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The Ligand Binding Site of the Neurokinin 2 Receptor

SITE-DIRECTED MUTAGENESIS AND IDENTIFICATION OF NEUROKININ BINDING RESIDUES IN THE HUMAN NEUROKININ 2 RECEPTOR* 

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Thirteen residues in the human neurokinin 2 (NK2) receptor were identified as potential ligand-binding residues by molecular modeling and amino acid sequence analysis. Site-directed mutagenesis was used to alter these residues in order to ascertain their importance in binding neurokinin A (NKA), the physiological peptide ligand for the NK2 receptor, and the non-peptide NK2 receptor selective antagonist SR48968. Four sites appear to be critical for NKA binding (Glu292 → His, Ile296 → His, Gly297 → Phe, and Gly372 → Thr) that maintain their affinity for SR48968, despite being unable to bind the peptide ligand, His296 → Ala and His386 → Leu no longer bind NKA or SR48968. We have also identified a residue (Leu297) which appears to play a minor role in the binding of substance P (SP) and neurokinin B (NKB) to the NK2 receptor. The mutant receptor Leu297 → Ser binds NKB and SP with approximately a 5-fold greater affinity in comparison with the wild type receptor while the affinity of NKA remains unaffected. The results suggest that intramembranous residues, as well as residues which lie close to the extracellular side of transmembrane helices 3, 5, and 6, form part of the NK2 receptor binding site. Binding of SP and NKB to the NK2 receptor may also be influenced by residues near the extracellular side of helix 7. These results suggest that some regions of the binding site for NKA in the NK2 receptor are not used for binding SP in the NK1 receptor. However, it also seems that the NKA binding site includes regions that are also used by other G-protein-coupled receptors such as rhodopsin and the β2-adrenergic receptors.

The neurokinins, substance P (SP),1 neurokinin A (NKA), and neurokinin B (NKB) are neuropeptides of 10–11 amino acid residues in length which share the conserved C-terminal sequence Phe-X-Gly-Leu-Met-NH2. They are widely distributed in both the central and peripheral nervous systems. These peptides each act upon three distinct membrane receptors, neurokinin 1 (NK1), neurokinin 2 (NK2), and neurokinin 3 (NK3), respectively, which belong to the superfamily of G-protein-coupled receptors (GPCRs) which possess seven transmembrane α-helices. However, despite the high degree of sequence identity between the three receptors (approximately 50–60%) and between the three ligands, each receptor displays a clear selectivity between the three peptides (1). The rank order of potency of the three neurokinin agonists for the NK2 receptor is NKA > NKB > SP, for the NK1 receptor, SP > NKA > NKB; and for the NK3 receptor, NKB > NKA > SP.

An understanding of the molecular basis of peptide-receptor interactions can contribute to the design of novel ligands with desirable pharmacological properties. Site-directed mutagenesis, coupled with innovative ligand design, has already been used to successfully identify the principle ligand binding residues in the β-adrenergic receptor, a member of a GPCR subfamily that bind a class of much smaller ligands (2, 3). The ligand binding site in this receptor subclass is believed to lie in the extracellular third of the seven transmembrane helical bundle. However, it is not clear whether such a binding site will be common to those receptors, such as the NK2 receptor, which bind much larger ligands. It seems that these larger peptide ligands might bind primarily to extramembranous regions of the receptor (4, 5). They also, presumably, form many more sites of receptor-ligand interaction compared to small ligands such as adrenaline.

In this paper, we have employed a three-dimensional model of a human NK2 receptor (based on Ref. 6), alongside an alignment of 10 neurokinin receptor sequences, in order to identify potential sites for NKA binding and selectivity. In order to reduce the likelihood of disrupting the structure of the receptor protein, these sites have been substituted either to residues with similar properties or else to residues found in the NK1 or NK3 receptors. Our results suggest the involvement of the transmembrane helices in NKA binding to the NK2 receptor.

EXPERIMENTAL PROCEDURES

Materials—NKA, NKB, and SP were purchased from Novabiochem. [125I]iodohistidyllNKA ([125I]NKA; specific activity = 1900–2200 Ci/mmol) was purchased from Amersham International and [3H]SR48968 (specific activity = 25 Ci/mmol) was purchased from Du Pont NEN. pEF-BOS was the kind gift of Dr. S. Nagata (7). cDNA for the human ileum NK2 receptor was kindly provided in the cloning vector pUC19 by Glaxo. General reagents were purchased from Sigma. Celi culture reagents were purchased from Life Technologies, Inc. and Boehringer Mannheim.

