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NEUROMEDIN K: A NOVEL MAMMALIAN TACHYKININ IDENTIFIED IN PORCINE SPINAL CORD

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SUMMARY: A new peptide, designated "neuromedin K" has been discovered and isolated from porcine spinal cord by using bioassays for a tachykinin-like effect on the contractility of smooth muscle preparation from guinea-pig ileum. Porcine neuromedin K has been identified by microsequencing as: Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2. The sequence thus determined has been confirmed by synthesis. Neuromedin K has been found to have not only a remarkable sequence homology to kassinin and substance P, but also a prompt stimulant activity on guinea-pig ileum in a manner similar to that of substance P, suggesting that neuromedin K may be involved in neural transmission.

The rapidly growing knowledge of neuropeptides has revealed the existence of a complex network of neural communications. In order to acquire further information about the mechanism of neurotransmission and neuromodulation, it is urgent that still unidentified neuropeptides be discovered. However, difficulties in obtaining the special tools necessary for finding undiscovered species in tissue extracts have been a major obstacle. Neuropeptides so far identified elicit a variety of biological or pharmacological actions according to the assay systems used. For instance, substance P, which is a neurotransmitter involved in the transfer of pain information, exhibits a wide spectrum of diverse activities such as smooth muscle stimulation, hypotensive, sialogogic reactions, etc. (1). These facts suggest that sensitive assay systems for effects on muscle contraction or blood pressure, even though they are not specific, would be well suited for detecting unknown neuropeptides. In a series of previous experiments isolating opioid peptides (2,3,4), we noted the presence of potent stimulant activity on guinea-pig ileum in the side-fractions of opioid peptides. Thus, in the present survey for novel neuropeptides in porcine spinal cord, we have used assays for a stimulant effect on the contractility of smooth muscle preparations from guinea-pig ileum and rat uterus. We have found a new peptide that is distinct from substance P but elicits a prominent stimulant activity of the ileum preparation in a manner similar to substance P. By structural analysis, the peptide is found to have a remarkable homology to amphibian kassinin. Therefore, we have designated it "neuromedin K". This paper describes the isolation and complete amino acid sequence of neuromedin K.

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METHODS AND MATERIALS

Isolation: Spinal cords were isolated from pigs within 1 hr of killing. The minced tissue was boiled for 12 min in two volumes of 1M AcOH containing 20mM HCl to inactivate intrinsic proteases. After cooling, extractions were performed at 4°C by homogenizing with a polytron. The supernatant of the extracts, obtained after 30-min' centrifugation at 12,000 x g, was desalted through an Amicon UM-2 membrane. The concentrate thus obtained was then subjected to acetone-precipitation at a concentration of 75%. After removal of the precipitates, the supernatant was evaporated in vacuo to dryness. The residual materials were dissolved in 1M AcOH and then adsorbed on a column (8.0 x 22 cm) of SP-Sephadex C-25 (H^+-form), preequilibrated with 1M AcOH. Successive elutions with 1M AcOH, 2M pyridine and then 2M pyridine-AcOH (pH 5.0) afforded three respective fractions of SP-I, SP-II and SP-III. The dry concentrate (SP-II) obtained by lyophilization of the above fraction SP-II was used as the starting material for the present purification. Gel-filtrations of SP-II were performed on a column (4.5 x 140 cm) of Sephadex G-50 (fine) and on a column (4.5 x 140 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 stimulant activity by using smooth muscle preparations isolated from guinea-pig ileum and rat uterus. Another aliquot of each fraction was subjected to radioimmunoassay for oxytocin. Fractions eliciting the ileum activity were diluted to a concentration of 0.5M AcOH by adding an equal volume of water. Peptides in the solution thus obtained were adsorbed on a reverse phase preparative column (8.0 x 500 mm) of Nucleosil 30 C-18 (Nagel), washed with 0.5M AcOH and eluted with a solution of H_2O:CH_3CN:10%TFA (40:60:1, v/v). Every 1/4 portion of the bioactive fraction thus obtained was further rechromatographed on the same column as above under the conditions described in the legend of Fig.2. Final purification of neuromedin K was performed on a μ-Bondapak C-18 column (3.9 x 300 mm) and its purity was checked by HPLC on a reverse phase column (4.6 x 75 mm)(Waters) of Chemcosorb 3 ODS-H (Chemco). Column effluents on HPLC were monitored by measuring absorbance at 210 nm and 280 nm, simultaneously.

