Structure–Activity Relationships of Neuromedin U. I. Contractile Activity of Dog Neuromedin U-Related Peptides on Isolated Chicken Crop Smooth Muscle

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Synthetic dog neuromedin U-25 (d-NMU-25), U-8 (d-NMU-8) and their fragments were examined for contractile activity on chicken crop smooth muscle preparation. The relative activities of d-NMU-25, d-NMU-25 (15—25)-NH_2 and d-NMU-8 to porcine neuromedin U-8 (p-NMU-8) were 1.69, 2.54 and 5.78, respectively. High activity of d-NMU-8 may be attributable to the N-terminal pGlu residue, which provides resistance to aminopeptidases. Various NMU-8 analogs, having various amino acids, N^{α}-acetylated amino acids, D-amino acids, or simple organic acids at position 1, were synthesized and evaluated for contractile activity. None of the substitutions caused a significant decrease of the biological activity. Modification at the N-terminal to give aminopeptidase resistance produced analogs with increased contractile activity, presumably because they were not degraded by soluble enzymes released into the bioassay fluid from isolated chicken crop tissue.

Key words neuromedin U; structure–activity relationship; smooth muscle contraction; chicken crop; enzymatic degradation; N-terminal

Porcine and dog neuromedin U (NMU) peptides were found in two molecular forms, 25-amino acid peptide amides (p-NMU-25 and d-NMU-25) and 8-amino acid peptide amides (p-NMU-8 and d-NMU-8).[1,2] Both NMU-25 peptides contain an Arg–Arg sequence preceding the C-terminal octapeptide portions. Thus, the dibasic sequence may be the processing site to produce p-NMU-8 and d-NMU-8. Other NMU peptides do not contain the dibasic structure. The amino acid sequence of the C-terminal heptapeptide amide is conserved among mammalian NMU peptides and this portion seems to be essential for the biological activity.[3,4] The NMU peptides have potent contractile activity on rat uterus,[1] chicken gastrointestinal tract,[5] and various other smooth muscle preparations, as reviewed by Nandha and Bloom.[6]

In our previous studies on the structure–activity relationships of NMU peptides, the importance of the side chain and optical configuration of each amino acid residue of p-NMU-8 were examined.[7] The minimum common structure of Phe–Leu–Phe–Arg–Pro–Arg for biological activity,[8] and the chain-length requirement of various NMU peptides for increased activity,[8,9] were also reported. Here, we describe the contractile activity of d-NMU-25, d-NMU-8 and their fragments (Fig. 1), together with various NMU-8 analogs modified at the N-terminal (Fig. 2), on isolated chicken crop. Proteolytic degradation of the synthetic NMU-8 peptides and the analogs by soluble enzymes of chicken crop was examined in order to elucidate the relationship between resistance to enzymatic hydrolysis and contractile potency.

Results and Discussion

The peptides shown in Figs. 1 and 2 were synthesized by solid-phase techniques[10] with Boc-amino acid on benzhydrylamine resin (1% divinylbenzene (DVB) polymer) or Merrifield resin using a peptide synthesizer. After HF deprotection and cleavage from the resin, each peptide was purified by reversed-phase HPLC, followed by gel filtration. Synthetic peptides were confirmed by FAB-MS and amino acid analysis of the acid hydrolysate, and the homogeneity was analyzed by analytical HPLC and high-performance TLC (Tables 1 and 2).

Contractile activity of the synthetic peptide was estimated on isolated chicken crop smooth muscle preparation as described in previous papers.[5,8] Representative concentration-response curves of NMU-related peptides are shown in Fig. 3. The activity of p-NMU-8 was taken as the standard, and the potency of each peptide is expressed in terms of relative activity (RA value: EC_{50} of p-NMU-8/EC_{50} of each peptide) and maximum contraction (maximal effect of analog/maximal effect of p-NMU-8) × 100 (Table 3).

No.	Peptide
3	dog NMU-25 H-Phe(1)-Arg-Leu-Asp-Glu(5)-Glu-Phe-Gln-Gly-Pro(10)-Ile-Ala(13)-Ser-Gln(14)-Val-Arg-Arg-Gln(18)-Phe-Leu-Phe-Arg-Pro-Arg-Asn(25)-NH_2
4	dog NMU-25 (15-25)-NH_2 H-Val-Arg-Arg-Gln-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH_2
1	dog NMU-8 pGlu(1)-Phe-Leu-Phe-Arg-Pro-Arg-Asn(8)-NH_2
2	porcine NMU-8 H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH_2
5	dog NMU-8 (1-7)-OH pGlu-Phe-Leu-Phe-Arg-Pro-Arg-OH
6	porcine NMU-8 (1-7)-OH H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg-OH