Site-directed Mutagenesis of NK2 Receptor cDNA—A 1260-base pair BamHI/Ndel restricted fragment of the NK2 receptor cDNA was introduced into the polylinker region of pEF-BOS using BstXI adapters. Point mutations were created by the method reported by Kunkel (8) using single stranded DNA of the NK2 receptor cdNA in pEF-BOS. All mutations were confirmed using dideoxy chain termination DNA sequencing (9). At least two individual clones were selected for binding assessment.

Cell Expression—Wild type and mutated receptors were expressed transiently in COS-1 cells by Lipofectamine lipoprecipitation of NK2 receptor cDNA in pEF-BOS. Transfections were performed using 12 μg/ml Lipofectamine and 1 μg/ml DNA in Opti-MEM for 5 h at 37 °C, 10% CO2, and terminated by the addition of Dulbecco's modification of Eagle's medium containing 20% fetal calf serum. The following
day, the transfected cells were plated at a density of 5 × 10^5 cells per well into 24-well plates and maintained for a further 2 days in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum at 37 °C and 10% CO2.

**Ligand Binding Assays**—Binding assays were performed on intact COS-1 cell monolayers, 72 h post-transfection. At this stage, the medium was removed and the cells were washed twice with phosphate-buffered saline (pH 7.4). For competition studies the cells were incubated for 90 min at room temperature in binding buffer (Dulbecco’s modified Eagle’s medium, 20 mM Hepes, 0.1% (w/v) bovine serum albumin, 400 μg/ml bacitracin) containing 0.1 nM [3H]NKA and varying concentrations of NKA, SP, or NKB. Nonspecific binding was determined in the presence of a 1000-fold excess of NKA. Scatchard analyses of [3H]NKA binding were performed for specific receptors with binding at 0.1 nM [3H]NKA was undetectable. The cells were incubated with binding buffer containing varying concentrations (0.025–2 nM) of [3H]NKA for 90 min at room temperature. For antagonist Scatchard analyses, concentrations of 0.25–20 nM [3H]-SR48968 were incubated with cell monolayers for 60 min at room temperature. In both cases nonspecific binding was determined on monolayers of cells transfected with the pEF-BOS vector not containing the cDNA for the receptor. Protein assays were performed on solubilized cell monolayers by the BCA method (Pierce, Ref. 10). To terminate binding, the ligand-buffer mixture was removed and the cells were washed manually three times with ice-cold phosphate-buffered saline, solubilized with 1% SDS, 0.1 M NaOH, neutralized with 0.1 M HCl, and collected for counting. For β counting scintillation fluid was added. Computerized data analysis was carried out using GraphPAD inplot software.

**Sequence Alignment**—The amino acid sequences of 10 neurokinin receptors were extracted from the OWL protein sequence data base (11) and aligned using the program MALIGN (12).

**RESULTS**

To identify residues with roles in NKA binding in the NK2 receptor, point mutations were introduced into the human ilum NK2 receptor cDNA. The resulting mutant receptors were transiently expressed in COS cells and their ability to bind [3H]NKA and the NK2 receptor selective non-peptidic antagonist [3H]-SR48968 was assessed. A typical example of the binding of [3H]NKA to the human NK2 receptor expressed in COS-1 cells is shown in Fig. 1. The Kd was estimated to be 0.8 ± 0.1 nM with a Bmax corresponding to 3 × 10^5 receptor binding sites per cell.

