Natural agonist enhancing bis-His zinc-site in transmembrane segment V of the tachykinin NK_3 receptor

Mette M. Rosenkilde, Maria Lucibello, Birgitte Holst, Thue W. Schwartz*

Laboratory for Molecular Pharmacology, Department of Pharmacology, The Panum Institute 18.6, Blegdamsvej 3B, DK-2200, Copenhagen, Denmark

Received 2 September 1998

Abstract In the wild-type tachykinin NK_{3A} receptor histidyl residues are present at two positions in TM-V, V:01 and V:05, at which Zn^{2+} functions as an antagonist in NK_1 and κ-opioid receptors with engineered metal-ion sites. Surprisingly, in the NK_{3A} receptor Zn^{2+} instead increased the binding of the agonist ^{125}I-[MePhe^7]neurokinin B to 150%. [MePhe^7]neurokinin B bound to the NK_{3A} receptor in a two-component mode of which Zn^{2+} eliminated the subnanomolar binding mode but induced a higher binding capacity of the nanomolar binding mode. Signal transduction was not induced by $ZnCl_2$ but 10 μM $ZnCl_2$ enhanced the effect of neurokinin B. Ala-substitution of HisV:01 eliminated the enhancing effect of Zn^{2+} on peptide binding. It is concluded that physiological concentrations of Zn^{2+} have a positive modulatory effect on the binding and function of neurokinin B on the NK_{3A} receptor through a bis-His site in TM-V.

© 1998 Federation of European Biochemical Societies.

Key words: Metal ion; Tachykinin receptor; Zinc; 7TM receptor

1. Introduction

In mammals, there are three types of receptors for the homologous tachykinin neuropeptides: substance P, neurokinin A (NKA), and neurokinin B (NKB) [1,2]. The NK_1 receptor, which is found both centrally and peripherally binds preferentially substance P but also acts as a high affinity receptor for the two other neurokinins, NKA and NKB [3]. The NK_2 receptor binds preferentially NKA and is found almost exclusively in the periphery [1,2]. In contrast, the two NK_3 receptor subtypes, which are selective for NKB, are almost exclusively expressed in the central nervous system, especially the cerebral cortex and hippocampus [1,2,4]. It was originally believed that there was only one NK_3 receptor type. However, recently Jim Krause and coworkers discovered that an orphan receptor which originally was suspected to be an opioid receptor [5] in fact could be activated by nanomolar concentrations of NKB [4]. Accordingly, it has been suggested that the two highly homologous NKB receptors, which mainly differ in the long N-terminal extension (see Fig. 1) should be called NK_{3A} and NK_{3B} [3].

Previously we have engineered metal-ion binding sites into other 7TM receptors by site directed mutagenesis. Initially this was performed in the NK_1 receptor by probing the binding site for a non-peptide antagonist systematically with histidine substitutions. In this way the antagonist binding site was both structurally and functionally converted into a high affinity zinc site [6]. The originally constructed tridentate metal-ion site, which consisted of two His residues located in an *i* and *i*+4 position in TM-V and a single His residue located across in TM-VI, could be moved to the κ-opioid receptor without loss of affinity for Zn^{2+} [7]. Artificially introduced metal-ion sites have subsequently been used as structural and functional probes, which have provided important distance constraints in molecular models of 7TM receptors and information about conformational changes occurring between helices [8–10]. In all cases, the binding of the metal ion has been antagonistic as it was blocking ligand binding and/or inhibiting signal transduction [10].

An optimal location of two histidyl residues for binding Zn^{2+} is in an *i* and *i*+4 position in an α-helix [11]. In accordance with this, Zn^{2+} binds with single digit μM affinity between histidyl residues introduced at position V:01 and V:05 both in the NK_1 and the κ-opioid receptor [6,7]. As shown in Fig. 1, in both of the NK_3 receptor subtypes histidyl residues are located at these two positions in the wild-type receptor proteins. Since zinc is stored and released from synaptic vesicles in the brain, and since zinc concentration in the extracellular fluid accordingly is high and regulated [12–14], we have studied here the effect of Zn^{2+} on ligand binding and signal transduction in the NK_{3A} receptor with and without substitution of one of the two histidyl residues in TM-V. Surprisingly, it is found that in the wild-type NK_{3A} receptor Zn^{2+} enhances ligand binding in contrast to the blocking effect which is observed in all the receptors where this metal-ion site has been artificially introduced.

