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Effect of single point mutations of the human tachykinin NK_1 receptor on antagonist affinity

Kenneth Lundstrom [a,b,*], Anthony B. Hawcock [a,1], Alfredo Vargas [b], Peter Ward [a], Pam Thomas [a], Alan Naylor [a]

[a] *Glaxo Wellcome, Medicines Research Centre, Gunnels Wood Road, Stevenage, Herts SG1 2NY, United Kingdom*
[b] *Glaxo Institute for Molecular Biology, 14. chemin de Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland*

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Abstract

Molecular modelling and site-directed mutagenesis were used to identify eleven amino acid residues which may be involved in antagonist binding of the human tachykinin NK_1 receptor. Recombinant receptors were expressed in mammalian cells using the Semliki Forest virus system. Wild type and mutant receptors showed similar expression levels in BHK and CHO cells, verified by metabolic labelling. Binding affinities were determined for a variety of tachykinin NK_1 receptor antagonists in SFV-infected CHO cells. The binding affinity for GR203040, CP 99,994 and CP 96,345 was significantly reduced by mutant Q165A. The mutant F268A significantly reduced the affinity for GR203040 and CP 99,994 and the mutant H197A had reduced affinity for CP 96,345. All antagonists seemed to bind in a similar region of the receptor, but do not all rely on the same binding site interactions. Functional coupling to G-proteins was assayed by intracellular Ca^{2+} release in SFV-infected CHO cells. The wild type receptor and all mutants except A162L and F268A responded to substance P stimulation. © 1997 Elsevier Science B.V.

Keywords: Semliki Forest virus system; Tachykinin NK_1 receptor; Mutagenesis, site-directed; Binding assays; Functional coupling

1. Introduction

The human tachykinin NK_1 receptor is a member of the superfamily of G-protein coupled receptors which are postulated to have a topology of seven transmembrane spanning helices (Yokota et al., 1989; Hershey and Krause, 1990). Radioligand binding studies have identified the tachykinin NK_1 receptor in both the peripheral and central nervous system (Takeda et al., 1991). The NK_1 receptor is thought to play an important role in pain transmission and neurogenic inflammatory diseases (Barnes et al., 1990; Helke et al., 1990).

The tachykinin NK_1 receptor has been the subject of intensive site-directed mutagenesis studies aimed at determining the regions of the receptor involved in ligand binding. By generating chimeric receptors between the tachykinin NK_1 and NK_3 receptors, the epitopes responsible for the specific binding of the non-peptide tachykinin NK_1 receptor antagonist CP 96,345 were identified. These epitopes lie in transmembrane domains V and VI at the extracellular side of the membrane (Gether et al., 1993). Mutation of the histidine residue at position 197 was shown to affect the binding of CP 96,345, but did not affect binding of the natural agonist of the receptor, substance P (Fong et al., 1993). It has also been shown that both the extracellular regions and the transmembrane domains are involved in substance P binding (Fong et al., 1992b). Extensive mutagenesis studies on the second and seventh transmembrane domains revealed that asparagine 85, asparagine 89, tyrosine 95, asparagine 96 and tyrosine 287 are required for high affinity binding of peptides (Huang et al., 1994). Asparagine 85 seems to interact with the C-terminus of substance P. Glutamine 78 in transmembrane domain II and tyrosine 205 in transmembrane domain V are involved in the receptor activation process. Huang et al. (1994) have also suggested that the key residues for peptide binding may lie close to residues which have been implicated in antagonist binding; histidine 197, histidine 265 and tyrosine 287. They also suggest that a volume exchange effect can explain competitive

* Corresponding author. Present address: F. Hoffmann-La Roche Ltd., CNS Department, Building 68/209, CH-4070 Basel, Switzerland. Tel.: (41-61) 687-8653; Fax: (41-61) 688-4575;
e-mail: kenneth.lundstrom@roche.com
[1] Present address: Pfizer Central Research, Sandwich, Kent, UK.

binding between substance P and non-peptide antagonists. Other studies have shown that amino acid substitutions in transmembrane domain II (glutamine 78, asparagine 85, asparagine 89, tyrosine 92 and asparagine 96) do not affect high affinity binding of substance P, but do block the ability of peptides to compete with non-peptide antagonists (Rosenkilde et al., 1994).

