Importance of the Aromatic Residue at Position 6 of [Nle^{10}]Neurokinin A(4–10) for Binding to the NK-2 Receptor and Receptor Activation

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Steric and electrostatic requirements at position 6 of [Nle^{10}]NKA(4–10), a full agonist of NK-2 receptors, for molecular recognition by the receptor were studied. Two series of peptide analogues, (a) *p*-substituted analogues, [*p*-X-Phe^6,Nle^{10}]NKA(4–10), where X = F, Cl, Br, I, NH_2, NO_2, and (b) [D-Phe^6,Nle^{10}]NKA(4–10), [Trp^6,Nle^{10}]NKA(4–10), and [Chex-Ala^6,Nle^{10}]-NKA(4–10), were synthesized, and their biological activity was examined. Competition binding experiments with [^{3}H]NKA were performed using cloned human NK-2 receptors expressed in CHO cells. Antagonistic and agonistic properties of the analogues were studied using an in vitro functional assay with hamster tracheal rings. The rank order of potency of agonists was [Nle^{10}]NKA(4–10) ≈ [*p*-F-Phe^6,Nle^{10}]NKA(4–10) > [*p*-NH_2-Phe^6,Nle^{10}]NKA(4–10) > [*p*-Cl-Phe^6,-Nle^{10}]NKA(4–10) > [*p*-NO_2-Phe^6,Nle^{10}]NKA(4–10) > [Trp^6,Nle^{10}]NKA(4–10). Size and planarity of the aromatic side chain were crucially important for the biological activity, whereas electron-donating and electron-withdrawing properties of the *para*-substituent were less important. The results favor the hypothesis that weakly polar $\pi-\pi$ interactions exist between the aromatic group and the receptor.

Introduction

NKA belongs to the tachykinin family of oligopeptides, including SP and NKB, which have 10–12 amino acid residues and share the common C-terminal sequence Phe-Xaa-Gly-Leu-MetNH_2.[1] They are widely distributed in the central nervous system as well as in peripheral organs and tissues. The term "tachykinins" reflects their contractile effect on smooth muscles.[2,3] SP, NKA, and NKB interact with at least three receptor subtypes. The rank order of potency for tachykinin receptors is SP > NKA > NKB for the NK-1 receptor, NKA > NKB > SP for the NK-2 receptor, and NKB > NKA > SP for the NK-3 receptor.[4] The receptors have been cloned and are members of the superfamily of G-protein-coupled receptors with seven putative membrane-spanning segments.[5]

The C-terminal heptapeptide, NKA(4–10), contains the minimal sequence required for NK-2 receptor activation.[6] Met^{10}→Nle replacement enhances selectivity of NKA(4–10) for the NK-2 receptor but with some reduction of potency.[7,8] The replacement also prevents oxidation of Met^{10}, and consequently, [Nle^{10}]NKA(4–10) (**1**) is more stable than NKA(4–10) and is the parent compound for this study.

A Phe residue at position 6 is common to all decapeptide tachykinins and is found at corresponding positions 7 in peptides containing 11 amino acid residues and 8 in kassinin. The Phe residue is crucially important since its replacement with either L-Ala or 1-amino-1-cyclohexyl carboxylic acid eliminates binding of NKA to all three receptor subtypes.[6,8]

Molecular dynamics simulations of the structures of both NKA and **1** in water, using a simulated annealing method, demonstrate that the aromatic ring of Phe^6 is located on the surface of the folded peptide backbone, forming a hydrophobic patch.[9] Weakly polar interactions, aromatic–aromatic ($\pi-\pi$) and amino–aromatic (N–π), make a significant contribution to the stability of protein–ligand complexes.[10,11] The peptide-binding site of the human NK-2 receptor includes hydrophobic residues on the extracellular third of helices 3, 5, 6, and 7, close to the lipid bilayer interface.[12,13] Consequently, the Phe^6 aromatic side chain of **1** could be involved in weakly polar interactions with a putative hydrophobic pocket in the NK-2 receptor.

