Localization of Agonist and Antagonist Binding Domains of the Human Neurokinin-1 Receptor

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To identify the molecular determinants of ligand-receptor interactions, the extracellular domain of the human neurokinin-1 receptor was systematically substituted with the corresponding sequences from the other two neurokinin receptor subtypes. Three residues within the first extracellular segment and 2 residues of the second segment are required for the optimal binding of all three natural peptide agonists. The divergent nature of 4 of the 5 residues supports the hypothesis that the peptide binding site on the neurokinin-1 receptor is not highly conserved in the other two receptor subtypes. In contrast, substitution of part of the third extracellular segment and the fourth extracellular segment with the corresponding amino acids of the human neurokinin-3 receptor results in an increase in neurokinin B affinity without affecting substance P binding, suggesting that the two peptides do not interact with the same set of functional groups on the receptor. Among the four extracellular regions, only parts of the third and fourth segments affect the binding of the quinuclidine antagonist L-703,606, and these two regions may partially account for the neurokinin-1 receptor subtype specificity of this non-peptide antagonist. These studies demonstrate that both the extracellular and transmembrane domains of the neurokinin-1 receptor are involved in the binding of substance P and related peptides.

The peptide neurotransmitters substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) are characterized by the common C-terminal sequence FXGLM-NH₂. The biological actions of neurokinins are mediated by three subtypes of the neurokinin receptor (NKR). These receptors are members of the G-protein coupled receptor family that is characterized by seven putative transmembrane helices (1-5). The rank order of potency of the neurokinin agonists for the neurokinin-1 receptor (NK1R) is SP > NKA > NKB, while the rank orders of potency for the other two subtypes (NK2R and NK3R) are NKA > NKB > SP and NKB > NKA > SP, respectively.

Previous studies have led to a message-address hypothesis for peptide-receptor interaction. This hypothesis envisions the conserved C-terminal portion of the peptides as an activation message that can be recognized by all three receptor subtypes, whereas the divergent N-terminal portion of the peptides acts as a recognition address to determine receptor subtype selectivity (6-8). Analysis of the amino acid sequences of the three NKR subtypes has revealed a high level of sequence similarity within the transmembrane domains, while the extracellular and cytoplasmic loops are more divergent (9, 10). Therefore, it can be postulated that the common C-terminal half of the peptides interacts with the conserved transmembrane domains of the receptors, while the unique N-terminal half of the peptides recognizes the more divergent extracellular domains.

Studies on other G-protein coupled receptors whose endogenous agonists are small molecules have demonstrated that the binding sites for these ligands are located within the transmembrane domains of the receptors (11-13). Because the neurokinin peptides are larger than these non-peptide ligands, it seems likely that the extracellular domains of the NK1R might comprise part of the ligand binding site. We have tested this hypothesis by systematically substituting the extracellular segments of the human NK1R with the corresponding sequence from the human NK3R. If the divergent extracellular sequences of the receptors determine the rank order of potency of peptide agonists, then substitution of the extracellular sequences in the NK1R by the homologous sequences from the NK3R should cause an increase in the affinity of NKB with a concomitant decrease in SP affinity. The present results confirm the contribution of the extracellular domains of the NK1R to peptide binding. However, the data also suggest that the two peptides do not necessarily interact with the same set of residues on the NK1R. Therefore, the peptide binding domains in the neurokinin receptors are more complicated than what the simple message-address model would predict.

EXPERIMENTAL PROCEDURES

All mutant receptors were constructed from the human NK1R by either the polymerase chain reaction method (Perkin-Elmer Cetus) or the uracil substitution method of site-directed mutagenesis (Bio-Rad). All mutated sequences and any sequence that was derived from polymerase chain reaction were confirmed by DNA sequencing (United States Biochemical Corp.). All receptors were expressed in COS cells to determine the ligand binding affinity. Some mutant receptors were also expressed in Xenopus oocytes to determine the functional activity (10, 14).

