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Connectivity and orientation of the seven helical bundle in the tachykinin NK-1 receptor probed by zinc site engineering

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A high affinity, tridentate metal ion site has been constructed previously by His substitutions in an antagonist binding site located between transmembrane segment (TM)-V and TM-VI in the substance P NK-1 receptor. Here, an attempt is made to probe helix–helix interactions systematically in the NK-1 receptor by engineering of bis-His Zn(II) sites. His residues were introduced at selected positions individually and in combinations in the exterior segments of TM-II, III and V in both the wild-type background and after Ala substitution of naturally occurring His residues, and the increase in the affinity for Zn(II) was monitored in competition binding experiments with iodinated substance P or a tritiated non-peptide antagonist. In this way, two high affinity bis-His sites were constructed between position 193 in TM-V (Glu193, GluV:01) and position 109 in TM-III (Asn109, AsnIII:05) as well as between the neighboring, naturally occurring His108 in TM-III (HisIII:04) and position 92 in TM-II (Tyr92, TyrII:24), respectively. Functionally, the coordination of zinc ions at these two sites blocked the receptor as it antagonized the substance P-induced increase in phosphatidylinositol turnover. It is concluded that the bis-His zinc sites from the central TM-III helix to TM-II and -V, respectively, together with the interconnected, previously constructed tridentate site between TM-V and -VI, constitute a basic network of distance constraints for the molecular models of receptors with seven transmembrane segments which, for example, strongly support an anti-clockwise orientation of the seven helical bundle as viewed from the extracellular space.

Keywords: G-protein-coupled receptors/membrane protein structure/metal ion site/substance P/7TM receptor

Introduction

G-protein-coupled receptors with their seven transmembrane segments (7TM) constitute the largest superfamily of proteins in our organism. Several hundreds of distinct and specific 7TM receptors are receivers for hormones, transmitters and other kinds of intercellular chemical messengers of a surprisingly broad chemical variety: monoamines, purines, amino acids, lipids, peptides and proteins (Watson and Arkinstall, 1994). 7TM molecules are also the key chemical and physical sensors in the nervous system. For example, rhodopsin and opsins function as light sensors in the eye, and ~1000 different receptors act as receptors for odors and pheromones in our olfactory system (Shepherd, 1994). Recently, 7TM receptors have been demonstrated also to be the crucial cellular entry factors for infectious agents such as malaria parasites and the HIV-1 virus (Deng *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996).

Surprisingly little experimentally based information is yet available concerning the three-dimensional structure of any member of this important superfamily of proteins. Although meticulous cryoelectron microscopy studies of rhodopsin have indicated that these proteins may fold into a seven helical bundle, the resolution of the structures does not yet allow the identification of individual helices (Schertler *et al.*, 1993; Schertler and Hargrave, 1995; Unger and Schertler, 1995). In fact we do not even know for certain whether the helical bundle is arranged in a clockwise or anti-clockwise manner, although much evidence does point in favor of an anti-clockwise arrangement as viewed from the outside of the cell (Baldwin, 1993). Thus, current molecular models of 7TM receptors are, to a large extent based on speculations and assumptions, however sound these may be (Baldwin, 1993; Schwartz, 1994; Ballesteros and Weinstein, 1995). Information concerning helix–helix interactions are inferred mainly from mutational studies where a particular substitution can revert the deleterious effect caused by another mutation (Zhou *et al.*, 1994; Liu *et al.*, 1995; Sealfon *et al.*, 1995; Mizobe *et al.*, 1996). Based on experience from classical protein engineering, such 'double revertant' experiments are generally taken as strong evidence indicating that the two original residues are facing each other in the three-dimensional structure of the receptor (Rao *et al.*, 1994; Zhou *et al.*, 1994; Liu *et al.*, 1995; Sealfon *et al.*, 1995; Sieving *et al.*, 1995; Mizobe *et al.*, 1996). In most cases, this is a reasonable assumption; however, it is difficult to exclude possible indirect effects in experiments performed in 7TM receptors without any real structural basis.

In the tachykinin NK-1 receptor, a mutationally identified, presumed binding site for a non-peptide antagonist (Fong *et al.*, 1993, 1994; Gether *et al.*, 1993, 1994) recently was both structurally and functionally exchanged by a metal ion site through systematic substitutions with His residues (Elling *et al.*, 1995). The tridentate zinc site was composed of two His residues located in an i and $i+4$ position at the exterior end of TM-V and a single His residue located in TM-VI (Figure 1). This metal ion site has been transferred to the distantly related κ-opioid receptor without loss of metal ion affinity, indicating that the interface between at least TM-V and -VI is similar in rhodopsin-like receptors (Thirstrup *et al.*, 1996). Due to the small size of the metal ion and the well characterized geometry of imidazole–Zn(II) interactions in proteins

