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The Third Extracellular Loop of the Human δ-Opioid Receptor Determines the Selectivity of δ-Opioid Agonists

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SUMMARY

In the present study, we replaced the third extracellular loop of the human δ-opioid receptor with that of the human μ-opioid receptor. A modified polymerase chain reaction overlap extension method was used to achieve the exact splicing in the chimera to show the importance of the extracellular loop in ligand binding without interference from transmembrane substitutions. The replacement of the third extracellular loop did not alter the affinity of [^{3}H]diprenorphine but caused a dramatic decrease in the affinity of both the δ-selective peptide agonists cyclo[D-Pen^2,4′Cl-Phe^4,D-Pen^5]enkephalin and deltorphin II and the δ-selective nonpeptide agonists SNC 121 and (−)TAN 67. The affinities of the μ-selective peptide agonist [D-Ala^2-MePhe^4-Gly-ol^5]enkephalin and the μ-preferring nonpeptide agonist morphine were not affected. Site-directed mutagenesis studies show that the mechanism of ligand recognition might be different for each structural class of opioid ligands.

The opioid peptides and opiate alkaloids mediate their physiological effects through membrane receptors. Three major types of opioid receptors [δ (1), μ(2), and κ (3)] from several species, including human, have been cloned. Predicted amino acid sequence analysis confirms that these receptors possess the characteristic features of the G protein-coupled receptor superfamily, including seven membrane-spanning helices connected with three intracellular and three extracellular loops. Site-directed mutagenesis studies in the cationic neurotransmitter (e.g., adrenergic, muscarinic) receptor family indicate that ligands bind in the cavity formed by the transmembrane helices (4). The topology of the binding pocket is conserved throughout the bacteriorhodopsin-rhodopsin-classic neurotransmitter receptor family (5). The ligand selectivity is determined by the amino acid substitutions inside the binding pocket. Affinity labeling experiments also confirm the role of the aspartate residue in the third transmembrane domain of the muscarinic cholinergic receptors as a counter-ion of the positively charged nitrogen of the ligand (6). The primary sequences of the three opioid receptor types show unusually high homology in the putative transmembrane helices and the intracellular loop regions, whereas the extracellular regions are considerably different (Fig. 1).

There is remarkable structural diversity among the δ-selective opioid agonists (Fig. 2). The structural classes include peptide ligands (pCl-DPDPE and deltorphin II) and the nonpeptide ligands SNC 121 and (−)TAN 67.

The present study was designed to examine the role of the third extracellular loop of the human δ-opioid receptor in determining the selectivity of the peptide and nonpeptide agonists. To distinguish the effects of the loop sequence from the possible effects of the conservative substitutions in the transmembrane helices, the δ-opioid receptor was spliced with the μ-opioid receptor using a PCR overlap extension method (7) at the beginning and the end of the putative extracellular loop, as determined by hydropathy analysis and sequence alignments. The chimeric construct was characterized by radioligand binding assays. The results show that the third extracellular loop of the hDOR is important in the the determination of the selectivity of the δ-opioid peptide and nonpeptide agonists but has no effect on the binding of the μ-selective peptide and nonpeptide agonists.

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ABBREVIATIONS: hDOR, human δ-opioid receptor; hMOR, human μ-opioid receptor; PCR, polymerase chain reaction; SNC 121, (+)-[(4α-*R*)-α(2*S*,5*R*)-4-propyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; TAN 67, 2-methyl-4aα-(3-hydroxyphenyl)-1,2,3,4a,5,12,12aα-octahydro-quinolino[2,3,3-*g*]isoquinoline; DAMGO, [D-Ala^2-MePhe^4-Gly-ol^5]enkephalin; pCl-DPDPE, cyclo[D-Pen^2,4′Cl-Phe^4,D-Pen^5]enkephalin (where Pen is ββ-dimethylcysteine); WT, wild type; NTI, naltrindole.

