ACCELERATED COMMUNICATION

Partial Agonism through a Zinc-Ion Switch Constructed between Transmembrane Domains III and VII in the Tachykinin NK₁ Receptor

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ABSTRACT
Partly due to lack of detailed knowledge of the molecular recognition of ligands the structural basis for partial versus full agonism is not known. In the β₂-adrenergic receptor the agonist binding site has previously been structurally and functionally exchanged with an activating metal-ion site located between AspIII:08—or a His residue introduced at this position in transmembrane domain (TM)-III—and a Cys residue substituted for AsnVII:06 in TM-VII. Here, this interhelical, bidentate metal-ion site is without loss of Zn²⁺ affinity transferred to the tachykinin NK₁ receptor. In contrast to the similarly mutated β₂-adrenergic receptor, signal transduction—i.e., inositol phosphate turnover—could be stimulated by both Zn²⁺ and by the natural agonist, Substance P in the mutated NK₁ receptor. The metal-ion acted as a 25% partial agonist through binding to the bidentate zinc switch located exactly one helical turn below the two previously identified interaction points for Substance P in, respectively, TM-III and -VII. The metal-ion chelator, phenantroline, which in the β₂-adrenergic receptor increased both the potency and the agonistic efficacy of Zn²⁺ or Cu²⁺ in complex with the chelator, also bound to the metal-ion site-engineered NK₁ receptor, but here the metal-ion chelator complex instead acted as a pure antagonist. It is concluded that signaling of even distantly related rhodopsin-like 7TM receptors can be activated through Zn²⁺ coordination between metal-ion binding residues located at positions III:08 and VII:06. It is suggested that only partial agonism is obtained through this simple well defined metal-ion coordination due to lack of proper interactions with residues also in TM-VI.

Mutational mapping and cross-linking experiments have provided much information concerning binding pockets for ligands in 7-transmembrane (TM) receptors (Schwartz, 1994; Strader et al., 1994). Nevertheless, very little detailed knowledge is in fact available concerning the actual molecular recognition of the ligands within these binding pockets. Only very few experiments have been performed where significant gain-of-function could be accounted for by proven point-to-point interactions between specific chemical groups on the ligand and receptor, respectively (Strader et al., 1991). However, in contrast to, for example, peptides and small organic compounds where our knowledge about their receptor recognition is still rather limited, the coordination chemistry of metal-ions by proteins in general is very well understood (Vallee and Auld, 1990; Albert et al., 1998). Based on this, metal-ion site engineering has been exploited as a useful technique to study helix-helix interactions in membrane proteins. Originally, the binding site for the prototype nonpeptide Substance P antagonist CP96,345 was structurally and functionally exchanged with a high affinity tridentate Zn²⁺ site in the tachykinin NK₁ receptor (Elling et al., 1995). This site could be transferred without loss of zinc affinity to the distantly related κ-opioid receptor, indicating that the overall structure within the family of rhodopsin like 7TM receptors is well conserved (Thirstrup et al., 1996). Subsequently, a number of interhelical bis-His metal-ion sites were engineered into the NK₁ receptor providing important structural information about the helical packing (Elling and Schwartz, 1996). Construction of inhibitory metal-ion sites have also been used to try to characterize the conformational changes associated with receptor activation (Sheikh et al., 1996). Importantly, however the zinc sites created in many different

ABBREVIATIONS: TM, transmembrane; BH, Bolton-Hunter.
locations in a number of different receptors have as yet all been antagonistic. Thus, either basically all the helices move upon activation, or it is possible to stabilize a multitude of inactive conformations, which implies that each of the inhibitory metal-ion sites then provides rather little information about the active conformation.