Bioassay: The effect of the sample on the contractility of freshly isolated preparations of rat uterus and guinea-pig ileum was examined according to the described method (1,5). Uterine contractility was examined in modified Locke-Ringer's solution, while the ileum was bathed in Tyrode's solution.

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 3N mercaptoethanesulfonic acid at 110°C for 20 hr. Analyses of amino acids released after enzymatic digestion were carried out on a picomole level with a pre-labeling analysis system (Waters). Released amino acids were subjected to fluorescence-labeling prior to the 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 (1.5 nmole) was carried out with 0.5 μg of the enzyme (Sigma) in 10 μl of 1% ammonium bicarbonate (pH 8.0) at 37°C for 3 hr. Thermolytic digestion of the peptide (1.5 nmole) was performed with 2 μg of the enzyme (Sigma) in 5 μl of a 0.2M N-ethylmorpholine buffer (pH 8.0) containing 10mM $CaCl_2$ at 37°C for 4 hr. Cyanogen bromide cleavage of the peptide (1.0 nmole) was carried out by the described method (6). The resulting homoserinelactone at the C-terminus was completely converted to the corresponding homoserine by incubating at 40°C for 24 hr in a solution of 1% ammonium bicarbonate (pH 8.0) and then subjected to HPLC. Amino terminal analyses of the native peptide and its N-terminal fragment peptides (100-200 pmole) were carried out by digestion with aminopeptidase M (Sigma : 50-200 ng) in a 0.2 M N-ethylmorpholine buffer (pH 8.0) at 37°C, followed by analyzing the released residue at appropriate time intervals with the pre-labeling

method. Otherwise, analyses were done by the dansylation method (7). The C-terminal amide in neuromedin K was determined by the method of Tatemoto and Mutt (8); methionine amide generated upon carboxypeptidase Y digestion was identified as its dansyl derivative. The dansyl amino acid and amide were identified on a polyamide sheet (Cheng Ching).

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

RESULTS AND DISCUSSION

Isolation: Acid extracts (ca. 40 L) of spinal cords (ca. 20 kg) isolated from 550 pigs were desalted by ultrafiltration on an Amicon UM-2 membrane. The resulting concentrate (ca. 3.3 L) was subjected to acetone-precipitation at a concentration of 75%, and then the precipitate was removed by centrifugation. Peptides in the resulting supernatant were adsorbed on SP-Sephadex (H^+-form) in the presence of 1M AcOH. Successive elutions with 1M AcOH, 2M pyridine and 2M pyridine-AcOH (pH 5.0) afforded three respective fractions, SP-I, SP-II and SP-III. Fraction SP-II that was eluted with 2M pyridine contained neuromedin K along with other slightly basic peptides including oxytocin, while fraction SP-III that was eluted with 2M pyridine-AcOH contained more basic peptides such as substance P. Lyophilization of fraction SP-II gave a dry concentrate of ca. 1.5 g that was used as the starting material for this purification. By the first gel-filtration on Sephadex G-50, remarkable stimulant activities on contractility of smooth muscle in both assay systems using guinea-pig ileum and rat uterus, were found in fractions corresponding to the range of Mr 800-2000 daltons. The bioactive fractions (Ve/Vo=1.83-2.13) thus obtained were further subjected to the second gel-filtration on Sephadex G-25. As shown in Fig.1, ileum activity was separated in fractions #75-92 from oxytocin immunoreactivity (#93-98), corresponding to the bulk of uterus activity. Peptides in fractions #87-92 corresponding to the main peak of ileum activity were adsorbed on a preparative column of Nucleosil 30 C-18 in the presence of 0.5M AcOH, washed thoroughly with 0.5M AcOH and then eluted with a solution of H_2O:CH_3CN:10%TFA=40:60:1 (v/v). The eluate thus obtained was subjected to preparative HPLC on the same column as above with a linear gradient elution as mentioned in the legend for Fig.2. As shown in Fig.2, efficient purification of the ileum activity was achieved at this step. The main peak of the activity emerged at the retention time of 92-94 min and was well separated from a large amount of inactive materials. Final purification by reverse phase HPLC on a μ-Bondapak column afforded pure neuromedin K with a prominent ileum activity as a single peptide peak at 53.5 min, as shown in Fig.3. Purity of the peptide was also confirmed by a different HPLC, as seen in Fig.4A.

