Contractile Activity of Porcine Neuromedin U-25 and Various Neuromedin U-Related Peptide Fragments on Isolated Chicken Crop Smooth Muscle

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Contractile activity of porcine neuromedin U-25 (p-NMU-25) and various neuromedin U (NMU) peptide fragment amides was examined on chicken crop smooth muscle preparation. The relative activity (expressed as RA value) of p-NMU-25 to porcine neuromedin U-8 (p-NMU-8) was 5.51±0.09, and p-NMU-25 (15—25) was the most potent fragment with an RA value of 7.78±0.05. All C-terminal 11-peptide amides of rat, rabbit and frog NMU peptides retained activity about three-fold higher than the corresponding C-terminal 8-peptide amides. The peptide segment Asn^{15}–Arg–Arg^{17} of p-NMU-25, as well as the corresponding positions of various NMU peptides: Ser^{13}–Gly–Gly^{15} of rat NMU and Ser^{15}–Arg–Gly^{17} of rabbit and frog NMUs, appeared to be involved in the structural requirements for increased contractile activity in the assay system.

Keywords neuromedin U; porcine; C-terminal undecapeptide amide; structure–activity relationship; smooth muscle contraction; chicken crop

Introduction

Neuromedin U (NMU) peptides as a new neuropeptide family were determined recently from porcine,[1] rat,[2,3] frog,[4] guinea pig,[5] dog[6] and rabbit.[7] The amino acid sequences of the C-terminal heptapeptide amides are conserved among mammalian NMUs, and this portion seems to be essential for the biological activity.[1,3] Porcine and dog NMUs were determined as two molecular forms, 25-peptide amides (p-NMU-25 and d-NMU-25) and 8-peptide amides (p-NMU-8 and d-NMU-8). Both NMU-25 peptides contain an Arg–Arg sequence preceding the C-terminal octapeptide portions, respectively. Thus, the dibasic structure may be a processing site to produce p-NMU-8 and d-NMU-8.[1,6] While the other NMUs do not contain the dibasic sequence (Fig. 1).

In our previous paper,[8] the relative importance of the side chain and optical configuration of each amino acid residue of p-NMU-8 was shown for the agonistic and antagonistic contractile activities on isolated chicken crop, and the minimum structural requirement for the activity was indicated to be Phe^{17}–Leu–Phe–Arg–Pro–Arg^{22} in the structure–activity relationship study of rat neuromedin U (r-NMU).[9] The present paper describes the activity of p-NMU-25, its N-terminal deletion peptide fragments, and C-terminal 9-, 10- and 11-peptide amides of various NMU peptides for contractile activity on an isolated chicken crop preparation.

Materials and Methods

The peptide amides shown in Fig. 2 were synthesized by solid phase techniques[10] with Boc-amino acid on benzhydrylamine (BHA) resin [1% divinylbenzene (DVB) polymer] using a peptide synthesizer. After HF deprotection and cleavage from the resin, each peptide was purified by reversed phase high performance liquid chromatography (RP-HPLC) followed by gel filtration. Homogeneity of the synthetic peptide was confirmed by analytical HPLC, high performance thin layer chromatography (HP-TLC) (Table I) and amino acid analysis of acid hydrolysates (Table II).

Contractile activity of the synthetic peptide was assayed on an isolated chicken crop preparation as described in the previous paper.[9] The pharmacological parameter of relative affinity (RA) (EC_{50} of p-NMU-8/EC_{50} of each peptide) was obtained from dose–response curves (n= 7—25) of each peptide (Table III).