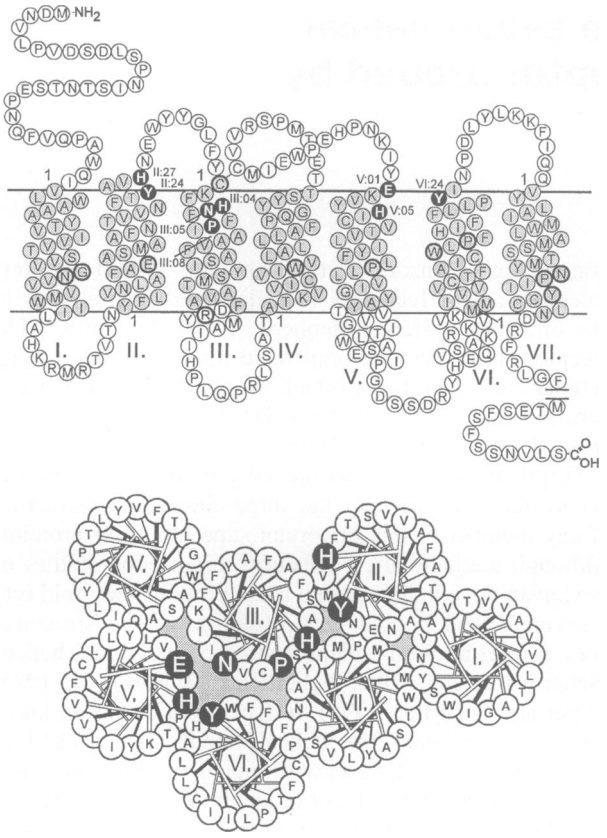

Fig. 1. Serpentine and helical-wheel diagram of the tachykinin NK-1 receptor. The helical wheels of the NK-1 receptor are placed in accordance with the classical Baldwin interpretation of the electron density map of bovine rhodopsin obtained by cryo-electron microscopy by Schertler and co-workers (Baldwin, 1993; Schertler *et al.*, 1993). The helical wheels here have been rotated slightly in comparison with similar figures from previous reports (Schwartz, 1994; Elling *et al.*, 1995) in accordance with the results from the bis-His zinc site constructions of the present investigation (Table I and Figure 2) and are presented in an anti-clockwise orientation as viewed from the extracellular space. The generic numbering and nomenclature system for residues in the transmembrane segments of 7TM receptors is indicated (Baldwin, 1993; Schwartz, 1994). This is based on alignment of multiple 7TM sequences according to the highly conserved 'fingerprint' residues of each transmembrane segment, which here are indicated with a bold circle. Indicated in white on black are residues probed by histidine or alanine substitution in the present study (generic nomenclature of residue followed by specific numbering in brackets): TyrII:24 (Tyr92); HisII:27 (His95); HisIII:04 (His108); AsnIII:05 (Asn109); ProIII:08 (Pro112); GluV:01 (Glu193); HisV:05 (His197); TyrVI:24 (Tyr272).

with known 3D structure (Chakrabarti, 1990; Arnold and Haymore, 1991; Christianson, 1991; Regan, 1995), artificially constructed metal ion sites could provide valuable information about the relative rotation and 'vertical' position of helices in 7TM receptors in general. In the present study, we try to construct bis-His Zn(II) sites reaching out from TM-III both towards the neighboring TM-II and across the presumed main ligand binding crevice towards TM-V at the location where a metal ion site previously was constructed (Elling *et al.*, 1995). This strategy is based on the assumption that TM-III is the 'central column' in the seven helical bundle and that the helices are arranged in a sequential manner in the bundle (Baldwin, 1993; Schwartz, 1994; Ballesteros and Weinstein, 1995).

Results

The generic numbering and nomenclature system for residues in the transmembrane helices of 7TM receptors (Baldwin, 1993; Schwartz, 1994) is recapitulated in Figure 1.

Zinc site construction between TM-III and TM-V

The first residue to be probed by His substitutions in TM-III was Pro112 (ProIII:08) as it corresponds to the notorious Asp (AspIII:08) which is the contact point for the amine function of the ligands in monoamine receptors (Strader *et al.*, 1989, 1991). It is assumed that, for example, catecholamines bind between this position in TM-III and two Ser residues in TM-V (Strader *et al.*, 1989, 1991). Four different constructs were tested where the [P112H] substitution was combined with various His residues positioned in the exterior part of TM-V and -VI. However, in all cases the zinc affinity, as determined by the ability of Zn(II) to inhibit substance P binding, was unaltered or even lower than in the corresponding constructs without a His residue at position 112 in TM-III (Table I). Thus, either the helical rotation or the relatively deep location of His112 (HisIII:08) in relation to His193 (HisV:01) and His197 (HisV:05) prevents His112 from forming a high affinity, bis-His zinc site with either of these residues in TM-V.

Introduction of a His residue one helical turn above Pro112 in TM-III at position 109 (Figure 1) gave more positive results. In this series, the construct with only a single His residue present in this region of the receptor, [N109H,H197A]NK-1, i.e. with the naturally occurring His residue at position 197 substituted with an Ala residue, gave an apparent Zn(II) affinity of 176 ± 16 μM. Importantly, this affinity was increased to 28 ± 1 μM when a second His residue was introduced at position 193, i.e. the [N109H,E193H,H197A]NK-1 construct of Table I and Figure 2. Thus, apparently a bis-His site can be formed between position 109 in TM-III and position 193 in TM-V. In contrast, there was no evidence that a similar connection could be established between His109 and His197 in TM-V as the affinity in the construct with a His residue located at both of these positions, i.e. [N109H]NK-1, was only marginally increased to 140 ± 6 μM. Although a presumed bis-His Zn(II) site could be established between residues located at positions 109 and 193, the geometry of this interaction was, however, apparently not optimal for a tridentate or a full tetrahedral coordination of the metal ion with additional His residues located at positions 197 and 272. In fact, the measured apparent zinc affinity was ~2-fold lower when His109 was introduced into constructs with two or three His residues located at these positions (Table I).