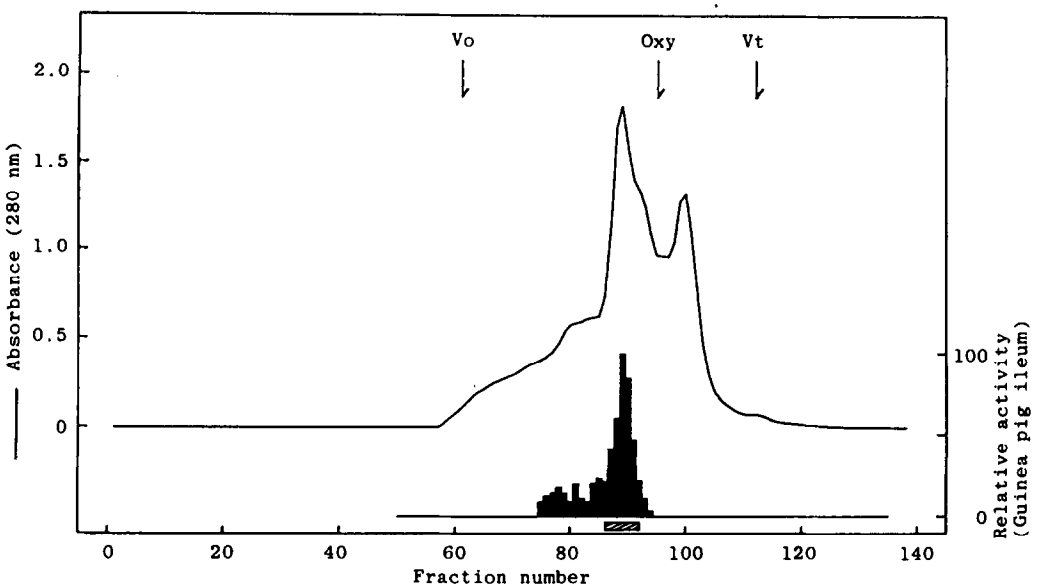

Fig. 1 Sephadex G-25 gel-filtration of fractions containing small peptides obtained from acid extracts of porcine spinal cord.
Sample : Fractions (#99-115, Ve/Vo=1.83-2.13) obtained by Sephadex G-50 gel-filtration of SP-II.
Column : Sephadex G-25 (fine), 4.5 x 140 cm.
Eluent : 1M AcOH. Fraction size : 20 ml/tube.
Guinea-pig ileum contractile activity is shown by the vertical black bar. Arrows indicate the elution positions of Vo) bovine serum albumin, Oxy) oxytocin and Vt) NaCl, respectively.

