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Point mutation increases a form of the NK_1 receptor with high affinity for neurokinin A and B and septide

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1 The binding modalities of substance P and neurokinin A on the wild type and Gly^{166} to-Cys mutant NK_1 receptors expressed on CHO cells were investigated in homologous and heterologous binding experiments using both radiolabelled substance P and neurokinin A.

2 On the wild type NK_1 receptor NKA displaces radiolabelled substance P with very low apparent affinity, despite its high-affinity binding constant (determined in homologous binding experiments). The Gly^{166} to-Cys substitution in the NK_1 tachykinin receptor greatly enhances the apparent affinity of neurokinin A in competition for radiolabelled substance P, but it does not change the binding constant of neurokinin A. The mutation, thereby, eliminates the discrepancy between the low apparent affinity and the high binding constant of neurokinin A.

3 On the wild type receptor the binding capacity of neurokinin A is significantly smaller than that of substance P. In contrast, the two tachykinins bind to approximately the same number of sites on the mutant receptor.

4 Simultaneous mass action law analysis of binding data in which multiple radioligands were employed in parallel demonstrated that a one-site model was unable to accommodate all the experimental data, whereas a two-site model provided a dramatically better description.

5 These two receptor-sites display equally high affinity for substance P, while neurokinin A strongly discriminates between a high and a low affinity component. The binding affinities of neurokinin A are not affected by the mutation, which instead specifically alters the distribution between receptor sites in favour of a high affinity neurokinin A binding form.

6 The low apparent affinity and binding capacity of neurokinin A on the wild type receptor results from neurokinin A binding with high affinity only to a fraction of the sites labelled by substance P. The mutation increases the proportion of this site, and consequently enhances the apparent affinity and binding capacity of neurokinin A.

7 The binding modalities of septide-like ligands (i.e. neurokinin B, SP(6-11), SP-methyl ester) are affected similarly to neurokinin A and are better resolved into two sites. The mutation leaves the affinity of these ligands for the two receptor forms unchanged, but increases the fraction of high-affinity sites. On the other hand, the binding of non-peptide and peptide antagonists (SR140.333 and FK888) behaved similarly to substance P with a single high affinity site that is unaffected by the mutation.

8 These findings may suggest that the NK_1 receptor exists in two different forms with similar affinity for substance P and NK_1 antagonists, but with a high and a low affinity for neurokinin A and septide-like ligands. Hence, the Gly^{166} in the NK_1 receptor would seem to control the distribution between a pan-reactive form and a substance P-selective form of the receptor.

Keywords: NK1 receptor; point mutation; high-affinity tachykinin binding; law of mass action; multiple radioligands; two-site analysis; multiple binding sites; neurokinins; septide

Introduction

Four mammalian tachykinin receptors have been identified that are activated by three distinct peptides, SP, NKA and NKB (Nakanishi, 1991; Maggi, 1995; Krause *et al*., 1997). The three peptides displayed distinct orders of potency for the receptors when assayed in heterologous binding experiments, SP appearing as the preferred ligand for NK_1.

The ligands share a conserved carboxy-terminal sequence ($FXGLM\text{-}NH_2$) that is believed to be the necessary element required to bind to tachykinin receptors. The amino-terminal part is variable among the three peptides and was initially believed to establish subtype specific interactions with divergent residues in the receptor, thus constituting the molecular basis underlying the apparent high selectivity in this system (Nakanishi, 1991; Maggi, 1995).

However, the selectivity of tachykinins for the receptors is associated with ambiguity as recently reviewed by Schwartz & Maggi (1997). Initially, studies on isolated organs indicated that NKA, NKB and truncated and constrained tachykinin peptides, SP(6–11) and septide, elicit functional responses in NK_1 receptor preparations with potencies far greater than their apparent affinities (Chassaing *et al*., 1992; Petitet *et al*., 1992; Hall *et al*., 1994; Meini *et al*., 1994; Torrens *et al*., 1994). To explain these paradoxical findings the existence of a 'septide-sensitive' receptor was proposed (reviewed by Glowinski, 1995).

The high potency of NKA, septide and an entire class of functionally similar tachykinins (termed *septide-like* ligands) was confirmed on the cloned NK_1 receptor expressed in cell lines (Pradier *et al*., 1994; Sagan *et al*., 1996; Riitano *et al*., 1997). Contrary to substance P, the *septide-like* ligands were only potent activators of G_q, not of G_s. Still, these ligands did not display any significant binding to the receptor in

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heterologous competition for radiolabelled SP or non-peptide antagonists. These observations argued against the existence of a 'septide sensitive' receptor and led to the proposal that septide-like ligands interact with a site different from that of SP on the NK_1 receptor (Pradier *et al*., 1994).

Recently, the binding constants of several *septide-like* ligands were measured directly in homologous binding experiments, which revealed that they do in fact bind to the NK_1 receptor with high affinity in agreement with their high potency to stimulate receptor mediated G_q activation. (Hastrup & Schwartz, 1996). Schwartz & Maggi (1997) interpreted these findings as an indication that the NK_1 receptor might have two different conformers, which bind SP equally well but interact distinctly with *septide-like* ligands. According to this interpretation it is not necessary to speculate the existence of a septide-receptor or a septide-site on the NK_1 receptor.

We have recently shown that a site around Gly^{166} at the extracellular surface of the fourth transmembranal domain in the NK_1 receptors is important for the selectivity of the receptor for tachykinins (Werge, 1994; Ciucci *et al*., 1997; Riitano *et al*., 1997). Mutation of this epitope enhances the apparent affinity of NK_1 for *septide-like* ligands measured in heterologous binding experiments. Since this class of tachykinins potently activates G_q – but not G_s – it is particularly interesting that the mutation also shifts G-protein selectivity of the receptor in favour of G_q (Riitano *et al*., 1997). Hence, the peculiar and complicated pharmacological properties of NK_1 seem to be dependent on the chemical nature of the residue found in this receptor site.

