0026-895X/94/040690-06$03.00/0

MOLECULAR PHARMACOLOGY, 45:690-695

Localization of the Ligand Binding Site of the Neurokinin-1 Receptor: Interpretation of Chimeric Mutations and Single-Residue Substitutions

RUEY-RUEY C. HUANG, HONG YU, CATHERINE D. STRADER, and TUNG MING FONG

Department of Molecular Pharmacology and Biochemistry, Merck Research Laboratories, Rahway, New Jersey 07065

Received September 16, 1993; Accepted January 20, 1994

SUMMARY

Previous studies have indicated that substitution of the third or fourth extracellular segment of the human neurokinin-1 receptor with the equivalent segment from the neurokinin-3 receptor affects the binding affinities of peptide agonists and/or nonpeptide antagonists. To elucidate the roles of the divergent residues within these domains in ligand binding, single-residue substitutions in these regions were analyzed. Neurokinin B affinity was increased by one single-residue substitution (E172K) in the third extracellular segment and one double-residue replacement (L279R+K280W) in the fourth extracellular segment, and the affinity for the antagonist L-703,606 was reduced by one single-residue substitution (Y272A) in the fourth extracellular segment. The effect of these three specific substitutions is consistent with the prediction of chimeric mutations. However, the substantial reduction in ligand binding affinity observed upon multiple-residue substitutions in the third extracellular segment (residues 176–183 or 187–195) has not been reproduced by eliminating potential electrostatic interactions or substituting with the corresponding residues from the neurokinin-3 receptor, suggesting that the reduction in ligand binding affinity observed with some chimeric receptors is not due to the loss of direct electrostatic interactions. These data indicate that other factors such as conformational effects may complicate the interpretation of data obtained with chimeric receptors, and they demonstrate the need to evaluate chimeric receptors along with single-residue substitutions in the same region to localize specific residues involved in ligand binding. Furthermore, the available data suggest that one major determinant of peptide selectivity in the neurokinin-1 receptor may be the conformational compatibility between a peptide and the receptor.

SP is involved in a wide spectrum of biological activities, such as pain modulation and neurogenic inflammation (1–5). It binds to the NK1R with high affinity. Two related NK peptides (NKA and NKB) bind preferentially to two other receptor subtypes, the NK2R and the NK3R, respectively. However, all three peptides share a common carboxyl-terminal sequence (FXGLM-NH_2) and bind to all three receptor subtypes, albeit with different affinities. The rank order of potency for the NK1R is SP > NKA > NKB, whereas it is NKA > NKB > SP for the NK2R and NKB > NKA > SP for the NK3R. These receptors belong to the G protein-coupled receptor family, characterized by seven hydrophobic transmembrane domains. The major sequence conservation among the three NK receptors lies within the transmembrane domain (58% amino acid similarity) (4, 6). Because of the sequence conservation among both the receptors and the ligands, the NK receptors provide a model system with which to study the molecular basis of biological recognition.

A fundamental goal in studies of ligand-receptor recognition is the identification of specific molecular interactions between the ligand and the receptor. To achieve this goal, mutational analysis of receptors has been widely used in systems where high-resolution three-dimensional structures are not available (7, 8). Several recent studies have attempted to localize the ligand-binding domain of the NK1R by analyzing chimeric receptors (9–12). Focusing on the extracellular domain of the human NK1R, we have previously reported that substitution of part of the third extracellular segment (residues 170–173) or of the fourth extracellular segment (residues 271–280) of the human NK1R with the homologous sequence of the human NK3R results in an increase in NKB affinity, without affecting SP affinity (9). On the other hand, substitution of residues 187–195 in the third extracellular segment or residues 271–280 in the fourth extracellular segment leads to a reduction in the affinity for L-703,606, which is an iodinated analog of the quinuclidine antagonist CP-96,345 (13, 14). In addition, substitution of residues 176–183 in the third extracellular segment results in substantial reduction in the affinity for peptides, without affecting antagonist affinity.

