

Structure–function analysis of the cloned opiate receptors: peptide and small molecule interactions

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Opiate receptors mediate the physiological actions of opioid peptides and the clinical effects of the synthetic opioid agonists and antagonists. Site-directed mutagenesis studies have revealed regions of opiate receptors that are essential for ligand recognition, and this could aid the design of more selective opioid ligands.

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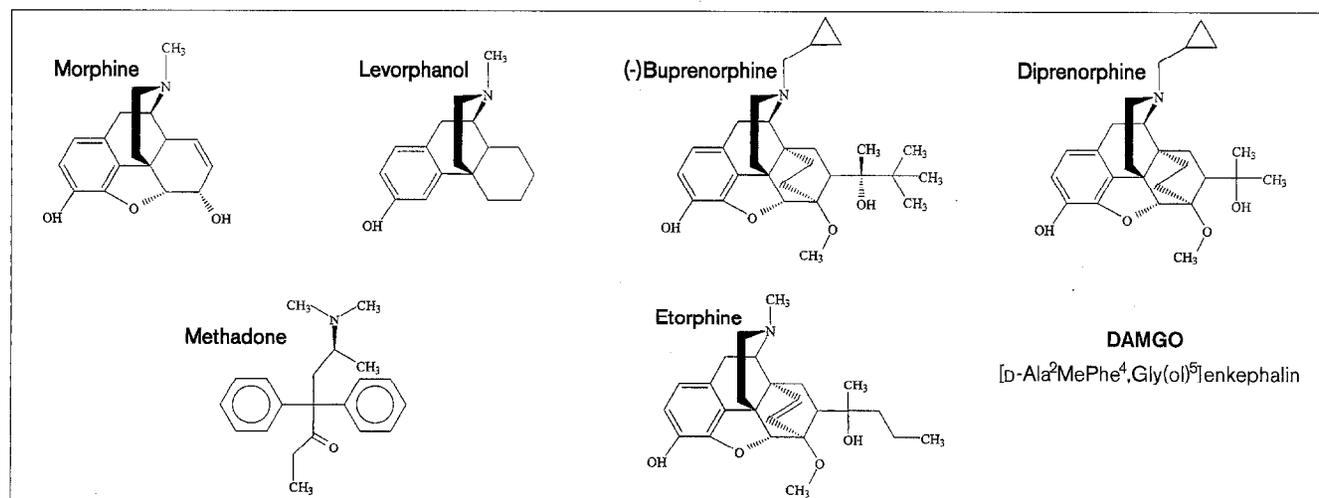
Opiates are used therapeutically to relieve pain and, in some cases, to produce euphoria. The finding that morphine and other opiates bind stereoselectively to sites in the brain led to a search for and eventual identification of the endogenous ligands for the opiate receptors, the enkephalins, endorphins and dynorphins. These endogenous opiates are peptides, whereas the common clinically used opiates (such as morphine, fentanyl and methadone) are alkaloid derivatives (see Fig. 1 for representative structures) [1], yet they can all produce analgesia and modulate motor activity and behavior.

Opiates induce their biological effects by interacting with membrane-associated receptors [2]. Pharmacological and physiological studies have suggested that multiple classes of opiate receptors exist in the body that respond

differently to morphine and its analogs. To date, three different types of opioid receptors have been defined and named either for the drugs used in the studies (μ for morphine and κ for ketocyclazocine) or for the anatomical location (δ for vas deferens). Molecular cloning studies confirmed that functionally distinct receptors are also genetically distinct, and it turns out that each receptor class has a number of subtypes that differ in their binding affinity for different drugs [1].

The distribution of opioid receptors in the central nervous system is complex, and considerable variations occur between different anatomical loci and species. The largest differences are observed with μ and κ receptors, especially κ . The distribution of δ receptors is well conserved across species. In general, δ receptors, which selectively bind agonists such as the peptide DPDPE and the antagonist naltrindole, are concentrated in the forebrain and spinal cord, and are relatively rare in midbrain and brainstem. In contrast, μ and κ receptors are widely distributed across tissues. The μ receptors, which have high affinity for most clinically used opiates and are characterized by their specific binding of the peptide DAMGO (see Fig. 1) and the antagonist peptide CTOP, are highly expressed throughout the basal ganglia and thalamic and spinal cord regions, where they mediate the analgesic effects of morphine. They are also expressed in brain regions involved in mood and motivation, and in brainstem regions that mediate the side effects (such as respiratory depression) of morphine.

