Gly^{166} in the NK_1 receptor regulates tachykinin selectivity and receptor conformation

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Abstract We have studied the pharmacological properties of genetically engineered human NK_1 tachykinin receptors in which residues at the extracellular surface of the fourth transmembranal domain were substituted with the corresponding amino acids from the NK_2 receptor. We show that substitution of G166C:Y167F in the human NK_1 receptor induces high affinity binding of a group of tachykinin ligands, known as 'septides' (i.e. neurokinin A, neurokinin B, [$pGlu^6$,Pro^9]-substance P^{6-11} and substance P-methylester). In contrast, binding of substance P and non-peptide antagonists is unaffected by these mutations. This effect parallels that found on the rat receptor and is therefore species specific. Second, we demonstrate that mutation of Gly^{166} to Cys alone is both necessary and sufficient to create this pan-reactive tachykinin receptor, whereas replacement of Tyr^{167} by Phe has no detectable effect on the pharmacological properties of the receptor. Furthermore, analysis of the effect of *N*-ethylmaleimide and dithiothreitol on binding of radiolabelled substance P documents differences in the mode in which this ligand interacts with wild-type and mutant receptors and supports the existence of a mutational induced change in the conformational status of the NK_1 receptor.

Key words: NK_1 receptor; Substance P; Neurokinin A; Selectivity; Affinity; Receptor conformation

1. Introduction

Mammalian tachykinins (SP, NKA and NKB) exert their physiological effects through activation of three distinct subtypes of receptors originally termed the substance P (NK_1), the substance K (NK_2) and the neurokinin B (NK_3) receptors [1,2]. Each peptide hormone is considered the preferential endogenous ligand for the respective receptor and this selectivity is believed to arise from subtype specific interactions between divergent residues in the N-terminal part of the peptides and variant elements in the receptor molecules. The preserved C-terminus of tachykinins constitutes a prerequisite for binding to the receptor, and its possible interaction with common epitopes in the NK_1 receptors has been proposed as the molecular basis underlying the significant crossreactivity observed within this system [3,4].

Various lines of evidence, however, indicate that this paradigm is too simplified. First, it is possible to build affinities and selectivities comparable or even greater than those of natural tachykinins into the fundamental C-terminal core element of these peptide ligands [5]. Second, receptor specificity for peptidic ligands may be essentially obliterated by substitutions of single or multiple residues that presumably change the conformation of the receptor molecule rather than affecting direct ligand receptor interactions [6–8].

In analogy to selectivity for peptide ligands, a number of non-peptidic antagonists display a dramatic species specificity towards various NK_1 receptors. A few key residues that likely do not form part of the docking site, have been shown to regulate the ability of the receptor molecule to bind these ligands through an indirect effect on receptor conformation [9–12].

Thus, control of the receptor conformation may constitute the principal mechanism that regulates affinity and selectivity in the tachykinin system. The interesting question is whether NK_1 binding is subject to a single central regulatory mechanism that governs overall affinity and selectivity or rather that multiple control systems exist. If so, do such mechanisms, controlled by physically separate sites in the receptor, operate in functional independent or interrelated modes?

We have recently identified a site in the rat NK_1 receptor which is important for the recognition and selectivity of tachykinin peptides [6]. Mutation of this epitope abolishes selectivity and results in receptor that binds SP, heterologous tachykinins as well as modified SP analogues with high affinity [6,13].

To investigate the mechanisms underlying the recognition and discrimination in the tachykinin system both multiple and single substitutions of residues at the interface between the fourth transmembranal domain and the second extracellular loop on the NK_1 receptor were analyzed. We show that these mutations affect selectivity towards peptidic ligands without changing specificity for non-peptides, and demonstrate that this effect is independent of the species related differences in structure among NK_1 receptors. Furthermore we identify the single amino acid substitution affecting receptor selectivity and present evidence which supports the notion that the mutation does induce a change in receptor conformation which may be detected also for binding of the otherwise mutationally inert SP ligand.