Fig. 1. Dog and Porcine NMU Peptides and Fragments

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1 2 3 4 5 6 7 8
X-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH_2

	X		X
7	Glu	**15**	Ac-Phe
8	Gly	**16**	D-Tyr
9	Ala	**17**	D-Ala
10	Phe	**18**	Benzoyl
11	Pro	**19**	Hexahydrobenzoyl
12	Ac (acetyl)-Tyr	**20**	Isobutyryl
13	Ac-Gly	**21**	Propionyl
14	Ac-Ala	**22**	Ac

Fig. 2. Synthetic [X^1]-NMU-8 (Analogs No. **7**—**22**)

Analogs **8**, **10** and **16** were reported in refs. 7 and 8.

Table 1. Characteristics of Synthetic Neuromedin U (NMU)-Related Peptides

Peptide	$[\alpha]_D^{23}$ ($c=0.5$, 2 M AcOH)	RP-HPLC[a] t_R (min)	HP-TLC[b]	
			Rf^1	Rf^2
1	−56.4	15.7	0.59	0.56
3	−67.3	17.1	0.40	0.06
4	−28.6	10.7	0.36	0.05
5	−42.9	16.7	0.53	0.56
6	−33.5	15.2	0.52	0.56
7	−43.3	12.4	0.44	0.51
9	−50.8	12.4	0.48	0.52
11	−60.8	13.1	0.47	0.50
12	−49.7	20.1	0.59	0.57
13	−56.7	16.8	0.54	0.54
14	−64.4	18.4	0.55	0.55
15	−52.8	23.0	0.60	0.58
17	−58.8	13.5	0.51	0.53
18	−59.7	23.1	0.59	0.58
19	−57.8	24.8	0.61	0.58
20	−57.9	21.0	0.60	0.58
21	−52.9	19.4	0.60	0.57
22	−49.5	17.6	0.59	0.57

a) Conditions: column, Puresil 5 μ C_{18} (4.6×250 mm); flow rate, 1 ml/min; detection, 210 nm; eluent system, linear gradient from 16% to 40% MeCN (20 min) in 0.1% TFA; t_R, retention time. *b*) Rf^1, *n*-BuOH–Pyridine–AcOH–H_2O (30:20:6:24); Rf^2, *n*-BuOH–AcOET–AcOH–H_2O (1:1:1:1).

RA of d-NMU-25 (**3**) to p-NMU-8 (**2**) was 1.69, which is significantly lower ($p<0.1$) than that of p-NMU-25 (RA 5.51[9]) or rat neuromedin U (r-NMU-23, RA 10.25[8]), and the RA value of d-NMU-25 (15—25)–NH_2 (**4**) was 2.54. While, d-NMU-8 (**1**) had 5.78-fold potent activity (Fig. 3 and Table 3), which is definitely higher ($p<0.01$) than p-NMU-8 (**2**). In previous papers[8,9] we found that the elongation of the peptide chain from the C-terminal of r-NMU-23 increased the contractile activity, and that the C-terminal 11-amino acid peptide amides of the porcine, rat, frog and rabbit NMU peptides had 3- to 8-fold more potent activity, suggesting the contribution of the N-terminal tripeptide portions of the 11-amino acid peptide amides to the increased activity. The present results indicated that the pGlu residue of d-NMU-8 (**1**) meets the requirement. O'Harte *et al.* also reported[2] that substitution of the Tyr^1 residue in p-NMU-8 (**2**) with a pGlu residue results in an enhanced ability to contract the rat uterus in terms of the maximum response, and the presence of an aminopeptidase-resistant structure of both pGlu and D-Tyr in a synthetic analog[7] was deduced to contribute to the activity by reducing the rate of peptide degradation by tissue enzymes. Many biologically active peptides contain an N-terminal pGlu residue, which is known to be a part of the structural requirement for biological activity, or to play a role in protection against aminopeptidases. Various NMU-8 analogs modified at the N-terminal were prepared to examine the biological activity and the stability against the soluble proteolytic enzymes of isolated chicken crop tissue, which was used in the bioassay system.