We have previously expressed the human tachykinin NK_1 receptor at high levels with the aid of the Semliki Forest virus (SFV) expression system (Lundstrom et al., 1994, 1995b). In this study, we have used the SFV system to express the wild type human tachykinin NK_1 receptor and a series of mutant receptors in Chinese hamster ovary (CHO) cells. The mutants were selected based on a model for the binding of CP 96,345 to a model of the human tachykinin NK_1 receptor. Comparative binding studies with several structurally different tachykinin NK_1 receptor agonists and antagonists were carried out to investigate whether these compounds bind to a similar site to that suggested for CP 96,345 (Gether et al., 1993). The functional coupling of human wild type and mutant tachykinin NK_1 receptors was studied by Fura-2 assays monitoring intracellular Ca^{2+} release.

2. Materials and methods

2.1. Materials

Plasmids were transformed into *Escherichia coli* DH5α cells and purified by standard methods (Maniatis et al., 1982). Baby hamster kidney (BHK) and Chinese hamster ovary (CHO) cells from the ATCC were grown in a 1:1 mixture of F-12 MEM and Iscove (Gibco-BRL) containing 10% fetal calf serum and 4 mM glutamine, Restriction enzymes were purchased from New England Biolabs, *Pyrococcus furiosus* DNA polymerase and pBluescript vector from Stratagene. The capping nucleotide m^7GpppG, SP6 RNA polymerase and RNase inhibitor were supplied by Pharmacia.

Substance P was purchased from Peninsula, Leupeptin, Pepstatin A, Phosphoramidon and PMSF from Sigma. GR82334 ([D-pro^9[Spiro-γ-Lactam]Leu^{10},Trp^{11}]physalemin-(1–11)) was purchased from Neosystem Laboratories. SR140333 ((*S*)1-[2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl-4-phenyl-1-azoniabicyclo[2.2.2]octane, chloride) was a gift from Sanofi Recherche. CP 96,345 (*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo-[2.2.2] octan-3-amine), CP 99,994 ((+)-(2*S*,3*S*)-3-(2-methoxybenzylamino)-2-phenylpiperi-dine), RP67580 (7,7-diphenyl-2[1-imino-2(2-methoxy-phenyl)-ethyl]perhydro-isoindol-4-one (3a*R*,7a*R*)) and GR203040 ((2*S*,3*S*)-2-methoxy-5-(tetrazol-1-yl)-benzyl-(2-phenyl-piperidin-3-yl)-amine) were synthesized in the Department of Medicinal Chemistry, Glaxo Wellcome Research and Development.

2.2. Cloning and site-directed mutagenesis

The cDNA for the coding region of the human tachykinin NK_1 receptor has previously been introduced into the pSFV1 vector (Lundstrom et al., 1994). Amino acid substitutions were carried out using the Megaprimer technique (Lundstrom et al., 1991). Oligonucleotide primers with mutated sequences were used with primers with *Bam*HI sites from either the 5′ or 3′ end of the coding region in a PCR (94°C 1 min, 60°C 2 min, 72°C 3 min for 35 cycles). The amplified fragments were gelpurified and used as megaprimers with 5′ and 3′ end primers, respectively, in a second PCR. The products from the second PCR were gelpurified and cloned into pBluescript and the accuracy of the mutations confirmed by DNA sequencing. The mutant tachykinin NK_1 receptor fragments were then introduced into the *Bam*HI site of the pSFV1 vector.

2.3. Expression of tachykinin NK_1 receptor mutants

*Spe*I linearized pSFV1-NK1 wildtype and mutant plasmids were in vitro transcribed and coelectroporated with pSFV-Helper2 RNA (Berglund et al., 1993) into BHK cells as earlier described (Lundstrom et al., 1994). In vivo packaged recombinant SFV particles were collected after 24 h. Confluent BHK and CHO cell cultures were infected with α-chymotrypsin (Boehringer-Mannheim) activated SFV-NK1 virus stocks at a multiplicity of infection (MOI) of 10. The protein expression was verified 16 h post-infection by metabolic labeling with [^{35}S]methionine (Amersham) followed by SDS-PAGE analysis (Laemmli, 1970) and autoradiography.

2.4. Intracellular Ca^{2+} release

CHO cells were infected with SFV-NK1 wild type and mutant virus stocks and substance P stimulated Ca^{2+} mobilization assayed with Fura-2 (1-[2-(5-Carboxy-azol-2-yl)-6 aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy) ethane-*N*,*N*,*N*′,*N*-tetraacetic acid) 16 h post-infection as earlier described (Lundstrom et al., 1995a). SFV-LacZ infections served as a control. Substance P was added at a final concentration of 10 nM and the tachykinin NK_1 antagonist CP 99,994 at 1 μM. Fluorimetric determinations were carried out in a JASCO FP777 spectrofluorimeter with an excitation at 340 nm and emission at 505 nm.