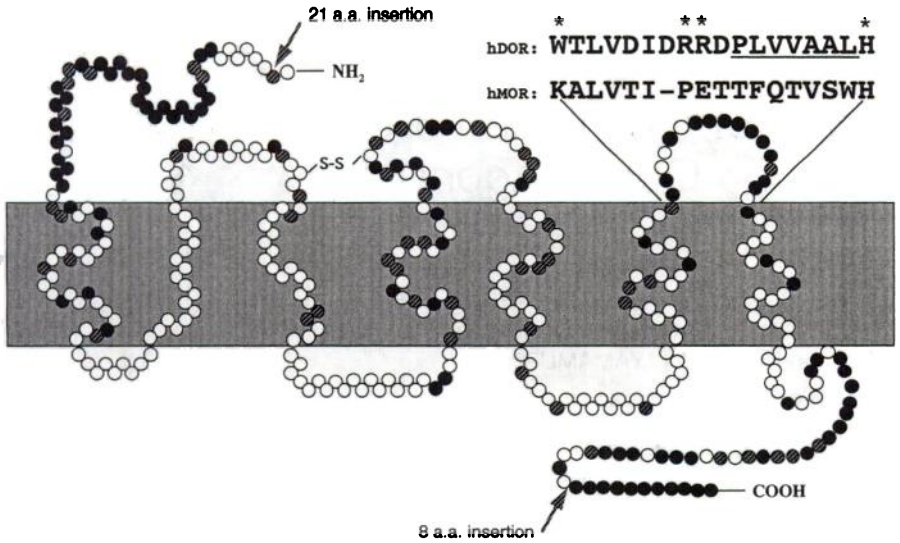

Fig. 1. The putative transmembrane topology of the chimeric receptor. The insert shows the amino acid sequence of the third extracellular loop of the hMOR that replaces the corresponding hDOR residues (*top*). *Underlined*, hydrophobic residues at the carboxyl terminus of the hDOR third extracellular loop; *, location of point mutations (Trp284, Arg291, Arg292, His301); ○, residues identical in the human δ- and μ-opioid receptors; ◍, conservatively substituted residues in the μ-opioid receptor; ●, nonconservative substitutions; *arrows*, amino acid insertions in the hMOR sequence.

