Construction of a High Affinity Zinc Switch in the \( \kappa \)-Opioid Receptor*

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Very limited structural information is available concerning the superfamily of G-protein-coupled receptors with their seven-transmembrane segments. Recently a non-peptide antagonist site was structurally and functionally replaced by a metal ion site in the tachykinin NK-1 receptor. Here, this Zn(II) site is transferred to the \( \kappa \)-opioid receptor by substituting two residues at the outer portion of transmembrane V (TM-V), Asp223 and Lys227, and one residue at the top of TM-VI, Ala298, with histidyl residues. The histidyl residues had no direct effect on the binding of either the non-peptide antagonist \([^{3}H]diprenorphine \) or the non-peptide agonist, \([^{3}H]CI977\), just as these mutations/substitutions did not affect the apparent affinity of a series of other peptide and non-peptide ligands when tested in competition binding experiments. However, zinc ions in a dose-dependent manner prevented binding of both agonist and antagonist ligands with an apparent affinity for the metal ion, which gradually was built up to \( 10^{-6} \) M. This represents an increase in affinity for the metal ion of about 1000-fold as compared with the wild-type \( \kappa \)-receptor and is specific for Zn(II) as the affinity for e.g. Cu(II) was almost unaffected. The direct transfer of this high affinity metal ion switch between two only distantly related receptors indicates a common overall arrangement of the seven-helix bundle among receptors of the rhodopsin-like family.

G-protein-coupled receptors with their seven-transmembrane segments (7TM) constitute a true superfamily of membrane proteins with hundreds of individual members. Among the three major families of 7TM receptors, the rhodopsin-like family is by far the quantitatively dominating. All types of chemical messengers act on these receptors, and they also serve as sensory molecules in our olfactory system and obviously in the eye. However, our knowledge about their molecular structure is quite limited. Rhodopsin has recently been characterized by cryoelectron microscopy, and a seven-helix bundle was observed (1, 2). Yet, their resolution does not allow for firm identification of which helix corresponds to which electron density. Thus, in fact we do not even yet know the helical connectivity for certain or whether the helical bundle is arranged in a clockwise or anti-clockwise manner, even though the general model proposed by Baldwin (3) has become widely accepted.

Although the mutations that affect ligand binding in different 7TM receptors appear to cluster on certain faces of the helices (4), we do not have any hard evidence that the many different receptors adopt a similar conformation. Ligand binding sites as such have not been transferred between receptors, although chimeric constructs and single substitutions in some cases have conveyed an increased affinity for a particular ligand (5–8).

Recently we introduced a high affinity zinc-binding site in the tachykinin NK1 receptor (9). This site was introduced by systematically replacing residues in the binding site for the prototype non-peptide antagonist, CP96,345, with His residues. In the final construct, the zinc ion appeared to be coordinated with a submicromolar affinity by two histidyl residues placed at the top of TM-V and one at the top of TM-VI (9). In the present study we have attempted to move this well defined zinc-binding site to the \( \kappa \)-opioid receptor, which is only 30% identical to the NK-1 receptor in the transmembrane domains. Amino acid residues located at the three equivalent positions at the top of TM-V and -VI were mutated to histidyl residues in the \( \kappa \)-opioid receptor. The 1000-fold increase in zinc affinity as determined by the ability of the metal ion to inhibit the binding of both radiolabeled opioid agonist and radiolabeled opioid antagonist supported the notion that the helical arrangement is similar among 7TM rhodopsin-like receptors.

**MATERIALS AND METHODS**

Site-directed Mutagenesis—The point mutations were introduced in the receptor by the polymerase chain reaction overlap extension technique (10) using as template the wild-type rat opioid receptor cDNA (kindly provided by David K. Grandy, Vollum Institute, Oregon). All reactions were carried out using the Phusion polymerase (Stratagene) under conditions recommended by the manufacturer. The generated fragments were then subcloned into the pTEJ-8 eukaryotic expression vector (11) containing the wild-type \( \kappa \)-opioid receptor cDNA by substituting the wild-type cDNA fragment with the mutated cDNA fragment. The mutations were verified by restriction endonuclease digestion and DNA sequencing (U. S. Biochemical Corp. Sequenase). Accordingly, the three following constructs were generated: A298H, D223H/K227H, and D223H/K227H/A298H.

