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ISOLATION AND STRUCTURAL DETERMINATION OF RAT NEUROMEDIN U

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SUMMARY: Rat neuromedin U was isolated from the small intestine using mainly immunoaffinity chromatography and radioimmunoassay for pig neuromedin U-8. The amino acid sequence of rat neuromedin U was determined by microsequence analysis to be Tyr-Lys-Val-Asn-Glu-Tyr-Gln-Gly-Pro-Val-Ala-Pro-Ser-Gly-Gly-Phe-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH_2, and this structure was confirmed by synthesis. Although the C-terminal heptapeptide amide structure of pig neuromedin U is completely conserved in rat neuromedin U, the remainder of the peptide reveals nine amino acid replacements and two amino acid deletions when compared to pig neuromedin U-25. Rat neuromedin U exerts two-fold potent uterus stimulant activity as compared to pig neuromedin U-25.

Neuromedin U-8 (NMU-8) and neuromedin U-25 (NMU-25) are novel bioactive peptides isolated from pig spinal cord using a bioassay for a stimulant effect on rat uterus (1). These two peptides induce hypertension in anesthetized rat in addition to exerting potent uterus contractile activity. NMU-8 is an octapeptide having an amide structure at its C-terminus, while NMU-25 is an N-terminally extented form of NMU-8 carrying paired Arg residues just before the NMU-8 sequence. In our systematic survey for unknown neuropeptides in pig spinal cord, we already isolated a series of new bioactive peptides: two tachykinins, two bombesin-like peptides and their big forms, and neurotensin-like peptide (2-7). Although these peptides have their counterparts in known peptides, neither NMU-8 nor NMU-25 shows significant structural homology to known members of neuropeptides or hormones (1). In order to elucidate the physiological significance of NMU-8 and NMU-25, we raised antisera against these peptides and investigated the regional distribution in rat brain and peripheral tissue using radioimmunoassay (RIA) and immunohistochemical method. The results demonstrated that rat small intestine contained neuromedin U (NMU)-like immunoreactivity (NMU-LI) at much higher concentration than any regions of rat brain or other peripheral tissue (8,9). However, chromatographic properties of NMU-LI from rat intestine and brain suggested that rat NMU may be different from pig NMU-25 and NMU-8 (to

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be reported elsewhere). Based on these results, we undertook the isolation of rat NMU from the rat intestine and determined its amino acid sequence.

MATERIALS AND METHODS

Materials: Pig NMU-8 and NMU-25 were synthesized and purified in our laboratory, as reported in the previous paper (1). Bradykinin was purchased from Protein Research Foundation (Osaka, Japan).

Isolation of rat NMU: Small intestines of male Wistar rats, including the duodenum, jejunum and ileum, were collected soon after decapitation and washed with cold saline. Rat intestines (117 g) from 17 animals were boiled in 5 volumes of water for 10 min to inactivate intrinsic proteases. After addition of glacial acetic acid (final concentration = 1.0 M), the boiled tissue was homogenized by a Polytron mixer for 10 min at 4°C. The supernatant obtained by centrifugation at 20,000 x g for 30 min was precipitated by addition of acetone (final acetone concentration = 75%). After removal of the precipitates by centrifugation, the supernatant was evaporated, diluted two-fold with water, and pumped onto a C_{18} silica column (90 ml, LC-SORB ODS, Chemco). The column was washed with 0.5M acetic acid, and then eluted with a solution of 60% CH_3CN containing 0.1% trifluoroacetic acid (TFA). The eluate was evaporated, reconstituted with 1M acetic acid, and then loaded onto a SP-Sephadex C-25 column (H^+-form, 1.7 x 13 cm, Pharmacia). Successive elutions with 1M acetic acid, 2M pyridine, and 2M pyridine-acetate (pH 5.0) yielded three respective fractions: SP-I, SP-II and SP-III. SP-III fraction containing basic materials was lyophilized and then subjected to Sephadex G-50 gel filtration (fine, 1.8 x 135 cm, Pharmacia). The fractions containing NMU-LI were pooled and loaded onto an anti-pig NMU-8 immunoaffinity column (see details below). The peptides eluted from the affinity column were finally purified by reverse phase HPLC on a μ-Bondasphere C_{18} column (3.9 x 150 mm, Waters) at a flow rate 1.0 ml/min with a linear gradient elution of CH_3CN in 0.1% TFA for 120 min. Column effluents from HPLC were monitored by measuring absorbance at 210 nm and at 280 nm.