Zinc site construction between TM-III and TM-II

Above, a possible spatial connection has been established between residue 109 (III:05) in TM-III and residue 193 (V:01) in TM-V. In order to obtain an additional, structural constraint in the receptor structure, the neighboring residue in TM-III, His108, was chosen as the basis for further engineering of bis-His sites. This residue corresponds to Glu113 of rhodopsin, which forms the Schiff-base connection to the retinal ligand (Khorana, 1992) and, in

Table I. Binding of Zn(II) and substance P to the wild-type and mutant NK-1 receptors

	His residues[a]	Helical segment	$ZnCl_2$ K_i ± SEM (μM)	(*n*)	Fold increase	Substance P K_d ± SEM (nM)	(*n*)	Fold decrease	B_{max} fmol/ 100 000 cells
hNK-1			384 ± 29	(17)	1	0.11 ± 0.01	(25)	1	88.0 ± 9.5
[E193H;H197A]	1 His (193)	TM-V	85 ± 6	(3)	5	0.07 ± 0.01	(3)	1	13.2 ± 1.9
[E193H][b]	2 His (193; 197)	TM-V	11 ± 2	(5)	35	0.12 ± 0.01	(8)	1	26.0 ± 3.6
[E193H;Y272H][b]	3 His (193; 197; 272)	TM-V and VI	0.62 ± 0.10	(7)	619	0.34 ± 0.04	(14)	3	23.5 ± 3.7
[P112H]	2 His (112; 197)	TM-III and -V	271 ± 54	(4)	1	0.07 ± 0.02	(4)	1	39.7 ± 4.5
[P112H;E193H; H197A]	2 His (112; 193)	TM-III and -V	94 ± 6	(4)	4	0.32 ± 0.05	(4)	3	22.6 ± 4.9
[P112H;E193H]	3 His (112; 193; 197)	TM-III and -V	76 ± 39	(4)	5	0.40 ± 0.07	(4)	4	32.3 ± 7.5
[P112H;E193H; Y272H]	4 His (112; 193; 197; 272)	TM-III, -V and VI	1.1 ± 0.1	(4)	349	1.12 ± 0.13	(4)	10	23.0 ± 5.2
[N109H;H197A]	1 His (109)	TM-III and -V	176 ± 16	(4)	2	0.21 ± 0.06	(3)	2	15.7 ± 4.4
[N109H]	2 His (109; 197)	TM-III and -V	140 ± 6	(3)	3	0.12 ± 0.03	(3)	1	21.0 ± 2.7
[N109H;E193H; H197A]	2 His (109; 193)	TM-III and -V	28 ± 1	(3)	14	3.7 ± 0.6	(9)	34	17.7 ± 6.0
[N109H;E193H]	3 His (109; 193; 197)	TM-III and -V	20 ± 3	(3)	19	9.8 ± 0.6	(3)	89	46.6 ± 9.8
[N109H;E193H; Y272H]	4 His (109; 193; 197; 272)	TM-III, -V and VI	1.5 ± 0.6	(3)	256	31 ± 7	(3)	282	52.1 ± 15.6
[H95A]	1 His (108)	TM-II and -III	211 ± 37	(4)	2	0.02 ± 0.01	(4)	0.2	23.8 ± 6.2
[Y92H;H95A]	2 His (92; 108)	TM-II and -III	16 ± 1	(3)	24	1.5 ± 0.4	(3)	14	3.9 ± 2.2
[Y92H]	3 His (92; 95; 108)	TM-II and -III	14 ± 1	(5)	27	0.28 ± 0.06	(8)	3	10.3 ± 4.0
hNK-1[c]			376 ± 29	(10)	1	1.8 ± 0.1	(12)	1	130 ± 17
[Y92H][c]	3 His (92; 95; 108)	TM-II and -III	30 ± 1	(5)	13	182 ± 49	(6)	101	58.5 ± 12.0
[Y92H;H95A][c]	2 His (92; 108)	TM-II and -III	32 ± 4	(4)	12	>1000	(4)	–	70.0 ± 15.0
[Y92H;H108A][c]	2 His (92; 95)	TM-II and -III	269 ± 25	(3)	1	>1000	(3)	–	86.2 ± 12.3

[a]Only His residues located in the specific exterior region which is subject to mutational analysis are indicated.
[b]Results concerning [E193H] and [E193H,Y272H] have been published previously (Elling *et al.*, 1995) and are included here for direct comparison only.
[c]The important control construct where His108 was substituted with Ala could not be analyzed with radiolabeled substance P; thus, this construct as well as a number of the related constructs were analyzed using the radiolabeled non-peptide antagonists, [^{3}H]LY303,870.

the NK-1 receptor, His108 previously has been implicated in the binding of the agonist, substance P (Fong *et al.*, 1992). In our molecular model, His108 would appear to be in relatively close contact with residues in TM-VII and TM-II (Figure 1). Mutational interference with the binding of the radioactive probes prevented further characterization of the relevant positions in TM-VII in the present study. However, a possible spatial connection between His108 and Tyr92 located on a hydrophilic, presumably inner face of TM-II was established through the construction of a bis-His zinc site. First, the possible interference from the naturally occurring His95 located either in extracellular loop 1 or in a putative helical extension of TM-II (as indicated in Figure 1) was eliminated through Ala substitution (Table I). The introduction of a His residue at position 92 in TM-II increased the zinc affinity 13-fold (from 211 ± 37 to 16 ± 1 μM), as determined in competition binding experiments using radiolabeled substance P, and increased the zinc affinity 12-fold (from 376 ± 29 to 32 ± 4 μM) in competition with the radiolabeled antagonist, [^{3}H]LY-303,870 (Table I, [H95A] versus [Y92H,H95A]). The notion that the increase in zinc affinity was in fact caused by a coordination of the metal ion between the introduced His residue at position 92 and the natural His residue at position 108 was confirmed through a reversal of the effect of the introduction of His92 following Ala substitution of His108 ([Y92H] versus [Y92H,H108A] in Table I and Figure 2). This latter experiment could only be performed with the radiolabeled antagonist as a probe since Ala substitution of His108 as expected (Fong *et al.*, 1992) eliminated the binding of the substance P tracer.