Recently an activating metal-ion site was designed in the $\beta_2$-adrenergic receptor (Elling et al., 1999). The $\beta_2$-receptor was chosen for this, because—like other monoamine receptors—it has a very well characterized agonist binding site located in the deep part of the main ligand binding pocket between TM-III, -V, -VI, and -VII (Strader et al., 1994). Moreover, it could be anticipated that the $\beta_2$-receptor would be relatively easy to activate because it already displays a fair amount of constitutive signaling activity and because a large number of different mutations have been described, which increase its constitutive activity (Lefkowitz et al., 1993). The activating metal-ion site was constructed between Asp$^{113}$ (AspIII:08), which is the key residue in monoamine agonist binding—or a His residue introduced at this position—and a Cys residue introduced in TM-VII for Asn$^{312}$ (AsnVII:06), which is well known interaction point especially for partial agonists (Suryanarayana et al., 1991) (Fig. 1). Recently, mutational analysis of supposedly opposing residues in TM-III and -VII of the $\alpha_{1b}$-adrenergic and $\delta$-opioid receptors have created constitutive activity (Befort et al., 1999; Porter and Perez, 1999); however, the basal signaling of the zinc site-engineered $\beta_2$-adrenergic receptor was similar to that of the wild-type receptor. Not only free zinc ions but also complexes between Zn$^{2+}$ or Cu$^{2+}$ and small hydrophobic aromatic chelators were potent activators of the metal-ion site-modified $\beta_2$-receptor. This activating metal-ion site created an important distance constraint between TM-III and TM-VII in the (an) active conformation of the $\beta_2$-receptor. However it was not possible to determine the actual efficacy of Zn$^{2+}$ in this receptor because the mutations that created the activating metal-ion site at the same time destroyed the binding of the catecholamine agonists (Elling et al., 1999).

As demonstrated by both mutational analysis and by cross-linking experiments in the tachykinin NK$\textsubscript{1}$ receptor, the endogenous agonist, the neuropeptide Substance P binds rather superficially in this receptor, i.e., to the N-terminal extension, extracellular loop two and to the extracellular ends of TM-III, -VI, and VII (Fig. 1) (Fong et al., 1992a,b; Strader et al., 1994; Boyd et al., 1996; Kage et al., 1996). Importantly, as shown even by steric hindrance mutagenesis, where large and chemically different residues were used to fill up the deep pocket of the main ligand-binding crevice, Substance P does not reach the more deeply located residues corresponding to the monoamine binding site (Holst et al., 1998). In this study the agonistic metal-ion site designed in this deep pocket of the $\beta_2$-adrenergic receptor is transferred to the only 24% identical NK$\textsubscript{1}$ receptor without loss of Zn$^{2+}$ affinity. In the NK$\textsubscript{1}$ receptor, stabilization of TM-III relative to TM-VII through metal-ion coordination between residues III:08 and VII:06 maximally results in 25% agonism. Moreover, the ability of the hydrophobic metal-ion chelators to function as agonists appears to be dependent on the structural context of the engineered metal-ion site, because only antagonism was observed with the metal-ion chelator complexes in the NK$\textsubscript{1}$ receptor.

**Materials and Methods**

**Ligands**

Substance P was purchased from Peninsula (St. Helens, Merseyside, UK) and 1,10-phenanthroline was obtained from Sigma Chemical Co. (St. Louis, MO). Zn$^{2+}$(phenanthroline)$_3$ and Cu$^{2+}$(phenanthroline)$_3$

\[ \text{Zn}^{2+}(\text{phenanthroline})_3 \]

\[ \text{Cu}^{2+}(\text{phenanthroline})_3 \]

Fig. 1. Serpentine diagram of the NK$\textsubscript{1}$ receptor. ProIII:08 (Pro$^{112}$) and MetVII:06 (Met$^{291}$), which are substituted by either Asp or His or by Cys, respectively, are indicated in white on red. The as yet identified supposed interaction points for the endogenous agonist, Substance P, are indicated in white on green. A few highly conserved “fingerprint” residues in each transmembrane segment are indicated in black on gray. The generic numbering system of Baldwin (Baldwin, 1993) for 7TM receptor residues is used in parallel with the specific numbering throughout the paper (Schwartz, 1994).
were prepared by dissolving phenylalanine in ethanol and mixing with aqueous solutions of ZnCl₂ or CuSO₄ to a final molar ratio of 3:1.

**Molecular Biology**

The point mutations were constructed using oligonucleotide-directed mutagenesis and recombinant polymerase chain reaction as previously described (Rosenkilde et al., 1994). cDNAs encoding wild-type and mutant receptors were cloned into the eukaryotic expression vector pTEJ-8; all mutations were verified by restriction endonuclease mapping and DNA sequencing (ALFExpress DNA Sequencer; Amersham Pharmacia Biotech, Uppsala, Sweden).

