Interaction of Substance P with the Second and Seventh Transmembrane Domains of the Neurokinin-1 Receptor

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ABSTRACT: The neurokinin-1 receptor is a member of the G-protein-coupled receptor family and has the highest affinity for the endogenous peptide transmitter substance P. Previous studies have indicated that several residues in the first and second extracellular segments, and at least part of the transmembrane domain, of the human neurokinin-1 receptor are involved in substance P binding to the receptor. To further map the peptide binding site, single-residue substitutions in the transmembrane domains were analyzed. Asn-85, Asn-89, Tyr-92, and Asn-96 in the second transmembrane domain and Tyr-287 in the seventh transmembrane domain are required for the high-affinity binding of peptides, with Asn-85 possibly interacting with the C-terminus of substance P. In addition, Glu-78 in the second transmembrane domain and Tyr-205 in the fifth transmembrane domain appear to be involved in the receptor activation process. Some of the key residues for peptide binding are likely to be near those residues that are required for the binding of competitive antagonists (such as His-197, His-265, and Tyr-287). These data suggest that a volume exclusion effect can explain the competitive antagonism of substance P binding by non-peptide antagonists. Furthermore, the key residues identified thus far are required for the high-affinity binding of all three neurokinin peptides, consistent with a hypothesis that the conformational compatibility between the receptor and the peptide agonist may be a major determinant of peptide recognition.

The undecapeptide neurotransmitter substance P (SP[1]) has been implicated in pain modulation and neurogenic inflammation (Helke et al., 1990; Henry, 1993; Iversen et al., 1987; Regoli et al., 1989). There are three mammalian neurokinin peptides, SP, neurokinin A (NKA), and neurokinin B (NKB), all of which share a consensus C-terminal sequence (FXGLM-NH_2) and have divergent N-terminal sequences. Three neurokinin receptor subtypes (designated NK1R, NK2R, and NK3R) have also been identified, which share significant sequence similarity and belong to the G-protein-coupled receptor family (Fong et al., 1992a; Hershey & Krause, 1991; Nakanishi, 1991). All three receptor subtypes bind all three neurokinins, with the NK1R preferring SP, the NK2R preferring NKA, and the NK3R preferring NKB. Like all G-protein-coupled receptors, the neurokinin receptors are characterized by seven hydrophobic domains which, by analogy with rhodopsin, are postulated to form transmembrane helices. Both tissue studies and heterologous expression of the cloned neurokinin receptors have demonstrated that agonist activation of these receptors leads to the stimulation of the phosphatidylinositol (PI) hydrolysis pathway (Fong et al., 1992a; Nakanishi, 1991; Womack et al., 1988).

To investigate the structure–function relationships of peptide–receptor interactions, we have used the NK1R as a model system. Previous studies have indicated that both the extracellular and transmembrane domains of the NK1R are required for the binding of SP, and several residues in the extracellular domain have been identified as being critical for SP binding, but not for SP-mediated receptor activation (Fong et al., 1992a,b). However, it was unclear which part of the transmembrane region is involved in SP binding and receptor activation. The present studies provide new evidence indicating that residues in the second, fifth, and seventh transmembrane domains play an important role in peptide binding and/or receptor activation. By substituting selected residues postulated to lie within the transmembrane domains and testing the functional effects of these substitutions, we have identified several residues in the helix 2 and helix 7 regions that are required for the high-affinity binding of peptides. Furthermore, Glu-78 in helix 2 and Tyr-205 in helix 5 are required for receptor activation. None of these substitutions affects the binding affinity of the quinuclidine antagonist L-703,606, which is an analog of CP-96,345 (Cascieri et al., 1992; Snider et al., 1991), while Tyr-287 in helix 7 apparently is involved in the binding of the perhydroisoindole antagonist RP67580 (Garret et al., 1991). Combined with previous results on the localization of peptide binding site (Fong et al., 1992a,b), these data suggest that peptide agonists interact with both the transmembrane and extracellular domains of the receptor.

MATERIALS AND METHODS

The human NK1R cDNA and NK2R cDNA were cloned into the pCDM9 vector as described (Fong et al., 1992a; Gerard et al., 1990). All mutations were constructed from human NK1R or NK2R by the uracil selection method of site-directed mutagenesis (Bio-Rad, Richmond, CA). All mutated sequences were confirmed by DNA sequencing. All receptors were expressed in COS cells for determination of ligand binding affinity (Fong et al., 1992a). Intact cells were dissociated nonenzymatically (Specialty Media, Lavallette, NJ) for assays using ^{125}I-labeled SP ([^{125}I]-BHSP, New England Nuclear, Boston, MA) or [^{3}H]-SR48968 (Amersham, Arlington Heights, IL), while membranes were prepared from intact cells and used in assays of [^{125}I]-L-703,606 (Cascieri et al., 1992).