Substitution of Tyr92 with His impaired the ability of substance P to compete with binding of the radiolabeled antagonist 100-fold, 1.8 ± 0.1 nM versus 182 ± 49 nM (Table I). However, in analogy with results obtained in a series of Ala substitutions of residues on this hydrophilic, presumed inner face of TM-II, including position 92, the actual affinity for substance P was in fact only minimally affected by the His92 substitution (0.28 ± 0.06 versus 0.11 ± 0.01 nM) as determined in homologous binding assays with radiolabeled substance P itself (Rosenkilde *et al.*, 1994).

Functional consequences of zinc binding

In the receptors with artificially constructed metal ion sites, Zn(II) functioned as an antagonist of the substance P-induced increase in phosphatidylinositol turnover, with IC_{50} values of 31 ± 7×10^{-6} M for the [Y92H]NK-1 and 42 ± 9×10^{-6} M for the [N109H,E193H,H197A]NK-1 constructs (Figure 3). For both of the inter-helical bis-His sites of the present study, i.e. the one between TM-II and -III, [Y92H], and the one between TM-III and -V, [N109H,E193H,H197A], there was a close correlation between the ability of Zn(II) to inhibit substance P binding and its ability to block the substance P-induced signal transduction events (Figures 2 and 3).

Discussion

In the present study, two bis-His metal ion sites have been constructed from TM-III to positions in TM-II and TM-V, respectively. These sites are located close to the

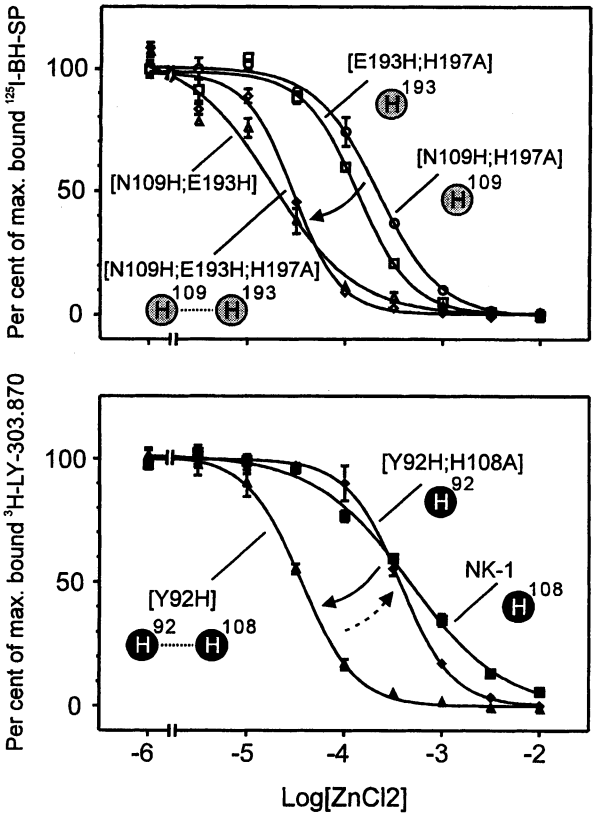

Fig. 2. Construction of bis-His Zn(II) sites between residues located at the exterior end of TM-III and residues in TM-II and -V respectively. For the bis-His zinc site between the naturally occurring HisIII:04 (His108) in TM-III and the substituted HisII:24 (His92) in TM-II the competition curves using [^{3}H]LY-303,870 are shown since Ala substitution of His108 affects substance P binding (Fong *et al.*, 1992). Top panel: competition binding experiments for $ZnCl_2$ using [^{125}I]BH-substance P in the [N109H;H197A]NK-1 (○), [E193H;H197A]NK-1 (□), [N109H;E193H]NK-1 (△) and [N109H;E193H;H197A]NK-1 (◇) constructs. Lower panel: competition binding experiments for $ZnCl_2$ using [^{3}H]LY303.870 in wild-type human NK-1 (■), [Y92H; H108A]NK-1 (◆) and [Y92H]NK-1 (▲).

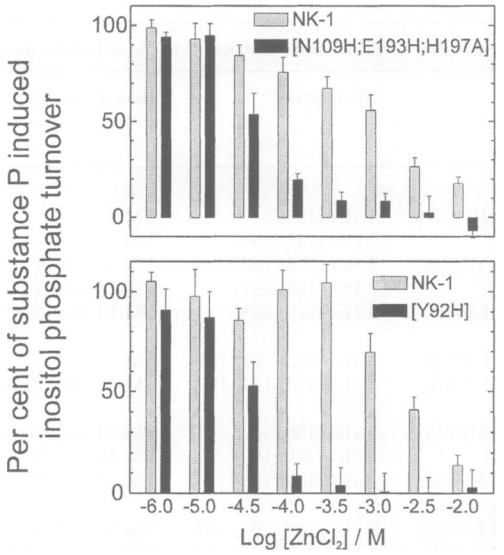

Fig. 3. Functional analysis of Zn(II) antagonism of substance P-induced phosphatidylinositol turnover in the constructs with engineered bis-His Zn(II) sites. [N109H,E193H,H197A]NK-1 presumably forms a bidentate site between TM-III and TM-V involving residues HisIII:05 (His109) and HisV:01 (His193). [Y92H] presumably forms a bidentate site between TM-II and TM-III involving residues HisII:24 (His92) and the naturally occurring His at position 108, HisIII:04, as shown in Figure 2. Data are expressed as mean ± SEM, n = 3–6. IC_{50} values for $ZnCl_2$: NK-1 (1 and 10 nM), >1 mM; [Y92H]NK-1, 31 ± 7 μM ; [N109H;E193H;H197A]NK-1, 42 ± 9 μM.

extracellular side of the receptor and it is therefore still uncertain whether these positions are located in the actual transmembrane helices or in the connecting loops. However, the high affinity of the previously generated bis-His site between residues at position V:01 and V:05 in the presumed TM-V (Elling *et al.*, 1995) does indicate that the residues are in fact located in the optimal i and i+4 position of a helix (Chakrabarti, 1990; Arnold and Haymore, 1991; Regan, 1995). The most exteriorly located of these residues, V:01, is part of one of the interhelical bis-His sites of the present study. Furtheremore, electron paramagnetic resonance (EPR) studies with spin-labeled rhodopsin (Altenbach *et al.*, 1996) show that the transmembrane helices may continue several helical turns outside the membrane, at least on the intracellular side.