Figure 1



The chemical structures of several different opioid ligands.

The κ receptors selectively bind the synthetic agonist U50 488 and the antagonist nor-BNI and they have high affinity for the endogenous peptide dynorphin A. They mediate the endocrine functions of opiates and are concentrated in the cerebral cortex, striatum and hypothalamus. The receptors in the limbic and mesolimbic regions are thought to be involved in the mood and motivational effects of opiates.

The signal transduction pathway of opiate receptors involves GTP binding (G) proteins that negatively couple the receptors to adenylyl cyclase [2] and regulate ionic conductances [3]. Opiates cause an inhibition of Ca^{2+} conductance and a potentiation of K^+ currents, hyperpolarizing neurons and resulting in an overall suppression of evoked and spontaneous neuronal activity.

The information available from structure–function studies on the opioid receptors has been limited by the fact that the levels of receptor expression found in native tissues and tumor cell lines that endogenously express the receptor are low. The recent cloning of the genes for the opiate receptors [4] has provided new tools to identify the domains of the receptors involved in ligand binding, G-protein coupling and agonist regulation. Here, we review what has been learnt about the structural requirements of opioid receptor function.

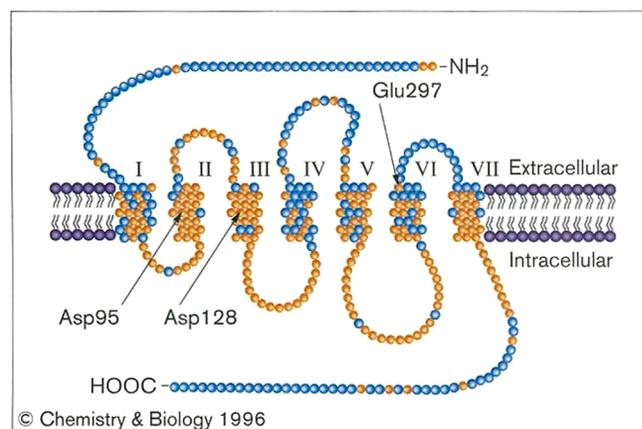
Cloning of the genes for the opiate receptors

The first opiate receptor gene to be cloned, the gene for the δ receptor, was cloned by expression [5,6]. The mouse κ and δ receptor genes were cloned by cross-hybridization [7], using genes for the somatostatin receptors, (a receptor family with high homology to the known opiate receptors [4]) as was the rat brain μ receptor [8]. The human opioid receptor genes have now been cloned [1], and are very similar to the rodent gene in amino acid sequence.

All the known opiate receptors are very similar to each other in amino acid sequence (60–70% identity) [4]. Regions of high similarity are shown in Figure 2. The most diverse segments of the receptors that differ significantly in amino acid sequence are located at the amino- and carboxyl termini, in the second and third extracellular loops and in transmembrane segment IV. These regions are suspected to be responsible for the functional diversity of these receptors.

When expressed in mammalian cells, the cloned receptors have similar pharmacological specificities to the endogenously expressed receptors [4]. The cloned κ receptor binds the synthetic compound κ elective ligand, U50 488, with high affinity, indicating that this receptor may correspond to the subtype referred to as the κ_1 receptor. As the cloned μ receptor has high affinity for naloxonazine it may correspond to the μ_1 subtype. These identifications are

Figure 2



Comparison of predicted amino acid sequences of the mouse δ and κ receptors and the rat μ receptor. The amino acid sequences of the cloned opioid receptors are presented as a seven-transmembrane-spanning model. Circles represent amino acid residues. Blue circles represent amino acids that differ between the three cloned receptors. Orange circles represent amino acids that are identical among the three receptors. Roman numerals refer to the transmembrane spanning segments. Mutations of aspartates 95 (Asp95) and 128 (Asp128) of the mouse δ receptor to asparagines are indicated. A critical residue involved in the binding of the κ antagonist nor-BNI is a glutamate at residues 297 (Glu297).

necessarily tentative, however, as the pharmacological specificities of the endogenously expressed μ and κ receptor subtypes are not clearly established [1].