In the present study, the importance of the aromatic residue is further investigated by examining steric and electrostatic requirements at position 6 of **1** both for binding to the NK-2 receptor and for receptor activation. For this purpose, the following peptides were synthesized: (a) *para*-substituted analogues, [*p*-X-Phe^6,Nle^{10}]-NKA(4–10), where X = F, Cl, Br, I, NH_2, NO_2, and (b) analogues in which the Phe^6 was replaced with a nonaromatic structure (Chex-Ala) and with different aromatic amino acids, D-Phe and Trp (Table 1). The substituents can be classified as either electron donating (NH_2) or electron withdrawing (NO_2 and halogens). The substituents should not only increase the van der Waals volumes of the Phe^6 derivatives but also perturb the electrostatic field around the aromatic ring. Replacements by D-amino acids introduce a conformational change in the peptide backbone and are therefore suitable for examination of the contribution of individual amino acids to the secondary structure of peptides required for activity.[14–16]

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Table 1. Analysis of [Nle^{10}]NKA(4−10) Analogues[a]

peptide	amino acid composition							MS results
	Ser	Asp	X[b]	Val	Gly	Leu	Nle	
2, [*p*-F-Phe^6,Nle^{10}]NKA(4−10)	0.82 (1)	1.02 (1)	0.95 (1)	0.99 (1)	1.10 (1)	1.10 (1)	1.02 (1)	767.0 (766.0)
3, [*p*-Cl-Phe^6,Nle^{10}]NKA(4−10)	0.88 (1)	1.01 (1)	0.92 (1)	0.95 (1)	0.97 (1)	1.06 (1)	0.97 (1)	783.0 (783.5)
4, [*p*-Br-Phe^6,Nle^{10}]NKA(4−10)	0.94 (1)	0.99 (1)	nd[c] (1)	1.04 (1)	0.95 (1)	0.99 (1)	1.09 (1)	827.3 (827.0)
5, [*p*-I-Phe^6,Nle^{10}]NKA(4−10)	0.83 (1)	0.99 (1)	nd (1)	1.05 (1)	0.99 (1)	0.98 (1)	0.97 (1)	875.0 (875.0)
6, [*p*-NH_2-Phe^6,Nle^{10}]NKA(4−10)	0.74 (1)	1.03 (1)	0.79 (1)	1.12 (1)	1.09 (1)	1.12 (1)	1.10 (1)	764.0 (764.0)
7, [*p*-NO_2-Phe^6,Nle^{10}]NKA(4−10)	0.71 (1)	0.98 (1)	nd (1)	1.08 (1)	1.05 (1)	1.11 (1)	1.08 (1)	794.0 (793.0)
8, [Chex-Ala^6,Nle^{10}]NKA(4−10)	0.75 (1)	1.01 (1)	1.01 (1)	1.09 (1)	1.05 (1)	1.05 (1)	1.05 (1)	754.5 (754.5)
9, [Trp^6,Nle^{10}]NKA(4−10)	0.85 (1)	0.96 (1)	− (1)	0.99 (1)	1.00 (1)	1.06 (1)	1.08 (1)	788.0 (788.0)
10, [D-Phe^6,Nle^{10}]NKA(4−10)	0.73 (1)	1.05 (1)	1.01 (1)	1.02 (1)	1.04 (1)	1.12 (1)	1.02 (1)	749.0 (749.0)

[a] Numbers in parentheses are the expected number of residues or the calculated masses. [b] Amino acid replacement at position 6. [c] nd, not determined.

Table 2. Competition Binding Potencies of Natural Neurokinins, [Nle^{10}]NKA(4−10), and [Nle^{10}]NKA(4−10) Analogues

peptide	log IC_{50} ± SEM (n = 3)	K_i (nM)
NKA	−8.77 ± 0.17	0.71
1	−7.51 ± 0.19	12.7
NKB	−6.99 ± 0.24	42.2
2	−6.97 ± 0.07	44.2
SP	−6.61 ± 0.20	102
6	−6.43 ± 0.14	156
3	−5.46 ± 0.05	1422
7	−	>2000
9	−	>2000
10	−	>2000

Table 3. Absolute Electronegativity (χ) of the *Para*-Substituent in the Aromatic Ring of Phe^6 in [Nle^{10}]NKA(4−10) Analogues and Volume Difference between the Parent Residue and Its Replacements

residue	χ^a	ΔV (Å^3)
p-F-Phe	10.41	1.6
p-Cl-Phe	8.31	11.7
p-Br-Phe	7.6	16.8
p-I-Phe	6.76	28
p-NH_2-Phe	6.07	11.6
p-NO_2-Phe	6.2	16.9
Chex-Ala	−	38.5
Trp	−	35.8

[a] Absolute electronegativity values were determined by Pearson.[22]

Human recombinant NK-2 receptors are used to screen the receptor binding activity of the analogues. The effects of *para*-substituents and replacements of Phe^6 on receptor activation are assessed by measuring contraction of hamster tracheal rings in vitro. The biological data could then be correlated with the topographic features of residues at position 6 of [Nle^{10}]NKA-(4−10) analogues using molecular modeling.