The binding affinities of various ligands for the NK1R and its mutants were determined using 125I-Bolton-Hunter labeled SP (BHSP) or [125I]L-703,606, which is an iodinated analog of the NK1-specific antagonist CP-96,345 (15, 16), in the presence of varying concentration of unlabeled ligands. 125I-BHSP was used when the Kᵰ value of SP was smaller than 10 nM, [125I]L-703,606 was used when the Kᵰ value of SP was larger than 10 nM, and [125I]Bolton-Hunter labeled edeloin (BHE) was used for the human NK3R and the E3 mutant of the human NK1R. The final concentration of radiolabeled ligand was 0.2 nM. Intact COS cells were used in the 125I-BHSP or 125I-BHE binding assay, whereas plasma membranes were used in the
[125]I-L-703,606 binding assay. In the case of [125]I-BHSP, the data were fitted to the equation (cpm(L) - cpm(1 μM SP))/cpm(0) = IC_{50}/(L + IC_{50}), in which cpm(L) and cpm(0) represent bound [125]I-BHSP in the presence and 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 [125]I-BHSP. In the case of [125]I-L-703,606 binding in the presence of unlabeled L-703,606, the data were fitted as described above. In the case of [125]I-L-703,606 binding in the presence of agonist, the data were fitted to the equation (cpm(L) - cpm(1 μM L-703,606))/(cpm(0) - cpm(1 μM L-703,606)) = F × IC_{50}/(L + K_{d}) + (1 - F) × K_{d}, in which F is the fraction of NK1R in the high affinity state, K_{d} is the agonist affinity for the high affinity state, and K_{d} is the agonist affinity for the low affinity state. The IC_{50} value was then solved numerically from the fitted curve.

RESULTS

To probe the role of the extracellular loops of the NK receptors in agonist and antagonist binding, these regions of the human NK1R were systematically replaced with the analogous regions from the human NK3R (Fig. 1). The resulting mutant receptors were transiently expressed in COS cells, and their ability to bind peptide agonists and a non-peptide quinuclidine antagonist was assessed. Substitution of the second extracellular loop of the NK1R (E2 mutants, residues 96-108) with the analogous region of the NK3R resulted in undetectable binding of [125]I-BHSP at 0.2 nM. In contrast, the non-peptide antagonist [125]I-L-703,606 bound to both the E2 mutant and the wild type NK1R with the same affinity (Fig. 2). The binding affinities of both SP and NK for the E2 mutant were greatly reduced (Fig. 2). However, G-protein activation by the E2 mutant was normal. In Xenopus oocytes expressing the E2 mutant, agonists elicited an oscillating calcium-activated chloride current that is characteristic of activation of the phospholipase C-mediated phosphatidylinositol pathway (14). As would be expected from the reduced binding affinities of agonists for the E2 mutant receptor, the dose-response curves for SP and NKB were shifted to the right compared to the wild type receptor (Fig. 3B).

Substitution of the entire third extracellular domain (E3) of the NK1R with the corresponding sequence of the NK3R resulted in a mutant receptor with no detectable binding of [125]I-BHSP or [125]I-L-703,606. In addition, the E3 mutant did not bind [125]I-BHE, which has high affinity for the NK3R. Because the separation of free and bound radioactive ligands by filtration is possible if the dissociation rate constant is smaller than 0.5 s^{-1} (equivalent to K_{d} < 20 nM), a lower limit of IC_{50} > 20 nM was assigned to the E3 mutant for all ligands. However, the E3 mutant receptor was fully functional in Xenopus oocytes (Fig. 3C), indicating that the receptor was correctly processed. The EC_{50} values for the E3 mutant were also consistent with reduced peptide binding affinities compared to the wild type. Three smaller substitutions in the E3 region were constructed in order to analyze the contribution of this extracellular loop to ligand binding. Substitution of residues 170-174 by the homologous NK3R residues (E3a mutant) resulted in a change in the rank order of agonist potency, with SP > NKB > NKA (Table I; Fig. 2). The affinity of SP for the E3a mutant was similar to that of the wild type NK1R. However, the affinity for NKB was increased 3-fold, suggesting that this region of the NK3R might be important for NKB binding. A second substitution in this region, in which residues 176-183 were replaced with the NK3R sequence (E3b mutant), resulted in a reduction in the binding affinities for all three peptide agonists, while the affinity for the antagonist L-703,606 was not affected (Table I; Fig. 2). Finally, when residues 187-195 of the NK1R were substituted with the analogous NK3R residues (E3c mutant), the binding affinities for the three peptides were not significantly affected. In contrast, the binding affinity of the antagonist L-703,606 was reduced 200-fold (Table I; Fig. 2). In the wild type NK3R, the K_{d} of L-703,606 was greater than 1 μM. Substitution of the fourth extracellular loop of the NK1R (E4 mutant, residues 271-280) with the corresponding NK3R...
sequence did not significantly affect SP binding affinity (Table I). However, both NKB and NKA exhibited 5-8-fold increases in binding affinity for the E4 mutant compared to the wild type NK1R. A parallel decrease in affinity of the NK1 selective antagonist L-703,606 was observed for this mutant receptor (Fig. 2). In addition, a point mutation in helix 7 was constructed to replace methionine 291 with the NK3R homolog phenylalanine. A 3-fold increase in NKB affinity was also observed for the M291F mutant (Table I).

