Vol. 119, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1984 Pages 14-20

NEUROMEDIN C: A BOMBESIN-LIKE PEPTIDE IDENTIFIED IN PORCINE SPINAL CORD

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Received January 11, 1984

SUMMARY: From a side fraction obtained in our previous isolation of neuromedin B from porcine spinal cord, we have purified another decapeptide that exhibits a potent stimulant effect on the smooth muscle preparation of rat uterus. By microsequencing and synthesis, the amino acid sequence of the peptide has been identified as: Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2. This peptide is found to be identical with the carboxy-terminal subsequence [18-27] of gastrin releasing peptide, and to display a potent contractile activity on rat uterus in the characteristic manner of bombesin. These facts strongly suggest that the peptide may be a neuromediator in the neural communication systems of mammals. We propose the name "neuromedin C" for this peptide, since it is closely related to "neuromedin B", recently identified as a bombesin-like mammalian peptide.

Bombesin is a tetradecapeptide identified in amphibian skin and is known to display various intense biological effects in mammals (1,2). It is a potent releaser of gastric acid, gastrin and other gastrointestinal hormones in mammals, as well as a powerful stimulant for gastrointestinal, uterine and urinary tract smooth muscles. In the central nervous system, it also exhibits potent effects on thermoregulation, glucoregulation, and release of anterior pituitary hormones. The effects which amphibian bombesin elicits in mammals strongly suggest a possibility that bombesin-like peptides may be acting in mammals as endogenous neuromediators. Actually, bombesin-like immunoreactivity has been demonstrated to exist in mammalian gastrointestinal tissues, brain and spinal cord (3,4). In mammalian intestine and stomach, gastrin releasing peptide (GRP) and its derivatives have been identified as bombesin-like peptides (5,6,7). However, nothing had been isolated from neural tissues, until our recent identification in porcine spinal cord of "neuromedin B", which has a surprising sequence homology to amphibian bombesins (8). We have further investigated still unidentified neuropeptides in porcine spinal cord, using bioassay for a stimulant effect on rat uterus contraction, and have isolated a novel peptide, designated "neuromedin C", from a side fraction of the previous purification of neuromedin B. This paper will report the isolation of neuromedin C from porcine spinal cord and its complete amino acid sequence determined by

0006-291X/84 $1.50

microsequencing as well as synthesis. Relative potency on rat uterus of neuromedin C, compared with neuromedin B and amphibian bombesins, will also be discussed.

MATERIALS AND METHODS

Isolation: The starting material used in the present purification of neuromedin C was a side fraction (fraction E) obtained in our previous isolation of neuromedin B (8). As described, peptides of Mr 700-5,000 daltons, that prepared from the acid extracts of porcine spinal cords (ca. 20 kg), were adsorbed on SP-Sephadex C-25, and eluted with 1M CH_3COOH (SP-I), 2M pyridine (SP-II) and 2M pyridine-acetate (pH 5.0) (SP-III), successively. Gel-filtrations of SP-III in 1M CH_3COOH were performed on a column (4.5 x 140 cm) of Sephadex G-50 (fine) and on a column (7.5 x 135 cm) of Sephadex G-25 (fine), successively. An aliquot of each fraction was subjected to bioassays for contractile activity using smooth muscle preparations isolated from guinea pig ileum and rat uterus. Column effluents of Sephadex G-25 gel-filtration were fractionated into fractions A-G, based on the results of bioassays (see Fig.1 in ref. 8) and pooled. Fraction E was used for the present purification. Peptides in fraction E were adsorbed on a reverse phase column of Nucleosil 30 C-18 (Nagel) in the presence of 0.5M CH_3COOH and then eluted with a solution of H_2O:CH_3CN:10%TFA = 40:60:1 (v/v). The peptides thus obtained were submitted to ion exchange high performance liquid chromatography (HPLC) on a column of TSK IEX-530 CM (4.0 x 300 mm, Toyosoda) under the conditions detailed in the legend of Fig.1. The uterus contractile fraction was further purified by reverse phase HPLC (Fig.2). Final purification of neuromedin C was performed on a column of μ-Bondapak C-18 (3.9 x 300 mm, Waters) using 10 mM $HCOONH_4$ buffer (pH 4.0) with a linear gradient elution of CH_3CN from 10% to 50%. Purity of neuromedin C was checked on two different HPLC systems using reverse phase and ion exchange columns (Fig.3). Column effluents on HPLC were monitored by measuring the absorbance at 210 nm or 280 nm.