Immunoaffinity chromatography: Immunoglobulin G (IgG) fraction was purified from 2 ml of antiserum #139-8 against pig NMU-8 by using a Protein A-Sepharose CL-4B column (0.8 x 5 cm, Pharmacia). IgG fraction thus obtained was coupled with 2 ml of AFFI-GEL 10 (BIO-RAD) in 0.1M sodium phosphate buffer (pH 7.4) at 4°C for 4hr. The NMU-LI fraction in the gel filtration was dissolved in 0.1M sodium phosphate buffer (pH 7.4), loaded onto the column (gel volume = 500 μl), and washed with the same buffer. The adsorbed peptides were then eluted with a solution of 1M CH_3COOH containing 10% CH_3CN.

RIA for NMU-8: Details for RIA of NMU-8 will be reported elsewhere. In brief, antiserum #139-8 could be used for RIA at a final dilution of 1:800,000, and the peptides were measurable in a range of 2-200 fmol/tube in this RIA system. The antiserum mainly recognized the C-terminal pentapeptide amide structure of NMU-8, and NMU-25 and N-terminal amino acid shortened NMU-8s had comparable affinities with the antiserum.

Sequence analysis: Sequence analysis was performed mainly by a gas phase sequencer (Model 470A/120A, Applied Biosystems). The resulting PTH-amino acids were analyzed by reverse phase HPLC in concert with the gas phase sequencer. PTH-amino acids were measurable as low as 1 pmol. Amino acid analysis was carried out with an amino acid analyzer (A-5550, IRICA) after hydrolysis of the peptide (ca. 250 pmol) in 6N HCl containing 0.1% phenol and 0.02% 2-mercaptoethanol at 110°C for 24 hr. Carboxy-terminal amino acid amide was determined by the method of Tatemoto and Mutt (10). Asparagine amide generated upon tryptic digestion was identified as its dansyl derivative on a polyamide sheet.

Synthesis: Rat NMU was synthesized by solid phase techniques conducted on p-methyl-benzhydrylamine resin with a peptide synthesizer (430A, Applied Biosystems) and purified by reverse phase HPLC. Correct synthesis was confirmed by amino acid analysis and sequencing.

Bioassay: The effect of rat NMU on the contractility of freshly isolated rat uterus was examined according to the reported method (11).

RESULTS AND DISCUSSION

From the acid extracts of 17 rat small intestines, the basic peptide fraction, which contained almost all of the NMU-LI, was effectively condensed through acetone precipitation, followed by step-wise chromatographies on reverse phase C_{18} silica gel column and on SP-Sephadex C-25 ion exchange column. The basic peptide fraction (dry weight; 91 mg) was then subjected to gel filtration on a Sephadex G-50 column, as shown in Fig. 1. More than 95% of the total NMU-LI were eluted in fractions #53-56 corresponding to synthetic pig NMU-25. A minor NMU-LI peak emerged around fraction #60, but was much earlier than that of pig NMU-8. Fractions #53-55 were pooled, lyophilized (dry weight; 4.5 mg), and subjected to anti-pig NMU-8 immunoaffinity chromatography. The adsorbed peptides on the immunoaffinity column were then subjected to reverse phase HPLC on a C_{18} column, and rat NMU was finally purified to a homogeneous state (Fig. 2). Purification by anti-pig NMU-8 immunoaffinity chromatography was highly efficient; about 70% of the peptides eluted from the affinity column was rat NMU, as calculated from the absorbance at 210 nm on reverse phase HPLC at the next purification step.

