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ACCELERATED COMMUNICATION

Analysis of Selective Binding Epitopes for the κ-Opioid Receptor Antagonist Nor-binaltorphimine

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SUMMARY

The structural determinants for the selective binding of the nonpeptide opioid receptor antagonist nor-binaltorphimine (nor-BNI) to the κ-opioid receptor were characterized using a systematic series of chimeras between the κ receptor and the homologous μ-opioid receptor. All 10 chimeric constructs bound the nonselective antagonists (−)-naloxone and diprenorphine with similar affinities, as did the two wild-type receptors. Introduction of amino-terminal segments of increasing length, extending to and including transmembrane segment VI, from the μ receptor into the κ receptor did not impair the high affinity binding of nor-BNI, and neither did introduction of the intracellular carboxyl-terminal extension of the μ receptor. In contrast, nor-BNI binding was impaired ≥600-fold in constructs in which extracellular loop 3 and transmembrane segment VII originated from the μ receptor. The exchange of a single residue within this region, Glu^{297}, for lysine, the corresponding residue from the μ receptor, reduced the binding affinity of nor-BNI 142-fold, without affecting the binding of the nonselective compounds (−)-naloxone and diprenorphine. It is concluded that the selective binding of nor-BNI to the κ-opioid receptor is determined by nonconserved residues located in extracellular loop 3 and transmembrane segment VII and that Glu^{297}, located just outside transmembrane segment VI, plays a major role in the κ-selective binding characteristics of nor-BNI.

The recent cloning of cDNAs encoding a number of opioid receptors (1–6) has demonstrated that the three most prevalent opioid receptor subtypes, κ, μ, and δ, all belong to the family of rhodopsin-like receptors within the superfamily of G protein-coupled receptors (7). The primary structures of the three receptor subtypes (47% overall identity) are characterized by a remarkably high degree of homology in the TMs (66% identity), in contrast to the highly divergent EC domains (12% identity).

Although the opioid receptor system features a myriad of chemically and pharmacologically distinct ligands, only a few of these compounds are highly selective for the individual opioid receptor subtypes. One exception is the κ receptor-selective nonpeptide antagonist nor-BNI (8, 9). Nor-BNI was developed as a 'bivalent ligand,' a term used to denote a molecule composed of two, covalently joined, recognition units (10). The underlying principle applied in the development of these bivalent ligands is the 'message-address' concept proposed by Schwyzer (11). According to this theory the 'address' moiety of the compound provides the selective binding properties of the molecule, whereas signaling by the receptor is mediated via the 'message' moiety of the ligand. Nor-BNI represents a prototypical compound within a series of nonpeptide opiates that were designed as bivalent ligands with the specific aim of attaining improved selectivity for the κ receptor.

In the present study we have used a systematic series of chimeric constructs between the rat κ- and μ-opioid receptors to locate the epitopes that confer upon the κ receptor selectivity for nor-BNI. The data suggest that nonconserved residues in EC loop 3 and TM-VII domains of the κ receptor are key structural elements involved the recognition of the address part of nor-BNI and that an acidic residue, Glu^{297}, located immediately above TM-VI is essential for receptor subtype-selective binding of nor-BNI.

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ABBREVIATIONS: TM, transmembrane segment; nor-BNI, nor-binaltorphimine; EC, extracellular.