2. Materials and methods

2.1. Materials

NKB and [MePhe^7]NKB were purchased from Peninsula (St. Helens, Merseyside, UK). The NK_{3A} non-peptide antagonist, SR142,271, was kindly provided by Drs. Xavier Edmons-Alt and Jean-Claude Brelière (Sanofi Recherche, Montpellier, France) [15]. *Pfu* polymerase was from Stratagene (La Jolla, CA, USA). AG 1-X8 anion-exchange resin was from Bio-Rad (Hercules, CA, USA). [^{3}H]*myo*-inositol (PT6-271); Bolton-Hunter reagent, specific activity 2000 Ci/mmol; and Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP were from Amersham (Little Chalfont, UK).

2.2. Construction of the [H244A]NK_{3A} mutant receptor

The cDNA encoding the wild-type human NK_{3A} receptor generously supplied by Dr. Cathrine Strader [16] was cloned into the eukaryotic expression vector pTEJ-8 [17]. The mutation was constructed by polymerase chain reaction (PCR) using the overlap extension method [18] and *pfu* polymerase according to the instruction of the manufacturer. The PCR product was digested with appropriate restriction endonucleases, purified, and cloned into the pTEJ8-NK_{3A}. The mutation was verified by restriction endonuclease mapping and subsequent DNA sequence analysis using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP on an

*Corresponding author. Fax: (45) 3535 2995.
E-mail: schwartz@molpharm.dk

0014-5793/98/$19.00 © 1998 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(98)01331-3

Alfexpress DNA sequencer according to manufacturers' instructions (Pharmacia Biotech, Uppsala, Sweden).

2.3. Transfections and tissue culture

COS-7 cells were grown in Dulbecco's Modified Eagle Medium 1885 supplemented with 10% fetal calf serum, 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 after transfection by the calcium phosphate precipitation method.

2.4. Binding experiments

Monoiodinated ^{125}I-[MePhe^7]NKB (NEX285) was purchased from New England Nuclear. Transfected COS-7 cells were transferred to culture plates one day after transfection. The number of cells per well, 20 000, 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 35 pM ^{125}I-[MePhe^7]NKB 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 1 μM [MePhe^7]NKB. Determinations were made in triplicate.

2.5. Phosphatidylinositol assay

One day after transfection COS-7 cells (0.3×10^6 cells/well) were incubated for 24 h with 5 μCi of [^{3}H]-*myo*-inositol in 0.5 ml inositol-free Dulbecco's 1885 medium supplemented with 10% fetal calf serum, 2 mM glutamine and 0.01 mg/ml gentamicin per well. Cells were washed twice in 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. After stimulation for 20 min at 37°C with increasing concentration of NKB with or without added $ZnCl_2$, or stimulation with $ZnCl_2$ with or without added NKB, the 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 [19]. Determinations were made in triplicate.

2.6. Calculations

IC_{50} and EC_{50} values were determined by non-linear regression using the Inplot 4.0 software (GraphPad Software, San Diego, CA, USA). K_d and B_{max} values were calculated from competition binding experiments with 10–12 different concentrations of cold ligand and using the equation $K_d = IC_{50} - L$ and $B_{max} = B_o(1+(K_d/L))$. K_i values were calculated using the equation $K_i = IC_{50}/(1+L/K_d)$ [20]. The homologous competition binding experiments with [MePhe^7]NKB in the presence of various concentrations of $ZnCl_2$ were further analyzed based on the two-component binding mode observed in the absence of the metal-ion using Inplot. These calculations were performed by holding the IC_{50} of the high affinity binding mode constant (although the binding capacity of this binding mode was gradually reduced in response to the increasing concentrations of $ZnCl_2$) and calculating the percentage distribution between the high affinity and the low affinity state as well as the affinity of the low affinity state.

3. Results

In mutated versions of both the NK_1 receptor and the κ-opioid receptor in which histidyl residues are introduced at positions V:01 and V:05, Zn^{2+} in concentrations from 10^{-6} to 10^{-4} M compete in a mono-component, dose-dependent manner for binding with radioactive ligands (dashed curves in Fig. 2A) [21,22]. In contrast, in the NK_{3A} receptor, where histidyl residues are located at these two positions already in the wild-type receptor (Fig. 1), Zn^{2+} influenced ligand binding in a biphasic manner. In concentrations from 10^{-6} to 10^{-5} M $ZnCl_2$ increased the binding of ^{125}I-[MePhe^7]NKB whereas concentrations from 10^{-4} to 10^{-2} M blocked ligand binding (Fig. 2A). A maximum of 140 ± 4% binding of ^{125}I-[MePhe^7]-NKB was found with 10^{-5} M of $ZnCl_2$ ($N = 24$). The agonist-enhancing effect of $ZnCl_2$ was conceivably mediated through Zn^{2+} binding to the bis-His site at the extracellular end of transmembrane segment V (Fig. 1) since the enhancing effect was not observed in the mutant receptor, where one of these histidyl residues had been mutated into an alanine, [H244A]NK_{3A} (Fig. 2B). In the mutant receptor, only the ligand-blocking effect at high Zn^{2+} concentrations was observed just as it is observed in several other wild-type receptors at similar high concentrations [6,7].