Structural Analysis: The amino acid composition of neuromedin K thus purified was determined after acid hydrolysis to be: Asp 1.99(2); Gly 1.15(1); Val 0.90(1); Met 1.73(2); Leu 1.00(1); Phe 2.05(2); His 0.95(1). Another amino acid analysis of the peptide by the pre-labeling method after exhaustive enzymatic digestion verified that neuromedin K contains no Asn residue, but two mole equivalents of Asp in the molecule. Based on the amino acid analysis, the yield of pure neuromedin K was estimated to be about 10 nmoles starting from 550 pigs. Thus, neuromedin K was proved to be a decapeptide consisting of Asp(2), Gly(1), Val(1), Met(2), Leu(1), Phe(2) and His(1). For the N-terminal analysis, neuromedin K (0.2 nmole) was digested with aminopeptidase M (0.1 μg) and the amino acid residues released at appropriate time intervals were analyzed by the pre-labeling method. It was observed that the first release of Asp was followed by the second release of Met. Thus, the N-terminal sequence of neuromedin K was deduced as NH_2-Asp-Met-. The presence of C-terminal Met-amide was verified by generation upon digestion with charboxypeptidase Y, followed by dansylation. Treatment of neuromedin K (1.0 nmole) with cyanogen bromide, followed by HPLC gave a fragment peptide (BrCN-1), as shown in Fig.4B. The N-terminal His residue of BrCN-1 was determined by dansylation. Its amino acid composition was also determined to

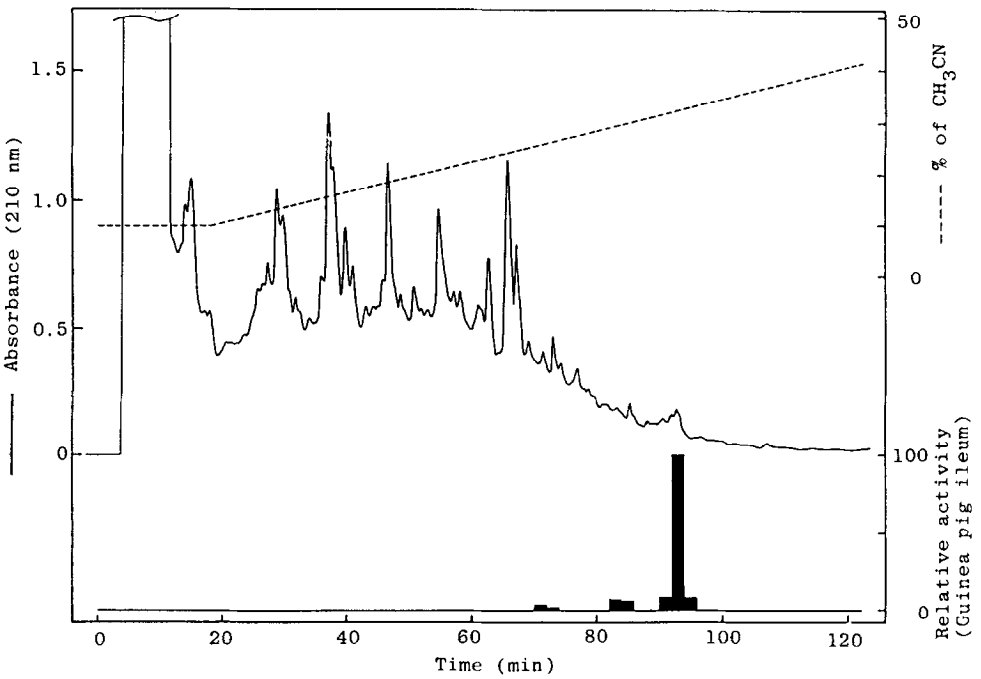

Fig. 2 Reverse phase HPLC of the fraction with guinea-pig ileum contractile activity obtained by Sephadex G-25 gel-filtration.
Sample : One-fourth of the bioactive fractions (#87-92 in Fig.1), after batch-wise treatment with C-18 resin.
Column : Nucleosil 30 C-18, 8.0 x 500 mm (Nagel). Flow rate : 4.0 ml/min.
Solvent system : Linear gradient elution from (a) to (b) (160 min).
(a) H_2O : CH_3CN : 10% TFA = 90 : 10 : 1 (v/v)
(b) H_2O : CH_3CN : 10% TFA = 40 : 60 : 1 (v/v)