**Cell Biology**

Cloned human NK₁ receptors were transiently expressed in COS-7 cells transfected 2 days before analysis. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 10 μg/ml gentamicin.

**Inositol Phosphate Turnover.** COS-7 cells were seeded in 12-well culture dishes 1 day after transfection at a density of 250,000 cells/well and supplemented with 10 μCi [³H]myoinositol/ml (Amersham Pharmacia Biotech). Two days after transfection, cells were washed twice with PI buffer (20 mM HEPES, pH 7.4, with 140 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 10 mM glucose, and 0.05% (w/v) bovine serum albumin) and were incubated in 0.5 ml of PI buffer supplemented with 10 mM LiCl at 37°C for 30 min. After stimulation with increasing concentration of Substance P for 45 min at 37°C, cells were extracted with 10% ice-cold perchloric acid followed by incubation on ice for 30 min. The resulting supernatant was neutralized with KOH in HEPES buffer, and the generated [³H]inositol phosphates were purified on Bio-Rad AG 1-X8 anion exchange resin (Berridge et al., 1983).

**Binding Experiments.** Monoiodinated [¹²⁵I]-Bolton Hunter (BH)-labeled Substance P was prepared and purified by high performance liquid chromatography (Gether et al., 1993). Transfected COS-7 cells were transferred to culture plates 1 day after transfection. The number of cells seeded per well was determined by the expression efficiency of the individual clones aiming at 5 to 10% binding of the added radioligand. Two days after transfection, cells were assayed by competition binding for 3 h at 4°C using 15 pM [¹²⁵I]-BH-Substance P 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 MnCl₂, 0.1% (w/v) bovine serum albumin, 40 μg/ml bacitracin.

**Data Analysis**

IC₅₀ and EC₅₀ values were determined by nonlinear regression using GraphPad Prizm (GraphPad Software, Inc., San Diego, CA). Data are presented as mean ± S.E. from three or more experiments carried out in duplicates. Kᵡ and Kᵣ were calculated from IC₅₀ using the Cheng-Prusoff equation Kᵡ = IC₅₀/(1 + [ligand]/Kᵣ) and Kᵣ = IC₅₀ - [ligand]. Bₘₐₓ values were estimated from competition binding experiments using the equation Bₘₐₓ = B₀ IC₅₀/[ligand], where B₀ is the specifically bound radioligand. Data were evaluated for statistical significance using the appropriated unpaired or paired two-sided t test, assuming Stuart distribution. Kᵡ, Kᵣ, IC₅₀, and EC₅₀ values were transformed to minus logarithm of the value before statistical analysis was performed.

**Results**

**Radioligand Binding Analysis.** The starting point for the metal-ion site engineering in the NK₁ receptor was the agonistic site recently constructed between position III:08 and VII:06 in the β₂-adrenergic receptor (Elling et al., 1999). In contrast to the β₂-adrenergic receptor, where AspIII:08 (Asp¹¹³) is highly important for catecholamine binding, the corresponding ProIII:08 (Pro¹¹²) in the NK₁ receptor is not involved in peptide agonist binding. Thus, it has previously been demonstrated that Pro¹¹² can be substituted with either Asp or His without affecting Substance P binding or its stimulation of inositol phosphate turnover (Holst et al., 1998). Here, a Cys residue was introduced in position VII:06 in the NK₁ receptor, [M291C]-hNK₁, and whole cell binding experiments performed on transiently transfected COS-7 cells revealed that the affinity for Zn²⁺ was increased significantly (Kᵡ = 59 ± 11) compared with wild-type (240 ± 60 μM) (P < .05) (Table 1). In fact, the affinity for Zn²⁺ was also increased in the [P112D]-hNK₁ construct (Kᵡ = 65 ± 1) (P < .05) (Fig. 2A). A further increase in Zn²⁺ affinity was observed in the double mutants [P112D;M291C]-hNK₁ (Fig. 2A) and [P112H;M291C]-hNK₁ where Zn²⁺ inhibited ¹²⁵I-BH-Substance P binding with Kᵡ values of 33 ± 12 μM and 41 ± 8 μM, respectively. This corresponds to an increase in apparent Zn²⁺ affinity of 7 fold compared with the wild-type NK₁ receptor. The metal-ion affinity in the NK₁ double mutants corresponds to the affinity obtained for Zn²⁺ in a number of antagonistic bis-His sites previously constructed between different helices in the NK₁ receptor (Elling and Schwartz, 1996), and the affinity corresponds to the potency for Zn²⁺—91 and 38 μM—determined in the corresponding two metal-ion sites in the β₂-adrenergic receptor (Elling et al., 1999). Not only the free zinc ion but also Zn²⁺ and Cu²⁺ in complex with the strong metal-ion chelator phenanthroline bound to the [P112D;M291C]-hNK₁ and the [P112H; M291C]-hNK₁ constructs with a rather similar affinity as free Zn²⁺ as demonstrated in the ability of the complexes to compete for ¹²⁵I-BH-Substance P binding (Fig. 2) (Table 1).