Results and Discussion

The contractile activity of synthetic peptide amides was estimated on isolated chicken crop smooth muscle preparations. The activity of p-NMU-8 was taken as the standard and the relative potency of each peptide is expressed as the RA value. p-NMU-25 and the N-terminal deletion fragments, p-NMU-25 (7—25), (12—25) and (15—25), showed significantly higher activity than p-NMU-8. The potency of p-NMU-25 was 5.5 times as high as that of p-NMU-8, and the elimination of N-terminal peptide segments seemed to increase the activity. The RA value of C-terminal 11-peptide amide p-NMU-25 (15—25) was 7.78, which was the highest value among the p-NMU-25 related

		1				5					10					15					20					25	
porcine	p-NMU-25	F	K	V	D	E	E	F	Q	G	P	I	V	S	Q	N	R	R	Y	F	L	F	R	P	R	N-NH_2
	p-NMU-8																		Y	F	L	F	R	P	R	N-NH_2
rat	r-NMU	Y	K	V	N	E	*	Y	Q	G	P	*	V	A	P	S	G	G	F	F	L	F	R	P	R	N-NH_2
dog	d-NMU-25	F	R	L	D	E	E	F	Q	G	P	I	A	S	Q	V	R	R	Q	F	L	F	R	P	R	N-NH_2
	d-NMU-8																		pE	F	L	F	R	P	R	N-NH_2
guinea pig	gp-NMU																	G	Y	F	L	F	R	P	R	N-NH_2
frog	f-NMU	L	K	P	D	E	E	L	Q	G	P	G	G	V	L	S	R	G	Y	F	V	F	R	P	R	N-NH_2
rabbit	rb-NMU-25	F	P	V	D	E	E	F	Q	S	P	F	G	S	R	S	R	G	Y	F	L	F	R	P	R	N-NH_2

Fig. 1. NMU Peptides from Various Species

*; deletion, pE; pyroglutamyl.

	1	5	10	15 (13)	16 (14)	17 (15)
p-NMU-25	H- Phe- Lys- Val- Asp-	Glu- Glu- Phe- Gln- Gly-	Pro- Ile- Val- Ser-	Gln-	Asn-	Arg- Arg- X
p-NMU-25 (7–25)		H- Phe- Gln- Gly-	Pro- Ile- Val- Ser-	Gln-	Asn-	Arg- Arg- X
p-NMU-25 (12–25)			H- Val- Ser-	Gln-	Asn-	Arg- Arg- X
p-NMU-25 (15–25)				H- Asn-	Arg-	Arg- X
p-NMU-25 (16–25)					H- Arg-	Arg- X
p-NMU-25 (17–25)						H- Arg- X
p-NMU-25 (18–25) (p-NMU-8)						H- X
r-NMU (13–23)				H- Ser-	Gly-	Gly- Y
r-NMU (14–23)					H- Gly-	Gly- Y
r-NMU (15–23)						H- Gly- Y
r-NMU (16–23)						H- Y
rb-NMU-25 (15–25)				H- Ser-	Arg-	Gly- X
rb-NMU-25 (16–25)					H- Arg-	Gly- X
rb-NMU-25 (17–25) (gp-NMU)						H- Gly- X
f-NMU (15–25)				H- Ser-	Arg-	Gly- Z
f-NMU (16–25)					H- Arg-	Gly- Z
f-NMU (17–25)						H- Gly- Z
f-NMU (18–25)						H- Z

X: Tyr- Phe- Leu- Phe- Arg- Pro- Arg- Asn- NH_2, Y: Phe- Phe- Leu- Phe- Arg- Pro- Arg- Asn- NH_2,