To investigate the mechanisms underlying the pharmacological properties of the tachykinin system, binding of radiolabelled substance P and neurokinin A to the wild type and the (Gly^{166} to Cys)-mutant NK_1 receptors was analysed in homologous and heterologous binding experiments. Also, the displacement of these tachykinin tracers by septide, septide-like ligands and NK_1 receptor antagonists was assessed. Despite that both radioligands are full agonists on the NK_1 receptor, they yielded significantly different binding capacities and contradictory results as to the effect of the mutation. We show by computer analysis that a two-site model is able to describe this very large set of binding data consistently. In this paradigm the mutation is seen to affect the distribution between the two receptor sites but not the ligand affinities of the receptor.

Methods

Materials

Monoiodinated ^{125}I-labelled Bolton-Hunter substance P ([^{125}I]-SP), 2000 Ci mmol^{-1}, and 2-^{125}I-iodohistidyl neurokinin A ([^{125}I]-NKA), 2000 Ci mmol^{-1}), were purchased from Amersham. All peptides were obtained from Sigma or Novabiochem. SR140333 (Emonds-Alt *et al*., 1993) was kindly provided by Dr Emonds-Alt, Senofi Recherche. FK888 (Fujii *et al*., 1992) was synthesized in the Department of Chemistry, Menarini Ricerche.

Cell culture

CHO cells were grown in DMEM:F12 (1 : 1) supplemented with 10% foetal calf serum. Stable expression of wild type and mutant receptors in CHO cells was obtained as described previously (Riitano *et al*., 1997).

Binding assay

Stable expressing cells were plated in 24 well plates (5×10^5 cells/well) and wild type and mutant receptors characterized using [^{125}I]-SP and [^{125}I]-NKA as radioligand. Cells were incubated until equilibrium (preliminary experiments, not shown) for 3 h at 4°C in presence of 10 pM radioligand 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^{-1}), leupeptin (5 μg ml^{-1}) and chymostatin (10 μg ml^{-1}) to prevent degradation. (A very low concentration of tracer was used to improve the possibility to visualise multiple binding components, which are easily masked at high concentrations of radioligand). Removal of the incubation medium and very rapid cell washing in ice-cold phosphate-buffered saline was performed simultaneously in all 24 wells using a cell harvester. The monolayer was lysed in 0.5 ml of 4%SDS, 0.4 M NaOH and counted. Each experimental curve contained six (Tables 1, 3 and 4) or 12 (Tables 2 and 5) data points as indicated and all determination were done in duplicate. Experiments including more radioligands were performed contemporary on sister plates using identical solutions of cold competitor.

All tables and figures present data, which are means of several independent experiments as indicated. Specific bound radioligand was less than 10% of total amount of radioligand in all experiments.

Data analysis and statistics

Binding isotherm for the experiments shown in Figures 1, 3 and 4 as well as in Tables 1, 3 and 4 were fitted using the computer program ALLFIT (DeLean *et al*., 1978) to determine IC_{50}, upper and lower asymptotes and the Hill coefficient.

Equilibrium binding experiments were performed in which multiple radiolabelled and cold ligands were used in parallel (Tables 2 and 5). Munson & Rodbard (1980) have extensively described the mathematical analysis of such complex binding systems. The computer program LIGAND allows sets of binding curves to be fitted simultaneously (to the law of mass action using non-linear least squares fitting procedures) according to models describing the interaction between multiple ligands and multiple independent binding sites. This approach consents sequential fitting of a given set of binding isotherms to increasingly complex models. The goodness of each fit (sum of squares) – and the statistical comparison of them (the *P*-value determined by the F ratio test using the 'extra sum of squares' principle) – indicates whether the experimental data is compatible with one or another physical arrangement.

The data presented in Table 2 are the results of simultaneous analysis of four binding isotherms, ^{125}I-SP vs SP, ^{125}I-SP vs NKA, ^{125}I-NKA vs SP and ^{125}I-NKA vs NKA, according to a two-site model. Table 5 reports the results of the analysis of a total of six binding isotherms: two binding isotherms of a cold ligand in competition for ^{125}I-SP and ^{125}I-NKA plus the four binding isotherms indicated above.

Results

As observed previously, mutations that substitute Gly^{166} in the NK_1 receptor induce a selective and significant increase in the low apparent affinity for *septide-like* ligands (e.g. NKA, NKB

and septide) in heterologous competition for radiolabelled SP, resulting in non-selective, high affinity tachykinin receptors (Werge, 1994; Riitano *et al*., 1997; Ciucci *et al*., 1997). This effect is particularly interesting in view of the recent finding that NKA and septide in homologous binding experiments are high affinity ligands also on the wild type NK_1 receptor (Hastrup & Schwartz, 1996). The identified receptor site is evidently involved in controlling the complicated interactions relating to ligand binding and selectivity.

To investigate these mechanisms we characterized the binding properties of the wild type receptor and single residue mutant, G166C employing two different radioligands; first, the classical NK_1 tracer, [^{125}I]-SP, and second, [^{125}I]-NKA that belongs to the class of *septide-like* ligands.

