Structure-Contractile Activity Relationships of Neurokinin A on Guinea Pig Tracheal Smooth Muscle

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In order to investigate the effect of the fourth and seventh positions from the C-terminus of NKA and NKA(4-10) in biological activity, NKA, NKA(4-10) and their analogues substituted with the other amino acid residues at these positions were synthesized by solid phase peptide synthesis. The contractile activities of these synthetic peptides on guinea pig tracheal smooth muscle known as the NK-2 receptor-specific tissue, were examined. Our results are indicated that the aliphatic amino acid (Val or Ile) and acidic amino acid (Asp) at the fourth and seventh positions from the C-terminus of NKA, respectively, are important for the binding with the NK-2 receptor present in the guinea pig trachea. In particular, the aliphaticity at the fourth position from C-terminus of NKA rather than the hydrophobicity is important for the NK-2 receptor binding affinity.

Introduction

Tachykinins are a family of neuropeptide sharing the common carboxyl terminal sequence, Phe-X-Gly-Leu-Met-NH_2. In mammals, the three tachykinin peptides containing substance P (SP) and neurokinin peptides [neurokinin A (NKA) and neurokinin B (NKB)] are known to exist.[1,2] Since these mammalian tachykinin peptides are widely distributed throughout the central and peripheral nervous systems, they are believed to act as putative neurotransmitters.[3] SP and neurokinin peptides produce a contractile effect in many smooth muscles and in some instances evidence is available to support their physiological role as mediators for non-cholinergic nerve-mediated excitation in smooth muscle. The diverse biological actions of SP, NKA and NKB are mediated by at least three distinct receptors which have been termed NK-1, NK-2 and NK-3.[4] They have different affinity for the three receptors, as measured in both binding and functional assays. SP, NKA, and NKB show binding preferences for NK-1, NK-2 and NK-3 receptor, respectively.

The C-terminal heptapeptide of SP and neurokinin peptides was known to be at least the shortest length to exhibit the contractile activity on the isolated rat vas deferens (RVD), guinea pig ileum (GPI) and guinea pig trachea (GPT).[5,6] The distinct difference between SP and neurokinin peptides in their active sequence of C-terminal heptapeptide are the amino acid residues at the two positions (fourth and seventh residues) from the C-terminus (Table 1). The fourth residue from the C-terminus in SP and neurokinin peptides is aromatic (Phe) and aliphatic (Val), respectively. The seventh residue from the C-terminus is acidic (Asp) and carboxyamide (Gln), respectively (Table 1).

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Table 1. Amino acid sequences of mammalian tachykinin peptides

Peptides	Amino acid sequences
SP	Arg-Pro-Lys-Pro-**Gln**-Gln-Phe-**Phe**-Gly-Leu-Met-NH_2
NKA	His-Lys-Thr-**Asp**-Thr-Phe-**Val**-Gly-Leu-Met-NH_2
NKB	Asp-Met-His-**Asp**-Phe-Phe-**Val**-Gly-Leu-Met-NH_2

Therefore, in this study, to investigate the effect of the these two residues (fourth and seventh residues) in NKA and NKA(4-10) on the contractile activity, we synthesized some analogues (Table 2) substituted with the other amino acid residues at these positions of NKA and NKA(4-10) by the solid phase method using Fmoc-chemistry.[7] The contractile activities of these synthetic peptides on guinea pig tracheal smooth muscle[8~10] known as the NK-2 receptor-specific tissue were examined.

Experimental Section

Peptide synthesis. Fmoc (9-fluorenylmethoxycarbonyl)-NH-SAL-Resin was used as the starting material for the