Expression of Mutant Receptor cDNAs in COS-7 Cells—COS-7 cells were grown in Dulbecco's modified Eagle's medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 10 \( \mu \)g/ml gentamicin. The wild-type \( \kappa \)-receptor and the mutant receptors were transiently transfected into COS-7 cells by the calcium phosphate precipitation method as described previously (12).

Ligands—The antagonist radioligand \([^{3}H]diprenorphine \) (1H1DIP) (specific activity, 30 Ci/mmol) and the agonist radioligand \([^{3}H]CI977\) (specific activity, 43 Ci/mmol) were obtained from Amersham Corp. The peptide agonists dynorphin A-(1–17) and DAKLI were purchased from Peninsula Laboratories (Belmont, CA). The non-peptide agonist CI977 was kindly provided by the Parke-Davis Neuroscience Research Centre. The antagonists Naloxone and Nor-binaltorphimine (Nor-BNI) were purchased from Research Biochemicals (Natick, MA).

Receptor Binding Assays—The transfected COS-7 cells were transiently transfected to 24-well culture plates 1 day after transfection and 24 h prior to the binding experiment. The cells were seeded at a density between \( 1 \times 10^5 \) and \( 1 \times 10^6 \) cells/well depending on the expression efficiency of....
and the choice of radioligand. The number of cells per well were adjusted aiming at 5–10% binding of the added radioligand. Binding was performed for 20 h at 4 °C with 0.25 nM [3H]DIP or 0.20 nM [3H]CI977 plus variable amounts of unlabeled peptide or non-peptide compounds present in 0.5 ml of 50 mM Tris-HCl, pH 7.4, containing 5 mM MgCl₂ and 0.1% (w/v) bovine serum albumin (Sigma) supplemented with 100 μg/ml bacitracin (Sigma).

Molecular Modeling—An initial model of the κ-opioid receptor was built using bacteriorhodopsin as a template. Initially, the a-helices corresponding to the transmembrane domains were built, and the amino acid side chains were oriented in their preferred positions and energy minimized using the consistent valence force field of DISCOVER ( Biosym Ltd., San Diego, CA). The κ-opioid receptor sequence was aligned to the bacteriorhodopsin sequence, and subsequently the a-helices were superimposed on the three-dimensional structure (13). The alignment and the superimposition were mutually changed until the model fit with the known mutagenesis data from other receptor systems like e.g. rhodopsin, monoamine receptors, and the tachykinin NK1 receptor. Then the a-helix distances were changed to fit with the proposed helical arrangement for rhodopsin as proposed by Baldwin (3). TM-V and TM-VI were further restrained based on the data from the introduction of the zinc binding in the NK1 receptor (9) and the κ-opioid receptor (this work). Finally the model was energy-minimized using the consistent valence force field of DISCOVER. The histidyl residues at the top of TM-V and TM-VI were oriented in a mutated receptor model (see Fig. 3) and manually restrained to accommodate the binding of a zinc ion. The histidyl residues and the metal ion were oriented to fit the known geometries as observed for other structurally resolved zinc ion-binding sites coordinated by histidyl residues (14).

**RESULTS**

The His residues were introduced in three stages in the κ receptor at positions corresponding to those previously identified in the NK-1 receptor (Fig. 1). First, a His residue was introduced at position 24 in TM-VI, A298H (concerning the generic nomenclature-numbering system for 7TM receptors; see Fig. 1 and Ref. 4). Second, a bis-His site was introduced at positions 1 and 5 in TM-V, D223H/K227H. Finally, the His residues in the two constructs were combined in the triple His construct, D223H/K227H/A298H.

As shown in Table I, although two of the substitutions could be considered to be rather dramatic alterations of the side chain in both size and charge, His for Ala and His for Asp, the introduction of the three His residues only had a minor effect on the binding affinity of both peptide agonists, dynorphin A and DAKLI, and non-peptide antagonists, naloxone and nor-BNI. In fact, the affinity of the non-peptide agonist CI977 was improved 6–7-fold in these constructs compared with the wild-type κ receptor (Table I).