The amino acid composition of rat NMU thus isolated was determined after acid hydrolysis to be Asp, 1.96(2); Ser, 0.92(1); Glu, 1.97(2); Pro, 3.17(3); Gly, 3.07(3); Ala, 0.98(1); Val, 1.86(2); Leu, 1.11(1); Tyr, 1.94(2); Phe, 2.90(3); Lys, 1.18(1); Arg, 1.94(2). Thus, rat NMU was found to consist of 23 amino acid residues, i.e., two amino acids less than pig NMU-25. Based on the

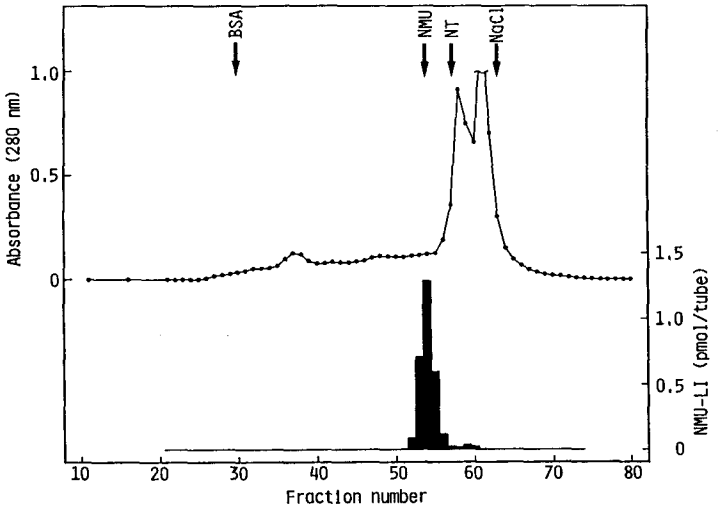

Figure 1. Sephadex G-50 gel filtration of the basic peptide fraction purified from rat small intestine.
Sample: Basic peptide fraction purified from 117g of rat small intestine.
Column: Sephadex G-50 fine (1.8 x 135 cm, Pharmacia).
Solvent: 1M acetic acid. Fraction size: 5 ml/tube. Flow rate: 8 ml/hr.
Arrows indicate the elution positions of bovine serum albumin (BSA), pig NMU-25 (NMU), neurotensin (NT) and NaCl, respectively.
Black columns represent NMU-LI of each fraction.

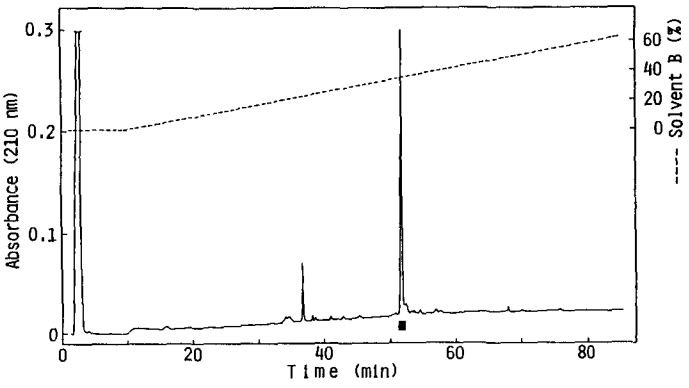

Figure 2. Final purification of rat neuromedin U by reverse phase HPLC.
Sample: Half of anti-pig NMU-8 immunoaffinity chromatography purified fraction obtained from fractions #53-55 in Sephadex G-50 gel filtration.
Column: μ-Bondashere C_{18} (100A, 3.9 x 150 mm, Waters).
Flow rate: 1.0 ml/min. Temperature: ambient.
Solvent system: Linaer gradient elution from (A) to (B) for 120 min.
H_2O:CH_3CN:10%TFA = (A) 90:10:1, (B) 40:60:1 (v/v).
NMU-LI was observed only at the black bar region.

amino acid analysis data, 2.60 nmol of rat NMU were purified from 117 g of rat small intestine. The average tissue concentration of NMU-LI in the rat duodenum, jejunum and ileum was about 35 pmol/g wet tissue. The yield of rat NMU in the present purification indicated that 65% of rat NMU in the small intestine was isolated as a pure peptide. This total recovery yield of rat NMU was significantly higher than those of our previous isolation of a series of neuromedins (5-20%) (2-7).

