The Extracellular Domain of the Neurokinin-1 Receptor Is Required for High-Affinity Binding of Peptides

Tung Ming Fong,* Hong Yu, Ruey-Ruey C. Huang, and Catherine D. Strader

Department of Molecular Pharmacology and Biochemistry 80M-213, Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065

Received March 31, 1992; Revised Manuscript Received September 4, 1992

ABSTRACT: The neurokinin-1 receptor binds neurokinin peptides with the potency order of substance P > substance K > neurokinin B. Elucidating the molecular basis of differential peptide selectivity will require the localization of the binding domain on the receptor. In the present report, mutagenesis and heterologous expression experiments reveal that a segment of the extracellular N-terminal sequence of the neurokinin-1 receptor is required for the high-affinity binding of substance P and related peptide agonists. Substitution of amino acid residues in the N-terminal region of the receptor affects the binding affinity of both intact peptides and a C-terminal substance P "analog", but not of a nonpeptide antagonist. Glycosylation of the receptor does not change the peptide binding affinity. In addition, substitution of the valine-97 residue in the rat neurokinin-1 receptor by a glutamate residue increases the binding affinity of neurokinin B but not substance P or substance K, suggesting that the second extracellular segment is involved in peptide selectivity. These results indicate that the extracellular domains of neurokinin-1 receptor play a critical role in peptide binding.

The peptide neurotransmitter substance P (SP)[1] binds preferentially to the neurokinin-1 receptor (NK1R) to elicit various biological responses (Helke et al., 1990; Iversen et al., 1987; Regoli et al., 1989). Molecular cloning has demonstrated that all neurokinin receptors belong to the superfamily of G protein coupled receptors whose structure is believed to be similar to the heptahelical structure of bacteriorhodopsin (Nakanishi, 1991). One of the physiological functions of the NK1R is the activation of a phospholipase C to increase the level of intracellular calcium. This response has been shown in cultured cells (Womack et al., 1988) and in heterologous expression systems such as *Xenopus* oocytes and CHO cells (Fong et al., 1992; Nakajima et al., 1992). In addition to binding SP, the NK1R also binds other neurokinin peptides with lower affinity. All neurokinins share the same consensus C-terminal sequence (FXGLM-NH_2), while the receptor specificity is provided by the divergent N-terminal sequence of neurokinins. Understanding the molecular basis of peptide–receptor interactions will require the localization of the peptide binding site on the NK1R.

Previous studies on other G protein coupled receptors that bind small molecules, such as the neurotransmitters acetylcholine and catecholamines or the chromophore retinal, have demonstrated that the ligand binding site is located within the transmembrane domain (Dohlman et al., 1988; Khorana, 1992; Strader et al., 1989; Wheatley et al., 1988). Because of the topological similarity among these heptahelical receptors, it is possible that the SP binding site on the NK1R also involves the transmembrane domain. Nonetheless, SP and related peptides are substantially larger than acetylcholine and catecholamines. The larger size of the peptides may necessitate a different binding mechanism and/or the involvement of other domains on the receptor. In the present study, we have used site-directed mutagenesis to investigate the possible involvement of the extracellular domains of the NK1R in peptide binding. These data indicate that part of the N-terminal sequence of the receptor is required for the high-affinity binding of peptides. In addition, the data suggest the involvement of the second extracellular segment in determining peptide selectivity.

MATERIALS AND METHODS

Construction of Mutant Receptors. The cDNA clones encoding the rat NK1R (Yokota et al., 1989), human NK1R, and their mutants were inserted into the expression vector pCDM9 as described (Fong et al., 1992). The deletion mutants, substitution mutants, and double amino acid mutant were constructed by polymerase chain reaction (PCR). The single amino acid mutant was constructed by the oligonucleotide-directed thionucleotide mutagenesis method (Amersham, Chicago, IL). The sequence of all mutations and the adjacent regions that were derived from PCR were confirmed using the dideoxy chain termination method (USBC, Cleveland, OH). In the substitution mutants rS(1–13) and rS(1–27), the indicated residues of the rat NK1R were substituted with the non-NK1R sequence MDYKDDDDK-PW which contained the FLAG antigenic sequence (IBI, New Haven, CT). The rD(2–7,14–27) mutant of the rat NK1R, the V97E mutant of the rat NK1R and S16A+N18A mutant of the human NK1R contained an antigenic fusion peptide (DSQGRNCSTNDSPL) at the C-terminus of the receptor for the purpose of receptor purification (Fong et al., manuscript in preparation). The presence of the C-terminal fusion peptide in the wild-type receptor has no effect on the peptide binding affinity, antagonist binding affinity, the maximal electrophysiological response, or the EC_{50} value of the electrophysiological response.

Expression. For binding assays, the receptor cDNA was expressed in COS cells (ATCC, Rockville, MD). Transfection of 20 μg of plasmid DNA into 10 × 10^6 cells was achieved by electroporation. Transfected cells were incubated in COS media (90% DMEM media, 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin) for 3 days in a 37

* Address correspondence to this author.