The affinity for Zn(II) was monitored indirectly through its effect on the binding of radiolabeled antagonist, [3H]DIP, and agonist, [3H]CI977, respectively. The apparent affinity for Zn(II) on the wild-type κ receptor was 1.2 × 10⁻³ M, when using [3H]DIP as radioligand (Table I and Fig. 2A). Introduction of the single His residue at position VI:24, A298H, increased the affinity for zinc ions 8-fold whereas in the construct with the bis-His substituents at the top of TM-V the affinity for Zn(II) was increased 160-fold to 7.5 × 10⁻⁸ M. When all three His residues were combined the affinity for Zn(II) was 1.2 × 10⁻⁹ M corresponding to a 10,000-fold increase in apparent affinity (Table I and Fig. 2A). In the wild-type receptor and in the

### Table 1

**Effect of histidyl substitutions at the outer segments of TM-V and TM-VI of the rat κ-opioid receptor.**

|          | WT κ-opioid | A298H | D223H/K227H | D223H/K227H/A298H |
|----------|-------------|-------|-------------|-------------------|
| IC₅₀ pm  | I₅₀ pm      | -Fold | I₅₀ pm      | -Fold             | I₅₀ pm         | -Fold         |
| Agonists |             |       |             |                   |                |               |
| Dynorphin A | 0.42 ± 0.15 (4) | 0.36 ± 0.09 (3) | 0.86           | 0.69 ± 0.23 (3) | 1.6            | 2.7 ± 1.4 (5) | 6.4         |
| DAKLI     | 0.21 ± 0.04 (4) | 0.14 ± 0.024 (5) | 0.67           | 0.50 ± 0.15 (4) | 2.4            | 0.61 ± 0.10 (4) | 2.9         |
| CI977     | 6.3 ± 1.0 (5) | 0.84 ± 0.22 (4) | 0.13           | 1.0 ± 0.29 (5) | 0.16           | 1.0 ± 0.26 (4) | 0.16       |
| Antagonists |            |       |             |                   |                |               |
| Naloxone  | 11 ± 1.2 (5) | 30 ± 8 (4) | 2.7           | 110 ± 10 (4) | 10            | 40 ± 9.4 (1) | 3.6         |
| Nor-BNI   | 0.42 ± 0.11 (4) | 0.35 ± 0.06 (3) | 0.83           | 0.85 ± 0.14 (4) | 2.0           | 1.0 ± 0.50 (3) | 2.4         |
| Metal ions |            |       |             |                   |                |               |
| ZnCl₂     | 1200 ± 80 (5) | 150 ± 23 (4) | 8             | 7.5 ± 0.5 (3) | 160           | 1.2 ± 0.15 (5) | 1000        |
| CuCl₂     | 77 ± 8 (4)  | 52 ± 6 (4)  | 1.5           | 11 ± 1.1 (4) | 7             | 8.1 ± 2.0 (4) | 9.5         |
| ZnCl₂⁺    | 1100 ± 170 (5) | 200 ± 60 (3) | 5.3           | 4.9 ± 0.9 (5) | 220           | 0.60 ± 0.22 (4) | 1800       |
|           |             |       |             |                   |                | 310 ± 120 (4) | 3.5        |

*Performed with radiolabeled agonist, [3H]CI977. For the triple mutant both the high and low affinity sites are indicated when using [3H]CI977.
mono- and bis-His constructs the results, obtained with the radiolabeled agonist, [3H]CI977, were very similar to the results obtained with the radiolabeled antagonist, [3H]DIP (Table I). The competition binding curve for Zn(II) was not monophasic when using the radiolabeled agonist, [3H]CI977. Whole cell binding experiments were performed in transiently transfected COS-7 cells.

We next used the information from this study to construct a molecular model of the κ-opioid receptor based on the rhodopsin template (1, 3) (Fig. 3). The orientation of the transmembrane domains, the relative orientation of the seven-helix bundle or at least of TM-V and -VI as shown in Figs. 1 and 3.

**DISCUSSION**

The transfer of the triple-His zinc site from the NK-1 receptor to the κ-opioid receptor, which results in similar, high affinity metal ion affinity in both receptors, indicates that the relative orientation of the seven-helix bundle or at least of TM-V and -VI is similar in these two proteins. Obviously we cannot exclude the possibility that the presence of the metal ion is able to distort two rather dissimilar receptor structures to form two equally high affinity complexes. However, we find it more likely that the almost identical, high affinity for zinc ions introduced by the three His residues in the two different receptors indicate that these residues are placed relatively similarly in relation to each other and in a rather similar environment in the two proteins. Since only 30% of the residues are identical in the NK-1 and the κ receptor in the transmembrane domains, the direct transfer of this zinc site supports the notion that despite the low degree of primary sequence conservation, rhodopsin-like receptors do share a common seven-helix bundle structure in the membrane. The high affinity coordination of a metal ion in a 7TM receptor and its ability to prevent ligand binding raises both structural and functional issues.