Thus, analysis of chimeric receptors has suggested that the

ABBREVIATIONS: SP, substance P; BH, Bolton-Hunter-labeled; NK, neurokinin; NK1R, neurokinin-1 receptor; NK2R, neurokinin-2 receptor; NK3R, neurokinin-3 receptor.

extracellular domain is important for ligand binding. However, because multiple residues are replaced in these chimeric receptors, these studies cannot identify the specific residues that interact directly with ligands. In the present study, the precise role of the divergent residues in the third and fourth extracellular segments was investigated by analyzing single-residue substitutions in these regions. The increased NKB affinity observed in the chimeric receptors can be partially reproduced by single- or double-residue substitutions in these regions, and one residue has been identified as playing a role in antagonist binding. However, the substantial reduction in peptide or antagonist affinity observed when residues 176–183 or 187–195 were substituted has not been mimicked by eliminating potential electrostatic interactions contributed by side chains in these regions. The results support a model for the peptide binding site that involves mainly the first and second extracellular segments and the transmembrane domain of the NK1R.

Materials and Methods

All mutants were constructed from the human NK1R by the uracil selection method of site-directed mutagenesis (Bio-Rad). All mutated sequences were confirmed by DNA sequencing. All receptors were expressed in COS cells for determination of ligand binding affinity (15). The nonpeptide antagonists used in the present studies were *cis*-racemates and were synthesized as described previously (13, 16, 17).

The binding affinities of various ligands for the wild-type and mutant receptors were determined using ^{125}I-BH-SP in the presence of varying concentrations of unlabeled ligands (15). Briefly, the binding reaction mixture contained 0.2 nM radiolabeled ligand, unlabeled ligands at various concentrations, and intact COS cells expressing the NK1R or the mutant receptor, 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, 0.01 mM phosphoramidon. The cell number was adjusted so that the radioactivity bound in the absence of unlabeled ligand was <10,000 cpm. Binding was performed at 4° for 1 hr and was terminated by filtration through GF/C filters. The data were fitted to the equation $[cpm(L) - cpm(1\ \mu M\ SP)]/[cpm(0) - cpm(1\ \mu M\ SP)] = IC_{50}/(L + IC_{50})$, in which $cpm(L)$ and $cpm(0)$ represent bound radiolabeled ligand in the presence or the 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. In the case of some mutant receptors where the level of ^{125}I-BH-SP binding was very low, membranes were prepared from the transfected cells and used for ^{125}I-L-703,606 binding (13, 15).

Results

We previously determined that substitution of residues 170–173 (TTET) in the third extracellular segment of the human NK1R with residues at similar positions in the human NK3R (KTKV) (the E3a mutant in Table 1; see Fig. 1) caused a 3-fold increase in the affinity of NKB without affecting the affinities of SP or the nonpeptide antagonist L-703,606. To investigate whether the lysine residues of the NK3R could contribute additional binding energy, Thr-170 and Glu-172 were substituted individually with lysine. Substitution of Thr-170 (T170K) did not increase the affinity of NKB for the NK1R (Table 1). On the other hand, substitution of Glu-172 by lysine (E172K) caused a 2-fold increase in NKB affinity, with a concomitant decrease in NKA affinity. However, replacement of Glu-172 by alanine did not affect the binding affinities of any of the ligands tested, suggesting that Glu-172 is not a critical determinant of the differential affinities of the three natural peptides for the NK1R.

Substitution of residues 176–183 (SRVVCMIE) in the third extracellular domain of the human NK1R with residues at similar positions in the human NK3R (GRTLCFVQ) (the E3b mutant in Table 1) resulted in a substantial reduction in the affinities of all three peptides, without affecting antagonist affinity. Such a reduction in peptide binding affinity could be due to the elimination of hydrogen bonds or other electrostatic interactions between peptides and the substituted NK1R. In the present study, three divergent residues capable of forming hydrogen bonds in this region of the NK1R were substituted individually with the corresponding NK3R residue (S176G, M181F, and E183Q). These mutant receptors bound all three peptides with the same affinities as did the wild-type NK1R (Table 1).

Substitution of residues 187–195 (HPNKIYEKV) in the third extracellular segment of the human NK1R with residues at similar positions in the human NK3R (GPKQHFT, which is two residues shorter than the segment of the NK1R) (the E3c mutant in Table 1) resulted in a 200-fold reduction in affinity of the antagonist L-703,606, without significantly affecting the peptide affinities. In the present study, all divergent residues in the E3c region were substituted or deleted individually or in pairs, guided by the alignment shown in Fig. 1, in which the highly conserved residues were anchored and the gap was placed in the middle. As shown in Table 1, deletion of two residues (ΔK190+I191), a double-residue substitution (H187G+N189K), and single-residue substitutions (Y192Q, E193A, or K194A) failed to reproduce the substantial reduction in the affinity of L-703,606. Similarly, we previously reported that Val-195 could be substituted with alanine without affecting the affinities of antagonists (18). Only one mutation in this region (E193H) resulted in a loss of detectable binding of both ^{125}I-BH-SP and ^{125}I-L-703,606. This was apparently a conformational effect specific to this substitution, because the E193A mutant bound all ligands with the same affinities as did the wild-type receptor. These data suggest that none of the divergent residues within the region of residues 187–195 in the NK1R is required for the high affinity binding of L-703,606.

