Vol. 114, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 29, 1983 Pages 541-548

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

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Received June 2, 1983

SUMMARY: In our survey for unknown neuropeptides in porcine spinal cord, we have purified a novel decapeptide that exhibits a potent stimulant effect on the smooth muscle preparation of rat uterus. By microsequencing and synthesis, the peptide has been identified as Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2. The peptide is found to have a surprising sequence homology to amphibian bombesins, and to display a potent contractile activity of rat uterus in the characteristic manner of the known bombesins. These facts strongly suggest that the peptide may be involved in the neural communication system of mammals as a neuromediator or hormone. Thus, we propose the name "neuromedin B" for the peptide.

The remarkable sequence homology of amphibian peptides with several mammalian neuropeptides or peptide hormones has been observed in cases such as amphibian tachykinins vs. substance P, caeruleins vs. CCK/gastrin, and sauvagine vs. corticotropin releasing factor (1). Thus, the phylogenetic barriers seem to be vanishing between amphibian and mammalian peptides. Interestingly, the bombesin peptide family identified in amphibian skin is known to display various and intense pharmacological effects in mammals (2). Bombesin is a moderate hypertensive agent in the dog and rat. It is a potent releaser of gastrin, gastric acid and cholecystokinin in mammals, as well as a powerful stimulant for intestinal, uterine and urinary tract smooth muscles. Especially in the rat central nervous system, it is the most potent agent acting on thermoregulation, glucoregulation, and release of prolactin and growth hormone. The effects of bombesin in mammals strongly suggest the possibility that bombesin-like peptides may be acting in mammals as endogenous hormones or neuromediators. Although bombesin-like immunoreactivity has been demonstrated in mammalian gut and brain (3,4), only gastrin releasing peptide (GRP) related to bombesin has been identified in mammalian gastric tissue and nothing had been isolated from neural tissues (5).

In our survey for unknown neuropeptides in porcine spinal cord, we have purified a new bombesin-like peptide, designated "neuromedin B", that elicits a stimulant effect on rat uterus preparation and have sequenced the new

0006-291X/83 $1.50

peptide. This paper will report the isolation of neuromedin B from porcine spinal cord and its complete amino acid sequence determined by microsequencing as well as by synthesis. The sequence homology of neuromedin B with amphibian bombesins will also be discussed.

METHODS AND MATERIALS

Isolation: The starting material used in the present purification of neuromedin B was a side fraction SP-III obtained in our previous isolation of neuromedin K (6). As described, a fraction containing peptides of Mr 700-5000 daltons was prepared by ultrafiltration, followed by acetone-precipitation from the acid extracts of spinal cords (ca. 20 kg) isolated from 550 pigs. The peptides in the above fraction were adsorbed on SP-Sephadex C-25, and eluted with 1M CH_3COOH, 2M pyridine and 2M pyridine-acetate (pH 5.0), successively. The fraction eluted with 2M pyridine-acetate was lyophilized to give an SP-III concentrate of ca. 10.5 g. The concentrate (SP-III), containing basic peptides including neuromedin B and substance P was the starting material in this purification. 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. Column effluents were monitored by measuring absorbance at 280 nm. An aliquot of each fraction was subjected to bioassays for contractile activity by using smooth muscle preparations isolated from guinea-pig ileum and rat uterus. Another aliquot of each fraction was used for radioimmunoassay for substance P.
Fractions A,B,C,D,E,F with uterus activity were pooled, respectively (Fig. 1). Fraction F was used for further purification. The peptides in fraction F were adsorbed on a reverse phase column of Nucleosil 30 C-18 (Nagel) in the presence of 0.5 M CH_3COOH, washed with 0.5M CH_3COOH and 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.2. Final purification of neuromedin B was performed on a reverse phase column of Chemcosorb 5ODS-H (4.6 x 250 mm, Chemco). 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 method (7,8). Uterine contractility was examined in Locke-Ringer's solution, while the ileum was bathed in Tyrode's solution.

Radioimmunoassay: Substance P contents in fractions were measured by radioimmunoassay according to the method of Nakazato, et al. (9).