[1] Abbreviations: NK1R, neurokinin-1 receptor; NK2R, neurokinin-2 receptor; NKB, neurokinin B; PCR, polymerase chain reaction; pGlu, pyroglutamyl; Septide, (pGlu^6,Pro^9)SP(6–11); SK, substance K; SP, substance P.

0006-2960/92/0431-11806$03.00/0

°C, 5% CO_2 incubator. For determination of intracellular effector activation, in vitro RNA transcript was synthesized from plasmid DNA using T7 RNA polymerase and injected into *Xenopus* oocytes at 2 ng per oocyte (Fong et al., 1988). Injected oocytes were incubated at 19 °C for 3 days.

Binding Assays. Binding of [^{125}I]Bolton–Hunter-labeled SP ([^{125}I]BHSP, from NEN, North Billerica, MA) was performed using intact transfected COS cells, 0.2 nM [^{125}I]-BHSP, and various concentrations of unlabeled peptides as described previously (Fong et al., 1992). The data were fitted to the equation [cpm(L) – cpm(1 μM SP)]/[cpm(0) – cpm(1 μM SP)] = $IC_{50}/(L + IC_{50})$, in which cpm(L) and cpm(0) represent bound [^{125}I]BHSP in the presence and the absence of unlabeled peptide, respectively; L represents the concentration of unlabeled peptide; and IC_{50} represents the concentration of unlabeled peptide that causes 50% inhibition of specifically bound [^{125}I]BHSP. The equilibrium dissociation constant (K_d) was then calculated from the IC_{50} values. For SP, $K_{d(SP)} = IC_{50} - [[^{125}I]BHSP]$. For other peptides, $K_d = IC_{50}/(1 + [[^{125}I]BHSP]/K_{d(SP)})$. The receptor number ($B_{max}$) was calculated according to the equation $B_{max} = B_0 \times IC_{50(SP)}/[[^{125}I]BHSP]$, in which B_0 is the amount of specifically bound radioligand in the absence of unlabeled ligand (DeBlasi et al., 1989).

Binding of [^{125}I]L-703,606 was performed using plasma membranes prepared from the transfected COS cells, 0.2 nM [^{125}I]L-703,606, and various concentrations of unlabeled ligands (Cascieri et al., 1992). All preparations were carried out at 4 °C. The transfected COS cell pellet was homogenized with a Dounce homogenizer in 8.5% sucrose, 25 mM HEPES, 5 mM EGTA, 5 mM $MgCl_2$, and 1 mM PMSF, pH 7.4. The cell lysate was centrifuged at 800g for 10 min. The supernatant was centrifuged at 160000g for 1 h. The membrane pellet was resuspended in 25 mM HEPES, 5 mM $MgCl_2$, and 0.1 mM EDTA, pH 7.4. The amount of membranes used in the assay was adjusted such that the specific binding of [^{125}I]L-703,606 in the absence of unlabeled ligand was less than 10 000 cpm (about 15% of total added cpm). The binding reaction mixture contained 0.02 mL of unlabeled ligand, 0.02 mL of [^{125}I]L-703,606, and membrane sample in a final volume of 0.24 mL in 50 mM Tris, pH 7.4, 5 mM $MnCl_2$, 150 mM NaCl, 0.04 mg/mL bacitracin, 0.004 mg/mL leupeptin, 0.2 mg/mL BSA, and 0.01 mM phosphoramidon. All subsequent steps were identical to the [^{125}I]BHSP binding assay. When using L-703,606 as the displacing ligand, the data were analyzed with the same equation described above for [^{125}I]-BHSP binding assay. When using peptide agonists as the displacing ligand, the data were analyzed by the equation [cpm(L) – cpm(1 μM L-703,606)]/[cpm(0) – cpm(1 μM L-703,606)] = $F(IC_{50H})/(L + IC_{50H}) + (1 - F)(IC_{50L})/(L + IC_{50L})$, in which F is the fraction of NK1R in the high-affinity state, IC_{50H} relates to the peptide affinity for the high-affinity state, and IC_{50L} relates to the peptide affinity for the low-affinity state.

Electrophysiological Assays. The activation of calcium-gated chloride channels mediated by the NK1R was measured by two-electrode voltage clamp (Fong et al., 1988). Each oocyte was exposed to agonist only once to avoid interference from desensitization. The peak current was determined at a holding potential of –80 mV.

RESULTS

Effects of Deleting the N-Terminal Sequence on Peptide Binding. To investigate the role of the N-terminal region of the NK1R in ligand binding, two substitution mutants of the rat NK1R were constructed and expressed in COS cells and