Sixteen analogs of NMU-8 were designed; analogs containing L-amino acids at the position 1, and analogs with aminopeptidase-resistant structure, including N^{α}-acetylated amino acids, D-amino acids and some organic acids. The activities of [Gly^1]-(**8**) and [D-Tyr^1]-NMU-8 (**16**) were re-examined in this study, because of a minor

Table 2. Amino Acid Analysis and FAB-MS Data for Synthetic NMU-Related Peptides

Peptide	Amino acid analysis[a]							FAB-MS	
	Asp	Pro	Leu	Phe	Arg	NH_3	Others[b]	$[M+H]^+$	Formula
1	1.01 (1)	0.98 (1)	1.01 (1)	1.96 (2)	1.97 (2)	4.14 (2)	Glu 1.08 (1)	1059	$C_{50}H_{74}N_{16}O_{10}$
3	2.05 (2)	1.85 (2)	2.03 (2)	3.95 (4)	4.90 (5)	5.49 (5)	[c]	3106.67[e]	$C_{140}H_{217}N_{45}O_{36}$
4	1.09 (1)	0.83 (1)	1.04 (1)	2.03 (2)	3.91 (4)	3.92 (3)	[d]	1487	$C_{67}H_{110}N_{26}O_{13}$
5	—	0.96 (1)	1.01 (1)	1.97 (2)	1.98 (2)	0.47 (0)	Glu 1.09 (1)	946	$C_{46}H_{67}N_{13}O_9$
6	—	0.95 (1)	1.04 (1)	2.02 (2)	1.99 (2)	0.77 (0)	Tyr 0.99 (1)	998	$C_{50}H_{71}N_{13}O_9$
7	1.02 (1)	0.94 (1)	1.01 (1)	1.98 (2)	1.93 (2)	2.80 (2)	Glu 1.11 (1)	1077	$C_{50}H_{76}N_{16}O_{11}$
9	1.06 (1)	1.02 (1)	0.95 (1)	1.86 (2)	2.05 (2)	1.95 (2)	Ala 1.07 (1)	1019	$C_{48}H_{74}N_{16}O_9$
11	1.04 (1)	1.90 (2)	1.04 (1)	2.02 (2)	2.00 (2)	2.87 (2)		1045	$C_{50}H_{76}N_{16}O_9$
12	1.02 (1)	1.00 (1)	1.03 (1)	1.95 (2)	2.00 (2)	2.26 (2)	Tyr 0.99 (1)	1153	$C_{56}H_{80}N_{16}O_{11}$
13	1.05 (1)	1.03 (1)	0.94 (1)	1.91 (2)	2.05 (2)	1.93 (2)	Glu 1.02 (1)	1047	$C_{49}H_{74}N_{16}O_{10}$
14	1.05 (1)	1.00 (1)	0.97 (1)	1.91 (2)	1.99 (2)	2.04 (2)	Ala 1.09 (1)	1061	$C_{50}H_{76}N_{16}O_{10}$
15	1.06 (1)	1.03 (1)	0.99 (1)	2.90 (3)	2.03 (2)	2.02 (2)		1137	$C_{56}H_{80}N_{16}O_{10}$
17	1.03 (1)	1.01 (1)	0.98 (1)	1.90 (2)	2.02 (2)	1.85 (2)	Ala 1.05 (1)	1019	$C_{48}H_{74}N_{16}O_9$
18	0.91 (1)	0.99 (1)	0.97 (1)	1.95 (2)	2.19 (2)	1.85 (2)		1052	$C_{52}H_{73}N_{15}O_9$
19	0.91 (1)	0.99 (1)	0.93 (1)	1.97 (2)	2.21 (2)	1.89 (2)		1058	$C_{52}H_{79}N_{15}O_9$
20	0.93 (1)	0.92 (1)	1.05 (1)	1.97 (2)	2.13 (2)	1.94 (2)		1018	$C_{49}H_{75}N_{15}O_9$
21	0.96 (1)	0.98 (1)	0.99 (1)	1.93 (2)	2.13 (2)	1.97 (2)		1004	$C_{48}H_{73}N_{15}O_9$
22	0.96 (1)	0.89 (1)	1.09 (1)	1.98 (2)	2.09 (2)	2.11 (2)		990	$C_{47}H_{71}N_{15}O_9$

a) Hydrolysis at 130 °C for 3.0 h by vapor of 6 N hydrochloric acid containing phenol (3%); numbers in parentheses are theoretical values. *b*) The other amino acid(s) analyzed. *c*) Ser: 0.88 (1), Glu: 5.46 (5), Gly: 1.02 (1), Ala: 1.01 (1), Val: 0.88 (1), Ile: 0.96 (1). *d*) Glu 1.18 (1), Val 0.91 (1). *e*) Found value for the most abundant isotopic variant was consistent with calculated value (3106.66) for the formula.

