Open areas, δ opioid receptor sequences; shaded areas, μ opioid receptor sequences; solid areas, junction sites.

Table 1. Affinity of [^{3}H]bremazocine for wild-type, chimeric, and mutated opioid receptors

Receptor	K_d, nM
Wild-type μ	0.8 ± 0.3
Wild-type δ	2.8 ± 0.4
D2M	0.6 ± 0.2
D3M	1.1 ± 0.4
D5M	0.5 ± 0.01
D6M	0.6 ± 0.2
D7M	1.8 ± 1.0
M2D	3.5 ± 1.0
M5D	5.3 ± 2.8
δRR>QQ	0.9 ± 0.4
δVRR>AQQ	5.4 ± 0.5

Data represent means ± SEM of two or three experiments.

of a high-affinity DAMGO binding site. Further substitution of δ receptor sequence into the μ receptor background (proceeding from D2M to D3M) led to a loss in the ability of the chimera to bind DAMGO (K_i > 1000 nM). Consistent with this finding, additional substitution of δ receptor sequences into the chimeras—e.g., D5M, D6M, and D7M—also totally abolished DAMGO binding. In contrast, DAMGO bound to the M5D chimera with a K_i of 23 nM. These results suggested that a major determinant of DAMGO high-affinity binding resided in a domain including TM2, the first extracellular loop, and the proximal portion of TM3.

DSLET displayed a marked preference for receptor chimeras that contained TM6, TM7, and the intervening third extracellular loop of the δ receptor, as was evident from the 50-fold increase in affinity when progressing from D6M to D7M (Table 2). Consistent with this observation, DSLET also exhibited high-affinity binding to the M2D and M5D constructs, which also contained this domain of the δ receptor. The affinity of DSLET for the D2M, D3M, D5M, and D6M chimeras was negligible.

The putative third extracellular loops of the δ and μ receptor are highly divergent (Fig. 1). One particularly striking difference is the pair of vicinal arginine residues at positions 291 and 292 in the δ receptor that are replaced by glutamic acid and threonine, respectively, in the μ receptor. Mutation of both arginines to glutamine in the δ receptor led to a dramatic decrease in the affinity of DSLET (K_i > 1000 nM), while not affecting the affinity of bremazocine or etorphine (Fig. 4; Tables 1 and 2). The δ-selective alkaloid antagonist naltrindole also displayed high-affinity binding to the R291Q/R292Q variant δ receptor, similar to the binding to the wild-type and D7M chimeric receptors (Fig. 4; Table 2). The importance of the vicinal arginines for DSLET binding was also confirmed with another δ receptor construct in which, in addition to the double arginine-to-glutamine mutation, Val-287 was changed to alanine. Again, DSLET displayed negligible affinity for this receptor variant, while bremazocine and etorphine binding were unaffected by the substitutions (Tables 1 and 2). This evidence indicated that Arg-291 and -292 in the putative third extracellular loop play a vital role in the interaction of DSLET with the δ receptor.

DISCUSSION

The major finding of the present study was that amino acids in putative extracellular loops of the δ and μ receptors play critical roles in high-affinity binding of DSLET and DAMGO, respectively. The domain delineated by TM2, the first extracellular loop, and the proximal amino acids in TM3 of the μ receptor are important for DAMGO binding, while Arg-291 and -292 in the putative third extracellular loop of the δ receptor are important for DSLET binding. In contrast, these

Table 2. Summary of ligand affinities for wild-type, chimeric, and mutated opioid receptors

Receptor	K_i, nM				
	DSLET	DAMGO	Etorphine	Naloxone	Naltrindole
Wild-type μ	>1000	3.0 ± 0.1	21 ± 9	0.5 ± 0.2	ND
Wild-type δ	33 ± 9	>1000	20 ± 5	12 ± 4	2.0 ± 0.3
D2M	>1000	32 ± 1	6.3 ± 3.6	2.2 ± 0.9	ND
D3M	670 ± 170	>1000	2.7 ± 0.4	5.2 ± 1.7	ND
D5M	>1000	>1000	5.3 ± 2	3.6 ± 0.2	ND
D6M	850	>1000	10 ± 5	3.0 ± 0.1	ND
D7M	15 ± 4.6	>1000	45 ± 2	ND	5.8 ± 0.1
M2D	43 ± 22	>1000	7.2 ± 1.6	124 ± 6	ND
M5D	16 ± 2.7	23 ± 3.5	ND	4.2 ± 0.9	ND
δRR>QQ	>1000	>1000	7.5 ± 4.4	ND	1.5 ± 0.1
δVRR>AQQ	>1000	>1000	30	ND	ND