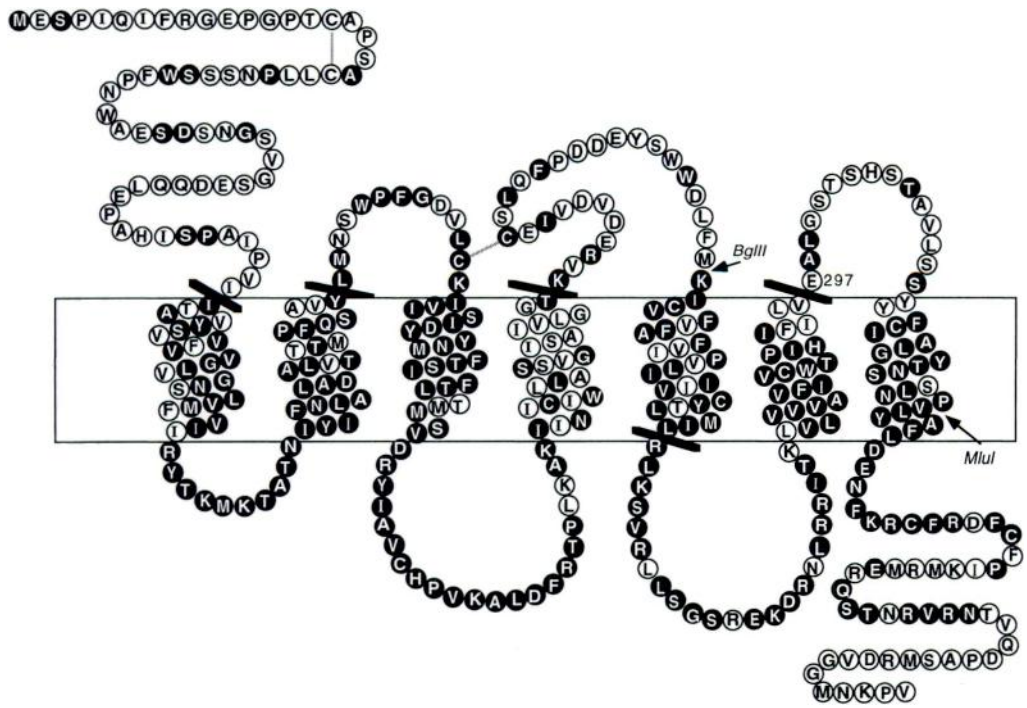


Fig. 1. Structure of the rat κ receptor. *Residues in white on black*, residues conserved between the μ and κ receptors. *Bars*, crossover points of the receptor chimeras. The highly conserved cysteine residues of EC loop 1 and EC loop 2 are shown linked by a disulfide bond. A putative disulfide bridge within the aminoterminal extension is indicated between non conserved cysteine residues. The corresponding positions in the cDNA for the restriction enzymes *Bgl*II and *Mlu*I are indicated. These sites were used for the construction of the complex chimeras κμκ and μκ μ (Table 1).

Experimental Procedures

Construction of mutant opioid receptor genes. The receptor chimeras were synthesized by the polymerase chain reaction overlap extension technique (12, 13), using as templates the wild-type rat κ- and μ-opioid receptor cDNAs. Polymerase chain reactions used *Pyrococcus furiosus* polymerase (Stratagene) and reaction conditions as recommended by the manufacturer. The crossover points in the mutant receptor constructs (Fig. 1) correspond to the following positions in the κ receptor cDNA (the adenine in the initiating codon ATG is defined as +1): κμ1, position 182; μκ3, position 357; μκ5 and κμ5, position 596; μκ6 and κμ6, position 752; μκ7 and κμ7, position 888. The complex chimeras μκμ and κμκ were constructed by the exchange of a *Bgl*II-*Mlu*I fragment (positions 680–990) between the two wild-type receptor cDNAs. The *Mlu*I site was preexistent in the μ receptor and was introduced by site-directed mutagenesis as a silent mutation at the corresponding position in the κ receptor. The structure of the mutant receptor cDNAs was verified by DNA sequencing (Sequenase; United States Biochemicals).

Expression of mutant receptor cDNAs in COS cells. COS cells were grown in Dulbecco's modified Eagle medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 10 μg/ml gentamicin. The wild-type κ and μ receptors and the mutant receptor constructs were transiently transfected into COS-7 cells by the calcium phosphate precipitation method, according to previously reported procedures (14).

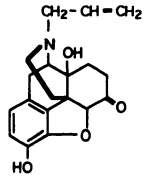
Ligands. The nonselective antagonist [^{3}H]diprenorphine (specific activity, 30 Ci/mmol) was obtained from Amersham (catalogue no. TRK.730). (−)-Naloxone and nor-BNI were purchased from Research Biochemicals (Natick, MA).