**Signal Transduction.** None of the single substitutions at either position III:08 or VII:06 affected the EC₅₀ for Sub-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Substance P</th>
<th>ZnCl₂</th>
<th>Zn²⁺(phenanthroline)₂</th>
<th>Cu²⁺(phenanthroline)₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kᵡ S.E.M.</td>
<td>Bₘₐₓ S.E.M. (n)</td>
<td>Kᵡ S.E.M. (n)</td>
</tr>
<tr>
<td>m⁶</td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
</tr>
<tr>
<td>-------------------</td>
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</tr>
<tr>
<td>hNK₁-wt</td>
<td>0.12 ± 0.04</td>
<td>39 ± 7.5 (7)</td>
<td>240 ± 60 (6)</td>
</tr>
<tr>
<td>P112H-hNK₁</td>
<td>0.08 ± 0.02</td>
<td>15 ± 2.3 (3)</td>
<td>280 ± 20 (3)</td>
</tr>
<tr>
<td>P112D-hNK₁</td>
<td>0.05 ± 0.02</td>
<td>6.7 ± 1.4 (3)</td>
<td>65 ± 1 (3)</td>
</tr>
<tr>
<td>M291C-hNK₁</td>
<td>0.16 ± 0.03</td>
<td>2.7 ± 0.5 (3)</td>
<td>59 ± 11 (3)</td>
</tr>
<tr>
<td>P112H,M291C-hNK₁</td>
<td>0.53 ± 0.13</td>
<td>7.5 ± 1.4 (6)</td>
<td>41 ± 8 (6)</td>
</tr>
<tr>
<td>P112D,M291C-hNK₁</td>
<td>4.8 ± 1.1</td>
<td>6.3 ± 0.8 (6)</td>
<td>33 ± 12 (6)</td>
</tr>
</tbody>
</table>

As opposed to Zn²⁺, Cu²⁺ cannot be used in these experiments; due to the general toxic effect of the free ion.
stance P in stimulating inositol phosphate turnover in transiently transfected COS-7 cells significantly (P > .05) (Table 2) (Holst et al., 1998). Compared with the β2-adrenergic receptor, where agonist binding and action was totally eliminated even by the single substitution in position VII:06, the EC50 for Substance P was only increased 6- to 7-fold in the two double mutants in the NK1 receptor (Table 2). Zn2+ did not by itself affect inositol phosphate turnover in COS-7 cells transfected with the wild-type NK1 receptor (Fig. 3). However, in cells transfected with the [P112D;M291C] or the [P112H;M291C] mutant forms of the NK1 receptor Zn2+ stimulated inositol phosphate turnover dose dependently and with EC50 values of 83 ± 17 and 84 ± 19 μM, respectively (Fig. 3).