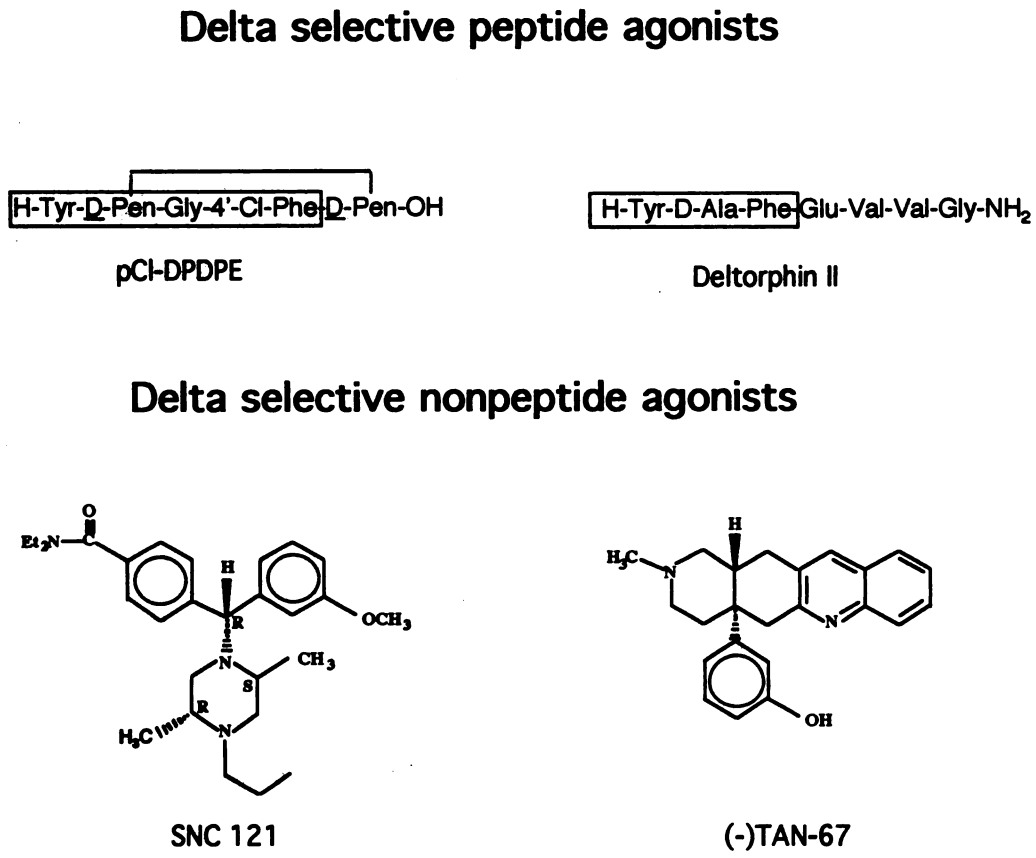

Fig. 2. Selected representatives of different structures of δ-selective agonists. *Boxed*, putative message sequences in the peptides.

Materials and Methods

Construction of the chimeric receptor. The human δ-opioid receptor clone was isolated in our laboratory as described previously (1). The human μ-opioid receptor/pcDNA I clone was a gift of Dr. J. B. Wang (University of Maryland, College Park, MD) (2). A *Hin*dIII fragment from the original clone was ligated into the pcDNA 3 vector for the transient transfection experiments. The low error rate *Pyrococcus furiosus* polymerase was used in all reactions except when stated otherwise.

Due to the lack of conserved restriction sites around the required junction site, the chimeric fragment for chimera [δ(1–282)-μ(304–320)-δ(301–372)] was assembled in three consecutive reactions. In the first and second PCRs, the hDOR/pcDNA 3 clone was used as a template. The sense primer (μ sequences underscored) was a chimeric primer: 5′ > GTT TCT TGG CAC TTC TGC ATC GCG CTG GGT > 3′. The antisense primer 5′ > CGG GTC TGG GGT CGT CGA AGT CGG C > 3′ is downstream from the unique *Not*I restriction site in the hDOR. The 149-base-pair product was isolated from an agarose gel. In the second PCR, the sense primer 5′ > CGG GTC TGG GGT CGT CGA AGT CGG C > 3′ carried the unique *Bst*EII restriction site. The antisense primer was the chimeric primer: 5′ > TTG TAA CCA AGG CTT TGA CGA TGA CGA AGA T > 3′. After 35 cycles, the amplification product (251 base pairs) was isolated from an agarose gel. The third amplification was a two-step process using

Taq polymerase and the hMOR/pcDNA3 clone as the template. In the first five cycles, the products from the two previous PCRs were used as primers with low stringency conditions (50° annealing temperature), and then 500 ng of the δ primers 5′ > CAG CTG GTA CTG GGA CAC GGT GAC CAA GAT > 3′ and 5′ > CGG GTC TGG GGT CGT CGA AGT CGG C > 3′ were added and the amplification was continued for another 35 cycles under high stringency conditions (annealing temperature, 65°). The product (423 base pairs) was isolated from the agarose gel, analyzed with the restriction enzyme *Sty*I, and subcloned into the PCR II vector by blunt-end ligation. The sequence of the PCR product was verified by dideoxy sequencing. The product was digested with *Bst*EII and *Not*I and ligated into the similarly digested hDOR/pcDNA3 fragment. Chimeric clones were selected using the restriction enzyme *Sty*I, and the correct insertion was verified by dideoxy sequencing.

Site-directed mutagenesis. Point mutations in the hDOR/pcDNA3 sequence were generated with the unique site elimination method (8) using the Transformer Site-Directed Mutagenesis kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. The point mutations were verified by dideoxy sequencing.