Bioassay: The effect of the sample on the contractility of freshly isolated preparations of guinea pig ileum and rat uterus was examined according to the described methods (9,10). Relative potency of the bombesin-like peptide was determined by rat uterus assay. Bombesin and bradykinin were purchased from Protein Research Foundation, and GRP, litorin and ranatensin from Peninsula.

Sequence analyses: Because of the very minute amount of the sample, all analyses were carried out on a subnanomole scale. Amino acid analyses were performed with Hitachi-835 amino acid analyzer, after hydrolysis of the peptide (ca. 0.4 nmol) in 3 M mercaptoethanesulfonic acid at 110°C for 20 hr. Amino acid sequence of the peptide was determined by an automated gas-phase sequenator (Applied Biosystems 470A) on a 100 pmole level with the program modified for small peptides. PTH-amino acids were analyzed on a Zorbax C-8 column (4.6 x 300 mm, Dupont), using a three-solvent program to give a complete baseline separation. The C-terminal amide in neuromedin C was determined by the method of Tatemoto and Mutt (11): Methionine amide generated upon chymotryptic digestion was identified as its dansyl derivative. Native and synthetic neuromedin C (0.5 nmol each) were each digested with 400 ng of chymotrypsin (Sigma) in 10 μl of 0.2 M N-ethyl-morpholine buffer (pH 8.0) at 37°C for 3 hr. After addition of 1 M CH_3COOH to terminate the reaction, the digests were separated by reverse phase HPLC. Chymotryptic peptides (ChT-1, -2, -3, -4) of synthetic neuromedin C were each isolated by the same HPLC system and identified on the basis of amino acid composition.

Synthesis of neuromedin C: The decapeptide amide according to the sequence determined for neuromedin C was synthesized by solid phase techniques, conducted on a p-methyl-benzhydrylamine resin. Purification was made by CM-52 ion exchange chromatography and reverse phase HPLC. Correct synthesis was confirmed by amino acid analysis and sequencing.

RESULTS AND DISCUSSION

A basic peptide fraction (SP-III) of Mr 700-5,000 daltons, obtained from acid extracts of porcine spinal cord (ca. 20 kg), was the starting material for the purification of neuromedin B and neuromedin C. After gel-filtration of SP-III on Sephadex G-50, fractions eliciting a contractile effect on rat uterus and guinea pig ileum were pooled and subjected to a second gel-filtration on Sephadex G-25. An appreciable uterus contractile activity distributed widely in fractions A-F (see Fig.1 in ref.8), while a major peak of ileum stimulant activity emerged at fractions C and D. As previously reported, neuromedin B was isolated from fraction F (8). The present purification was concerned with fraction E, which exhibited a potent effect on rat uterus.

Peptides in fraction E were adsorbed on a reverse phase column of Nucleosil 30 C-18 and eluted with a solution of H_2O:CH_3CN:10%TFA = 40:60:1 (v/v). After lyophilization, the resulting peptides were separated by cation exchange HPLC on a column of TSK IEX-530 CM. As shown in Fig.1, four peaks of uterus contractile activity were observed. Neuromedin C was isolated from the bioactive peak eluted at 83-85 min. Purification and characterization of