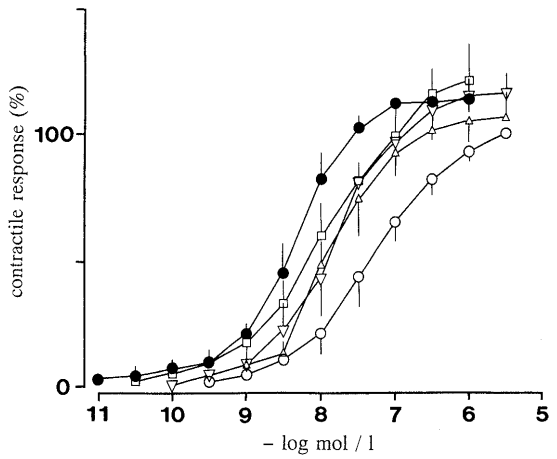

Fig. 3. Concentration–Response Curves of NMU-8 Analogs for the Contraction of Isolated Chicken Crop Preparation

▽, d-NMU-8 (**1**); ○, p-NMU-8 (**2**); □, [Ac-Tyr^1]-NMU-8 (**12**); △, [D-Tyr^1]-NMU-8 (**16**); ●, isobutyryl-NMU-8 (2—8)–NH_2 (**20**). The number of experiments is shown in Table 3, except for **2** ($n=31$).

Table 3. Contractile Activity of NMU-Related Peptides on Isolated Chicken Crop Smooth Muscle Preparations

Peptide	RA[a]	Max. contraction[b]	n[c]
1	5.78±0.86	126±5	14
3	1.69±0.34	116±4	18
4	2.54±0.40	112±4	13
5	NA[d]	0	6
6	NA	0	6
7	7.18±1.68	114±2	6
8	0.82±0.08	112±4	7
9	0.69±0.15	99±4	7
10	0.88±0.19	111±3	9
11	1.95±0.23	114±4	14
12	10.2 ±1.55	121±7	6
13	8.40±1.82	133±8	6
14	7.74±1.27	119±7	5
15	2.80±0.34	95±7	5
16	5.04±0.56	106±6	5
17	12.4 ±1.12	117±3	5
18	8.94±3.65	103±8	6
19	11.1 ±5.67	105±3	6
20	15.7 ±5.10	114±3	6
21	22.0 ±5.65	125±7	5
22	16.3 ±3.17	128±5	6

a) RA was calculated as EC_{50} of p-NMU-8/EC_{50} of each analog. *b*) Maximum contraction (%) was calculated as (maximal effect of analog/maximal effect of p-NMU-8) × 100. *c*) Number of experiments. *d*) Not assessed.

change of the estimation method from that used in the previous report[7] for the contractile activity on isolated chicken crop. These analogs are shown in Fig. 2, and the biological activities are summarized in Table 3.

All the synthetic analogs examined retained considerable contractile activity. The RA values of [L-Amino acid^1]-NMU-8 analogs, such as [Glu^1]-, [Gly^1]-, [Ala^1]- and [Pro^1]-NMU-8, are 0.69—7.18 in comparison with p-NMU-8 ([Tyr^1]-NMU-8). The RA of [Phe^1]-NMU-8 (**10**), corresponding to r-NMU-23 (16—23)–NH_2, was 0.88.[8] The results suggested that replacement of the N-terminal tyrosine residue of p-NMU-8 (**2**) by any other amino acid caused no significant decrease of the activity. It seems likely that the N-terminal amino acid residue is not important to elicit contraction, in contrast to the C-terminal asparagine amide, which can not be replaced with any other amino acid without a great decrease of the activity.[11]

Acetylation of the α-amino groups of [Tyr^1]-, [Phe^1]-, [Gly^1]- and [Ala^1]-NMU-8 brought about a 3- to 11-fold increase of the contractile activity compared with the corresponding N^{α}-free parent peptide amides. The biological potency of analogs with D-Tyr and D-Ala at the N-terminal was also high (RA values of **16** and **17**: 5.04 and 12.4, respectively). Even the introduction of simple organic acids, such as benzoyl, hexahydrobenzoyl, isobutyryl, propionyl and acetyl, produced analogs with higher activity (RA values of 8.94 to 22.0) than that of p-NMU-8 itself. These analogs correspond to N-terminally acylated heptapeptide amides of NMU-8 (2—8)–NH_2. The N-terminally free heptapeptide amide had an RA value of 0.61.[8] Thus, the introduction of any residue, able to provide protection against aminopeptidase digestion, at position 1 of NMU-8 seemed to enhance the biological activity.

