Is the C-terminal flanking peptide of rat cholecystokinin double sulphated?

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Received 27 November 1985

A specific radioimmunoassay was developed to the predicted nine amino acid C-terminal flanking peptide of cholecystokinin (peptide serine serine, PSS). In aqueous extracts of rat brain, PSS was undetectable unless the extracts were first treated with arylsulphatase, which also resulted in desulphation of cholecystokinin. The reverse-phase HPLC analysis of partially desulphated extracts showed the presence of two peaks intermediate to the naturally occurring and the completely desulphated forms. It is therefore proposed that the CCK-flanking peptide PSS has both tyrosine residues sulphated.

Cholecystokinin *Flanking peptide* *Radioimmunoassay* *(Rat brain)* *Desulfation* *HPLC*

1. INTRODUCTION

The structure of rat pre-pro-cholecystokinin was recently elucidated from cloned mRNA [1,2]. The known sequence of cholecystokinin is flanked at the C-terminal end by a 12-amino-acid residue (Gly-Arg-Arg-Ser-Ala-Glu-Asp-Tyr-Glu-Tyr-Pro-Ser). It is likely that the first 3 amino acids would be lost following cleavage and that a nonapeptide (peptide serine serine, PSS) would be produced. All the naturally occurring forms of cholecystokinin have a sulphated tyrosine residue, which is essential for biological activity [3]. Failure to detect PSS immunologically unless first treated with arylsulphatase suggests possible sulphation of its tyrosine residues.

2. MATERIALS AND METHODS

The whole brain from 200 g Wistar rats was rapidly removed after decapitation and immediately minced and extracted by plunging into water (10 ml/g) containing 65 mM dithiothreitol (DTT) in a vigorously boiling water bath [4]. After maintenance at 100°C for 10 min the extracts were cooled, centrifuged and partially purified by loading 5 ml onto reverse-phase octadecylsilica cartridges (Sep-Pak, Waters), washing with 20 ml water and eluting with 2 ml of 40% acetonitrile/water containing 0.05% trifluoroacetic acid (TFA) and 65 mM DTT. The eluate was then diluted to give a final acetonitrile concentration of 8% and possible particulate matter removed by centrifugation at 12000 × *g* for 5 min. 1 ml of this extract was incubated with 100 μl of 2 M acetate buffer (pH 5.0) and 2.5 mg arylsulphatase (Sigma 8629) dissolved in 500 μl of 0.2% sodium chloride for 2 h at 37°C. A second aliquot was treated identically but with one-tenth the amount of arylsulphatase (250 μg) and incubated for only half the time (1 h).

Samples of both extracts treated with arylsulphatase and the untreated extract were subject to reverse-phase high-pressure liquid chromatography (HPLC) on a 5 μm techsil C18 column eluted at 1 ml per min with water/acetonitrile containing 0.05% TFA and 65 mM DTT. The column was first eluted with 10% acetonitrile for 10 min and then a gradient was developed from 10 to 13% acetonitrile over

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

60 min. This was followed after 2 min at 13% acetonitrile with a gradient from 13 to 25% over 10 min and then after 2 min at 26% with a gradient from 26 to 29%, followed by 2 min at 29%, then a gradient from 29 to 42% acetonitrile. Fractions (2 ml) were collected and 1 ml of each was treated with 100 μl of 2 M sodium acetate buffer (pH 5.0) and 250 μg arylsulphatase dissolved in 100 μl of 0.2% sodium chloride and the whole incubated at 37°C for 2 h.

PSS concentrations were measured using an antiserum (PSS-4) raised in a rabbit immunized with synthetic non-sulphated PSS (2 μmol) conjugated with glutaraldehyde to bovine serum albumin (0.5 μmol). Rabbits received a primary immunization of 200 nmol conjugated PSS in 2 ml Freund's complete adjuvant and 8 boosts of 50 nmol conjugated PSS at monthly intervals.

