

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 β_2 -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 β_2 -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, phenanthroline, which in the β_2 -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

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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 β_2 -adrenergic receptor (Elling et al., 1999). The β_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 β_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 Asp¹¹³ (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 Asn³¹² (AsnVII:06), which is a 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 α_{1b} -adrenergic and δ -opioid receptors have created constitutive activity (Befort et al., 1999; Porter and Perez, 1999); however, the basal signaling of the zinc site-engineered β_2 -adrenergic receptor was similar to that of the wild-type receptor. Not only free zinc ions but also complexes between Zn²⁺ or Cu²⁺ and small hydrophobic aromatic chelators were potent activators of the metal-ion site-modified β_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 β_2 -receptor.

However it was not possible to determine the actual efficacy of Zn²⁺ 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 NK₁ 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 β_2 -adrenergic receptor is transferred to the only 24% identical NK₁ receptor without loss of Zn²⁺ affinity. In the NK₁ 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 NK₁ 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). Zn²⁺(phenanthroline)₃ and Cu²⁺(phenanthroline)₃

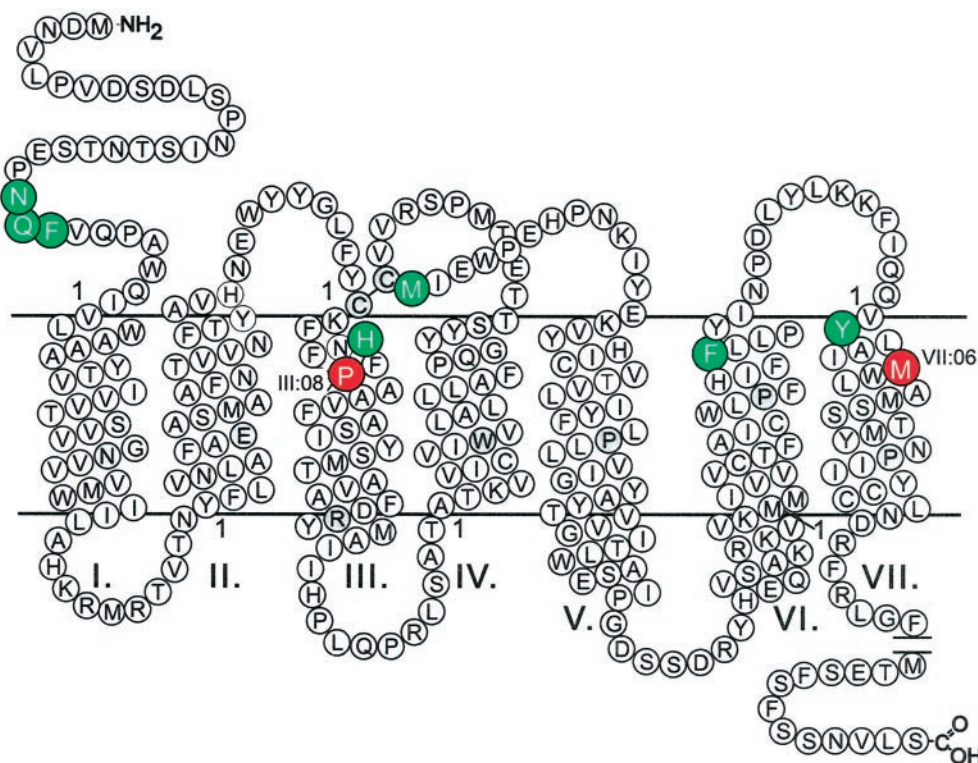


Fig. 1. Serpentine diagram of the NK₁ receptor. ProIII:08 (Pro¹¹²) and MetVII:06 (Met²⁹¹), which are substituted by either Asp or His or by Cys, respectively, are indicated in white on red. The as yet identified supposed interaction points for the endogenous agonist, Substance P, are indicated in white 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).

tical significance using the appropriated unpaired or paired two-sided t test, assuming Stuart distribution. K_i , K_d , IC_{50} , and EC_{50} values were transformed to minus logarithm of the value before statistical analysis was performed.