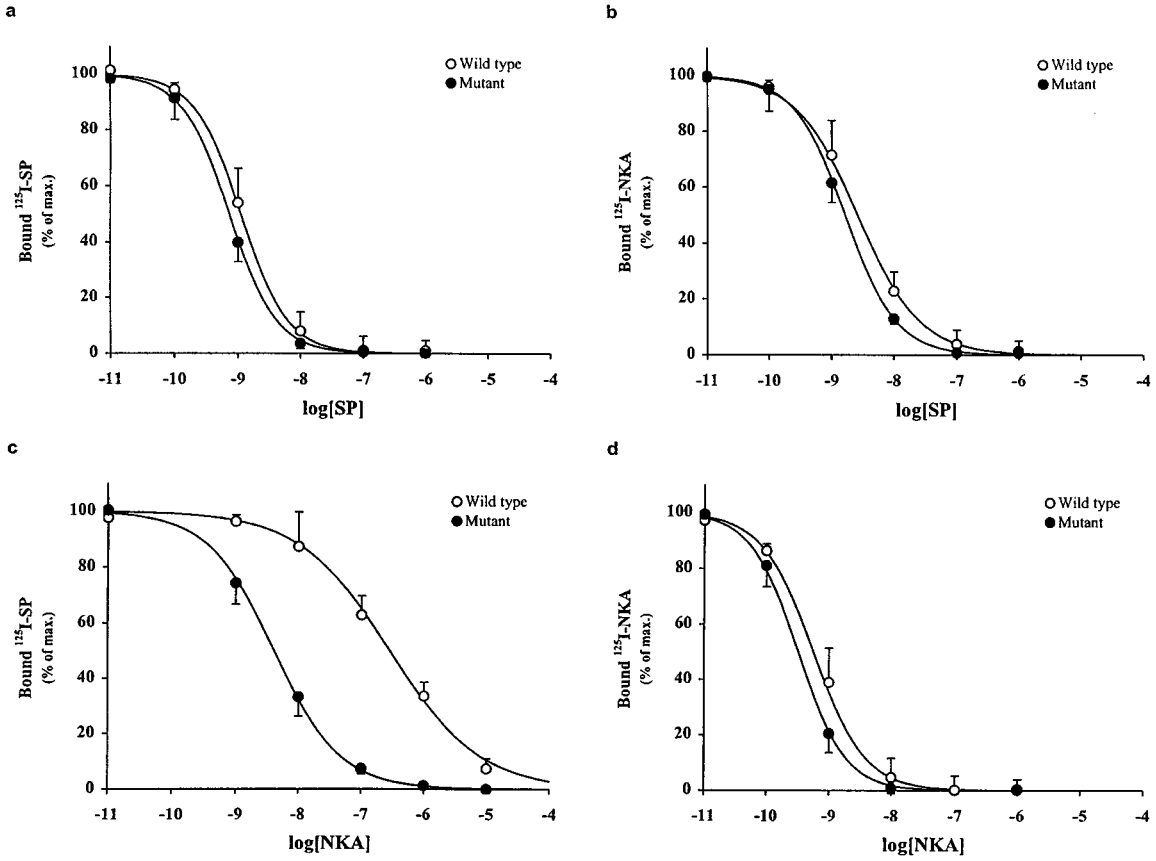

Figure 1 Binding isotherms of SP and NKA in competition for [^{125}I]-SP and [^{125}I]-NKA on the wild type and mutant (G166C) NK_1 receptor. The ability of SP (a and b) and NKA (c and d) to compete with [^{125}I]-SP (a and c) and [^{125}I]-NKA (b and d) for binding to wild type (○) and mutant (●) NK_1 receptor was determined. The data are expressed as percent of radioligand specifically bound to the receptor in absence of cold competitor. The curve and data points are means of three different experiments performed in parallel. An error bar for each point indicates the standard deviation. The mutation induced a significant change in ligand affinity only in (c) ($P<0.001$).

Table 1 Inhibition constants of SP and NKA in competition with [^{125}I]-SP and [^{125}I]-NKA on wild type and mutant (G166C) NK_1 receptor

	Wild type				*Mutant*			
Competitor:	*SP*		*NKA*		*SP*		*NKA*	
Tracer:								
^{125}I-SP	1.1	(±0.11)	280	(±22)	0.72	(±0.12)	4.1	(±0.38)
	1.2	*(±0.069)*	*0.60*	*(±0.125)*	*1.1*	*(±0.13)*	*0.82*	*(±0.088)*
^{125}I-NKA	0.56	(±0.17)	2.6	(±0.58)	0.32	(±0.034)	1.6	(±0.38)
	1.1	*(±0.13)*	*0.89*	*(±0.22)*	*1.2*	*(±0.18)*	*1.1*	*(±0.12)*
Effect of tracer:								
(^{125}I-SP/^{125}I-NKA)	2.0		110		2.3		2.6	
	P<0.05		*P<0.001*		*P<0.05*		*P<0.01*	

The inhibition constants (IC_{50}) and the Hill coefficient (in italics) of SP and NKA in competition with [^{125}I]-SP and [^{125}I]-NKA on wild type and mutant (G166C) NK_1 receptor were determined using the computer program ALLFIT (DeLean *et al*., 1978). The results are given in nanomolar (nM) and are means of three independent experiments. The standard deviation is given in parenthesis. The discrepancy between the affinities determined in homologous and heterologous binding is presented and its statistical significance determined.

Wild type and G166C mutant receptors stably expressed at comparable receptor density (0.91 ($\pm$0.15) and 1.1 ($\pm$0.13) pmol mg^{-1} respectively) in CHO cells were used for these studies. SP and NKA were studied in competition for the binding of the homologous and the heterologous tracer.

We found that the mutation dramatically enhances the ability of NKA to displace radiolabelled SP (Figure 1c). In contrast, the mutation does not affect the binding properties of NKA in homologous binding experiments, in which NKA displays a high-affinity interaction to both wild type and mutant receptor (Figure 1d). Accordingly, on the mutant receptor there is no discrepancy between the affinities of NKA measured in heterologous competition for [^{125}I]-SP and homologous binding experiments (Table 1).