Expression of the WT and chimeric receptor proteins. COS-7 cells were cultured as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and transfected transiently by the DEAE-dextran transfection method using 5 μg of DNA for the WT hDOR and the third-loop chimera and 10 μg of DNA for the hMOR. After 48–72 hr of incubation at 37°, crude cell membranes were prepared as described previously (1).

Radioligand binding assays. Tissue linearity studies [0.1–0.6% (w/v) tissue] were used to determine the tissue dilution factor to give comparable specific [^{3}H]diprenorphine binding (0.4 nM, 23°, 3-hr incubation) to the WT and chimeric constructs. Nonspecific binding was determined in the presence of naltrexone (10 μM). [^{3}H]Diprenorphine (39 Ci/mmol) was from Amersham (Arlington Heights, IL). Saturation binding studies (three experiments) consisted of duplicate total and nonspecific binding samples for six concentrations (0.1–5.0 nM) of [^{3}H]diprenorphine. The binding of [^{3}H]diprenorphine was inhibited by 10 concentrations of deltorphin II, pCl-DPDPE (9), DAMGO (Research Biochemicals, Natick, MA), morphine (Sigma Chemical, St. Louis, MO), and SNC 121 (10). (−)TAN 67, the active conformer of (±)TAN 67 (11), was synthesized by H.N. The results are from three independent experiments performed in duplicate. The K_i values for drugs were calculated according to the Cheng-Prusoff equation in Table 2 (12).

Expression and characterization of the mutant receptors. The hDOR point mutants were expressed transiently in COS-7 cells as described for the chimeric proteins. Radioligand binding experiments (tissue linearity, saturation isotherms, and binding inhibition studies) were performed as described using [^{3}H]NT1 (30 Ci/mmol; DuPont-New England Nuclear, Boston, MA) and [^{3}H]pCl-DPDPE (41 Ci/mmol; DuPont-New England Nuclear) to label the WT and mutant receptors.

Results

Construction of the chimeric receptors. The third extracellular loop of the human δ-opioid receptor was replaced with the respective fragment of the human μ-opioid receptor. A chimeric fragment carrying a conserved restriction site at each end was spliced together by a modified PCR overlap extension method and ligated into the digested WT hDOR using the same unique restriction sites. Fig. 1 shows the putative secondary structure of the chimera and the location of the point mutations in the hDOR. The WT, chimeric, and mutant receptors were expressed transiently in COS-7 cells and characterized by radioligand binding experiments.

Binding of [^{3}H]Diprenorphine to the WT and chimeric receptors. To ascertain that the exchange of the extracellular loop did not affect the overall secondary structure of the receptor protein, we determined the affinity of the nonselective opiate, [^{3}H]diprenorphine, to the WT and chimeric receptors in saturation binding experiments. As shown in Table 1, [^{3}H]diprenorphine bound with similar high affinity to the hDOR (K_d = 1.48 ± 0.8 nM), to the hMOR (K_d= 0.55 ± 0.3 nM), and to the chimera (K_d = 1.15 ± 0.5 pM). The difference in the K_d values was not significant ($p > 0.54$). Because the B_{max} values were different in the saturation experiments, the receptor densities were set to a comparable level in further experiments by adjusting the amount of DNA used in the transfections, and the tissue dilution factors as determined by tissue linearity experiments.

Binding affinity of δ-selective peptide agonists. The inhibition of [^{3}H]diprenorphine binding by various opioid ligands was used to further characterize the ligand selectivity of the chimeric receptor. As shown in Table 2 and Fig. 3A, the affinity of pCl-DPDPE, a δ-selective cyclic opioid peptide was reduced markedly from 1.7 ± 0.5 nM (hDOR) to 3,190 ± 450 nM (chimera). The affinity of the δ-selective linear peptide deltorphin II was similarly reduced from 20 ± 0.5 nM (hDOR) to >10,000 nM (chimera) by the exchange of the third extracellular loop (Table 2). Site-directed mutagenesis of single positive residues in this region of the hDOR (R291S, R292E, H301N) had no effect on the affinity of [^{3}H]pCl-DPDPE or [^{3}H]NTI (Table 3). The mutation of the aromatic residue in the third extracellular loop of the hDOR (W284L) also showed no effect on the affinity of [^{3}H]pCl-DPDPE or [^{3}H]NTI (Table 3).

