Hypothesis

On the role of extracellular loops of opioid receptors in conferring ligand selectivity

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Abstract Based on an analysis of results taken from site-directed mutagenesis studies performed on opioid receptors, a role for the extracellular loops in conferring opioid subtype selectivity is proposed. It is suggested that the extracellular loop regions (which represent the region of highest sequence variability among opioid subtypes) interact with opioid ligands in a primarily non-specific fashion. Although these interactions are non-specific, they appear to play a discriminatory role in ligand binding and, in certain cases, prevent particular ligands from binding among receptor subtypes. We propose that selectivity may be imparted through a mechanism of exclusion, rather than specific pharmacophore recognition within the extracellular loops and N-terminal domain. This hypothesis is supported by a careful analysis of the binding profiles of several selective and non-selective ligands to a variety of chimeric mutants. These results, when combined with results taken from single-point mutation experiments point to the existence of a high affinity binding pocket within the transmembrane region which may be common among the opioid subtypes.

Key words: Opioid receptor; Extracellular loops; Chimeric receptor; Ligand binding; DAMGO

1. Introduction

Over the years, a great deal of effort has been devoted to understanding the structure–activity relationships of opioid ligands, especially as they relate to selectivities among the known receptor subtypes (δ, μ, and κ). Although binding data for a wide variety of peptide and non-peptide ligands has produced great insight to the basis for molecular recognition, a detailed understanding of the structural requirements for ligand binding has not yet emerged. Using the 'message-address' concept of Schwyzer [1], Takemori and Portoghese have suggested ligand binding and selectivity may be conferred through the recognition of two distinct structural units [2]. According to this concept, opioid ligands possess both an 'address' and a 'message' that play unique functional roles in ligand binding and receptor function. Although both elements are presumed to bind to the receptor, the address is thought to impart selectivity while the message is common. This concept is exemplified by the structures of endogenous opioid peptides enkephalin and dynorphin, where the N-terminal tyrosine residue may be considered the common message and the C-terminal domain presents the variable address. It has been proposed that the selectivity of dynorphin for κ is conferred through the presence of several basic amino acid residues in the address [2]. This address, when appended to the C-terminus of the δ-selective enkephalin, has also been shown to increase the κ affinity, lending some support to the generality of a subtype address [3].

Although this concept has been used to rationalize binding data for a number of opioid ligands, the absence of structural data relating the receptor and ligand has proven problematic in interpreting the results. The relatively recent cloning and sequencing of the opioid receptors, however, has begun to provide new insight to mechanism of ligand binding and receptor function [4–8]. Simple sequence analyses of the receptors has revealed that these proteins, as many G-protein coupled receptors, show very high sequence homology within the putative transmembrane domain (for review see [9]), suggesting a common binding pocket among opioid subtypes. The extracellular loops and N-terminal domains, on the other hand, show limited sequence identity. While indirectly, these observations support the general hypothesis that the common 'message' of opioid ligands may bind within the transmembrane domain of the receptor. Furthermore, the extracellular loops which are different among subtypes may recognize the variable 'address', thereby imparting the selectivities known for various ligands.

In order to isolate regions of the opioid receptor subtypes which play a role in selectivity, various studies involving chimeric mutants which allow for the exchange of various extracellular loops among subtypes have been reported [3,10–16]. Chimeric mutants between all possible combinations (δ/κ, δ/μ, and κ/μ) have been generated and in several studies, potential interactions between selective ligands and extracellular loop regions have been suggested [3,13,16]. In this report, we review the results of various chimeric studies performed on opioid receptors and interpret them in combination with results involving single-point mutations. The analysis presented correlates the binding data of several opioid ligands across all mutant sequences to determine the role that the extracellular loops and the N-terminus play in ligand recognition. The results also emphasize the importance of sites of recognition in the transmembrane domain that may be common among all subtypes.