Sequence analyses: Because of the very minute amount of the sample, all analyses were carried out on a subnanomole scale. Amino acid analyses were usually performed with Hitachi-835 amino acid analyzer, after hydrolysis of the peptide (ca. 0.5 nmole) in 3M mercaptoethanesulfonic acid at 110°C for 20 hr. Analyses of amino acids released after aminopeptidase M digestion were carried out on a picomole level with a pre-labeling analysis system (Waters). Amino acids released after enzymatic digestion of the peptide were subjected to fluorescence-labeling prior to analysis by treating with o-phthalaldehyde in the presence of ß-mercaptoethanol. By this analysis, Asp and Asn were discriminated from each other.
Chymotryptic digestion of the peptide (5 nmole) was carried out with 2 µg of the enzyme (Sigma) in 25 µl of 1% ammonium bicarbonate (pH 8.0) at 37°C for 3 hr. Thermolytic digestion of the peptide (2 nmole) was performed with 1 µg of the enzyme (Sigma) in 10 µl of 50mM Hepes buffer (pH 7.5) containing 10mM $CaCl_2$ at 37°C for 4 hr. Fragment peptides generated from enzyme digestion were isolated by reverse phase HPLC on a Chemcosorb 3ODS-H column (4.6 x 75 mm, Chemco) eluting with the solvent system of H_2O-CH_3CN-TFA.

Sequence analyses of the chymotryptic peptides (ChT-1 and ChT-2) were performed by the dansyl-Edman method, mainly according to the report of Hartley (10). Three nanomoles of the peptides were used for sequence analyses. N-terminal amino acids of neuromedin B and its thermolytic peptides (Th-1 and Th-2) were identified by the dansyl method. C-terminal analyses of chymotryptic peptides were carried out by the carboxylpeptidase method. ChT-1 (0.5 nmol) was digested with CPase Y (Oriental Yeast) in 20 µl of 0.1 M pyridine-acetate (pH 5.5) and ChT-2 (0.5 nmol) was treated with CPase A (Sigma) in 20 µl of 0.2 M N-ethylmorpholine-acetate (pH 8.0). The released amino acids were analyzed by an amino acid analyzer. An amino acid amide in neuromedin B was determined by the method of Tatemoto and Mutt (11). Methionine amide generated upon chymotryptic digestion was identified as its dansyl derivative.

Synthesis of neuromedin B: The decapeptide amide according to the sequence determined for neuromedin B 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

The SP-III concentrate obtained from porcine spinal cord in our preceding isolation of neuromedin K was the starting material for the present purification (6). Acid extracts (40 L) of isolated spinal cords (ca. 20 kg) collected from 550 pigs were desalted by ultrafiltration on an Amicon UM-2 membrane. The concentrated extract (3.3 L) thus obtained was subjected to acetone precipitation at a concentration of 75% and the resulting precipitate was removed by centrifugation. After evaporation of the supernatant, the residual material was dissolved in 1M CH_3COOH (2 L), adsorbed on SP-Sephadex C-25 (H^+-form) and then eluted with 1M CH_3COOH, 2M pyridine and 2M pyridine-acetate (pH 5.0), successively. The SP-III fractions eluted with 2M pyridine-acetate were pooled. Lyophilization afforded a dry concentrate, SP-III (ca. 10.5 g). The SP-III concentrate contained neuromedin B and other basic peptides including substance P, but no peptides of less basicity such as oxytocin and neuromedin K.

The first gel-filtration of SP-III was performed on a Sephadex G-50 (fine) column and eluted with 1M CH_3COOH. Aliquots of fractions were assayed for contractile activity of smooth muscle preparations utilizing guinea-pig ileum and rat uterus. Smooth muscle stimulant activities in both assays were observed in the region of molecular weight corresponding to 800-2500 daltons. Bioactive fractions thus obtained were pooled and further subjected to a second gel-filtration with Sephadex G-25 (fine). An appreciable uterus activity distributed in a wide range with fractions A-F, as seen in Fig.1, while a major peak of ileum activity emerged at the position between fraction C and D. This peak of ileum activity was overlapped with substance P immunoreactivity (data not shown). The present purification is concerned with fraction F, which exhibits a potent stimulant effect on rat uterus but a weak effect on guinea-pig ileum.