Competition binding experiments performed with [MePhe^7]-NKB at various Zn^{2+} concentrations confirmed the enhancing effect of the metal ion on peptide binding (Fig. 3A). An increase in binding capacity from 194 ± 17 in the absence to 486 ± 64 fmol/10^5 cells in the presence of $10^{-4.5}$ M $ZnCl_2$ was found for the wild-type receptor, whereas no change in binding capacity was observed in the [H244A] mutant form of the NK_{3A} receptor (Table 1). More detailed analysis of the binding curves revealed that [MePhe^7]NKB in the absence of Zn^{2+} interacted with the wild-type NK_{3A} receptor in a two-component fashion with 62 ± 5% being bound in a very high affinity mode ($K_i = 0.20 \pm 0.03$ nM) and 38% being bound in a nanomolar mode ($K_i = 3.2 \pm 0.6$ nM), calculated by individual analysis of 24 experiments (Fig. 3B). Surprisingly, while Zn^{2+} increased the total amount of bound ^{125}I-[MePhe^7]NKB (Fig. 3A, Table 1) the metal ion at the same time in a dose-dependent manner eliminated the binding of the radioactive peptide ligand to the very high affinity site (table inserted in Fig. 3B). This elimination of the very high affinity state was also re-

Table 1
Competition binding experiments for the peptide agonist [MePhe^7]NKB and for the non-peptide antagonist SR142,271 in the wild-type human NK_{3A} receptor and in the [H244A] mutant form of this

NK_{3A}	$ZnCl_2$ (M)	B_{max} (fmol/10^5 cell)	[MePhe^7]NKB		SR142,271	
			K_i (nM) (n)	Hill	K_i (nM) (n)	Hill
Wild type	0	194 ± 17	0.53 ± 0.06 (24)	−0.71 ± 0.02	1.47 ± 0.16 (8)	−1.19 ± 0.14
	10^{-6}	234 ± 24	0.98 ± 0.14 (6)	−0.77 ± 0.07	2.47 ± 0.34 (5)	−1.27 ± 0.14
	10^{-5}	449 ± 78	1.01 ± 0.18 (9)	−0.91 ± 0.05	1.35 ± 0.17 (8)	−1.12 ± 0.11
	$10^{-4.5}$	486 ± 64	1.54 ± 0.20 (7)	−1.12 ± 0.09	2.02 ± 0.18 (6)	−1.30 ± 0.10
[H244A]	0	397 ± 49	1.15 ± 0.14 (4)	−0.95 ± 0.08	0.94 ± 0.10 (5)	−1.26 ± 0.13
	10^{-6}	405 ± 80	1.56 ± 0.29 (5)	−0.89 ± 0.04	0.80 ± 0.06 (3)	−0.95 ± 0.03
	10^{-5}	431 ± 91	1.62 ± 0.40 (5)	−0.97 ± 0.12	0.93 ± 0.10 (3)	−0.98 ± 0.18
	$10^{-4.5}$	422 ± 100	1.72 ± 0.24 (5)	−0.95 ± 0.09	0.82 ± 0.15 (3)	−1.15 ± 0.09

The receptors were expressed transiently in COS-7 cells and competition binding experiments were performed as described in the text using ^{125}I-[MePhe^7]NKB.