Results

Disulfide 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 β_2 -adrenergic receptor (Elling et al., 1999). In contrast to the β_2 -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 ($K_i = 59 \pm 11$) compared with wild-type (240 ± 60 μ M) ($P < .05$) (Table 1.). In fact, the affinity for Zn²⁺ was also increased in the [P112D]-hNK₁ construct ($K_i = 65 \pm 1$) ($P < .05$) (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 K_i values of 33 ± 12 μ M and 41 ± 8 μ M, 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 μ M—determined in the corresponding two metal-ion sites in the β_2 -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.).¹

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

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 μ Ci [3 H]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 MgSO_4 , 1 mM CaCl_2 , 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 [3 H]inositol phosphates were purified on Bio-Rad AG 1-X8 anion exchange resin (Berridge et al., 1983).

Binding Experiments. Monoiodinated ^{125}I -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 ^{125}I -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 MnCl_2 , 0.1% (w/v) bovine serum albumin, 40 $\mu\text{g/ml}$ bacitracin.

¹ As opposed to Zn^{2+} , Cu^{2+} cannot be used in these experiments; due to the general toxic effect of the free ion.

IC₅₀ and EC₅₀ values were determined by nonlinear regression using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Data are presented as mean ± S.E. from three or more experiments carried out in duplicates. K_d and K_i were calculated from IC₅₀ using the Cheng-Prusoff equation $K_i = IC_{50}/(1 + [ligand]/K_d)$ and $K_d = IC_{50} - [ligand]$. B_{max} values were estimated from competition binding experiments using the equation $B_{max} = B_0 IC_{50}/[ligand]$, where B₀ is the specifically bound radioligand. Data were evaluated for statis-

K_d , K_i , and B_{max} of Substance P, Zn^{2+} , and metal-ion chelator complexes in the wild-type receptor and mutant forms of the NK₁ receptor. Receptors were expressed transiently in COS-7 cells, and competition binding experiments were performed as described in the text using [¹²⁵I]-BH-Substance P as radioligand. Determinations were performed in duplicate and n indicates the number of experiments.

	Substance P		ZnCl ₂	Zn ²⁺ (phenantroline) ₃	Cu ²⁺ (phenanthroline) ₃
	<i>K_d</i> S.E.M.	<i>B_{max}</i> S.E.M. (<i>n</i>)	<i>K_i</i> S.E.M. (<i>n</i>)	<i>K_i</i> S.E.M. (<i>n</i>)	<i>K_i</i> S.E.M. (<i>n</i>)
	<i>nM</i>	<i>fmol / 10⁵ cells</i>	<i>μM</i>	<i>μM</i>	<i>μM</i>
hNK1-wt	0.12 ± 0.04	39 ± 7.5 (7)	240 ± 60 (6)	860 ± 140 (7)	338 ± 110 (6)
P112H-hNK1	0.08 ± 0.02	15 ± 2.3 (3)	280 ± 20 (3)	910 ± 30 (3)	400 ± 40 (3)
P112D-hNK1	0.05 ± 0.02	6.7 ± 1.4 (3)	65 ± 1 (3)	43 ± 4 (3)	53 ± 1 (3)
M291C-hNK1	0.16 ± 0.03	2.7 ± 0.5 (3)	59 ± 11 (3)	90 ± 8 (3)	53 ± 8 (3)
P112H;M291C-hNK1	0.53 ± 0.13	7.5 ± 1.4 (6)	41 ± 8 (6)	63 ± 11 (5)	45 ± 12 (4)
P112D;M291C-hNK1	4.8 ± 1.1	6.3 ± 0.8 (6)	33 ± 12 (6)	42 ± 6 (6)	44 ± 15 (5)

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 β_2 -adrenergic receptor, where agonist binding and action was totally eliminated even by the single substitution in position VII:06, the EC_{50} for Substance P was only increased 6- to 7-fold in the two double mutants in the NK₁ receptor (Table 2). Zn^{2+} 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 Zn^{2+} stimulated inositol phosphate turnover dose dependently and with EC_{50} values of 83 ± 17 and $84 \pm 19 \mu M$, respectively (Fig. 3).