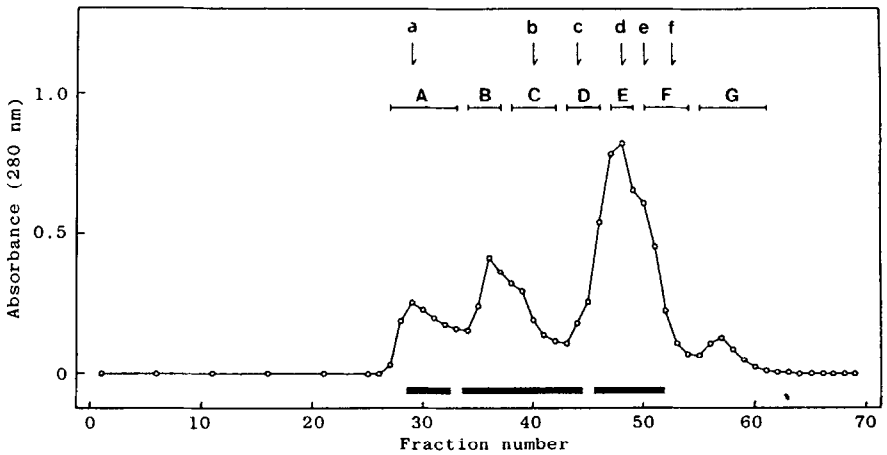

Fig. 1 Sephadex G-25 gel-filtration of the basic peptide fraction obtained from acid extracts of porcine spinal cord.
Sample : Ileum and uterus contractile fractions purified from SP-III by Sephadex G-50 gel-filtration. Fraction size : 100 ml/tube.
Column : Sephadex G-25 fine, 7.5 x 135 cm. Eluent : 1M CH_3COOH.
Uterus contractile activity was observed at the black bar regions.
Arrows indicate elution positions of a) bovine serum albumin, b) substance P sulfoxide, c) substance P, d) α-neo-endorphin, e) somatostatin and f) NaCl, respectively.

The peptides in fraction F were adsorbed on a reverse phase column of Nucleosil 30 C-18 in the presence of 0.5M CH_3COOH then eluted with 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 seen in Fig.2, a major peak of uterus activity was eluted at 55-58 min. The main peak of ileum activity was separated in the fraction eluted at 81-83 min (data not shown). Final purification of the major peak of uterus activity thus obtained was achieved by reverse phase HPLC on a column of Chemcosorb 5ODS-H. Thus, neuromedin B eliciting uterus activity was isolated in a single peak, as shown in Fig.3. The purity of the peptide was confirmed by a different HPLC, as shown in Fig.4A. The amino acid composition of neuromedin B was determined after acid hydrolysis to be: Asp 1.05, Thr 1.00, Gly 2.14, Ala 1.08, Met 0.91, Leu 1.07, Phe 1.14, His 0.96, Trp 0.75, indicating its decapeptide structure. Amino acid analysis by the pre-labelling method after exhaustive digestion of the peptide verified that neuromedin B contains no Asp but only one Asn in the molecule. Based on the amino acid analysis, it was estimated that 18 nmoles of neuromedin B were purified from 550 pigs. Due to the very limited amount of the peptide, sequence analyses were performed on a subnanomole scale. The N-terminal of neuromedin B was identified as Gly by the dansyl method.

Chymotryptic digestion of neuromedin B, followed by reverse phase HPLC afforded two fragment peptides, ChT-1 and ChT-2, as shown in Fig.4B. Amino