Due to the small size of the zinc ion and the inherent geometry of the imidazole metal ion coordination (Chakrabarti, 1990; Arnold and Haymore, 1991; Christianson, 1991; Regan, 1995), the bis-His sites of the present study impose important distance constraints on the α-carbons of the involved residues in molecular models of 7TM receptors. Thus, both the relative helical rotation and the vertical translocation of the involved helices have in this way been restricted. Each of these bis-His sites can obviously be satisfied in many different possible models and, therefore, are not of much value individually. However, it takes relatively few distance constraints to fix the overall, general structure of the molecule and its helical components. Importantly, the two bis-His sites of the present study are connected since they originate from neighboring residues on the TM-III helix. Moreover, one of the bis-His sites reaches over to a residue on TM-V which previously has been implicated in a tridentate zinc site connecting the outer segment of TM-V and TM-VI (Elling *et al.*, 1995). Thus, a 'network' of interconnected metal ion sites is emerging which could possibly serve as a constraining scaffold in the molecular models of 7TM receptors in general.

One of the very basic structural questions concerning 7TM receptors is whether the helical bundle is arranged in a clockwise or an anti-clockwise manner (Baldwin, 1993; Schwartz, 1994). It is generally assumed that the seven helical bundle is arranged with the transmembrane segments placed sequentially after each other in analogy with, for example, bacteriorhodopsin, i.e. that TM-I is followed by TM-II followed by TM-III, etc. until TM-VII closes the 'circle' (Baldwin, 1993; Schwartz, 1994; Ballesteros and Weinstein, 1995). One argument in favor of a sequential arrangement is that the loops are too short to allow for a pass over of intervening helices or to cross over each other (Baldwin, 1993). Furthermore, in rhodopsin, the loops can be shortened without loss of retinal binding or function (Franke *et al.*, 1992). However, a sequentially arranged helical bundle can be formed either clockwise or anti-clockwise. As shown in Figure 4, the four residues which in the present study have been

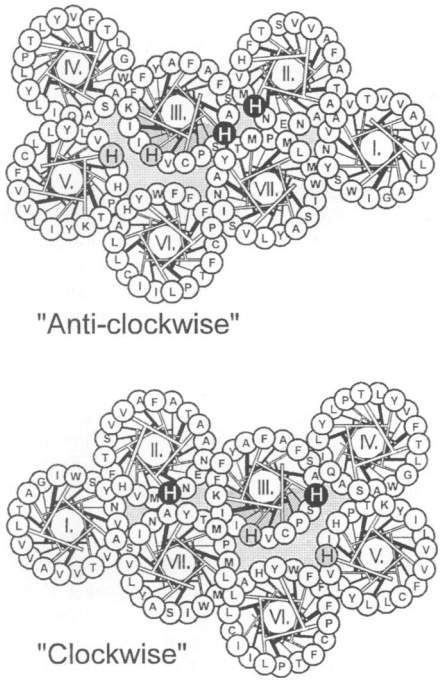

Fig. 4. The bis-His Zn(II) sites support an anti-clockwise arrangement of the transmembrane helices of the NK-1 receptor. Helical-wheel diagram of the NK-1 receptor shown in an anti-clockwise and in a clockwise arrangement as viewed from the extracellular space. The His residues presumed to be involved in the bis-His metal ion sites presented here are indicated. Both models are built according to the same general principles (Schwartz, 1994). The dark gray arc in TM-III indicates the face which, according to results from substituted Cys accessability studies, should be facing inwards in the dopamine D_2 receptor (Javitch *et al.*, 1995).

shown to be pair-wise able to form bis-His metal ion sites are only in close spatial proximity in an anti-clockwise model, as viewed from the extracellular space. It would obviously be possible to bring one of the pairs into reasonably close proximity even in the clockwise model, for example through rotation of TM-III. However, it is not possible to satisfy both of the pairs at the same time, even with such manipulations, without violating the basic assumption, that the connectivity of the helices is sequential (Baldwin, 1993; Schwartz, 1994; Ballesteros and Weinstein, 1995). Importantly, the structural constraint imposed by the bis-His site between positions 92 and 108 in TM-II and -III, respectively, is almost impossible to satisfy in a clockwise model, as any (in)appropriate rotation of TM-III inevitably will place both residue 109 ('His109') and importantly residue 112 (corresponding to the notorious Asp in the monoamine receptors) facing towards the membrane. These residues are both located on a presumed water-exposed, inner face of TM-III, which recently was probed extensively by Javitch and co-workers in the dopamine D_2 receptor by a substituted-cysteine accessibility method (Javitch *et al.*, 1995). An anti-clockwise orientation of the helices in 7TM receptors is also supported by different kinds of mutational exchanges of supposedly interfacing residues on TM-I, TM-II and TM-VII in the muscarinic and adrenergic receptors (Liu *et al.*, 1995; Mizobe *et al.*, 1996). Activating mutations in rhodopsin also suggest a close interaction between TM-II and TM-VII (Rao *et al.*, 1994; Sieving *et al.*, 1995). Moreover, the seven helices of the bacterial proton pump, bacteriorhodopsin, are arranged in an anti-clockwise manner (Engelman *et al.*, 1980; Henderson *et al.*, 1990), although this protein is unrelated to G-protein-coupled receptors with regard both to sequence and function.