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Abbreviations: NK_1, neurokinin-1 receptor; SP, substance P; NKA, neorokinin A or substance K; NKB, neorokinin B; G-protein, guanyl nucleotide binding regulatory protein; PI, polyphosphoinositide; cAMP, cyclic adenylate mono-phosphate; [^{125}I]-SP, monoiodinated [^{125}I]-Bolton-Hunter substance P; [^{125}I]-NKA, 2-^{125}I-iodohistidyl neurokinin A; septide, [$pGlu^6$,Pro^9]-substance P^{6-11}, ; NEM, *N*-ethylmaleimide; DTT, dithiothreitol

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2. Materials and methods

2.1. Materials

Monoiodinated ^{125}I-labelled Bolton-Hunter substance P ([^{125}I]-SP), 2000 Ci/mmol, and 2-^{125}I-iodohistidyl neurokinin A ([^{125}I]-NKA), 2000 Ci/mmol, were purchased from Amersham. All peptides were obtained from Sigma or Novabiochem. SR140333 [14] was kindly provided by Dr. Emond-Alt, Senofi Recherche. FK888 [15] was synthesized in the Department of Chemistry, Menarini Ricerche Sud.

2.2. Construction of mutant receptors

The cDNA encoding the human NK_1 receptor was kindly provided by Dr. Schwartz, University of Copenhagen. The cDNA was subcloned into the replicative form of M13mp19 DNA, and site-directed mutagenesis performed using the *oligonucleotide-directed in vitro mutagenesis system* from Amersham according to the instructions of the supplier. The oligonucleotides used were: the C166G mutation 5′-ggccttccccagtgctactactcaacc-3′; the Y167F mutation 5′-ccttcccccaggcttctactcaaccac-3′; the G166C:Y167F mutation 5′-ggccttcccccagtgcttctactcaaccac-3′. They were purchased from Primm. The nucleotide sequence was verified by sequencing using the Sequenase sequencing system from U.S. Biochemical Corp. The cDNA was subsequently subcloned into the mammalian expression vector, pcDNA3 (Invitrogen).

2.3. Cell culture and transfection

COS1 cells were grown in DMEM supplemented with 10% fetal calf serum, and CHO cells were grown in DMEM:F12 (1:1) supplemented with 10% fetal calf serum. COS1 cells were transiently transfected as previously described [6]. Stable expression in CHO cells was obtained by transfection of cDNA encoding wild-type or mutant NK_1 receptors using Lipofectamine (Gibco, BRL.) and stable expressing clones were identified following selection with 400 μg/ml G418 (Geneticin, Gibco, BRL).

2.4. Binding assay

Transiently or stable expressing cells were plated in 24 well plates and wild-type and mutant receptors characterized using [^{125}I]-SP and [^{125}I]-NKA as radioligand. Cells were incubated for 3 h at 4°C (until equilibrium) in presence of 10 pM radiolabelled substance P and increasing concentrations of cold competing ligands in 0.5 ml of 50 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM $MnCl_2$, 0.1% BSA supplemented with bacitracin (100 μg/ml), leupeptin (5 μg/ml) and chymostatin (10 μg/ml) to prevent degradation. The incubation medium was removed and the cells washed with ice-cold phosphate-buffered saline. The monolayer was lysed in 0.5 ml of 4% SDS, 0.4 M NaOH and counted. All determinations were done in duplicate.

2.5. Data analysis and statistics

Equilibrium binding parameters were computed by the analysis of the binding isotherms according to the mass action law using non-linear least squares fitting procedures with the computer program LIGAND (as described in [16]). All binding isotherms of a given experiment were fitted simultaneously; hence the data set of the homologous binding of radiolabelled and cold substance P as well as that of the heterologous competition between radiolabelled substance P and a given cold ligand were concurrently loaded into the computer program and used in the fitting routine.