A number of studies have suggested that subtypes of δ receptors can be distinguished by the antagonists BNTX and NTB [9]. Based on these selectivities, the cloned receptor corresponds to the δ_2 receptor [4]. Either there is another δ receptor subtype gene still to be cloned or the δ_1 subtype results from differences in mRNA or variations in protein processing.

When studying recombinant proteins, especially in protein families where several members of a family may have similar characteristics, it is always important to check that the recombinant protein has the characteristics of the endogenous protein. The three recombinant opiate receptors all couple to adenylyl cyclase, inhibiting forskolin-stimulated cAMP accumulation [5–8], like the endogenous proteins. The κ receptor couples to an endogenous omega conotoxin sensitive (N-type) Ca^{2+} channel when expressed in PC12 cells [10], as it does when endogenously expressed in neurons where it inhibits neurotransmitter release [10]. The cloned μ receptor has been reported to mediate agonist-induced increases in K^+ conductance via an inwardly rectifying K^+ channel [11], a channel important in regulating membrane potential and activity of neurons. Studies using oocytes have shown that the cloned receptors couple with the

recently cloned inward rectifier GIRK1 [11], and previous studies [3] have reported that endogenously expressed μ , δ and κ receptors are coupled to an inward rectifier to modulate neuronal excitability and firing rates. Thus, both the pharmacological and functional characteristics of the recombinant and endogenously expressed opiate receptors are similar, and data on the relationship between structure and function in the recombinant proteins can be taken as a good guide to the *in vivo* situation.

Structure-function analysis

Once the receptor genes were cloned, the obvious step was to perform mutagenesis studies to identify the domains of the receptors that are involved in different functions, especially ligand binding. Such studies may be useful in the rational design of new and better opiates for therapeutic uses.

The first mutagenesis studies simply tested whether the regions that are involved in ligand binding in other G-protein-linked receptors were also involved in drug binding to opiate receptors. Most G-protein-linked receptors have conserved aspartic acid residues in transmembrane-spanning regions II and III [12]. Mutations of these negatively charged residues to neutral asparagine have been found to diminish drug binding in non-opiate receptors [12,13]. It has been proposed that these residues serve as counter-ions to the basic regions of ligands and thus facilitate binding through electrostatic interactions.

First, the functional role of a conserved aspartate (residue 95) in the second transmembrane-spanning region of the δ receptor in the binding of ligands was tested [14] (Fig. 2). Asp95 had been reported to be critical for Na^+ to regulate agonist binding to other receptors, including the α_2 -adrenergic receptor [13]. Indeed, the Asp95→Asn mutant of the δ receptor no longer showed Na^+ regulation of agonist binding [14], consistent with the notion that this residue is involved in recognition. The mutant receptor also had much lower affinity than the wild-type receptor for selective agonists such as DPDPE, but its affinity for antagonists was normal. Thus, Asp95 is unlikely to be part of the recognition site of the receptor for selective agonists, as the sequence of transmembrane-spanning region II is very similar in all three opioid receptors. It may, however, be important in establishing the high affinity of the receptor for selective agonists by slowing the rate of dissociation of the agonists from the receptor through electrostatic interactions. As the mutation Asp95→Asn has limited effect on antagonist binding, agonists and antagonists must have different determinants for binding to the δ receptor.

Asp128, in transmembrane-spanning region III, is another conserved residue that is also involved in agonist binding to the δ receptor [15] (Fig. 2). The Asp128→Asn receptor mutant no longer showed high affinity agonist binding to

the receptor, although the effect of the mutation on antagonist binding was less profound. Mutations of this residue in the β -adrenergic receptor [16] also significantly affected agonist binding. Like Asp95, Asp128 of the δ receptor may selectively enhance the binding affinity of agonists for the receptor by reducing their rates of dissociation from the receptor. It is unclear, however, why antagonist binding is relatively unaffected by the Asp95→Asn and Asp128→Asn mutations, since antagonists too have cationic groups that are required for high-activity binding.