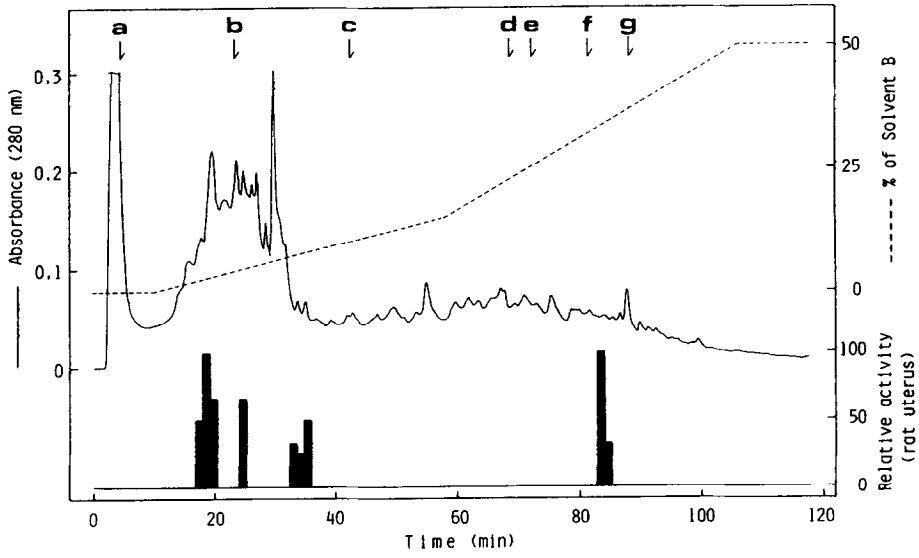

Fig. 1 Ion exchange HPLC of fraction E.
Sample : One-third of fraction E obtained by Sephadex G-25 gel-filtration, after batch-wise treatment with C-18 resin (see Fig.1 in ref.8).
Column : TSK IEX-530 CM, 4.0 x 300 mm (Toyosoda). Flow rate : 1.0 ml/min.
Solvent system : (A) 10 mM $HCOONH_4$ (pH 6.5) : CH_3CN = 90 : 10 (v/v)
(B) 1.0 M $HCOONH_4$ (pH 6.5) : CH_3CN = 90 : 10 (v/v)
Linear gradient elution from A:B = 100:0 to A:B = 85:15 (48 min), followed by that from A:B = 85:15 to A:B = 50:50 (48 min).
Relative contractile activity on rat uterus is shown by the black bars.
Arrows indicate elution times of a) Leu-enkephalin, b) [Arg^6]-Leu-enkephalin, c) LH-RH, d) PH-8P, e) somatostatin, f) substance P, g) α-neo-endorphin, respectively.

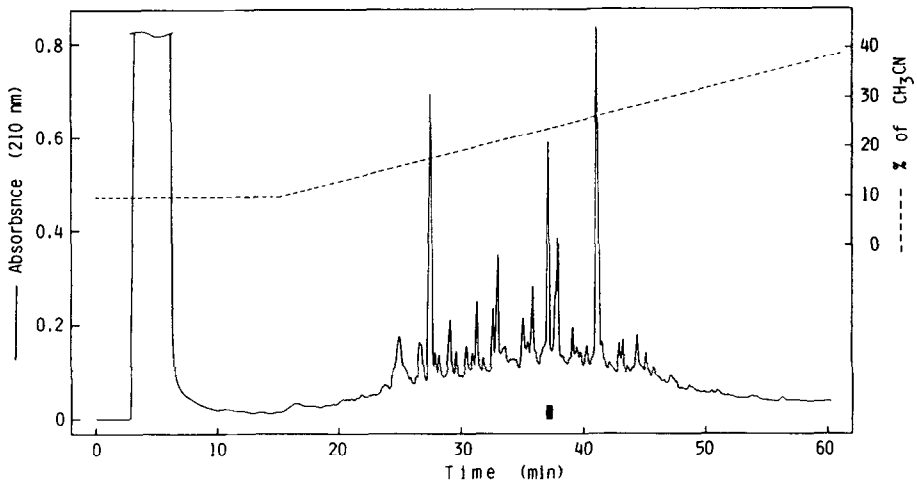

Fig. 2 Reverse phase HPLC of the bioactive fraction.
Sample : The rat uterus contractile fraction eluted at 83-85 min on ion exchange HPLC (Fig.1). Flow rate : 1.0 ml/min.
Column : Chemcosorb 5ODS-H, 4.6 x 250 mm (Chemco).
Solvent system : Linear gradient elution from (A) to (B) (80 min).
(A) H_2O : CH_3CN : 10% TFA = 90 : 10 : 1.0 (v/v)
(B) H_2O : CH_3CN : 10% TFA = 40 : 60 : 1.0 (v/v)
Uterus contractile activity was observed at the black bar region.

other peaks with uterus activity is now going on. The major peak of uterus contractile activity eluted at 83-85 min was further purified by reverse phase HPLC on a Chemcosorb 5ODS-H column, as shown in Fig.2. Uterus contractile activity was observed only at the region indicated with a black bar, being well separated from inactive materials. Final purification of the uterus active fraction to a homogeneous state was achieved by reverse phase HPLC on a column of μ-Bondapak C-18. Purity of the peptide was confirmed by reverse phase HPLC as well as ion exchange HPLC, as shown in Fig.3A and 3B. The amino acid composition of neuromedin C was determined after acid hydrolysis to be : Asp 1.2 (1), Gly 2.2 (2), Ala 1.2 (1), Val 0.9 (1), Met 1.0 (1), Leu 1.1 (1), His 1.8 (2), Trp 0.7 (1), indicating its decapeptide structure. Based on the amino acid analysis data, it was estimated that only 2.6 nmol of neuromedin C were purified from 20 kg of spinal cord. The recovery yield of neuromedin C in the course of repeated HPLC was lower than that of other neuropeptides which we have isolated. The low yield was likely due to its adsorptive property, associated with the basicity of the peptide and existence of Trp residue in the molecule. As a matter of fact, neuromedin C was eluted later on cation exchange HPLC, compared with other peptides (Fig.1). Therefore, the actual content of neuromedin C in spinal cord is thought to be higher than the yield observed in the present purification.