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[1] Abbreviations: BHSP, Bolton–Hunter-labeled substance P; NK1R, neurokinin-1 receptor; NK2R, neurokinin-2 receptor; NK3R, neurokinin-3 receptor; NKA, neurokinin A; NKB, neurokinin B; PI, phosphatidylinositol; SP, substance P; SPOME, substance P methyl ester.

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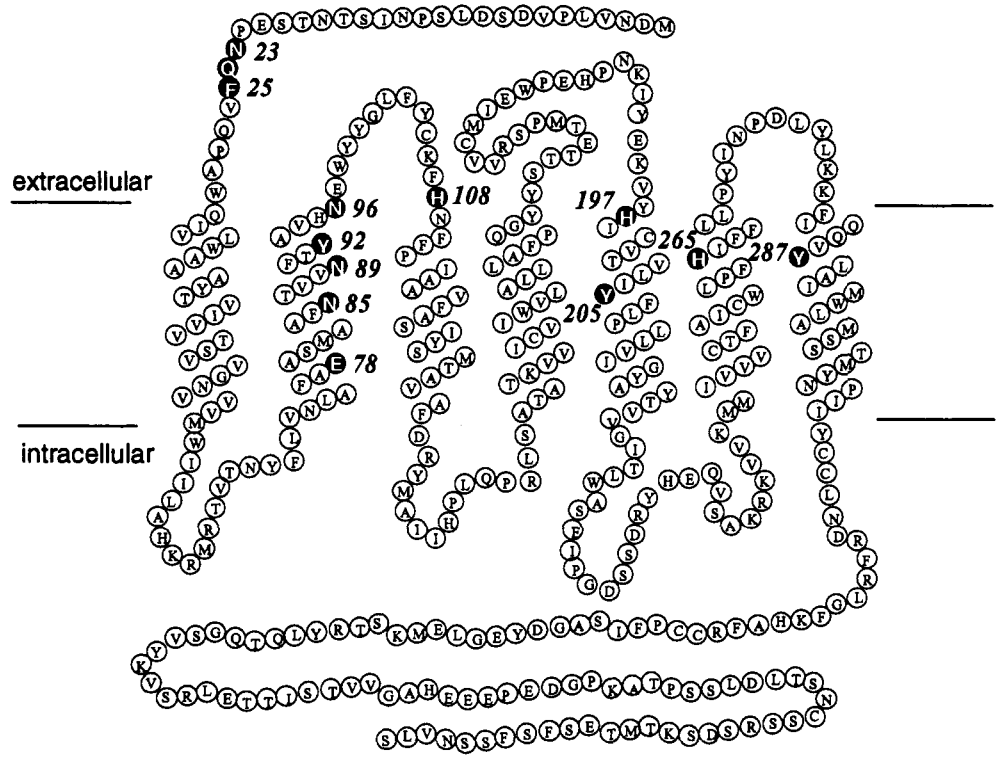

FIGURE 1: Schematic model of the human NK1R showing the postulated transmembrane topology. The locations of mutated residues in the present and previous studies are highlighted.

The binding affinities of various ligands for the wild-type and mutant human NK1R were determined using [^{125}I]-BHSP and intact COS cells in the presence of varying concentrations of unlabeled ligands or using [^{125}I]-L-703,606 and membrane samples in the presence of varying concentrations of unlabeled ligands (Fong et al., 1992a). Briefly, the binding reaction mixture contained radiolabeled ligand (0.2 nM for [^{125}I]-SP or [^{125}I]-L-703,606), unlabeled ligands at various concentrations, and intact COS cells expressing the wild-type or the mutant receptor (or membranes prepared from these cells in the case of [^{125}I]-L-703,606) in 0.24 mL of 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM $MnCl_2$, 0.04 mg/mL bacitracin, 0.004 mg/mL leupeptin, 0.2 mg/mL bovine serum albumin, and 0.01 mM phosphoramidon. The receptor concentration in the binding mixture was adjusted so that the bound counts per minute (cpm) in the absence of unlabeled ligand were less than 10 000 cpm. Binding was performed at 4 °C for 1 h and terminated by filtration through GF/C paper. The data were fit to the equation, $[\text{cpm}(L) - \text{cpm}(1\ \mu\text{M SP})]/[\text{cpm}(0) - \text{cpm}(1\ \mu\text{M SP})] = IC_{50}/(L + IC_{50})$, in which cpm($L$) and cpm(0) represent bound radiolabeled ligand in the presence or absence of unlabeled ligand, respectively, L represents the concentration of unlabeled ligand, and IC_{50} represents the concentration of unlabeled ligand that causes 50% inhibition of the specifically bound radiolabeled ligand.

The binding affinities of the wild-type and mutant human NK2R were measured in a manner similar to those of NK1R with a few modifications. Intact cells and 0.5 nM [^{3}H]-SR48968 were used in a total volume of 0.5 mL. The incubation was performed at 23 °C for 1 h. The receptor concentration in the binding mixture was adjusted such that the bound cpm in the absence of unlabeled ligand was less than 1000 cpm.