Substitution of residues 271–280 (PYINPDLYLK) in the fourth extracellular domain with residues at similar positions in the NK3R (TAIYQQLNRW) (the E4 mutant in Table 1) led to an 8-fold increase in the affinity for NKB, with a concomitant 8-fold decrease in antagonist affinity. However, this substitution did not affect the affinity of SP. When each of the divergent residues in this region was substituted individually or in pairs, only one mutant (Y272A) exhibited reduced antagonist affinity, without changes in peptide binding, whereas another mutant (L279R+K280W) exhibited a slight increase in NKB affinity (Table 1). Whereas the affinity of L-703,606 was reduced 12-fold with the Y272A mutant, the affinities of other related quinuclidine analogs were reduced 4–5-fold (Table 2). In contrast, the affinity of the perhydroisoindole antagonist RP67580 was not affected by the Y272A substitution (Fig. 2).

Discussion

Construction of chimeras between two related proteins has been widely used to identify functionally important domains, as exemplified by recent studies of the ligand binding sites of

TABLE 1

Binding affinities of the wild-type human NK1R and mutants of the human NK1R

Mean ± standard error are listed, with the number of independent determinations given in parentheses.

Receptors	IC_{50}			
	SP	NKA	NKB	L-703,606
	nM			
Wild-type human NK1R[a]	0.6 ± 0.15 (7)	31 ± 5 (5)	81 ± 13 (5)	1.0 ± 0.3 (4)
Wild-type human NK1R[b]	3.9 ± 0.7 (4)	89 ± 31 (3)	370 ± 106 (3)	0.3 ± 0.1 (2)
E3a [(170–173)NK3R][a,c]	1.0 ± 0.1 (2)	63 ± 34 (2)	25 ± 5 (2)	1.2 ± 0.4 (2)
T170K[a]	0.5 ± 0.1 (2)	88 ± 2 (2)	128 ± 8 (2)	ND[d]
E172K[a]	0.7 ± 0.1 (2)	378 ± 150 (2)	37 ± 6 (2)	ND
E172A[a]	0.4 ± 0.1 (2)	69 ± 32 (3)	92 ± 24 (3)	ND
E3b [(176–183)NK3R][b,c]	470.0 ± 250 (2)	500 ± 30 (2)	10,000 ± 700 (2)	1.4 ± 0.6 (2)
S176G[a]	0.4 ± 0.1 (2)	78 ± 22 (2)	94 ± 3 (2)	ND
M181F[a]	0.4 ± 0.1 (2)	18 ± 6 (2)	105 ± 45 (2)	ND
E183Q[a]	0.9 ± 0.2 (3)	50 ± 10 (2)	88 ± 29 (2)	ND
E3c [(187–195)NK3R][a,c]	1.3 ± 0.6 (4)	67 ± 16 (4)	130 ± 31 (4)	200.0 ± 5 (2)
H187G+N189K[a]	1.0 ± 0.2 (2)	54 ± 12 (2)	230 ± 100 (2)	4.0 ± 1.6 (2)
Δ(K190+I191)[a]	0.4 ± 0.1 (2)	81 ± 14 (2)	178 ± 35 (2)	2.0 ± 1 (2)
Y192Q[a]	0.4 ± 0.2 (2)	36 ± 3 (2)	63 ± 15 (2)	3.0 ± 2 (2)
E193H[d,e]	>20	ND	ND	>20
E193A[a]	0.6 ± 0.1 (2)	28 ± 12 (2)	75 ± 6 (2)	3.0 ± 0.2 (2)
K194A[a]	0.5 ± 0.1 (2)	22 ± 12 (2)	171 ± 6 (2)	2.0 ± 0.3 (2)
E4 [(271–280)NK3R][a,c]	1.4 ± 0.1 (2)	6 ± 1.5 (3)	11 ± 1.5 (3)	8.5 ± 0.5 (2)
Y272A[a]	0.5 ± 0.1 (3)	22 ± 4 (2)	115 ± 40 (2)	12.0 ± 4 (5)
N274Y[a]	0.6 ± 0.2 (2)	40 ± 15 (2)	141 ± 40 (2)	1.0 ± 0.3 (2)
P275Q[a]	0.6 ± 0.1 (2)	43 ± 22 (2)	71 ± 15 (2)	0.9 ± 0.2 (2)
D276Q[a]	0.7 ± 0.1 (2)	40 ± 9 (2)	87 ± 13 (2)	0.6 ± 0.1 (2)
Y278N[a]	0.6 ± 0.2 (2)	39 ± 7 (2)	105 ± 30 (2)	1.2 ± 0.1 (2)
L279R+K280W[a]	1.8 ± 0.6 (2)	30 ± 17 (2)	38 ± 5 (3)	0.5 ± 0.2 (2)