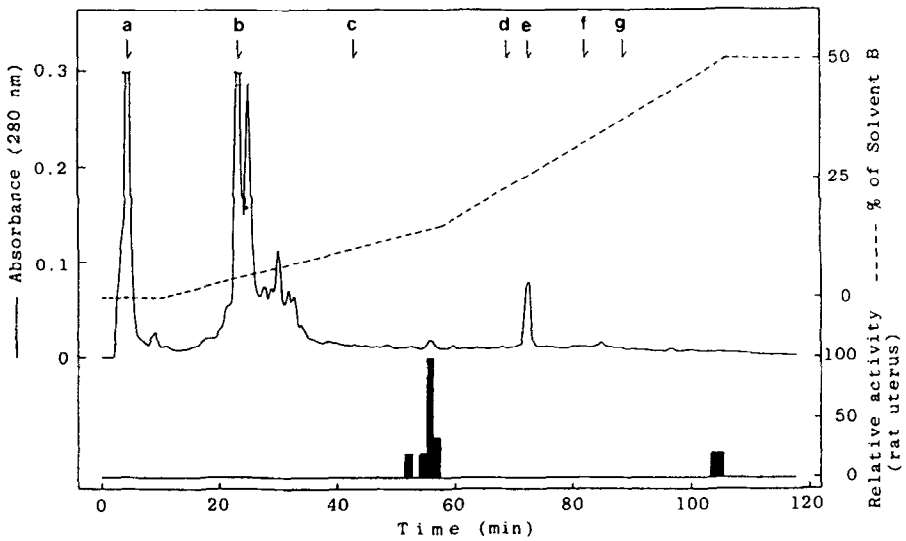

Fig. 2 Ion exchange HPLC of fraction F.
Sample : One-third of fraction F that was treated batch-wise with C-18 resin.
Column : TSK IEX-530 CM, 4.0 x 300 mm (Toyosoda). Flow rate : 1.0 ml/min.
Solvent system : (A) 10 mM $HCOONH_4$ (pH 6.5) : CH_3CN = 90 : 10 (v/v)
(B) 1.0 M $HCOONH_4$ (pH 6.5) : CH_3CN = 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 of rat uterus is also shown in the figure.
Arrows indicate elution times of a) Leu-enkephalin, b) [Arg^6]-Leu-enkephalin, c) LH-RH, d) PH-8P, e) somatostatin, f) substance P and g) α-neo-endorphin, respectively.

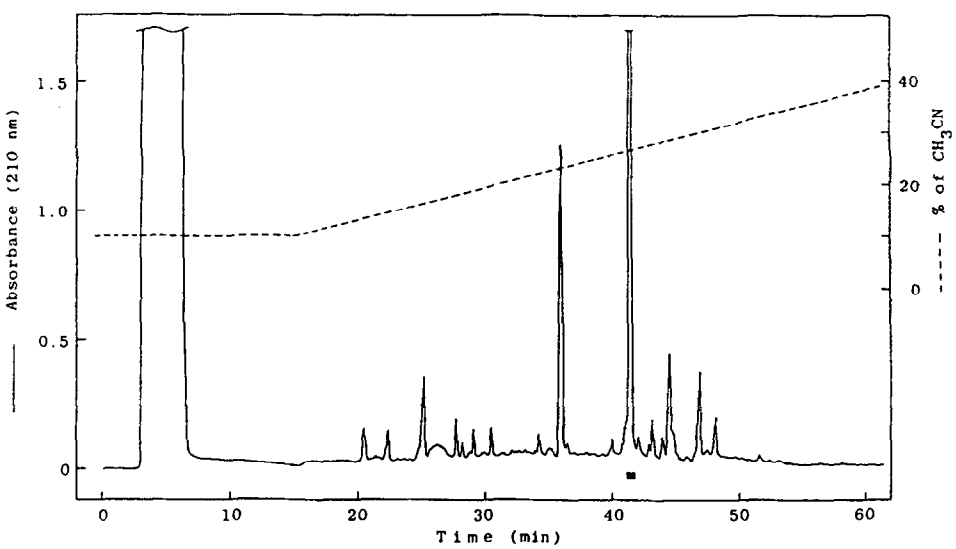

Fig. 3 Final purification of the bioactive fraction by HPLC.
Sample : The rat uterus contractile fraction eluted at 55-58 min on ion exchange HPLC (Fig. 2). Flow rate : 1.0 ml/min.
Column : Chemcosorb 5ODS-H, 4.6 x 250 mm (Chemco).
Solvent system : A linear gradient elution from (A) to (B) (80 min).
(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)