The formation of total inositol phosphates (inositol mono-, bis-, and trisphosphates) was measured essentially as described by Yokota et al. (1992) and Zhu et al. (1992) with minor modifications. Briefly, 10^7 COS cells were electroporated in the presence of 10 μg of the plasmid DNA and distributed evenly to 12-well plates at about 50 000 cells per well in 10% FBS and 90% DMEM supplemented with 100 units/mL penicillin–0.1 mg/mL streptomycin. Each well also contained 10 μL of [^{3}H]inositol (1 mCi/mL, 24.4 Ci/mmol). Two days after the transfection, the media were removed, each well was washed twice with PBS containing 10 mM LiCl, and LiCl was loaded into cells by incubation in the washing buffer at 37 °C for 30 min. Fresh washing buffer (1 mL) containing agonist (or agonist plus antagonist) at various concentrations was added to each well and incubated at 37 °C for 30 min. The reaction was stopped by removing the buffer, adding 500 μL of ice-cold 5% TCA, and standing at 4 °C for 10 min. The solubilization mixture was applied to a column containing Dowex AG1-X8 (formate form). The column was washed with 10 mL of 5 mM inositol, and total inositol phosphates were eluted with 4 mL of 1 M ammonium formate–0.1 M formic acid. The total inositol phosphates were quantitated by scintillation counting.

The non-peptide antagonists used in the present studies were cis racemates and were synthesized as described previously (Cascieri et al., 1992; Lowe et al., 1992; Peyronel et al., 1992). All peptides were purchased from Peninsula Laboratories.

RESULTS

Effects of Receptor Mutations on Binding Affinities of Peptides for the NK1R. To investigate the role of the transmembrane domain of the human NK1R in peptide binding, we have substituted several residues in the helical regions (Figure 1) and analyzed the ligand binding properties of the resulting mutant receptors. As shown in Figure 2, individual substitution of three conserved residues in the second transmembrane domain (Asn-85, Asn-89, or Tyr-92) with Ala resulted in the loss of high-affinity binding of SP, while the binding affinities of the antagonists L-703,606 and

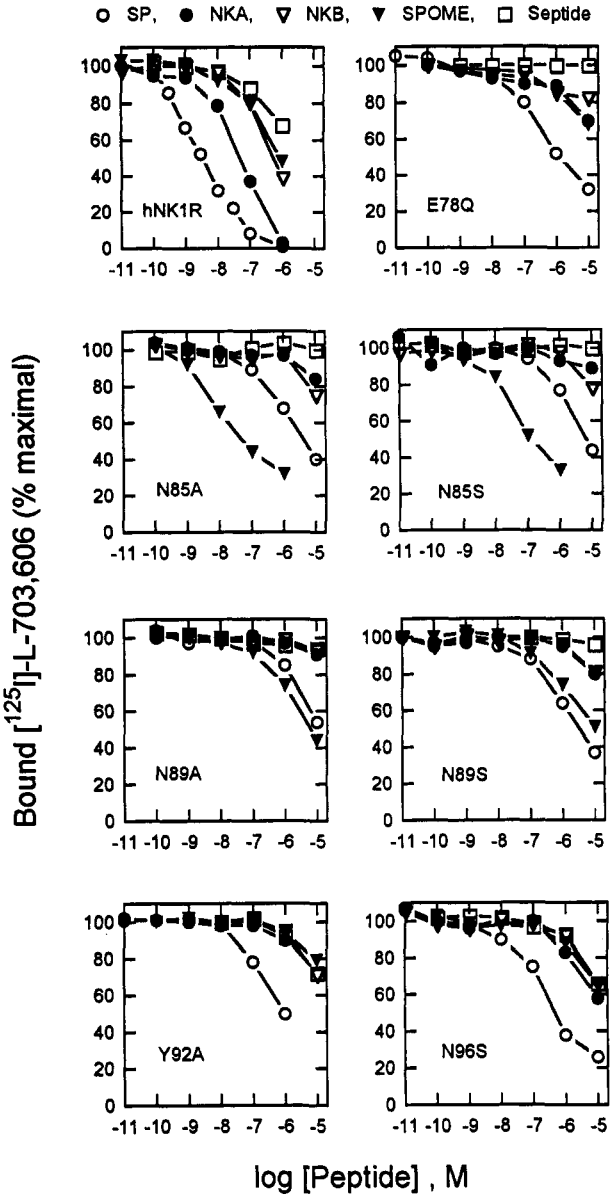

FIGURE 2: Inhibition of [^{125}I]-L-703,606 binding to the wild-type or mutant NK1R by various peptides: o, SP (RPKPQQFFGLM-NH_2); ●, NKA (HKTDSFVGLM-NH_2); ▽, NKB (DMHDFFVGLM-NH_2); ▼, SPOME (RPKPQQFFGLM-OCH_3); □, septide ([pGlu^6,Pro^9]SP_{6-11}). The 100% control is the maximal level of bound [^{125}I]-L-703,606 in the absence of unlabeled ligands. Each curve is the average of 2–5 independent experiments. The identity of each receptor is indicated inside each graph.