2. Binding of DAMGO as a prototype for an alternative opioid ligand–receptor interaction model

The μ selective peptide agonist, DAMGO ([D-Ala^2, MePhe^4, Gly(ol)^5]enkephalin), has been the subject of several recent reports involving chimeric opioid receptors [10–13]. DAMGO

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is highly selective for the μ receptor, showing minimal affinity for δ or κ receptors (K_i > 1000 nM). [17]. In the first study, chimeric receptors are made which possess elements of δ and μ receptors and the most striking results are shown for receptors in which the first extracellular loop is exchanged. In particular, when the first extracellular loop (EL-1) from the δ receptor is incorporated into the μ receptor, DAMGO binding is significantly decreased. When the reciprocal chimeric receptor is constructed (EL-1 of μ is incorporated into the δ receptor) specific high affinity binding of DAMGO to the mutant receptor occurs [10]. Another study involving δ/μ chimera identified EL-1 as playing a key role in conferring the μ selectivity of DAMGO [11]. Based on these results, it is reasonable to suggest that EL-1 of the μ receptor is involved in DAMGO binding.

A third study compares the binding of μ selective agonists (including DAMGO) to two κ/μ chimeras. Interestingly, it was found that a κ/μ chimera in which only helix 6, EL-3, helix 7, and the C-terminus were taken from the μ receptor bound DAMGO with a K_i value of 11.8 nM whereas the native μ receptor gave an affinity of 3.3 nM. The reciprocal chimera which possessed the latter portions of the κ receptor bound DAMGO with a K_i value of 363 nM. The native κ receptor showed no affinity for DAMGO [12]. Another study involving DAMGO binding to κ/μ chimera has identified the third extracellular loop as the region of discrimination between μ and κ opioid receptors [13]. The results of these studies contrast sharply with the concept of specific high affinity contacts between DAMGO and EL-1 of the μ receptor. Rather than providing specific ligand binding sites, it is possible that the extracellular loops act via an exclusion mechanism whereby particular elements of the loops have unfavorable interactions with selective ligands preventing binding. In the case of DAMGO, its μ selectivity may be conferred by unfavorable interactions with the extracellular loops of δ and κ, rather than specific favorable contacts between DAMGO and the extracellular loops of m. This notion is in harmony with an opioid binding pocket wherein the 'message' portion of opioid ligands binds inside the receptor cavity formed by the 7 transmembrane (TM) domains which possess high sequence homology among the opioid receptor subtypes. In this view, the extracellular loop regions act as a barrier to the high affinity ligand binding region which resides in the transmembrane domain.

This view is also supported by results of single-point site-directed mutagenesis studies on opioid receptors. An analysis of these results shows a trend that selective opioid ligands are more sensitive to point mutations in the transmembrane region than are non-selective ligands [18–20]. In other words, binding of selective ligands is typically reduced by such point mutations whereas non-selective ligands remain relatively unaffected. This is well demonstrated by DAMGO binding to the μ receptor. Several studies have shown that single-point mutations of aspartate residues in TM 2 and 3 as well as the histidine in TM 6 each cause dramatic decreases in DAMGO binding to the μ receptor [19,21]. Such results are not compatible with a model in which specific favorable interactions with the loop regions stabilize ligand binding and confer selectivity. However, if the path of the selective ligand to the receptor cavity is inhibited by the loops (as suggested in the above model) it is possible that this limits the possible orientations of the ligand in approach to the receptor cavity, making it highly dependent on favorable interactions found inside the ligand binding pocket. Thus, even though DAMGO binds the μ receptor with high affinity, its path to the binding pocket inside the receptor may still be restricted by the extracellular loops such that loss of any binding sites in the cavity via mutation would cause a significant decrease in ligand binding.

By contrast, relatively non-selective opioid ligands, which tend to be small rigid molecules possessing the characteristic tyramine or 'message' moiety (e.g. ethyl ketocyclazocine, bremazocine, diprenorphine), give a much different binding profile to mutant receptors. In particular, several studies have demonstrated that these ligands bind virtually all chimeric receptors with affinities equal to that of wild type receptors [10–13,16]. This strongly suggests that high affinity binding is conferred in regions of the TM domains of the receptor regardless of the subtype (i.e. binding to residues conserved among receptor subtypes). The non-selectivity of these ligands is, in our view, due to their ability to essentially bypass the extracellular loops and enter into the transmembrane region. This lack of restriction of the loops on these ligands gives them more freedom of mobility which would make them less dependent on individual interactions in the ligand binding site. This additional freedom would explain their relative insensitivity to single-point mutations compared to selective ligands.