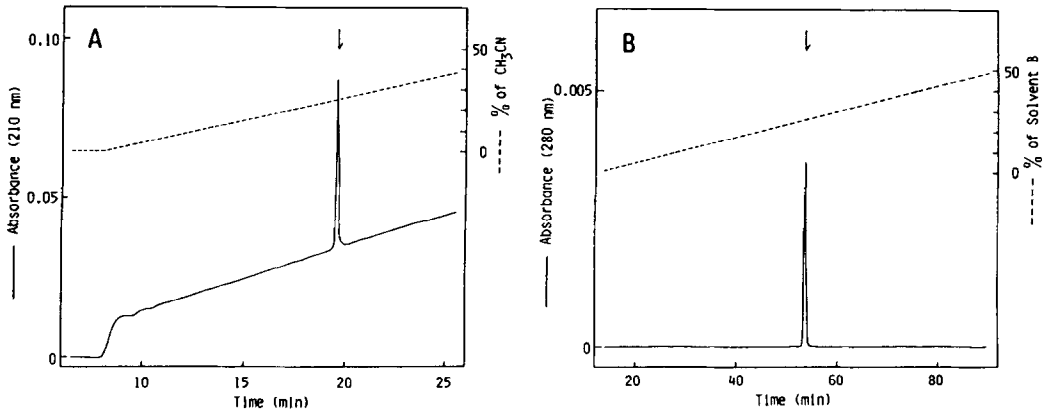

Fig. 3 (A) Reverse phase HPLC of native neuromedin C.
Sample : Neuromedin C (150 ng). Flow rate 1.0 ml/min.
Column : Chemcosorb 30DS-H, 4.6 x 75 mm (Chemco)
Solvent system : Linear gradient elution from (a) to (b) (30 min).
(a) H_2O : CH_3CN : 10% TFA = 90 : 10 : 1.0 (v/v)
(b) H_2O : CH_3CN : 10% TFA = 40 : 60 : 1.0 (v/v)
(B) Ion exchange HPLC of native neuromedin C.
Sample : Neuromedin C (0.4 nmol). Flow rate : 1.0 ml/min.
Column : TSK CM-2SW, 4.6 x 250 mm (Toyosoda).
Solvent system : (a) 10 mM $HCOONH_4$ (pH 6.5) : CH_3CN = 90 : 10 (v/v)
(b) 1.0 M $HCOONH_4$ (pH 6.5) : CH_3CN = 90 : 10 (v/v)
Linear gradient elution from (a):(b) = 100:0 to (a):(b) = 50:50 (80 min).
Arrows indicate elution times of synthetic neuromedin C.

The amino acid sequence of neuromedin C was determined by an automated gas-phase sequenator starting with 500 pmol of the peptide. Edman degradation afforded 390 pmol of PTH-Gly at the first cycle. Liberated PTH-amino acids were successfully identified up to the 8th step and the yield of PTH-His at the 8th step was about 40 pmol. Thus, sequence analysis provided unambiguous assignment to the 8th residue as Gly-Asn-His-Trp-Ala-Val-Gly-His---. The presence of methionine amide at the C-terminal was verified by generation upon chymotryptic digestion, followed by dansylation in a manner similar to the method of Tatemoto and Mutt (11). From the results described above, the complete amino acid sequence of neuromedin C was determined to be : Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2, as shown in Fig.4.

Further structural confirmation was provided by comparison with the decapeptide amide synthesized according to the sequence of neuromedin C determined above. Both native and synthetic peptides migrated together on

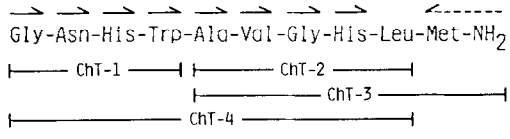

Fig. 4 Complete amino acid sequence of neuromedin C.
(——>) : By the sequenator. (<---) : By the method of Tatemoto and Mutt.
ChT : Chymotryptic peptides.

