Isolation, structure and properties of the C-terminal flanking peptide of preprocholecystokinin from rat brain

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The C-terminal flanking peptide of preprocholecystokinin has been isolated from rat brain. Micro-sequence analysis revealed the primary structure: Ser-Ala-Glu-Asp-Tyr-Glu-Tyr-Pro-Ser. Arylsulphatase and mild acid hydrolysis suggested that both tyrosine residues are sulphated. The peptide was not active in bioassay systems that respond to CCK8; the significance of the conserved tripeptide Ser-Ala-Glu is discussed.

Cholecystokinin *Flanking peptide* *Sulfated tyrosine*

1. INTRODUCTION

Cholecystokinin (CCK) acts in the gut as a hormone controlling the gall bladder and pancreas, and in the brain it is a possible peptide transmitter produced by various neurones including mesolimbic dopaminergic neurones and some cortical GABA neurones [1,2]. Recently the cDNA sequences complementary to the mRNA encoding rat, mouse, human and porcine preprocholecystokinin (preproCCK) have been elucidated [3–7]. Although these data reveal the primary amino acid sequence of the precursor they provide little insight into post-translational processing pathways. It has been clear for some time that there are differences in post-translational processing of the CCK precursor in brain and gut [1,2], but the mechanisms involved are uncertain. In both cases, processing of the precursor at the sequence -Phe-Gly-Arg-Arg-Ser- is expected to generate the Phe-amide which is the common C-terminal residue of the known biologically active forms (fig.1). Cleavage here should also liberate a C-terminal flanking peptide, which is of interest because it should contain a tripeptide sequence (Ser-Ala-Glu) identical to that in the corresponding region of the precursor for the related hormone gastrin [8–10]. We have recently developed a radioimmunoassay (RIA) for the C-terminal flanking peptide of rat, human and mouse preproCCK [11] and we have now used it to monitor the isolation of this peptide from rat brain.

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2. MATERIALS AND METHODS

2.1. *Isolation*

Rats were decapitated and the brains quickly removed and frozen on dry ice. They were later extracted in boiling water, centrifuged (50000 × *g*, 30 min) and the supernatants lyophilized and stored at −20°C. The extracts were subsequently dissolved in water (20 $g \cdot l^{-1}$), and fractionated by gel filtration on Sephadex G50 fine (5 × 100 cm) in 0.05 M ammonium bicarbonate. Tubes containing the immunoreactivity were then applied to an amino ethyl (AE) cellulose ion-exchange column (1 × 15 cm) equilibrated with 0.05 M triethylamine (pH 7.0 after gassing with CO_2) and eluted with a gradient to 0.5 M triethylamine (pH 11.5). Tubes containing immunoreactivity were lyophilized and

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further purified on HPLC, using three reversed-phase systems in turn. (1) A μBondapak C18 cartridge in Waters Z-module equilibrated with 0.1% heptafluorobutyric acid (HFBA) and eluted with a gradient of acetonitrile using an Altex HPLC system. (2) A μBondapak C18 cartridge in a Waters Z-module equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with an acetonitrile gradient. (3) Techsil C_{18} 5 μm column (5 × 250 mm) eluted with an acetonitrile gradient in 0.1% TFA. At each step the purification was monitored by RIA using an antibody raised to the extreme C-terminal pentapeptide of preproCCK [11]. The label was iodinated synthetic YEYPS

Predicted sequence Trp - Met - Asp - Phe - Gly - Arg - Arg - **Ser - Ala - Glu - Asp - Tyr - Glu - Tyr - Pro - Ser.**

Observed sequence **SER- ALA-GLU-ASP- TYR-GLU-TYR-PRO-SER.**

SO_3H SO_3H

Fig.1. Amino acid sequence of the C-terminal region of rat preproCCK predicted from the cDNA sequence (top) and the observed sequence of the nonapeptide isolated in this study. The antibody used in RIA was raised to the C-terminal pentapeptide and reacts with both the synthetic and natural peptides.