The present study indicates that engineering of metal ion sites can be employed to probe helix–helix interactions in 7TM receptors and conceivably also other membrane proteins where biophysical, 'real' structural information is still almost absent. In the *Escherichia coli* lactose permease, artificial Mn(II) sites were built recently; however, this was based on information of spatial proximity of specific residues from site-directed excimer fluorescence experiments (He *et al.*, 1995a,b; Jung *et al.*, 1995), and in the NK-1 receptor the previous tridentate metal ion site was built on the basis of mutational mapping of an antagonist binding site (Elling *et al.*, 1995). Another method for probing spatial proximity of residues in membrane helices is to construct artificial disulfide bridges, which has been used extensively in, for example, the bacterial chemotaxis receptors (Lynch and Koshland, 1991; Pakula and Simon, 1992; Chervitz and Falke, 1995). This method recently has been applied to rhodopsin, where a disulfide bridge was built successfully between two of the positions in TM-V and -VI (V:05 and VI:24) (Yu *et al.*, 1995), which previously had been probed by metal ion site construction (Elling *et al.*, 1995).

An interesting possibility is to use the constructed intra- and inter-helical metal ion sites of the present study to measure distances in 7TM receptors by EPR determinations of metal–nitroxyl interactions after introduction of spin labels at other positions in the receptor (He *et al.*, 1995a,b; Jung *et al.*, 1995) or in the ligand.

Materials and methods

Materials

Substance P was purchased from Peninsula (St Helens, Merseyside, UK). $ZnCl_2$ (minimum 98% pure) was from Merck. *Pfu* polymerase was from Stratagene (La Jolla, CA). Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP was from Amersham (Little Chalfont, UK). AG 1-X8 anion-exchange resin was from Bio-Rad Laboratories (Hercules, CA). [^{3}H]*myo*-inositol (PT6-271) was from Amersham (Little Chalfont, UK). Bolton–Hunter reagent, sp. act. 2000 Ci/mmol, was from Amersham.

Construction of mutant receptors

The cDNA encoding the wild-type NK-1 receptor (Gerard *et al.*, 1991) was cloned into the eukaryotic expression vector pTEJ-8 (Johansen *et al.*, 1990). Mutant receptors were constructed using PCR methods (Ho *et al.*, 1989; Horton *et al.*, 1989). The PCR products were digested with appropriate restriction endonucleases, purified and cloned into the pTEJ8-NK-1. All PCRs were performed using *Pfu* polymerase according to the manufacturer's instructions. All mutations were verified by restriction endonuclease mapping and subsequent DNA sequence analysis using a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP on an Alfexpress™ DNA sequencer according to the manufacturer's instructions (Pharmacia Biotech, Uppsala, Sweden).

Transfections and tissue culture

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) 1885 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 0.01 mg/ml gentamicin. The expression plasmids containing the cDNAs encoding the wild-type or mutant receptors were transiently expressed in COS-7 cells transfected by the calcium phosphate precipitation method according to previously described methods (Johansen *et al.*, 1990; Gether *et al.*, 1992).

Binding experiments

Monoiodinated [^{125}I]Bolton–Hunter-labeled substance P ([^{125}I]BH-SP) was prepared and purified by high performance liquid chromatography (Gether *et al.*, 1992). [^{3}H]LY303.870, sp. act. 31.4 Ci/mmol, was a generous gift from Dr Philip Hipskinds, Eli Lilly, Indiana, USA. Transfected COS-7 cells were transferred to culture plates 1 day after transfection. The number of cells per well was determined by the apparent expression efficiency of the individual clones aiming at 5–10% binding of the added radioligand. Two days after transfection, cells were assayed by competition binding for 3 h at 4°C using 40 pM [^{125}I]BH-SP or 75 pM [^{3}H]LY303.870 plus variable amounts of unlabeled ligand in 0.5 ml of a 50 mM Tris–HCl buffer, pH 7.4, supplemented with 150 mM NaCl, 5 mM $MnCl_2$, 0.1% (w/v) bovine serum albumin, 40 μg/ml bacitracin. Non-specific binding was determined as the binding in the presence of either 1 μM SP or 1 μM LY303.870. Determinations were made in triplicate (SP) or duplicate ($ZnCl_2$).

Phosphatidylinositol assay

To assay phosphatidylinositol turnover, transfected COS-7 cells (0.1–1.6×10^6 cells/well) were incubated for 24 h with 5 μCi of [^{3}H]*myo*-inositol in 1 ml of inositol-free DMEM 1885 supplemented with 10% FCS, 2 mM glutamine and 0.01 mg/ml gentamicin per well. Cells were washed twice in PI buffer: 20 mM HEPES, pH 7.4, supplemented with 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 0.05% (w/v) bovine serum albumin; and were incubated in 0.5 ml PI buffer supplemented with 10 mM LiCl at 37°C for 30 min. Phosphatidylinositol turnover was stimulated by submaximal concentrations of substance P, i.e. 1 nM for 20 min in the [Y92H] construct and 10 nM for 45 min in the [N109H;E193H;H197A] construct in the presence of increasing Zn(II) concentrations. Cells were extracted with 10% ice-cold perchloric acid, neutralized with KOH in HEPES buffer, and the generated [^{3}H]inositol phosphates were purified on Bio-Rad AG 1-X8 anion-exchange resin as described (Zoffmann *et al.*, 1993). Determinations were made in duplicate.