Amino acid sequence analysis was performed by a gas-phase sequencer and PTH-amino acid liberated at each cycle of Edamn degradation was successfully identified up to the C-termianl residue, as shown in Fig. 3a. Carboxy-terminal Asn-NH_2 was also clearly identified as its dansyl derivative by the method of Tatemoto and Mutt (10). Thus, the amino acid sequence of rat NMU was determined, as shown in Fig. 3b. Positive confirmation of the sequence thus determined was provided by chemical synthesis of rat NMU. The native and synthetic rat NMUs were co-eluted as a single peak on reverse phase HPLC under identical conditions used for Fig. 2.

Biological activity of rat NMU was measured by a bioassay for the stimulant effect on rat uterus. Figure 4 shows that rat NMU also elicits a repeated and sustained uterus contraction in a manner similar to that of pig NMU-25. Furthermore, potency of rat NMU was about twice as high as that of pig NMU-25, when rat uterus was used for the assay.

In order to maximize the sequence homology between rat NMU and pig NMU-25, spaces must be inserted in rat NMU at position 6 and at position 11, respectively, of the pig NMU-25 numbering system (Fig. 3b). Except for these amino acid deletions, the other amino acid replacements observed between rat

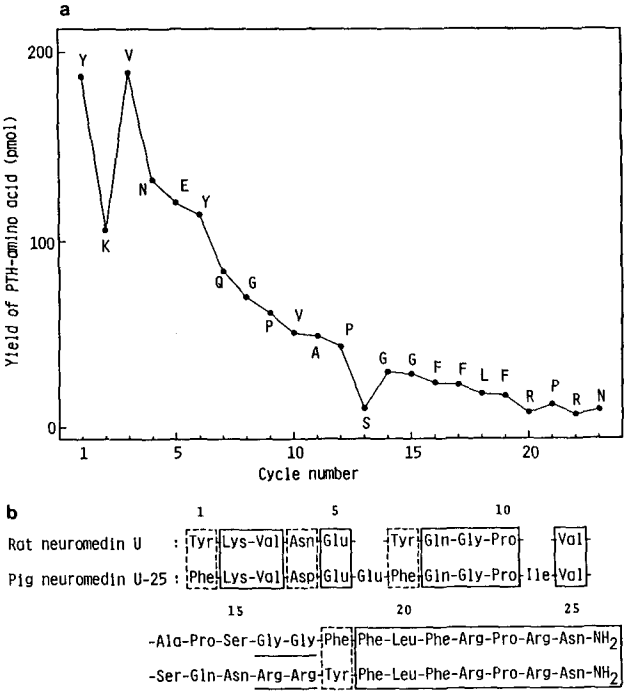

Figure 3. (a) Yield of PTH-amino acid at each cycle of Edman degradation. (b) Amino acid sequences of rat neuromedin U and pig neuromedin U-25. Identical residues and homologous residues are boxed with solid lines and dashed lines, respectively.

NMU and pig NMU-25 could occur by one base substitution of each amino acid codon. The carboxy-terminal heptapeptide sequence of pig NMU-25, which is essential for biological activity, is completely conserved in rat NMU.