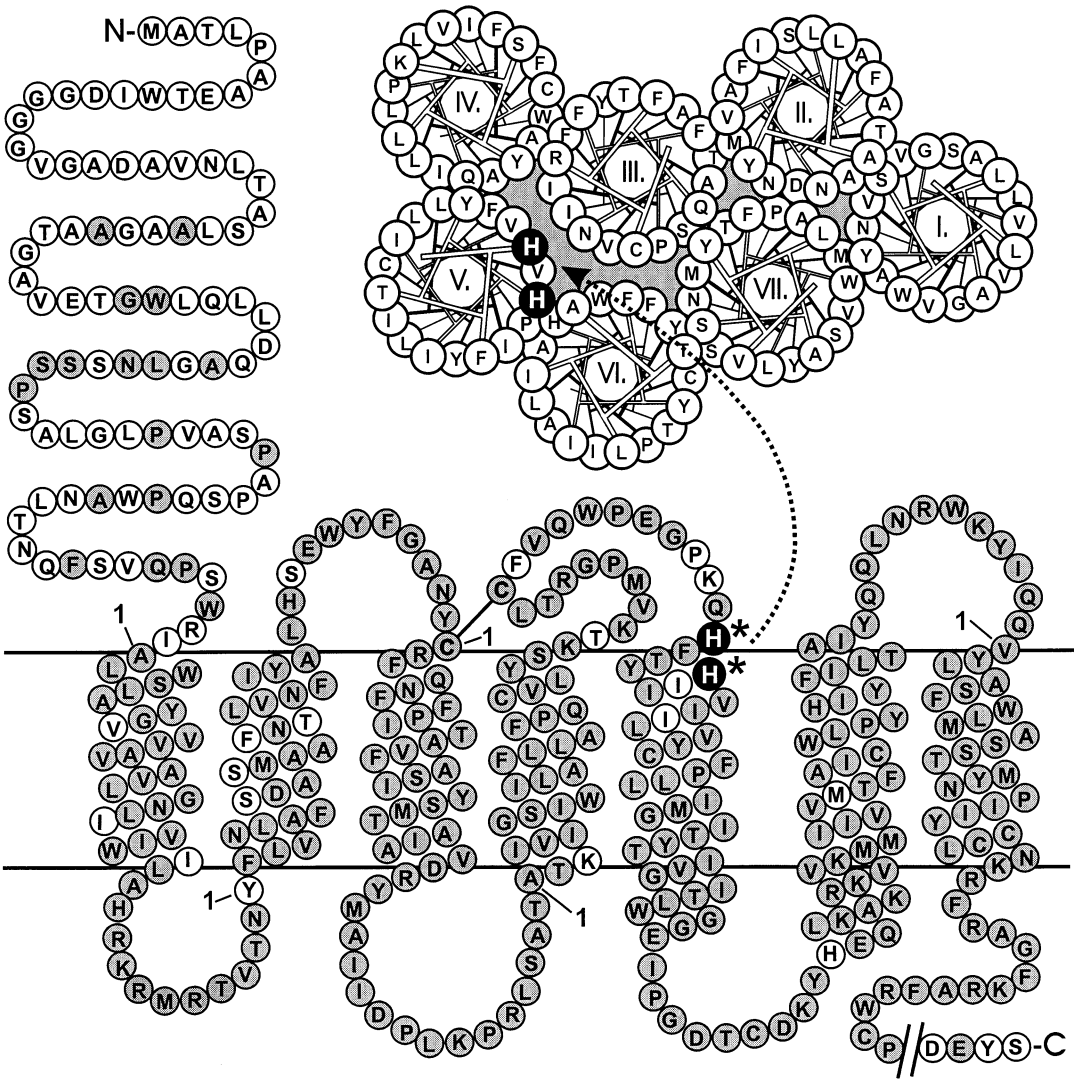


Fig. 1. Serpentine and helical wheel diagram of the tachykinin NK_{3A} receptor. The helical wheel diagram is built over the rhodopsin structure [28] as interpreted by Baldwin [29] with TM-III as the central helix and in anti-clockwise orientation as viewed from outside the cell, which recently has been probed experimentally [8]. Helix-helix interaction has been optimized on the basis of metal-ion site engineering [10]. Residues which are conserved between the NK_{3A} and NK_{3B} receptors are shown in black on grey. However, of these conserved residues HisV:01 (His^{244}) and HisV:05 (His^{248}) are highlighted in white on black. The generic numbering system for residues in 7TM receptors with the helix number in roman followed by the residue number in Latin separated by a ':' is used here and in the text [29].

flected in the gradual change in Hill coefficient from −0.71 towards unity (Table 1). Thus, $ZnCl_2$ eliminates the very high, sub-nanomolar affinity binding state of the NK_{3A} receptor while it increases the binding capacity of the nanomolar affinity state of the receptor.

The very high affinity state of peptide binding was not observed in the [H244A]NK_{3A} receptor as ^{125}I-[$MePhe^7$]NKB bound with an affinity (K_i) of 1.15 nM in a mono-component fashion with a Hill coefficient of −0.95 (Table 1). Zn^{2+} did not affect the binding affinity and it did not affect the binding capacity for [$MePhe^7$]NKB in the [H244A]NK_{3A} receptor as determined in competition binding experiments (Table 1).