The relationship between the N-terminal structure of NMU-8-related peptides and the resistance to proteolytic degradation in the assay system was examined. The soluble enzyme solution was prepared from freshly isolated chicken crop. The tissue was cut along the longitudinal smooth muscle and washed three times with Tyrode's solution at 30 °C in the same manner as used in the bioassay procedure. The tissue strips were incubated for 15 min with fresh Tyrode's solution. The tissue was removed, and the solution was used as soluble enzyme extract of isolated chicken crop preparation. Representative HPLC chromatograms of the 3 h incubation mixture (0.2 μmol peptide/enzyme extract from 1 g tissue/2 ml Tyrode's solution) of p-NMU-8 (**2**) and d-NMU-8 (**1**) are shown in Fig. 4, A and B. The main cleavage products were isolated by HPLC and identified by amino acid analysis of acid hydrolysates of the collected peak materials, as well as by comparison of the retention times with those of authentic synthetic peptides on HPLC, or co-elution with synthetic peptides. The results are shown in Fig. 4, C and D. Various cleavage peaks from p-NMU-8 (**2**) were found (Fig. 4, A). The biggest six peaks, excluding the starting material (peak h), were of p-NMU-8 (2—8)–NH_2 (peak f), (3—8)–NH_2 (peak d), (4—8)–NH_2 (peak b), (2—7)–OH (peak g), tyrosine (peak a) and phenylalanine (peak c). The proteolytic cleavage occurred mainly at Tyr^1–Phe^2 of p-NMU-8 (**2**), followed by Phe^2–Leu^3 and Leu^3–Phe^4. Partial hydrolysis of the Arg^7–Asn^8 bond is evidenced by the appearance of considerable amounts of p-NMU-8 (1—7)–OH (peak i) and (2—7)–OH (peak g). The results suggested that the degradation of p-NMU-8 (**2**) was mainly due to aminopeptidase-like enzymes. On the other hand, the main proteolytic degradation products of d-NMU-8 (**1**) (Fig. 4, B) were eluted after the starting material (peak n). These degradation products were identified as d-NMU-8 (1—7)–OH (peak o), d-NMU-8 (1—6)–OH (peak p) and (1—4)–OH (peak q), respectively, which correspond to peaks i, k, and l derived from p-NMU-8. None of these peaks was generated by the action of aminopeptidase. Both

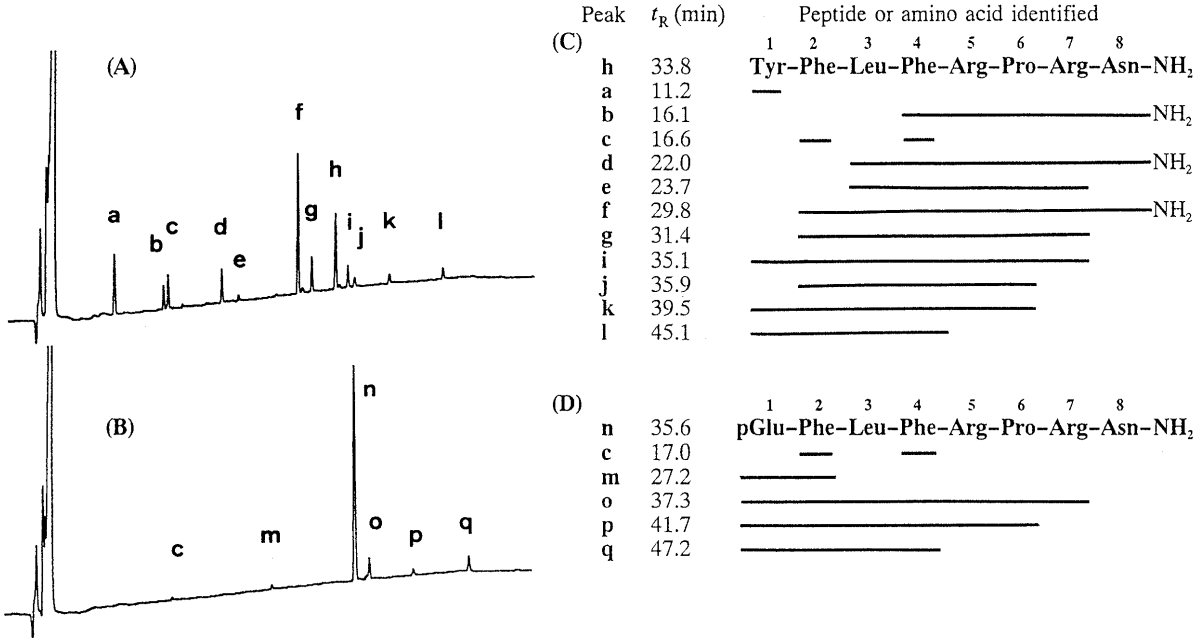

Fig. 4. HPLC Profiles of the Hydrolysate Mixture with the Soluble Enzyme Extract of Isolated Chicken Crop and the Degradation Products of p-NMU-8 (A) and (C), and d-NMU-8 (B) and (D)