**TABLE I**

| Receptor          | NKA IC50 (nM) ± S.E. | NKB IC50 (nM) ± S.E. | SP IC50 (nM) ± S.E. |
|-------------------|----------------------|----------------------|---------------------|
| Wild type         | 1.1 ± 0.1 (10)       | 15 ± 2 (10)          | 273 ± 43 (10)       |
| Glu265−His        | NB                   | NB                   | NB                  |
| Met1−Leu          | 1.3 ± 0.1 (4)        | 24 ± 2 (4)           | 644 ± 60 (4)        |
| Leu190−Phe        | 0.9 ± 0.2 (6)        | 21.6 ± 2.3 (6)       | 275 ± 39 (6)        |
| Cys197−Gly        | 1.7 ± 0.2 (9)        | 22.4 ± 2.9 (9)       | 242 ± 49 (9)        |
| Gly199−Asn        | 2.2 ± 0.2 (9)        | 30 ± 6 (9)           | 386 ± 81 (9)        |
| Gly201−Lys        | 1.8 ± 0.3 (9)        | 18 ± 0.2 (9)         | 332 ± 71 (9)        |
| Gly204−Lys        | 1.4 ± 0.1 (6)        | 11 ± 2 (6)           | 176 ± 23 (6)        |
| Leu205−Glut       | 2.0 ± 0.4 (6)        | 21 ± 5 (6)           | 200 ± 19 (6)        |
| Leu204−Thr        | 1.1 ± 0.2 (9)        | 8 ± 0.1 (9)          | 212 ± 44 (9)        |
| His202−Ala        | NB                   | NB                   | NB                  |
| His199−Leu        | NB                   | NB                   | NB                  |
| Met1−Thr          | 1.2 ± 0.1 (12)       | 30 ± 4 (12)          | 289 ± 17 (12)       |
| Gly201−Phe        | 1.0 ± 0.1 (6)        | 12 ± 0.6 (6)         | 235 ± 38 (6)        |
| Ser21−Thr         | 1.0 ± 0.1 (4)        | 16 ± 3 (4)           | 232 ± 65 (4)        |
| Leu202−Ile        | 0.9 ± 0.1 (6)        | 13 ± 0.9 (6)         | 174 ± 9 (6)         |
| Leu202−Ser        | 1.4 ± 0.2 (10)       | 5 ± 0.8 (10)         | 35 ± 4 (10)         |

*NB, no detectable [3H]NKA binding.

**Fig. 1.** Scatchard analysis of [3H]NKA binding to the wild type human NK2 receptor expressed in COS cells. [3H]NKA binding to COS-1 cells expressing human NK2. Scatchard analysis is shown with the binding isotherm inset (see "Experimental Procedures" for details). Each data point represents the average of triplicates fitted by computerized linear regression. S.E. is represented by error bars. Bound [3H]NKA is expressed as specifically bound femtomoles per mg of total cell protein.

**Fig. 2.** Measurement of agonist affinity for the wild type and Leu202 → Ser NK2 receptors. Competition curves for wild type NK2 receptor (unfilled) for NKA, NKB, and SP compared with the Leu202 → Ser mutant (filled) (see "Experimental Procedures" for details). Each data point is the average of triplicates. The curves are representative of 10 experiments performed in triplicate.
Fig. 3. Scatchard analysis of \(^3\)H-SR48968 binding to the wild type NK2 receptor and mutant receptors which show no NKA binding.

\(^3\)H-SR48968 Scatchard plots for wild type (with binding isotherm inset), Gln\(^{109}\) → His, Ile\(^{202}\) → Val, Gly\(^{273}\) → Pro, and Gly\(^{273}\) → Thr (see "Experimental Procedures" for details). Triplicate values ± S.E. per data point. B, femtomoles of \(^3\)H-SR48968 specifically bound per mg of total cell protein. Fitted by computerized linear regression.

19 mutant NK2 receptors. Values for the Gln\(^{109}\) → His, His\(^{198}\) → Ala, His\(^{198}\) → Leu, Ile\(^{202}\) → Val, Gly\(^{273}\) → Pro, and Gly\(^{273}\) → Thr mutants were unobtainable since they no longer bound detectable levels of \(^{125}\)I-NKA at 0.1 nM. Indeed, no specific binding of \(^{125}\)I-NKA was detectable up to 2 nM (data not shown). However, Scatchard analysis shows that, with the exception of His\(^{198}\) → Ala and His\(^{198}\) → Leu, these mutants maintained their ability to bind the NK2 receptor selective non-peptidic antagonist \(^3\)H-SR48968 as illustrated in Fig. 3 (\(K_g\) and \(B_{max}\) values are shown in Table II). \(K_g\) values for \(^3\)H-SR48968 binding are comparable to those obtained for the wild type receptor. \(B_{max}\) values indicate reasonable levels of the receptors are present in the plasma membrane.

Further substitutions were made in helix 3 (Met\(^{17}\) → Leu), at the extracellular side of helix 4 (Leu\(^{160}\) → Phe and Cys\(^{167}\) → Gly), in the second extracellular loop (Gly\(^{190}\) → Asn/Lys, Gly\(^{191}\) → Lys, and Leu\(^{194}\) → Glu/Thr), and at the top of helix 6 (Ser\(^{274}\) → Thr/Tyr). These mutant receptors had binding profiles similar to the wild type receptor (Tables I and II).