Z: Tyr- Phe- Val- Phe- Arg- Pro- Arg- Asn- NH_2

Fig. 2. Synthetic Peptide Amides Related to NMU

TABLE I. Characteristics of Synthetic p-NMU-25 and NMU-Related Peptide Amides

Peptide	Yield[a] (%)	$[\alpha]_D^{23}$ ($c=0.5$) (2 M AcOH)	HPLC[b] t_R (min)	HP-TLC[c] Rf^1	HP-TLC[c] Rf^2
p-NMU-25	9.1	−71.4	28.72	0.44	0.50
p-NMU-25 (7—25)	11.2	−66.7	17.18	0.48	0.49
p-NMU-25 (12—25)	13.3	−50.3	6.79	0.42	0.18
p-NMU-25 (15—25)	31.6	−41.4	6.65	0.40	0.20
p-NMU-25 (16—25)	28.6	−34.9	7.07	0.31	0.08
p-NMU-25 (17—25)	32.5	−29.2	8.99	0.41	0.31
p-NMU-25 (18—25) (p-NMU-8)	23.4	−36.4	13.00	0.45	0.44
r-NMU (13—23)	14.2	−41.7	25.00	0.40	0.43
r-NMU (14—23)	27.9	−50.0	25.33	0.45	0.35
r-NMU (15—23)	24.0	−47.7	25.37	0.49	0.35
r-NMU (16—23)	13.6	−40.3	21.15	0.45	0.45
rb-NMU-25 (15—25)	39.7	−41.9	10.20	0.30	0.32
rb-NMU-25 (16—25)	32.5	−36.1	10.72	0.39	0.35
rb-NMU-25 (17—25) (gp-NMU)	15.0	−40.5	13.50	0.47	0.14
f-NMU (15—25)	29.3	−50.2	5.78	0.35	0.16
f-NMU (16—25)	30.9	−35.0	6.11	0.38	0.34
f-NMU (17—25)	18.5	−48.9	8.07	0.44	0.35
f-NMU (18—25)	22.1	−42.3	6.59	0.48	0.48

a) Based on the C-terminal amino acid incorporated on BHA resin. *b*) Column: YMC-Pak ODS-AM (4.6 × 150 mm), elution: linear gradient (30 min) from 21.0 to 25.9% MeCN in 0.1% TFA, flow rate: 1 ml/min; detection: 210 nm. *c*) Rf^1; *n*-BuOH : pyridine : AcOH : H_2O (30 : 20 : 6 : 24), Rf^2; *n*-BuOH : AcOEt : AcOH : H_2O (1 : 1 : 1 : 1).

peptides examined. The results indicated that the N-terminal portion of the p-NMU-25 molecule, positions 1—17, contributes to the enhanced potency on smooth muscle, and the peptide segment Asn^{15}–Arg^{16}–Arg^{17}, which precedes the p-NMU-8 sequence, may be most important in the molecule for the activity enhancement. In other words, further elongation of the peptide chain from the C-terminal 11-peptide amide toward the N-terminal is not necessary to increase the activity. Thus it can be assumed that the dibasic Arg^{16}–Arg^{17} portion plays an important role not only as a processing site to produce p-NMU-8, but also for increasing the affinity to NMU receptor(s).

In the r-NMU molecule, the whole amino acid sequence was required for the potent activity, and a special importance of the Ser^{13}–Gly^{14}–Gly^{15} portion was demonstrated for the increased contractile activities on chicken crop and rat uterus smooth muscles.[9] Thus, both C-terminal 11-peptide amides of rat and porcine NMUs retained the increased activity, and the tripeptide segments preceding the 8-peptide amides of NMUs seemed to play a role in the enancement of biological activities. On the other hand, amino acid sequence variation was observed in this portion in NMU family peptides; *i.e.*, Asn–Arg–Arg in p-NMU-25, Ser–Gly–Gly in r-NMU, Val–Arg–Arg in d-NMU-25, and Ser–Arg–Gly in rabbit and frog NMUs. Therefore, the contribution of each amino acid residue of the tripeptide segments of p-NMU-25, r-NMU, rb-NMU and f-NMU for the activity was examined using the various synthetic 9-, 10- and 11-peptide amides shown in Fig. 2.