2.6. Effect of NEM and DTT treatment

CHO clones stably expressing wild-type or G166C mutant receptor were seeded in 24 well plates (2×10^5 cells/well) and assayed after 24–48 h essentially as described [17]. Briefly, the cells were incubated in HEPES buffer (20 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM KCl, 2.2 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mg/ml glucose, 0.6 mg/ml BSA) containing NEM or DTT at indicated concentrations for 1 h at 4°C or 37°C, respectively. Cells were subsequently washed and incubated in radioligand buffer (10 pM [^{125}I]-SP or [^{125}I]-NKA in 0.5 ml HEPES buffer) at 4°C for 3 h to reach equilibrium. The cells were washed with PBS, lysed and counted as above. Specific binding was determined in the presence of excess cold SP.

3. Results

3.1. The G166C mutation of the human NK_1 receptor induces a selective increase of affinity for a class of NKA-like tachykinin ligands

As observed previously for the rat receptor [6,13], the double residue substitution of the human receptor greatly enhanced the affinity of the heterologous tachykinins, NKA and NKB, while it did not affect the binding of SP, and thus, resulted in a non-selective high affinity tachykinin receptor (Table 1). N- and C-terminally modified SP analogues, such as septide and SP-methylester, also displayed a significant increase in affinity for the mutant receptor, whereas the mutant affinities of the NK_3 agonist, [MePhe^7]-NKB and of the NK_1 receptor antagonists, FK888 and SR140333, were similar to those for wild-type receptors (Table 1). This double residue mutation, therefore, identifies two classes of neurokinin ligands: the mutation sensitive NKA-like agonists and the mutation insensitive SP-like ligands, which include agonists and antagonists. To identify which of the two residues was responsible for this class specific change of affinity we prepared and studied the corresponding single site mutations, Y167F and G166C of the NK_1 receptor: The Tyr to Phe transmutation of residue 167 resulted in little or no change in affinity for the set of studied ligands compared to wild-type NK_1 receptor (Table 1). In contrast, replacement of Gly by Cys in position 166, caused a change of binding affinities that was very similar to that observed for the G166C:Y167F dou-

Table 1
Dissociation constants of tachykinin ligands for binding to wild-type, G166C, Y167F and G166C:Y167F NK_1 receptors

Ligands	Dissociation constants (K_d)								Ratio (wild-type/mutants)		
	Wild-type (nM)	(*n*)	G166C (nM)	(*n*)	Y167F (nM)	(*n*)	G166C:Y167F (nM)	(*n*)	G166C	Y167F	G166C:Y167F
SP	0.53	(9)	0.41	(5)	0.24	(4)	0.40	(3)	1.3	2.7	1.4
NKA	120	(4)	6.5	(5)	54	(2)	3.8	(2)	18	2.2	32
NKB	310	(4)	5.1	(4)	240	(2)	3.4	(2)	61	1.3	91
SP-methylester	20	(8)	1.3	(2)	45	(2)	1.1	(2)	15	4.4	18
Septide	250	(7)	4.5	(3)	75	(2)	20	(2)	56	3.3	13
[Sar^9]-SP	5.1	(2)	3.8	(2)	4.7	(2)	4.2	(2)	1.3	1.1	1.2
[MePhe^7]-NKB	420	(2)	270	(2)	1200	(2)	360	(2)	1.6	0.35	1.2
FK888	1.0	(3)	0.23	(2)	0.55	(2)	0.24	(2)	4.3	1.7	4.0
SR140333	4.8	(3)	3.8	(2)	6.2	(2)	1.8	(2)	1.3	0.77	2.7

Binding isotherms for tachykinins and non-peptide ligands in competition for [^{125}I]-SP were generated as described in Section 2. Data were analyzed with the computer program Ligand [15] to calculate binding affinities. Results are presented as nanomolar dissociation constants (K_d) and all reported data have a standard error of the mean lower than 25% which are not included in the table for clarity. The number of individual experiments (*n*) is indicated. The effect of the mutation is highlighted by taking the ratio between the affinities on wild-type the NK_1 receptor and each mutant receptor.