The effect of $ZnCl_2$ on non-peptide antagonist binding was tested with SR142,271 in competition binding experiments against ^{125}I-[$MePhe^7$]NKB. Zn^{2+} did not affect the high affinity, mono-component binding of the non-peptide antagonist neither in the wild-type nor in the mutant receptor (Table 1).

Dose-response curves for induction of phosphatidylinositol turnover by NKB through the NK_{3A} receptor performed in the absence and in the presence of 10^{-5} M $ZnCl_2$ were almost superimposable with an EC_{50} of 1.4 ± 0.4 nM and 1.1 ± 0.3 nM, respectively (Fig. 4A). However, when dose-response experiments for $ZnCl_2$ were performed in the presence of low, sub-maximal doses of NKB, a small potentiating effect of 10^{-5} M Zn^{2+} upon the signalling of NKB was revealed (Fig. 4B).

4. Discussion

4.1. Positions V:01 and V:05 are located optimally for Zn^{2+} binding in 7TM receptors

Previously we have built histidyl residues into these two positions in various 7TM receptors: the NK_1 [6], the κ-opioid [7], the NK_2 (M. Lucibello, M.M. Rosenkilde, B. Holst, S.A. Hjorth and T.W. Schwartz, unpublished observation), and in chemokine receptors (M.M. Rosenkilde, T.N. Kledal and

T.W. Schwartz, unpublished observation). In all these cases Zn^{2+} at single digit μM concentrations inhibits the binding of ligands and/or the signaling of the receptor (see for example Fig. 2A). This effect is not observed in the corresponding wild-type receptors. The high affinity binding of Zn^{2+} to these two histidyl residues and the fact that interhelical bis-His zinc sites can be constructed from these positions in TM-V to positions in TM-III and TM-VI [8,10] indicate that: (i) positions V:01 and V:05 in 7TM receptors are located in an α-helical structure; (ii) these residues are facing inward towards TM-III and VI; and (iii) side chains at these positions are relatively freely accessible for ions in solution.

4.2. Zn^{2+} binds to the NK_{3A} wild-type receptor with single digit μM affinity

The high affinity Zn^{2+} binding to the NK_{3A} receptor can be observed indirectly in the dose-dependent positive effect of low concentrations of the metal ion on the peptide binding capacity (Fig. 3A, Table 1) and in the dose-dependent negative effect of the metal ion on the very high affinity binding mode for the peptide (Fig. 3B). More than half of the very high affinity, subnanomolar binding sites for [MePhe^7]NKB are eliminated by 10^{-5} M $ZnCl_2$ (insert in Fig. 3B). This is an effect which is rather similar to that observed with $ZnCl_2$ on, for example, ^{125}I-substance P binding in the mutant NK_1 receptor in which two histidines were introduced in TM-V (dashed curve in Fig. 2A).