2.5. Radioligand binding assays

CHO cells were infected with SFV-NK1 wild type and mutant virus stocks and harvested after 16 h in phosphate buffered saline (PBS) containing 0.02% EDTA by centrifugation at 500 × *g* for 5 min. Cells were resuspended in 10 vol of HEPES (50 mM) buffer (pH 7.4), containing

Leupeptin (0.1 mM), Bacitracin (25 μg/ml), EDTA (1 mM), PMSF (1 mM) and Pepstatin A (2 μM) and homogenised with an Ultra Turrax homogeniser. After centrifugation at 48 000 × *g* for 30 min, the pellet was resuspended in HEPES buffer (without PMSF and Pepstatin A) by vortexing for 5 s, forced through 0.8 mm and then 0.6 mm bore syringe needles. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard. Membrane suspensions were stored at −80°C.

In the tachykinin NK_1 receptor binding assays CHO cell membranes were incubated for 40 min at room temperature in a buffer containing HEPES (50 mM), $MnCl_2$ (3 mM), pH 7.4 and [^{3}H]substance P (0.3–0.7 nM, final concentration). Non-specific binding was defined by the addition of cold substance P (1 μM). Reactions were terminated by rapid filtration through Wallac A filtermats. Filters were washed with ice cold HEPES buffer and radioactivity counted in a Wallac plate counter. Tissue linearity binding curves were constructed for all membrane preparations, and a protein concentration that lay on the linear portion of the curve chosen for subsequent assays.

For saturation binding assays membranes were incubated with a range of concentrations of [^{3}H]substance P (0.005–10 nM) as described above. For competition binding studies, membranes were incubated with 11 concentrations of competing compounds to define the IC_{50} values.

2.6. Data analysis

Inhibition curves were analyzed and pIC_{50} values calculated using the curve fitting program SAGE (Glaxo Wellcome VAX Library). pIC_{50} values were converted to inhibition constants (pK_i values) using the Cheng Prusoff equation ($K_i = IC_{50}/(1 + L/K_d)$, where L is the ligand concentration and K_d is the dissociation constant (Cheng and Prusoff, 1973). The K_d and B_{max} (maximum number of binding sites per mg of tissue) values determined from saturation curves were analysed using the curve fitting program Origin (Glaxo Wellcome VAX library). Values were expressed as arithmetic means ± s.e.m. Significance was determined using Dunnett's multiple comparison test.

2.7. Modelling of the receptor and selection of mutants

A model of the putative membrane spanning regions of the human tachykinin NK_1 receptor was constructed using the published sequence (Takeda et al., 1991). The membrane spanning regions were located in this sequence using the hydrophobicity profiling method of Kyte and Doolittle (1982). The structure of bacteriorhodopsin (Henderson et al., 1990) was used as a structural template for the model. As there is no detectable homology between bacteriorhodopsin and the human tachykinin NK_1 receptor, the sequences were aligned using helical wheels to match the