Results

[^{3}H]NKA bound to membranes prepared from CHO cells expressing the NK-2 receptor in a saturable and displaceable manner with K_d of 0.71 nM (Table 2). The Hill coefficient was close to 1.0 in all experiments. The cloned receptors showed the correct rank order of potency for natural neurokinins (Table 2). **1** inhibited the binding of [^{3}H]NKA to the NK-2 receptor with an inhibitory constant (K_i) of 12.7 nM.

The rank order of potency of the synthetic analogues, parent peptide, and natural neurokinins, in binding to the NK-2 receptor, is shown in Table 2. In general, substitutions of hydrogen in the *para*-position of the aromatic ring of Phe^6 led to a reduction of binding, but *p*-F substitution resulted in only a moderate decrease, with K_i still in the nanomolar range. The next most potent analogue was **6** with K_i of 0.15 μM. Substitution with Cl gave rise to an analogue with K_i of 1.42 μM. Introduction of a nitro group was even more detrimental to binding. Analogues with the *p*-Br or *p*-I substitution, at concentrations up to 0.1 mM, did not compete with [^{3}H]NKA for binding to the NK-2 receptors.

Introduction of F in the *para*-position of the aromatic ring of Phe^6 had no significant influence on the ability of the peptide to stimulate contractile activity of tracheal rings. Moreover, curves, reflecting agonistic activity of **1** and of **2**, were virtually superimposable. The efficacy (114.38 ± 4.81) of **2** was not significantly different from that (116.99 ± 3.01) of **1**. The K_A values were 70.3 ± 15.7 and 126 ± 42.78 nM for **2** and **1**, respectively. The efficacy of **6**, 95.77 ± 2.73, was significantly lower than that of **1**, and the K_A was 663.1 ± 27.54 nM. The efficacy, 97.78 ± 2.73, of **3** was also significantly lower than that of **1**. The K_A was 2250 ± 71 nM, corresponding with a reduction in potency. Similarly, **7** caused smaller maximal contraction (efficacy 84.6 ± 4.64) than did **1**. The analogue still displayed agonism; the K_A was 2490 ± 77 nM. **4** and **5** displayed neither agonism nor antagonism.

Replacement of Phe^6 with either D-Phe or Trp resulted in a pronounced decrease in the binding affinity (Table 2). **8** did not inhibit [^{3}H]NKA binding to the NK-2 receptors at concentrations up to 0.1 mM. **9** had some agonism, but the efficacy (56.64 ± 6.24) was reduced to nearly one-half that of the parent peptide. The K_A of this partial agonist was 941 ± 30 nM. **10** had no agonism but had low antagonist activity (data not shown). Replacement with Chex-Ala led to an analogue devoid of both agonism and antagonism.

Calculated volume difference (ΔV) between the Phe^6 residue and its replacements is shown in Table 3.

Discussion

The substituent at the *para*-position of the aromatic ring of Phe^6 in **1** appears to be crucially important for interactions with the NK-2 receptor. It was assumed that *para*-substitution would not change the overall backbone conformation. This hypothesis was proven when ΔV and χ of substituents were correlated with biological activity of analogues. The binding affinity and agonism of analogues with *p*-halogen substitution seem to correlate inversely with the van der Waals volume of the halogens. The similarity of receptor activity of **2** with that of **1** (Table 2) is probably a reflection of the

small size of F (Table 3). The absence of receptor activity following substitution with Br, but not with smaller halogens, indicates that the substituent must be smaller than Br to allow the "hydrophobic patch" of the peptide[9] to fit a putative hydrophobic pocket in the receptor.

Significant differences in the binding affinity and agonism between **2** and **3** indicate that a 10-fold increase approximately in the size of the substituted group (Table 3) can have a detrimental effect on biological activity. It might have been expected that the higher electronegativity of F compared with Cl (Table 3) would have an influence on the peptide binding and agonism. The observation, however, that **7** is less potent than **6** indicates that the electronegativity of the substituent is unlikely to be crucially important for binding and receptor activation (Tables 2 and 3). Escher and coworkers[17] similarly demonstrated that [*p*-NO_2-Phe^7,Nle^{11}]SP was less potent than [*p*-NH_2-Phe^7,Nle^{11}]SP, confirming the primary importance of the size of the *para*-substituent for activation of NK-1 receptors in guinea pig ileum.

p-NH_2-Phe^6 has a ΔV close to that of *p*-Cl-Phe^6, and the ΔV of *p*-NO_2-Phe^6 is close to that of *p*-Br-Phe^6 (Table 3). **6**, however, is more potent than **3**, and **7** is active despite the ΔV of *p*-NO_2-Phe being as large as that of *p*-Br-Phe. Evidently, NH_2 and NO_2 groups have more planar structures than Cl and Br, respectively, and assuming planarity of the binding pocket in the receptor (see below) enhanced affinity of peptides with NH_2 and NO_2 substitution might be expected.