The relative activity of C-terminal 9-, 10- and 11-peptide amides of porcine, rat and rabbit NMUs on chicken crop smooth muscle is also summarized in Table III. The amino acid sequence of the C-terminal 8-peptide amides of p-NMU-25 and rb-NMU is common. Successive intro-

TABLE II. Amino Acid Analysis of Synthetic Peptide Amides Related to NMU

Peptide	Asp	Ser	Glu	Pro	Gly	Val	Ile	Leu	Tyr	Phe	Lys	Arg	NH^3	Recovery (%)
p-NMU-25	2.98 (3)	1.00 (1)	4.09 (4)	1.96 (2)	1.04 (1)	1.58 (2)	0.66 (1)	1.04 (1)	0.97 (1)	3.93 (4)	0.95 (1)	4.05 (4)	4.55 (5)	80.7
p-NMU-25 (7—25)	1.96 (2)	0.78 (1)	2.07 (2)	1.92 (2)	1.00 (1)	0.83 (1)	0.76 (1)	1.04 (1)	0.95 (1)	2.89 (3)	—	4.42 (4)	4.44 (5)	79.6
p-NMU-25 (12—25)	2.06 (2)	0.83 (1)	1.05 (1)	0.98 (1)	—	0.98 (1)	—	1.01 (1)	0.98 (1)	2.03 (2)	—	4.08 (4)	3.71 (4)	76.0
p-NMU-25 (15—25)	2.02 (2)	—	—	0.93 (1)	—	—	—	1.03 (1)	0.99 (1)	2.00 (2)	—	4.04 (4)	2.45 (3)	66.9
p-NMU-25 (16—25)	1.01 (1)	—	—	0.98 (1)	—	—	—	1.04 (1)	0.97 (1)	2.03 (2)	—	4.14 (4)	2.11 (2)	67.9
p-NMU-25 (17—25)	1.05 (1)	—	—	0.92 (1)	—	—	—	1.00 (1)	0.97 (1)	1.99 (2)	—	3.07 (3)	2.12 (2)	72.0
r-NMU (14—23)	1.03 (1)	—	—	0.93 (1)	2.09 (2)	—	—	1.00 (1)	—	2.90 (3)	—	2.04 (2)	2.11 (2)	79.0
r-NMU (15—23)	1.02 (1)	—	—	0.97 (1)	1.06 (1)	—	—	0.99 (1)	—	2.92 (3)	—	2.04 (2)	2.10 (2)	82.0
rb-NMU-25 (15—25)	1.03 (1)	0.87 (1)	—	0.92 (1)	1.03 (1)	—	—	1.03 (1)	0.96 (1)	2.03 (2)	—	3.12 (3)	1.89 (2)	79.8
rb-NMU-25 (16—25)	1.04 (1)	—	—	0.92 (1)	1.02 (1)	—	—	1.02 (1)	0.98 (1)	2.01 (2)	—	3.01 (3)	1.73 (2)	62.4
rb-NMU-25 (17—25)	1.03 (1)	—	—	0.93 (1)	1.08 (1)	—	—	1.01 (1)	0.96 (1)	1.98 (2)	—	2.01 (2)	1.75 (2)	71.2
f-NMU (15—25)	1.08 (1)	0.92 (1)	—	0.95 (1)	1.12 (1)	0.93 (1)	—	—	0.97 (1)	1.93 (2)	—	3.10 (3)	1.94 (2)	80.1
f-NMU (16—25)	1.06 (1)	—	—	0.95 (1)	1.06 (1)	0.94 (1)	—	—	0.96 (1)	1.94 (2)	—	3.09 (3)	1.84 (2)	64.7
f-NMU (17—25)	0.97 (1)	—	—	0.98 (1)	1.06 (1)	0.93 (1)	—	—	0.99 (1)	1.92 (2)	—	2.07 (2)	2.05 (2)	77.9
f-NMU (18—25)	1.05 (1)	—	—	0.97 (1)	—	0.97 (1)	—	—	0.98 (1)	1.95 (2)	—	2.07 (2)	1.96 (2)	80.3

Hydrolysis: at 130 °C for 3.0 h by vapor of 6 N hydrochloric acid containing phenol (3%). Numbers in parentheses are theoretical values.