Binding assays. Radioligand binding asssays were performed using intact cells, as previously described in detail for binding analysis of the angiotensin type 1 receptor (15). Transfected cells were transferred to 12- or 24-well culture plates (1–5 × 10^5 cells/well), 1 day after transfection and 24 hr before binding analysis. Competition binding was carried out at 4° for 16 hr using 0.15 pmol of the radioligand [^{3}H]diprenorphine and variable amounts of unlabeled ligands, in a total binding volume of 0.5 ml. The number of cells/well was adjusted according the expression level observed for the individual constructs, aiming at binding of 5–15% of the added radioligand. Nonspecific binding was determined in the presence of 10 μM (−)-naloxone. The binding buffer used consisted of 25 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 0.1% bovine serum albumin, and 100 μg/ml bacitracin. All determinations were done in triplicate. Binding data were analyzed using the Inplot 4.0 program (GraphPAD Software, San Diego, CA). Dissociation and inhibition constants were estimated from competition binding curves using the equation $K_d = IC_{50} - [L]$ and the Cheng-Prusoff equation, $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] represents the concentration of radioligand in the binding assay.

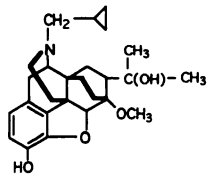
Results

Expression and binding analysis of wild-type κ and μ receptors. The nonselective antagonist diprenorphine bound well to the κ and μ wild-type receptors, with K_d values

Nonselective antagonists

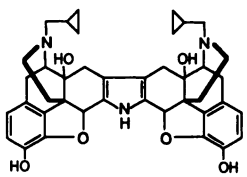


(-)Naloxone



Diprenorphine

κ receptor selective antagonist



nor-binaltorphimine (nor-BNI)

Fig. 2. Structures of the chemically similar, nonselective, nonpeptide opioids diprenorphine and naloxone and of the bivalent κ receptor-selective compound nor-BNI.

TABLE 1

Binding affinities of diprenorphine, naloxone, and nor-BNI for the wild-type κ and μ receptors and for the chimeric receptor constructs

Data are presented as the mean ± standard error with the number of experiments indicated in parentheses. For those constructs that exhibited high affinity binding of nor-BNI, K_i values were derived from competition binding curves fitted to a two-site binding model. The K_i value for the high affinity site is shown; the affinity of the low affinity site is in the micromolar range. The effect upon nor-BNI binding affinity is indicated for each chimeric construct in terms of a mutational factor, F_{mut}, calculated as K_i (mutant receptor)/K_i (wild-type receptor).

	Diprenorphine K_d	Naloxone K_i	nor-BNI K_i	nor-BNI F_{mut}
	nM	*nM*	*nM*	
κ-Wild-type	0.35 ± 0.05 (3)	7.0 ± 0.8 (12)	0.10 ± 0.04 (6)	1.0
μκ3	1.3 ± 0.2 (3)	21 ± 2.0 (12)	0.37 ± 0.1 (4)	3.7
μκ5	0.6 ± 0.2 (3)	5.6 ± 1.0 (10)	0.09 ± 0.04 (4)	0.9
μκ6	1.2 ± 0.1 (3)	7.8 ± 0.3 (9)	0.18 ± 0.05 (4)	1.8
μκ7	0.5 ± 0.2 (3)	5.6 ± 0.5 (11)	0.23 ± 0.04 (6)	2.3
μκμ	1.5 ± 0.3 (3)	8.3 ± 0.6 (3)	0.24 ± 0.05 (4)	2.4
κμ7	1.60 ± 0.26 (3)	37.6 ± 5.3 (10)	61 ± 6.5 (4)	610
κμ6	0.48 ± 0.15 (3)	5.0 ± 1.7 (9)	119 ± 15 (3)	1190
κμ5	0.32 ± 0.02 (3)	3.7 ± 0.3 (13)	135 ± 36 (4)	1350
κμκ	0.15 ± 0.02 (3)	1.8 ± 0.2 (6)	293 ± 121 (3)	2930
κμ1	0.26 ± 0.13 (3)	2.7 ± 0.2 (10)	72 ± 19 (4)	720
μ-Wild-type	0.25 ± 0.06 (3)	2.9 ± 0.2 (9)	102 ± 9.5 (4)	1020

