Involvement of Trp-284, Val-296, and Val-297 of the Human δ-Opioid Receptor in Binding of δ-Selective Ligands*

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Given the high homology in amino acid sequence between the δ-opioid receptor and the two other types (μ and κ), distinct residues in this receptor may confer its selectivity to some ligands. In order to identify molecular determinants in the human δ receptor responsible for the selectivity of δ-selective ligands, two different δ/μ chimeras were constructed. In the first one, the δ sequence from the top of transmembrane 5 to the C terminus was replaced by the equivalent μ sequence, and in the second one, 13 consecutive residues in the third extracellular loop region of the δ receptor were replaced by the μ counterpart. These two chimeras retained the ability to bind the nonselective bremazocine but completely lost the ability to bind different δ-selective ligands. These results suggested that the region of the third extracellular loop of the δ receptor is crucial for the type selectivity. Furthermore, an alanine scan was performed by site-directed mutagenesis of 20 amino acids located in or proximal to the third extracellular loop. Among all the point mutations, only mutations of Trp-284, Val-296, and Val-297 significantly decreased the binding of δ-selective ligands tested. Moreover, combined mutation of Trp-284, Val-296, and Val-297 considerably decreased the affinities of the receptor for δ-selective ligands compared with the single point mutations. These findings suggest that Trp-284, Val-296, and Val-297 are crucial residues involved in the δ receptor type selectivity.

Opioid receptors are cell surface glycoproteins that constitute specific binding sites for a variety of compounds used for treating pain. Extensive pharmacological studies led to the definition of three opioid receptor types, μ, κ, and δ (1, 2). Indeed, the availability of highly selective ligands permitted a better characterization of these three types of receptors. In the past few years, cDNAs encoding the μ, κ, and δ of different species have been cloned (reviewed in Ref. 3). However, human receptors represent the ultimate therapeutic targets. Thus, the cloning of human μ, κ, and δ cDNAs (4–6) provided particularly relevant tools for opioid drug discovery.

Analysis of the predicted amino acid sequences of the three human opioid receptors has shown that these receptors have characteristics common to the guanine nucleotide-binding regulatory protein-coupled receptors with an extracellular N-terminal domain, a cytoplasmic C-terminal domain, and seven putative transmembrane domains (7, 8). Moreover, given their high degree of identity (approximately 60%) with the highest similarity in the transmembrane domains and intracellular loops, distinct residues in these receptors may confer their selectivity to some ligands. Binding sites of selective ligands most probably reside, at least in part, in the divergent regions that are the extracellular loops and the N-terminal domain. The C-terminal domain is also a divergent region, but its involvement in binding of ligands is unlikely.

All of the opioid analgesics currently used clinically interact with the μ receptor and are known to induce severe side effects and addiction (9, 10). These major disadvantages were less pronounced with the use of a number of agonists selective for the κ receptor which also display analgesic properties (11–13), suggesting that some effective analgesics with minimal side effects, selectively interacting with one of the three types of opioid receptors, may be developed. Particularly, δ receptors that bind enkephalin-like peptides with high affinity have been proposed to mediate analgesia (14–16) and to induce weak opiate physical dependence (17, 18), making them an interesting target for drug design.

In an effort to elucidate the binding sites of δ-selective ligands, construction of chimeric δ/μ receptors and site-directed mutagenesis on the human δ-opioid receptor cDNA were performed. The ability of these mutated receptors to bind δ-selective ligands was evaluated by studying competition of [3H]bremazocine binding with four δ-selective ligands, i.e. SNC-80, DPDPDE, 1 deltorphin II, and naltrindole. Three hydrophobic residues, Trp-284, Val-296, and Val-297, were identified as essential residues for normal binding of δ-selective ligands.

Experimental Procedures

pDNA3 vector was purchased from Invitrogen. 293S cells were obtained from Cold Spring Harbor. [3H]Bremazocine was purchased from DuPont NEN. Naloxone and naltrindole are from Research Biochemicals Int. (RBII). SNC-80 is from Torris Cookson (Bristol, United Kingdom). DPDPDE and deltorphin II were purchased from Bachem Bioscience.