We did not observe any effect of the mutation on the interaction of SP neither in homologous (Figure 1a) nor heterologous (Figure 1b) in binding experiments (Table 1).

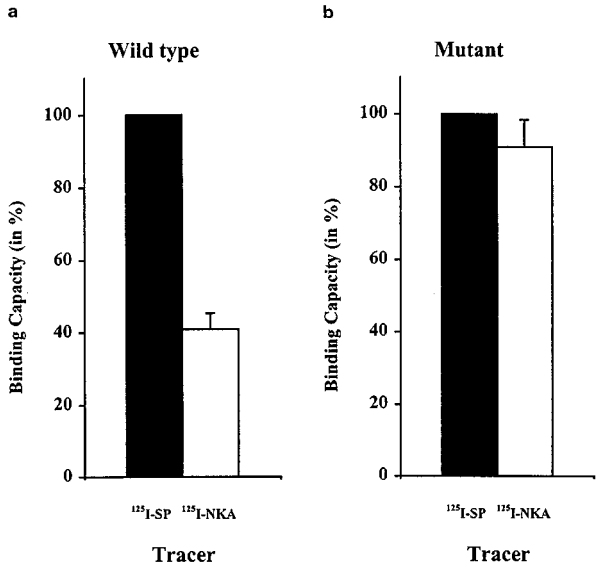

Figure 2 Binding capacity of substance P and neurokinin A on the wild type and mutant (G166C) NK_1 receptor. The binding capacity of radiolabelled substance P (black columns) and neurokinin A (white columns) on wild type (a) and mutant (b) was determined in three independent experiments. Data are expressed in per cent of the binding capacity of substance P on the examined receptor (wild type: 0.91$\pm$0.15 pmol/mg; mutant 1.1$\pm$0.13 pmol mg^{-1}). Error bars indicate the standard deviation.

Interestingly, on the wild type receptor the Hill coefficient of NKA in heterologous competition for [^{125}I]-SP is considerably smaller than unity, whereas those of the other binding isotherms are all close to unity (Table 1). However, on the mutant receptor no such difference is observed, as none of the four Hill coefficients is notably different from unity.

Mass action law analysis of the binding isotherms shown in Figure 1 revealed that the binding capacity of [^{125}I]-NKA is considerably smaller than that of [^{125}I]-SP on the wild type receptor (Figure 2a). No such difference is observed on the mutant receptor (Figure 2b)

Since the mutation affects the discrepancies in binding capacity and NKA affinity, we analysed receptor binding using two different radioligands to generate sets of four detailed competition binding isotherms in parallel experiments ([^{125}I]-SP and [^{125}I]-NKA versus both SP and NKA). To equally consider all four isotherms the binding data were analysed simultaneously according to the law of mass action using the computer program LIGAND; see Methods (Munson & Rodbard, 1980).

While a one-site model does not adequately accommodate the experimental data, a two-site model provides a dramatic improvement in the description of the data that statistically is highly significant ($P<0.0001$, wild type, and $P<0.001$, mutant; $n=7$). Hence, our analysis constitutes an objective statistical method with which to reject the hypothesis of a single receptor site.

According to this analysis NKA binding curves were resolved into a high and a low affinity component, while SP exhibited equal affinities for the two sites. Therefore, the discrepancy in affinity of NKA between homologous or heterologous binding experiments on the wild type receptor (Figure 1c and d) is due to the fact that a fraction of [^{125}I]-SP is bound to a site that has low affinity for NKA. Likewise, the lower binding capacity measured by [^{125}I]-NKA compared to [^{125}I]-SP on wild type expressing cells is due to the fact that in the homologous binding assay only the high affinity component for NKA can be detected. In fact, the fraction of total SP binding sites determined by radiolabelled NKA in homologous competition (41% = 0.37 pmol mg^{-1}) corresponds very closely to the fraction of high affinity sites determined by unlabelled NKA in heterologous competition for [^{125}I]-SP (31% = 0.28 pmol mg^{-1}; Table 2). In cells expressing mutant receptors, only the relative proportion of high and low affinity sites for NKA was significantly changed and the high affinity site became dominant (Table 2). The binding constants of SP and NKA were equal on the wild type

Table 2 Dissociation constants of SP and NKA for binding to wild type and mutant (G166C) NK_1 receptors

	(K_d, nM)			
	Wildtype		*Mutant*	
	K_d^H	K_d^L	K_d^H	K_d^L
Ligands:				
Sub. P	0.54 ($\pm$0.12)	0.54 ($\pm$0.12)	0.25 ($\pm$0.071)	0.25 ($\pm$0.071)
NKA	3.7 ($\pm$0.17)	860 ($\pm$120)	1.4 ($\pm$0.16)	1200 ($\pm$360)
B_{max} (%)	31 ($\pm$4)	69 ($\pm$4)	96 ($\pm$1)	4 ($\pm$1)

Four detailed binding isotherms for SP and NKA in competition for [^{125}I]-SP and [^{125}I]-NKA were generated in parallel and analysed simultaneously with the computer program Ligand (Munson & Rodbard, 1980) according to a two-site model and the dissociation constants (K_d) of SP and NKA determined. NKA binding was resolved into both a high affinity (K_d^H) and a low affinity (K_d^L) component while SP displayed equal affinities for these sites. The results are given as nanomolar (nM) and are means of seven independent experiments. The standard error of the mean is given in parenthesis. The relative contributions of the high and low affinity component of NKA binding are reported in % of the total B_{max} on the wild type and mutant receptors (0.9 and 1.1 pmol mg^{-1}, respectively).