of 0.35 nM (κ) and 0.25 nM (μ) (Table 1). Another, chemically related (Fig. 2), nonselective opioid receptor antagonist, (−)-naloxone, also competed with [^{3}H]diprenorphine binding to the wild-type κ and μ receptors, with similar affinities [K_i values of 7.0 nM (κ) and 2.9 nM (μ)]. Furthermore, (−)-naloxone appeared to bind to the receptor with a single-site mode of interaction (Fig. 3).

Construction and analysis of chimeric κμ and μκ receptors. Genetic crossover points were placed at five different positions in the receptor molecules, corresponding to boundaries between the putative TMs and the EC and intracellular domains of the two receptors (Fig. 1). To assess the importance of the carboxyl-terminal, intracellular domain of the receptor, two complex chimeras, κμκ and μκμ, were constructed. No major structural incompatibilities were apparent in any of these receptor constructs, as evidenced by their high affinity binding of both nonselective antagonists, diprenorphine and naloxone (Table 1).

Binding of nor-BNI. Introduction of amino-terminal segments of increasing length from the μ receptor into the κ receptor did not affect the high affinity binding of the κ-selective antagonist nor-BNI (Table 1). Even the chimeric construct μκ7, in which the first six TMs originate from the μ receptor, bound nor-BNI with subnanomolar affinity (K_i = 0.23 nM). Introduction of the carboxyl-terminal intracellular segment of the μ receptor into the κ receptor, as in construct μκμ, similarly did not impair the binding of nor-BNI (K_i = 0.24 nM). Together, these constructs delineate nor-BNI-selective binding epitopes in EC loop 3 and TM-VII (Table 1). In accordance with this, the constructs in which the EC loop 3 and TM-VII sequences are derived from the μ receptor (κμ7, κμ6, κμ5, κμκ, and κμ1) displayed affinities for nor-BNI that

were >600-fold reduced, compared with the wild-type κ receptor (K_i values ranging from 61 to 293 nM) (Table 1). Conversely, high affinity binding of nor-BNI was conveyed to the μ receptor by genetic exchange of κ receptor EC loop 3 and TM-VII with the corresponding segments of the μ receptor, as in construct μκ7 (Table 1). Binding of nor-BNI to the wild-type κ receptor conforms to a two-site binding mode, with 81% of the receptor population corresponding to a high affinity site (K_i = 0.10 nM) and the remainder, 18%, of low affinity (K_i = 638 nM) (Fig. 3). This two-site binding mode was retained in those receptor constructs that exhibited high affinity binding of nor-BNI (e.g., μκ) (Fig. 3). High affinity binding of another κ receptor antagonist, U69,593 (which is also considered κ selective, although less so than nor-BNI), classified the κ receptor as being of the κ_1 subtype (16) (data not shown).

Effect of Glu^{297} exchange on the binding of nor-BNI. In view of the positive charge present at the N17-position of nor-BNI and the previously suggested need for an anionic counterpart in the receptor (17), Glu^{297} was subjected to point substitution, because this amino acid represents the only acidic residue within the previously identified segment (EC loop 3 and TM-VII). This residue was exchanged with a lysine residue, the amino acid present at the corresponding position in the μ-opioid receptor. The binding affinities of the two nonselective compounds, diprenorphine and naloxone, were unaffected by the E297K substitution; however, the binding of nor-BNI was reduced by 2 orders of magnitude (K_i = 14.2 nM) (Fig. 4).