In the β_2 -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 Zn^{2+} , are administered together. As shown in Fig. 4A, Zn^{2+} 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 Zn^{2+} was rather similar (Table 2). In agreement with basic pharmacological theory the partial agonist, Zn^{2+} , 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 IC_{50} for Zn^{2+} inhibition of Substance P induced signaling through the [P112D;M291C]-hNK₁ receptor was $26 \pm 5 \mu M$, which corresponds relatively closely to the affinity measured in the binding assay, $K_i = 33 \pm 12 \mu M$. How-

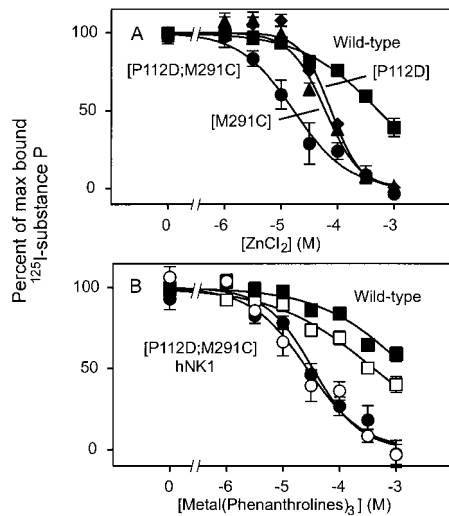


Fig. 2. Competitions binding experiments. Panel A, affinity for $ZnCl_2$ in wild-type NK₁ (■) and after introduction of aspartic acid in TM III:08 [P112D]-NK₁ (◆), cysteine in TM VII:06 [M291C]-NK₁ (▲) and the two mutation in combination [P112D;M291C]-NK₁ (●). Panel B, binding of Zn^{2+} (phenanthroline)₃ (closed symbol) and Cu^{2+} (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 [^{125}I]-BH-labeled Substance P as a radioligand. Data are mean \pm S.E. from three or more separate experiments carried out in duplicate.

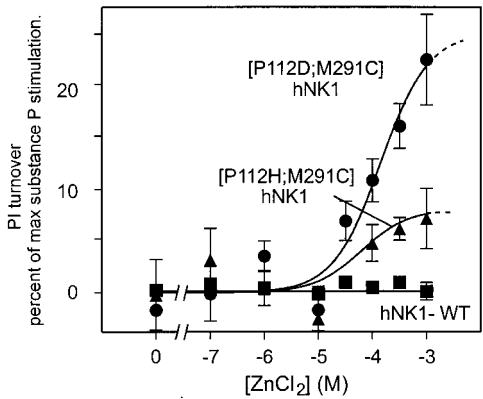


Fig. 3. Inositol phosphate turnover in response to $ZnCl_2$ in the wild-type NK₁ receptor (■), the [P112D;M291C]-NK₁ (●), and the [P112H;M291C]-NK₁ (▲), expressed in COS-7 cells. The $ZnCl_2$ -induced stimulation is expressed as percentage of the maximal Substance P response in the respective constructs. Data are mean \pm S.E. from eight (wild-type) or six (mutants) separate experiments carried out in duplicate. For both mutants, the agonist effect of $ZnCl_2$ concentrations above 10^{-5} M were significant ($P < .05$, two-tailed paired t test) and for the [P112D;M291C]-NK₁ construct the $ZnCl_2$ stimulation at concentrations above 10^{-4} M was highly significant ($P < .001$).

TABLE 2

Inositol phosphate turnover

Stimulatory effect of Zn^{2+} and Substance P and inhibitory effect of Zn^{2+} , Zn^{2+} (phenanthroline)₃ and Cu^{2+} (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.