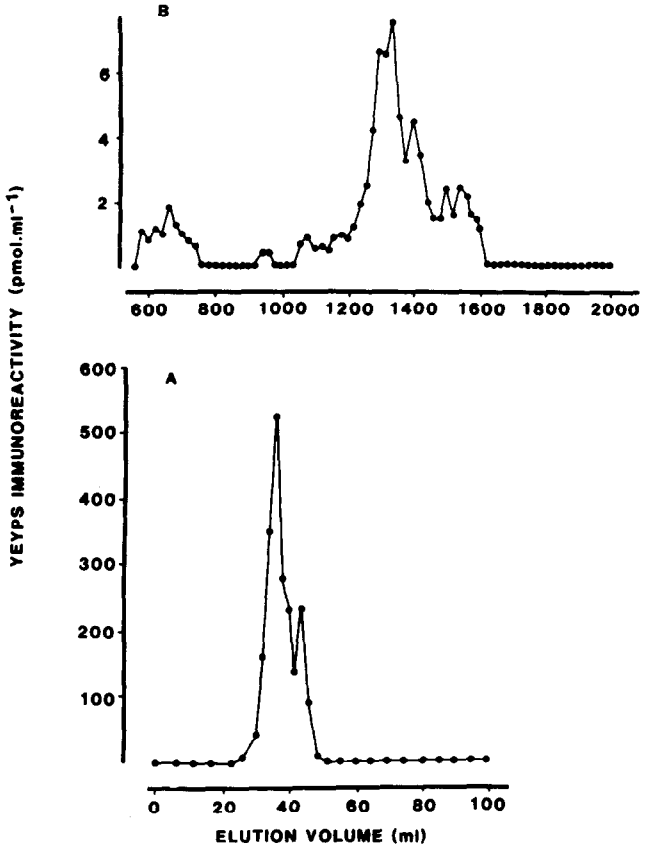

Fig.2. Purification by gel filtration and ion-exchange chromatography of rat brain boiling water extracts. (B) Sephadex G50 (5 × 100) eluted with 0.05 M ammonium bicarbonate. Tubes with immunoreactivity (1250–1420 ml) were applied to AE cellulose ion-exchange chromatography columns (A) eluted with a gradient of triethylamine.

(obtained from CRB, Harston, Cambridge, England), and this peptide was also used as standard.

2.2. *Amino acid sequence*

Approx. 2 nmol of homogeneous peptide was obtained from the final HPLC step. A sample of 500 pmol was taken for microsequence analysis using an Applied Biosystems gas-phase sequenator, and HPLC detection of phenylthiohydantoin (PTH) derivatives. The possible sulphation of tyrosine residues was examined by mild acid hydrolysis in 0.1 M HCl (100°C; 5 min) or digestion with 200–400 μg arylsulphatase (Sigma, VIII) dissolved in 0.2 M sodium acetate buffer (pH 5.0, 2 h, 37°C).

2.3. *Bioassays*

The actions of the purified peptide were studied on guinea pig gall bladder in vitro, guinea pig ileum in vitro, rat pancreatic acinar cells in vitro (kindly performed by Dr J. Calam), and rat stomach relaxation in vivo. Concentration or doses of the purified peptide were determined by RIA and were similar to those of CCK8 (1–20 nM) giving responses in the same systems.

3. RESULTS AND DISCUSSION

A single major peak of activity was found in eluates of the gel filtration and ion-exchange columns using the present RIA (fig.2). This material was recovered in good yields (>80%) as a single peak after each of three HPLC steps. The final stage gave approx. 2 nmol of material from 50 g rat brain in a form suitable for structural studies (fig.3). Microsequence analysis of 500 pmol revealed a primary amino acid sequence identical to that of the C-terminal nonapeptide of preproCCK predicted from the cDNA sequence (fig.1). HPLC detection of the PTH derivatives identified tyrosine in the unsulphated form at the appropriate cycles. The sequence analysis involved exposure of the peptide to gaseous TFA at 45°C; it seems likely that these conditions might desulphate previously sulphated tyrosines. Consistent with this, incubation of the pure native peptide with arylsulphatase converted into the immunoreactive material to a more hydrophobic peptide on HPLC through at least one intermediate, suggesting that both tyrosines occurred in the sulphated form. Similar results were obtained with hydrolysis by 0.1 M HCl which is known to