TABLE III. Contractile Activity of p-NMU-25 and Various NMU Fragments on Isolated Chicken Crop

Peptide	RA	*n*
p-NMU-8 [p-NMU-25 (18—25)]	1.00	
p-NMU-25	5.51±0.09	21
p-NMU-25 (7—25)	3.87±0.68	18
p-NMU-25 (12—25)	6.20±1.29	25
p-NMU-25 (15—25)	7.78±0.05	19
p-NMU-25 (16—25)	3.15±1.62	23
p-NMU-25 (17—25)	2.25±0.17	7
r-NMU (13—23)	2.81±0.52[9]	9
r-NMU (14—23)	3.08±0.61	7
r-NMU (15—23)	0.79±0.20	8
r-NMU (16—23)	0.88±0.19[9]	9
rb-NMU-25 (15—25)	3.36±1.08	8
rb-NMU-25 (16—25)	1.01±0.14	8
rb-NMU-25 (17—25) (gp-NMU)	1.43±0.33	8
f-NMU (15—25)	3.97±0.61	13
f-NMU (16—25)	1.77±0.37	12
f-NMU (17—25)	1.65±0.42	9
f-NMU (18—25)	1.45±0.21	9

Isolated chicken crop was suspended in an organ bath at 28 °C containing Tyrode's solution under 0.5 g tension. Peptide dissolved in saline was added cumulatively to obtain a dose–response curve, from which the RA to p-NMU-8 was calculated. *n*: number of experiments.

duction of Arg to the N-terminal of p-NMU-8 produces p-NMU-25 (17—25) and (16—25), which increased the activity by two and three times. Successive elongation of p-NMU-8 with Gly and Arg gives rb-NMU (17—25) (or guinea pig NMU) and (16—25), which did not significantly increase the activity (RA values of 1.43 and 1.01 respectively), however, marked enhancement of the activity appeared following the introduction of Ser (RA 3.36). f-NMU (18—25), which corresponds to [Val^3]-p-NMU-8 with an RA value of 1.45, did not significantly increase the activity by successive elongation with Gly and Arg: RA values of f-NMU (17—25) 1.65 and f-NMU (16—25) 1.77. However, further elongation with Ser did increase the activity: RA of f-NMU (15—25) 3.97. r-NMU (16—23), which corresponds to [Phe^1]-p-NMU-8 with RA 0.88,[9] also failed to increase the activity by elongation with Gly. Further elongation with the second Gly increased the activity, *i.e.*, r-NMU (14—23), RA 3.08, which is rather higher than the 11-peptide, r-NMU (13—23). Results showed that the peptide segment Asn^{15}–Arg–Arg^{17} of p-NMU-25, as well as the corresponding positions of various NMU peptides: Ser^{13}–Gly–Gly^{15} of r-NMU and Ser^{15}–Arg–Gly^{17} of rb- and f-NMUs, appeared to be involved in the structural requirements for the increased contractile activity in the assay system. Introduction of an 11th amino acid residue to the C-terminal 10-peptide amides is effective for increasing the activity in all species of NMU peptides examined, except r-NMU, of which the C-terminal 10-peptide amide has 3.5 times higher activity than 8- or 9-peptide amides. Considering the heterogeneity of this portion of NMU molecules, it seems that not a special sequence but just a di- or tri-peptide chain length from the C-terminal octapeptides is necessary. Since these hydrophilic tripeptide portions in all species of NMU are located at the N-terminal of the extremely hydrophobic sequence, Tyr(or Phe)–Phe–Leu(or Val)–Phe, which is followed by the second hydrophilic and basic portion of C-terminal Arg–Pro–Arg–Asn–NH_2, an amphiphilic structure might be involved in the enhancement of the activity on chicken crop preparation.