RP67580 were not affected (Table 1). Substitution of Asn-85 or Asn-89 with Ser (Figure 2) or Gln (data not shown) did not restore the high-affinity binding of SP. On the other hand, substitution of Tyr-92 by Phe completely restored the high-affinity binding of all neurokinin peptides (Tables 1 and 2). Another residue located near the top of helix 2, Asn-96, is not conserved among the three subtypes of NK receptors. When Asn-96 was substituted with Ser (the NK3 receptor homolog), the binding affinities of SP, NKA, and NKB were all reduced, whereas the antagonist binding affinity was not affected (Table 1 and Figure 2). Similar to the Asn-85 and Asn-89 residues, substitution of Asn-96 with Gln did not restore the high-affinity binding of peptide agonists (data not shown). These data suggest that the side chains of Asn-85, Asn-89, Tyr-92, and Asn-96 are required for the high-affinity binding of SP to the NK1R. The absence of an effect of these substitutions on antagonist binding affinity indicates that these

Table 1: Comparison of Binding Affinities of SP and Antagonists for the Wild-Type NK1R and Substitution Mutants[a]

	IC_{50} (nM)		
receptor	SP	L-703,606	RP67580
hNK1R	4 ± 0.7 (4)	0.3 ± 0.1 (2)	14 ± 2 (3)
E78A	110 ± 10 (2)	0.7 ± 0.2 (2)	40 (2)
E78Q	1517 ± 500 (4)	0.6 ± 0.1 (2)	90 (2)
N85A	3550 ± 650 (2)	0.3 ± 0.1 (2)	8 ± 2 (2)
N89A	9000 ± 500 (2)	0.3 ± 0.1 (2)	24 ± 5 (2)
Y92A	1000 ± 300 (2)	0.3 ± 0.1 (2)	12 ± 2 (2)
Y92F	10 ± 5 (2)	0.3 ± 0.1 (2)	25 ± 3 (2)
N96S	180 ± 12 (3)	0.3 ± 0.1 (3)	11 ± 3 (2)
Y205A	150 ± 50 (2)	0.9 (2)	15 (2)
Y287A	10000 ± 1000 (2)	1.0 ± 0.3 (2)	170 ± 20 (2)
Y287F	325 ± 125 (2)	0.3 ± 0.1 (2)	28 ± 3 (13)
Y287Q	>1000 (2)	1.0 ± 0.1 (2)	139 ± 43 (2)
Y287W	>1000 (2)	0.4 ± 0.1 (2)	527 ± 85 (2)
Y287S	>1000 (2)	1.1 ± 0.2 (2)	225 ± 5 (2)
Y287H	20 ± 6 (2)	0.6 ± 0.2 (2)	37 ± 7 (3)

[a] The IC_{50} values were derived from the inhibition of [^{125}I]-L-703,606 binding. Mean ± SEM is shown. The number of independent experiments is included in parentheses.

amino acid substitutions do not cause a large-scale distortion of the receptor conformation.

In order to localize which part of the peptide may interact with residues in helix 2, the affinity of SP was compared with those of NKA, NKB, SP methyl ester (SPOME), and septide. SPOME is similar in structure to SP, with the exception of the C-terminal amide to ester substitution. Septide ([pGlu^6,Pro^9]SP_{6-11}) is an analog of the C-terminal half of SP. For the human NK1R, the rank order of potency for peptide agonists in inhibiting antagonist [^{125}I]-L-703,606 binding is SP > NKA > NKB = SPOME > septide (Figure 2). When Asn-85 was substituted with Ala or Ser, the binding affinity of SPOME for the N85A or N85S mutant was increased compared to that for the wild-type receptor, while the affinities of all other peptides were reduced compared to those of the wild-type receptor. When Asn-89, which is one helical turn above Asn-85, was substituted with Ala or Ser, the affinity of SPOME was slightly reduced, whereas the affinities of all other peptides were substantially reduced. For other mutant receptors, such as Y92A or N96S, the affinities of all peptides tested here were substantially reduced (Figure 2).

Previous studies have suggested that one divergent residue at position 291 of helix 7 can slightly modulate the peptide binding affinity (Fong et al., 1992b). To further investigate the role of helix 7 in peptide binding, a nearby residue (Tyr-287) was substituted. As shown in Table 1, the Y287A substitution resulted in a substantial reduction in SP affinity without affecting the affinity of L-703,606. This substitution also reduced the affinity of the perhydroisoindole antagonist, RP67580. When Tyr-287 was substituted by other residues, only the Y287H substitution restored the high-affinity binding of SP, while both the Y287H and Y287F substitutions restored the high-affinity binding of RP67580 (Tables 1 and 2).