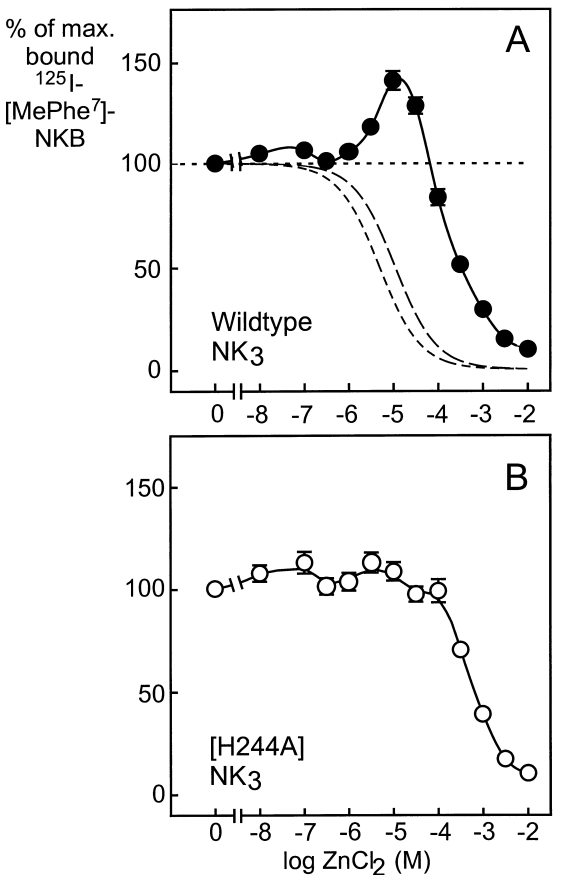


Fig. 2. Dose-response curves for $ZnCl_2$ on the binding of ^{125}I-[MePhe^7]NKB to the human NK_{3A} receptor. Binding experiments were performed as whole cell binding on transiently transfected COS-7 cells at 4°C. Panel A shows binding experiments performed in the wild-type NK_{3A} receptor (● – ●). For comparison the dose-response curves for $ZnCl_2$ against ^{125}I-substance P binding in the mutated NK_1 receptor (long dashes) and against [^{3}H]diprenorphine binding in the mutated κ-opioid receptor (short dashes) are shown. In both cases two histidines were present at positions V:01 and V:05, respectively [6,7]. Panel B shows binding experiments performed in the [H244A]NK_{3A} receptor (○ – ○) where HisV:01 had been exchanged.

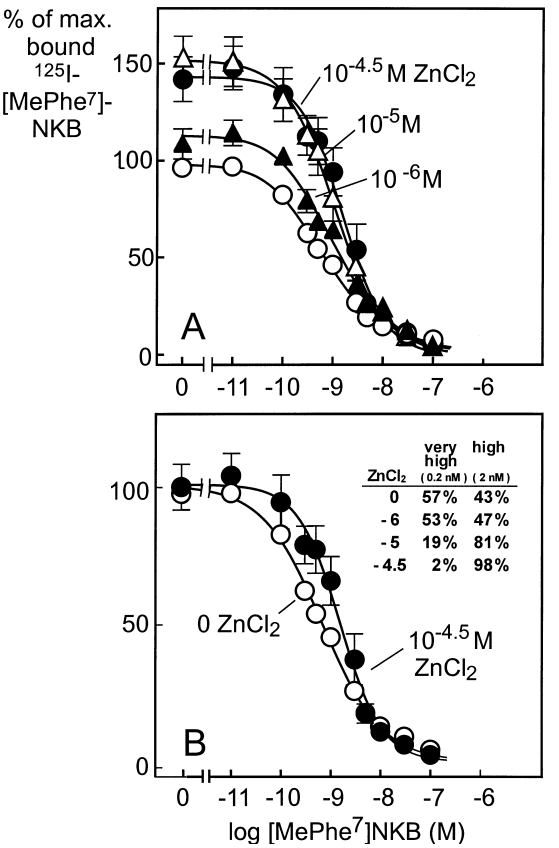


Fig. 3. Competition binding curves for [MePhe^7]NKB in the wild-type NK_{3A} receptor at various $ZnCl_2$ concentrations. Panel A: Binding of ^{125}I-[MePhe^7]NKB expressed as % of maximally bound radioligand in the absence of $ZnCl_2$ (○ – ○) and in the presence of 10^{-6} M (▲ – ▲), 10^{-5} M (△ – △), and $10^{-4.5}$ M (● – ●) $ZnCl_2$. Panel B: Binding of ^{125}I-[MePhe^7]NKB expressed as % of maximally bound radioactive ligand in the presence of the indicated $ZnCl_2$ concentrations. The summarized binding curve ($N = 24$) for [MePhe^7]NKB in the absence of $ZnCl_2$ had a Hill coefficient of −0.7 and analysis based on a two-component binding mode gave a very high (0.2 nM) and a high affinity (2.0 nM) sites. In the inserted table the relative distribution of these two affinity sites in the absence and presence of $ZnCl_2$ is shown. The summarized binding curves for [MePhe^7]NKB in the presence of $ZnCl_2$ 10^{-5} and $10^{-4.5}$ M were analyzed based on a two-component binding mode where the affinity of the very high affinity site was kept constant at 0.2 nM.

The effect of Zn^{2+} binding to the NK_{3A} receptor mimics the effect of mutating one of the two histidyl residues in TM-V at least to a certain degree. Thus, both Zn^{2+} binding and mutation of HisV:01 (His^{244}) eliminate the very high affinity binding of [MePhe^7]NKB. The location of HisV:01 (His^{244}) at the exterior part of the main ligand binding crevice suggests that the imidazole side chain of HisV:01 (His^{244}) could be more or less directly involved in binding the peptide ligand. Importantly, however, the very high affinity binding mode for NKB does not appear to be necessary for activation of the NK_{3A} receptor since normal stimulation of phosphatidylino-