ND, not determined. Data are means ± SEM of 2–5 experiments.

extracellular domains could be swapped without affecting high-affinity binding of bremazocine, etorphine, and naloxone.

There are seven amino acids that differ between the D2M and D3M receptor chimeras (Fig. 1). These differences account for a >30-fold higher affinity of DAMGO for the D2M receptor relative to D3M. Three μ/δ amino acid substitutions alter the electrostatic charge of the first extracellular loop: Asn-127 is lysine in the δ receptor, Gly-131 is glutamic acid in the δ receptor, and Thr-137 is glutamic acid in the δ receptor. The other μ/δ amino acid substitutions between the junction sites in D2M and D3M are more conservative: Val-126 (μ) is alanine (δ), Ile-138 (μ) is leucine (δ), Ile-142 (μ) is alanine (δ), and Ile-144 (μ) is leucine (δ). Site-directed mutagenesis will be necessary to distinguish which of these substitutions are major determinants of the receptor selectivity displayed by DAMGO.

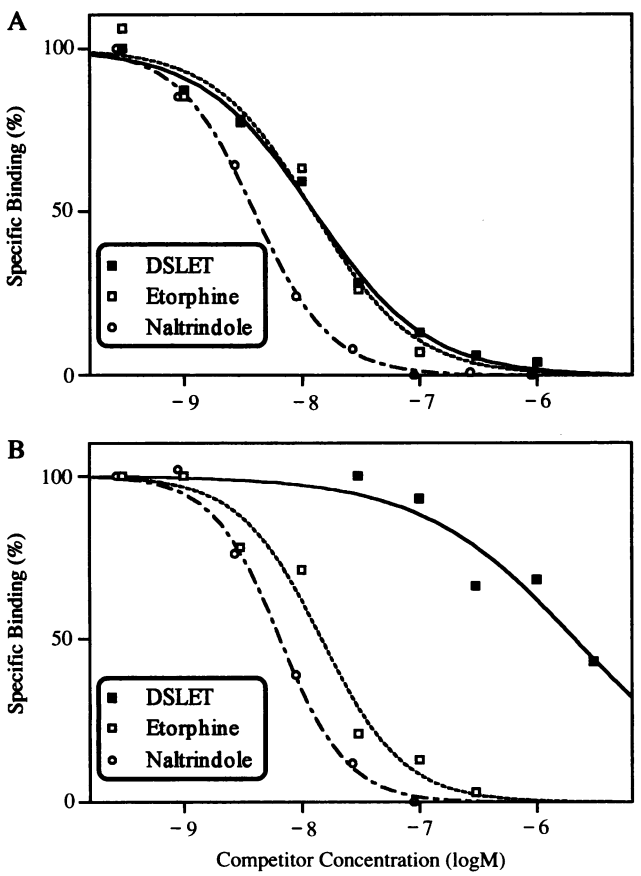

FIG. 4. Competition of [^{3}H]bremazocine binding to wild-type δ and R291Q/R292Q double mutant δ receptors by DSLET, etorphine, and naltrindole. Membranes from human embryonic kidney 293 cells stably expressing either wild-type δ (*A*) or R291Q/R292Q mutant δ (*B*) receptor were used. Data points are averages of duplicate determinations; a representative data set is shown.

