Isolation and characterization of cholecystokinin-58 (CCK-58) from porcine brain

Kazuhiko Tatemoto, Hans Jörnvall+, Sirkka Siimesmaa, Gunnel Halldén and Viktor Mutt

Departments of Biochemistry II and +Chemistry I, Karolinska Institute, S-104 01 Stockholm, Sweden

Received 28 June 1984

A 58-residue peptide has been isolated from extracts of porcine brain and shown to be an N-terminally extended cholecystokinin (CCK). The amino acid sequence of this peptide is: Ala-Val-Gln-Lys-Val-Asp-Gly-Glu-Ser-Arg-Ala-His-Leu-Gly-Ala-Leu-Leu-Ala-Arg-Tyr-Ile-Gln-Gln-Ala-Arg-Lys-Ala-Pro-Ser-Gly-Arg-Val-Ser-Met-Ile-Lys-Asn-Leu-Gln-Ser-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr(SO_3)-Met-Gly-Trp-Met-Asp-Phe-NH_2. The peptide was found to induce contraction of the guinea-pig gallbladder, but the pattern of this action seemed to differ from those of CCK-8 and CCK-33.

Brain peptide *Gut peptide* *Brain CCK* *CCK-58* *Amino acid sequence* *C-terminal amide*

1. INTRODUCTION

Cholecystokinin (CCK), originally isolated in [1] from porcine intestine as a 33-residue peptide, has been shown to display various biological effects including stimulation of both pancreatic exocrine secretion and gallbladder contraction [2]. Later, its larger molecular forms, CCK-39 [3] and CCK-58 [4], were isolated from porcine and canine intestine, respectively. CCK-like immunoreactivity has been found to occur not only in the intestine but also in the brain [5,6]. Subsequently, an 8-residue peptide has been isolated from sheep brain and shown to be identical to the C-terminal octapeptide of CCK-33 [7]. We have reported that porcine brain contains substantial amounts of a CCK molecule with 58 amino acid residues [8,9]. This paper describes the isolation and complete amino acid sequence of this brain peptide.

Preliminary communications on this study have been published; Tatemoto, K., First International Meeting of Interdisciplinary Neuroendocrinology, June 1983, Graz, Austria; Tatemoto, K., Shizuoka Symposium on Brain-Gut Peptides, July 1983, Shizuoka, Japan

2. MATERIALS AND METHODS

Thermolysin was obtained from Daiwa Kasei K.K., Osaka, and trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated) from Worthington. CCK-58 was assayed by the biological method using contraction of the guinea-pig gallbladder in vivo [10] and by the chemical method based on the detection of C-terminal amide (phenylalanine amide) released by treatment of samples with thermolysin [11]. High-performance liquid chromatography (HPLC) was performed in a Waters instrument using reversed-phase μBondapak C_{18} columns under chromatographic conditions described in the legends to fig.1–3. N-terminal amino acids were determined by the dansyl method [12]. Amino acid analyses were preformed with a Beckman 121 M amino acid analyser after hydrolysis of samples in 5.7 M HCl containing 0.5% phenol at 110°C for 24 h. The tryptophan content was determined after hydrolysis in 3 M mercaptoethanesulfonic acid [13]. Step-wise sequencer degradation of the intact peptide was carried out in a Beckman 890 D liquid-phase sequencer in the presence of glycine-precycled polybrene [14]. Phenylthiohydantoin derivatives were identified by HPLC [15].