DNA Construction and Site-directed Mutagenesis—Both δ and μ receptor cDNAs were subcloned in pDNA3 expression vector (Invitrogen) at EcoRI + XhoI sites and at HindIII site, respectively. δ1–214/μ215–381 chimera was created using two distinct BglII restriction sites conserved in the δ and μ cDNA containing vector. The first site is located in the sequence encoding Lys-214 at the most C-terminal part of the second extracellular loop of the δ receptor and the second site is located in the vector. The 1.8-kilobase fragment generated by BglII digestion of μ encoding plasmid was ligated with the 5-kilobase fragment generated by the same digestion of the δ encoding plasmid. δ1–288–300 chimera and point mutations were created using Clontech site-directed mutagenesis kit which is based on the method of Deng and Nickoloff (19). Mutations and constructions were verified and confirmed by restriction enzyme mapping and DNA sequencing.

Cal Culture and Transfer—Human embryonic kidney 293S cells (Cold Spring Harbor) were grown as monolayers in 150-mm Petri dishes containing Dulbecco’s modified Eagle’s medium supplemented with

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The abbreviations used are: DPDPDE, cyclic [d-penicillamine]d-d-penicillamine; TM, transmembrane domain; hDOR, human δ-opioid receptor.
RESULTS

In order to identify specific residues involved in binding of \( \delta \)-selective ligands, an alanine scan was performed for 20 amino acids located in or proximal to the third extracellular loop (Fig. 5). These point mutations on the wild-type \( \delta \) receptor were made using site-directed mutagenesis. Saturation binding experiments with \( [\text{H}] \)bremazocine and competition curves with SNC-80, DPDPE, deltorphin II, and naltrindole on the two chimeras demonstrated that their affinities for the four \( \delta \)-selective ligands were considerably decreased (Fig. 4A). A representative experiment of competition binding is shown in Fig. 4B. These results suggest that residues located downstream from the second extracellular loop and more particularly around the third extracellular loop of the hDOR are involved in binding of \( \delta \)-selective ligands.

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three amino acids Trp-284, Val-296, and Val-297 to the binding of δ-selective ligands, two different combinations of point mutations were generated. In one mutant the two valines were replaced with alanines (V296A + V297A), and in the other mutant all three amino acids as well were replaced with alanines (W284A + V296A + V297A). As shown in Table II, these combined mutations did not affect the binding of the nonselective ligand bremazone. Simultaneous mutations of V296A and V297A led to a greater effect on binding of SNC-80 and DPDPE compared with the single point mutations but did not significantly change the binding of deltorphin II and naltrindole. Moreover, combined mutations of W284A, V296A, and V297A greatly decreased the affinities of the receptor for all four δ-selective ligands (Table II). However, this triple point
mutation did not lead to an effect comparable with the chimeras, suggesting that more residues may be involved or that the mutations may be too conservative.

In the chimeras, residues of the δ sequence were replaced for residues of the μ sequence, whereas in the triple point mutant (W284A + V296A + V297A), alanines were used to replaced the δ residues. Thus, to determine if the substitution of alanines for Trp-284, Val-296, and Val-297 account for the smaller effect on binding of selective ligands compared with the chimeras, the same three amino acids were replaced with the equivalent residues of the μ. In the first mutant, only the Trp-284 was replaced with a lysine (W284K). In the other mutant, in addition to W284K mutation, Val-296 and Val-297 were replaced with a glutamic acid and a threonine, respectively (W284K + V296Q + V297T). Nonlinear regression analysis of the [3H]bremazocine saturation curves showed that these two other mutants maintained their ability to bind bremazocine to a comparable extent to the wild-type δ receptor (Table III). However, the mutation of Trp-284 for a lysine produced an effect of over 10-fold on the binding of DPDPE, deltorphin II, and naltrindole but led to a particularly pronounced effect on SNC-80 binding (373-fold). Substitution of μ residues for Trp-

Fig. 3. **Schematic representation of the δ/μ288–300 chimera.** A, amino acid sequence of the wild-type δ receptor in the proposed transmembrane topology. Shaded residues were replaced by the corresponding μ sequence shown by the arrow. The single letter amino acid code is used. B, three-dimensional model of the δ/μ288–300 chimera viewed from the extracellular surface. Parts in red represent δ residues; parts in green represent Ile-289, a μ residue which is conserved in δ receptor, and the purple residue is Asp-290 of the δ receptor that corresponds to a gap in the μ receptor. Only TM’s and extracellular loops backbone are illustrated.
284, Val-296, and Val-297 did not increase the loss of binding for the δ-selective ligands that were observed with the alanine substitutions.