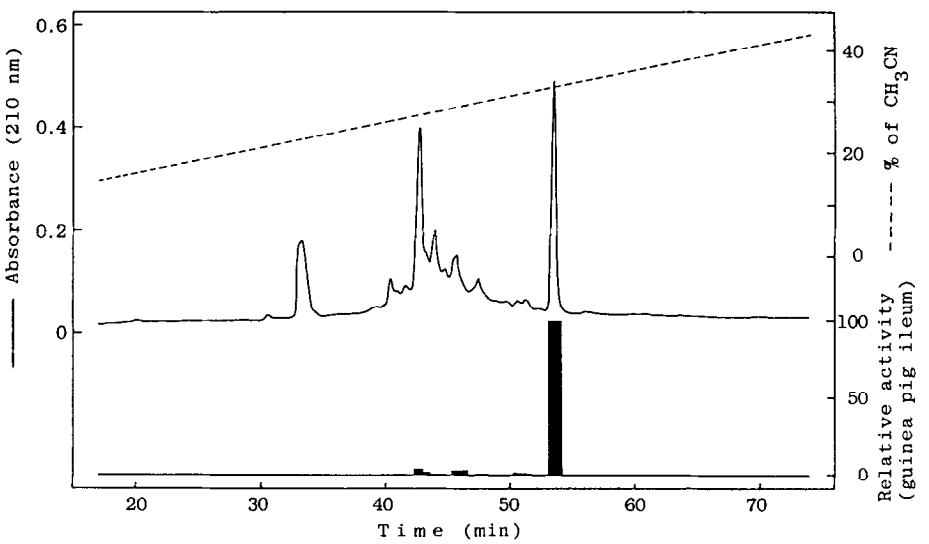

Fig. 3 Final purification of neuromedin K by HPLC.
Sample : Fraction with ileum activity eluted at 92-94 min on Nucleosil 30 C-18 (Fig.2).
Column : μ-Bondapak C-18, 3.9 x 300 mm (Waters). Flow rate : 2.0 ml/min.
Solvent system : Linear gradient elution from (a) to (b) (80 min).
(a) H_2O : 100 mM $HCOONH_4$ (pH 4.0) : CH_3CN = 79 : 11 : 10 (v/v)
(b) H_2O : 100 mM $HCOONH_4$ (pH 4.0) : CH_3CN = 40 : 10 : 50 (v/v)