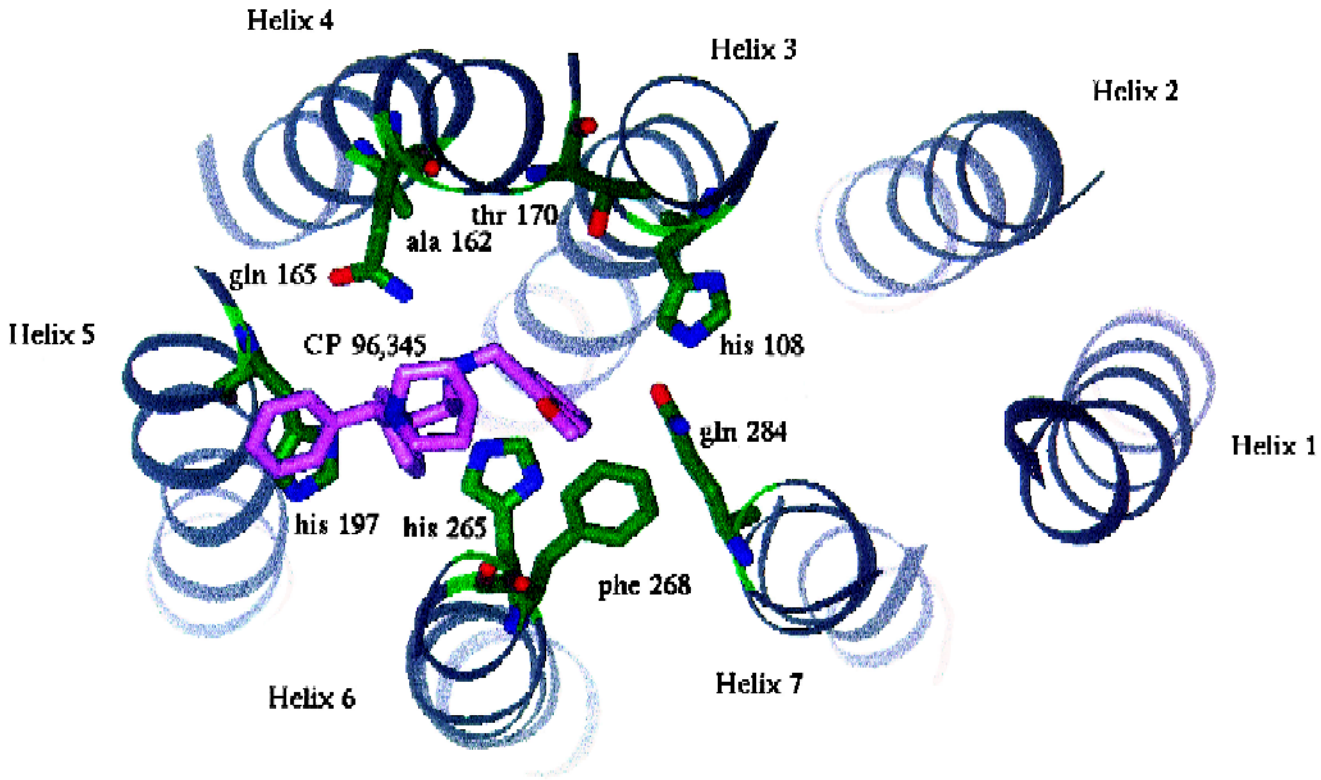

Fig. 1. Model of the binding mode for the antagonist CP 96,345 at the human tachykinin NK_1 receptor.

Table 1
Mutations of the human tachykinin NK_1 receptor based on the model of CP 96,345 binding shown in Fig. 1

Mutation	Selection criteria	Published observation
E97V	Possible site of interaction between quinuclidine nitrogen of CP 96,345 and the human NK_1 receptor	Interacts with some peptide ligands, not others (Fong et al., 1992a)
H108A	Close to proposed antagonist binding site	Affects agonist, not CP 96,345 binding (Fong et al., 1995)
A162L	Occludes possible binding pocket for benzylamine substituent of CP 96.345	
Q165A	Possible hydrogen bond donor to amine nitrogen of benzylamine substituent of CP 96,345	Affects agonist, not antagonist binding (Fong et al., 1995)
T170A	Possible hydrogen bond donor to secondary amine of CP 96,345	
E172Q	Possible site of interaction between quinuclidine nitrogen of CP 96,345 and the human NK_1 receptor	
E193Q	Possible site of interaction between quinuclidine nitrogen of CP 96,345 and the human NK_1 receptor	
H197A	Known to affect binding of CP 96,345	Affects agonist, not antagonist binding (Fong et al., 1993)
H265A	Known to affect binding of CP 96,345	Affects agonist, not antagonist binding (Fong et al., 1995)
F268A	Possible contact to ring benzyl-amine substituent of CP 96,345	
Q284A	Possible hydrogen bond donor to secondary amine of CP 96,345	Weak or no effect on both agonist and antagonist binding (Fong et al., 1993)

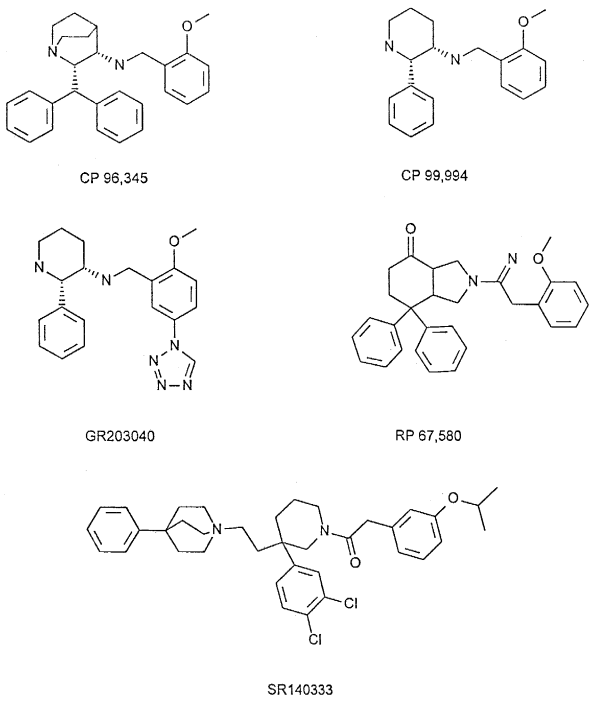

Fig. 2. Structures of the tachykinin NK_1 receptor antagonists.

pore facing and lipid facing residues of the two proteins. The model was then constructed by swapping the side chains of bacteriorhodopsin for the appropriate side chain from the sequence of the tachykinin NK_1 receptor.