**DISCUSSION**

In the present study, we used chimeric constructs between δ and μ-opioid receptors as an initial approach to locate critical regions involved in the selectivity displayed by the δ receptor. δ1-214/μ215-381 and δ/μ288-300 chimeric receptors allowed us to show that the region of the third extracellular loop is essential to the binding of tested δ-selective ligands. Then, using site-directed mutagenesis of individual residues in or surrounding the third extracellular loop, we have identified Trp-284, Val-296, and Val-297 of the human δ-opioid receptor as important molecular determinants for the normal binding of SNC-80, DPDPE, deltorphin II, and naltrindole. Furthermore, simultaneous mutation of these three hydrophobic residues considerably altered the binding of the four δ-selective ligands. All the chimeric or mutated receptors displayed high affinity binding of bremazocine, a nonselective ligand.

Replacement of Trp-284 by either an alanine or a lysine led to the most important effect on the binding of δ-selective ligands compared with the other point mutations, suggesting that Trp-284 plays a major role in determining the selectivity for ligands. In a recent study, Hjorth and co-workers (24) proposed that Glu-297 of the κ-opioid receptor plays an important role in the κ-selective binding characteristics of nor-binaltorphimine. Interestingly, this residue is located at the corresponding position of Trp-284 of the δ receptor, suggesting that the residue at this position (δ, Trp; μ, Lys; and κ, Glu) may represent a crucial recognition site for opioid ligands. This hypothesis may explain why the δ/μ288-300 chimera has higher affinities for the four δ-selective ligands than the δ1-214/μ215-381 chimera. Indeed, the presence of Trp-284 in the δ/μ288-300 chimera may contribute to restore in part the

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**TABLE 1**

| Ligand       | Kd (nM)  | Ki (nM)   |
|--------------|----------|-----------|
|              | [3H]Bremazocine | SNC-80 | DPDPE | DELT II | NALT |
| Wild-type δ  | 3.05 ± 0.37 | 1.32 ± 0.42 | 4.42 ± 0.79 | 0.95 ± 0.16 | 0.12 ± 0.03 |
| δ1-214/μ215-381 | 2.19 ± 0.09 | >1000 | >1000 | >1000 | 24.24 ± 5.92 |
| δ/μ288-300    | 1.30 ± 0.07 | 954 ± 258 | 633 ± 143 | 614 ± 128 | 8.23 ± 0.71 |

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**Fig. 4.** Binding parameters for wild-type δ and chimeric receptors. A, saturation and competition binding assays were performed as described under “Experimental Procedures.” Data from five distinct saturation and competition experiments were analyzed using the nonlinear least-squares regression curve-fitting program GraphPad Prism. Results of the computer analysis are presented as means ± S.E. K_d is the dissociation constant for [3H]bremazocine binding, and K_i is the dissociation constant for the competitors. B, competition of [3H]bremazocine with SNC-80, DPDPE, deltorphin II, and naltrindole in cells expressing wild-type δ and chimeric receptors. The curves shown are representative of the five distinct experiments conducted in duplicate presented in A.
Molecular Determinants for Selectivity of δ-Opioid Receptor

Previous studies using κ/δ or δ/μ chimeric receptors revealed that δ-selective ligands interact mainly with the C-terminal half of the δ receptor (25, 26) which is in accordance with results presented in this work. Moreover, it has been shown that mutation of Asp-95 located in TM2 of the δ receptor reduced the affinity of the receptor for δ-selective agonists but not for antagonists or nonselective ligands (27). This suggests that binding of different classes of ligands may involve distinct residues. More recently, Wang and co-workers (28) proposed that vicinal arginine residues in the putative third extracellular loop of the δ receptor are important for high affinity binding of δ-serine, leucine, enkephalin-threonine which is a δ-selective ligand. In contrast, in our study, mutation of these two arginines did not affect the binding of δ-selective ligands with the exception of Arg-291 which seems important for the binding of deltorphin II. This discrepancy may be due to the different amino acids chosen to replace the arginines and to the ligand tested.

The sequence analysis suggested that residue Trp-284 is located at the most N-terminal position of the third extracellular loop and that residues Val-296 and Val-297 are located at the extracellular surface of TM7. Based on our three-dimensional model, these three residues are pointing toward the inside of the putative binding pore formed by the seven TMs (Fig.6A). Positions of Trp-284, Val-296, and Val-297 in the sequence of the receptor along with the orientation of TM6 and TM7 in the three-dimensional model suggest that these three residues are involved in the discrimination of δ-selective ligands from agonists and antagonists.