Binding affinity of δ-selective nonpeptide agonists. As shown in Fig. 3, C and D, the affinities of both SNC 121 and (−)TAN 67 were markedly reduced from 9.3 ± 0.3 nM (hDOR) to >10 μM (chimera) and from 2.4 ± 0.5 nM (hDOR) to 1060 ± 30 nM (chimera), respectively. Interestingly, the mutation of Trp284 to an aliphatic amino acid (leucine) resulted in a 42-fold decrease in the affinity of SNC 121 (13), whereas this mutation caused only a 5-fold decrease in the affinity of (±)TAN 67 in competing for specific [^{3}H]pCl-DPDPE binding. The respective K_i values for (±)TAN 67 were 2.0 ± 0.7 nM (hDOR) and 10 ± 1.5 nM (mutant). The decrease in (±)TAN 67 affinity was 3.6-fold when competing for specific [^{3}H]NTI binding.

Binding affinity of μ-selective ligands. The exchange of the third extracellular loop of the hDOR with that of the hMOR did not affect the affinity of the μ-selective peptide agonists DAMGO (Fig. 3B) or dermorphin (Table 2). Both peptides had K_i values in the micromolar range similar to that of the WT hDOR. Interestingly, the affinity of naloxone, a μ-preferring opiate antagonist, increased from 230 ± 4 nM

TABLE 1

Saturation analysis of specific [^{3}H]diprenorphine binding to COS-7 cell membrane expressing the WT hDOR, WT hMOR, or the chimeric receptor

The K_d and B_{max} (mean ± standard error) values are from three independent determinations performed in duplicate.

Receptor	K_d	B_{max}
	nM	*pmol/mg protein*
hDOR	1.48 ± 0.8	2.58 ± 0.9
hMOR	0.55 ± 0.3	0.45 ± 0.1
Chimeric receptor	1.15 ± 0.5	4.15 ± 1.2

TABLE 2

Summary of the ligand affinities for the WT and chimeric receptors

COS-7 cells were transfected with the cDNAs encoding the WT hDOR, WT hMOR, or the chimeric opioid receptor. The specific [^{3}H]diprenorphine binding to crude membrane preparations was inhibited by increasing concentrations of the opioid ligands. The K_i values were calculated according to the Cheng-Prusoff equation (12), using the individual K_d values from Table 1. The data are the mean of three independent determinations performed in duplicate.

Drug	K_i		
	hDOR	hMOR	Chimera
	nM		
δ-agonists			
pCl-DPDPE	1.7	1,630	3,190
Deltorphin II	20.0	>10,000	>10,000
SNC 121	9.3	>10,000	>10,000
(−) TAN 67	2.4	590	1,060
μ-agonists			
DAMGO	2,780	27	1,570
Dermorphin	620	7	2,280
Morphine	543	29	357
Antagonist			
Naloxone	230	43	49

(WT hDOR) to 49 ± 16 nM (chimera) by the exchange of the third extracellular loop (Table 2; $p < 0.005$, significant).

Discussion

The major findings of this study are: 1) the third extracellular loop of the human δ-opioid receptor plays an important role in the selectivity of both the enkephalin agonists and nonpeptide δ-agonists of different structural classes; 2) the replacement of the third extracellular loop of the hDOR with that of the hMOR has no effect on the affinity of the μ-selective peptide and nonpeptide agonists.

