Structure-Activity Study of Guinea Pig Trachea Tachykinin NK-2 Receptor: Effect of Substitution at the Seventh Position from C-Terminus of Neurokinin A

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Received May 19, 2000

Substance P (SP: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), neurokinin A (NKA: His-Lys-Thr-Asp-Thr-Phe-Val-Gly-Leu-Met-NH_2) and neurokinin B (NKB: Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2) are mammalian tachykinin peptides that have similar biological actions, such as smooth muscle contraction, salivation, vasodilatation and neuronal excitation.[1~3] These mammalian tachykinin peptides are characterized by the C-terminal common sequence, Phe-Xaa-Gly-Leu-Met-NH_2, where Xaa represents an aromatic (Phe, Tyr) or branched aliphatic (Val, Ile). Since SP, NKA and NKB are widely distributed throughout the central and peripheral nervous systems, they are believed to act as putative neurotransmitters.[4] 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.[5] The receptors have been cloned and are members of the superfamily of G protein-coupled receptors with seven putative membrane-spanning segments.[6]

The carboxy-terminal heptapeptide of SP, NKA, and NKB was known to be at least the shortest length to display the contractile activity on various isolated muscles such as guinea pig ileum (GPI), guinea pig trachea (GPT), and rat vas deferens (RVD) containing tachykinin receptors.[7~9] The difference between SP and neurokinin peptides (NKA and NKB) in their active sequence of C-terminal heptapeptide is the fourth and seventh positions from C-terminus. The fourth position from C-terminus of SP and neurokinin peptides is aromatic (Phe) and aliphatic (Val), respectively. The seventh position from C-terminus is acidic (Asp) and carboxyamide (Gln), respectively. In this study, in order to investigate the effects of the seventh residue C-terminus of NKA in the binding NK-2 receptor and design more selective agonist against NK-2 receptor than NKA, we synthesized three analogues substituted with Glu, 2-aminobutyric acid (Abu), or Ala at this position of NKA. The biological activity of the peptides was examined by the contractile activity of the smooth muscle of guinea pig trachea (GPT) known as the NK-2 receptor-specific tissue.

Experimental Section

Peptide synthesis. Fmoc (9-fluorenylmethoxycarbonyl)-NH-SAL-Resin was used as the starting material to obtain peptide amide by solid phase peptide synthesis.[10] The coupling of Fmoc-amino acid in each step was performed using DIPCI (diisopropylcarbodiimide)-HOBT(1-hydroxybenzotriazole) in NMP (1-methyl-2-pyrridone). After chain elongation, the side chain deprotection and cleavage of the peptides from resins were achieved in a single-step reaction by stirring the peptide-resin in the solution of trifluoroacetic acid/1, 2-ethanedithiol/H_2O/triisopropylsilane (92.5/2.5/2.5/2.5, v/v/v/v) for 2 hr at room temperature. After removing volatile materials under vacuum, the crude residues were washed with cold diethyl ether to remove trace amount of scavengers, and then the crude product was lyophilized. The fluffy product was purified by a preparative C_{18} RP-HPLC (Delta Pak, 5mm particle size, 3.9×150 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 ^oC) 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

The smooth muscle contractile response to exogenously added mammalian tachykinin peptides in the guinea pig, rat, sheep, and human airways appears to be mediated by tachykinin receptors.[11~13] Of the endogenous ligands, only NKA was known to be potent contractile agents of human airway smooth muscle involved with NK-2 receptor.[11~13] Thus,

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Table 1. Amino acid sequences of NKA and its analogues synthesized in this study

Peptides	Amino acid sequences
NKA	His-Lys-Thr-Asp-Thr-Phe-Val-Gly-Leu-Met-NH_2
[Glu^4]-NKA	His-Lys-Thr-**Glu**-Thr-Phe-Val-Gly-Leu-Met-NH_2
[Ala^4]-NKA	His-Lys-Thr-**Ala**-Thr-Phe-Val-Gly-Leu-Met-NH_2
[Abu^4]-NKA	His-Lys-Thr-**Abu**-Thr-Phe-Val-Gly-Leu-Met-NH_2

The bolded amino acids indicate the substituted residue in this study. Abu: 2-aminobutyric acid

structure-activity study of NKA and its analogues in the contractile activity mediated by the NK-2 receptor-specific tissue such as GPT will provide an useful information in the understanding the contraction mechanism in human bronchus.