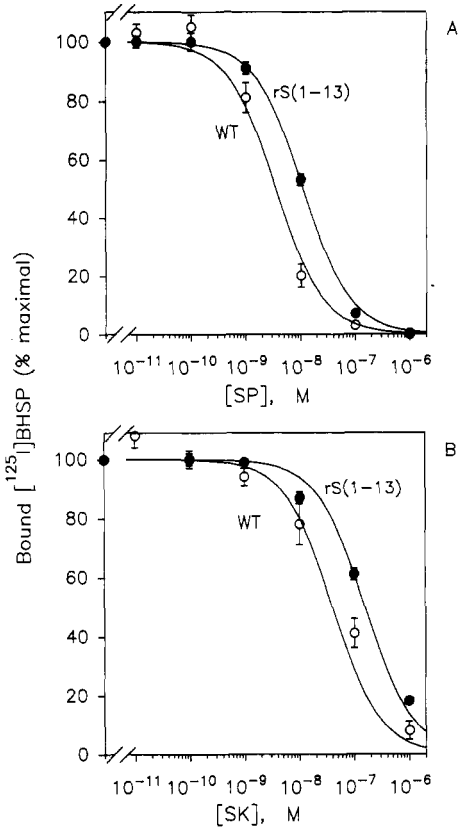

FIGURE 1: Inhibition of [^{125}I]BHSP binding to the wild type (WT) or rS(1–13) mutant of the rat NK1R by SP or SK. Each point represents the average of triplicate measurements. The error bar represents standard error. The data shown are representative of 2–5 similar experiments.

Xenopus oocytes. In the rS(1–13) mutant, the N-terminal residues 1–13 of the rat NK1R were substituted by the non-NK1R sequence MDYKDDDDKPW. A 3-fold decrease in ligand binding affinity was observed for the rS(1–13) mutant compared to the wild-type receptor (Figure 1; Table I). In contrast, the rS(1–27) mutant, where the N-terminal residues 1–27 of the rat NK1R were substituted by the same non-NK1R sequence, did not exhibit specific binding of [^{125}I]-BHSP at 0.2 nM. These data led to the construction of the rD(2–7,14–27) mutant in which residues 2–7 and 14–27 of the rat NK1R were deleted. The rD(2–7,14–27) mutant did not exhibit specific [^{125}I]BHSP binding at 0.2 nM. Because the separation of free and bound radioactive ligand by filtration is only possible if the dissociation rate constant of the ligand is less than 0.5 s^{-1} (equivalent to $K_d < 20$ nM), the K_d values of the rS(1–27) and rD(2–7,14–27) mutants were estimated to be greater than 20 nM. However, both of these mutant receptors were present at the plasma membrane on the basis of functional assays (see below).

Effects of Deleting the N-Terminal Sequence on Receptor Activation. Previous studies have demonstrated that, for the wild-type NK1R, the EC_{50} values determined from the functional assay in oocytes approximate the equilibrium dissociation constant (Fong et al., 1992). To estimate the binding affinity of mutants having reduced affinity for [^{125}I]-BHSP, these mutants were expressed in *Xenopus* oocytes. The magnitude of the response elicited by 30 nM SP was proportional to the injected amount of NK1R RNA in the range of 0–2 ng/oocyte (correlation coefficient = 0.97, data not shown), demonstrating that the maximal response is proportional to the receptor number under the present experimental conditions. Bath application of SP elicited an oscillating inward current from oocytes injected with RNA

Table I: Agonist Binding Affinity and EC_{50} of Electrophysiological Response for Wild Type, Two Substitution Mutants, and One Deletion Mutant of the Rat NK1R[a]

receptor	K_d, nM		EC_{50}, nM		N-terminal sequence
	SP	SK	SP	Septide	
					1 28
rNK1R	3.2 ± 0.7 (5)	55 ± 26 (3)	4 (3)	50 (2)	MDNVLPMDSDLFPNISTNTSESNQFVQP
rS(1-13)	10.7 ± 2.6 (2)	157 ± 30 (2)	9 (2)	-	MDykdddkpwNISTNTSESNQFVQP
rS(1-27)	> 20 (2)	-	-	-	MDykdddkpwP
rD(2-7,14-27)	> 20 (2)	-	500 (2)	> 10000 (2)	MDSDLFPP

[a] The mean ± SEM are listed. The number of experiments is given in parentheses. Figures 1 and 3 show representative experiments. All receptors differ only in their N-terminal sequence as shown in the last column. Upper-case letters represent sequences endogenous to the rat NK1R while lower-case letters represent non-NK1R sequences.

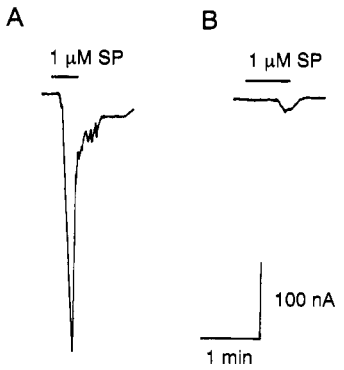

FIGURE 2: Current traces of voltage-clamped oocytes expressing the rD(2–7,14–27) mutant (A) or the rS(1–27) mutant (B). The horizontal bar represents the duration of SP application at 1 μM.

transcripts encoding the mutant receptors (Figure 2). The wild type and the rS(1–13) mutant have EC_{50} values for SP of 4 and 9 nM, respectively, consistent with the binding affinity measurements (Table I). In contrast, the EC_{50} value for the rD(2–7,14–27) mutant in response to SP was increased about 100-fold compared to the wild type (Figure 3). The maximal response mediated by the rD(2–7,14–27) mutant was approximately equal to that of the wild type. The rS(1–27) mutant elicited only a small response in the presence of 1 μM SP (Figure 2).