The construction of chimeric proteins is a powerful tool for mapping regions responsible for a biochemical function. Recently, a number of chimeric receptors have been constructed between the opioid receptor types. It has been shown (14) that DAMGO, a μ-selective peptide, differentiates between the μ- and δ-opioid receptors in the region, including the first intracellular loop, whereas the selectivity determinants for the same peptide between the μ- and κ-opioid receptors was mapped to the region from the fifth transmembrane domain to the carboxyl terminus of the receptor (15). The κ-to-μ (16) or the κ-to-δ (17) preference of the dynorphin peptides is determined by the region around the second extracellular loop, whereas the δ-to-κ (17) and δ-to-μ(18) preference of the enkephalins is governed by the carboxyl-terminal half of the receptor. It was also shown (19) that the binding determinants for the nonselective opiates are at least partially different from that of the selective ligands.

To study the role of the extracellular loops without interference from the transmembrane substitutions, we used the PCR overlap extension method to achieve an exact splicing site at the border of the third loop region and the transmembrane regions. The exchange of the third extracellular loop of the hDOR with that of the hMOR diminished the affinity of both the δ-selective peptide agonists and the nonpeptide δ ligands. However, the same chimeric substitution had no effect on the affinities of the μ-preferring agonists.

We have shown in this study and previously (20) that the replacement of the second or third extracellular loop has no effect on the affinity of morphine. On the other hand, the affinity of naloxone, a μ-preferring opiate antagonist, increased in the chimeric receptor, reaching the WT hMOR value. This increase shows that the μ preference of naloxone is determined by an interaction with the third extracellular loop of the μ-opioid receptor.

Interestingly, the affinities of the δ-selective nonpeptide agonists SNC 121 and (−)TAN 67 are profoundly affected by the exchange of the third extracellular loop. The selection mechanism, however, seems to be different for these ligands. The decrease in the affinity of SNC 121 is at least partially accounted for by the loss of an aromatic stacking interaction. Trp284 is at the border of the sixth transmembrane domain and the third extracellular loop of the hDOR. The respective residue is lysine in the μ-opioid receptor. Mutation of Trp284 to leucine causes a 42-fold decrease in the affinity of SNC 121 (13). The δ-selectivity of (±)TAN 67, however, is not determined clearly by Trp284 because this mutation causes only a 5-fold decrease in the affinity of this ligand. Possible mechanisms (aromatic-amino interactions with positively charged residues or the interaction of the quinoline nitrogen of the ligand with hydrogen-bond donors) are currently under investigation.

It should also be emphasized that in the chimera constructed in this study, the third extracellular loop of the hMOR was inserted into the human δ-opioid receptor. However, there is a seven-amino-acid sequence at the carboxyl terminus of the third extracellular loop of the μ-opioid receptor that is hydrophilic and clearly part of the extracellular loop. This portion of the δ-opioid receptor, on the other hand, is strongly hydrophobic (Fig. 1) and presumably is already part of the seventh transmembrane helix. The change in the length of the third extracellular loop may affect the positioning of the seventh transmembrane domain, causing an alteration in the shape of the binding pocket. The exclusion of water molecules from the cleft may change the local pK_a values of even remote residues inside the binding pocket. Further chimeric studies are under way to investigate these mechanisms.

The affinity of the δ-selective peptides (pCl-DPDPE and deltorphin II) was also decreased dramatically by the exchange of the third extracellular loop, whereas the affinity of the μ-selective peptides (DAMGO and dermorphin) was not affected. Although these data are similar to the results obtained with the nonpeptide ligands, site-directed mutagenesis studies indicate that the underlying mechanisms are quite different. It was shown (21) that the mutation of a single Asn residue (Asn127) in the first extracellular loop of the μ-opioid receptor to the corresponding δ residue (Lys108) decreases the affinity of DAMGO to the WT δ-value while having no significant effect on the affinity of DPDPE or morphine. Data presented in this study indicate that the third extracellular loop of the δ-opioid receptor determines the affinity of the δ-selective peptide agonists just as robustly as the first extracellular loop of the μ-opioid receptor determines the affinity of DAMGO. According to the message-address theory (22), we mutated the positively charged residues in this region of the δ-opioid receptor, which can interact with the negative charge of the enkephalins or interact with the phenyl ring (amine-π interaction). We also mutated the only aromatic residue in the third extracellular loop of the δ-opioid receptor to an aliphatic residue to investigate the