Table 2. Amino acid sequences of NKA, NKA(4-10) and their analogues

Peptides	Amino acid sequences
NKA	His-Lys-Thr-Asp-Thr-Phe-Val-Gly-Leu-Met-NH_2
NKA(4-10)	Asp-Thr-Phe-Val-Gly-Leu-Met-NH_2
[Gln^4]-NKA	His-Lys-Thr-**Gln**-Thr-Phe-Val-Gly-Leu-Met-NH_2
[Gln^4]-NKA(4-10)	**Gln**-Thr-Phe-Val-Gly-Leu-Met-NH_2
[Phe^7]-NKA	His-Lys-Thr-Asp-Thr-Phe-**Phe**-Gly-Leu-Met-NH_2
[Phe^7]-NKA(4-10)	Asp-Thr-Phe-**Phe**-Gly-Leu-Met-NH_2
[Ile^7]-NKA	His-Lys-Thr-Asp-Thr-Phe-**Ile**-Gly-Leu-Met-NH_2
[Ile^7]-NKA(4-10)	Asp-Thr-Phe-**Ile**-Gly-Leu-Met-NH_2

The bolded amino acids indicate the substituted residue in this study.

Table 3. Amino acid compositions of the synthetic peptides

Peptides	Asp	Ser	Thr	Gln	Gly	Val	Met	Ile	Leu	Phe	His	Lys
NKA	1.11 (1)	0.84 (1)	0.75 (1)	–	1.11 (1)	1.06 (1)	0.88 (1)	–	1.00 (1)	1.03 (1)	1.08 (1)	1.09 (1)
[Gln^4]-NKA	–	0.86 (1)	0.83 (1)	0.95 (1)	1.10 (1)	1.12 (1)	1.10 (1)	–	1.00 (1)	0.97 (1)	0.97 (1)	0.97 (1)
[Gln^4]-NKA(4-10)	–	0.86 (1)	–	1.10 (1)	1.01(1)	1.11 (1)	1.01 (1)	–	1.00 (1)	1.00 (1)	–	–
[Phe^7]-NKA	0.97 (1)	1.01 (1)	0.86 (1)	–	1.00 (1)	–	1.10 (1)	–	1.00 (1)	2.02 (2)	1.01 (1)	1.00 (1)
[Phe^7]-NKA(4-10)	0.90 (1)	0.91 (1)	–	–	0.92 (1)	–	1.02 (1)	–	1.00 (1)	2.03 (2)	–	–
[Ile^7]-NKA	0.96 (1)	0.94 (1)	0.84 (1)	–	0.98 (1)	–	0.91 (1)	1.06 (1)	1.00 (1)	0.98 (1)	1.01(1)	1.02(1)
[Ile^7]-NKA(4-10)	1.08 (1)	0.88 (1)	–	–	1.02 (1)	–	0.92 (1)	0.98 (1)	1.00 (1)	1.02 (1)	–	–

Acid hydrolysis of the synthetic peptides was done with 6N-HCl in the presence of 2% phenol at 110 °C for 22 h. Numbers in the parenthesis as the theoretical values.

solid phase peptide synthesis.[7] The coupling of Fmoc-amino acid in each step was performed using DIPCI(diisopropyl-carbodiimide)-HOBt(1-hydroxybenzotriazole) (1 : 1) in NMP (1-methyl-2-pyrridone). After chain elongation, the protected-peptide-NH-SAL-Resin was treated with trifluoroacetic acid / 1,2-ethandithiol / H_2O / triisopropylsilane (92.5 / 2.5 / 2.5 / 2.5, vol/vol) at room temperature for 2 hr. After removing volatile materials under a vacuum, the crude residues were washed with cold ethylether 3 times to remove trace amount of scavengers and then the crude product was lyophilized. The crude product was purified by a preparative C_{18} RP-HPLC (Delta Pak, 5 μm, 3.9150 mm). The amino acid composition of the purified peptides was identified by amino acid analysis (Pharmacia Biochrom. 20). The molecular weight of the purified peptides was checked by fast atom bombardment mass spectrometry (FAB-MS) (VG70-VSEG Mass Spectrometer).