To investigate which region of the SP peptide might interact with the N-terminus of the receptor, the efficacy of a C-terminal analog of SP in activating the NK1R was determined. As shown in Figure 3A, the hexapeptide (pGlu^6,-Pro^9)SP(6–11) (commonly known as Septide) activated the wild-type NK1R with an EC_{50} value of 50 nM. In contrast, the potency of Septide for the rD(2–7,14–27) mutant was greatly reduced (the EC_{50} value was estimated to be about 10 μM by extrapolation of the data shown in Figure 3B). Furthermore, the potency of SK or NKB in activating the rD(2–7,14–27) mutant was also lower than that of the wild type. The relative order of potency was qualitatively the same in both the wild-type and rD(2–7,14–27) mutant receptors (i.e., SP > SK > NKB). Quantitative estimates of EC_{50} values for the mutant receptor were not obtained because of solubility limit.

Effects of Substitution of Residues 21–29 on Peptide Binding and Receptor Activation. The newly discovered nonpeptide SP antagonist CP-96,345 binds to the human NK1R with high affinity (K_d = 0.3 nM) while it has a low affinity for the rat NK1R (K_d = 30 nM; Cascieri et al., 1992; Snider et al., 1991). An iodinated analog of CP-96,345, [^{125}I]L-703,606, has been synthesized in which the methoxy group in CP-96,345 was replaced by an ^{125}I-iodo substituent

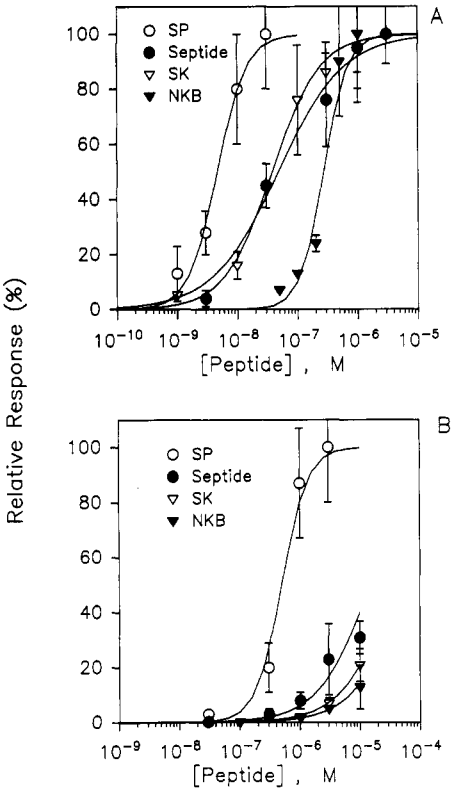

FIGURE 3: Dose–response curves of the agonist-elicited chloride current from oocytes expressing the wild type (A) or the rD(2–7,-14–27) mutant (B) of the rat NK1R. Each point represents the average of at least 3 measurements. The error bar represents standard error. The data shown are representative of 2–3 similar experiments. For the wild-type receptor, 30 nM SP elicited a chloride current of 1425 ± 343 (n = 3) nA. For the rD(2–7,14–27) mutant receptor, 3000 nM of SP elicited a chloride current of 1500 ± 353 (n = 4) nA.

(Cascieri et al., 1992). To further characterize the involvement of the N-terminal sequence of the NK1R in ligand binding, the substitution mutant hS(21–29) of the human NK1R was constructed in which amino acids 21–29 of the human NK1R (EPNQFVQPA) were substituted by the homologous region of the human NK2 receptor (GITAFSMPS). Like the rD-(2–7,14–27) mutant of the rat NK1R, the hS(21–29) did not display significant binding of [^{125}I]BHSP at 0.2 nM, indicating a reduced binding affinity for SP. However, the hS(21–29) mutant bound [^{125}I]L-703,606 with the same affinity and B_{max} as the wild-type human NK1R (Figure 4).

The inhibition of [^{125}I]L-703,606 binding to the wild-type human NK1R by SP and related peptides was biphasic, and the data were best fitted by an equation describing two populations of receptors having high affinity and low affinity for agonists, respectively. Curve fitting of the SP inhibition

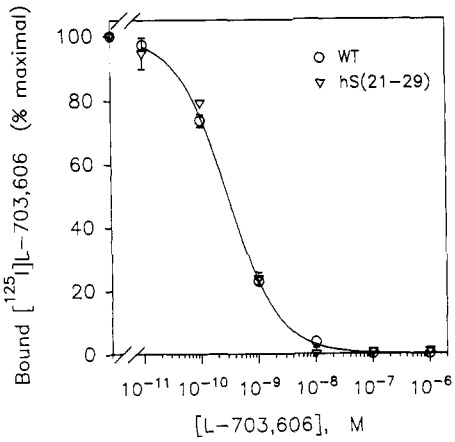

FIGURE 4: Inhibition of [^{125}I]L-703,606 binding to the wild type (WT) or the hS(21–29) mutant of the human NK1R by L-703,606. Each point is the average of duplicate measurements. The error bar represents the range of variation. The data shown here are representative of 2 similar experiments.