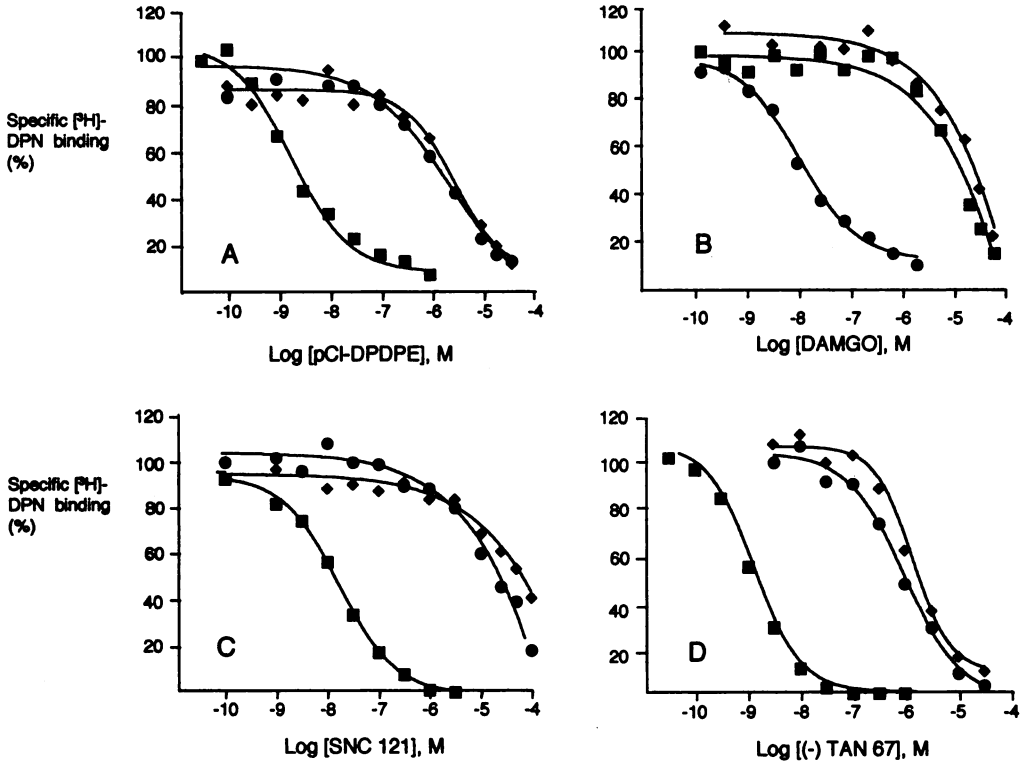

Fig. 3. The inhibition of specific [³H]diprenorphine binding by selected opioid agonists. COS-7 cells were transfected with the cDNAs encoding the WT hDOR (■), WT hMOR (●), and the chimeric (♦) opioid receptor. The specific [³H]diprenorphine binding to crude membrane preparations was inhibited by increasing concentrations of pCl-DPDPE (A), DAMGO (B), SNC 121 (C), and (−)TAN 67 (D). The data are the mean of three independent determinations performed in duplicate. The specific [³H]diprenorphine binding for different transfections was: 2,890 ± 1140 dpm (hDOR); 1400 ± 610 dpm (hMOR); and 2940 ± 1180 dpm (chimera). The nonspecific binding was less than 10%.

TABLE 3

Saturation analysis of specific [³H]NTI and [³H]pCl-DPDPE binding to COS-7 cell membranes expressing the WT and mutant human δ-opioid receptors

The K_d and B_{max} (mean ± standard error) values are from three independent determinations performed in duplicate.