Pharmacological assay. Hartley guinea pig (280-320 g) was sacrificed by blow on the head and the trachea was rapidly dissected at 1.5-2.0 mm intervals and put in cold Krebs solution (NaCl 119.0 mM, KCl 3.5 mM, KH_2PO_4 1.5 mM, $CaCl_2$ 1.25 mM, $NaHCO_3$ 25.0 mM, Glucose 11.0 mM, pH 7.4). Two dissected muscles were connected to steel hook vertically jointed in series and incubated in the 5 mL organ bath filled with warm (37 °C) oxygenated (95% O_2, 5% CO_2) Krebs solution. The muscle was equilibrated, under a tension of 1.0 g prior to the assay for 60-90 min., while being washed every 20 minutes with the Krebs solution. The preparated muscles were excised by the addition of 10^{-6} M carbachol. Contractions were recorded isotonically under a resting tension of 1.0 g *via* FD-pick up (TB-612T, Nihon Koden) connected to amplifier (AP 601G, Nihon Koden) and recorder (WI-621G, Nihon Koden). Peptides were dissolved in saline and were applied at intervals of 10-15 min, consecutively.

Results and Discussion

SP and NKA have been known to co-localize in a population of primary sensory C-fibres which have peripheral branches in various tissues including the respiratory tract.[10] Local release of SP and/or NKA from sensory fibres seems to play an important role in neurogenic inflammation, airway oedema and branchoconstraction.[12~15] NKA was more active than SP in causing bronchocontraction *in vivo* in the guinea pig, rat, sheep, and man.[16~19] Similarly, NKA appears to be particularly active on the guinea pig isolated trachea which has been particularly useful in understanding the mechanisms that could airway narrowing.[20, 21]

It has been known that the heptapeptide in carboxyl side and C-terminal amide (-CO-NH_2) of SP, NKA and NKB are requisite for their biological activity.[5,6] This biologically-active part of SP, NKA and NKB is called message sequence. The N-terminal tri- or tetra peptides of SP, NKA and NKB did not exert considerable effects on biological activity, such as the contraction of smooth muscle. In particular, the C-terminal heptapeptide of NKA, NKA(4-10), showed about two-fold higher activity than NKA in the contractile activity of the NK-2 specific smooth muscle, such as rabbit pulmonary artery (RPA) or guinea pig trachea (GPT).[6, 23] This N-terminal tri- or tetrapeptide parts of SP, NKA and NKB called as address sequence were suggested to be related to the interaction with the phospholipid bilayer on the cell surface.[22,23]

In this study, in order to examine the effect of the fourth and seventh residues from the C-terminus of NKA and NKA(4-10) on contractile activity of the isolated guinea pig tracheal smooth muscle, some analogues substituted with other amino acid at these positions of NKA and NKA(4-10) were synthesized (Table 2). The purity of the synthetic peptides was confirmed by the elution profile in analytical C_{18} reversed-phase HPLC (data not shown). The correct amino acid composition of the synthetic peptides was confirmed by amino acid analysis (Table 3). The experimental values of the molecular weight of the synthetic peptides determined

Table 4. Molecular weights of the synthetic peptides determined by FAB-MS

Peptides	Theoretical values	Experimental values
NKA	1134.0	1134.0
[Gln^4]-NKA	1146.3	1145.5
[Gln^4]-NKA(4-10)	780.0	780.3
[Phe^7]-NKA	1182.0	1181.2
[Phe^7]-NKA(4-10)	816.2	816.4
[Ile^7]-NKA	1147.4	1146.9
[Ile7]-NKA(4-10)	781.1	781.8