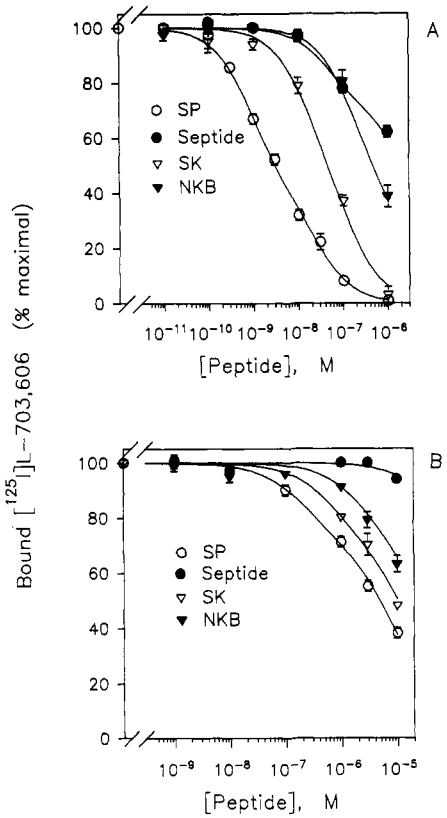

FIGURE 5: Inhibition of [^{125}I]L-703,606 binding to the wild type (A) or the hS(21–29) mutant (B) of the human NK1R by various peptides. Each point is the average of duplicate measurements. The error bar represents the range of variation. The data shown here are representative of 3 similar experiments.

data in Figure 5A yielded an IC_{50H} value of 0.8 nM for the high-affinity state and an IC_{50L} value of 24 nM for the low-affinity state. In contrast, the SP inhibition curve for the hS(21–29) mutant was shifted to the right, yielding 234 nM for the high-affinity state and 10 μM for the low-affinity state (Figure 5B). Similarly, the IC_{50} values of Septide, SK, and NKB for the hS(21–29) mutant were all shifted to the right compared to the wild type (Figure 5), indicating that the binding affinities for all four peptides were reduced in the hS(21–29) mutant.

Although the hS(21–29) mutant displayed a lower binding affinity for SP and related peptides than the wild-type receptor, it was fully active in the electrophysiological assay in oocytes.

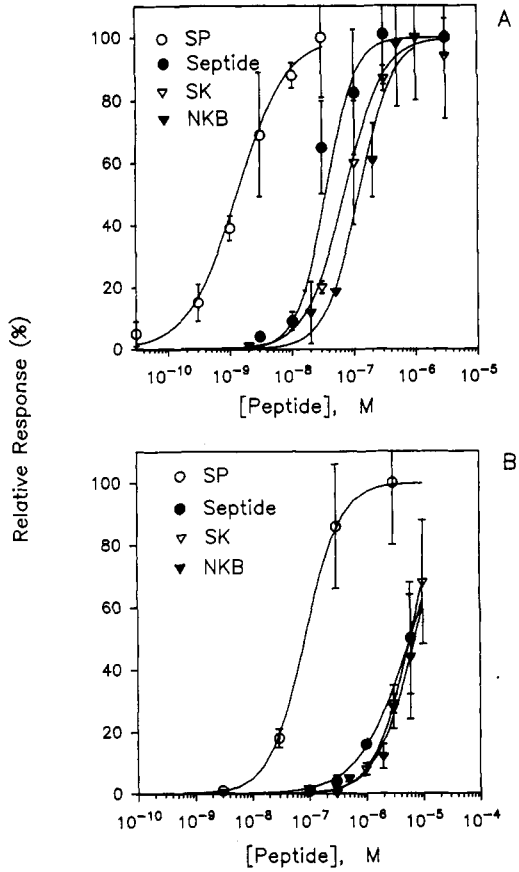

FIGURE 6: Dose–response curves of the agonist-elicited chloride current from oocytes expressing the wild type (A) or the hS(21–29) mutant (B) of the human NK1R. Each point is the average of at least 3 measurements. The error bar represents standard error. The data shown here are representative of 3–4 similar experiments. For the wild-type receptor, 30 nM SP elicited a chloride current of 1768 ± 245 (n = 7) nA. For the hS(21–29) mutant, 3000 nM SP elicited a chloride current of 2017 ± 154 (n = 3) nA.

The maximal amplitude of the response to SP was equivalent to that of the wild-type human NK1R. However, the EC_{50} values for all four peptides were about 100-fold higher than those for the wild type (Figure 6). These functional data are consistent with the binding data and demonstrate that residues 21–29 of the NK1R are required for the high-affinity binding of peptides but not the nonpeptide antagonist L-703,606. There was a discrepancy between the EC_{50} value from the functional assay and the IC_{50} value from the binding assay for Septide. This is probably due to the fact that the EC_{50} value for the functional assay is a function of both binding affinity and intrinsic efficacy (Kenakin & Morgan, 1989), and the intrinsic efficacy of Septide may be higher than that of SK or NKB.