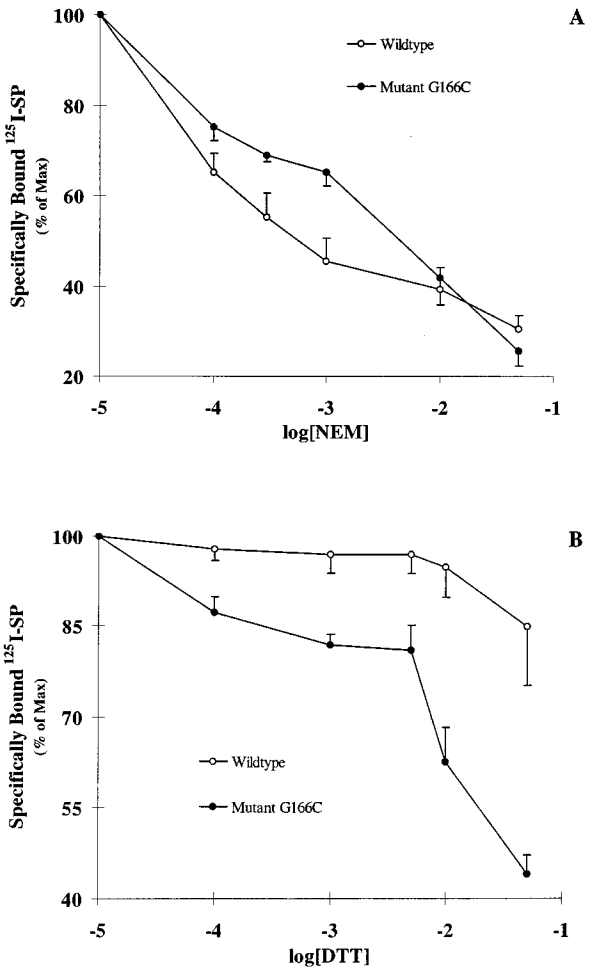

Fig. 1. Effect of NEM (panel A) and DTT (panel B) on binding of [^{125}I]-SP on wild-type (open circles) and mutant (closed circles) NK_1 receptor. Specifically bound radioligand is expressed in percent of bound tracer in absence of NEM or DTT, and is the mean of three similar experiments. Error bars reporting the standard error of the mean is given in each point. X-axis is given in logarithm to the molar (M) concentrations of NEM and DTT.

ble mutation. Thus, the glycine for cysteine substitution at position 166 in the NK_1 receptor is the necessary and sufficient change to endow the NK_1 receptor with enhanced affinity for NKA-like ligands.

3.2. *NEM and DTT differentially affects substance P binding to wild-type and mutant receptor*

It is known that the binding of SP to NK_1 receptor is sensitive to thiol reagents [17]. To further characterize the difference between wild-type and G166C mutant receptors, we examined the sensitivity of SP binding to wild-type and mutant receptors after exposure to increasing concentrations of either *N*-ethylmaleimide (NEM), or dithiothreitol (DTT).

In cells expressing wild-type NK_1 receptor the binding of [^{125}I]-SP was inhibited considerably more by NEM over a wide range of concentrations than in mutant expressing cells. Hence, as shown in Fig. 1A, 1 mM NEM inhibited 55% of SP binding on wild-type but only 35% on mutant NK_1 receptor. Fig. 1B shows that also the effect of DTT discriminates between binding of [^{125}I]-SP to wild-type and mutant receptors. However, these experiments document that the mutation renders SP binding to the receptor more sensitive to the action of DTT. The application of sulfhyldryl reagents, therefore, allows us to verify the mutational induced alteration in the receptor molecule.

4. Discussion

As shown previously, two-amino acid substitution in the extracellular surface of the fourth transmembranal helix of the rat NK_1 receptor selectively enhances the apparent binding affinity for a heterogeneous group of tachykinins [6,13], all of which share the ability to preferentially induce PI signaling rather than cAMP responses upon binding to NK_1 receptor [18]. The same mutation was found to potentiate selectively the ability of the NK_1 receptor to trigger PI signaling presumably by shifting its G protein preference, which suggested that the replacement of two residues had an influence on the overall conformation of the molecule rather than only affecting the interactions at the ligand binding site [13].

In this study we report three additional observations that are important to understand the role of this locus in the NK_1 receptor and the mechanism of the change in binding affinity.