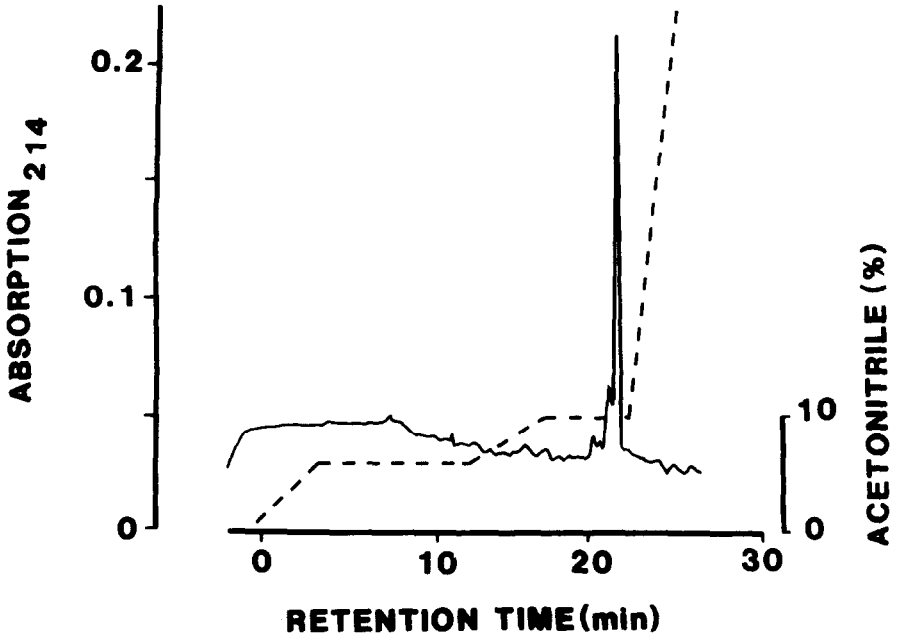

Fig.3. Final separation of the C-terminal flanking peptide of preproCCK by HPLC on Techsil C_{18} 5 μm (5 × 200 mm) equilibrated with 0.1% TFA and eluted with a gradient of acetonitrile (broken line).

desulphate CCK8 and caerulein (fig.4) [12]. Even the very mild acid conditions (pH 5, 37°C, 2 h) needed for optimal arylsulphatase activity produce partial desulphation, suggesting that at least one of the sulphated tyrosines is particularly labile. While the present work was in progress, two other laboratories described assays using antibodies specific for the unsulphated C-terminal proCCK fragment [13,14]. In these studies, desulphation of the native peptide was necessary to generate immunoreactive material. In contrast, the antibody used to monitor purification in this study appears

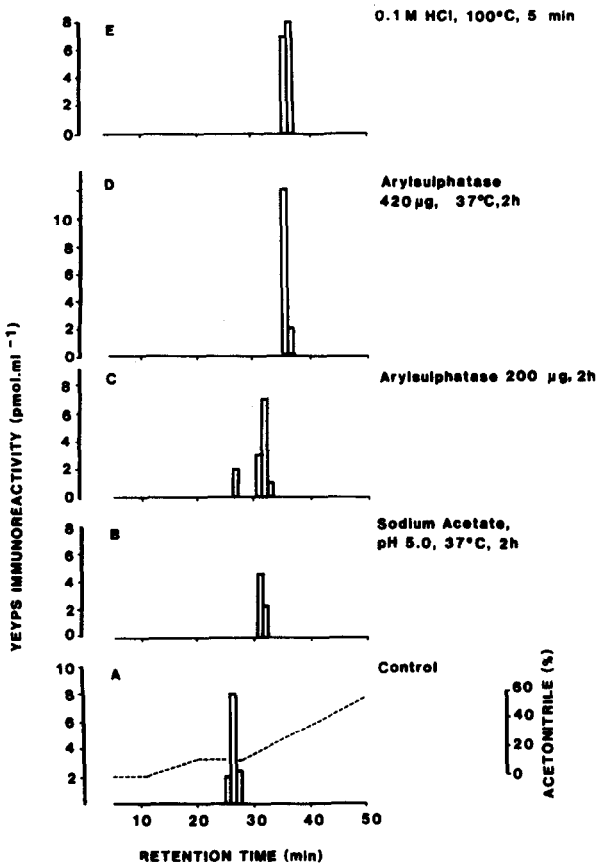

Fig.4. HPLC profiles of acid hydrolysis and arylsulphatase-treated proCCK flanking peptide. Samples were run on a Techsil C_{18} 5 μm column equilibrated with 0.1% TFA and eluted with a gradient of acetonitrile. Panels: A, intact peptide; B, mild acid hydrolysis, control incubation for arylsulphation (sodium acetate pH 5.0 for 2 h at 37°C); C and D, arylsulphatase 200 μg and 400 μg, respectively (2 h at 37°C in sodium acetate buffer pH 5.0); E, acid hydrolysis (boiling in 0.1 M HCl for 5 min).

to react equally with the sulphated and desulphated peptides.

The purified rat brain peptide was without activity on four tissues all of which respond to CCK8: guinea pig gall bladder and ileum, and rat pancreas acinar cell and stomach. These experiments were carried out using quantities of flanking peptide similar to those of CCK8, both alone and together with CCK8. The present assay systems would have detected peptides not only with CCK-like activity, but also with opioid-, VIP-, bombesin- and tachykinin-like properties [15]. The evident lack of activity of the C-terminal fragment of proCCK is of interest in view of the fact that the initial tripeptide sequence, Ser-Ala-Glu-, is identical to that in the corresponding region of the precursor for the related hormone, gastrin. Conservation is presumably related to biological importance; one possibility is that this region is important for the appropriate post-translational processing at the adjacent Gly-Arg-Arg sequence. The Ser-Ala-Glu tripeptide may facilitate the action of the enzyme that carries out the initial cleavage between Arg-Ser.

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