Antagonist binding to κ receptors

To investigate which regions of the κ receptor are involved in antagonist binding, Kong *et al.* [17] generated a chimeric κ/δ receptor, using the sequence from the amino terminus to halfway down transmembrane region I, and found that the amino-terminal region of the κ receptor is important in the binding of the non-selective antagonist naloxone to the κ receptor. Similarly, a series of κ/μ receptor chimeric receptors were used to show that nor-BNI, the κ selective antagonist, interacts with regions in the vicinity of the third extracellular loop and transmembrane segment VII [18]. It was therefore proposed that Glu297, at the border between transmembrane segment VI and the third extracellular loop, is a critical amino acid in the κ receptor for nor-BNI binding (Fig. 2). Mutation of this negatively charged residue to a positively charged lysine reduced the binding of nor-BNI to the κ receptor by 100-fold. The affinity of nor-BNI for the μ receptor is 100-fold lower than its affinity for the κ receptor and, interestingly, the μ receptor has a lysine at residue 303, corresponding to Glu297 in the κ receptor. Hjorth *et al.* [18] suggested that the Lys303 in the μ receptor may serve to repel nor-BNI, lowering its affinity for the μ receptor, while in the κ receptor Glu297 may serve as a counter-ion to attract the nor-BNI. The corresponding amino acid in the δ receptor is a tryptophan [4]. Nor-BNI has very little affinity for the δ receptor, perhaps because the bulky side group of the tryptophan prevents nor-BNI binding.

Agonist binding to κ receptors

Chimeric receptors in which the second extracellular loop of the κ receptor is exchanged with that of the μ receptor have higher affinity for the κ agonist dynorphin A than does the μ receptor [19]. This may be rationalized by observing that the second extracellular loop of the κ receptor has many more negatively charged amino acids than the corresponding region of either the μ or δ receptors, and that dynorphin A has an excess of basic charges in its carboxy-terminal region. The specificity of this interaction may also come down to electrostatic interactions.

The role of the second extracellular loop of the κ receptor in agonist binding is so far unique among the opiate receptors. It is not unprecedented in the wider G-protein-coupled receptor family, however; recent studies

have suggested that this region may be involved in the binding of selective peptide agonists to the somatostatin receptor SSTR1 [20]. But the use of an extracellular loop to form part of a recognition site for small synthetic drugs is rare. Studies of the adrenergic receptors [12] suggested that the transmembrane-spanning regions form hydrophobic pockets embedded in the membrane to which agonists and antagonists bind. Small molecules such as norepinephrine or isoproterenol, the ligands for the adrenergic receptors would easily fit into such pockets. Large peptides, such as dynorphin A (17 amino acids) or β -endorphin (31 amino acids) might be expected to fit into small pockets less well, however. Extracellular loops of receptors may therefore be more important as recognition sites for large peptide agonists than they are for smaller classical transmitters such as norepinephrine.

The mutagenesis studies performed on the κ receptor suggest that antagonists and the agonist bind to physically distinct sites within the receptor (Fig. 3a). These drugs are competitive in their interaction with the receptor, however. This implies that either the distinct recognition sites overlap in three-dimensional space, or binding to one site induces conformational changes in the receptor that hinder binding to the other site. The precise way in which the interactions of the agonists and antagonists with the receptor are distinguishable is important, as differences here may explain differences in the functional consequences of their binding.