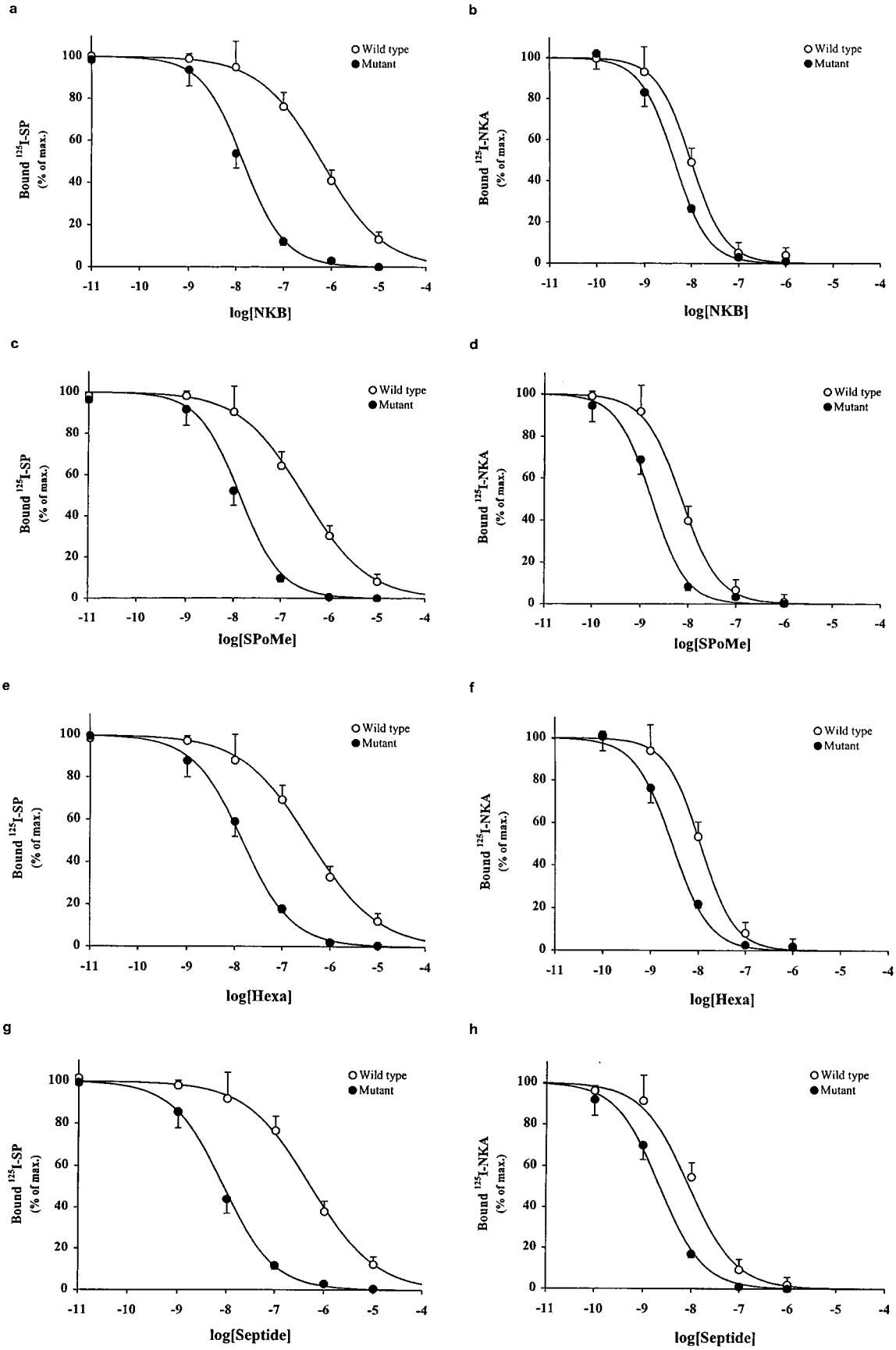

Figure 3 Binding isotherms of septide-like ligands in competition for [^{125}I]-SP and [^{125}I]-NKA on the wild type and mutant (G166C) NK_1 receptor. The ability of NKB (a and b), SP methyl-ester 'SPoMe' (c and d), SP(6-11) 'Hexa' (e and f) and septide (g and h) to compete with [^{125}I]-SP (a, c, e and g) and [^{125}I]-NKA (b, d, f and h) for binding to wild type (○) and mutant (●) NK_1 receptor was determined. The data are expressed as per cent of radioligand specifically bound to the receptor in absence of cold competitor. The curve and data points are means of three different experiments performed in parallel. An error bar for each point indicates the standard deviation. The mutation induced a significant change in ligand affinity in (a) and (c) ($P<0.01$), in (e) and (g) ($P<0.05$), and in (b), (d), (f) and (h) ($P<0.05$).

Table 3 Inhibition constants of *septide-like* ligands in competition with [^{125}I]-SP and [^{125}I]-NKA on wild type and mutant (G166C) NK_1 receptor

	Wild type					*Mutant*				
Competitor:	*NKB*	*SPoME*	*Hexa*	*Septide*	*NKB*	*SPoME*	*Hexa*	*Septide*		
Tracer:										
^{125}I-SP	620	(±220)	290	(±100)	370	(±53)	530	(±91)	14	(±3.0)	14	(±3.5)	14	(±3.1)	8.6	(±1.9)
	0.67	*(±0.23)*	*0.66*	*(±0.31)*	*0.61*	*(±0.076)*	*0.66*	*(±0.11)*	*0.97*	*(±0.32)*	*0.98*	*(±0.26)*	*0.82*	*(±0.17)*	*0.83*	*(±0.19)*
^{125}I-NKA	9.6	(±2.3)	7.2	(±1.7)	11	(±1.7)	8.9	(±2.6)	4.2	(±1.3)	1.7	(±0.26)	2.8	(±0.58)	2.2	(±0.41)
	1.2	*(±0.33)*	*1.1*	*(±0.42)*	*1.2*	*(±0.39)*	*0.89*	*(±0.33)*	*1.2*	*(±0.26)*	*1.2*	*(±0.40)*	*1.1*	*(±0.31)*	*0.96*	*(±0.33)*
Effect of tracer:																
(^{125}I-SP/^{125}I-NKA)	65		40		32		60		3.3		7.9		5.1		3.9	
	P<0.005		*P<0.005*		*P<0.001*		*P<0.001*		*P<0.01*		*P<0.01*		*P<0.01*		*P<0.01*	