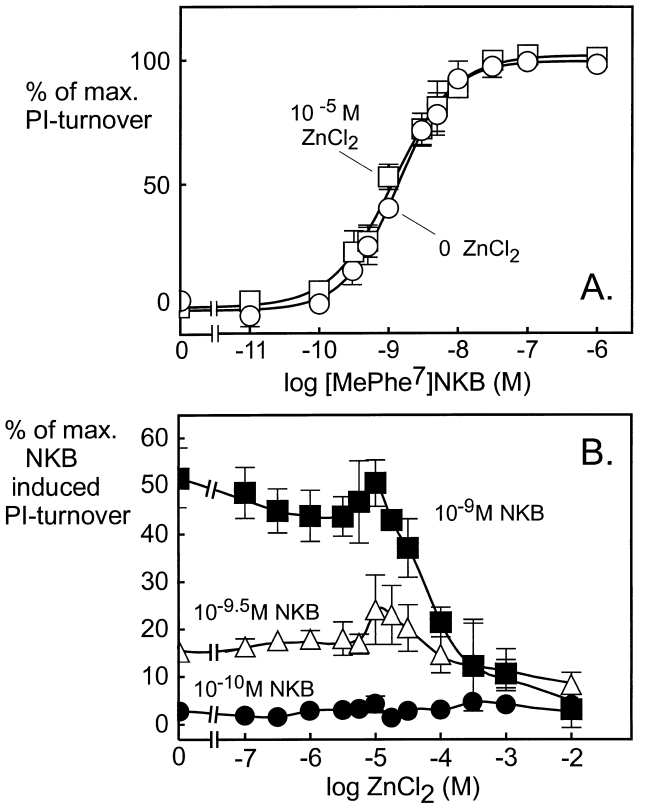


Fig. 4. The effect of $ZnCl_2$ on the signaling of the NK_{3A} receptor. Panel A: Dose-response curves for stimulation of phosphatidylinositol turnover in response to NKB in the absence (○ – ○) and in the presence (□ – □) of $ZnCl_2$, 10^{-5} M. Panel B: Dose-response curves for stimulation of phosphatidylinositol turnover in response to $ZnCl_2$ in the wild-type NK_{3A} receptor in the presence of NKB at 10^{-10} M (● – ●), $10^{-9.5}$ M (△ – △), and 10^{-9} M (■ – ■).

sitol turnover is observed in the presence of 10^{-5} M $ZnCl_2$ (Fig. 4A). Under these circumstances, the very high affinity binding mode is basically eliminated (insert in Fig. 3B).

4.3. How does Zn^{2+} enhance agonist binding?

Since Zn^{2+} eliminates one binding mode of the peptide agonist, it could have been anticipated that the amount of bound radioactive peptide would decrease. On the contrary, an increase in bound peptide is observed. Thus it must be concluded that the zinc ions stabilize the nanomolar affinity agonist binding state of the NK_{3A} receptor, and that it consequently is able to pull more receptors into this conformation. In this context it is important to recall that in 7TM receptor systems the B_{max} values calculated on the basis of binding experiments performed with agonists are often lower than those based on binding with radioactive antagonists [23]. This at least indicates that there is a potential to increase the binding capacity for agonists. The actual conformational change which is stabilized or induced by Zn^{2+} is obviously today unclear. However, the binding of the zinc ion between HisV:01 and HisV:05 will very likely stabilize the helical structure of the extracellular end of TM-V since these two residues are located one helical pitch apart in the presumed α-helical structure of the transmembrane segment. Cysteine availability scanning experiments have indicated that the helical structure in this part of 7TM receptors can be rather unstable [24].

4.4. Is the effect of Zn^{2+} of physiological importance?

Zinc is rather abundant in the brain [12,13], where the NK_{3A} receptors are expressed. Importantly, a number of specific metal-ion transporters ensure that zinc ions are stored in synaptic vesicles, especially in glutaminergic neurons [13]. Zinc ions have been described to modulate the glutaminergic transmission both as positive and negative modulators. Thus, physiological concentrations of Zn^{2+} inhibit the NMDA receptor function and potentiate the non-NMDA receptors [25–27]. The concentration of Zn^{2+} in the synaptic cleft has been estimated to reach more than 10^{-4} M during stimulation [14]. If this is correct, then the agonist enhancing phenomenon described for Zn^{2+} in the present study for the NK_{3A} receptor could very well be of physiological importance. It should be noted that the two histidyl residues which constitute the metal-ion site in the NK_{3A} receptor are conserved in the NK_{3B} receptor (Fig. 1) [4]; it is therefore likely that Zn^{2+} will bind to this receptor with single digit μM affinity also.