**DISCUSSION**

Fig. 4 shows the alignment of the neurokinin receptor sequences. The asterisks (*) show the positions that are completely conserved. The N and C termini show the greatest degree of variability along with the central region of the third cytoplasmic loop and the second extracellular loop. The crosses (+) show the positions substituted in this work. The region of the second extracellular loop on the N-terminal side of helix 5 is particularly attractive as a target for mutagenesis since the residues are highly variable and an optimal sequence alignment is only possible by including gaps. It is a prime target for probing the receptor in search of residues or epitopes that might confer the specificity that each particular neurokinin receptor has for its ligand. However, the substitution of Gly\(^{190}\) and Gly\(^{191}\) for residues found at the equivalent positions in the NK1 and NK3 receptors had little effect on the binding affinities of the peptide agonists even though these positions were substituted to residues with very different properties (i.e. Asn and Lys). It may be that the entire structure of this loop is
important rather than the side chains of one or two individual residues. In the NK1 receptor, several regions of the N terminus and the second and third extracellular loops have been shown to affect high affinity binding of the three neurokinins (13).

### Table II

| Receptor       | $K_i$ (nM) | $B_{max}$ (fmol/mg) |
|----------------|------------|---------------------|
| Wild type      | 2.7 ± 0.3  | 2322 ± 550          |
| Gln$^{109}$ → His | 2.5 ± 0.5  | 705 ± 203           |
| Met$^{117}$ → Leu | 11.9 ± 2.1 | 1234 ± 205          |
| Leu → Phe      | 1.3 ± 0.2  | 462 ± 109           |
| Cys$^{167}$ → Gly | 2.7 ± 0.7  | 1854 ± 872          |
| Gly$^{190}$ → Asn | 3.2 ± 0.7  | 2674 ± 1842         |
| Gly$^{190}$ → Lys | 3 ± 0.4    | 1285 ± 352          |
| Leu$^{194}$ → Ala | 1.4 ± 0.1  | 1104 ± 02           |
| Leu$^{194}$ → Thr | 3.8 ± 0.8  | 482 ± 98            |
| His$^{196}$ → Ala | NB$^*$   | NB                  |
| His$^{196}$ → Leu | NB        | NB                  |
| Ile$^{199}$ → Val | 4 ± 0.3    | 762 ± 332           |
| Tyr$^{204}$ → Phe | 2.7 ± 0.4  | 590 ± 112           |
| Gly$^{273}$ → Thr | 10.7 ± 0.1 | 2808 ± 219          |
| Gly$^{273}$ → Thr | 2.0 ± 0.5  | 1169 ± 459          |
| Ser$^{274}$ → Thr | 5.9 ± 2.5  | 1943 ± 953          |
| Ser$^{274}$ → Tyr | 2.9 ± 0.5  | 651 ± 192           |
| Leu$^{292}$ → Ile | 9.5 ± 1.3  | 1117 ± 22           |
| Leu$^{292}$ → Ser | 2.4 ± 0.3  | 885 ± 114           |

* NB$^*$, no detectable $^*$H-SR48968 binding.

The remaining residues targeted for site-directed mutagenesis are on the predicted internal face of the extracellular third of helices 3–7 (Fig. 5). Met$^{117}$ lies deep in the membrane helix 3. The substitution of this residue for Leu has no effect on receptor binding characteristics. Likewise, the residues Leu$^{160}$ and Cys$^{166}$ (helix 4) and Leu$^{194}$ (helix 5) do not appear to be involved in NKA binding.

Gln$^{109}$ at the top of helix 3, Ile$^{202}$ on helix 5, and Gly$^{273}$ at the top of helix 6, all seem to play important roles in NKA-receptor interaction but not in the binding of the selective non-peptidic antagonist SR48968. The maintenance of SR48968 binding shows that the mutants are fully expressed and strongly suggests that the receptor structure is not significantly disrupted. This result is interesting since two of these residues are at equivalent positions to putative ligand binding site residues in other GPCR classes. Gln$^{109}$ is in the equivalent position to the putative Schiff base counterion (Glu$^{143}$) in rhodopsin which is thought to be responsible for the absorption characteristics of this GPCR (14, 15). Within the neurokinin group of receptors, the equivalent residue to Gln$^{109}$ in the NK1 receptor (His$^{109}$) has been shown to be involved in high affinity agonist binding (13). Ile$^{202}$ on the other hand is equivalent to Ser$^{202}$ in the $\beta$-adrenergic receptor which has been implicated in binding the catechol moiety of adrenaline (2). This suggests that the different GPCRs use a similar region as a ligand binding site despite the diversity of their ligands.