In the β2-adrenergic receptor, the construction of the metal-ion site between positions III:08 and VII:06 was deliberately performed as an exchange of crucial parts of the agonist binding site, and accordingly the mutations eliminated both catecholamine binding and action. In contrast, in the NK1 receptor the agonist, Substance P, was still able to activate the metal-ion site-engineered receptor mutants, which allowed for comparison of efficacies and for studies where the two agonists, Substance P and Zn2+, are administered together. As shown in Fig. 4A, Zn2+ acted as a 25% partial agonist in the [P112D;M291C] mutant form of the NK1 receptor compared with Substance P but only as a 5 to 10% agonist in the [P112H;M291C] construct. However, in absolute numbers the measured phosphate inositol turnover induced by Zn2+ was rather similar (Table 2). In agreement with basic pharmacological theory the partial agonist, Zn2+, also behaved as an antagonist by dose dependently bringing the inositol phosphate turnover down to its own maximal stimulatory level in competition for receptor occupancy against the full agonist, Substance P (Fig. 4A) (Kenakin, 1993). The IC50 for Zn2+ inhibition of Substance P induced signaling through the [P112D;M291C]-hNK1 receptor was 26 ± 5 μM, which corresponds relatively closely to the affinity measured in the binding assay, Kd = 33 ± 12 μM. How-

**Fig. 2.** Competitions binding experiments. Panel A, affinity for ZnCl2 in wild-type NK1 (■) and after introduction of aspartic acid in TM III:08 [P112D]-NK1 (○); cysteine in TMVII:06 [M291C]-NK1 (▲) and the two mutation in combination [P112D;M291C]-NK1 (●). Panel B, binding of Zn2+ (phenanthroline)3 (closed symbol) and Cu2+ (phenanthroline)3 (open symbol) in the wild-type NK1 (■) receptor and in the metal ion binding site-engineered mutant [P112D;M291C]-NK1 (●). Whole cell binding experiments were performed in transiently transfected COS-7 cells, using [125I] -BH-labeled Substance P as a radioligand. Data are mean ± S.E. from three or more separate experiments carried out in duplicate.

**Fig. 3.** Inositol phosphate turnover in response to ZnCl2 in the wild-type NK1 receptor (■), the [P112D;M291C]-NK1 (○), and the [P112H; M291C]-NK1 (▲), expressed in COS-7 cells. The ZnCl2-induced stimulation is expressed as percentage of the maximal Substance P response in the respective constructs. Data are mean ± S.E. from eight (wild-type) or six (mutants) separate experiments carried out in duplicate. For both mutants, the agonist effect of ZnCl2 concentrations above 10-5 M were significant (P < .05, two-tailed paired t test) and for the [P112D;M291C]-NK1 construct the ZnCl2 stimulation at concentrations above 10-4 M was highly significant (P < .001).

**TABLE 2**

<table>
<thead>
<tr>
<th>Inositol phosphate turnover</th>
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</thead>
<tbody>
<tr>
<td>Stimulatory effect of Zn2+ and Substance P and inhibitory effect of Zn2+, Zn2+(phenanthroline)3 and Cu2+(phenanthroline)3 on Substance P (30 nM) induced stimulation. Receptors were transiently expressed in COS-7 cells, and inositol phosphate accumulation was performed as described. Determinations were performed in duplicate and n indicates the number of experiments.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substance P</th>
<th>ZnCl2</th>
<th>Zn2+(phenanthroline)3</th>
<th>Cu2+(phenanthroline)3</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 S.E.M.</td>
<td>Fmax S.E.M. (n)</td>
<td>EC50 S.E.M. (n)</td>
<td>Fmax S.E.M. (n)</td>
</tr>
<tr>
<td>hNK1-wt</td>
<td>NS</td>
<td>0.36 ± 0.1 (5)</td>
<td>38 ± 5 (8)</td>
</tr>
<tr>
<td>P112H-hNK1</td>
<td>NS</td>
<td>0.54 ± 0.1 (4)</td>
<td>32 ± 4 (3)</td>
</tr>
<tr>
<td>P112D-hNK1</td>
<td>NS</td>
<td>0.29 ± 0.03 (4)</td>
<td>25 ± 8 (3)</td>
</tr>
<tr>
<td>M291C-hNK1</td>
<td>NS</td>
<td>0.55 ± 0.1 (4)</td>
<td>26 ± 7 (4)</td>
</tr>
<tr>
<td>P112H,M291C- hNK1</td>
<td>84 ± 19</td>
<td>4.1 ± 1.1 (5)</td>
<td>1.70 ± 0.5 (4)</td>
</tr>
<tr>
<td>P112D,M291C- hNK1</td>
<td>83 ± 17</td>
<td>5.8 ± 1.5 (6)</td>
<td>2.50 ± 0.3 (4)</td>
</tr>
</tbody>
</table>