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

3. RESULTS

3.1. Isolation procedures

Porcine brain (1000 kg) was boiled (10 min), frozen, and extracted at 4–5°C with 0.5 M acetic acid (2000 l) overnight without stirring and then for 5–6 h with stirring. After filtration, the peptides in the extract were adsorbed onto alginic acid (30 kg wet wt) at pH 2.7, eluted with 0.2 M HCl, precipitated by NaCl at salt saturation and collected by filtration. The peptide precipitate (970 g wet wt), containing the CCK-like bioactivity (0.2 CCK units/mg), was dissolved in water (10 l) and peptides in the solution were re-precipitated by NaCl at pH 4.0 and collected by filtration. This second precipitate (800 g wet wt) was suspended in methanol (40 l) containing 0.05% mercaptoethanol and extracted for 10 min with vigorous stirring. After filtration, the methanol-insoluble material was dried under vacuum overnight. The dried material (254 g, 0.7 CCK units/mg) was dissolved in water (2.5 l) and the pH of the solution was adjusted to 7.2. After removal by centrifugation of the precipitate formed, 2 vols of ethanol were added to the supernatant and this ethanol precipitate was collected by suction filtration. This precipitate was suspended in water (5 l) and the pH of the suspension was slowly adjusted to 4.2 by addition of acetic acid under constant stirring. The insoluble material was removed by centrifugation and the supernatant was lyophilized. The lyophilized material (49.5 g, 2.0 CCK units/mg) was chromatographed on Sephadex G-25 (fine) in 0.2 M acetic acid. The fractions containing the CCK-like activity were pooled and lyophilized. These fractions (a total of 15.4 g) were further purified by ion exchange chromatography on CM–cellulose with a step-wise elution by 0.04, 0.1 and 0.2 M ammonium bicarbonate. The CCK fraction, eluted at 0.1 M ammonium bicarbonate, was lyophilized and the lyophilized material (554 mg) was chromatographed on Sephadex G-50 (fine) in

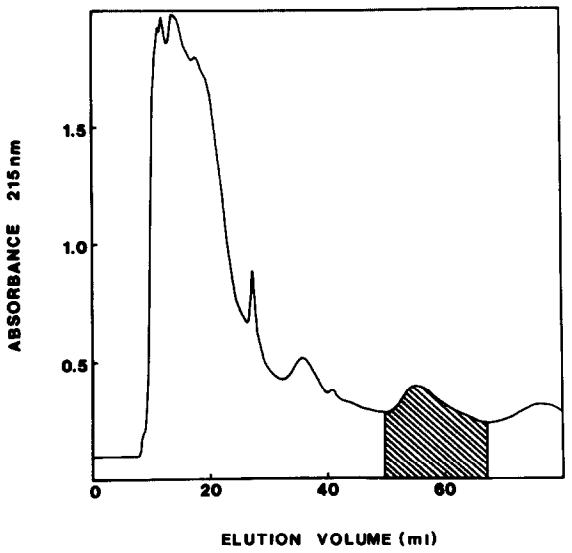

Fig.1. HPLC profile of the CCK fraction from CM–cellulose chromatography. An aliquot (10 mg) of the fraction (121 mg) was applied to a reversed-phase μBondapak C_{18} column (7.8 × 300 mm) and eluted at a flow rate of 2 ml/min with 40% ethanol containing 5 mM ammonium acetate and 0.2% acetic acid. The fraction containing CCK (hatched area) was detected by using both the biological and chemical assay methods.

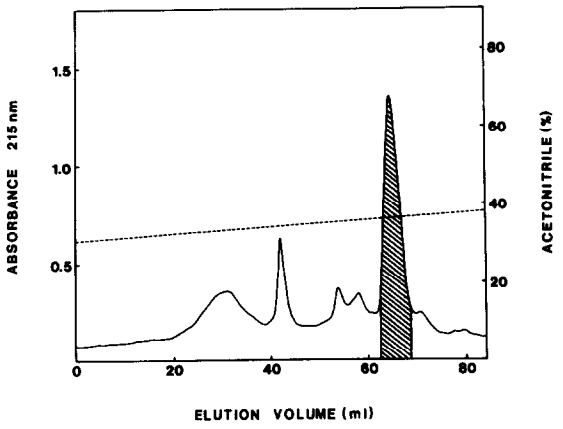

Fig.2. HPLC profile of the CCK fraction (fig.1). The CCK-containing fraction (a total of 300 ml) was concentrated under vacuum to half the volume to remove the ethanol. An aliquot (50 ml) of the solution (150 ml) was then applied to a reversed-phase μBondapak C_{18} column (7.8 × 300 mm) for the second time through the solvent delivery system at a flow rate of 2 ml/min. The HPLC column was then washed with 40 ml of 25% acetonitrile containing 0.1% trifluoroacetic acid at the same flow rate. The peptides, concentrated on the HPLC column, were eluted at a flow rate of 2 ml/min with a linear gradient system (90 min) from 25% to 40% acetonitrile containing 0.1% trifluoroacetic acid. The major peak (hatched area) contained CCK and this peak fraction was subjected to structural and biological studies after lyophilization.