Calculations

Binding data were analyzed and IC_{50} values determined by non-linear regression using the Inplot 4.0 software (GraphPad Software, San Diego). Values of the dissociation and inhibition constants (K_d and K_i) were estimated from competition binding using the equations $K_d = IC_{50}-L$ and $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of radioligand.

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References

Altenbach,C., Yang,K., Farrens,D., Khorana,H.G. and Hubbell,W.L. (1996) Structural features and light-dependent changes in the E–F interhelical loop region of rhodopsin: a site-directed spin labeling study. *Biochemistry*, in press.

Arnold,F.H. and Haymore,B.L. (1991) Engineered metal-binding proteins: purification to protein folding. *Science*, **252**, 1796–1797.

Baldwin,J.M. (1993) The probable arrangement of the helices in G protein-coupled receptors. *EMBO J.*, **12**, 1693–1703.

Ballesteros,J.A. and Weinstein,H. (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure–function relations in G protein-coupled receptors. In Sealfon,S.C. (ed.), *Receptor Molecular Biology*. Academic Press, San Diego, CA, pp. 366–428.

Chakrabarti,P. (1990) Geometry of interaction of metal ions with histidine residues in protein structures. *Protein Engng*, **4**, 57–63.

Chervitz,S.A. and Falke,J.J. (1995) Lock on/off disulfides identify the transmembrane signalling helix of the aspartate receptor. *J. Biol. Chem.*, **270**, 24043–24053.

Christianson,D.W. (1991) Structural biology of zinc. *Adv. Protein Chem.*, **42**, 281–355.

Deng,H. *et al.* (1996) Identification of a major co-receptor for primary isolates of HIV-1. *Nature*, **381**, 661–666.

Dragic,T. *et al.* (1996) HIV-1 entry into $CD4^+$ cells is mediated by the chemokine receptor CC-CKR-5. *Nature*, **381**, 667–673.

Elling,C.E., Nielsen,S.M. and Schwartz,T.W. (1995) Conversion of antagonist-binding site to metal-ion site in the tachykinin NK-1 receptor. *Nature*, **374**, 74–77.

Engelman,D.M., Henderson,R., McLachlan,A.D. and Wallace,B.A. (1980) Path of the polypeptide in bacteriorhodopsin. *Proc. Natl Acad. Sci. USA*, **77**, 2023–2027.

Feng,Y., Broder,C.C., Kennedy,P.E. and Berger,E.A. (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science*, **272**, 872–877.

Fong,T.M., Huang,R.C. and Strader,C.D. (1992) Localization of agonist and antagonist binding domains of the human neurokinin-1 receptor. *J. Biol. Chem.*, **267**, 25664–25667.

Fong,T.M., Cascieri,M.A., Yu,H., Bansal,A., Swain,C. and Strader,C.D. (1993) Amino-aromatic interaction between histidine 197 of the neurokinin-1 receptor and CP 96345. *Nature*, **362**, 350–353.

Fong,T.M., Yu,H., Cascieri,M.A., Underwood,D., Swain,C.J. and Strader,C.D. (1994) The role of histidine 265 in antagonist binding to the neurokinin-1 receptor. *J. Biol. Chem.*, **269**, 2728–2732.

Franke,R.R., Sakmar,T.P., Graham,R.M. and Khorana,H.G. (1992) Structure and function in rhodopsin. *J. Biol. Chem.*, **267**, 14767–14774.

Gerard,N.P., Garraway,L.A.,Jr, Eddy,R.L., Shows,T.B., Iijima,H., Paquet,J.L. and Gerard,C. (1991) Human substance P receptor (NK-1): organization of the gene, chromosome localization, and functional expression of cDNA clones. *Biochemistry*, **30**, 10640–10646.

Gether,U., Marray,T., Schwartz,T.W. and Johansen,T.E. (1992) Stable expression of high affinity NK_1(substance P) and NK_2 (neurokinin A) receptors but low affinity NK_3 (neurokinin B) receptors in transfected CHO cells. *FEBS Lett.*, **296**, 241–244.

Gether,U., Johansen,T.E., Snider,R.M., Lowe,J.A.,III, Nakanishi,S. and Schwartz,T.W. (1993) Different binding epitopes on the NK1 receptor for substance P and a non-peptide antagonist. *Nature*, **362**, 345–348.

Gether,U., Nilsson,L., Lowe,J.A. and Schwartz,T.W. (1994) Specific residues at the top of transmembrane segment V and VI of the NK-1 receptor involved in binding of the nonpeptide antagonist CP 96,345. *J. Biol. Chem.*, **269**, 23959–23964.

He,M.M., Voss,J., Hubbell,W.L. and Kaback,H.R. (1995a) Use of designed metal-binding sites to study helix proximity in the lactose permease of *Escherichia coli*. 1. Proximity of helix VII (Asp237 and Asp240) with helices X (Lys319) and XI (Lys358). *Biochemistry*, **34**, 15661–15666.

He,M.M., Voss,J., Hubbell,W.L. and Kaback,H.R. (1995b) Use of designed metal-binding sites to study helix proximity in the lactose permease of *Escherichia coli*. 2. Proximity of helix IX (Arg302) with helix X (His322 and Glu325). *Biochemistry*, **34**, 15667–15670.

Henderson,R., Baldwin,J.M., Ceska,T.A., Zemlin,F., Beckmann,E. and Downing,K.H. (1990) Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J. Mol. Biol.*, **213**, 899–929.

Ho,S.N., Hunt,H.D., Horton,R.M., Pullen,J.K. and Pease,L.R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene*, **77**, 51–59.