Ile$^{202}$ was targeted due to the significance of Ser$^{202}$ in the $\beta$-adrenergic receptor. However, the equivalent residue in the rat and mouse NK2 receptors is a phenylalanine. Hence it is surprising that the conservative substitution Ile$^{202}$ → Val has such a dramatic effect on NKA binding without affecting $\beta$-adrenergic receptor binding.

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**Fig. 4. Sequence alignment of 10 neurokinin receptors extracted from the OWL protein sequence database (11). The asterisks (*) mark positions conserved between the neurokinin receptors. The crosses (+) mark positions targeted for substitution.**
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Fig. 5. Figure depicting the model of the human NK2 receptor based upon Ref. 6. The putative peptide binding residues are shown with black circles. Other residues mutated are shown in grey circles. Residues predicted to be on the internal face of the helices are in uppercase and project out of the plane of the page.

Fig. 6. Three-dimensional NK2 receptor model based on Ref. 6. The three-dimensional model of the human NK2 receptor (Ca form) showing the location of the putative NKA binding residues (dot surfaces).

SR48968 binding. This may indicate a possible point of a direct peptide receptor interaction. However, this seems unlikely since the residue is not conserved in the rodent NK2 receptors. Another possibility is that the mutation indirectly affects a nearby ligand-receptor interaction. In our three-dimensional model, Ile<sup>292</sup> contacts the conserved residue His<sup>267</sup> on helix 6 which has been implicated in NK1 receptor ligand binding (16).

Substitution of the equivalent residue to Ile<sup>292</sup> in the NK1 receptor (Thr<sup>291</sup> → Ala) shows no effect on agonist or non-peptide antagonist binding (17), suggesting different sites of peptide-receptor interaction for these two neurokinin receptors. The difference between the NK1 and NK2 receptors in this region is further emphasized since substitution of the conserved histidine on helix 5 (His<sup>158</sup>) to either alanine or leucine abolishes both NKA and SR48968 binding. Substitution of this histidine in the NK1 receptor abolishes non-peptide antagonist binding but does not significantly affect SP binding (17). However, it is yet to be determined whether the effect of the His<sup>158</sup> mutations in the NK2 receptor are a result of the disruption of correct protein folding rather than directly effecting ligand-receptor interactions.

The substitution of Gly<sup>273</sup> to proline or threonine, the equivalent residues in NK1 and NK3, respectively, abolishes NKA but not SR48968 binding indicating it as a potentially critical residue in NKA binding. The substitution of the small glycine residue by proline or threonine in the NK2 receptor may sterically hinder NKA binding. Alternatively, the more conformationally flexible glycine residue may be necessary for maintaining the correct local structure in this region of NK2. The substitution of the adjacent residue Ser<sup>274</sup>, however, does not seem to affect NKA binding, even when substituted for tyrosine, a much larger residue. In our three-dimensional model Gly<sup>273</sup> faces helix 7 whereas Ser<sup>274</sup> faces toward helix 5.

Analysis of the sequence alignment (Fig. 4) reveals that a 27-residue region that includes helix 7 (residues 285–311; human NK2 receptor numbering) is completely conserved in the neurokinin subfamily of GPCRs apart from two positions (292 and 293) which lie near the extracellular side of the helix. The substitution of Leu<sup>292</sup> → Ser slightly affects the affinity of the NK2 receptor for NKB and SP despite not altering NKA or SR48968 binding. The substitution Leu<sup>292</sup> → Ile, however, has no significant effect on agonist selectivity. This may imply that this residue is part of the NKB and SP binding site in the NK2 receptor. In the NK1 receptor, substitution of the equivalent residue (Ile<sup>290</sup>) has no effect on agonist selectivity (18).

It seems, therefore, that the neuropeptide binding site in the NK2 receptor involves residues on the extracellular third of helices 3, 5, 6, and 7 close to the bilayer interface. This is in common with other GPCR classes (19). Fig. 6 shows where
these residues lie in our current three-dimensional model which will be used to identify further sites that may be involved in ligand binding.

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