The model was used to examine possible modes of binding of the non-peptide antagonist CP 96,345 at the tachykinin NK_1 receptor. Based on observed differences in antagonist binding at rat and human tachykinin NK_1 receptors (Gitter et al., 1991), initial interest focused on residue 97 (part of the loop joining transmembrane domains II and III), which is a glutamate in the human receptor and a valine in the rat receptor. However, the basis for the species selectivity has been shown to be due to substitutions between the rat and human tachykinin NK_1 receptors at positions 116 and 290 (Fong et al., 1992a). Following the work of Fong et al. (1993), interest was focused on the region of the receptor around histidine residues 197 and 265 on transmembrane helices V and IV, respectively. Modelling the mode of binding for CP 96,345 at this site (Fig. 1) led to the identification of other residues which might affect antagonist binding. The eleven mutants suggested and the criteria for their mutagenesis are summarized in Table 1. Many of these mutations have been described by other authors and references to these findings are included in the table. In addition to the extensively studied antagonist CP 96,345, a selection of other antagonists were also examined against the mutated receptors to assess if all compounds shared a common binding site or whether there were detectable differences in antagonist binding. The compounds used are shown in Fig. 2.

3. Results

3.1. Site-directed mutagenesis and expression of the tachykinin NK_1 receptor

The amino acid substitutions in the tachykinin NK_1 receptor gene were introduced by PCR using the

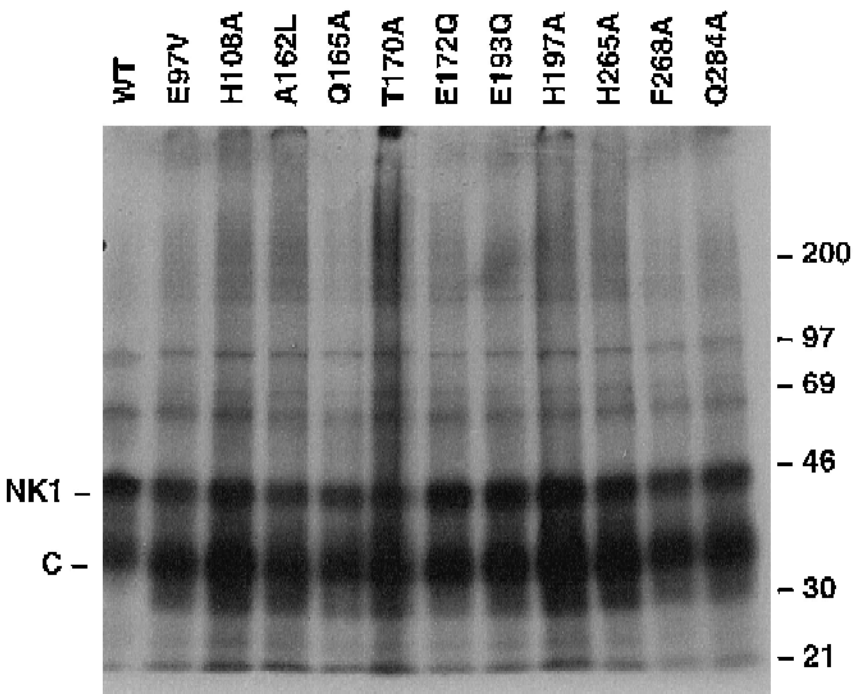

Fig. 3. BHK cells infected with SFV-NK1 wild type and mutant virus stocks were labeled with [^{35}S] methionine 16 h post-infection. SFV capsid, C was expressed as an internal control. The molecular weight standard on the right in kDa.

Megaprimer technique described in Section 2. The accuracy of the mutations was confirmed by DNA sequencing. No additional changes in the sequences were obtained from the PCRs. The mutated tachykinin NK_1 receptor fragments were then introduced into the pSFV1 vector. SFV-NK1 wild type and mutant virus stocks were generated with estimated titers of 1×10^9 virus particles per ml. BHK and CHO cells were infected with these virus stocks and expression of recombinant wild type and mutant tachykinin NK_1 receptors verified by metabolic labeling followed by SDS-PAGE and autoradiography. High-levels of expression of all mutant tachykinin NK_1 receptors were obtained (Fig. 3). No differences in the expression levels compared to the wild type could be detected for any of the mutants. The SFV capsid protein was expressed as an internal standard.