Experimental

All reagents and solvents for peptide synthesis were obtained from Watanabe Chem. Ind. Ltd. or Wako Pure Chem. Ind. Ltd., Japan, unless otherwise mentioned, and were used without further purification. Evaporation of organic solvents was carried out *in vacuo* below 40 °C in a rotary evaporator.

Peptide Synthesis Peptides were synthesized in the same manner as described previously[9] by a solid-phase method[10] on BHA resin with N^{α}-Boc amino protection employing a model 990C peptide synthesizer (Beckman Instruments Ltd., U.S.A.). Anchoring of the first amino acid was achieved through dicyclohexylcarbodiimide (DCC) coupling of 0.4 mmol eq of N^{α}-Boc–Asn (Peptide Institute Inc., Japan) with 1 g eq of BHA resin (1% DVB polymer, available amine of the resin; 0.66 mmol/g; Peptide Institute Inc., Japan), in the presence of 0.8 mmol eq of 1-hydroxybenzotriazole (HOBt), followed by acetylation with acetic anhydride (2 mmol eq)–pyridine (1 mmol eq) in *N,N'*-dimethylformamide (DMF). Deprotection of the N^{α}-Boc group was accomplished with 33% trifluoroacetic acid (TFA) in dichloromethane (DCM) for 30 min. N^{α}-Boc–amino acids (2.5 eq) were coupled for 1—2 h *via* DCC (2.5 eq) for the first coupling, or DCC (2.5 eq)–HOBt (2.5 eq) for further repeated coupling in DCM and/or DMF. Every introduction reaction of an amino acid was repeated until the resin became negative to the Kaiser test,[11] then the acetylation procedure with acetic anhydride–pyridine described above

was perfomed. N^{α}-Boc–Gln was coupled in the presence of a 2-fold excess of HOBt. Side-chain protection of N^{α}-Boc–amino acids was as follows: Arg(Tos), Ser(Bzl), Tyr(Cl_2–Bzl), Glu(OBzl) and Lys(ClZ). Final deprotection and cleavage from the resin were achieved in HF in the presence of 10% anisole at 0 °C for 45 min. After removal of HF *in vacuo*, the residue was washed with ethyl acetate–ether (1 : 1) and extracted with diluted acetic acid. Crude lyophilized peptides were purified by semi-preparative RP-HPLC using a column of μ-Bondasphere C_{18} (19 × 150 mm) (Waters) or YMC-Pack D-ODS-5-A (20 × 250 mm) (YMC Co., Japan). The purified peptide was lyophilized from diluted HCl (about a 5-fold excess) and finally gel-filtered on a Toyopearl HW-40 superfine column (1.5 × 58 cm) using 5% MeCN in 5 mM HCl as an eluent, and the desired fractions were lyophilized. The final yields of the peptides based on the C-terminal amino acid on the resin are shown in Table I.

Acid hydrolysis of synthetic peptides was carried out with 6 N HCl vapor containing 3% phenol at 130 °C for 3 h. Amino acid analysis of the acid hydrolysate was performed on a Beckman model 7300 amino acid analyzer system (Table II). HPLC analysis of the peptides was carried out using a YMC-Pack ODS-AM column (4.6 × 150 mm) with a linear gradient elution of 21.0—25.9% MeCN over a period of 30 min in 0.1% TFA. Optical rotations of the peptides were measured with a DIP-370 digital polarimeter (Nippon Bunko Co., Ltd., Japan) employing a 3 × 50 mm cell. Peptides were dissolved in 12% AcOH at a concentration of 0.50% of peptide. The *Rf* values in HP-TLC, performed on precoated silica gel (Kieselgel 60; Merck), refer to the following solvent systems: Rf^1, *n*-BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24) and Rf^2, *n*-BuOH–AcOEt–AcOH–H_2O (1 : 1 : 1 : 1). These analytical data are shown in Table I.

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