Receptor	[³H]NTI		[³H]pCl-DPDPE	
	K_d	B_{max}	K_d	B_{max}
	nM	*pmol/mg protein*	*nM*	*pmol/mg protein*
WT hDOR	0.16 ± 0.05	5.2 ± 1.6	0.50 ± 0.02	2.0 ± 0.5
W284L mutant	0.16 ± 0.04	3.7 ± 1.0	0.60 ± 0.03	2.1 ± 0.3
R291S mutant	0.14 ± 0.03	3.8 ± 0.8	0.27 ± 0.06	2.5 ± 0.8
R292E mutant	0.16 ± 0.03	6.6 ± 2.2	0.51 ± 0.03	2.2 ± 0.6
H301N mutant	0.13 ± 0.03	3.0 ± 1.3	0.50	1.0

possibility of an aromatic stacking interaction in the δ message selection. The results show that the replacement of the aromatic (Trp284) or individual positive (Arg291, Arg292, His301) residues of the hDOR have no effect on the affinity of the δ-selective peptides. However, it was reported recently (23) that simultaneous mutation of Arg291 and Arg292 to neutral residues (glutamine) decreased the affinity of DSLET to the δ-opioid receptor more than 300-fold. It is possible, due to the mobility of the third extracellular loop, that either of the two arginine residues can interact alternatively with the carboxyl terminus of the enkephalins. Another possibility is that the two positively charged arginine residues shield the negative charge of the two neighboring aspartate residues, and it is the lack of this repulsive force (the corresponding four-amino-acid stretch is negative in the μ-opioid receptor) that determines the affinity of δ ligands. It is interesting to note that although deltorphin II and dermorphin carry the same putative message sequence (Tyr-D-Ala-Phe), their affinity seems to be determined by different parts of the receptor. Further site-directed mutagenesis experiments are necessary to study the possibility that the interplay of the first and the third extracellular loops governs the entrance of both the μ- and δ-selective peptides into the binding pocket.

In conclusion, the third extracellular loop of the hDOR plays an important role in determining the selectivity of the δ ligands. The interacting residue seems to be different for each structural class of ligands.

Acknowledgments

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Errata

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The article by Varga *et al.* [Varga, E. V., X. Li, D. Stropova, T. Zalewska, R. S. Landsman, R. J. Knapp, E. Malatynska, K. Kawai, A. Mizusura, H. Nagase, W. R. Roeske, and H. I. Yamamura. The third extracellular loop of the human δ opioid receptor determines the selectivity of δ opioid agonists. *Mol. Pharmacol.* **50:** 1619–1624 (1996)] has been reported by its authors to contain some errors in the PCR primer sequences.

On p. 1620, in Materials and Methods, the correct primer sequences are as follows:

In the first PCR:

```
5′ > GTT TCT TGG CAC TTC TGC ATC GCG CTG GGC T > 3′
5′ > CGG CTG AAG CTG CTG GGG TCT GGG C > 3′
```

In the second PCR:

```
5′ > CAG CTG GTA CTG GGA CAC GGT GAC CAA GAT > 3′
5′ > TTG TAA CCA AGG CTT TGA CGA TGA CGA AGA T > 3′
```

On p. 1621, the correct sequences of the δ primers used in the second step of the third PCR are:

```
5′ > CAG CTG GTA CTG GGA CAC GGT GAC CAA GAT > 3′
5′ > CGG CTG AAG CTG CTG GGG TCT GGG C > 3′
```

These errors do not affect the results or conclusions of the article. The authors apologize for any confusion the errors may have caused.

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There is an incorrect use of one of the references in the article by Ma *et al.* [Ma, J. F., G. Grant, and P. W. Melera. Mutations in the sixth transmembrane domain of P-glycoprotein that alter the pattern of cross-resistance also alter sensitivity to cyclosporin A reversal. *Mol. Pharmacol.* **51:**922–930 (1997)]. With the exception of its use on page 922, all citations of Ref. 1 should be replaced with a citation of Ref. 22.

The authors apologize for any confusion this error may have caused.