[a] Data were determined using ^{125}I-BH-SP.
[b] Data were determined using ^{125}I-L-703,606.
[c] The data for the E3a, E3b, E3c, and E4 mutants were taken from Ref. 9.
[d] ND, not determined.
[e] This mutant did not have detectable binding of either ^{125}I-BH-SP or ^{125}I-L-703,606, indicating that either the receptor folding is impaired or the binding affinities of both ligands are too low (K_d > 20 nM) (see Ref. 25).

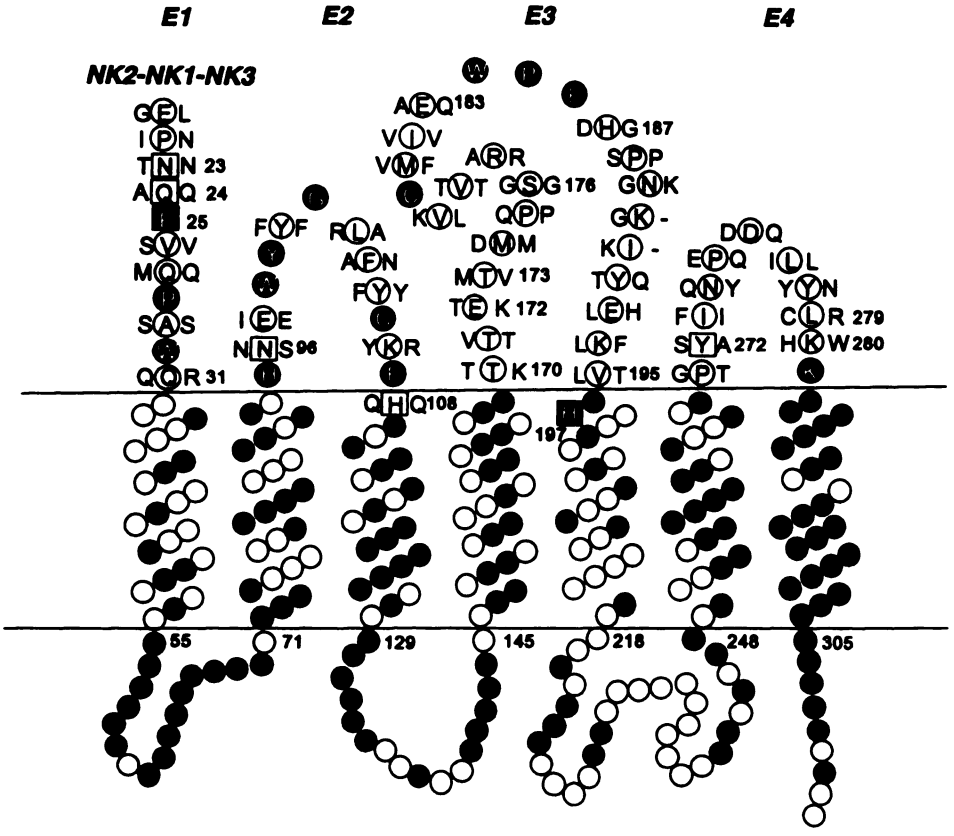
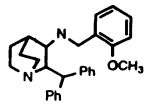
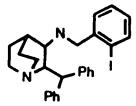
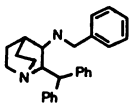
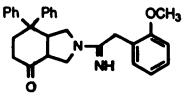


Fig. 1. Schematic model of the NK receptors, incorporating the sequence alignment of the human NK1R, NK2R, and NK3R in the extracellular domain. The numbering is based on the human NK1R, and only residues 21–313 are presented. In the extracellular regions (*E1-E4*), the amino acids of the human NK1R are represented in the *middle*, with the amino acids of the human NK3R on the *right* and the amino acids of the human NK2R on the *left*. *Filled circles* and *filled squares*, conserved residues. *Squares*, positions where substitution of one residue changes the binding affinity of at least one agonist or one antagonist by >10-fold.