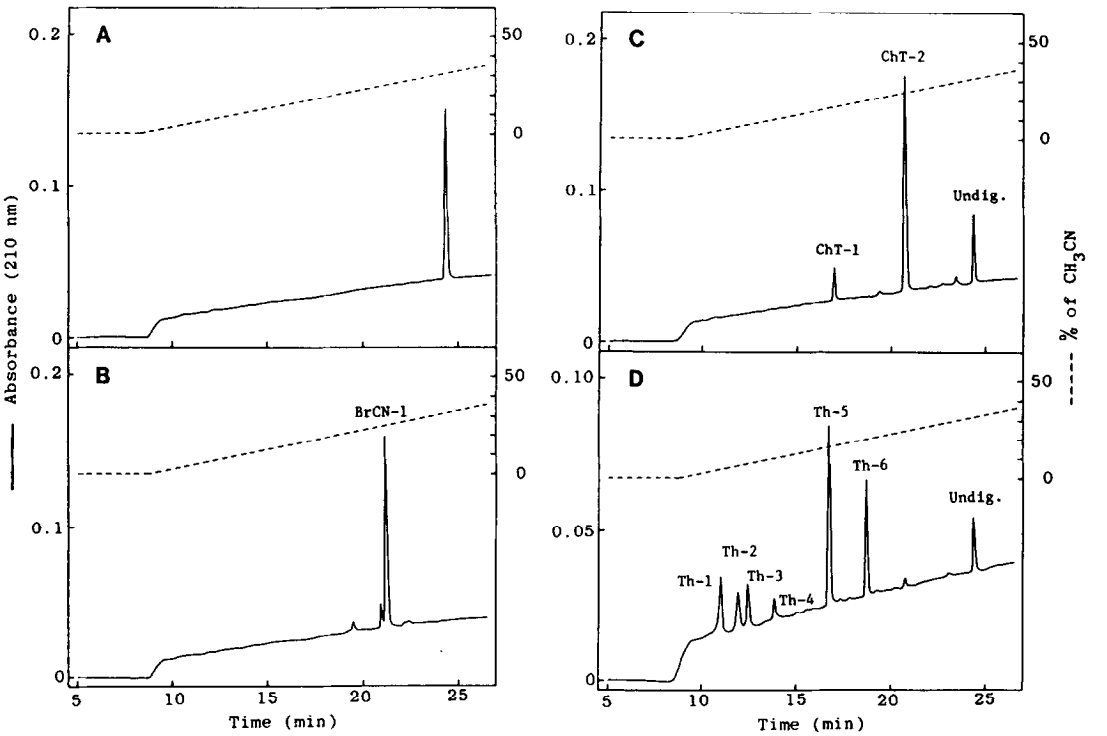

Fig. 4 Reverse phase HPLC of purified native neuromedin K, and its fragments obtained by BrCN cleavage, chymotryptic and thermolytic digestions.
Sample : (A) Purified native neuromedin K on μ-Bondapak C-18 (eluted at 53.5 min in Fig.3) (0.4 nmol)
(B) BrCN peptide of native neuromedin K (1.0 nmol)
(C) Chymotryptic digests of native neuromedin K (1.5 nmol)
(D) Thermolytic digests of native neuromedin K (1.5 nmol)
Column : Chemcosorb 3 ODS-H, 4.6 x 75 mm (Chemco). Flow rate : 1.0 ml/min.
Solvent system : Linear gradient elution from (a) to (b) (30 min).
(a) H_2O : CH_3CN : 10% TFA = 100 : 0 : 1 (v/v)
(b) H_2O : CH_3CN : 10% TFA = 40 : 60 : 1 (v/v)
Undig. represents undigested neuromedin K.

be: Asp(1), Gly(1), Val(1), Met(1), Leu(1), Phe(2) and His(1), indicating that the N-terminal dipeptide Asp-Met was cleaved off from neuromedin K. Thus, BrCN-1 was proved to be His-(Asp, Gly, Val, Leu, Phe_2)-Met-NH_2, which corresponded to the C-terminal fragment of neuromedin K. Although Asp-HSer corresponding to the N-terminal dipeptide could not be isolated by HPLC, the N-terminal sequence of Asp-Met-His in neuromedin K was confirmed. Chymotryptic digestion afforded two fragments (ChT-1 and -2), which were separated by HPLC (Fig.4C). The N-terminal residues of ChT-1 and ChT-2 were identified as Val by the dansyl method and Asp by the aminopeptidase method, respectively. Based on their amino acid compositions, the ChT-1 and ChT-2 fragments were found to be: Val-(Gly,Leu)-Met-NH_2 and Asp-Met-His-(Asp, Phe_2), respectively. Further structural information was provided by thermolysin digestion of neuromedin K, which gave six fragments (Th-1-Th-6) along with the undigested

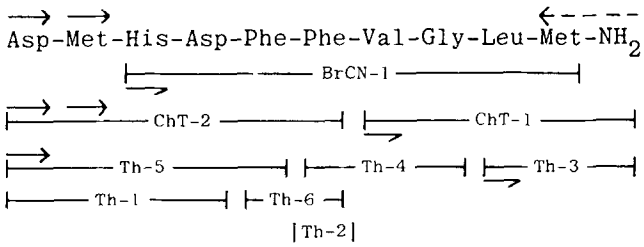

Fig. 5 Complete amino acid sequence of neuromedin K.
(⟶) : By the aminopeptidase method. (⟶) : By the dansyl method.
(⟵) : By the method of Tatemoto and Mutt.
BrCN : BrCN peptide. ChT : Chymotryptic peptides. Th : Thermolytic peptides.