**Metal Ion Sites and 7TM Receptor Structure**—In the present study we chose a helical arrangement guided by the rhodopsin structure of Schertler and co-workers and by the helical assignment of Baldwin (3). The rotation of the helices was optimized in accordance with accumulated data from mutational analysis of multiple 7TM receptors (4). Subsequently the structure was refined in the interface between TM-V and -VI by the constructed zinc site. The two His residues placed at the top of TM-V are situated in the i and i + 4 positions in the amino acid sequence, which is optimal in an α-helical structure (20, 21). The relative position of this bis-His site at the top of TM-V to
the single His at the top of TM-VI imposes important conformational constraints upon the receptor model, both in respect to helical rotation and vertical shift.

Construction of metal-binding sites has been used extensively in the last few years in protein engineering, for example in de novo designed proteins (22, 23), in modulating the enzymatic activity of proteins by metal ion switches (24), and in modulating the activity of monodonal antibodies (25, 26). However, this has mainly been performed in proteins where a high resolution x-ray structure was available or in de novo designed proteins. Not until recently have metal ion sites started to be used in the structural and functional analysis of membrane proteins where basically no structural information is available (9, 27). In the lactose permease a bis-His Mn(II) site was introduced on the basis of site-directed excimer fluorescence information of the spatial proximity of the two side chains in TM-VIII and -X (27). In our case the mutational mapping of residues in the presumed binding pocket for a non-peptide antagonist was the starting point (8, 9, 28, 29). Also it has recently been shown that introducing cysteine residues at these positions in rhodopsin can enable the formation of disulfide bridges (30).

Molecular Mechanism of Zinc Ions in Preventing Ligand Binding—In the NK-1 receptor the zinc site was constructed in the presumed binding pocket for the prototype non-peptide antagonist CP96,345 (9). These mutations did not per se affect the binding of the agonist, substance P, which was expected on the basis of the fact that previous mutational mapping of the CP96,345 pocket did not impair substance P binding (8, 28, 29). It was suggested that Zn(II) and CP96,345 acted in a similar fashion as antagonists on the mutated and wild-type receptors, respectively, by binding between the outer segments of TM-V and -VI and thereby stabilizing a conformation of the receptor that could not bind the agonist (9, 32).

The introduction of the histidyl residues between TM-V and TM-VI in the κ-opioid receptor could be considered to be a rather dramatic alteration of the presumed binding pocket. However, from previous studies (33–36) it could be anticipated that the histidyl residues introduced at the top of TM-V and TM-VI might only have a minimal effect on the binding of the ligands, and this was also found to be true (Table I). For instance, using chimeric receptors between the κ- and δ-opioid receptor, it has been found that the selectivity of non-peptide κ agonist is mainly achieved by interactions with structural elements within TM-I to TM-IV and the second extracellular loop (36), whereas the C-terminal portion of TM-IV and the second extracellular loop are important for peptide agonist binding (33, 34, 36). In contrast, the region responsible for the selectivity of the nonpeptide antagonist, nor-BNI, has been located in and around the third extracellular loop (34, 35). From these studies it appears that TM-V and TM-VI are not directly involved in the binding of the various κ ligands as also found in the study presented here (Table I).

The unchanged affinity for both agonist and antagonist binding in the triple-His construct and the small size of the metal ion, in our opinion, support an allosteric mechanism for the function of the zinc ions on the mutated receptors. Thus, Zn(II) may exclude the other ligands from binding by constraining the receptor in a conformation that excludes their binding, i.e., stabilize a non-permissive conformation (9, 32). It cannot be excluded that Zn(II) prevents agonist and antagonist binding by a volume exclusion effect, as suggested for the molecular function of non-peptide versus peptide ligands (37). However, the small size of the metal ion and the presumed localization of ligand-binding sites to other positions within the receptor make this rather unlikely.

Interestingly, a clear biphasic competition curve is observed for Zn(II) when [3H]CI977, the agonist, is used as a radioligand as opposed to the classical monophasic competition curve found with [3H]DIP, the antagonist. This indicates that there is an important difference in the binding mode between antagonists and agonists. The two-component Zn(II) curve observed with the agonist tracer suggests the occurrence of two different states of the receptor in the presence of bound agonist, at least in the presence of Zn(II). On the other hand the displacement curve for antagonist tracer reflects a single high affinity zinc-binding site. It could then be envisioned that the antagonist keeps the receptor in a single stringent conformation.

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