0.2 M acetic acid containing 0.02% ethylmercaptan. The major peak in the chromatogram contained the CCK activity and this fraction was lyophilized. An aliquot (121 mg) of the lyophilized fraction (206 mg) was then subjected to successive HPLC on a reversed-phase μBondapak C_{18} column. Fig.1 illustrates a typical HPLC elution profile of the CCK fraction in 40% ethanol containing 5 mM ammonium acetate and 0.2% acetic acid under isocratic conditions. The CCK-containing fraction was detected by both the biological and the chemical methods, and was concentrated under vacuum to remove the ethanol. An aliquot (50 ml) of the concentrated solution (total, 150 ml) was applied to the HPLC column using the solvent delivery system and the HPLC column was washed with 25% acetonitrile containing 0.1% trifluoroacetic acid. The peptides were eluted from the column with increasing concentration of acetonitrile using a linear gradient system (fig.2). The major peak in the HPLC chromatogram contained a CCK-like peptide, measured by the assay methods, and the fraction containing this peak was lyophilized. It was noted that this CCK-like peptide was eluted in more hydrophobic HPLC solvent systems than that for CCK-8, CCK-33 or CCK-39. The lyophilized preparation (total, 1.0 mg) was found to be homogeneous and was subjected to structural analysis.

3.2. *Structural analysis*

The results of amino acid analysis indicated that the brain peptide consisted of 58 residues: Ala_6-Arg_6-Asx_6-Glx_5-Gly_4-His_2-Ile_3-Leu_5-Lys_3-Met_3-Phe_1-Pro_2-Ser_6-Trp_1-Tyr_2-Val_3, as reported [9]. The N-terminal amino acid of the peptide was found to be alanine. The results of C-terminal amide determination [11] indicated that the brain peptide, like other CCK molecules, has a C-terminal phenylalanine amide. Treatment of the peptide with trypsin yielded a total of nine fragments which were separated by HPLC, as shown in fig.3. When the elution volumes in the HPLC of these fragments were compared with those of tryptic fragments of CCK-39, it was found that 6 of the 9 fragments were eluted in identical positions to the corresponding fragments of CCK-39. Moreover, these 6 fragments were identical in amino acid composition to the corresponding fragments of CCK-39. These results indicate that the brain peptide may be an N-terminally extended form of CCK-39. The C-terminal tryptic fragment (T-9) of the brain peptide had an amino acid composition, terminal structure and biological activity identical to the octapeptide CCK-8. Edman degradation of the intact peptide in a Beckman 890 D liquid-phase sequencer yielded the amino acid sequence up to residue 57, establishing the brain peptide to be a CCK with 58 amino acid residues. The tyrosine *O*-sulfate residue at position 52 of the brain peptide was identified as a phenylthiohydantoin tyrosine in the sequencer analysis, probably due to loss of the sulfate group during the repetitive degradation procedures. However, since the C-terminal tryptic fragment of the brain CCK was eluted in the HPLC at an identical volume to CCK-8 and was highly biologically active, the residue at position 52 should be a tyrosine *O*-sulfate. The repetitive yield of the sequence analysis was 94–95%. From the results of amino acid analysis, C-terminal determination and sequence analysis, the complete amino acid sequence of brain CCK-58 was deduced to be as in fig.4.