The amino acid sequence of the mutant receptors submitted to an alanine scan is shown in Table I. Saturation and competition binding assays were performed as described under “Experimental Procedures.” Data from three to seven distinct saturation and competition experiments were analyzed using the nonlinear least-squares regression curve-fitting program GraphPad Prism. Results of the computer analysis are presented as means ± S.E. Kᵢ is the dissociation constant for [³H]bremazocine binding and, Kᵢ is the dissociation constant for the competitors. WT, wild type; DELT, deltorphin; NALT, naltrindole.

### Table I

|         | Kᵢ (nM) [³H]Bremazocine | SNC-80 | DPDPE | DELT II | NALT |
|---------|------------------------|--------|-------|---------|------|
| WT hDOR| 3.05 ± 0.37            | 2.33 ± 0.24 | 3.68 ± 0.41 | 0.80 ± 0.17 | 0.06 ± 0.02 |
| A275G  | 0.80 ± 0.11            | 1.25 ± 0.24 | 1.17 ± 0.14 | 0.43 ± 0.13 | 0.07 ± 0.04 |
| F280A  | 1.28 ± 0.33            | 1.99 ± 0.66 | 4.53 ± 1.14 | 1.16 ± 0.30 | 0.07 ± 0.05 |
| V283A  | 1.65 ± 0.05            | 1.62 ± 0.73 | 5.37 ± 1.49 | 0.94 ± 0.26 | 0.06 ± 0.00 |
| W284A  | 3.67 ± 0.32            | 59.33 ± 10.21 a | 31.91 ± 3.81 a | 8.24 ± 1.05 a | 0.29 ± 0.07 a |
| T285A  | 2.45 ± 0.44            | 4.37 ± 1.18 | 6.84 ± 1.55 | 1.07 ± 0.38 | 0.16 ± 0.05 |
| D288A  | 2.00 ± 0.69            | 2.08 ± 0.61 | 2.69 ± 0.93 | 0.92 ± 0.16 | 0.08 ± 0.06 |
| I289A  | 2.10 ± 0.49            | 5.39 ± 0.55 | 12.65 ± 1.89 a | 3.84 ± 0.60 a | 0.17 ± 0.02 |
| D290A  | 1.38 ± 0.56            | 1.15 ± 0.32 | 1.09 ± 0.48 | 1.07 ± 0.32 | 0.06 ± 0.01 |
| R291A  | 1.97 ± 0.28            | 3.86 ± 0.87 | 2.43 ± 0.64 | 11.83 ± 3.73 a | 0.13 ± 0.01 |
| R292A  | 2.80 ± 0.34            | 3.27 ± 0.61 | 4.91 ± 1.56 | 2.53 ± 0.64 a | 0.06 ± 0.00 |
| D293A  | 1.37 ± 0.41            | 1.42 ± 0.44 | 1.63 ± 0.40 | 1.53 ± 0.29 | 0.11 ± 0.01 |
| P294A  | 2.08 ± 0.48            | 2.28 ± 1.10 | 2.99 ± 0.19 | 0.88 ± 0.17 | 0.09 ± 0.05 |
| L295A  | 3.57 ± 0.37            | 3.98 ± 0.87 | 4.79 ± 0.25 | 1.12 ± 0.34 | 0.36 ± 0.03 a |
| V296A  | 3.47 ± 0.57            | 13.81 ± 3.34 a | 11.87 ± 1.39 a | 3.77 ± 0.49 a | 0.14 ± 0.05 |
| V297A  | 4.25 ± 0.52            | 7.18 ± 1.62 a | 4.65 ± 0.70 | 8.90 ± 1.68 a | 0.23 ± 0.07 a |
| A298G  | 2.63 ± 0.38            | 3.11 ± 0.25 | 2.98 ± 0.13 | 0.80 ± 0.12 | 0.29 ± 0.10 a |
| A299G  | 1.74 ± 0.44            | 1.82 ± 0.08 | 4.10 ± 0.80 | 0.84 ± 0.17 | 0.13 ± 0.07 |
| L302A  | 2.29 ± 0.34            | 2.42 ± 0.52 | 2.71 ± 0.42 | 0.86 ± 0.05 | 0.14 ± 0.05 |
| A309G  | 1.12 ± 0.23            | 0.93 ± 0.21 | 2.42 ± 0.53 | 0.52 ± 0.07 | 0.10 ± 0.04 |
| S312A  | 6.98 ± 3.08            | 5.88 ± 2.08 | 10.37 ± 2.11 a | 3.64 ± 0.80 a | 0.23 ± 0.11 |

*Significantly higher than wild-type with p < 0.05.