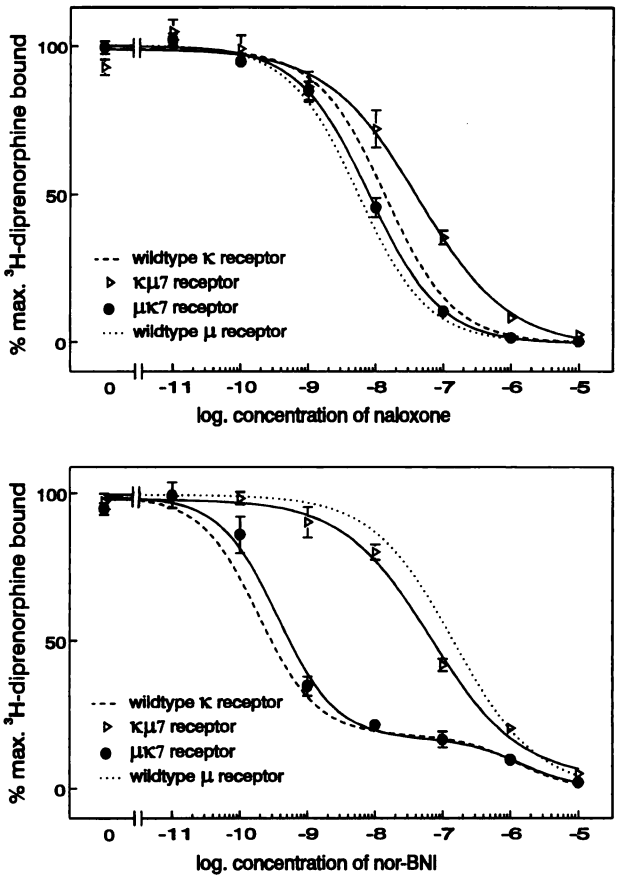


Fig. 3. Competition binding curves for the wild-type κ- and μ-opioid receptors and for the chimeric receptor constructs. [^{3}H]Diprenorphine was used as the radioligand in binding analysis of COS-7 cells transiently transfected with the receptor constructs, as described in Experimental Procedures. The structures of the receptor constructs are presented in Table 1. The nonselective antagonist (−)-naloxone (*upper*) and the κ-selective antagonist nor-BNI (*lower*) were used for competition. For those constructs that bound nor-BNI with high affinity, the competition is presented as a two-site fitting of the binding curves. K_i values are given in Table 1.

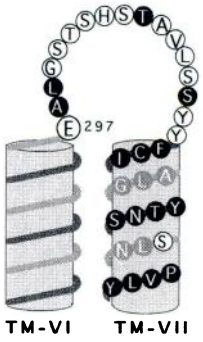
Discussion

Chimeric constructs between homologous receptors have been used extensively as an initial systematic approach to locate structural elements that are responsible for the phenotypic specificity displayed by receptor subtypes (18). For example, in the tachykinin system this approach was used to identify the outer portion of TM-V, TM-VI, and TM-VII as being crucial for the binding of a series of nonpeptide antagonists to both neurokinin type 1 and type 2 receptors (19, 20). Subsequently, systematic point mutagenesis of these ligand-binding epitopes has identified specific residues that serve to coordinate the antagonists (21). A similar approach using human and *Xenopus laevis* angiotensin type 1 receptors led to the identification of a residue in TM-VII that was potentially important for the interaction with several nonpeptide antagonists (13).

In the present investigation a 1000-fold loss of affinity, as well as a gain of affinity (with respect to ligand binding affinity), was observed using constructs formed between the κ- and μ-opioid receptors. Together, these chimeric exchanges indicated that important epitopes for the selective binding of the nonpeptide antagonist nor-BNI are located in a segment of the κ-opioid receptor that includes EC loop 3 and TM-VII. The identification of this domain as being essential for nor-BNI binding is in agreement with the results of a separate chimeric κ-μ receptor analysis, in which a κ receptor domain encompassing TM-VI, EC loop 3, and TM-VII correlated with high affinity binding of nor-BNI (22).

A positively charged group at the N17′-position of nor-BNI has previously been suggested to be of primary importance for the activity of this compound (23) and is proposed to interact with acidic residues present within the EC domain of the receptor (17). In the present study we found that the binding of nor-BNI was reduced by 2 orders of magnitude upon substitution of one residue, Glu^{297} (presumed to be positioned at the interface between TM-VI and EC loop 3), to a lysine, the corresponding μ receptor residue. We therefore suggest that the carboxylate group of the Glu^{297} residue interacts with the positively charged N17-position of nor-BNI.