The striking difference in affinity of DSLET for the D6M and D7M receptor chimeras suggested that an important component of the binding site for the δ-selective peptide resided in TM6, TM7, or the intervening third extracellular loop. We reasoned that of the 23 amino acid substitutions in this region (Fig. 1), the vicinal arginines at positions 291 and 292 of the δ receptor (which are replaced with glutamic acid and threonine, respectively, in the μ receptor) might play a significant role as counterions to the negatively charged C terminus of DSLET. This supposition was based on structure/activity studies, which have found that C-terminal-amidated peptides display a concomitant decrease in affinity for δ receptors and an increase in affinity for μ receptors (cf. ref. 24). In agreement with our prediction, double mutation of both arginines to glutamine in the wild-type δ receptor abolished high-affinity DSLET binding, while not affecting bremazocine, etorphine, and naltrindole binding significantly. It is also possible that the arginines do not interact with DSLET directly but are in contact with other amino acids in the δ receptor that contribute to the conformation of the binding site. Further analysis is necessary to determine whether both Arg-291 and Arg-292 play a role in high-affinity DSLET binding. In addition, it is not known at present whether a positive charge at position 291, 292, or both is sufficient (e.g., whether lysine can substitute for arginine).

The data from these studies add to the growing body of knowledge regarding the constituents of opioid receptor binding sites. Previous mutagenesis experiments have highlighted the importance of Asp-95 in TM2 of the δ receptor, and the corresponding Asp-114 in the μ receptor, for high-affinity selective agonist binding (25, 26). Mutation of Asp-147 in TM3 and His-297 in TM6 of the μ receptor inhibited both agonist and antagonist binding (26). In addition to these charged amino acids within TM, analysis of μ/κ and δ/κ receptor chimeras revealed that the second extracellular loop of the κ receptor was required for high-affinity binding of prodynorphin-derived peptides—i.e., dynorphin-(1–17), dynorphin-(1–13), α-neoendorphin, and dynorphin B (27–29). Evidence has also been provided that the binding site for antagonists in the κ opioid receptor differs substantially from the antagonist site of the μ and δ opioid receptors (30). The N terminus of the κ opioid receptor was found to be necessary for high-affinity naloxone binding and for reversal of κ agonist-mediated inhibition of forskolin-stimulated cAMP accumulation by naloxone. In contrast, Glu-297 in the putative third extracellular loop of the κ receptor plays a major role in binding the κ-selective antagonist, norbinaltorphimine (31). Meng *et al.* (29) and Fukuda *et al.* (32) reported recently that a major

binding determinant for δ-selective peptides resides in the region spanning TM5–TM7 of the δ receptor, in excellent agreement with our present data regarding the role of the arginine residues in the putative third extracellular loop. Our finding on the importance of the putative first extracellular loop for DAMGO binding with μ/δ receptor chimeras has also been recently reported independently (32, 33). In contrast, Xue *et al.* (34) found that the third extracellular loop of the μ receptor was important for agonist selectivity using μ/κ receptor chimeras. This discrepancy was clarified recently with the interesting finding that DAMGO distinguishes between μ and δ opioid receptors at a site different from that for the distinction between μ and κ opioid receptors (35).

An inherent difficulty in using analysis of receptor chimeras and site-directed mutagenesis is the ability to distinguish between specific local effects on receptor–ligand interactions and changes in overall receptor conformation. We cannot rule out effects on receptor tertiary structure due to mutations or chimera construction in the absence of biophysical data to the contrary. However, the selective effects on DSLET and DAMGO binding, coupled with the absence of effects on bremazocine, etorphine, naloxone, and naltrindole binding, suggest that the conformations of the chimeric and mutated receptors have not been grossly altered.

There are biochemical and immunological data that members of the G-protein-coupled receptor family share topographical features, such as an extracellular N terminus, an intracellular C terminus, and seven transmembrane helices connected by loops with unknown structures. The data presented in this report, in conjunction with evidence from other laboratories, suggest that the binding sites of δ and μ receptors for DSLET and DAMGO, respectively, are formed from both transmembrane and nonidentical extracellular components. Until high-resolution experimental data are obtained from crystallography, insights from analysis of receptor chimeras and mutagenesis will provide information for molecular modeling and computer simulation of opioid receptors. The molecular mechanisms involved in receptor activation and G-protein coupling triggered by agonist engagement of the opioid receptor binding site remain as long-term goals of these studies. It is hoped that an understanding of opioid receptor structure and function will lead to the development of therapeutic agents for pain management as well as other clinical situations.

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