3. Discussion

Although the above conceptual model has been discussed with respect to only a single ligand, it was derived from an analysis of a variety of results. As such, we will now discuss its application to the interpretation of other site-directed mutagenesis results performed on opioid receptors. Several reports involving chimeric receptors have focused attention on EL-2 of the κ receptor as being important in determining the κ selectivity of dynorphin peptides [3,14–16]. One study using a μ/κ chimera in which EL-2 of the κ receptor is inserted into the μ receptor demonstrates a marked increase in affinity for α-neoendorphin, dynorphin A(1–13), and dynorphin A(1–17) [14]. A similar pattern of results emerged from a study of enkephalin and dynorphin fragments interacting with δ and κ receptors and four δ/κ chimeric receptors. This study emphasized the role of coulombic interactions between the basic residues of the dynorphin fragments and the acidic residues located on EL-2 of the κ receptor [3]. These interactions appear to be crucial to conferring κ selectivity since EL-2 of the κ receptor possesses five acidic residues which are not found in δ and μ.

Recently, a more comprehensive study of the molecular basis of affinity and selectivity between δ and κ receptors has appeared which examines the binding of various selective and non-selective, peptide and non-peptide, agonist and antagonist opioid ligands to δ, κ, and δ/κ chimeric receptors [15]. Some of the most interesting results from this study regard the selectivity of enkephalin and dynorphin to δ and κ receptors. In particular, dynorphin A(1–13) shows high affinity for both wild type receptors (K_i values of 8.7nM and 0.9 nM for δ and κ respectively) while the enkephalins are very δ selective (3.4 nM and >10000 nM for Met-enkephalin, 2.2 nM and 1300 nM for Leu-enkephalin). Analysis of the binding studies on chimeric receptors indicates enkephalins bind with reasonable affinity to chimera having only the C-terminal portion (helices 5, 6, 7, their interconnecting loops, and the C-terminus) from the δ receptor. To explain these results, it has been suggested that the δ recep-

tor contains a high affinity binding pocket for the 'opioid core' (defined as Tyr-Gly-Gly-Phe) in the C-terminal domain capable of binding both enkephalins and dynorphin. This pocket is then said to be relatively weak in the κ receptor and the binding of dynorphin to κ is dependent on the coulombic interactions between the basic residues of the peptide and the anionic residues found in EL-2 of the κ receptor [15].

According to our conceptual model given above these results can be explained in an alternative fashion. For example, it is possible that unfavorable interactions between enkephalins and extracellular loop 3 of the κ receptor prevent enkephalin binding to the κ receptor. The sequences of helices 5, 6, and 7 of the δ and κ opioid receptors are very highly homologous. Thus, the enkephalin binding of the κ/δ chimera possessing the latter portion of the δ receptor is very likely due to differences in EL-3 between κ and δ where there is limited sequence homology. Furthermore, in our interpretation, it is not evident that κ and δ bind the opioid core differently. As stated above, the high homology among opioid receptor subtypes in the transmembrane region suggests a common binding pocket for common structural elements found in opioid ligands. If enkephalin binding to the κ receptor is indeed prevented by EL-3 of κ then it is very possible that the pocket in κ is very similar to that of δ. Mutational studies performed on opiate receptors to date have provided no powerful evidence that the tyramine moiety of small non-selective ligands binds in a different manner than the N-terminal tyrosine of peptide ligands regardless of the receptor subtype.

With respect to the importance of coulombic interactions between dynorphin and anionic residues on EL-2 of the κ receptor, two points should be raised. First, although dynorphin possesses highest affinity for κ receptors, it has been shown to have K_i values in the nanomolar range for both δ and μ [14,15]. This demonstrates that interactions between the C-terminal end of dynorphin and the anionic residues found on EL-2 of κ are not required for opioid binding. Secondly, mutation of the aspartate residue in helix 3 of the κ receptor causes a 600-fold decrease in dynorphin binding (T. Reisine, unpublished results). This again emphasizes the importance of transmembrane regions in ligand binding and casts some doubt on the importance of the contribution of the proposed coulombic interactions in dynorphin binding to the κ receptor.