The κ antagonist nor-BNI is the prototypical antagonist for the κ receptor system (11), and the identification of residues in the receptor that serve as points of interaction with this compound could provide insight, at the molecular level, into the putative significance of the bivalent nature of this compound. A bivalent compound could be envisioned to bind to the receptor either by binding to two separate subsites on a single receptor molecule or by simultaneously binding to identical sites located on separate receptor molecules. In the latter case, the ligand would serve as a dimerization agent for the receptors. The analysis of a series of nor-BNI-related compounds was previously taken as evidence that the ligand does, in fact, not dimerize the receptor, because only one of



	wildtype κ receptor			κ receptor – E297K		
	K_i (nM)±s.e.m.	(n)	F_{mut}	K_i (nM) ± s.e.m.	(n)	F_{mut}
Diprenorphine	0.35 ± 0.05	(3)	1.0	0.53 ± 0.10	(3)	1.5
Naloxone	7.0 ± 0.75	(12)	1.0	6.5 ± 0.29	(4)	0.9
nor-BNI	0.10 ± 0.04	(6)	1.0	14.2 ± 2.2	(3)	142

Fig. 4. Effects of Glu^{297} substitution on the binding of the nonselective antagonists diprenorphine and naloxone and the selective κ-opioid receptor antagonist nor-BNI. The receptor segment encompassing TM-VI, EC loop 3, and TM-VII is represented schematically to indicate the helical structure of the TMs and the putative location of Glu^{297} at the interface between TM-VI and the EC receptor domain. *White circles*, differences between the κ and μ receptors; *black and gray circles*, positions of identity. F_{mut}, mutational factor; *n*, number of experiments.

the two pharmacophore halves of the nor-BNI molecule was shown to be required for κ-selective binding. Furthermore, an isomeric form of nor-BNI in which one half of the molecule was substituted with an inactive pharmacophore also acted as a κ-selective antagonist (24). Although the data presented here suggest that essential interaction points on the receptor for the binding of nor-BNI are located in a rather confined segment of the receptor in EC loop 3 and TM-VII, with Glu^{297} being of particular importance, multiple interaction points within this segment could easily be envisioned. In view of the size of the nor-BNI molecule, it is quite probable that additional, and possibly conserved, ligand interaction points exist elsewhere in the κ receptor. The two-site mode of receptor binding exhibited by nor-BNI might thus reflect different sets of ligand-receptor interaction points.

The high affinity binding of the nonselective compounds diprenorphine and (−)-naloxone by all of the κ and μ chimeric receptor constructs emphasizes that (−)-naloxone is a nonselective opiate ligand with respect to these two receptor subtypes. In fact, naloxone in particular has served as the nonselective pharmacophore in the design of the bivalent and κ-selective ligand nor-BNI. In some studies naloxone has, however, been reported to exhibit some preference for the κ and μ receptor subtypes, relative to the δ receptor (3), and essential epitopes have been proposed to be localized in the amino-terminal and highly divergent part of the δ receptor (25). However, in a separate study naloxone binding was retained by a μ receptor mutant in which essentially the entire amino-terminal EC domain had been deleted (26). These differences may be ascribed to the different cell lines used (27); also, as was recently demonstrated, the choice of a radioligand is in some instances critically important for a true comparison of ligand affinities for different receptor molecules (28).

Although the μ receptor is most often associated with the analgesic properties of opioid compounds, the undesirable side effects, such as addiction, are also a characteristic of μ-selective compounds. In contrast, a number of agonists selective for the κ receptor also display analgesic properties, with a reduced potential for abuse (29, 30). Additional insight into the molecular mechanism of action of these various opioid receptor ligands is therefore clearly of interest with regard to the development of highly selective compounds that exhibit potent analgesic properties together with reduced side effect liabilities.

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