p-NMU-8 and d-NMU-8 (10 nmol each) were incubated with the soluble enzyme extract of isolated chicken crop (wet tissue 50 mg) in Tyrode's solution (100 μl) for 3 h at 30 °C. HPLC conditions: column, Puresil C_{18} (4.6 × 250 mm); elution, linear 40 min gradient elution from 4.0 to 36.0% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, UV at 210 nm. Isolated material from each peak (a—q) was identified by means of amino acid analysis of the acid hydrolysate and co-elution with authentic materials on HPLC.

p-NMU-8 (**2**) and d-NMU-8 (**1**) were found to be susceptible to trypsin-like enzymes at their C-terminal and to chymotrypsin-like enzymes, although the rates of hydrolysis were very slow. Thus, d-NMU-8 (**1**) was less rapidly degraded than p-NMU-8 (**2**).

The time courses of the proteolytic degradation of seven NMU-8 analogs were examined. Decrease of the peptides was analyzed by means of HPLC to determine the amount of the starting material at various incubation times with the soluble enzyme extract of isolated chicken crop tissue. As can be seen in Fig. 5, more than 80% of d-NMU-8 (**1**) remained intact after a 3 h incubation, while more than 80% of p-NMU-8 (**2**) was degraded in 2.5 h under the conditions examined. The half life of p-NMU-8 (**2**) was 1.4 h. [Ac-Tyr^1]-NMU-8 (**12**) and [D-Tyr^1]-NMU-8 (**16**) remained almost intact, with similar degradation curves to that of d-NMU-8 (**1**). The results reflect the potent biological activity of analogs **12** and **16** (RA 10.2 and 5.04, respectively). Similarly, [Ala^1]-NMU-8 (**9**), with contractile activity of RA 0.69, was susceptible to enzymatic degradation, while [Ac-Ala^1]-NMU-8 (**14**) and [D-Ala^1]-NMU-8 (**17**) were highly resistant to proteolysis, being accompanied by potent biological activity (RA values of **14** and **17** were 7.74 and 12.4, respectively). The results indicated that analogs with an aminopeptidase-resistant structure at the N-terminal remained intact.

The elimination of the N-terminal amino acid residue by an aminopeptidase-like enzyme is not so important in the inactivation of p-NMU-8 as that of the C-terminal, because synthetic NMU-8 (2—8)–NH_2 and NMU (3—8)–NH_2 retained considerable activity, namely, their RA values were 0.61 and 0.05, respectively.[8] In contrast, proteolytic removal of the C-terminal asparagine amide of NMU peptides causes complete loss of the biological

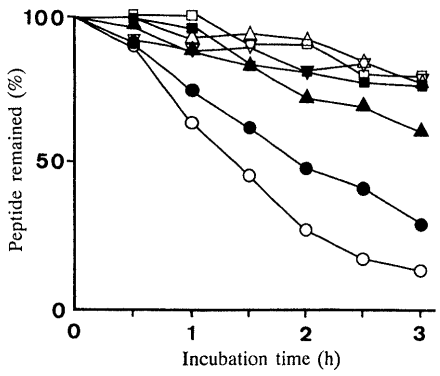

Fig. 5. Proteolytic Degradation Curves of NMU-8 Analogs Incubated at 30 °C with Soluble Enzyme Extract of Isolated Chicken Crop

▽, d-NMU-8 (**1**); ○, p-NMU-8 (**2**); □, [Ac-Tyr^1]-NMU-8 (**12**); △, [D-Tyr^1]-NMU-8 (**16**); ●, [Ala^1]-NMU-8 (**9**); ■, [Ac-Ala^1]-NMU-8 (**14**); ▲, [D-Ala^1]-NMU-8 (**17**).

activity; synthetic peptides, d-NMU-8 (1—7)–OH (**5**) and p-NMU-8 (1—7)–OH (**6**) had no activity (Table 3). A trypsin-like enzyme seems to be involved in the most important inactivation step of NMU peptides. However, only small amounts of trypsin-like enzymes seemed to be released from the chicken crop tissue. Consequently, the increased potency of d-NMU-8 (**1**) may be attributable to the prolonged half-life of the peptide amide (reduced proteolytic degradation) in the assay fluid.