Effect of Glycosylation on Peptide Binding Affinity. Both the rat and human NK1Rs have potential N-linked glycosylation sites at asparagine-14 and asparagine-18. To determine whether the glycosylation state of the receptor affects the ligand binding affinity, the double mutant S16A+N18A of the human NK1R was constructed and expressed in COS cells. These substitutions would remove both of the potential glycosylation sites from the human NK1R. As shown in Table II, the binding affinities of all three peptide agonists were not reduced by the mutation.

Effects of Valine-97 to Glutamate Mutation on Peptide Binding. The human NK1R and rat NK1R share a high degree of sequence similarity, with only 22 amino acids differing between the two species. However, the human receptor exhibits a higher affinity for all three neurokinin peptides than the rat receptor (Table II). The most striking

Table II: Agonist Binding Affinities of the Wild Type and Mutant Receptors[a]

receptor	K_d, nM SP	SK	NKB	*t* test[b]
rNK1R	3.2 ± 0.7 (5)	55 ± 26 (3)	179 ± 11 (3)	$p = 0.02$
rNK1R V97E	2.9 ± 0.7 (4)	58 ± 6 (3)	94 ± 22 (4)	
hNK1R	0.7 ± 0.2 (4)	25 ± 6 (4)	57 ± 9 (4)	$p = 0.1$
hNK1R S16A+N18A	0.4 ± 0.1 (3)	13 ± 3 (3)	65 ± 18 (2)	

[a] The K_d value is calculated from the IC_{50} value as described in Materials and Methods. The mean ± SEM are listed. The number of experiments is given in parentheses. Figures 1 and 7 show representative experiments. The mutants are as described in the text, with r representing rat origin and h representing human origin. [b] Student's *t* test of the level of significant difference for the K_d value of NKB compared to that of the rNK1R V97E mutant.

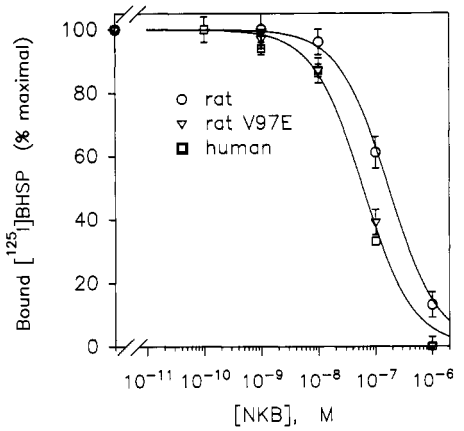

FIGURE 7: Inhibition of [^{125}I]BHSP binding to rat NK1R, V97E mutant of rat NK1R, or human NK1R by NKB. Each point represents the average of triplicate measurements. The error bar represents standard error. The data shown are representative of 3–4 similar experiments.

sequence divergence between the human NK1R and rat NK1R is at position 97 where the rat NK1R contains a valine residue while the human NK1R contains a glutamate residue. To investigate the functional effects of this sequence diversity on binding affinity, a single amino acid substitution of the rat NK1R was constructed to replace the valine-97 residue with a glutamate residue (V97E mutant). This amino acid substitution had no effect on the binding affinity of SP or SK. In contrast, a small but significant increase in the affinity of NKB for the V97E mutant of the rat NK1R was observed, such that the K_d value approached that of NKB binding to the human NK1R (Figure 7; Table II).

DISCUSSION

A central question in receptor research revolves around the mechanism of agonist-induced receptor activation. Before such a question can be answered, it is necessary to locate the ligand binding domain and define the various molecular interactions involved in binding. The NK1R is a member of the G protein coupled receptor family which also includes adrenergic receptors and muscarinic acetylcholine receptors. These receptors share characteristic structural features and a similar route of cellular activation via G proteins. The topological similarity among these receptors would suggest that the ligand binding domain may also be conserved. Previous studies on adrenergic and muscarinic receptors and rhodopsin have demonstrated that the binding sites for these small molecules are located in the bilayer domain (Dohlman et al., 1988; Khorana, 1992; Strader et al., 1989; Wheatley et al., 1988). Because neurokinin peptides are substantially larger than acetylcholine and catecholamines, we have investigated whether the peptide binding domain involves additional residues on the NK1R.

In the present study, substitution of the residues 1–13 of the rat NK1R with an unrelated sequence exerts a small effect on peptide binding. If the substitution is extended to include residues 1–27, however, the high-affinity binding of SP is lost. This decrease in binding affinity could result from the removal of residues critical for peptide binding, or from the introduction of the non-NK1R residues at positions homologous to residues 14–27. Therefore, the deletion mutant rD(2–7,14–27) of the rat NK1R was analyzed, which is similar to the rS(1–27) mutant in length but lacks the acidic substitution. The rD(2–7,14–27) mutant has a reduced affinity for SP as estimated from the EC_{50} for receptor activation, but retains the same maximal level of functional activity in oocytes as the wild type. The equal level of maximal electrophysiological response implies that the receptor number is not affected by the deletion of the N-terminal sequence. Therefore, residues in the region of 14–27 in the NK1R are required for the high-affinity binding of peptides. This is in contrast to the β-adrenergic receptor where deletion of residues before the first transmembrane segment did not affect the binding of catecholamines (Dixon et al., 1987).