Effects of Receptor Mutations on NK1R Activation. To test whether any of the above amino acid substitutions affect the receptor activation process, the agonist-stimulated PI hydrolysis was measured. As shown in Figure 3A, the accumulation of total inositol phosphates was approximately linear with up to 1-h exposure to SP. Thus, a single time point measurement after 30 min of incubation would give an estimate of the initial rate of PI hydrolysis. Although the substitution of Asn-85, Asn-89, Tyr-92, Asn-96, or Tyr-287 with Ala resulted in substantial reduction in the affinity of

Table 2: Comparison of Binding Affinities of Agonists and Antagonists for the Wild-Type NK1R and Two Substitution Mutants[a]

	IC_{50} (nM)				
receptor	SP	NKA	NKB	L-703,606	RP67580
hNK1R	0.6 ± 0.2 (7)	31 ± 5 (5)	81 ± 13 (5)	1.0 ± 0.3 (4)	18 ± 3 (4)
Y92F	0.5 ± 0.2 (4)	22 ± 2 (2)	33 ± 5 (2)	0.7 ± 0.1 (2)	28 ± 5 (2)
Y287H	0.6 ± 0.3 (2)	50 ± 20 (2)	117 ± 5 (2)	0.9 ± 0.1 (2)	36 ± 5 (2)

[a] The IC_{50} values were derived from the inhibition of [^{125}I]-BHSP binding. Mean ± SEM is shown. The number of independent experiments is included in parentheses.

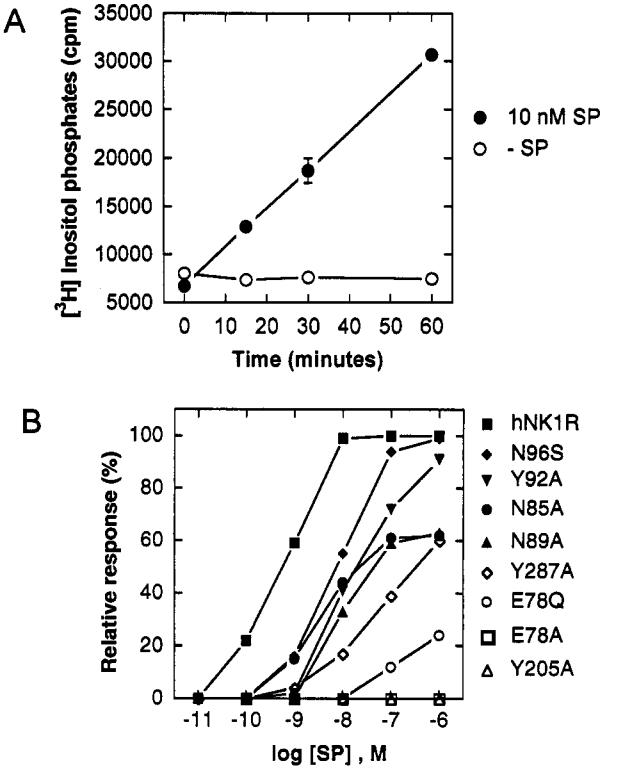

FIGURE 3: (A) Time course of stimulation of PI hydrolysis in response to 10 nM SP mediated by the wild-type human NK1R. ○ represents data in the absence of SP, and ● represents data in the presence of 10 nM SP. (B) Dose-response curves of SP-stimulated PI hydrolysis for the wild-type and various mutant NK1Rs. The accumulated inositol phosphates were measured after exposure to SP for 30 min. The relative response is defined as the specific increase in [^{3}H]inositol phosphates in response to SP and is normalized to the response mediated by the wild-type human NK1R in response to 1 μM SP. Each curve is the average of 3–5 independent experiments. ■, wild-type human NK1R; ◆, N96S mutant; ▼, Y92A mutant; ●, N85A mutant; ▲, N89A mutant; ◇, Y287A mutant; ○, E78Q mutant; □, E78 A mutant; △, Y205A mutant.