The present study has revealed that the contractile activity of d-NMU-25 (**3**) is not as high as those of p-NMU-25 and r-NMU-23, while that of d-NMU-8 (**1**) was definitely higher than that of p-NMU-8 (**2**) on the isolated chicken crop smooth muscle preparation. The NMU receptor of rat uterus was characterized recently, and the involvement of N-terminal regions of r-NMU-23

in the binding to the receptor was suggested.[12] However, it can be said that the elongation of chain-length from C-terminal octapeptide amide[9] is not always required for increased activity; rather, pGlu and other simple amino acid residues such as Ac–Ala, D-Ala, or just acyl groups may substitute for the whole N-terminal region structural requirements of r-NMU-23 or the other NMU-25 peptides. The N-terminal Tyr residue of p-NMU-8 (**2**) may not play an important role in the receptor activation, because no loss of the activation ability was apparent in spite of diverse structural changes at the N-terminal. The increased activity of most of the N-terminally modified analogs may be explained by a greater resistance to aminopeptidases, or possibly to some extent by an increase of affinity to the neuromedin U receptor. Although the reason for the slightly higher activity of [Glu[1]]-NMU-8 (**7**) than [pGlu[1]]-NMU-8 (**1**) is unknown, and the factor(s) producing the various RA values from 2.80 to 22.0 among the analogs bearing aminopeptidase-resistant structures remains obscure, the strategy of making N-terminally modified peptides of NMU-8 may afford superagonists, and stable antagonists may be designed by modifying the structure of the active site with N-terminal blocking groups.

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[8] by a solid-phase method[10] on benzhydrylamine (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 (2 mmol eq) in *N*,*N*-dimethylformamide (DMF). Boc–Arg(Tos)-Merrifield resin was purchased from Watanabe Chem. Ind., Ltd. Deprotection of the N^{α}-Boc group was accomplished with 33% trifluoroacetic acid (TFA) in dichloromethane (DCM) for 30 min. The peptide chain was elongated by coupling reaction for 1—2 h employing N^{α}-Boc–amino acids (2 eq) *via* benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate[13] (BOP reagent, 2 eq) in the presence of *N*-methylmorpholine (3 eq) in *N*-methylpyrrolidone or *N*,*N*-dimethylacetamide. DCC-HOBt in DMF was used for the second coupling, and further repeated coupling by BOP or symmetrical anhydride prepared *in situ* was performed, if necessary. N^{α}-Boc–Gln was coupled by using DCC–HOBt in the same manner as Boc–Asn. Every introduction reaction of an amino acid was repeated until the resin became negative to the Kaiser test.[14] Side-chain protection of N^{α}-Boc–amino acids was as follows: Arg(Tos), Ser(Bzl), Tyr(Cl_2–Bzl), Glu(OBzl) and Lys(Cl–Z). 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 dilute acetic acid (AcOH). The crude peptide was lyophilized and 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) with isocratic elution using a 0.1% TFA–MeCN solvent system at the flow rate of 8 ml/min. The purified peptide was lyophilized from dilute HCl (about 5-fold excess) and finally gel-filtered on a Toyopearl HW-40 superfine column (1.5 × 58 cm) using 25% MeCN in 5 mM HCl as an eluent, and the desired fractions were lyophilized.

Acid hydrolysis of synthetic peptides was carried out with vapor of 6 N HCl 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 2). HPLC analysis of the peptides was carried out on a system composed of two 510 pumps, a model U6K injector, a model 680 gradient controller, a model 730 data module (Waters) and a UV-8011 spectrophotometer (Tosoh). Analysis was performed on a column of Puresil C_{18} (4.6 × 250 mm; Waters) with a linear gradient of 16.0—40.0% MeCN over a period of 20 min in 0.1% TFA (Table 1). The fast atom bombardment mass spectrum (FAB-MS) of synthetic peptides was measured with a JMS-DX-300 mass spectrometer connected with a JMA-DA 5000 mass data system, except for dog NMU-25 (**3**), which was analyzed by a JMS-SX-102A unit (JEOL) (Table 2). Optical rotations of 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% peptide. The *Rf* values in HP-TLC, performed on precoated silica gel plates (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). The analytical data are shown in Table 1.