IP, inositol phosphate; NS, no stimulation in IP turnover was observed for 10-4 M and 10-3 M concentrations of ZnCl2.
ever, it is interesting that no inhibition of Substance P-induced signaling by Zn\(^{2+}\) was observed with the testable concentrations in the two single mutants [P112D] and [M291C], which both showed relative high affinity for Zn\(^{2+}\) as determined in competition binding assays (Tables 1 and 2).

The metal-ion chelator complexes, Zn\(^{2+}\)- and Cu\(^{2+}\)-phenanthroline, which competed for Substance P binding with similar affinity as the free zinc-ion (Fig. 2), were in contrast to Zn\(^{2+}\) not able to activate the metal-ion site-engineered NK\(_{1}\) receptor (Fig. 4, B and C). This lack of agonist activity of the chelator complexes is surprising because the chelators in fact stimulated signaling more efficaciously in the corresponding sites in the \(\beta_2\)-adrenergic receptor than the free metal-ions did.

In accordance with the fact that they did bind to the mutated NK\(_{1}\) receptor with high affinity (Fig. 2B), these metal-ion complexes acted as antagonists of the Substance P-induced inositol phosphate turnover in the [P112D;M291C]-hNK\(_{1}\) receptor (Fig. 4, B and C) as well as in the [P112H;M291C] construct (Table 2). However, where chelation of Zn\(^{2+}\) with phenanthroline increased the affinity approximately 10-fold in the corresponding metal-ion site-engineered \(\beta_2\)-adrenergic receptor, it had the opposite effect in the metal-ion site-engineered NK\(_{1}\) receptor, where the IC\(_{50}\) value for Zn\(^{2+}\)-phenanthroline inhibition of Substance P signaling was almost 10-fold lower than the IC\(_{50}\) value for free Zn\(^{2+}\) (Fig. 4, Table 1). Moreover, no inhibition of Substance P-induced signaling by metal-ion chelator complexes with the testable concentrations in the two single mutants [P112D] and [M291C], which both showed relative high affinity for these complexes as determined in competition binding assays (Tables 1 and 2).

**Discussion**

In this study, a high affinity agonistic metal-ion site is successfully transferred from the \(\beta_2\)-adrenergic receptor to the NK\(_{1}\) receptor.

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**Fig. 4.** Inositol phosphate turnover in response to ZnCl\(_2\) (panel A), Zn-(phenanthroline)\(_3\) (panel B), or Cu(phenanthroline)\(_3\) (panel C) on the wild-type NK\(_{1}\) receptor (■) and the [P112D;M291C]-NK\(_{1}\) (●) construct in COS-7 cells. Curves with open symbols represent inhibition experiments where the compounds are administered together with a submaximal dose of Substance P (30 nM). The dotted line in panel A marks the 25% stimulation induced by zinc ions in the metal binding site-engineered receptor. Data are mean ± S.E. in duplicate from three or more experiments.

**Fig. 5.** Low resolution model of the NK\(_{1}\) receptor as viewed from outside the cell and a helical wheel diagram of the most extracellularly located 18 residues of each helix. The as yet identified supposed interaction points for the endogenous agonist, Substance P, are indicated in white on green (Fong et al., 1992b; Strader et al., 1994; Boyd et al., 1996; Kage et al., 1996). ProII:08 (Pro112) and MetVII:06 (Met291), which in the present study are substituted by either Asp or His or by Cys, respectively, making an activating metal-ion site, are indicated in white on red. The helical wheel diagram has been constructed in accordance with the general model of Baldwin and coworkers (Baldwin, 1993), and the helices are arranged counter-clockwise as viewed from the outside-in according to previous studies using interhelical, inhibitory bis-His sites (Elling et al., 1996).
the only 24% identical tachykinin NK1 receptor. Importantly, in the NK1 receptor—as opposed to the adrenergic receptor—activation of the mutated receptor by the endogenous agonist can be obtained because the metal-ion site is introduced at a relatively deep location in the receptor structure compared with the Substance P binding site. These data add further evidence to the notion that rhodopsin-like 7TM receptors have a common molecular activation mechanism; but, that the active conformation of the seven helical bundle can be stabilized through ligand binding at very different sites (Schwartz and Rosenkilde, 1996). Moreover, because the rather complicated and ill-defined chemical recognition of a normal agonist now can be mimicked by a well defined simple molecular interaction between a metal-ion and two or three specific residues in the receptor, the basic pharmacological issue of the structural basis of, for example, full versus partial agonism may now start to be addressed systematically.