Non-sulphated synthetic PSS (2 nmol) was iodinated by conventional chloramine-T oxidation using 0.4 nmol $Na^{125}I$ (Amersham IMS 30) and 70 nmol chloramine-T over 10 s. The product was purified by isocratic elution on a 5 μm techsil C-18 (HPLC technology) column with 13% acetonitrile in water containing 0.05% TFA. The specific activity of iodinated PSS measured by self-displacement was 75 $Bq \cdot fmol^{-1}$. The brain extract column eluants were assayed in 0.8 ml of 0.5 M phosphate buffer (pH 7.4) containing 0.15 mM bovine albumin, 2 fmol labelled PSS and antiserum at a final dilution of 1:24000. After incubation at 4°C for 5 days the free peptide was separated from bound by the addition of 4 mg charcoal (Norit GSX, BDH) with 400 μg dextran (70T, Sigma) in 0.5 ml assay buffer followed by centrifugation. The assay was capable of detecting changes between adjacent tubes of 10 fmol non-sulphated PSS with 95% confidence. C-terminal CCK-like immunoreactivity was measured using an antiserum (02) which fully reacted with all molecular forms of CCK, both sulphated and non-sulphated [5]. Specific measurement of sulphated natural CCK-8 was made using an antiserum (OT10) which did not cross-react with non-sulphated CCK-8 [6].

3. RESULTS

Concentrations of PSS-like immunoreactivity, C-terminal CCK-like immunoreactivity (total CCK) and sulphated CCK-8 specific immunoreactivity are shown in table 1. It is notable that in the untreated aqueous extract the concentrations of C-terminal CCK-like immunoreactivity and sulphated CCK-8 specific immunoreactivity are similar, whereas PSS is undetectable. In arylsulphatase-treated extracts the C-terminal antibody detects similar concentrations of CCK to those in untreated extracts whereas CCK-8 sulphated concentrations are much lower. PSS-like immunoreactivity is revealed in arylsulphatase-treated extracts in molar quantities which are similar to those of sulphated CCK-8 in untreated extracts.

The nature of the presumed sulphated PSS-like immunoreactivity both untreated and treated with low-dose and high-dose arylsulphatase was further investigated using reverse-phase HPLC. The high-dose arylsulphatase, which resulted in complete desulphation of CCK-8, gave a peak of PSS-like immunoreactivity in the same position as synthetic non-sulphated PSS standard (fig.1). In the same column run C-terminal CCK-like immunoreactivity eluted in the position of non-sulphated CCK-8 but this peak was not detected by the specific sulphated CCK-8 assay. When untreated aqueous rat brain extracts were columned PSS-like im-

Table 1

Immunoreactive PSS, C-terminal CCK and specific sulphated CCK-8-like immunoreactivity in aqueous extracts of rat brain before and after treatment with arylsulphatase

	CCK-8 (pmol/g)	PSS (pmol/g)
Untreated	79.4 ± 6.1	nil detected
Sulphatase-treated (complete)	4.1 ± 1.3	72.2 ± 5.9
Untreated	76.6 ± 5.8	nil detected
Sulphatase-treated (mild)	27.7 ± 2.4	41.5 ± 6.8

Concentrations of CCK-8 were measured with specific antibody (OT10) and non-sulphated PSS with antibody PSS 4 in aqueous extracts of rat brain before and after complete and mild desulphation (mean ± SE, n = 10 per group)

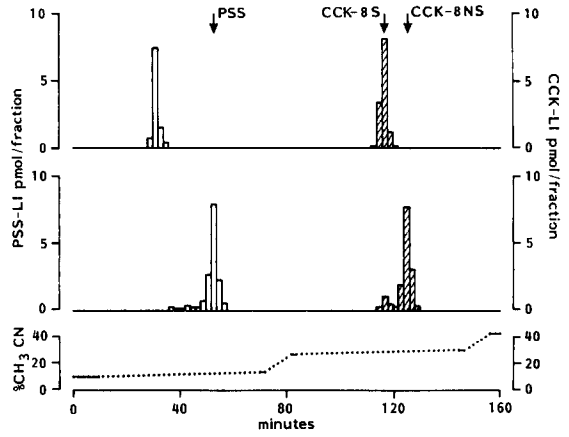

Fig.1. The top panel shows the elution profile of aqueous rat brain extract. PSS-like immunoreactivity (PSS-4) is revealed in the desulphated extracts only and depicted by the open bars. C-terminal CCK-like immunoreactivity (02), also detected in these extracts, is shown in the hatched bars. The antibody specific for sulphated CCK-8 (OT10) only detected the CCK peak in non-sulphatase-treated column fractions. The bottom panel shows the elution profile of the same aqueous brain extract as above, pre-treated with arylsulphatase before columning. Sulphated CCK-8 was not detected in this pretreated extract column using antibody OT10.