3.2. Functional activity of the tachykinin NK_1 receptor

To study the functional coupling of the expressed tachykinin NK_1 wild type and mutant receptors to G proteins, SFV-NK1 infected CHO cells were subjected to substance P induced Ca^{2+} mobilization 16 h post-infection. These studies allowed the grouping of the tachykinin NK_1 mutant receptors into three categories: those with high, low or no response to the ligand (Table 2, Fig. 4). The results are based on comparison of the relative fluorescence intensity of the responses to substance P in three independent experiments. The wild type tachykinin NK_1 receptor showed a very strong response as did four of the mutants. Five other mutants gave weak but significant responses, while substance P stimulation had no effect on mutants A162L and F268A, as well as on control SFV-LacZ infected CHO cells. The response to substance P could be blocked by addition of 1 μM tachykinin NK_1 receptor antagonist CP 99,994.

Table 2
Functional coupling of recombinant tachykinin NK_1 receptors to G-protein after substance P stimulation assayed by the Fura-2 Ca^{2+} mobilization

Mutation	Functional response
E97V	+ +
H108A	+
A162L	−
Q165A	+ +
T170A	+ +
E172Q	+ +
E193Q	+
H197A	+
H265A	+
F268A	−
Q284A	+
Wild type	+ +

Based on relative intensity of responses to stimulation the mutants could be divided into three groups: + + = strong; + = weak; − = no response.

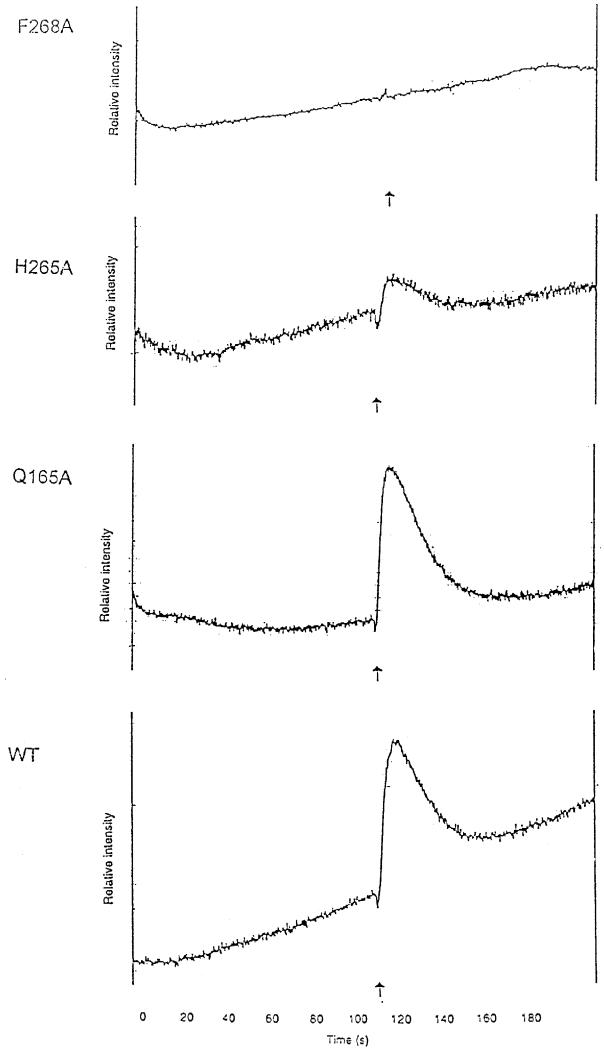

Fig. 4. Functional response to substance P in CHO cells infected with SFV-NK1 virus stocks. Fura-2 Ca^{2+} release was measured 16 h post-infection after stimulation (indicated by arrows) with 10 nM substance P.

3.3. Radioligand binding of the tachykinin NK_1 receptor

Saturation binding experiments demonstrated that there was no significant difference ($P > 0.05$) in the affinity of [^{3}H]-substance P for the wild type and mutant tachykinin NK_1 receptors examined in this study. The affinity of [^{3}H]-substance P was slightly lower than that shown in membranes prepared from CHO cells stably transfected with the tachykinin NK_1 receptor (Hawcock, unpublished observation). However, there were significantly less ($P < 0.01$) [^{3}H]-substance P binding sites in membranes prepared from CHO cells infected with SFV-NK1 mutant receptor virus stocks than with wild type receptor virus stock (Table 3).

