Syntheses and Biological Activities of Neurokinin A Analogs Substituted with Glycine

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Synopsis. Five neurokinin A (NKA) analogs substituted with glycine at position 3, 4, 5, 6, or 7 were synthesized by Merrifield's solid phase method. The contractile activities of the synthetic analogs were assayed on isolated guinea pig ileum and rat vas deferens. The results suggest that Asp^4, Phe^6, and Val^7 residues of NKA may be essential for the intrinsic activity on guinea pig ileum and on rat vas deferens, respectively.

Neurokinin A (NKA, also called neuromedin L or substance K) and neurokinin B (NKB, also called neuromedin K) are novel neuropeptides isolated from porcine spinal cord. Structure elucidation[1] revealed that NKA and NKB have close structural homologies with tachykinin such as substance P (SP), kassinin and physalaemin (Fig. 1). Decapeptide amides, NKA and NKB, have the same sequence between Phe^6 and Met^{10} and are very similar to the mammalian tachykinin SP, particularly in the sequence of C-terminal region.

Our study[3] on the structure activity relationship of NKB provides clear indication on the location of the active site, that is, the replacement of Phe^6 or Val^7 residue with Gly moiety brings drastic decrease of the contractile activities on isolated guinea pig ileum (GPI) and rat vas deferens (RVD), while the substitution of Gly for Phe^5 enhances their activities. In other words, NKB related octapeptide analogs [Gly^6]-NKB (3-10) and [Gly^7]-NKB (3-10) have no intrinsic activity on GPI and RVD. Furthermore, it has been revealed that the replacement of Phe^6 in NKB with Gly moiety changes the pharmacological spectrum of NKB from that of an agonist to that of an antagonist.[4] The knowledge has been utilized in the design of NKB analogs, and we have succeeded in the development of several specific antagonists against NKB.[5] Therefore glycine substitution seems to be useful tool for the study of the structure activity relationship of NKA.

In the present investigation, in order to examine the biological effect of the substitution of Gly moiety for Thr^3, Asp^4, Ser^5, Phe^6, and Val^7 residues of NKA and the amino acid residues for recognition of SP-P and SP-E type receptors, we synthesized [Gly^3]-, [Gly^4]-, [Gly^5]-, [Gly^6]-, and [Gly^7]-NKA (Fig. 2), and submitted them for biological assay.

Synthesis of NKA analogs used in this study was performed on an automated peptide synthesizer by standard solid phase method[6] starting from *t*-butoxycarbonyl (Boc)-Met benzhydrylamine-resin. Boc protection was used for α-amino function. The side-chain protective groups used were cyclohexyl for Asp, tosyl for His, benzyloxycarbonyl for Lys and benzyl for Ser and Thr. Deprotection and dicyclohexylcarbodiimide (DCC) coupling were programed as given in Table 1.

The second coupling was repeated with additive of one equivalent of 1-hydroxybenzotriazole (HOBt) according to a program without step 1—4. A 2.5 fold excess of amino acid derivatives was used for all couplings. The coupling reaction was monitored by the ninhydrin color test procedure of Kaiser et al.[7] A trace of unreacted amino group was acetylated with acetic anhydride and pyridine. After the completion of the

	1 3 4 5 6 7 10
NKA	H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH$_2$
NKB	H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH$_2$
	1 11
SP	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$
Kassinin	H-Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH$_2$
Physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH$_2$

Fig. 1. Amino acid sequences[2] of tachykinin peptides.

1	[Gly^3]-NKA	H-His-Lys-Gly-Asp-Ser-Phe-Val-Gly-Leu-Met-NH$_2$
2	[Gly^4]-NKA	H-His-Lys-Thr-Gly-Ser-Phe-Val-Gly-Leu-Met-NH$_2$
3	[Gly^5]-NKA	H-His-Lys-Thr-Asp-Gly-Phe-Val-Gly-Leu-Met-NH$_2$
4	[Gly^6]-NKA	H-His-Lys-Thr-Asp-Ser-Gly-Val-Gly-Leu-Met-NH$_2$
5	[Gly^7]-NKA	H-His-Lys-Thr-Asp-Ser-Phe-Gly-Gly-Leu-Met-NH$_2$

Fig. 2. Synthetic NKA analogs.

Table 1. Program for Solid Phase Synthesis

Step	Reagent	Operation	Mix time/min
1	CH_2Cl_2	3×Wash	1.5
2	TFA (25% in CH_2Cl_2)	Prewash	1.5
3	TFA (25% in CH_2Cl_2)	Deprotection	30
4	CH_2Cl_2	3×Wash	1.5
5	CH_2Cl_2	3×Wash	1.5
6	TEA (10% in CH_2Cl_2)	3×Neutralization	1.5
7	CH_2Cl_2	6×Wash	1.5
8	Boc-Amino Acid (in CH_2Cl_2)	Mix	5
9	DCC	Mix	180
10	CH_2Cl_2	6×Wash	1.5