The systematic replacement of the extracellular loops of the NK1R described above implicates the E2 loop in the N-terminal domain (El region) also indicated that residues 21-29 are important for high affinity peptide binding (17). To elucidate the precise role of individual residues in these segments in peptide binding, point mutations were performed in vivo. Point mutations were determined using 125I-BHSP. The data are expressed as percent of the wild type human NK1R. The present study was designed to identify the contribution of the extracellular domains of the NK1R to agonist and antagonist binding. None of the extracellular loop replacements described here resulted in a reversal in the relative affinities of SP and NKB. Therefore it is probable that some residues in the transmembrane domain of the receptor may confer SP specificity on the NK1R. On the other hand, the present results indicate that several residues in the N-terminal domain (N23, Q24, P25), loop E2 (N96, H108), and loop E3 (176-183) are required for the high affinity binding of all three peptides. However, these mutations do not affect the rank order of potency of the three neurokinin peptide agonists, and they are not required for the binding of the NK1 selective antagonist L-703,606. The absence of a decrease in the affinity of the antagonist argues against any effect of these mutations on the overall conformation of the receptor, although local conformational effects within the loops cannot be determined at present. The data suggest that these residues do not significantly affect SP binding affinity (Table I). Likewise, substitution of the conserved proline 28 and tryptophan 30 with alanine did not affect the ligand interactions. In contrast, substitution of the conserved phenylalanine 25 with alanine resulted in a dramatic decrease in affinity for all three neurokinin peptides. L-703,606 binding was not affected by this substitution. Asparagine 23 and glutamine 24 are conserved between the NK1R and NK3R, while the NK2R contains threonine and alanine, respectively. Substitution of asparagine 23 and glutamine 24 also resulted in a large reduction in the affinities of all three peptides for the NK1R. These substitutions did not affect the binding of the antagonist L-703,606. In the second extracellular loop, 6 residues are divergent between the NK1R and NK3R (Fig. 1). Substitution of the non-conserved asparagine 96 and histidine 108 with their NK3R homologs (serine and glutamine, respectively) resulted in a substantial reduction in the affinities for all three agonist peptides, although the antagonist affinity was not affected by these point mutations (Table I).

**DISCUSSION**

The present study was designed to identify the contribution of the extracellular domains of the NK1R to agonist and antagonist binding. None of the extracellular loop replacements described here resulted in a reversal in the relative affinities of SP and NKB. Therefore it is probable that some residues in the transmembrane domain of the receptor may confer SP specificity on the NK1R. On the other hand, the present results indicate that several residues in the N-terminal domain (N23, Q24, P25), loop E2 (N96, H108), and loop E3 (176-183) are required for the high affinity binding of all three peptides. However, these mutations do not affect the rank order of potency of the three neurokinin peptide agonists, and they are not required for the binding of the NK1 selective antagonist L-703,606. The absence of a decrease in the affinity of the antagonist argues against any effect of these mutations on the overall conformation of the receptor, although local conformational effects within the loops cannot be determined at present. The data suggest that these residues did not significantly affect SP binding affinity (Table I). However, both NKB and NKA exhibited 5-8-fold increases in binding affinity for the E4 mutant compared to the wild type NK1R. A parallel decrease in affinity of the NK1 selective antagonist L-703,606 was observed for this mutant receptor (Fig. 2). In addition, a point mutation in helix 7 was constructed to replace methionine 291 with the NK3R homolog phenylalanine. A 3-fold increase in NKB affinity was also observed for the M291F mutant (Table I).

The systematic replacement of the extracellular loops of the NK1R described above implicates the E2 loop in the binding of all three peptide agonists. Our previous analysis of the N-terminal domain (El region) also indicated that residues 21-29 are important for high affinity peptide binding (17). To elucidate the precise role of individual residues in these segments in peptide binding, point mutations were analyzed. The three non-conserved residues at positions 21, 22, and 29 in the El region of the human NK1R can be substituted with the corresponding amino acids from the NK3R without any effect on agonist or antagonist binding (Table I).