An analysis of the results of δ/κ and μ/κ chimera highlights the putative role of extracellular loop 3 of the κ receptor in preventing the binding of both δ and μ selective ligands to the κ receptor. In order to understand the δ/κ selectivity of endogenous opioid peptides, it is perhaps more useful to investigate why enkephalins do not bind to the κ receptor rather than why dynorphin does since both peptides appear to bind the δ receptor with high affinity [15]. A chimeric receptor possessing the N-terminus, EL-1, and EL-2 from the δ receptor and EL-3 from the κ receptor showed minimal, if any, affinity for enkephalins as well as other δ selective peptides. Importantly, the reciprocal chimera, in which κ/EL-3 is absent, showed markedly increased affinities for δ selective peptides even though the majority of the chimeric construct is from the κ receptor [15]. It is apparent that the presence of EL-3 from the κ receptor is sufficient to inhibit the binding of the δ selective peptides. Results from μ/κ are similar. In the μ/κ case, it has been shown the presence of EL-3 from κ (in a chimera with the N-terminus, EL-1 and EL-2 from the μ receptor) gives notable decreases in binding affinity for μ-selective ligands DAMGO, PLO17, sufentanil, and morphine [12]. As indicated above, results from μ/δ chimera have shown that EL-3 of the μ receptor is not required for specific, high affinity binding of DAMGO thus supporting the exclusionary effect of κ/EL-3 [10,11].

Another class of opioid ligands that displays an interesting pattern of site-directed mutagenesis results is that of the κ selective arylacetamides such as U50,488. A set of six μ/κ chimera was generated and the binding of arylacetamides U50,488 and U69,593 was tested across each chimera as well as the wild type (μ and κ) receptors [16]. It was found that the incorporation of EL-2 of the κ receptor into the μ receptor gave κ-like binding of opioid peptides such as dynorphin A and B, and α-neo-endorphin (as had been shown previously [14]). By contrast, U50,488 and U69,593 did not bind this chimera. The arylacetamides did show high affinity binding for the reciprocal chimera in which EL-2 of the μ receptor was inserted into the κ receptor. Interestingly, another pair of reciprocal chimera was generated in which neither receptor showed affinity for U50,488 and U69,593. The authors of this study concluded that U50,488 and U69,593 appear to require the whole κ receptor except EL-2 to attain high affinity binding. In a study of δ/κ chimera, it was found that a chimeric mutant possessing the C-terminal portion (from helix 5 to the C-terminus) of the δ receptor could bind U50,488 with affinities very similar to that of the κ wild type. Other δ/κ chimera showed decreased binding for U50,488 relative to the wild type κ receptor [15].

These results, when interpreted within our conceptual framework, are consistent with several aspects mentioned previously. It is possible that the extracellular loops of δ and μ prevent binding of arylacetamides to the transmembrane region and that this is the basis of their high κ selectivity. The fact that U50,488 shows no affinity (K_i>10,000 nM) for four of six μ/κ chimeric receptors [16] is consistent with the idea that the extracellular loops of the κ receptor do not possess binding determinants specific for this ligand. Apparently, the loops of the κ receptor present a barrier insufficient to prevent binding of arylacetamides to the receptor yet they provide enough of a barrier to make binding highly dependent on favorable interactions in the binding pocket. Such dependence is evidenced by the dramatic decreases in binding of U50,488 and U69,593 upon mutation of aspartate residues in helices 2 and 3 of the κ receptor ([20]; T. Reisine, unpublished results).