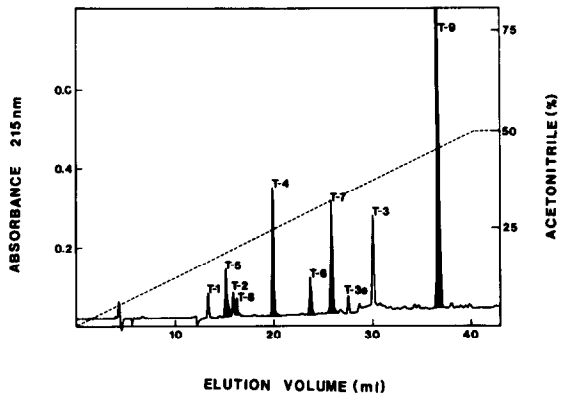

Fig.3. HPLC separation of tryptic fragments (T-1–T-9) of brain CCK-58. CCK-58 (60 μg) was dissolved in 30 μl of 1% NH_4HCO_3 and 1 μl trypsin solution (2 mg/ml) was added. After incubation at room temperature for 18 h, boiling for 6 min, and lyophilization, the tryptic peptides were separated on a reversed-phase HPLC column (μBondapak C_{18}, 3.9 × 300 mm, Waters) with a linear gradient of 0.12% trifluoroacetic acid/water and 0.1% trifluoroacetic acid/acetonitrile at a flow rate of 1 ml/min. The dashed line indicates the gradient profile. The tryptic fragments (T-4–T-9), shown in the black peaks, are identical to tryptic fragments of CCK-39.

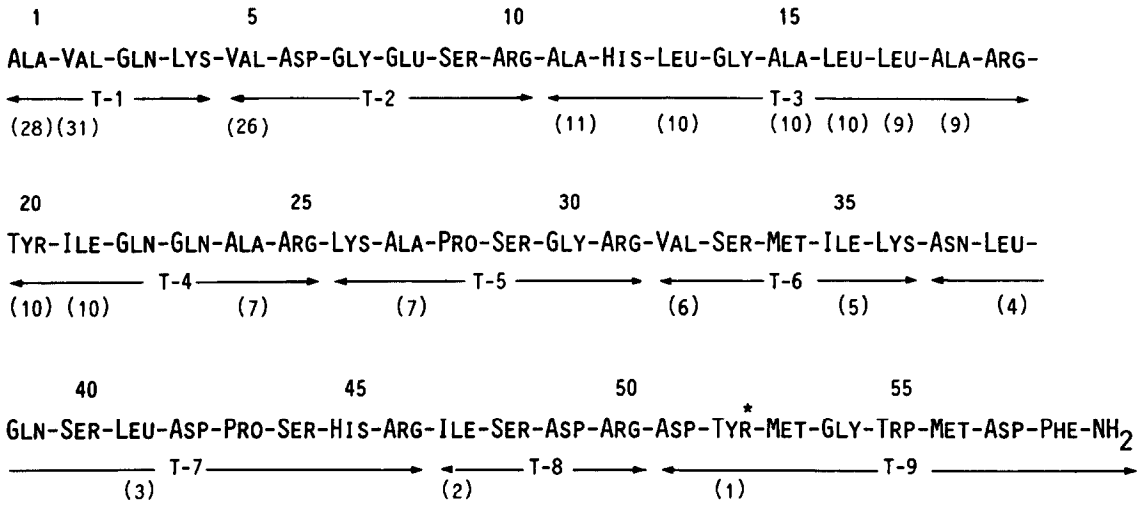

Fig.4. The complete amino acid sequence of CCK-58 (porcine brain). T-1 to T-9 are tryptic fragments. Figures in parentheses denote nmoles of stable phenylthiohydantoin amino acids recovered from sequencer degradation. Tyr* indicates tyrosine *O*-sulfate.

4. DISCUSSION

Fig.5 shows a comparison of the primary structures of porcine CCK-58 with other known CCK molecules. A high degree of sequence homology exists between porcine brain and canine intestinal CCK-58. However, there are differences, particularly at the N-terminal region (position 1–9). The structural differences may be due to species variations.

Recently, authors in [16] have isolated CCK-8 and N-terminal fragments of CCK-33, CCK-39 and CCK-58 from porcine brain. They reported that the fragments, CCK-33 desnonapeptide, CCK-39 desnonapeptide and desdecapeptide, and CCK-58 desnonapeptide, were about equally prominent, but, no intact molecule of CCK-33, CCK-39 or CCK-58 was detected in the brain [16]. The present study, however, clearly shows the occurrence of substantial amounts of intact CCK-58. The results of the biological and the chemical assays suggest that CCK-58, but not CCK-33 and CCK-39, is present as one of the major CCK forms in the brain. Here, the brains were boiled prior to the extraction in order to inactivate proteolytic enzymes in the tissue. Since this precaution was not taken in