The total loss of peptide activity in the functional assay, when the aromatic side chain at position 6 was replaced with a cyclohexyl group, favors the hypothesis that weakly polar $\pi-\pi$ interactions exist between the Phe^6 residue and the receptor. Huang and co-workers[13] identified 15 NK-2 receptor residues which are involved in the binding of peptide ligands; 10 are aromatic. The attractive orientation in the $\pi-\pi$ interaction is either edge-to-face or offset geometry, whereas face-to-face geometry leads to a repulsive orientation.[11] Thus, the $\pi-\pi$ interactions should be facilitated by planarity as well as by rigidity of the binding pocket of the receptor. This concept is supported by our results.

The retention of some, although low, peptide agonism when Phe^6 is replaced by Trp may be due to the planar nature of the Trp side chain. By contrast, the cyclohexyl ring in **8** adopts a chair conformation in energy minimization. This structure may not be fully complementary with the hydrophobic pocket and might only partially occupy the receptor-essential volume. Similarly, replacement of the Phe^6 with *p*-Br-Phe is not conducive to a planar side group conformation, since it causes total loss of peptide binding even though the ΔV of **4** is much lower than the ΔV of **9**.

The reduction in binding affinity and loss of all agonism but acquisition of some antagonism, when L-Phe^6 is replaced by D-Phe, may be a reflection of the totally different conformation of **10** from that of **1**. The D-Phe might interact with groups in the receptor other than those which could interact with an L-amino acid at position 6. The D-Phe appears to confer antagonism on the peptide with concomitant loss of agonism.

Conclusion

The results show that the size of the *para*-substituent in the aromatic ring at position 6 of [Nle^{10}]NKA(4−10) analogues is more important than electron-donating or -withdrawing properties. The planarity of the aromatic ring in Phe derivatives and Trp appears to be conducive to peptide activity, indicating a complementary planar pocket in the NK-2 receptor. The results also favor the hypothesis that the Phe^6 residue binds to the receptor through weakly polar $\pi-\pi$ interactions.

Experimental Section

Materials. Boc-Asp(OBzl)-OH, Boc-Leu-OH, Boc-Met-OH, Boc-Nle-OH, Boc-Phe-OH, Boc-D-Phe-OH, Boc-Ser(Bzl)-OH, Boc-Trp-OH, Boc-Val-OH, Boc-*p*-Bzl-NH_2-Phe-OH, Boc-*p*-F-Phe-OH, Boc-*p*-Cl-Phe-OH, Boc-*p*-I-Phe-OH, Boc-*p*-NO_2-Phe-OH, and *p*-methylbenzhydrylamine resin were purchased from Bachem California Inc. (Torrance, CA). Boc-*p*-Br-Phe-OH and Boc-Ala-OH were from Aldrich Chemical Co. (St. Louis, MO). TFMSA was from Applied Biosystems/Perkin-Elmer (Foster City, CA). NKA, NKB, and SP were purchased from Peninsula Laboratories Inc. (Belmont, CA). [4,5-^{3}H-Leu^9]Neurokinin A ([^{3}H]NKA; specific activity 170 Ci/mmol) was purchased from Cambridge Research Biochemicals (London, U.K.). Human recombinant NK-2 receptors expressed in CHO cell membranes were obtained from Biosignal Inc. (Montreal, Canada). Tris-HCl, Hepes (enzyme grade), DIC, TFA, DMF (peptide synthesis grade), DCM, acetonitrile, and triethylamine were purchased from Fisher Scientific (St. Louis, MO), and phenoxybenzamine (PBZ) was purchased from SmithKline Beecham Pharmaceuticals (Brentford, U.K.). Other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Peptide Synthesis. Peptides were synthesized on a 430A Applied Biosystems peptide synthesizer (Perkin-Elmer, Foster City, CA). Syntheses were performed on a 0.5-mmol scale on *p*-methylbenzhydrylamine resin. The reactive side chains in Asp and Ser were protected with a β-benzyl group. The amino group of *p*-NH_2-Phe was protected with a benzyloxycarbonyl group. *N*-α-Boc-protected amino acid derivatives were coupled as their 1-hydroxybenzotriazole active esters. Removal of *N*-α-Boc groups was performed in 50% TFA/DCM for 2 min and then 30% TFA in DCM for 17 min. DIEA was used to neutralize the resin. The resin was washed with DCM and NMP. Side chain deprotection and cleavage of peptides from the resin were achieved in a single-step reaction by stirring the peptide-resin in a solution of trifluoromethanesulfonic acid/thioanisole/ethanedithiol/trifluoroacetic acid (2:2:1:20, v/v/v/v) for 30 min at −10 °C followed by 90 min at room temperature.