The first relates to the significant differences in binding properties observed among NK_1 receptors from various species. It has been shown previously that numerous tachykinin ligands, in particular non-peptide antagonists, greatly discriminate between human and rat NK_1 receptors; a phenomenon that has been attributed to conformational differences between these receptors [9–12], and thus represents a naturally occurring system of conformational regulation of receptor selectivity. We find that the double mutation affects specificity towards tachykinins independently of the species origin of the receptor and, hence, its ability to discriminate among non-peptidic ligands, which suggests that affinity and selectivity in the tachykinin system are regulated through multiple indirect and non-interrelated mechanisms that control conformation of the receptor molecule.

The second point is the comparison of the binding characteristics of the double mutation and the two single mutations performed here in the human NK_1 receptor. As shown here, the ability to induce a class specific increase of affinity for NKA-like tachykinins is entirely due the replacement of Gly^{166} by Cys, while mutation of the adjacent residue in position 167 produces no detectable change. This indicates that Cys^{166} plays a primary role in the conformational change that results in enhanced affinity for the class of NKA-like ligands. Additional substitutions with other residues in this position will be necessary to clarify whether the introduction of a thiol function or other physicochemical properties of the replaced side-chain constitute the fundamental perturbation responsible for the phenomenon.

It is important to note that a Gly by Cys replacement in position 166 occurs naturally in both the NK_2 and NK_3 receptor sequences and represents a non-conservative substitution with respect to the NK_1 receptor sequence. It can be hypothesized that this spontaneous mutation plays a potential role in the divergent ligand binding characteristics that the three tachykinin receptor subtypes have acquired in the course of evolution. The fact that the introduction of this single residue is sufficient to enhance the affinity in the NK_1 receptor for heterologous tachykinins to the point that the apparent binding selectivity for SP is largely abolished, strongly support this hypothesis. Since a similar class specific enhancement of

affinity have been reported following an Ala substitution of Tyr^{216} [7] or transplantation of a NK_3 segment [8] in NK_1 receptors, it will be important to attempt to map such key regulatory elements in the receptor and study their interrelatedness by analyzing genetically engineered receptors bearing multiple mutations in these residues. Another important implication of the finding that the mutation only affects one specific class of tachykinin ligands, is that these NKA-like agonists bind to the NK_1 receptor in a fundamentally different manner than SP.

A third finding of this study is that the mutational effect on ligand binding to NK_1 receptors and the perturbation induced by the G166C mutation can be easily underscored and independently validated by chemical modification of the thiol groups in the NK_1 receptor. It was shown previously [17] that alkylation of NK_1 receptor by progressively increasing concentrations of NEM produces a complex pattern of inhibition of ligand binding, which probably reflects the contributions of several cysteine residues in maintaining the ligand binding conformation of the receptor. Since the individual sensitivity to alkylation of thiol residues in the protein depends on the microenvironment, which, in turn, can be altered by changes of conformations, we exploited NEM-mediated disruption of the binding interactions as a rough measurement of the effect of the mutation on the overall conformational stability of the NK_1 receptor. The data presented here indicate that the G166C mutation produces a clear cut shift in sensitivity to NEM even when the binding is monitored by radiolabelled SP, i.e. a ligand whose binding affinity is not altered by the mutation. This supports the idea that the mutation induces a conformational change of the receptor regardless of whether this can also be detected as a change of apparent binding affinity by some ligands and not by others. We also show here that mutant and wild-type receptors exhibit an opposite pattern of sensitivity to the action of the reducing agent DTT, indicating that both the reactivity of thiol groups and the resistance of disulfide bridges to reduction have been altered as a result of the conformational change promoted by the point mutation. The fact that the introduction of a single cysteine residue in the transmembrane region of the receptor can produce a global shift in the pattern of sensitivity to chemical modification of thiol groups is intriguing. We may speculate the introduction of Cys^{166} in the NK_1 receptor can alter the orientation of several thiol residues and even affect the configuration of possible disulfide bridges. Systematic mutagenesis of additional cysteine residues and the assessment of their role in the binding of both SP-like and NKA-like ligands will be necessary to evaluate this hypothesis.

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