peptide, as shown in Fig.4D. In a manner similar to that above, thermolytic fragments were found to be as follows: Th-1: Asp-Met-His-Asp, Th-2: free Phe, Th-3: Leu-Met-NH_2, Th-4: Phe-Val-Gly, Th-5: Asp-Met-His-Asp-Phe and Th-6: Phe-Phe. As summarized in Fig.5, the complete amino acid sequence of neuromedin K thus ascertained, is Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2. For structural confirmation, the decapeptide amide, according to the sequence determined above, was synthesized by solid phase techniques. Its amino acid analysis and sequencing confirmed the correct synthesis of neuromedin K. Native and synthetic neuromedin K migrate together on different HPLC systems under the conditions described in the legends of Figs.3 and 4A. There is also evidence that native neuromedin K undergoes chymotryptic as well as thermolytic digestion in exactly the same manner as the synthetic specimen. Thus, the complete sequence of neuromedin K is established without any ambiguity. As clearly seen in Fig.6, neuromedin K is found to have a remarkable sequence homology to substance P and the other known members of the tachykinin family, which in common have a similar C-terminal sequence represented as Phe-X-Gly-Leu-Met-NH_2 (X: hydrophobic or aromatic amino acid residue)(9). Especially, the C-terminal pentapeptide amide structure of

Molluscans:		
pGlu-Pro-Ser-Lys-Asp-Ala	Phe-Ile-Gly-Leu-Met-NH_2	Eledoisin
Amphibians:		
pGlu-Ala-Asp-Pro-Asn-Lys	Phe-Tyr-Gly-Leu-Met-NH_2	Physalaemin
pGlu-Pro-Asp-Pro-Asn-Ala	Phe-Tyr-Gly-Leu-Met-NH_2	Uperolein
pGlu-Asn-Pro-Asn-Arg	Phe-Ile-Gly-Leu-Met-NH_2	Phyllomedusin
Asp-Val-Pro-Lys-Ser-Asp-Gln	Phe-Val-Gly-Leu-Met-NH_2	Kassinin
Mammalians:		
Arg-Pro-Lys-Pro-Gln-Gln	Phe-Phe-Gly-Leu-Met-NH_2	Substance P
Asp-Met-His-Asp-Phe	Phe-Val-Gly-Leu-Met-NH_2	Neuromedin K

Fig. 6 Amino acid sequences of the tachykinin family.

kassinin is exactly conserved in neuromedin K. Such a structural resemblance indicates that neuromedin K is a member of the tachykinin family. As listed in Fig.6, a variety of tachykinins have been identified in molluscans and amphibians. To date, substance P has been regarded as the only peptide belonging to the tachykinin family identified in mammalians, although immunoreactivity resembling those of amphibian tachykinins has been demonstrated in mammalians (10). Therefore, neuromedin K is a new member of the tachykinin family identified in mammals. In addition, it should be mentioned that the dipeptide sequence of Phe-Phe observed in substance P also occurs at the 6th and 7th positions in neuromedin K.

Biological Activity: Tachykinins including substance P have been known to generally share a common spectrum of biological activities, such as a quick stimulant action on smooth muscle and a prompt hypotensive effect, although there are quantitative differences between them (9,11). It has been found in our preliminary assay that neuromedin K possesses a remarkable stimulant effect on guinea-pig ileum with a potency and pattern similar to substance P, while neuromedin K and substance P both exhibit a weaker effect on rat uterus. An appreciable hypotensive effect of neuromedin K, although weaker than substance P, has also been found in the bioassay utilizing rabbit. The detailed features of biological actions of neuromedin K must await further examination, but it is obvious that the peptide elicits typical tachykinin-like activity. Substance P is now considered to be a neurotransmitter involved in the pain transfer mechanism in mammalians. Occurrence in mammalians of neuromedin K resembling substance P in structure and activity, strongly suggests a possibility that neuromedin K may also participate in the neural network in mammalians. Attempts to differentiate the distribution and biological activities of neuromedin K from those of substance P are now going on.

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