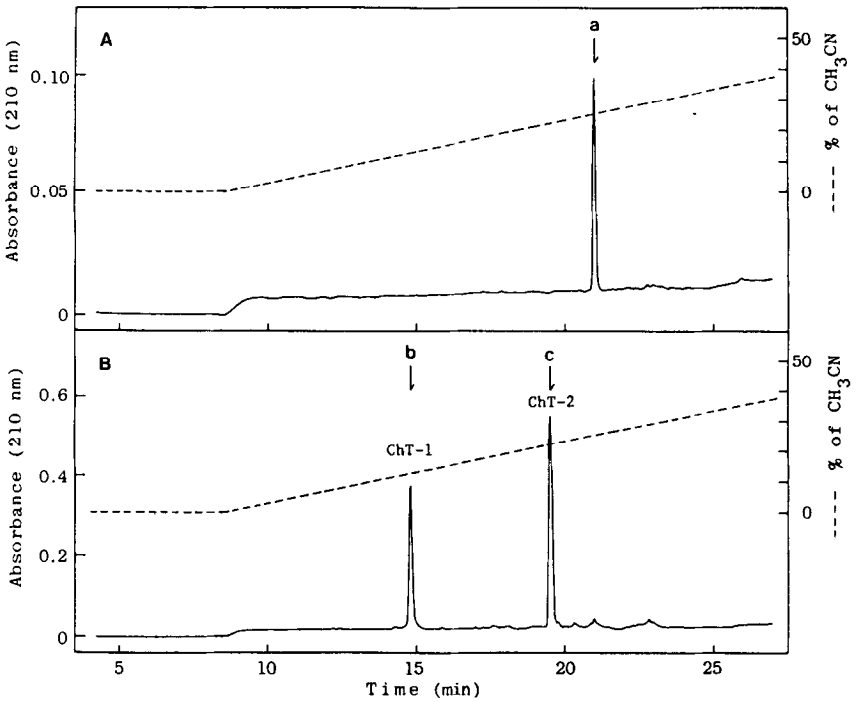

Fig. 4 (A) Reverse phase HPLC of natural neuromedin B
Sample : Neuromedin B (400 ng). Flow rate : 1.0 ml/min.
Column : Chemcosorb 30DS-H, 4.6 x 75 mm (Chemco).
Solvent system : Linear gradient elution from (I) to (II) (30 min).
(I) H_2O : CH_3CN : 10% TFA = 100 : 0 : 1.0 (v/v)
(II) H_2O : CH_3CN : 10% TFA = 40 : 60 : 1.0 (v/v)
(B) Reverse phase HPLC of chymotryptic digests of neuromedin B.
Sample : Chymotryptic digests of neuromedin B (5 nmol).
Chromatographic conditions were exactly the same as in Fig. 4A.
Arrows indicate elution times of a) synthetic neuromedin B.
b) Ala-Thr-Gly-His-Phe and c) Gly-Asn-Leu-Trp, respectively.

acid analyses indicated that ChT-1 was a pentapeptide composed of Thr 0.98, Gly 0.99, Ala 1.01, Phe 1.05, His 0.96; and that ChT-2 was a tetrapeptide composed of Asp 1.05, Gly 0.98, Leu 0.99, Trp 0.79. By analyses performed by the dansyl-Edman method and the carboxypeptidase method, the sequences of ChT-1 and ChT-2 were determined to be Ala-Thr-Gly-His-Phe and Gly-Asn-Leu-Trp, respectively, as shown in Fig.5. The only residue which could not be detected

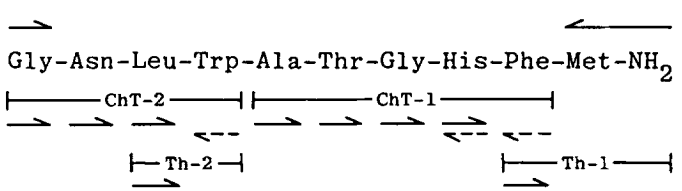

Fig. 5 Complete amino acid sequence of neuromedin B
(⟶) : By the dansyl-Edman method. (⟵) : By the carboxypeptidase method. (⟵) : By the method of Tatemoto and Mutt.
ChT-1, ChT-2 : Chymotryptic peptides. Th-1, Th-2 : Thermolytic peptides.

in the chymotryptic peptides was Met, suggesting that Met should be a C-terminal residue of neuromedin B. 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). Additionally, two peptides (Th-1 and Th-2) in the thermolytic digests of neuromedin B were isolated by reverse phase HPLC. Amino acid compositions were determined to be Met 0.97, Phe 1.03 for Th-1 and Leu 1.00, Trp 0.75 for Th-2. The N-terminal residues of Th-1 and Th-2 were identified as Phe and Leu, respectively. Thus, the sequences of Th-1 and Th-2 were deduced to be Phe-Met-NH_2 and Leu-Trp, respectively. The formation of Th-1 confirmed the C-terminal sequence of neuromedin B.