TABLE 2

Binding affinities of antagonists for the wild-type human NK1R and the Y272A mutant

Mean ± standard error are listed, with the number of independent determinations given in parentheses.

Compounds[a]		IC_{50}		Ratio of IC_{50} values
		Wild-type	Y272A	
		nM		
	CP-96,345	0.45 ± 0.1 (4)	2 ± 0.1 (3)	4
	L-703,606	1.0 ± 0.3 (4)	12 ± 4 (5)	12
	L-703,605	21 ± 5 (12)	102 ± 30 (6)	5
	RP67580	18 ± 3 (4)	23 ± 2 (2)	1

[a] Ph, phenyl.

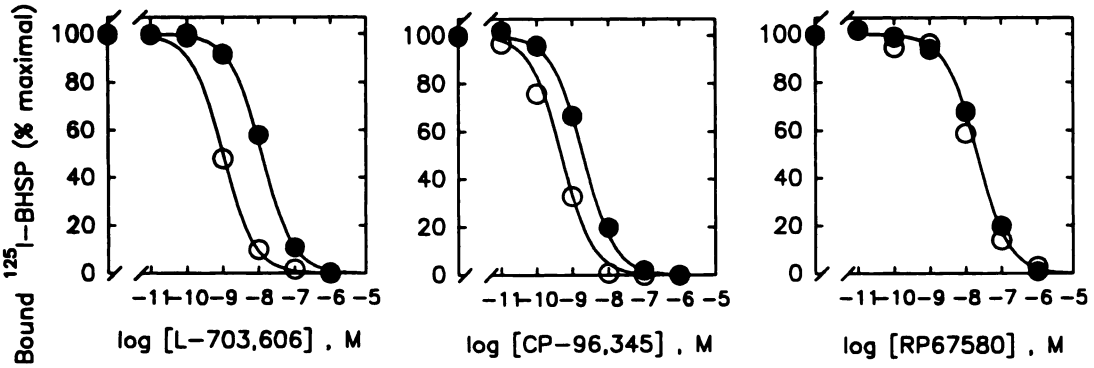


Fig. 2. Measurement of binding affinity of L-703,606, CP-96,345, and RP67580 for the human NK1R (○) and its Y272A mutant (●). Experimental procedures are described in Materials and Methods.

NK receptors (9–12) and other G protein-coupled receptors (19–22). However, it is difficult to determine whether changes in ligand binding affinity upon such "domain swapping" are due to (i) long-range conformational perturbation of the receptor, (ii) local conformational changes in the binding pocket, or (iii) alteration of direct interactions between the receptor and the ligand (23). Such ambiguities may lead to uncertain assignment of the ligand binding domain. The ability to mimic the effects of chimeric receptors with single-residue substitutions would favor the hypothesis that those residues are spatially close to the ligand binding site (case ii or iii). On the other hand, the lack of effect of single-residue substitutions on ligand binding affinity would argue against a direct interaction between that residue and the ligand. For example, substitution of the first or second extracellular segments in the human NK1R leads to a substantial reduction in the affinities for all three natural peptides, without affecting antagonist binding, and this effect can be mimicked by several single-residue substitutions within the same region (9, 15). These results indicate that the first and second extracellular segments of the NK1R either interact directly with peptides or are critical for maintaining the geometry of the binding pocket.

Our previous studies on NK1R/NK3R chimeric receptors also suggested a role for the third and fourth extracellular segments in the binding of peptides and antagonists. The present studies were designed to characterize the precise role of residues in these regions by single-residue substitution. Within the third extracellular segment, the modest increase in the affinity of NKB and the lack of effect on SP affinity observed for the E3a mutant could be partially mimicked by the E172K mutant, in which lysine is the corresponding residue in the NK3R. Because the E172K substitution also reduced NKA affinity, we replaced Glu-172 with alanine to determine the contribution of the carboxylate side chain to peptide binding. The affinities of the E172A mutant for all peptides were similar to those of the wild-type receptor, indicating that Glu-172 does not contribute directly to peptide binding. A small increase in the affinity for NKB was also observed when Val-97 in the second extracellular segment of the rat NK1R was substituted with glutamate, which is found in both the human NK1R and the human NK3R (15). However, the small magnitude of the increase in NKB affinity for the mutant receptors would not be consistent with strong ionic interactions contrib-

uted by the lysine or glutamate residue, although other weaker interactions are possible. For example, the charged residues in the second and third extracellular segments may play a role in the binding of NKB to the NK3R through electrostatic guidance.