In the region of residues 14–27 in the NK1R, there are two potential N-linked glycosylation sites. Previous photoaffinity labeling experiments indicated that the nonglycosylated rat NK1R binds SP with the same affinity as glycosylated NK1R (Kage et al., 1991), suggesting that the carbohydrate substituents on the receptor do not contribute to the high-affinity binding of peptides. Additional support for this conclusion arises from the observation in the present study that eliminating the glycosylation at asparagine-14 and asparagine-18 by site-directed mutagenesis did not reduce the peptide binding affinity (Table II). Therefore, the contribution of residues 14–27 to high-affinity peptide binding reflects a contribution of the amino acid side chains rather than the carbohydrates.

To provide direct evidence supporting the important role of extracellular residues in peptide binding, substitution mutation was introduced into the human NK1R in which antagonist binding as well as agonist binding can be analyzed. The recently reported nonpeptide SP antagonist CP-96,345 possesses a high affinity at the human NK1R, but a reduced affinity at the rat NK1R. An iodinated analog of CP-96,345, [^{125}I]L-703,606, has been shown to be a high-affinity antagonist for the human NK1R (Cascieri et al., 1992). Therefore, we constructed the substitution mutant hS(21–29) in which the extracellular residues 21–29 of the human NK1R were substituted by the analogous residues from the human NK2 receptor. The hS(21–29) mutant was found to exhibit a binding affinity for the antagonist L-703,606 equal to that of the wild-type receptor (Figure 4). In addition, the B_{max} for [^{125}I]L-703,606 binding to the hS(21–29) mutant was equivalent to that of the wild-type receptor, demonstrating that the residues 21–29 are not essential for receptor expression and nonpeptide antagonist binding. In contrast, the hS(21–29) mutant possesses a reduced affinity for SP and related peptides compared to the wild-type receptor (Figure 5). Despite its lower binding affinity for SP, hS(21–29) mutant gives rise to the same maximal level of electrophysiological response in oocytes as the wild-type receptor. Because the substitution in the 21–29 region does not affect the affinity and B_{max} of L-703,606 binding, and the mutations in this region do no affect the maximal physiological response to agonists, it is concluded that these mutations do not affect the overall conformation of the receptor. Therefore, the reduced

affinity for neurokinin peptides binding to the hS(21–29) mutant suggests that amino acids 21–29 are required for the high-affinity interaction between the NK1R and the peptides.

Previous reports have demonstrated that the C-terminal residues 6–11 of SP are minimally required for agonist activity (Cascieri et al., 1985; Wormser et al., 1986). A slight modification of SP(6–11) yielded (pGlu^6,Pro^9)SP(6–11), which is commonly called Septide (Wormser et al., 1986). To investigate which part of the SP peptide might be interacting with residues 21–29 of the NK1R, the activity of Septide was compared to that of SP using the wild type and the hS(21–29) mutant of the human NK1R. Septide is about 20-fold less potent than SP at both the wild type and the hS(21–29) mutant (Figure 6). Therefore, the increased affinity of the undecapeptide SP versus the hexapeptide fragment for the NK1R does not require residues 21–29 in the receptor, assuming that the conformation of Septide is reasonably similar to that of the C-terminal half of SP. In addition, all four peptides tested here displayed reduced potency at the hS(21–29) mutant compared to the wild-type receptor (Figures 5 and 6). The simplest explanation for all the present data is that residues 21–29 in the NK1R interact directly with the conserved C-terminal half of SP. It should be pointed out that there are no 3-D structural data on the complex of receptor and SP, and it is possible that residues 21–29 may stabilize other residues in the receptor which in turn interact with SP. Of the 9 residues in region 21–29 of the human NK1R, only 2 residues are conserved between NK1R and NK2R. Further studies using sited-directed mutants of NK1R and various substituted SP analogs are required to identify any specific interaction between the receptor and peptides.

The present data also suggest that the molecular determinants on the receptor that recognize the divergent N-terminal half of neurokinin peptides are not contained within the N-terminal region of the NK1R. Further insight into the binding domain of the NK1R can be obtained by comparison of the human NK1R with the rat NK1R. Although both receptors bind neurokinin peptides with the same potency order (SP > SK > NKB), the absolute values of their affinities are higher for the human NK1R than the rat NK1R (Table II; Fong et al., 1992). This increased affinity suggests the presence of additional interactions in the human NK1R that are not present in the rat NK1R. Sequence comparison of the receptors from the two species reveals 22 amino acid substitutions. The most structurally divergent substitution is at position 97, where the rat NK1R contains a valine residue while the human NK1R contains a glutamate residue. The V97E mutant of the rat NK1R binds SP and SK with the same affinities as the wild-type rat NK1R. However, the binding affinity of NKB is increased by this substitution (Table II). Hydrophobicity analysis of the NK1R predicts the 97th residue to be located in the second extracellular segment of the receptor. Because the SP and SK binding affinities are not affected by the substitution, it appears that the substitution at the 97th residue does not affect the overall binding site conformation. Rather, the carboxyl group of glutamate at position 97 may interact directly with part of the NKB peptide to increase its binding affinity. This is consistent with the observation that the NK3 receptor from human and rat, which is characterized by its high affinity for NKB, contains a glutamate residue at the homologous position (Huang et al., 1992; Shigemoto et al., 1990). On the basis of these data, it is postulated that the second extracellular segment participates in peptide discrimination by the neurokinin receptors.