Table 2. Amino Acid Analyses of Synthetic NKA Analogs

Analog	Found (Calcd)										
	Asp	Thr	Ser	Gly	Val	Met	Leu	Phe	His	Lys	NH_3
1	1.04(1)	—	0.89(1)	2.02(2)	1.03(1)	1.00(1)	1.02(1)	1.00(1)	1.01(1)	1.00(1)	1.32(1)
2	—	0.92(1)	0.87(1)	2.04(2)	1.03(1)	0.99(1)	1.03(1)	1.02(1)	1.05(1)	1.04(1)	1.33(1)
3	1.02(1)	0.94(1)	—	1.97(2)	1.01(1)	1.01(1)	1.02(1)	1.01(1)	1.01(1)	1.01(1)	1.37(1)
4	1.02(1)	0.94(1)	0.89(1)	2.00(2)	1.03(1)	1.01(1)	1.04(1)	—	1.03(1)	1.03(1)	1.88(1)
5	1.06(1)	0.96(1)	0.89(1)	2.00(2)	—	1.01(1)	1.04(1)	1.01(1)	1.01(1)	1.02(1)	1.32(1)

Table 3. Physical Properties and Yield of Synthetic NKA Analogs

Analog	$[\alpha]_D^8$/° (*c* 0.2, 50% AcOH)	Retention time[a]/min	R_f^1	R_f^2	Yield/%
1	−20.5	13.3	0.00	0.32	11.0
2	−26.0	12.7	0.00	0.49	9.9
3	−24.5	13.4	0.00	0.43	7.8
4	−32.0	12.7[b]	0.00	0.38	10.9
5	−30.5	10.3	0.00	0.39	6.7

a) Condition of analytical HPLC: column, NOVA-PAK C_{18}; flow rate, 1 ml min^{-1}; Detection, 210 nm; eluent system, linear gradient from 14% [b) 4.2%] to 35% CH_3CN (15 min) in 20 mM phosphate buffer (pH 3.0).

chain elongations, the peptide resin was treated with liquid hydrogen fluoride (HF)[8] in the presence of anisole for 30 min at −20 °C and then 30 min at 0 °C to cleave the peptide from the resin with simultaneous removal of the protecting groups and formation of the carboxyl terminal amide. The excess HF was removed in vacuo, and the resulting material was washed with ethyl acetate prior to extraction of the peptide with aq acetic acid (50%). The crude product was purified by preparative reverse-phase high-performance liquid chromatography (HPLC) using aq trifluoroacetic acid (0.1%) in acetonitrile as eluent. The desired fractions were collected and desalted on a column of Sephadex LH-20 eluted with aq *N*,*N*-dimethylformamide (90%) containing 0.02% 1,2-ethanedithiol, giving pure peptide. The yield was sacrificed for the purity test of the fractions selected. Acid hydrolysates of the peptides contained the constituent amino acids in the ratios predicted by theory (Table 2). Homogeneity of the peptides was demonstrated by analytical HPLC and thin-layer chromatography (TLC) (Table 3). When single peaks and single spots were observed for a peptide in all chromatographic systems, the sample was considered appropriately pure for bioassay.

Biological properties of the synthetic NKA analogs were examined on GPI having SP-P type receptor and RVD having SP-E type receptor. The contractile activities of the analogs were compared with that of our synthetic NKA. The results are shown in Table 4.

On GPI assay, analog **1**, **2**, **3**, and **5** possessed about 25, 10, 20, and 10%, respectively, of the contractile activity of NKA, while analog **4** had little activity. The potency of analog **1** was as high as the intrinsic activity of NKA itself and analog **3** possessed about 80% of the activity on RVD assay, but analogs **2**, **4**, and **5** showed no agonistic activity. Analogs **4** on GPI, and **2**, **4**, and **5** on RVD, were tested for their antagonistic effects against NKA respectively. None of the analogs **2**, **4**, and **5** was found to act as an antagonist of NKA on both assays. These data suggest that the Phe[6] of NKA as well as NKB is essential for binding or stimulation of SP receptors in the two smooth muscle preparations. In contrast to the results obtained in NKB, whereby the substitution of Gly for Phe[6] changes the pharmacological spectrum of NKB from that of an agonist to that of an antagonist, the Gly[6] analog of NKA shows no antagonism to NKA. In comparison with the results that the Gly analogs of NKB provided consistent indications of the active sites, Phe[6] and Val[7], in GPI and RVD,[3] the Gly analogs of NKA indicated the location of the additional active site of Asp[4] in RVD. In other words, Asp[4] moiety as well as Phe[6] and Val[7] in NKA seems to be important for the recognition of SP-E type receptors. The results well agrees with the observation of Osakada et al.,[9] Asp[4] and Val[7] moieties being important for the recognition of SP-E type receptors.

Table 4. Biological Activities of Synthetic NKA Analogs

Analog	GPI		RVD	
	RA[a]	ANT[b]	RA	ANT
1	0.25	NT[c]	1.01	NT
2	0.12	NT	0	—[d]
3	0.20	NT	0.78	NT
4	<0.003	—	0	—
5	0.13	NT	0	—

a) Relative contractile activity to NKA=1. b) Antagonistic activity against NKA. c) Not tested. d) No antagonistic activity.