No “Common Lock for All Keys” in 7TM Receptors. It has generally been believed that there should exist a common active site or “lock” that all agonist “keys” should fit into or touch to activate 7TM receptors. The relatively well characterized and rather small catecholamine binding site was originally thought to represent this common lock (Hibert et al., 1993). However, based on the significantly different maps of binding sites, which subsequently were generated for peptide and monoamine agonists, respectively, it was argued that there may not be a common lock for all agonist keys in 7TM receptors (Schwartz and Rosenkilde, 1996; Holst et al., 1998). Such a common lock was not even needed in the various allosteric models for 7TM receptor function. It has, however, been difficult to prove this point, although mapping of different binding sites in different receptors by site-directed mutagenesis has supported this notion (Schwartz et al., 1995). For example, in the angiotensin AT1 receptor, which can be activated zinc switch located deep between TM-III and -VII. In contrast, mutational mapping—including steric hindrance mutagenesis—as well as cross-linking experiments indicates that Substance P activates this receptor through binding to extracellular epitopes as well as the most exterior parts of TM-III, -VI, and VII (Fong et al., 1992a,b; Strader et al., 1994; Boyd et al., 1996; Kage et al., 1996). Thus, the present study in which two chemically different agents, a metal-ion and a peptide, activates a common receptor through interactions at distinct binding sites is a strong argument in favor of the notion that there is no common lock for all agonist keys in 7TM receptors (Schwartz and Rosenkilde, 1996).

Interestingly, the most “deeply” located interaction points for Substance P, residues III:04 (His108) and VII:02 (Tyr287), are located exactly one helical turn “above” the two positions—residues III:08 and VII:06, respectively—at which the activating zinc site was introduced (Figs. 1 and 5). Thus, although the two agonist “keys” fit into two different locks in the zinc site-engineered NK1 receptor, the location of supposedly crucial residues of each of these locks above each other at the interface between TM-III and -VII indicates that, although the locks are clearly different, a common mechanism of activation of 7TM receptors does exist (Fig. 5).

Full versus Partial Agonism. Electron paramagnetic resonance studies of site-directed spin-labeled rhodopsin have shown that during receptor activation the most conspicuous conformational change appears to occur between TM-III and -VI (Farrens et al., 1996). Several other studies using various methodological approaches support this notion (Sheikh et al., 1996; Gether et al., 1997). Both electron paramagnetic resonance studies of rhodopsin (Sieving, 1995) as well as the substituted cysteine accessibility method (Engelman et al., 1980) and general fluorescence spectroscopy (Gether et al., 1995) combined with, for example, constitutively active mutants have previously been used to address the issue of partial versus full agonism. A major advantage in using a metal-ion as an agonist is that its binding mode is very well defined as opposed to the larger and chemically much more complicated monoamine or peptide agonists. In our case, it is clear that Zn2+ must activate the receptor through binding to residues III:08 and VII:06. However, Zn2+ acts as a 25% partial agonist in the NK1 receptor (Fig. 4), which probably is also the case in the mutated β2-receptor (Elling et al., 1999). In view of the biophysical observation of Farrens and coworkers (1996) that receptor activation is associated with mainly a movement of TM-III and -VI relative to each other, it is very interesting that the full agonist, Substance P, in addition to its interaction points in TM-III and -VII, also has apparent interactions with the extracellular end of TM-VI, i.e., residue VI:20 (Phe268) (Holst et al., 1998). The full agonists for the β-receptor, isoproterenol has also been shown to interact with residue VI:20 (Asn293) in addition to interactions with the earlier recognized more deeply located residues VI:16 (Phe280) and VI:17 (Phe281) (Schwartz, 1994; Wieland et al., 1996). Thus, we would suggest that the reason Zn2+ is only a relatively weak partial agonist is that it is only able to stabilize the right conformation between TM-III and TM-VI and that the metal-ion lacks key interactions in TM-VI, which are required for stabilizing a conformation associated with full agonism. It should be noted that this argument does not address the basic question whether partial agonism is obtained through stabilization of a conformation that is partially active in signaling or through stabilization of only part of the receptors in a fully signaling conformation at a given time. The metal-ion stabilized complex of TM-III and -VII could, for example, simply constitute a good firm scaffold for the final “docking” of TM-VI in a putative, signaling conformation. Importantly, this is still merely speculation, which nevertheless possibly could be experimentally addressed through further attempts in engineering of activating metal-ion sites in this region of the receptors.