HPLC Experiments. For analytical HPLC, Vydac 218TP54 columns (C18, 5-mm particle size, 250 mm × 4.6 mm; The Separations Group, Hesperia, CA) were irrigated for 35 min at 1 mL/min with a 3−60% linear gradient of 0.09% TFA in CH_3CN added to 0.1% TFA in H_2O. A_{220} of effluent fractions was recorded. For preparative HPLC, the above solvents were used at a flow rate of 4.5 mL/min, on a Vydac 218TPB1015 coulmn (C18, 15-mm particle size, 500 mm × 10 mm) packed in-house.

Mass Spectrometry. FAB-MS analyses were carried out by the Midwest Center for Mass Spectrometry (Department of Chemistry, University of Nebraska-Lincoln).

Amino Acid Analysis. The hydrolysates of purified peptides (6 N HCl, 110 °C, 24 h) were analyzed using a Waters AccQTag amino acid analysis system.

Ligand Binding Assay. Binding assays were performed with a cytoplasmic membrane fraction of CHO cells which had been transfected with human NK-2 receptor cDNA and selected for stable expression of NK-2 receptors.[18] The incubation media consisted of 20 mM Hepes buffer, pH 7.4, supplemented with 2 mM $MnCl_2$, 0.01% bovine serum albumin (protease-free), 4 μg/mL leupeptin, 4 μg/mL chymostatin, 0.1 mM bestatin, and 10 μM phosphoramidon. For competition

studies, the membrane suspension (protein concentration 15.6 μg/mL) was incubated for 60 min at 27 °C with 1 nM [^{3}H]NKA and various concentrations of the peptides to be tested. Nonspecific binding was determined in the presence of a 1000-fold molar excess of unlabeled NKA. The reaction was terminated by a rapid filtration through Whatman GF/B glass filters presoaked in 0.3% poly(ethylenimine), using a Brandel cell harvester (Biomedical R&D Laboratories Inc., Gaithersburg, MD). Then the filters were washed three times with ice-cold 50 mM Tris-HCl buffer, pH 7.4. The bound radioactivity was measured with a 1209 Rackbeta liquid scintillation counter (Wallac Inc., Gaithersburg, MD). Data were analyzed using GraphPad Prizm software (GraphPad Software Inc., San Diego, CA).

Assay with Isolated Hamster Tracheal Ring. Tracheae were obtained from male Syrian hamsters (90−120 g) which had been sacrificed by CO_2 intoxication. The tracheal rings (diameter 3−4 mm) were rapidly prepared and suspended at 37 °C in an organ bath containing 4 mL of Krebs solution[19] supplemented with 10 μM phosphoramidon and 1 μM indomethacin. The contractile activity was recorded by isometric transducers after a 60-min equilibration period which followed two or more reproducible responses to 1 mM carbachol. Cumulative responses to increasing concentrations of peptides were registered. Agonist dissociation constants (K_A) and relative efficacies were determined by the method of partial irreversible blockade with the alkylating agent PBZ as described elsewhere.[20,21]

Volume Calculation. The differences in the van der Waals volumes (ΔV) of the parent residue and its replacements were determined using the SYBYL molecular modeling package (Tripos Inc., St. Louis, MO).

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(23) Abbreviations used for amino acids and peptides follow the recommendations of the IUPAC−IUB Commission of Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9−37). Other abbreviations: Boc, *tert*-butyloxycarbonyl; Chex, cyclohexyl; CHO, Chinese hamster ovary; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DMF, *N,N*-dimethylformamide; [^{3}H]-NKA, [4,5-^{3}H-Leu^9]neurokinin A; NKA, neurokinin A; NKB, neurokinin B; NMP, *N*-methylpyrolidone; PBZ, phenoxybenzamine; SP, substance P; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid.

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