Smooth Muscle Contraction Assay on Isolated Chicken Crop Preparation A chicken (age, 8—14 d) was decapitated after having been starved for 12 h and the crop was rapidly excised. The tissue was cut vertically and rinsed well with Tyrode's solution. The connecting tissue and the blood vessels were trimmed off. Then the tissue was cut along the longitudinal smooth muscle to prepare two strips (3 × 20 mm). The tissue strip preparation was mounted at a resting tension of 0.5 g in an organ bath (10 ml) containing Tyrode's solution of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.4, $NaHCO_3$ 12, glucose 5.6. This was kept at 30 °C under slow bubbling with a mixture of 95% O_2 and 5% CO_2, and the solution was changed every 10—15 min. The tissue preparation was equilibrated for 60 min, challenged twice with carbachol (2 μM), and once with p-NMU-8, added cumulatively up to 3 μM, then re-equilibrated for 30—40 min. Increasing concentrations of p-NMU-8 were added to the bath at 2—4 min intervals until the maximal responses developed, and an analog was similarly added after 30 min resting period. The cumulative contractile responses were recorded using a model TD-111T isotonic transducer (Nihon Koden, Japan) with a model 561-3003 recorder (Hitachi). The pharmacological parameters, RA value (relative activity; EC_{50} of NMU-8 (**2**)/EC_{50} of an analog) and maximum contraction (%) [maximum effect induced by an analog/maximum effect induced by p-NMU-8 (**2**) (3 μM) × 100] were calculated from the concentration–response curves, representative examples of which are shown in Fig. 3. An experiment to determine the RA value and the maximum contraction of an analog was performed once on a given tissue preparation, and repeated five to eighteen times using different tissue preparations. The RA and maximum contraction values of p-NMU-8 (**2**) are taken as 1.00 and 100, respectively. The EC_{50} value of p-NMU-8 (**2**) was 80.8 ± 7.3 nM, obtained from 80 experiments. The assay results are summarized on Table 3.

Enzymatic Degradation of NMU-Peptides Isolated chicken crop tissue (790 mg, wet, from two animals) was cut in 2—3 mm widths along the longitudinal smooth muscle in a similar manner to that used for the preparation of strips for bioassay. The strips of the tissue bound to a fine glass tube were kept in Tyrode's solution in a test tube at 30 °C under slow air bubbling. The solution was changed every 10 min and after three washes with the solution, the crop strips were incubated with Tyrode's solution (1.422 ml) for 20 min. The tissue strips were removed, and the Tyrode's solution was placed in ice bath and used as a soluble enzyme extract of chicken crop. Each of the following synthetic peptides, d-NMU-8 (**1**), p-NMU-8 (**2**), [Ac–Tyr[1]]-NMU-8 (**12**), [D-Tyr[1]]-NMU-8 (**16**), [Ala[1]]-NMU-8 (**9**), [Ac–Ala[1]]-NMU-8 (**14**) and [D-Ala[1]]-NMU-8 (**17**), was dissolved in saline to prepare a 1 mM solution. The peptide solution (10 μl) and the soluble enzyme extract (90 μl) were mixed in a polypropylene tube (2 ml) with tight lid (0.2 μmol of peptide/enzymes from 1 g wet crop strip/2 ml of Tyrode's solution). Seven aliquots were prepared for each peptide and the tubes were incubated in a bath at 30 °C. An incubation tube was taken out from the bath at 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h and put in an ice bath. The digestion solution (100 μl) was acidified with 20 μl of 10% TFA solution and centrifuged at 12000 rpm at 4 °C for 20 min. The supernatant (24 μl) was subjected to RP-HPLC analysis to determine the amount of starting material that remained. The experiments on the time-course of the proteolytic digestion of each peptide were repeated 2—4 times. HPLC analysis was performed on a Puresil C_{18} (4.6 × 250 mm) column using linear gradient elution

from 4.0 to 36% MeCN over a period of 40 min in 0.1% TFA at the flow rate of 1 ml/min. Absorbance was measured at 210 nm. The peak area of the starting material obtained from each aliquot was compared with that from the aliquot at zero incubation time. The degradation curves are shown in Fig. 5. Typical chromatograms of p-NMU-8 (**2**) and d-NMU-8 (**1**) after 3 h incubation with the soluble enzyme extract are shown in Fig. 4, A and B. To identify the degradation products, 1 mg of p-NMU-8 (**2**) or d-NMU-8 (**1**) was incubated with soluble enzyme extract (2 ml) for 4 and 8 h, respectively. The products were separated by HPLC on a YMC C_{18}-AM (1 × 15 cm) column employing a linear 40 min gradient from 4.0 to 36% MeCN in 0.1% TFA at the flow rate of 3 ml/min. Each peak was collected and the product was lyophilized. Part of the product was hydrolyzed with 6 N HCl vapor at 130°C and the hydrolysate was analyzed with an amino acid analyzer to give a theoretical ratio of amino acids corresponding to the fragments shown in Fig. 4, C and D. Peaks a, b, c, d, f and i were confirmed by comparison of the retention times with those of the corresponding amino acids or synthetic peptides, and by co-elution on HPLC.

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