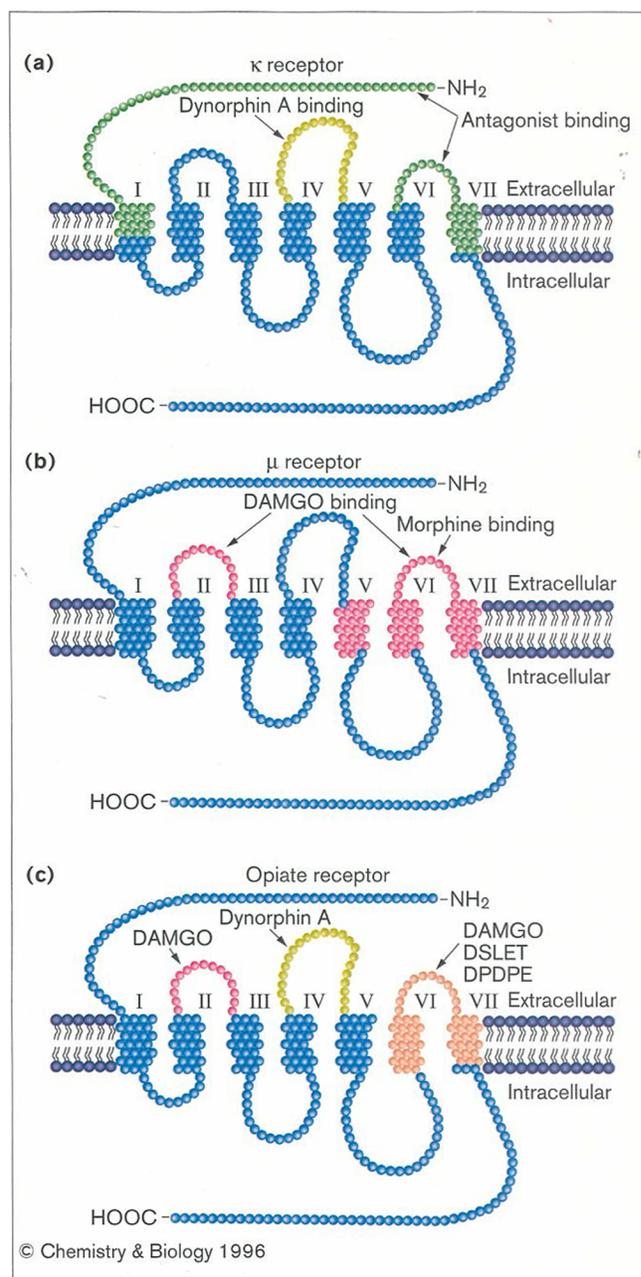
μ and δ receptor agonist recognition sites

Mutagenesis studies have also been conducted to identify regions of the μ receptor involved in agonist binding. Wang *et al.* [21] have generated a series of μ/δ receptor chimeras and reported that DAMGO, a peptide that is a selective μ agonist, bound to the first extracellular loop and transmembrane segment III of the μ receptor, whereas morphine and some other alkaloids bound to regions encompassing transmembrane segments V–VII and the third extracellular loop (Fig. 3b). These findings suggest that peptide and non-peptide agonists for the μ receptor bind to physically distinct sites within the receptor.

This study [21] also suggested that the selective δ receptor agonists DPDPE and DSLET bind to a region encompassing transmembrane segments V–VII and the third extra-cellular loop (Fig. 3c). Furthermore, two arginine residues in the third extracellular loop of the δ receptor were essential for DSLET binding since mutations of those residues to glutamines abolished DSLET binding [21]. Trp284, Val296 and Val297 in the third extracellular loop are critical for δ -selective agonists to bind to the receptor [22].

Very recently, it has been reported that aromatic amino acids in the transmembrane spanning regions of the

Figure 3



Agonist and antagonist receptor binding sites. (a) Potential agonist (yellow circles) and antagonist (green circles) binding sites of the κ receptor. (b) Potential agonist recognition sites (magenta circles) of the μ receptor. (c) Peptide agonist recognition sites are different in the three cloned opiate receptors. Dynorphin A may bind to a region of the second extracellular loop of the κ receptor. DSLET and DPDPE may bind primarily to the third extracellular loop of the δ receptor. DAMGO may bind to both the first and third extracellular loops of the μ receptor.

δ receptor also contribute to the binding of peptide δ -selective agonists [23]. Interestingly, the binding of the non-peptide selective agonist BW373U86 to the δ receptor was not clearly dependent on these aromatic amino

acids, nor was the binding of selective antagonists such as naltrindole or TIPP. The non-peptide ligands might be expected to fit more easily into a hydrophobic pocket and therefore be more dependent on the presence of aromatic amino acids than the large peptides which carry more charge.