**TABLE I**

| Receptors          | SP IC50 (nM) | NKA IC50 (nM) | NKB IC50 (nM) | L-703,606 IC50 (nM) |
|--------------------|--------------|---------------|---------------|---------------------|
| hNK1R wild type   | 0.63 ± 0.15  | 31 ± 5        | 81 ± 13       | 1.4 ± 0.5           |
| hNK1R mutant (B)  | 3.9 ± 0.74   | 89 ± 31       | 370 ± 108     | 0.3 (2)             |
| E2 = (96-108)NK3a | 6,000 ± 2,000 | ND            | >10,000 (2)   | 0.6 ± 0.2           |
| E3 = (176-183)NK3a| >20          | >20           | >20           |                     |
| E3 = (176-183)NK3b| 1 ± 2        | 63 ± 34       | 25 ± 5        | 1.2 ± 0.4           |
| E3 = (176-183)NK3c| 470 ± 250    | 500 ± 30      | 10,000 ± 700  | 1.4 ± 0.6           |
| E3 = (176-183)NK3b| 1 ± 2        | 67 ± 16       | 130 ± 31      | 200 (2)             |
| E3 = (176-183)NK3c| 1 ± 2        | 6 ± 1.5       | 11 ± 1.5      | 8.5 ± 0.5           |
| hNK3R wild type   | 42 ± 6       | 19 ± 4        | 2.3 ± 1.3     | >1,000 (2)          |
| EPA2(22,24,29)NSa | 0.45 ± 0.02  | 20 ± 1        | 64 ± 27       | ND                  |
| N23Tb              | 750 ± 250    | >10,000 (2)   | >10,000 (2)   | ND                  |
| Q24A               | 90 ± 10      | 5,000 ± 120   | 5,700 ± 600   | 0.4 ± 0.1           |
| P25A               | >10,000 (2)  | >10,000 (2)   | >10,000 (2)   | 0.45 (2)            |
| P28A               | 0.45 ± 0.02  | 36 ± 4        | 62 ± 23       | ND                  |
| W30A               | 0.45 ± 0.02  | 33 ± 12       | 105 ± 4       | ND                  |
| FPW(25,28,30)AAA   | >10,000 (2)  | ND            | ND            | 0.4 ± 0.2           |
| N96S               | 180 ± 22     | >1,000 (2)    | >1,000 (2)    | 0.3 (2)             |
| H108Q              | 180 ± 50     | >1,000 (2)    | >1,000 (2)    | 0.5 (2)             |
| M291F              | 0.3 (2)      | 14 ± 1.5      | 30 ± 2        | 1 (2)               |

* Data are determined using 125I-BHSP.
† Data are determined using 125I-L-703,606.
ND, not determined.
‡ Data are determined using 125I-BHE.
Ligand Binding Site of NK1R

in the external loops of the NK1R may interact with the conserved C-terminal half of the neurokinin peptides, or they may be required to maintain the local conformation of the peptide binding site. The divergent nature of these residues implies that the peptide binding sites of different receptor subtypes may not be very conserved.

Substitution of residues 170–174 in E3 and 271–280 in E4 by the corresponding NK3R sequence increases the affinity of the NK1R for NKB. However, the SP binding affinity is not significantly affected by these substitutions, suggesting that divergent residues in E3 and E4 affect NKB binding but not SP. Factors other than amino acid side chains may also contribute to ligand binding specificity, as it was demonstrated in the arabinose binding protein where substrate specificity and affinity are determined by bound water molecules (18). The present observations imply that different peptides may not interact with the same set of functional groups on the receptor, and therefore the active conformations of different neurokinin peptides may be different. Consistent with this hypothesis are NMR experiments demonstrating that different neurokinin peptides adopt different conformations in solution (19, 20). Analysis of conformationally constrained SP analogs has also suggested that different peptide conformations are recognized by different NKR subtypes (21).

Taken together, the results presented above indicate that peptide binding involves regions within all four of the extracellular domains of the NK1R. The transmembrane domain is still important for peptide binding as illustrated by the point mutation M291F. In contrast, substitutions in the E3c and E4 region reduce the binding affinity of the quinuclidine antagonist L-703,606 without affecting SP binding. This is different from the β-adrenergic receptor, where deletion of residues in the extracellular segments did not affect the binding of small molecule ligands (22). Overlapping but non-identical agonist and antagonist binding sites have been described for the β2-adrenergic receptor and α2-adrenergic receptor (23, 24). Because the specificity of L-703,606 for the NK1R subtype cannot be completely accounted for by the substitution in E3c and E4 regions, we hypothesize that amino acid residues within the transmembrane domain of the NK1R are also involved in the binding of this quinuclidine antagonist. Further studies will be required to identify specific residues that interact directly with functional groups on the agonist or antagonist molecule.

The involvement of all four extracellular loops of the NK1R in the binding of peptide agonists permits a relatively large surface area for the recognition of peptides. This is the first demonstration for a role of all four extracellular domains in the binding of small peptides to a G-protein coupled receptor. The involvement of the extracellular N-terminal residues in the binding of glycoprotein hormones has also been demonstrated in a subgroup of the G-protein-coupled receptors that contain a very large N-terminal domain (25). However, none of the mutations analyzed here affected the intrinsic activation of the intracellular effector. By analogy with the β-adrenergic receptor, one might speculate that residues in transmembrane helices 5 and 6 and their connecting third intracellular loop might be involved in agonist-mediated G-protein activation. The present study supports the hypothesis that the structures of the peptide binding sites differ for the three neurokinin receptor subtypes.

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Note Added in Proof—Some of the data were presented in abstract form (26). After the submission of the present report, another abstract appeared that described the binding properties of chimeric mutants between the rat NK1X and NK3R (27).

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