### Table II

|         | Kᵢ (nM) [³H]Bremazocine | SNC-80 | DPDPE | DELT II | NALT |
|---------|------------------------|--------|-------|---------|------|
| WT hDOR| 3.05 ± 0.37            | 2.19 ± 0.29 | 3.25 ± 0.84 | 0.49 ± 0.13 | 0.04 ± 0.02 |
| W284A  | 3.67 ± 0.32            | 59.33 ± 10.21 | 31.91 ± 3.81 | 8.24 ± 1.05 | 0.29 ± 0.07 |
| V296A  | 3.47 ± 0.57            | 13.81 ± 3.34 | 11.87 ± 1.39 | 3.77 ± 0.49 | 0.14 ± 0.05 |
| V297A  | 4.25 ± 0.52            | 7.18 ± 1.62 | 4.65 ± 0.70 | 8.90 ± 1.68 | 0.23 ± 0.07 |
| V296A + V297A | 3.74 ± 0.89 | 31.48 ± 10.45 | 19.73 ± 5.71 | 8.85 ± 4.45 | 0.14 ± 0.05 |
| W284A + V296A + V297A | 6.12 ± 0.59 | 306 ± 59 | 281 ± 62 | 424 ± 23 | 0.9 ± 0.2 |

*Data for these three mutants were transposed from Table I for better comparison.
TABLE III
Effect of \( \mu \) residue substitution on ligand affinities
Saturation and competition binding assays were performed as described under "Experimental Procedures." Data from three independent saturation and competition experiments were analyzed using the nonlinear least-squares regression curve-fitting program GraphPad Prism. Results of the computer analysis are presented as means ± S.E. \( K_d \) is the dissociation constant for \(^{3}H\)bremazocine binding, and \( K_i \) is the dissociation constant for the competitors. WT, wild type; DELT, deltorphin; NALT, naltindole.

|        | \( K_d \) (nM) | \( K_i \) (nM) |
|--------|----------------|----------------|
|        | \(^{3}H\)Bremazocine | SNC-80 | DPDPE | DELT II | NALT |
| WT hDOR| 3.05 ± 0.37     | 2.19 ± 0.29   | 3.25 ± 0.84 | 0.49 ± 0.13 | 0.04 ± 0.02 |
| W284K  | 7.21 ± 0.43     | 817 ± 365     | 38.10 ± 10.91 | 45.80 ± 3.39 | 0.6 ± 0.3 |
| W284K + V296Q + V297T | 4.05 ± 0.75 | 248 ± 38      | 122 ± 15     | 125 ± 28     | 0.49 ± 0.19 |

**Fig. 6. Three-dimensional model of the \( \delta \)-opioid receptor.** Side chains of Trp-284 and both Val-296 and Val-297 are highlighted in green and red, respectively, to emphasize their position at the top of the binding pore. Only TMs and extracellular loops backbone are illustrated. A, view from the extracellular surface. B, view from the side.
residues are most likely participating directly in the binding processes instead of only stabilizing the structure of the helices. The fact that bremazocine binding is not affected by the mutations supports this hypothesis.

Very recently, Befort and co-workers (29) have proposed that aromatic residues located in the TMs of the \( \delta \)-opioid receptor contribute to ligand recognition. They suggested that these aromatic residues constitute major anchor points for nonspecific opioids, whereas additional interactions may take place for \( \delta \)-selective ligands in other regions of the receptor. Supporting this idea, residues they have identified are conserved across \( \delta \), \( \mu \), and \( \kappa \) receptors. Their results combined with our findings led to our formulation of an hypothesis that binding of \( \delta \)-selective ligands may be achieved in two steps. The first step would be the recognition of the \( \delta \) type by ligand interaction with Trp-284, Val-296, and Val-297 located at the entrance of the binding pore formed by the seven TMs (Fig. 6B). The second step would be the interaction with the general binding domain formed by the aromatic residues proposed in the study of Befort et al. (29).

Supporting this hypothesis, the effect of Trp-284, Val-296, and Val-297 mutations on binding of a ligand seems to correlate with its degree of selectivity. The effect seems more important for highly selective ligands like SNC-80 and deltorphin II than for naltrindole, a weakly selective ligand.

Our study is the first extensive site-directed mutagenesis study aimed at identifying critical residues for \( \delta \)-selective ligand-receptor interaction. Even though Trp-284, Val-296, and Val-297 have been identified as recognition elements determining the selectivity, more studies are needed to elucidate the overall binding pathway for selective ligands.

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