In competition studies the binding affinities of a range of peptide and non-peptide antagonists and cold substance P were determined in membrane preparations from CHO cells infected with SFV-NK1 wild type and mutant receptor virus stocks (Table 4). The affinity of cold substance P

Table 3
Estimation of affinity (K_d) and receptor number (B_{max}) for [^{3}H]substance P on membranes prepared from CHO cells infected with SFV-NK1 wild type and mutant receptor virus stocks

Mutation	K_d (nM)	B_{max} (pmol/mg protein)
E97V	0.36 ± 0.16	3.80 ± 1.63
H108A	ND	ND
A162L	ND	ND
Q165A	0.65 ± 0.15	3.80 ± 1.00
T170A	0.42 ± 0.14	15.0 ± 3.00
E172Q	0.24 ± 0.07	3.20 ± 0.83
E193Q	0.35 ± 0.06	17.6 ± 4.60
H197A	0.49 ± 0.19	26.2 ± 2.70
H265A	0.39 ± 0.06	5.00 ± 2.10
F268A	0.33 ± 0.11	1.97 ± 0.58
Q284A	0.31 ± 0.09	3.10 ± 0.42
Wild type	0.29 ± 0.08	61.0 ± 6.00

Data are mean K_d ± SEM and B_{max} ± SEM ($n = 3–4$). ND indicates estimates were too low to measure.

was similar for all the receptor preparations, although it was lower than that for [^{3}H]-substance P. Significant changes in the binding affinities of some of the ligands tested were seen for three of the tachykinin NK_1 receptor mutants (Table 4). Mutation Q165A showed a significant ($P < 0.01$) decrease in affinity for GR203040, CP 99,994 and CP 96,345 and a small decrease in affinity for SR140333 that did not achieve significance. Likewise mutation H197A showed a significant ($P < 0.01$) affinity decrease for CP 96,345 and as for Q165A showed a small decrease for SR140333. Additionally, a significant decrease ($P < 0.05$) in GR203040 and CP 99,994 binding affinities were obtained for mutation F268A. This mutation also showed a slight but non-significant reduction in the binding affinity for CP 96,345. The RP67580 showed a slight but non-significant reduction in the binding affinity for mutations T170A, E193Q and H265A.

3.4. Statistical analysis and model analysis

The results of these binding studies indicated that most of the point mutations examined do not cause a statistically significant change in compound binding. Only Q165A, H197A and F268A had a significant effect on tachykinin NK_1 receptor antagonist binding, while slight but non-significant decreases in affinity were seen for some compounds for some of the other mutants (Table 4).

4. Discussion

The binding of the natural agonist, substance P, appears to be largely unaffected by the mutations of the tachykinin NK_1 receptor analysed in this study. This suggests that these residues are not required for agonist binding. Interestingly, the affinity was lower than that observed in membranes prepared from stably transfected cells (pK_i 9.6 ± 0.05; Hawcock, unpublished results). This difference could possibly be due to the fact that the SFV infection caused the cells to overexpress large numbers of receptors at the expense of other proteins, for example G-proteins, leading to an increased number of uncoupled receptors that would not recognize an agonist ligand. All mutants displayed significantly ($P < 0.001$) lower B_{max} values than the wild type. However, mutants T170A, E193Q and H197A had significantly higher B_{max} values than the other mutants studied. The most likely explanation for this is that the mutations affected the folding of the receptor and reduced the efficiency of transport to the membrane.

We expected the affinities for the tachykinin NK_1 receptor antagonists to be similar for the wild type expressed with SFV and in stable cell lines. However, the values were lower for SFV-expressed receptors. Obviously, due to the overexpression a percentage of the receptor population might be in an uncoupled state and as such would not recognize an agonist ligand. The antagonist ligands will, however bind to coupled and uncoupled receptors with equal affinity and the agonist–antagonist equilibrium would therefore be shifted. When the SFV expression was down-regulated by using a lower multiplicity of infection (MOI) saturation binding analysis demonstrated a lower B_{max}

Table 4
Affinity estimation (pK_i ± SEM; $n = 3–5$, except $^{\#}$ where $n = 2$) for a range of peptide and non-peptide antagonists and substance P on membranes prepared from CHO cells expressing wild type and mutant tachykinin NK_1 receptors