	Stimulation of IP Turnover						Inhibition of Substance P Induced IP Turnover					
	ZnCl ₂			Substance P			ZnCl ₂			Zn ²⁺ (phenanthroline) ₃		
	EC ₅₀	S.E.M.	<i>n</i>	EC ₅₀	S.E.M.	<i>n</i>	IC ₅₀	S.E.M.	<i>n</i>	IC ₅₀	S.E.M.	<i>n</i>
	μM	$fmol/10^5$ cells		nM	$fmol/10^5$ cells		μM			μM		
hNK1-wt	NS		(8)	0.36 ± 0.1	(5)	38 ± 3	>1000	(4)	>1000	(3)	>1000	(3)
P112H-hNK1	NS		(8)	0.54 ± 0.1	(4)	32 ± 4	550 ± 100	(3)	>1000	(3)	>1000	(3)
P112D-hNK1	NS		(8)	0.29 ± 0.03	(4)	25 ± 8	770 ± 180	(3)	>1000	(3)	>1000	(3)
M291C-hNK1	NS		(8)	0.55 ± 0.1	(4)	26 ± 7	210 ± 73	(3)	>1000	(3)	>1000	(3)
P112H;M291C-hNK1	84 ± 19	4.1 ± 1.1	(5)	1.70 ± 0.5	(4)	38 ± 5	39 ± 11	(3)	120 ± 12	(3)	237 ± 64	(3)
P112D;M291C-hNK1	83 ± 17	5.8 ± 1.5	(6)	2.50 ± 0.3	(4)	27 ± 2	26 ± 5	(4)	190 ± 96	(3)	36 ± 10	(3)

IP, inositol phosphate; NS, no stimulation in IP turnover was observed for 10^{-4} M and 10^{-3} M concentrations of $ZnCl_2$.

ever, it is interesting that no inhibition of Substance P-induced signaling by Zn²⁺ was observed with the testable concentrations in the two single mutants [P112D] and [M291C], which both showed relative high affinity for Zn²⁺ as determined in competition binding assays (Tables 1 and 2).

The metal-ion chelator complexes, Zn²⁺- and Cu²⁺-phenanthroline, which competed for Substance P binding with similar affinity as the free zinc-ion (Fig. 2), were in contrast to Zn²⁺ not able to activate the metal-ion site-engineered NK₁ 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 β_2 -adrenergic receptor than the free metal-ions did. In accordance with the fact that they did bind to the mutated NK₁ 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]-hNK₁ receptor (Fig. 4, B and C) as well as in the [P112H;M291C] construct (Table 2). However, where chelation of Zn²⁺ with phenanthroline *increased* the affinity approximately 10-fold in the corresponding metal-ion site-engineered β_2 -adrenergic receptor, it had the opposite effect

in the metal-ion site-engineered NK₁ receptor, where the IC₅₀ value for Zn²⁺-phenanthroline inhibition of Substance P signaling was almost 10-fold *lower* than the IC₅₀ value for free Zn²⁺ (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 β_2 -adrenergic receptor to

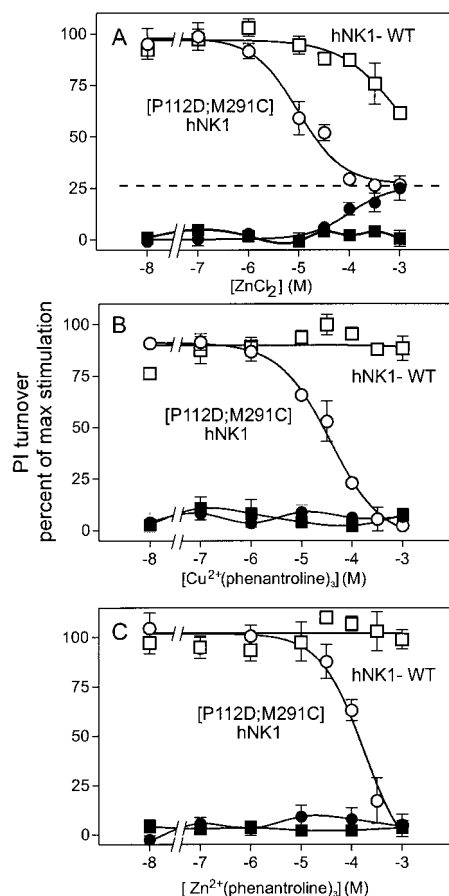


Fig. 4. Inositol phosphate turnover in response to ZnCl₂ (panel A), Zn(phenanthroline)₃ (panel B), or Cu(phenanthroline)₃ (panel C) on the wild-type NK₁ receptor (■) and the [P112D;M291C]-NK₁ (●) construct in COS-7 cells. Curves with open symbols represent inhibition experiments where the compounds are 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 \pm S.E. in duplicate from three or more experiments.