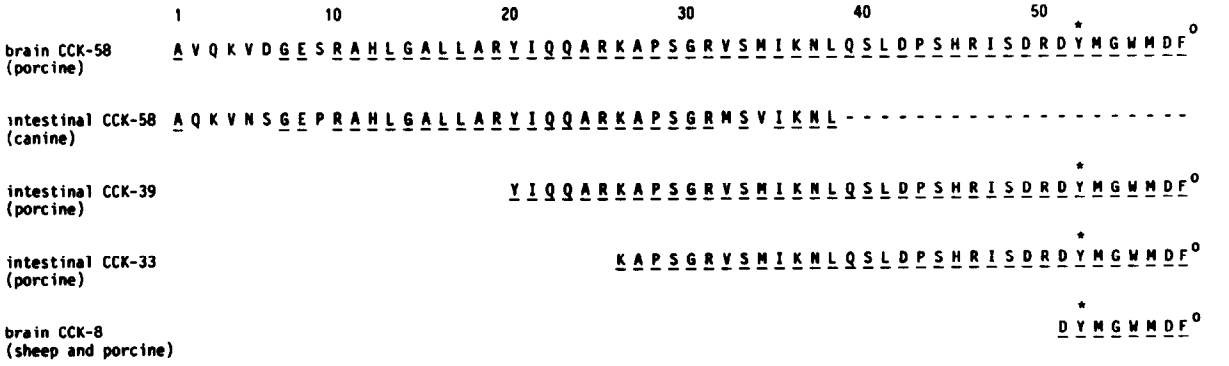

Fig.5. Comparison of the primary structures of CCK-58 (porcine brain) and known CCKs. Identities are underlined. Y*, tyrosine *O*-sulfate. F°, phenylalanine amide.

[16], it is possible that CCK-58 might have been degradated to CCK-8 and the N-terminal fragments during the extraction procedures.

CCK-58 (porcine brain) contracts the smooth muscle of the guinea-pig gallbladder. But, the pattern of the action seems to be different from those of CCK-8, CCK-33 and CCK-39: CCK-58 had a slower onset and prolonged duration as compared with the patterns of the gallbladder contraction elicited by other CCKs. A recent study indicated that CCK-58 selectively increased dopamine turnover in certain CCK-positive dopamine nerve terminals in which other known CCK molecules did not (Fuxe et al., personal communication). It may therefore be speculated that CCK-58 has different biological roles from those of other known CCK molecules.

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish Medical Research Council (Project 13x-1010 and 13x-3532), the Knut and Alice Wallenberg Foundation and the Nordisk Insulinfond.

REFERENCES

[1] Mutt, V. and Jorpes, J.E. (1968) Eur. J. Biochem. 6, 156–162.
[2] Mutt, V. (1980) in: Gastrointestinal Hormones (Glass, G.B.J. ed.) pp. 169–221, Raven, New York.
[3] Mutt, V. and Jorpes, E.J. (1971) Biochem. J. 125, 57–58p.
[4] Eysselein, V., Reeve, J.R., Shively, J.J.E., Hawke, D. and Walsh, J.H. (1982) Peptides 3, 687–691.
[5] Vanderhaeghen, J.J., Signeau, J.C. and Gepts, V. (1975) Nature 257, 604–605.
[6] Dockray, G.J. (1976) Nature 264, 568–570.
[7] Dockray, G.J., Gregory, R.A. and Hutchison, J.B. (1978) Nature 274, 359–361.
[8] Tatemoto, K. (1984) Biomed. Res., in press.
[9] Tatemoto, K. (1984) in: Karger Front. Horm. Res. vol. 12, in press.
[10] Ljungberg, S. (1969) Acta Pharm. Suecia 6, 599–606.
[11] Tatemoto, K. and Mutt, V. (1978) Proc. Natl. Acad. Sci. USA 75, 4115–4119.
[12] Hartley, B.S. (1970) Biochem. J. 119, 805–822.
[13] Penke, B., Ferenczi, R. and Kovacs, K. (1974) Anal. Biochem. 60, 45–50.
[14] Jörnvall, H. and Philipson, L. (1980) Eur. J. Biochem. 104, 237–247.
[15] Zimmerman, C.L., Appella, E. and Pisano, J.J. (1977) Anal. Biochem. 77, 569–573.
[16] Eng, J., Shiina, Y., Pan, Y.-C.E., Blacher, R., Chang, M., Stein, S. and Yalow, R.S. (1983) Proc. Natl. Acad. Sci. USA 80, 6381–6385.