In summary, the present results provide evidence that the extracellular domains of the NK1R are required for the high-affinity binding of peptides. Amino acid substitution in the region of 21–29 can greatly affect the peptide binding, but has no effect on the binding of the nonpeptide antagonist L-703,606. These data would be consistent with a model in which both the extracellular domain and the transmembrane domain contribute to peptide binding and selectivity, while the transmembrane domain controls receptor activation., Such a model of peptide receptors represents an extension of the model previously developed for the small molecule receptors in the G protein coupled receptor family.

ACKNOWLEDGMENT

We thank Drs. D. Burns, B. Frances, E. Seward, and C. Swain for synthesizing L-703,606.

REFERENCES

DeBlasi, A., O'Reilly, K., & Motulsky, H. J. (1989) *Trends Pharmacol. Sci. 10*, 227–229.
Cascieri, M. A., Chicchi, G. G., & Liang, T. (1985) *J. Biol. Chem. 260*, 1501–1507.
Cascieri, M. A., Ber, E., Fong, T. M., Sadowski, S., Bansal, A., Swain, C., Seward, E., Frances, B., Burns, D., & Strader, C. D. (1992) *Mol. Pharmacol. 42*, 458–463.
Dixon, R. A. F., Sigal, I., Candelore, M. R., Register, R. B., Scattergood, W., Rands, E., & Strader, C. D. (1987) *EMBO J. 6*, 3269–3275.
Dohlman, H. G., Caron, M. G., Strader, C. D., Amlaiky, N., & Lefkowitz, R. J. (1988) *Biochemistry 27*, 1813–1817.
Fong, T. M., Davidson, N., & Lester, H. A. (1988) *Synapse 2*, 657–665.
Fong, T. M., Anderson, S. A., Yu, H., Huang, R.-R. C., & Strader, C. D. (1992) *Mol. Pharmacol. 41*, 24–30.
Helke, C. J., Krause, J. E., Mantyh, P. W., Couture, R., & Bannon, M. J. (1990) *FASEB J. 4*, 1606–1615.
Hershey, A. D., & Krause, J. E. (1990) *Science 247*, 958–962.
Haung, R. R. C., Cheung, A. H., Mazina, K. E., Strader, C. D., & Fong, T. M. (1992) *Biochem. Biophys. Res. Commun. 184*, 966–972.
Iversen, L. L., Watling, K. J., McKnight, A. C., Williams, B. J., & Lee, C. M. (1987) *Top. Med. Chem. 65*, 1–9.
Kage, R., Hershey, A. D., Krause, J. E., Boyd, N. D., & Leeman, S. E. (1991) *Soc. Neurosci. Abstr. 17*, 805.
Kenakin, T. P., & Morgan, P. H. (1989) *Mol. Pharmacol. 35*, 214–222.
Khorana, H. G. (1992) *J. Biol. Chem. 267*, 1–4.
Nakajima, Y., Tsuchida, K., Negishi, M., Ito, S., & Nakanishi, S. (1992) *J. Biol. Chem. 267*, 2437–2442.
Nakanishi, S. (1991) *Annu. Rev. Neurosci. 14*, 123–136.
Regoli, D., Drapeau, G., Dion, S., & D'Orléans-Juste, P. (1989) *Pharmacology 38*, 1–15.
Shigemoto, R., Yokota, Y., Tsuchida, K., & Nakanishi, S. (1990) *J. Biol. Chem. 265*, 623–628.
Snider, R. M., Constantine, J. W., Lowe, J. A., III, Longo, K. P., Lebel, W. S., Woody, H. A., Drozda, S. E., Desai, M. C., Vinick, F. J., Spencer, R. W., & Hess, H. J. (1991) *Science 251*, 435–437.
Strader, C. D., Sigal, I. S., & Dixon, R. A. F. (1989) *FASEB J. 3*, 1825–1832.
Wheatley, M., Hulme, E. C., Birdsall, N. J. M., Curtis, C. A. M., Eveleigh, P., Pedder, E. K., & Poyner, D. (1988) *Trends Pharmacol. Sci.* (Feb 1988 Suppl.), 19–24.
Womack, M. D., MacDermott, A. B., & Jessell, T. M. (1988) *Nature 334*, 351–353.
Wormser, U., Laufer, R., Hart, Y., Chorev, M., Gilon, C., & Selinger, Z. (1986) *EMBO J. 5*, 2805–2808.
Yokota, Y., Sasai, Y., Tanaka, K., Fujiwara, T., Tsuchida, K., Shigemoto, R., Kakizuka, A., Ohkubo, H., & Nakanishi, S. (1989) *J. Biol. Chem. 246*, 17649–17652.

Registry No. SP, 33507-63-0; SK, 86933-74-6; NKB, 86933-75-7; Septide, 79775-19-2.