It is noteworthy that in each of the studies discussed above the ligands involved are agonists. This obviously raises questions pertaining to antagonist binding. Of course, it is unlikely that antagonists bind to opioid receptors in a fashion identical to that of agonists since agonists can effect G-protein coupling while antagonists do not. The opioid antagonist naloxone has been shown to bind all three opioid receptor subtypes with relatively high affinity (K_i of 17 nM, 2.3 nM and 0.93 nM for δ, κ and μ respectively) [17]. Similarly, naloxone binds to chimeric receptors with affinities similar to that of wild type receptors [14,15]. Also, naloxone binding to the μ receptor can be reduced by single-point mutations in the transmembrane region [21]. These results suggest that opioid binding of naloxone must be largely conferred by regions of the transmembrane domain common to opioid receptor subtypes similar (but not identical) to the non-selective agonists discussed above. The binding of selective antagonists to chimeric receptors has been much less

well studied than that of agonists. From the studies that have been done, it is found that selective antagonists have somewhat different binding profiles to chimeric receptors than their agonist counterparts [12,15,16]. For example, the κ selective antagonist, norbinaltorphimine, binds in a different manner to δ/κ and μ/κ chimera than do the κ selective arylacetamide agonists [15,16]. It has been noted that it is not clear if this is due to their difference in structure (arylacetamide versus alkaloid dimer) or function (agonist versus antagonist) [15]. In light of the lack studies performed on antagonists to date, it is not clear if or how the concepts outlined above apply to selective antagonists.

4. Summary and conclusion

In this report, a conceptual model for opioid selectivity based on the interpretation of results of single-point and chimeric mutations of opioid receptors has been presented. According to our analysis, we hypothesize that the extracellular loops of opioid receptors confer selectivity primarily by a mechanism of exclusion. This is not to say that favorable interactions between opioid ligands and receptor extracellular loops do not occur. Undoubtedly, such interactions do occur and play some role in ligand recognition and specificity. However, the results of the various mutation experiments performed to date have not demonstrated the existence of a specific, high affinity binding site on the extracellular loops. Indeed, analysis of some particular cases provides strong evidence toward the exclusion of this possibility.

Our proposal for the basis of opioid selectivity also has important consequences for ligand design. It is difficult to rationally design a selective ligand if there is no particular 'address' binding site that confers specificity to a given receptor. For example, highly selective ligands such as DAMGO and U50,488 do not appear to have high affinity binding sites on receptor loops. To understand specific ligand-receptor interactions, it may be as useful to assess which elements of the 'address' moiety prevent binding to a particular receptor subtype as it is to look for receptor sites that enhance binding. Again, our analysis does not exclude the possibility of 'address' binding subsites on particular receptors. It has been noted previously that selectivity can be conferred by enhanced binding as well as prevention of binding to a given subtype [2]. The site-directed mutagenesis studies performed to this point, however, emphasize the importance of an exclusion mechanism.

In a broad sense, it is possible that the role of the extracellular loops of opioid receptors is to exclude binding of ligands to the relatively promiscuous binding site located within the transmembrane domain. In its simplest form, opioid receptor binding would involve binding of the tyramine moiety of opioid ligands to a pocket formed by the transmembrane helices. This pocket may be common to all opioid receptor subtypes. Selectivity is conferred by the interplay between the ligand and the extracellular loops as the ligand attempts to get its 'message' to the binding pocket. The extracellular loops thus act as a gate which allows for the passage of certain ligands while excluding others. While this model is very likely overly simplified, it is consistent with our analysis of results taken from site-directed mutagenesis experiments on opioid receptors. In addition, this hypothesis is further supported by the following fundamental observations. First is the existence of relatively non-selective opioid ligands such as bremazocine and ethylketocyclazocine. These ligands tend to be small and rigid enabling them to more easily bypass extracellular loops than bulkier ligands. Also, they possess the critical tyramine moiety which most likely binds in the receptor cavity. This is demonstrated by the fact that these ligands bind virtually all chimeric receptors with equal affinity. Second, the high sequence identity of transmembrane regions among opioid receptor subtypes is suggestive of a common binding site within this region. Although it has by no means been demonstrated, there is, as yet, no convincing evidence that the common elements of opioid ligands do not bind elements common among the opioid receptor subtypes in the transmembrane domain. Studies involving single-point mutations of residues in the transmembrane domain of opioid receptors would be instrumental in learning more about this binding site.

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