The complete structure of neuromedin B was thus determined to be Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2, as shown in Fig.5.

Further structual confirmation was provided by synthesizing the decapeptide amide according to the sequence of neuromedin B determined above. Amino acid analysis and sequencing confirmed the correct synthesis of the peptide. Both native and synthetic peptide migrated together on reverse phase HPLC (Fig.4A) as well as on ion exchange HPLC under the same conditions described in the legend for Fig.2 (data not shown). It was also evidenced by reverse phase HPLC that native neuromedin B underwent chymotryptic digestion in exactly the same manner as the synthetic specimen (Fig.4B). Thus, the complete amino acid sequence of neuromedin B was unambiguously established to be Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2.

As clearly seen in Fig. 6, a surprising sequence homology is observed between neuromedin B and the known members of the bombesin family (1,2). The entire sequence of neuromedin B is conserved in that of the C-terminal decapeptide of bombesin, except that Leu^3, Thr^6 and Phe^9 of neuromedin B are replaced by Gln, Val and Leu in bombesin, respectively. Among these three differences mentioned above, two replacements of Val → Thr and Leu → Phe at the positions 6 and 9 in neuromedin B also occur in the same manner even

	Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2	Neuromedin B
---Ala-Lys-Met-Tyr-Pro-Arg-	Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	G R P
pGlu-Gln-Arg-Leu-	Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	Bombesin
pGlu-Gly-Arg-Leu-Gly-Thr-Gln-	Trp-Ala-Val-Gly-His-Leu-Met-NH_2	Alytesin
pGlu-Gln-	Trp-Ala-Val-Gly-His-Phe-Met-NH_2	Litorin
pGlu-Val-Pro-Gln-	Trp-Ala-Val-Gly-His-Phe-Met-NH_2	Ranatensin
Ser-Asn-Thr-Ala-Leu-Arg-Arg-Tyr-Asn-Gln-	Trp-Ala-Thr-Gly-His-Phe-Met-NH_2	Ranatensin-R
X-Glx-Thr-Pro-Gln-	Trp-Ala-Thr-Gly-His-Phe-Met-NH_2	Ranatensin-C

Fig. 6 Amino acid sequences of bombesin-like peptides

within the bombesin family, as shown in the case of ranatensin-R or -C vs. bombesin. In fact, the C-terminal heptapeptide of neuromedin B is exactly identical with those of ranatensin-R and -C. The deviation at position 3, being a leucine for a glutamine residue of bombesin, is compatible with one base difference in the genetic code. The striking homology mentioned above suggests that neuromedin B could be expected to elicit bioactivity similar to that of bombesin. In our preliminary experiments, neuromedin B elicits behavior quite similar to that of bombesin in the contractile reaction of rat uterus and guinea-pig ileum, distinct from that of the tachykinins.

Recent studies on the structure-activity relationship of bombesin show that the C-terminal nonapeptide of bombesin, and even more so the decapeptide, possesses the full spectrum of bombesin activity, even from a quantitative point of view (1,2). Accordingly, the structure of neuromedin B seems to be the minimum molecule for eliciting the full spectrum of bombesin-like activity. GRP consisting of 27 amino acid residues, the only peptide belonging to the bombesin family hitherto identified in mammals (5), shares the C-terminal decapeptide with amphibian bombesin, with a single difference at position 8 from the C-terminus, where it has a histidine for a glutamine residue. The existence of neuromedin B suggests the possibility that GRP may be not the mature hormone, but rather a large precursor from which the C-terminal decapeptide corresponding to neuromedin B is split off as its mature form.

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