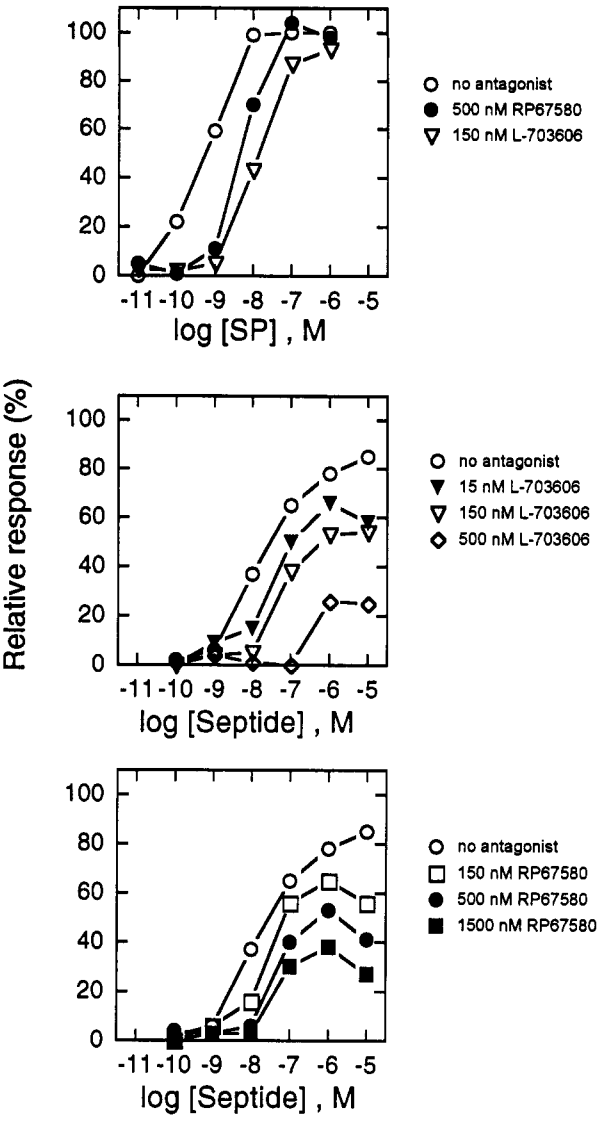

FIGURE 4: Dose-response curves of agonist-stimulated PI hydrolysis mediated by the wild-type human NK1R. The top panel represents SP-stimulated response in the absence (○) or presence of antagonist (●, 500 nM RP67580; ▽, 150 nM L-703,606). The middle panel represents septide-stimulated response in the absence (○) or presence of L-703,606 (▼, 15 nM L-703,606; ▽, 150 nM L-703,606; ◇, 500 nM L-703,606). The bottom panel represents septide-stimulated response in the absence (○) or presence of RP67580 (□, 150 nM RP67580; ●, 500 nM RP67580; ■, 1500 nM RP67580). The data represent the average of 2–5 independent experiments, and all data are normalized to the response in the presence of 1 μM SP.

SP, these mutant receptors were functional in mediating agonist-stimulated PI hydrolysis (Figure 3B). The increased EC_{50} values of the dose-response curves for these mutant receptors were consistent with the reduced SP binding affinity as measured with [^{125}I]-L-703,606. In contrast, two residues appear to be critical for receptor activation. Substitution of Glu-78 with Ala (the E78A mutant in Table 1) resulted in a reduction in the affinity of SP, while the binding affinity for the antagonist L-703,606 was not significantly affected. More importantly, several agonists (SP, NKA, NKB, SPOME, or septide at concentrations up to 1 μM) did not stimulate PI hydrolysis mediated by the E78A mutant. On the other hand, the E78Q mutant showed a weak response with high concentrations of SP (Figure 3B). Another residue, Tyr-205 in helix 5, appears to play a role similar to that of Glu-78. The Y205A mutant receptor did not mediate a PI response and displayed a reduced affinity for SP, although the antagonist binding affinity was identical to that of the wild-type receptor (Table 1). These data suggest that Glu-78 and Tyr-205 are involved in the peptide-induced receptor activation process.

Inhibition of Agonist-Elicited NK1R Activation by Antagonists. Of those residues that have been identified as being important for the binding of peptide agonists or non-peptide antagonists, Tyr-287 has been found to be involved in the binding of both peptide agonists and the perhydroisoindole antagonist RP67580. In contrast, these residues do not appear to interact with the quinuclidine antagonist CP-96,345 (or L-703,606). However, ligand dissociation experiments and Schild analysis of functional assays indicate that CP-96,345, L-703,606, and RP67580 are competitive antagonists of SP binding (Cascieri et al., 1992; Garret et al., 1992; Snider et al., 1991). As shown in Figure 4 and by Cascieri et al. (1992), both L-703,606 and RP67580 shifted the dose-response curve

Table 3: Binding Affinities for the Human NK2R and Two Substitution Mutants[a]

receptor	IC_{50} (nM)	
	NKA	SR48968
hNK2R	219 ± 52 (6)	1.2 ± 0.2 (5)
H198A	2000 ± 1000 (2)	1.5 ± 0.5 (2)
H267Y	1380 ± 700 (2)	1.8 ± 0.2 (2)

[a] The IC_{50} values were derived from inhibition of [^{3}H]-SR48968 binding. Mean ± SEM is shown. The number of independent experiments is included in parentheses.

of SP-stimulated PI hydrolysis for the wild-type NK1R to the right without affecting the maximal level of response. In contrast, the effect of these antagonists on septide-stimulated PI hydrolysis was more complex. The addition of RP67580 or L-703,606 decreased the maximal response and increased the EC_{50} values of the septide dose-response curves. While septide appears to be a partial agonist compared to SP, these data are consistent with a competitive antagonism of SP activation by these non-peptide antagonists and a noncompetitive mechanism of antagonism of septide activation.