Taken together, the mutagenesis studies suggest that the different opiate receptors have differences in their ligand binding domains, especially the peptide binding domains (Fig. 3c). The κ selective agonist dynorphin A has potential binding sites in the second extracellular loop. DAMGO, the selective μ receptor agonist, may have recognition sites in the first extracellular loop and transmembrane V and VII. The δ -selective agonists DPDPE and DSLET appear to have sites of interaction in transmembrane segments V–VII and the third extracellular loop of the δ receptor. Thus all of the opiate receptors appear to enlist extracellular domains to give tight and specific peptide binding, although different extracellular loops are used for different peptides. The drug recognition sites of the opiate receptors are also found in the extracellular domains, which are the regions that differ most in amino acid sequence, consistent with the hypothesis that regions of sequence divergence of the receptors are responsible for the functional differences of the receptors.

Intracellular domains of the opiate receptors

Although the extracellular loops of the opiate receptors vary in amino acid sequence, the sequences of the intracellular loops are very similar. As expected, given this structural similarity, all three opiate receptors couple to similar cellular effector systems and modulate those effectors in a similar way. The only intracellular domain of the opiate receptors that differs in amino acid sequence is the carboxyl terminus [4]. This region may not be necessary for coupling the opiate receptor to cellular effector systems, however, since in preliminary studies (A.D.B., G.B., J. Freeman, S. Li and T.R., submitted) truncation of the μ receptor carboxyl terminus did not prevent the receptor from coupling to adenylyl cyclase nor did truncation of 37 amino acids from the carboxyl terminus of the δ receptor [24].

Even though the three opiate receptors all couple to similar effector systems, their abilities to be regulated differ. Desensitization and down regulation of opiate receptors may be critical adaptive responses in the development of tolerance to opiates. For many G-protein-linked receptors, these responses involve post-translational modifications of intracellular domains. The coupling of κ and δ opiate receptors to adenylyl cyclase has been reported to undergo desensitization, which may be manifest as an uncoupling of the receptor from G proteins [25,26]. In both cases, the enzyme β -adrenergic receptor kinase has been proposed to serve as an intermediary in the phosphorylation of the

receptor, an event believed to facilitate the uncoupling of the receptor from adenylyl cyclase [25,26]. The μ receptors are much more resistant to desensitization, since μ agonists such as morphine and DAMGO do not appear to easily uncouple the receptor from the adenylyl cyclase response [27]. Since intracellular loops of the receptors are very similar in amino acid sequence, it may be that the carboxy-terminal region determines, at least in part, the differences in sensitivity to agonist regulation.

Amino acids in the carboxyl terminus of the δ receptor have been shown to be critical for the down regulation of the receptor following prolonged agonist treatment. The wild-type δ receptor is down regulated following agonist treatment, but a receptor truncated by 37 amino acids from its carboxyl terminus was less sensitive to agonist regulation [24]. Thr353 was also shown to be critical for the down regulation of the δ receptor.

The μ receptor also down regulated following treatment with agonists such as DADLE or etorphine [28]. However, preliminary studies (A.D.B., G.B. and T.R., unpublished results) with a truncated μ receptor also showed that it was down regulated following agonist pretreatment, suggesting that the carboxy-terminal amino acids of this receptor were not essential for the down regulation of this opiate receptor. These studies suggest that there may be structural differences in the opiate receptors that may contribute to their differential regulation by agonists.

Conclusion

Site-directed mutagenesis studies of the opiate receptors have revealed critical domains necessary for ligand binding. Modeling of these sites together with the known three-dimensional structure of the synthetic opiates should facilitate the rational design of a new generation of drugs with improved therapeutic potency and limited side effects. Opiates are the most effective analgesics available. Their use is limited by the development of tolerance and dependence, however. Furthermore, they inhibit respiration and gut motility and cause nausea, which diminishes their therapeutic usefulness [1]. New drug design could overcome these limitations.

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