The inhibition constants (IC_{50}) and the Hill coefficient (in italics) of *septide-like* ligands in competition with [^{125}I]-SP and [^{125}I]-NKA on wild type and mutant (G166C) NK_1 receptor were determined using the computer program ALLFIT (DeLean *et al*., 1978). The results are given nanomolar (nM) and are means of three independent experiments. The standard deviation is given in parenthesis. The discrepancy between the affinities determined in homologous and heterologues binding is presented and its statistical significance determined.

and mutant receptors. This suggests that the mutation enhances the proportion of the receptor form having high affinity for NKA, and consequently, the affinity of NKA in displacing [^{125}I]-SP increases and approaches that determined in homologous binding experiments using [^{125}I]-NKA. Hence, our analysis resolve the paradoxical findings of contradictory affinities of NKA and varying B_{max} determinations that associate with a single receptor site hypothesis in a manner that indicates the mechanism by which the mutation affects these pharmacological properties of the receptor.

We examined the binding profiles of additional tachykinin ligands in competition for [^{125}I]-SP or [^{125}I]-NKA. Figure 3 shows that among the examined binders, all *septide-like* ligands display dramatically higher affinity in competition for [^{125}I]-NKA than in competition for [^{125}I]-SP on the wild type receptor. Also, the Hill coefficients of *septide-like* ligands, as that of NKA, were significantly lower than unity in competition for radiolabelled SP (Table 3). The two NK_1 antagonists, FK888 and SR140333, competed with equal affinity for [^{125}I]-NKA and [^{125}I]-SP binding to the wild type receptor with Hill coefficients close to unity (Figure 4; Table 4).

On the mutant receptor septide-like ligands and tachykinin antagonists competed for [^{125}I]-NKA and [^{125}I]-SP binding with similar high affinity (Figures 3 and 4). Also, all ligands displayed Hill coefficients close to unity on this receptor (Tables 3 and 4). Thus, the mutation increases the ability of septide-like ligands to displace radiolabelled SP, but it leaves the potency of these ligands to compete for NKA binding unchanged.

Detailed binding isotherms of these ligands in competition for [^{125}I]-NKA and [^{125}I]-SP were determined in parallel experiments and analysed simultaneously with LIGAND. The binding isotherms of all *septide-like* ligands – similarly to NKA – displayed a high and a low affinity component in binding to the wild type NK_1 receptor (Table 5). Tachykinin antagonists were analysed and – in analogy to SP – shown to bind with equal affinity to both sites.

On the mutant receptor, the binding isotherms of *septide-like* ligands were resolved into a high and a low affinity site, and the affinity constants were shown to be equal to those determined on the wild type receptor (Table 5). The antagonist binding data were consistent with a single receptor site with affinities that were unchanged by the mutation.

Thus, the mutational induced increase in apparent affinity of septide-like ligands is not accounted for by a change in binding constant of these ligands but seems to result exclusively from the shift in the ratio between a high and low affinity form of the receptor.

Discussion

We have previously shown that mutations that replace Gly^{166} by Cys in the NK_1 receptor abolish the apparent selectivity towards tachykinins, shift G-protein coupling pattern of the receptor and change ligand sensitivity to chemical modification of the thiol-groups in the receptor molecule (Werge, 1994; Riitano *et al*., 1997; Ciucci *et al*., 1997). This implies that this site in the receptor plays an essential role in the regulation of the overall receptor conformation.

The analysis reported in this study have two principal implications on our understanding of the mechanisms that regulate the fundamental pharmacological properties of the NK_1 receptor.

The first point arises from the analysis of classic SP and septide-like ligands in competition for the binding sites labelled