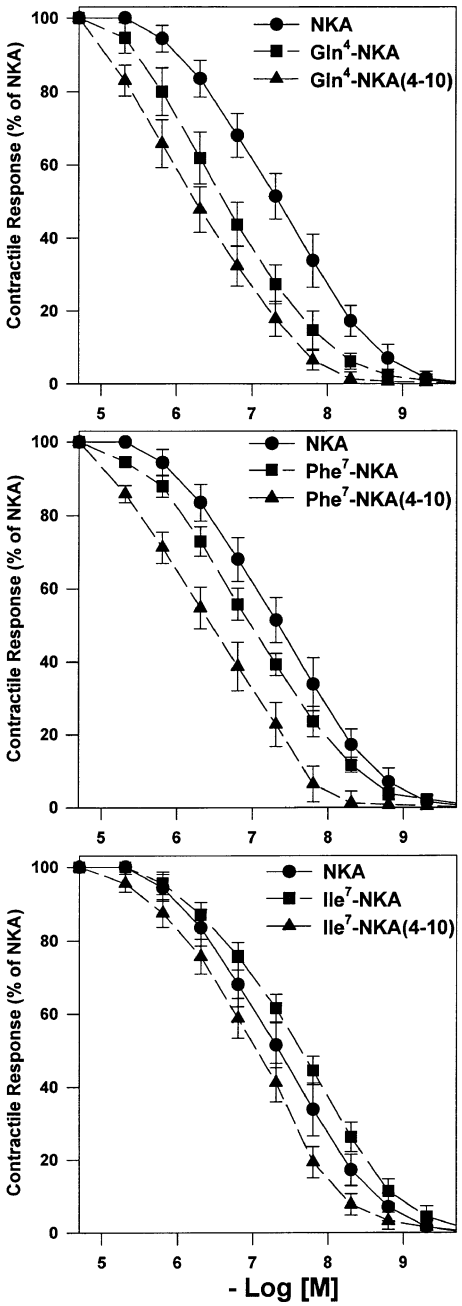

Figure 1. Concentration-response curves for the contraction of guinea pig trachea induced by NKA, NKA(4-10) and their analogues. Each value represents the mean of at least six experiments.

by FAB-MS were consistent with the calculated values (Table 4). The concentration-response curves for the contraction of guinea pig tracheal smooth muscle induced by NKA and its analogues are given in Figure 1. The concentration-response curves were obtained from at least six muscle preparation. The contraction activity of the synthetic peptides was described as the pD_2 value. The pD_2 value (-Log of molar concentration of peptide needed to obtain 50% of the maximal response) of the peptides were calculated from the concentration-response curves (Table 5).

The Gln replacement ([Gln^4]-NKA and [Gln^4]-NKA(4-10)) of the seventh residue (Asp) from the C-terminus of

Table 5. Contractile activities of NKA, NKA(4-10) and their analogues on the isolated guinea pig trachea

Peptides	pD_2[a]	R. A. (%)[b]	E_{max}[c]
NKA	7.35±0.04	100.0	1.0
NKA(4-10)[d]	7.77±0.05	265.0	1.0
[Gln^4]-NKA	6.55±0.06	15.8	1.0
[Gln^4]-NKA(4-10)	6.55±0.09	7.8	1.0
[Phe^7]-NKA	6.82±0.06	29.5	1.0
[Phe^7]-NKA(4-10)	6.47±0.08	13.2	1.0
[Ile^7]-NKA	7.39±0.10	109.6	1.0
[Ile^7]-NKA(4-10)	7.13±0.05	60.3	1.0

[a] pD_2: -log of molar concentration of agonist needed to 50% of the maximal response. Each value is the mean ± S.E. M. of eight experiment. [b] R.A.: Relative affinity expressed as a fraction of the affinity of NKA (NKA=100%). [c] E_{max}: Maximal effect expressed as a fraction of the maximal response of NKA (NKA=1.0). [d]: data is cited from reference 6.

NKA produced a remarkable decrease (15.8 and 7.8% of NKA) in the contractile activity. Thus, this result suggests the acidic residue at this position of NKA is essential for the contractile activity of guinea pig tracheal smooth muscle. Phe substitution ([Phe^7]-NKA and [Phe^7]-NKA(4-10)) of the fourth residue (Val) from the C-terminus of NKA caused a significant decrease (29.5 and 13.2% of NKA) in the contractile activity. In contrast, [Ile^7]-NKA showed a slightly higher activity than NKA (109.6% of NKA). In particular, the C-terminal heptapeptide, [Ile^7]-NKA(4-10) retained 60% activity of NKA. This result indicated that the aliphaticity at this position of NKA rather than the hydrophobicity is important for the contractile activity. Therefore, the acidic amino acid (Asp) and aliphatic amino acid (Val or Ile) at the seventh and fourth residues from the C-terminus of NKA, respectively, have a significant effect for the interaction with the NK-2 receptor.

In conclusion, our structure-activity study of NKA indicated that the aliphatic and acidic residue at the fourth and seventh residue from C-terminus of NKA, respectively, are important for the NK-2 receptor binding. In particular, the aliphaticity at the fourth position from C-terminus of NKA rather than the hydrophobicity is important for the NK-2 receptor binding affinity.

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