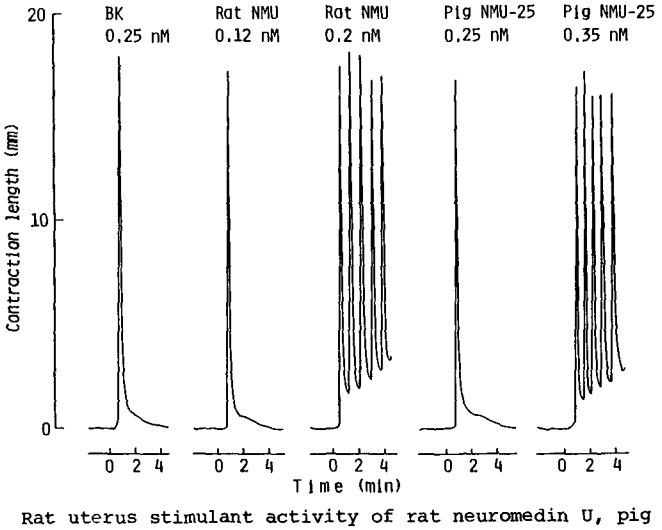

Figure 4. Rat uterus stimulant activity of rat neuromedin U, pig neuromedin U-25 and bradykinin.
Freshly isolated rat uterus was bathed at 32-35°C in modified Locke-Ringer solution with a tension of 0.5-1.0 g. Peptides dissolved in 0.9% saline were added at 0 min. BK: bradykinin.

Moreover, the 8th residues from the C-terminus are aromatic amino acids in both peptides. In the N-terminal region, amino acid sequences are homologous up to the 12th residue. However, five amino acid residues at the middle portion are quite different. Of note is that the Arg-Arg of paired basic amino acids, often observed as a signal for proteolytic processing, is replaced by Gly-Gly. The absence of a low molecular weight form in rat corresponding to pig NMU-8 and the relatively large difference in the elution time between rat NMU-LI and pig NMU-25 on reverse phase HPLC are probably due to these replacements (9,12).

NMU is a novel type of brain-gut peptide, and its physiological function has not been elucidated as yet. The present identification of rat NMU indicates that the C-terminal heptapeptide amide structure may be conserved among mammalian species and is essential in eliciting bioactivity. Therefore, this heptapeptide amide structure can be recognized as the minimum unit to exert the physiological functions of neuromedin U.

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REFERENCES

1. Minamino, N., Kangawa, K. & Matsuo, H. (1985) Biochem. Biophys. Res. Commun., 130, 1078-1085.
2. Kangawa, K., Minamino, N., Fukuda, A. & Matsuo, H. (1983) Biochem. Biophys. Res. Commun., 114, 533-540.
3. Minamino, N., Kangawa, K., Fukuda, A. & Matsuo, H. (1984) Neuropeptides, 4, 157-166.
4. Minamino, N., Kangawa, K. & Matsuo, H. (1984) Biochem. Biophys. Res. Commun., 122, 542-549.
5. Minamino, N., Kangawa, K. & Matsuo, H. (1983) Biochem. Biophys. Res. Commun., 114, 541-548.
6. Minamino, N., Kamgawa, K. & Matsuo, H. (1984) Biochem. Biophys. Res. Commun., 119, 14-20.
7. Minamino, N., Sudoh, T., Kangawa, K. & Matsuo, H. (1985) Biochem. Biophys. Res. Commun., 130, 685-691.
8. Honzawa, M., Sudoh, T., Minamino, N., Tohyama, M. & Matsuo, H. (1987) Neuroscience, 23, 1103-1122.
9. Sudoh, T., Honzawa, M., Minamino, N., Kangawa, K. & Matsuo, H. (1986) Seikagaku (in Japanese), 58, 987.
10. Tatemoto, K. & Mutt, V. (1978) Proc. Natl. Acad. Sci. USA, 75, 4115-4119.
11. Carraway, R. & Leeman, S.J. (1973) J. Biol. Chem., 248, 6854-6861.
12. Domin, J., Ghatei, M., Chohan, P. & Bloom, S.R. (1986) Biochem. Biophys. Res. Commun., 140, 1127-1134.