Comparison of the Metal-Ion Switch in Two Different Receptors. Although similar metal-ion affinities were found in the zinc site-engineered β2 and NK1 receptors, some interesting differences were observed between the two metal-ion sites. First, in the β2-receptor, Zn2+ was more efficacious in the construct where His was placed at
position III:08 than with the natural Asp at this location (Elling et al., 1999). In contrast, in the NK1 receptor, Zn\(^{2+}\) prefered an Asp at position III:08 (Fig. 3). Second, in the β2-adrenergic receptor, the additional binding of small aromatic metal-ion chelators, phenantroline or bipyridine, through a bridging zinc- or copper-ion resulted in both a higher affinity and higher efficacy for the metal-ions (Elling et al., 1999). In contrast, in the NK1 receptor the metal-ion chelator decreased the zinc potency, and the complex was devoid of agonistic properties (Fig. 4). Thus, in the β2-receptor, the aromatic metal-ion chelator must make some favorable interactions that are not possible in the NK1 receptor. Several residues could be involved. For example, TrpIII:04 of the β-receptor—which is a His in NK1—could make an aromatic-aromatic interaction with the chelator. The presence of this potentially metal-ion binding His residue at position III:04 in the NK1 receptor may also be the reason why Zn\(^{2+}\) and the metal-ion chelator complexes do bind with increased affinity in the two single mutants where Asp or Cys residues are introduced individually at the closely located III:08 and VII:06 positions, respectively (Figs. 1 and 2A and Table 1). Moreover, in the NK1 receptor, PheVI:16 could be imagined to sterically interfere with the binding of the chelator, which in the β-receptor could be favored by the smaller Asn residue. Conceivably, through additional substitutions in the NK1 receptor along these lines, it should be possible to make the metal-ion chelator complexes bind better and act as agonists also in this receptor.

Implications for Potential Use of Metal-Ion Site-Engineered Receptors in Transgenic Animals. In this study an activating metal-ion switch was built into the NK1 receptor and previously inactivating switches have been constructed in various other receptors (Thirstrup et al., 1996; Rosenkilde et al., 1999; Sheikh et al., 1999; Lu Zhi-Liang and Hulme, 2000). In most of these receptors the metal-ion sites function as real silent switches because the endogenous peptide ligand can bind and activate the receptor normally. Receptors with such silent switches could become useful in transgenic animal models aiming at pharmacologically controlling “knock-out” experiments. Due to the normal binding of the endogenous ligand, problems such as embryonic lethality and up-regulation of compensatory mechanisms could possibly be surmounted, because the introduced receptor would be perceived by the animal as being normal. However, because of general toxicity, free metal-ions cannot be used in vivo to turn on and off the metal-ion switches in the mutated 7TM receptors. For this purpose, the metal-ion chelator complexes are very interesting because the chelators generally render the metal-ions relatively atoxic. As shown in this present study, although the metal-ion sites can be moved relatively freely between rhodopsin-like 7TM receptors, the surrounding residues in the receptor may interfere with the binding and pharmacological properties of the metal-ion chelator complexes. Thus, in vitro optimization of metal-ion sites and chelator complexes needs to be performed in the individual receptor to obtain the desired pharmacological phenotype.

References


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