Analysis of chimeric receptors has also indicated that the middle portion of the third extracellular segment (E3b) may be critical for peptide binding and that the carboxyl-terminal portion of this loop (E3c) may be important for antagonist binding. However, neither the substantially decreased affinities of peptides for the E3b mutant nor the substantially decreased affinity of antagonist for the E3c mutant could be reproduced by elimination of potential electrostatic interactions or substitution with the corresponding NK3R residues (Table 1). The different effects of multiple-residue substitutions (residues 176–183 or 187–195) and single-residue substitutions suggest that substitution of the E3b and E3c regions in chimeric receptors affects ligand binding affinity either through conformational effects or through removal of nonelectrostatic interactions. These data further suggest that the ligand binding domain probably involves regions in close proximity to residues 176–195, i.e., either the nearby loops or the transmembrane domain.

Chimeric receptor studies on the rat NK1R have also indicated that exchanging residues 183–195 with the corresponding residues of the rat NK3R results in substantial changes in the affinity for CP-96,345 (10). Because substitutions of individual residues in the human NK1R (Table 1) have not revealed any divergent residues within the region of residues 183–195 that can cause substantial changes in the affinity for quinuclidine antagonists, it is possible that a specific conformational effect of these substitutions in both the rat and human NK1R could affect the precise position of nearby conserved residues (such as His-197) that have been shown to interact directly with the antagonists (24).

Within the fourth extracellular segment, the increased affinity of NKB for the E4 mutant can be partially mimicked by the double-residue mutation L279R+K280W, whereas the decreased affinity for the antagonist L-703,606 can be reproduced by the single-residue substitution Y272A. It is interesting to note that Tyr-272 is predicted by modeling to be close to His-197, which has been shown to interact with the benzhydryl moiety of quinuclidine antagonists such as CP-96,345 and L-703,606 (24). Overall, these single-residue substitutions confirm the role of the E4 domain in agonist and antagonist binding that was suggested by the chimeric receptor studies.

The present study indicates that, although the construction of chimeric receptors is a useful approach for localizing functional domains of a protein for further analysis and for identifying divergent residues that do not contribute to ligand interactions, the results from such studies should be interpreted with caution. In the absence of single-residue substitutions, chimeric receptor studies can not distinguish between direct interactions with ligands and conformational effects. Conformational effects have also been observed with chimeric β_1/β_2-adrenergic receptors, for which regions in the fourth and fifth transmembrane helices were implicated as determinants of subtype specificity (20, 21). However, the effects of the chimeric receptors were not reproduced by smaller molecular replacements in those regions, suggesting the presence of conformational effects (21). In the case of the NK1R, divergent residues in the third extracellular segment appear not to contribute significantly to direct interactions with ligands, although this region may be important for maintaining the active conformation of nearby domains.

Overall, the systematic replacement of most of the divergent residues in the extracellular domains of the NK1R indicates that the receptor subtype selectivity does not appear to result from direct interactions between divergent residues of the peptides and divergent residues in the extracellular domain of the NK1R. At least five residues in the first and second extracellular segments of the NK1R have been shown to play a critical role in the binding of all three NKs, and these residues apparently do not contribute to the differential peptide binding affinity (9). On the other hand, different NKs adopt different conformations in solution (26, 27). Furthermore, analysis of constrained peptides suggested that different receptor subtypes could recognize different peptide conformations (28). These data are consistent with the hypothesis that different NKs may adopt different conformations when binding to the NK1R, and they leave open the possibility that the conformational compatibility between a peptide and a receptor may determine the differential peptide selectivity of the NK receptors.

Acknowledgments

We thank Drs. D. Burns and B. Frances for synthesizing ^{125}I-L-703,606 and Drs. T. Ladduwahetty, V. Sabin, E. Seward, C. Swain, and B. Williams for synthesizing other antagonists.

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Send reprint requests to: T. M. Fong, Mail Code 80 M-213, Merck Research Laboratories, P.O. Box 2000, Rahway, NJ 07065.