Effects of Receptor Mutations on Binding Affinities of Peptides for the NK2R. We have previously shown that substitution of His-197 with Ala, or substitution of His-265 with Tyr, in the human NK1R results in a reduced binding affinity for non-peptide antagonists (such as CP-96,345 or RP67580) but not peptide agonists (Fong et al., 1993, 1994). Both of these His residues are conserved among the three subtypes of neurokinin receptors. To test whether the two conserved residues serve a similar function in other neurokinin receptor subtypes, we have constructed analogous substitutions in the human NK2R. In contrast to the human NK1R, both the H198A and H267Y mutants of the human NK2R exhibited negligible levels of [^{125}I]-NKA binding, while the binding affinity of the NK2 antagonist [^{3}H]-SR48968 was not affected in either mutant receptor (Table 3). The reduced affinity for NKA in both mutant receptors was further confirmed by the decreased potency of NKA in inhibiting the binding of [^{3}H]-SR48968 compared to the wild-type NK2R.

DISCUSSION

Mutagenesis studies of the extracellular domain of the NK1R have indicated that several residues in the first and second extracellular segments are specifically involved in peptide binding (Fong et al., 1992a,b). To further map the peptide binding domain in the NK1R, several residues in the transmembrane domain were substituted with Ala or other amino acids in the present study. Five residues in helix 2 (Glu-78, Asn-85, Asn-89, Tyr-92, and Asn-96) and one residue (Tyr-205) in helix 5 have been identified as playing a role in the high-affinity binding of peptide agonists or agonist-mediated receptor activation. The five residues in the second transmembrane domain would be located on the same hydrophilic face of a putative α-helical structure, but they appear to play different roles in receptor function. For the E78A and Y205A mutant receptors, the ability of SP to activate PI hydrolysis is completely abolished in these two mutant receptors. The lack of high-affinity SP binding in the E78A or Y205A mutants is also consistent with the failure of these mutant receptors to interact with G-proteins. Taken together, these data suggest that Glu-78 and Tyr-205 are mainly involved in receptor activation, although an additional role in peptide binding cannot be ruled out. An Asp (or Glu) residue in helix 2 and a Tyr (or Phe) residue in helix 5 are present at analogous positions of most G-protein-coupled receptors, suggesting that these two residues may participate in receptor activation in general. Mutagenesis studies on other G-protein-coupled receptors have also indicated that the homologous Asp residue in the helix 2 region is involved in receptor activation (Strader et al., 1988; Perlman et al., 1992; Neve et al., 1991; Bihoreau et al., 1993). The role of the conserved aromatic residue in helix 5 in the activation of other G-protein-coupled receptors remained to be explored.

In contrast to the substitution of Glu-78 or Tyr-205, substitution of Asn-85, Asn-89, Tyr-92, or Asn-96 in the second transmembrane domain with either Ala or Ser reduces the SP binding affinity, while these mutant receptors can still be activated. The EC_{50} values for SP-stimulated PI hydrolysis are increased by these substitutions, consistent with the reduced affinity of these mutant receptors for SP. Therefore, these four residues in the second transmembrane domain of the NK1R appear to be primarily involved in peptide binding. To identify which part of the SP peptide may interact with these residues, analogs of SP were analyzed. When Asn-85 is substituted with Ala, the affinity of SPOME is increased while the affinity of SP is decreased, suggesting that the C-terminus of SP may bind at or near Asn-85. This hypothesis is consistent with the larger size of methyl ester compared to amide, which may explain the reduced affinity of SPOME for the wild-type receptor compared to SP. With the N85A or N85S mutant receptor, the larger methyl ester group could be better accommodated due to the smaller side chains of Ala or Ser, perhaps leading to a new interaction between the methyl ester moiety and the receptor. The increased affinity of SPOME is not observed when other residues above Asn-85 are substituted. For example, the affinity of SPOME is slightly reduced in the N89A mutant and substantially reduced in the Y92A or N96S mutant. Furthermore, substitution of Tyr-92 or Asn-96 results in reduced affinities for all peptides tested, suggesting that these two residues probably interact with a group(s) common to all neurokinins, but not the C-terminal amide. The nature of the interactions between the peptides and Asn-85, Asn-89, or Asn-96 of the NK1R is likely to be hydrogen-bonding interactions with stringent requirements for intermolecular distance, because neither Ser nor Gln could substitute for Asn at any of these positions. On the other hand, Tyr-92 appears to be involved in an aromatic interaction with the peptides because the Y92F mutant receptor is functionally identical to the wild-type receptor. However, the present results cannot rule out the possibility that Asn-96 and Tyr-92 may be important for maintaining the local conformation of the peptide binding site.