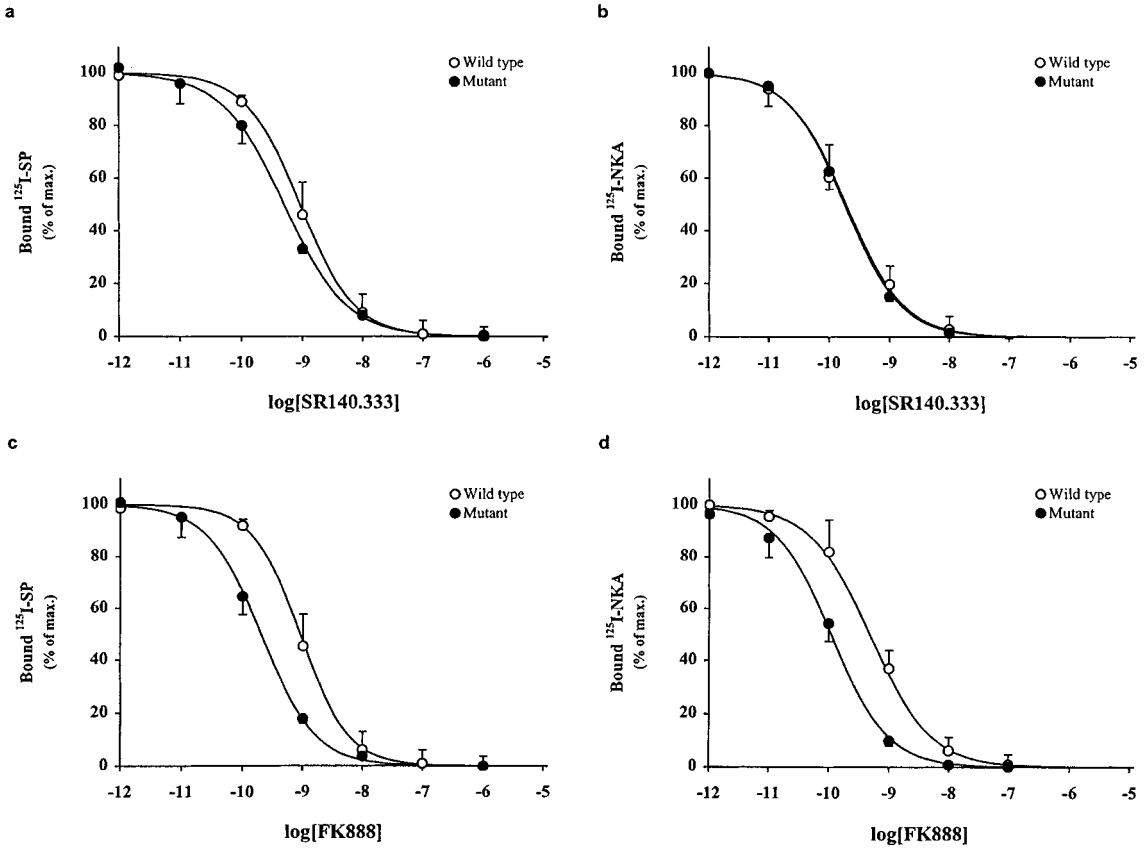

Figure 4 Binding isotherms of NK_1 antagonists in competition for [^{125}I]-SP and [^{125}I]-NKA on the wild type and mutant (G166C) NK_1 receptor. The ability of SR140.333 (a and b) and FK888 (c and d) to compete with [^{125}I]-SP (a and c) and [^{125}I]-NKA (b and d) for binding to wild type (○) and mutant (●) NK_1 receptor was determined. The data are expressed as percent of radioligand specifically bound to the receptor in absence of cold competitor. The curve and data points are means of three different experiments performed in parallel. An error bar for each point indicates the standard deviation. The mutation did not induce a significant change in ligand affinity for any of the two antagonists.

Table 4 Inhibition constants of NK_1 antagonists in competition with [^{125}I]-SP and [^{125}I]-NKA on wild type and mutant (G166C) NK_1 receptor

	Wild type				*Mutant*			
Competitor:	*SR140.333*		*FK888*		*SR140.333*		*FK888*	
Tracer:								
^{125}I-SP	0.90	(±0.33)	0.89	(±0.16)	0.49	(±0.091)	0.20	(±0.045)
	0.96	*(±0.094)*	*1.1*	*(±0.2)*	*0.85*	*(±0.12)*	*(0.98)*	*(±0.20)*
^{125}I-NKA	0.19	(±0.014)	0.48	(±0.030)	0.18	(±0.036)	0.11	(±0.018)
	0.92	*(±0.11)*	*0.87*	*(±0.17)*	*0.94*	*(±0.16)*	*0.94*	*(±0.14)*
Effect of tracer:								
(^{125}I-SP/^{125}I-NKA)	4.7		1.9		2.7		1.9	
	P<0.1		*P<0.05*		*P<0.05*		*P<0.1*	

The inhibition constants (IC_{50}) and the Hill coefficient (in italics) of NK_1 antagonists in competition with [^{125}I]-SP and [^{125}I]-NKA on wild type and mutant (G166C) NK_1 receptor were determined using the computer program ALLFIT (DeLean *et al*., 1978). The results are given in nanomolar (nM) and are means of three independent experiments. The standard deviation is given in parenthesis. The discrepancy between the affinities determined in homologous and heterologous binding is presented and its statistical significance determined.

by either [^{125}I]-SP or [^{125}I]-NKA. We have initially introduced a single receptor type (wild type or mutant) into CHO cells, which do not express any endogenous tachykinin receptor. This would prompt the expectation of binding data consistent with a single receptor site. However, this hypothesis clearly collides with the observed discrepancies in ligand affinities and binding capacity (Hastrup & Schwartz, 1996; Tables 1 and 3; Figure 2). We show here that this peculiar complexity in the binding characteristics of these tachykinins to NK_1 receptors is dramatically affected by the mutation of Gly^{166}. Computer analysis of this phenomenon utilizing multiple radioligand binding data showed that the binding isotherms of SP and NK_1 antagonists were always consistent with a single population of high-affinity sites. However, the binding isotherms of NKA and other septide-like ligands could only be adequately described assuming two independent binding sites that have equal affinities for SP but different affinities for the septide-like ligands. This is illustrated in Figure 5.