Acknowledgements: Tina Jakobsen, Lisbet Elbak and Dorte Frederiksen are thanked for expert technical assistance. The study was supported by grants from the Danish Medical Research Council, the Carlsberg Foundation and the Biotechnology Research Unit for Molecular Recognition.

References

[1] Regoli, D., Boudon, A. and Fauchere, J.L. (1994) Pharmacol. Rev. 46, 551–599.
[2] Maggi, C.A. (1995) Gen. Pharmacol. 26, 911–944.
[3] Maggi, C.A. and Schwartz, T.W. (1997) Trends Pharmacol. Sci. 18, 351–356.
[4] Krause, J.E., Staveteig, P.T., Mentzer, J.N., Schmidt, S.K., Tucker, J.B., Brodbeck, R.M., Bu, J.-Y. and Karpitskiy, V.V. (1997) Proc. Natl. Acad. Sci. USA 94, 310–315.
[5] Xie, G.-X., Miyajima, A. and Goldstein, A. (1992) Proc. Natl. Acad. Sci. USA 89, 4124–4128.
[6] Elling, C.E., Nielsen, S.M. and Schwartz, T.W. (1995) Nature 374, 74–77.
[7] Thirstrup, K., Elling, C.E., Hjorth, S.A. and Schwartz, T.W. (1996) J. Biol. Chem. 271, 7875–7878.
[8] Elling, C.E. and Schwartz, T.W. (1996) EMBO J. 15, 6213–6219.
[9] Sheikh, S.P., Zvyaga, T.A., Lichtarge, O., Sakmar, T.P. and Bourne, H.R. (1996) Nature 383, 347–350.
[10] Elling, C.E., Thirstrup, K., Nielsen, S.M., Hjorth, S.A. and Schwartz, T.W. (1997) Fold. Design 2, S76–S80.
[11] Chakrabarti, P. (1990) Protein Eng. 4, 57–63.
[12] Frederickson, C.J. (1989) Int. Rev. Neurobiol. 31, 145–238.
[13] Huang, E.P. (1997) Proc. Natl. Acad. Sci. USA 94, 13386–13387.
[14] Assaf, S.Y. and Chung, S.-H. (1998) Nature 308, 734–736.
[15] Emonds-Alt, X., Bichon, D., Ducoux, J.P., Heaulme, M., Miloux, B., Poncelet, M., Proietto, V., Broeck, D.V., Vilain, P., Neliat, G., Soubrie, P., Fur, G.L. and Breliere, J.C. (1995) Life Sci. 56, PL27–PL32.
[16] Huang, R.C., Cheung, A.H., Mazina, K.E., Strader, C.D. and Fong, T.M. (1992) Biochem. Biophys. Res. Commun. 184, 966–972.
[17] Johansen, T.E., Schüller, M.S., Tolstroy, S. and Schwartz, T.W. (1990) FEBS Lett. 267, 289–294.
[18] Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Gene 77, 61–68.
[19] Berridge, M.J., Dawson, M.C., Downes, C.P., Heslop, J.P. and Irvin, R.F. (1983) Biochem. J. 212, 473–482.
[20] Cheng, Y. and Prusoff, W. (1973) Biochem. Pharmacol. 22, 3099–3108.
[21] Sipos, L. and Von Heine, G. (1993) Eur. J. Biochem. 213, 1333–1340.
[22] Cronet, P., Sander, C. and Vriend, G. (1993) Protein Eng. 6, 59–64.

[23] Rosenkilde, M.M., Cahir, M., Gether, U., Hjorth, S.A. and Schwartz, T.W. (1994) J. Biol. Chem. 269, 28160–28164.
[24] Javitch, J.A., Fu, D. and Chen, J. (1995) Biochemistry 34, 16433–16439.
[25] Rassendren, F.-A., Lory, P., Pin, J.-P. and Natgeot, J. (1990) Neuron 4, 733–740.
[26] Hollmann, M., Boulter, J., Maron, C., Beasley, L., Sullivan, J., Pecht, G. and Heinemann, S. (1993) Neuron 10, 943–954.
[27] Chen, N., Moshaver, A. and Raymond, L.A. (1997) Mol. Pharmacol. 51, 1015–1023.
[28] Schertler, G.F.X., Villa, C. and Henderson, R. (1993) Nature 362, 770–772.
[29] Baldwin, J.M. (1993) EMBO J. 12, 1693–1703.