The seventh transmembrane domain is also important for the binding of peptides because the Y287A substitution reduces the affinities of SP, NKA, and NKB without affecting the affinity of L-703,606. The Y287H mutant receptor has the same affinity for peptides as the wild-type receptor, while Phe, Gln, Trp, or Ser could not substitute for Tyr-287 in restoring the high-affinity binding of peptides. These data indicate that a small aromatic residue at position 287 is required for the high-affinity binding of peptides, with possible hydrogen-bonding interactions between Tyr-287 and peptide agonists. In contrast, the perhydroisoindole antagonist RP67580 requires either Tyr, His, or Phe at position 287 for its high-affinity binding. These data would be consistent with an aromatic interaction rather than a hydrogen-bonding interaction between Tyr-287 and RP67580. The observation that residues in both helix 2 and helix 7 are required for peptide binding to the NK1R is also consistent with a hypothetical

model of the NK1R in which the seven transmembrane domains are arranged as a bundle, with helix 2 and helix 7 within the limit of flexibility of SP. Because all of the single amino acid substitutions analyzed to date [also see Fong et al. (1992b)] lead to either substantially reduced binding affinities for all three neurokinin peptides or only slightly increased affinities for some peptides, it is possible that the differential peptide binding affinities of the NK1R result from the conformational compatibility between each peptide and the NK1R. This type of conformation-dependent binding mechanism has also been shown for the growth hormone–receptor complex, in which each of the homodimer binding sites is composed of identical residues but the two sites adopt slightly different conformations upon ligand binding (De Vos et al., 1992). Furthermore, the solution conformations of neurokinin peptides are dependent on the peptide sequence (Levian-Teilelbaum et al., 1989; Saviano et al., 1991), providing a potential mechanism for conformation-dependent peptide recognition.

Mutational analysis of the NK2R also indicates that conformational effects may contribute to the receptor selectivity of a given peptide as well. The NK1R and NK2R share 39% overall sequence similarity, with 63% amino acid identity within the transmembrane domain. The sequence and functional similarities between these two receptors suggest that their overall structures are likely to be similar. However, His-198 and His-267 of the NK2R are required for the high-affinity binding of NKA (Table 3), whereas the analogous residues in the NK1R (His-197 and His-265) are not involved in the binding of neurokinin peptides (Fong et al., 1993, 1994). These data are consistent with the hypothesis that the distance between a bound peptide and the two His residues is dependent on the peptide conformation and/or receptor binding site conformation, and such a difference in intermolecular distance would be reflected in the sensitivity of peptide binding affinity to the amino acid substitution.

The present and previous studies (Fong et al., 1992b, 1993, 1994) on the ligand binding site of the NK1R have demonstrated that the peptide binding site probably involves at least nine residues in the transmembrane and extracellular domains (Asn-23, Gln-24, Phe-25, Asn-85, Asn-89, Tyr-92, Asn-96, His-108, and Tyr-287), while the binding site for small-molecule antagonists includes at least three residues in the transmembrane domains (His-197, His-265, and Tyr-287). Chimeric receptor studies are also consistent with the nonidentical nature of agonist and antagonist binding sites (Fong et al., 1992b; Gether et al., 1993). Therefore, it appears that peptide agonists and non-peptide antagonists do not utilize the same set of amino acid residues for their binding. Nevertheless, the available non-peptide antagonists appear to be competitive antagonists of SP binding to the NK1R (Snider et al., 1991; Cascieri et al., 1992). These data would suggest that the competitive behavior can arise from a volume exclusion effect, in which the binding pockets for agonists and antagonists spatially overlap, even though agonists and antagonists do not utilize the same set of residues for their interactions with the NK1R. The overlap of the agonist-occupied space and the antagonist-occupied space is indirectly supported by the observation that His-198 and His-267 in the NK2R are required for neurokinin binding, suggesting that the NK1R-bound neurokinin is in the vicinity of His-197 and His-265 of the NK1R. This conclusion is also supported by the data in Figure 4, in which the inhibition of SP-elicited responses by L-703,606 or RP67580 can be completely surmounted by higher concentrations of SP [also see Cascieri et al. (1992)]. In contrast, the inhibition of septide-elicited responses by antagonists cannot be fully surmounted by higher concentrations of septide. Since the undecapeptide SP is about twice as large as the hexapeptide septide, it is likely that there is relatively less spatial overlap between bound septide and L-703,606 than between bound SP and L-703,606, thus giving rise to the apparently noncompetitive relationship of septide and L-703,606. It is also important to point out that the nonidentical nature of agonist and antagonist binding sites does not rule out any possibility that SP and these antagonists interact with some as yet unidentified common groups in the NK1R.

In summary, these studies demonstrate the important role of the transmembrane domains of the NK1R in both peptide binding and receptor activation. Analysis of several SP analogs further suggests that the C-terminal amide group of SP may interact with Asn-85 in the second transmembrane domain, whereas Glu-78 and Tyr-205 in the second and fifth transmembrane domains are critical for receptor activation. In contrast, the fifth, sixth, and seventh transmembrane domains appear to make direct contact with non-peptide antagonists and are likely to be near the bound peptides. These results will facilitate the development of a structural model for other peptide receptors in the G-protein-coupled receptor family.

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