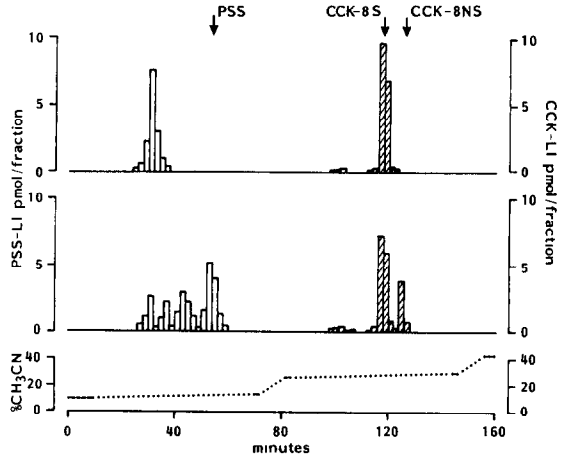

Fig.2. Reverse-phase HPLC showing effect of mild desulphation of PSS and CCK-8 in an aqueous rat brain extract. This was carried out as in fig.1. In the top panel the brain extract was columned without prior sulphatase treatment. In the bottom panel the extract was subjected to mild sulphatase pre-treatment before columning. PSS immunoreactivity (open bars) eluted in 4 peaks after this mild pretreatment of extract with sulphatase. Some C-terminal CCK-like immunoreactivity was also generated and eluted in the position of non-sulphated CCK, but this peak was not seen by the specific anti-sulphated CCK-8 antibody even in untreated column fractions.

munoreactivity was only revealed when column fractions were subsequently treated with arylsulphatase. The immunoreactivity thus demonstrated eluted earlier (fraction 16) than the synthetic non-sulphated PSS standard (fraction 27).

Similarly, CCK-like immunoreactivity eluted earlier (fraction 59) compared with the position of the peak when extract treated with arylsulphatase before columning was analysed (fraction 63).

When low-dose arylsulphatase pre-treatment of brain extracts was examined only a partial desulphation of CCK-8 was observed (fig.2). Under these conditions 4 peaks of immunoreactive PSS became apparent, the fractions all being treated after columning with arylsulphatase. The first peak appeared in the position of natural PSS (fraction 16) as seen in untreated extracts. Two small peaks then preceded the final peak which itself eluted in the position of the pure synthetic PSS standard.

4. DISCUSSION

These studies thus suggest the existence in rat brain of the predicted C-terminal flanking peptide of CCK-8, denoted here as PSS. The finding that, at a concentration of arylsulphatase sufficient to desulphate CCK-8 only partially, PSS immunoreactivity eluted in 4 separate positions on HPLC suggests the possibility that both tyrosines are sulphated. Thus the product presumably was a mixture of double-sulphated PSS, Tyr 5 sulphated PSS, Tyr 7 sulphated PSS and desulphated PSS.

Sulphation is now thought to be a widespread post-translational modification of peptides and proteins and is sometimes essential for biological activity, as in the case of cholecystokinin [7,8]. It is notable that a sulphated tyrosine residue is invariably preceded by an acidic region in the peptide sequence and most often the tyrosine itself follows an aspartate or glutamate residue (J.E. Dixon, personal communication). This acidic region may

prove to be an important recognition site for the cellular sulphotransferase involved.

In PSS both tyrosine residues are preceded by acidic amino acids in the sequence. It is of some interest that the sequence Glu-Asp-Tyr-Glu-Tyr in PSS is identical to residues 1397–1401 of complement component C4 which is also sulphated on these two tyrosine residues [9].

Recently, the predicted sequence for human pre-pro-cholecystokinin was reported and the C-terminal flanking region of this molecule was found to be closely identical to that of the rat, differing only in a glutamate substitution for aspartate at position 4, retaining, unchanged, the acidic arrangement of the molecule [10]. Indeed this C-flanking region of cholecystokinin has close sequence similarities with the C-flanking region of pre-pro-gastrin. It has become commonplace to find that more than one biologically active peptide is coded for in a single messenger RNA sequence [11–13]. As the C-flanking sequence of cholecystokinin appears to be highly conserved between man and rat and is sulphated in a similar manner to cholecystokinin (where the sulphate group is essential for biological activity), it is tempting to speculate that PSS may also be found to have a significant biological role.

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