	GR203040	CP 99,994	CP 96,345	RP67580	SR14033	GR82334	SP
E97V	9.7 + 0.11	9.5 ± 0.12	9.0 ± 0.08	7.7 ± 0.20	9.4 ± 0.11	7.4 ± 0.3 $^{\#}$	8.9 ± 0.1 $^{\#}$
Q165A	8.2 + 0.26 **	7.2 ± 0.14 **	7.4 ± 0.27 **	7.4 ± 0.39	8.1 ± 0.25	6.6 ± 0.15	8.6 ± 0.2 $^{\#}$
T170A	9.7 ± 0.08	9.3 ± 0.29	8.7 ± 0.20	6.9 ± 0.17	9.2 ± 0.34	7.2 ± 0.48	8.7 ± 0.16
E172Q	9.5 ± 0.09	9.0 ± 0.14	8.7 ± 0.16	7.5 ± 0.16	9.0 ± 0.23	7.2 ± 0.30	8.8 ± 0.11
E193Q	9.3 ± 0.19	8.6 ± 0.22	8.5 ± 0.22	6.9 ± 0.16	8.6 ± 0.19	7.0 ± 0.25	8.6 ± 0.28
H197A	9.4 ± 0.16	8.6 ± 0.25	7.5 ± 0.21 **	7.2 ± 0.33	8.1 ± 0.18	6.7 ± 0.36	8.7 ± 0.04
H265A	9.5 ± 0.25	8.4 ± 0.05	8.4 ± 0.07	7.0 ± 0.25	8.7 ± 0.06	7.7 ± 0.06	8.8 ± 0.09
F268A	8.7 ± 0.13 *	8.4 ± 0.05 *	7.9 ± 0.12	7.7 ± 0.20	8.2 ± 0.22	6.7 ± 0.27	8.8 ± 0.09
Q284A	9.2 ± 0.14	9.0 ± 0.11	8.7 ± 0.10	7.8 ± 0.08	8.9 ± 0.16	6.6 ± 0.28	9.0 ± 0.05
WT	9.4 ± 0.17	9.0 ± 0.19	8.6 ± 0.19	7.4 ± 0.19	8.8 ± 0.21	7.0 ± 0.23	8.7 ± 0.16

SP, Substance P; WT, wild type.
Significance $^{*} = P < 0.05$, $^{**} = P < 0.01$.

value. In experiments using these membrane preparations, similar affinity estimates were observed for a range of tachykinin NK_1 receptor antagonists as when using membranes prepared from stably transfected cells.

The differences in the levels for several of the mutants and total absence of functional response for the A162L and F268A mutants could not be correlated to the data from the binding studies. Obviously the coupling to the G-proteins must have been affected by a different folding of the mutant receptors compared to the wild type. However, in this study, our main interest was the mutational effect on the antagonist binding site in relation to our constructed model.

Interestingly, we could clearly show that all mutant NK_1 receptors were synthesized equally well as the wild type receptor in the mammalian cells by metabolic labeling coexpressing the SFV capsid protein as an internal standard. This observation is of great importance as the basis for the binding and functional studies, suggesting that the mutagenesis has affected the folding and transport of the receptor molecules and not the primary expression events.

It is evident that all the non-peptide tachykinin NK_1 receptor antagonists with the exception of RP67580 appear to be affected by the mutation Q165A. This residue is suggested to form a hydrogen bond to the amine nitrogen of the benzylamine substituent of CP 96,345 (Fong et al., 1995). The presence of the same group in CP 96,345, CP 99,994 and GR203040 would support this conclusion. All three of these compounds could therefore form a similar interaction with this glutamine residue in the receptor and therefore share an overlapping binding site. Mutation F268A significantly affects the binding of CP 99,994 and GR203040, but no other compounds. In the model from which the suggested mutations were derived, this residue lies close to the phenyl ring of the benzylamine substituent of the piperidine. However, none of the parameters measured in this study indicate the nature of this interaction.

The mutation H197A appears only to significantly affect the binding of CP 96,345. It has been suggested that this histidine forms an amino–aromatic interaction with one of the phenyl groups of the methyl biphenyl substituent of CP 96,345 (Fong et al., 1993). The data in this study support the suggestion that this histidine may be involved in the binding of this compound, but are not indicative of the nature of this interaction. Mutation H265A has been suggested to lie near the benzylamine ring of receptor bound CP 96,345 (Fong et al., 1995). It has also been noted that mutation of this residue has differing effects on closely related compounds and when mutated in rat and human receptors. The mutation of this residue in the present study does not cause a significant change in the affinity of any of the compounds tested.

None of the mutations have a significant effect on binding of SR140333 or RP67580. The data for SR140333 show two mutations, however, which whilst not significant do cause a ten-fold decrease in affinity of this compound for the mutated receptors. This could be taken as evidence for this compound overlapping in part with the binding sites of CP 96,345, CP 99,994 and GR203040. RP67580 on the other hand shows little evidence of communality between its binding site and that of the other compounds tested.

Overall the lack of statistical significance in these results makes them difficult to interpret in terms of the modes of binding of the compounds concerned. They do suggest that there is some overlap of the sites at which CP 96,345, CP 99,994 and GR203040 and possibly SR140333 bind and that the site at which RP 67580 binds may be somewhat different.

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