Horton,R.M., Hunt,H.D., Ho,S.N., Pullen,J.K. and Pease,L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene*, **77**, 61–68.

Javitch,J.A., Fu,D., Chen,J. and Karlin,A. (1995) Mapping the binding-site crevice of the dopamine D2 receptor by the substituted-cysteine accessibility method. *Neuron*, **14**, 825–831.

Johansen,T.E., Schøller,M.S., Tolstoy,S. and Schwartz,T.W. (1990) Biosynthesis of peptide precursors and protease inhibitors using new constitutive and inducible eukaryotic expression vectors. *FEBS Lett.*, **267**, 289–294.

Jung,K., Voss,J., He,M., Hubbel,W.L. and Kaback,H.R. (1995) Engineering a metal binding site within a polytopic membrane protein, the lactose permease of *Escherichia coli*. *Biochemistry*, **34**, 6272–6277.

Khorana,H.G. (1992) Rhodopsin, photoreceptor of the rod cell. *J. Biol. Chem.*, **267**, 1–4.

Liu,J., Schöneberg,T., Rhee,M.V. and Wess,J. (1995) Mutational analysis of the relative orientation of transmembrane helices I and VII in G protein-coupled receptors. *J. Biol. Chem.*, **270**, 19532–19539.

Lynch,B.A. and Koshland,D.E.,Jr (1991) Disulfide cross-linking studies of the transmembrane regions of the aspartate sensory receptor of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **88**, 10402–10406.

Mizobe,T., Maze,M., Lam,V., Suryanarayana,S. and Kobilka,B.K. (1996) Arrangement of transmembrane domains in adrenergic receptors. *J. Biol. Chem.*, **271**, 2387–2389.

Pakula,A.A. and Simon,M.I. (1992) Determination of transmembrane protein structure by disulfide cross-linking: the *Escherichia coli* Tar receptor. *Proc. Natl Acad. Sci. USA*, **89**, 4144–4148.

Rao,V.R., Cohen,G.B. and Oprian,D.D. (1994) Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. *Nature*, **367**, 639–642.

Regan,L. (1995) Protein design: novel metal-binding sites. *Trends Biochem. Sci.*, **20**, 280–285.

Rosenkilde,M.M., Cahir,M., Gether,U., Hjorth,S.A. and Schwartz,T.W. (1994) Mutations along transmembrane segment II of the NK-1 receptor affect substance P competition with non-peptide antagonists but not substance P binding. *J. Biol. Chem.*, **269**, 28160–28164.

Schertler,G.F.X. and Hargrave,P.A. (1995) Projection structure of frog rhodopsin in two crystal forms. *Proc. Natl Acad. Sci. USA*, **92**, 11578–11582.

Schertler,G.F.X., Villa,C. and Henderson,R. (1993) Projection structure of rhodopsin. *Nature*, **362**, 770–772.

Schwartz,T.W. (1994) Locating ligand-binding sites in 7TM receptors by protein engineering. *Curr. Opin. Biotechnol.*, **5**, 434–444.

Sealfon,S.C., Chi,L., Ebersole,B.J., Rodic,V., Zhang,D., Ballesteros,J.A. and Weinstein,H. (1995) Related contribution of specific helix 2 and 7 residues to conformational activation of the serotonin 5-HT_{2A} receptor. *J. Biol. Chem.*, **270**, 16683–16688.

Shepherd,G.M. (1994) Discrimination of molecular signals by the olfactory receptor neuron. *Neuron*, **13**, 771–790.

Sieving,P.A., Richards,J.E., Naarendorp,F., Bingham,E., Scott,K. and Alpern,M. (1995) Dark–light: model for nightblindness from the human rhodopsin Gly-90→Asp mutation. *Proc. Natl Acad. Sci. USA*, **92**, 880–884.

Strader,C.D., Candelore,M., Hill,W.S., Sigal,I.S. and Dixon,R.A.F. (1989) Identification of two serine residues involved in agonist activation of the β-adrenergic receptor. *J. Biol. Chem.*, **264**, 13572–13578.

Strader,C.D., Gaffney,T., Sugg,E.E., Candelore,M.R., Keys,R., Patchett,A.A. and Dixon,R.A.F. (1991) Allele-specific activation of genetically engineered receptors. *J. Biol. Chem.*, **266**, 5–8.

Thirstrup,K., Elling,C.E., Hjorth,S.A. and Schwartz,T.W. (1996) Construction of a high-affinity zinc-switch in the kappa-opioid receptor. *J. Biol. Chem.*, **271**, 7875–7878.

Unger,V.M. and Schertler,G.F.X. (1995) Low resolution structure of bovine rhodopsin determined by electron cryo-microscopy. *Biophys. J.*, **68**, 1776–1786.

Watson,S. and Arkinstall,S. (1994) *The G-protein Linked Receptor Factsbook*. Academic Press Ltd, London, UK.

Yu,H., Kono,M., McKee,T.D. and Oprian,D.D. (1995) A general method for mapping tertiary contacts between amino acid residues in membrane-embedded proteins. *Biochemistry*, **46**, 14963–14969.

Zhou,W., Flanagan,C.A., Ballesteros,J.A., Konvicka,K., Davidson,J.S., Weinstein,H., Millar,R.P. and Sealfon,S.C. (1994) A reciprocal mutation supports helix 2 and helix 7 proximity in the gonadotropin-releasing hormone receptor. *Mol. Pharmacol.*, **45**, 165–170.

Zoffmann,S., Gether,U. and Schwartz,T.W. (1993) Conserved His VI-17 of the NK-1 receptor is involved in binding of non-peptide antagonists but not substance P. *FEBS Lett.*, **336**, 506–510.

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