Experimental

Amino acid derivatives were purchased from Peptide Institute, Inc., Osaka, and benzhydrylamine (BHA) resin (available amine of the resin: 0.6 mmol g^{-1} of support) from Beckman Inc. The solid phase synthesis was carried out using a Beckman System 990C Peptide Synthesizer. Optical rota-

tions were measured in a Nipponbunkoh DIP-4 Polarimeter. Amino acid analyses on samples previously hydrolyzed with 6 M HCl (1 M=1 mol dm^{-3}) (110 °C, 24 h) were performed on a Beckman System 7300 Amino Acid Analyzer. TLC were carried out on silica-gel plates (Merck). The following solvent systems were used and allowed to ascend for 10 cm: R_f^1, *n*-BuOH-AcOH-H_2O (4:1:5, upper phase); R_f^2, *n*-BuOH-pyridine-AcOH-H_2O (30:20:6:24). Analytical HPLC were effected on the following systems: column, NOVA-PAK C_{18} (3.9×150 mm); flow rate, 1 ml min^{-1}, detection, 210 nm; eluent system, linear gradient of CH_3CN in 20 mM phosphate buffer (pH 3.0).

General Procedure for the Preparation of Analog. The first Boc-amino acid was coupled to the BHA-resin (0.60 meq. N g^{-1}) as follows. A half gram of the BHA-resin hydrochloride was neutralized with a solution of 10% (by volume) triethylamine (TEA) in dichloromethane (10 ml). Then the resin was washed with 10 ml of dichloromethane, and was mixed with 2.5 equiv of Boc-Met-OH and DCC in dichloromethane. The quantitation of coupling reaction was carried out by a negative ninhydrin color test. The unreacted amino groups were blocked by acetylation using Ac_2O (5 equiv) and pyridine (1 equiv) in dichloromethane for 20 min. For the coupling reactions after second Boc-amino acids, deprotection and DCC coupling were programed as given in Table 1. For second coupling, a programed without steps 1—4 was used. A 2.5 fold excess of DCC and the amino acid derivative dissolved in dichloromethane were used for all couplings. One equivalent of HOBt was used only for the second DCC-coupling. The protected peptide resin was simultaneously cleaved and deblocked with anhydrous liquid HF containing 10% anisole. Approximately 5 ml of liquid HF was used for 0.25 g resin, and the reaction mixture was kept at −20 °C for 30 min and then 0 °C for 30 min to prevent the succinimide formation in Asp-Gly sequence. After evaporation of HF in vacuo with ice-cooling, the peptide-resin mixture was washed with AcOEt. The peptide was extracted with three portions of 50% AcOH and the extracts combined were lyophilized.

Purification of the Peptides. The crude peptide was subjected to HPLC which is composed of a model 590 pump and an U6K injector (Waters) connecting with a column (20×300 mm) of Chemcosorb ODS (Chemco). The eluates were monitored with an UV detector S-310A model-II (Soma) at 210 nm wavelength. CH_3CN-0.1% TFA solvent system was used as eluent at flow rate 10 ml min^{-1}. Each peptide was emerged at 40—60 min by isocratical elution with 14—20% CH_3CN contents of the solvent system. The desired fraction was desalted on a Sephadex LH-20 column (15×500 mm) eluted with DMF-H_2O (9:1) containing 0.02% 1,2-ethanedithiol and the product was lyophilized. Homogeneity of the peptides was analyzed by analytical HPLC on a column of NOVA-PAK C_{18} (3.9×150 mm) with gradient elution using 20 mM phosphate buffer (pH 3.0)/CH_3CN and by TLC on silica-gel plate. The peptides on silica-gel plate were detected with ninhydrin and chlorine-*o*-tolidine reagents. When single peaks and single spots were observed for a peptide in all chromatographic systems and amino acid composition after hydrolysis of the peptides were consistent with the calculated values, the sample was submitted for bioassay.

Bioassay. The biological activities of NKA analogs were measured on GPI and RVD. Male guinea pig (weighing 200—250 g) and male rat (weighing 250—300 g) were stunned by a blow to the neck and exsanguinated. Ileum and vas deferens were rapidly isolated and kept for dissection in Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.6 mM glucose) at room temperature. Both organs were suspended in a 30 ml organ bath containing Tyrode's solution thermostated at 30 °C and bubbled with 95% O_2 and 5% CO_2 gas for 1 h. The contraction was recorded by means of an isotonic transducer (Nippon Kohden, TD-111T) with load of 2 or 1 g (for ileum and vas deferens respectively) on a Servocorder (Watanabe Instruments, SR6204). Concentration-response curves were obtained using a cumulative dose-assay, the time between two consecutive dose-response curves was longer than 10 min. In the tests for antagonistic activity on GPI and RVD, analog was added 10 min before NKA was added. The cumulative dose-response curve of NKA in the presence or the absence of the analog was obtained. In the presence of analogs at concentration 2×10^{-6} M, the maximal response induced by NKA was almost the same response without analogs.

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