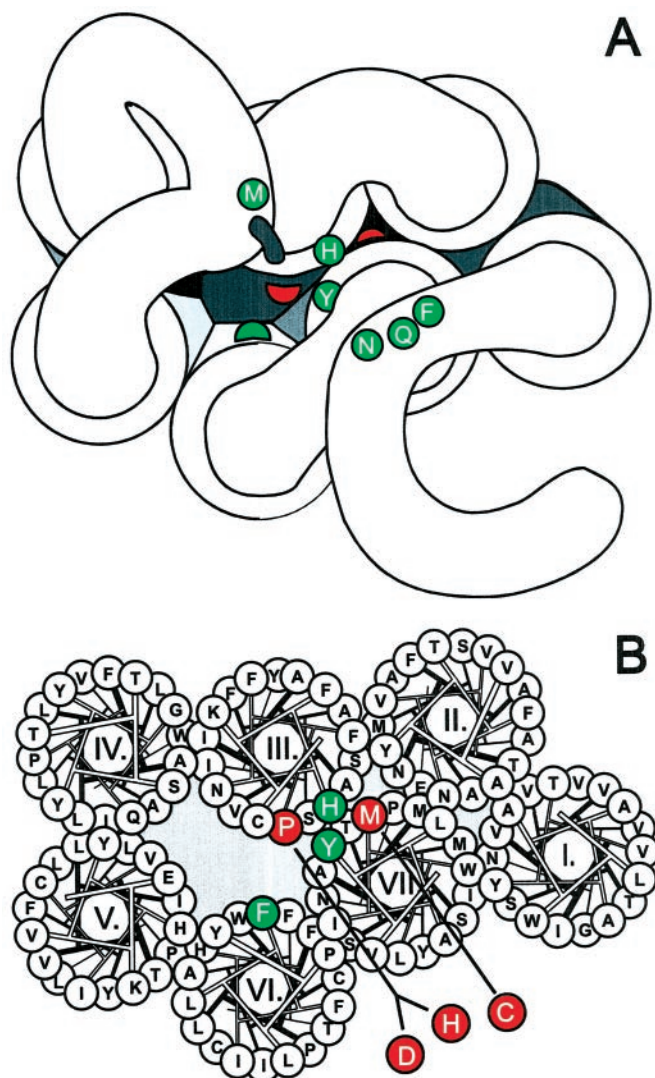


Fig. 5. Low resolution model of the NK₁ receptor as viewed from outside the cell and a helical wheel diagram of the most extracellularly located 18 residues of each helix. The as yet identified supposed interaction points for the endogenous agonist, Substance P, are indicated in white on green (Fong et al., 1992b; Strader et al., 1994; Boyd et al., 1996; Kage et al., 1996). ProIII: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 stimulated both by the endogenous peptide agonist angiotensin and by nonpeptide, biphenyl-imidazole agonists, mutational analysis has generated substantially different maps of supposed molecular interactions for these two types of agonists (Hjorth et al., 1994; Perlman et al., 1995, 1997). But, unfortunately no definitively clear picture of the binding site for the nonpeptide agonists was obtained. Importantly, in the zinc site-engineered NK₁ receptor of this study, Zn²⁺ most certainly activates the receptor through binding at the introduced 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 β_2 -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 residue VI:16 (Phe²⁹⁰) and VI:17 (Phe²⁹¹) (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-VII 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 β_2 and NK₁ receptors, some interesting differences were observed between the two metal-ion sites. First, in the β_2 -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, Zn²⁺ preferred 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 Zn²⁺ 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 conditioned “knock-out” experiments. Due to the normal binding of the endogenous ligand, problems such as embryologic 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.

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