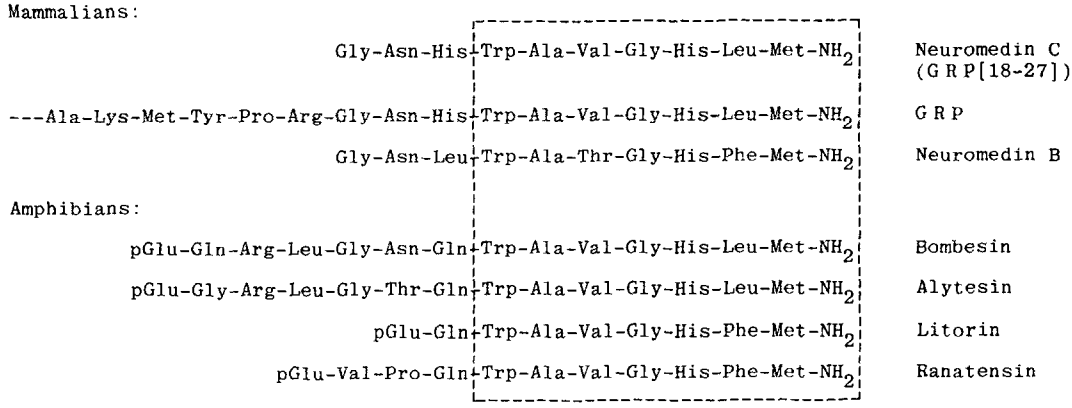

Fig. 5 Amino aicd sequences of bombesin-like peptides.

reverse phase HPLC as well as on ion exchange HPLC, as shown in Fig.3A and 3B. Reverse phase HPLC also provided evidence that native neuromedin C underwent chymotryptic digestion in exactly the same manner as the synthetic specimen. The resulting chymotryptic fragments were each identified as shown in Fig.4. Thus, the complete amino acid sequence of neuromedin C was established to be : Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2.

As clearly seen in Fig.5, neuromedin C is found to be identical with the C-terminal subsequence [18-27] of GRP, and quite homologous to bombesin. Actually, the entire sequence of neuromedin C is conserved in the C-terminal decapeptide of bombesin, except that His at position 3 of neuromedin C is replaced by Gln in bombesin. Our recently isolated novel mammalian bombesin-like peptide, neuromedin B, also has a surprising sequence homology to bombesin. The structural resemblance of the present peptide to neuromedin B and bombesin is the reason for naming it neuromedin C, the next letter in alphabetical sequence.

As expected from its remarkable homology to bombesin, neuromedin C elicits a typical bombesin-like behavior in contractile reaction on rat uterus and guinea pig ileum, distinct from that of tachykinin. Biological activity of neuromedin C was compared with other known bombesin-like peptides by using a bioassay for the contraction of rat uterus. As shown in Table 1, bombesin, litorin and ranatensin have almost the same contractile effect on rat uterus. Neuromedin C has about 60% of the potency of bombesin, and is a little more potent than GRP, an N-terminal extended peptide of neuromedin C. On the other hand, neuromedin B has only 5% of the biological activity of bombesin on rat uterus, indicating that such a decrease of potency can be ascribed to substitutions at the 3rd and 6th positions of neuromedin B. The replacement of Leu with Phe at the 8th position does not alter the activity, as observed between bombesin and ranatensin.

Table 1. Relative potency of bombesin-like peptides on rat uterus

Peptide	Relative potency
Bombesin	100
Neuromedin C (GRP[18-27])	61
GRP	48
Neuromedin B	4.9
Litorin	113
Ranatensin	109
Bradykinin	32

Relative potency was calculated on molar basis by taking bombesin as 100. (n=8)

Studies of the structure-activity relationship of bombesin reveal that the C-terminal nonapeptide of bombesin is essential in eliciting the full spectrum of bombesin activity (12). Accordingly, the decapeptide structure of neuromedin C seems to be the minimum molecule able to elicit the full spectrum of bombesin-like activity. The existence of neuromedin B, which is also a decapeptide with bombesin-like activity identified in spinal cord, further supports the significance of neuromedin C as a mature form of GRP in the neural tissues. Recently, a number of neuropeptides have been shown to be generated from their precursors by proteolytic processing at a single Arg residue as well as at paired basic amino acids. Neuromedin C, preceded by an Arg residue in the GRP molecule, is assumed to be programmed to be processed out of GRP or its precursor in the neural tissues.

ACKNOWLEDGEMENTS : We are grateful to Dr. I. Kubota, Suntory Institute for Biomedical Research, for sequence determination by gas-phase sequenator. We also thank Miss A. Fukuda for her skillful technical assistance.

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