It has been reported that the heptapeptide and C-terminal carboxyamide (-NH_2) of SP, NKA and NKB is essential for their biological activity.[3,7~9,14] These biologically active parts of SP, NKA and NKB are called the message sequence. The N-terminal tri- or tetra peptides of SP, NKA and NKB (SP: Arg-Pro-Lys-Pro-, NKA: His-Lys-Thr-, NKB: Asp-Met-His-) were known to play a significant role in the receptor recognition due to their electrostatic charges, a tendency to form an α-helical structure, hydrophilicities *etc*. These N-terminal tri- or tetra sequence of SP, NKA and NKB are called the address sequence.[3] In our previous study, Phe-substitution of the fourth position (Val) from C-terminus of NKA induced a significant reduction on the contractile activity of the smooth muscle of GPT. This result suggested that the branched aliphaticity is important for the NK-2 receptor binding affinity.[15] Gln-substitution of the seventh position (Asp) from C-terminus of NKA induced a remarkable reduction in the GPT contractile activity.[11] In the present study, three analogues substituted with Glu, 2-aminobutyric acid (Abu), and Ala at the seventh position (Asp) from C-terminus of NKA were synthesized by the solid phase method using Fmoc-chemistry (Table 1).[10]

The purity of the synthetic peptides was confirmed by showing a single peak in the elution profile of the analytical C_{18}-RP-HPLC (data not shown). The correct amino acid composition of the synthetic peptides was confirmed by amino acid analysis (data not shown). The experimental values of the molecular weight of the synthetic peptides determined by FAB-MS were consisted with the calculated values (Table 2). Concentration-contractile response curves were obtained from at least six preparations by the cumulative injection of the peptides (Figure 1). Apparent affinities

Table 2. Molecular weights of the synthetic peptides determined by FAB-MS

Peptides	Theoretical values	Experimental values
NKA	1134.0	1134.2
[Glu^4]-NKA	1147.7	1147.7
[Ala^4]-NKA	1090.0	1090.3
[Abu^4]-NKA	1126.0	1126.4

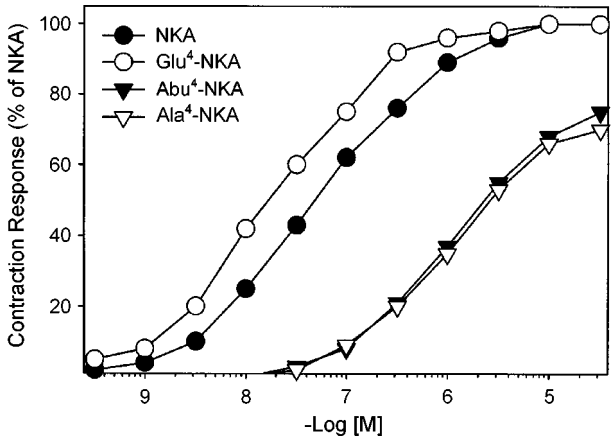

Figure 1. Concentration-response curves for the contraction of guinea pig trachea induced by NKA and its analogues. Each value represents the mean of at least six experiments.

Table 3. Contractile activities of NKA and its analogues on GPT

Peptides	PD_2[a]	R. A. (%)[b]	E_{max}[c]
NKA	7.35	100.0	1.00
[Glu^4]-NKA	7.78	309.9	1.00
[Ala^4]-NKA	5.94	4.5	0.75
[Abu^4]-NKA	5.49	5.0	0.70

[a] pD_2: -log of molar concentration of agonist needed to 50% of the maximal response. [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=1.00).

(pD2 values) of the peptides were calculated from the concentration-response curves and the relative affinities are expressed in percent of NKA (Table 3). The elimination of the negative charge by Ala or Abu-substitution of the seventh position (Asp) from C-terminus of NKA (Ala^4-NKA and Abu^4-NKA) produced a remarkable decrease (5.0% and 4.5% of NKA) in the contractile activity. In contrast, Glu-substitution (Glu^4-NKA) of this position in NKA caused 3-fold increased activity as compared to NKA. These results suggested that the negative charge at the seventh position from C-terminus of NKA is essential for interaction with NK-2 receptor.

In conclusion, the structure-activity study of NKA indicated that acidic residue at the seventh position from C-terminus of NKA is requisite for binding with NK-2 receptor. Moreover our results in this study may provide useful information in understanding of the ligand-NK-2 receptor interaction involved in the bronchoconstriction in mammalian airway system.

Acknowledgment. The authors wish to acknowledge the financial support of the Korea Research Foundation made in the program year of 1998 (Project No. 1998-15-D00243) and Matching Fund Programs of Research Institute for Basic Sciences, Pusan National University, Korea, 1999 (Project No. RIBS-PNU-99-401). We are deeply grateful to Mass Spectrometry Analysis Group of Korea Basic Science Institute for the mass spectra of the synthetic peptides.

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