Table 5 Dissociation constants of tachykinin ligands for binding to wild type and mutant (G166C) NK_1 receptors

	Wildtype		*Mutant*	
	K_d^H	K_d^L	K_d^H	K_d^L
Ligands:				
NKB	2.8 (±1.1)	730 (±270)	7.6 (±1.9)	2900 (±420)
SP methyl-ester	8.6 (±1.0)	470 (±89)	5.7 (±1.6)	980 (±160)
SP(6–11)	3.7 (±0.89)	840 (±157)	4.2 (±1.7)	440 (±180)
Septide	2.9 (±1.1)	750 (±157)	3.6 (±1.2)	220 (±85)
SR140333	0.72 (±0.11)	0.72 (±0.11)	0.19 (±0.018)	0.19 (±0.018)
FK888	0.61 (±0.13)	0.61 (±0.13)	0.17 (±0.021)	0.17 (±0.021)

Six detailed binding isotherms for SP, NKA and the indicated tachykinin ligand in competition for [^{125}I]-SP and [^{125}I]-NKA were generated in parallel and analysed simultaneously with the computer program Ligand (Munson & Rodbard, 1980) according to a two-site model and the dissociation constants (K_d) of the three ligands determined. Binding of NKA and *septide-like* ligands were resolved into both a high affinity (K_d^H) and a low affinity (K_d^L) component while SP and other ligands displayed equal affinities for these sites. The results are given as nanomolar (nM) and are means of three independent experiments. The standard error of the mean is given in parenthesis. The dissociation constants for SP and NKA and the relative contributions of the high and low affinity components were equal to the values reported in Table 2.

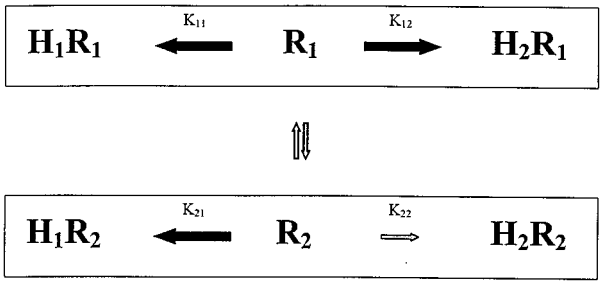

Figure 5 Schematic representation of two ligands interacting with two different binding sites. The NK_1 receptor is shown to exist in two forms (R_1 and R_2), that bind SP (ligand H_1) with equal affinity. Hence, the equilibrium constants, K_{11} and K_{21}, which control these interactions, are identical. In contrast, NKA (ligand H_2) discriminates between the two receptor-forms, reflected by a 100–1000 fold difference between the corresponding equilibrium constants, K_{12} and K_{22}. Whether the two receptor forms are physically distinct due to truncation or different glycosylation, stabilized by stable complex formation with e.g. G-proteins or interchanging with a very slow rate constant remains to be shown.

Importantly, these analyses indicated that the mutation did not notably affect the affinity of the receptor for any of the examined ligands.

This explains the inconsistency in binding affinities between homologous and heterologous binding assays reported recently for tachykinin ligands (Hastrup & Schwartz, 1996; Sagan *et al*., 1997). It also explains the discrepancy in receptor density when either [^{125}I]-SP or [^{125}I]-NKA is employed to saturate NK_1 receptors. In fact, since NKA has high affinity for only a fraction of the sites labelled by SP, the total number of high affinity binding detected by [^{125}I]-NKA is always lower than that measured by [^{125}I]-SP. This finding may also apply to the variant B_{max} observed between SP and the minimal tachykinin peptide, ALIE-124 (Sagan *et al*., 1997). Using this analysis, we found that the mechanism by which the mutation enhances the apparent affinity of NKA and septide-like ligands is primarily due to an increase in the proportion of high affinity sites. This explains why the mutation has a class-specific effect on the apparent affinity of a set of ligands. In fact only those ligands that discriminate two affinity components among the sites labelled by SP can display a leftward shift in the competition curves for this radiolabelled ligand.

The experimental set-up employing two different radioligands produces a phenomenology consistent with two independent receptor sites. We may speculate that this finding reflects the actual existence of two different forms of the single transfected NK_1 receptor, as suggested by Schwartz & Maggi (1997), but our analysis provide no proof of this. The two hypothetical forms of the NK_1 receptor could be generated by variant post-translational modifications or direct receptor-receptor interactions may result in such multimeric forms, as recent evidence suggests for some types of seven transmembrane receptors (Hebert *et al*., 1996; Romano *et al*., 1996). Alternatively, two receptor forms might be stabilized by either some sort of intramolecular constraint or by the interaction of the receptor with accessory membrane proteins, such as, for example different types of G protein sub-units. The latter scenario would constitute a possible explanation to our previous observation that the mutation enhances the potency of the receptor to activate G_q, implying that the mutation shifts the ratio between a G_q and a G_s coupled form of the receptor in favour of the G_q preferring form. However, if the two hypothetical receptor forms are thought to interconvert into each other, they must do so with a very slow time constant in order to generate data in an equilibrium-binding assay consistent with two independent binding sites as observed in this study. The possibility of interconversion among the receptor forms is illustrated in Figure 5.

While any inference on the nature of the mechanism that generates two affinity-forms remains pure speculation in the absence of additional experiments, the finding that the mutation dramatically changes the proportion of affinity forms suggests, that the residue in this point of the receptor plays a crucial role in the stabilization of the two receptor-sites. Substitutions in other areas of the NK_1 receptor have significantly altered the apparent receptor selectivity (Huang *et al*., 1995; Tian *et al*., 1996) and an important task will be to re-examine several mutants employing both radiolabelled SP and NKA in order to obtain a complete map of the molecular domains involved in this phenomenon.

Another interesting conclusion that can be drawn from this study relates to the differences in binding mode between natural tachykinins. On one hand, unselective high affinity binding of tachykinins is an intrinsic feature of the NK_1 receptor (Hastrup & Schwartz, 1996), on the other hand, our findings suggest that one form of the NK_1 receptor discriminate strongly among these mammalian neuropeptides. Therefore, selectivity in the tachykinin system cannot be rationalized merely in terms of subtype specific interactions (Yokota *et al*., 1992) or conformations (Huang *et al*., 1995). Rather, the apparent ligand-receptor specificity can be thought of as the result of a complex interplay between the

conformational space represented by the ligand and a subtype specific distribution among different receptor forms.

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