Post-translational processing of preprotachykinins

Isolation of protachykinin-(1–37)-peptide from human adrenal-medullary pheochromocytoma tissue

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The biosynthetic precursors of the mammalian tachykinins, α -, β - and γ -preprotachykinins, contain a common *N*-terminal region of 74 amino acids. A polyclonal antiserum was raised against a synthetic peptide representing *N*-tyrosylated β -preprotachykinin-(48–56)-peptide as predicted from the nucleotide sequence of cloned DNA complementary to human β -preprotachykinin mRNA. By using this antiserum in radioimmunoassay, a single immunoreactive peptide was identified in an extract of a human pheochromocytoma that produced substance P and neurokinin A. Partial microsequencing and determination of the amino acid composition of the peptide indicated identity with preprotachykinin-(20–56)-peptide. Thus the data demonstrate that the Ala¹⁹–Glu²⁰ bond in preprotachykinin is the site of cleavage of the signal peptide.

INTRODUCTION

Sequence analysis of cloned cDNAs from bovine (Nawa *et al.*, 1983) and rat (Kawaguchi *et al.*, 1986; Krause *et al.*, 1987) brain and from a human laryngeal tumour (Harmar *et al.*, 1986) has identified mRNAs directing the synthesis of three preprotachykinins. In the bovine species, the three mRNAs are the product of a single gene and are generated by alternative RNA splicing events (Nawa *et al.*, 1983, 1984). β - and γ -preprotachykinins contain the sequence of substance P and neurokinin A, but α -preprotachykinin contains the sequence of substance P only. The *N*-terminal region (residues 1–74) of the three preprotachykinins contains the substance P sequence and is the same in the three precursors. Substance P is flanked at its *N*-terminal side by the sequence:

As part of a programme of studies to investigate the pathways of post-translational processing of the preprotachykinins, it was decided to develop a specific radioimmunoassay for this region of the precursors. The removal of *C*-terminal basic amino acids by the action of carboxypeptidase H (Fricker *et al.*, 1986) after cleavage of a prohormone by an endopeptidase has been described for several systems (Loh *et al.*, 1984). It was argued, therefore, that the *C*-terminal arginine residue in the *N*-flanking peptide may be rapidly removed by endogenous carboxypeptidase H, and so an antiserum was raised against a synthetic peptide that represents preprotachykinin-(48–56)-peptide.

Synthesis of substance P by human neoplastic tissue has been extensively documented, and tumours studied include carcinoid tumours of the foregut, midgut, lung,

rectum, ovary and testis (reviewed by Conlon *et al.*, 1986), neuroendocrine carcinomas of the skin (Gould *et al.*, 1980) and thyroid (Skrabanek *et al.*, 1979) and pheochromocytomas (Bucsis *et al.*, 1981). In the present study, chromatographic analysis of an extract of adrenal-medullary pheochromocytoma tissue synthesizing substance P and neurokinin A has led to the isolation of a peptide fragment from the common *N*-terminal region of the preprotachykinins. Structural characterization of this fragment provides insight into the pathway of post-translational processing of human preprotachykinin in tumour tissue.

EXPERIMENTAL

Materials

The peptide Tyr-Phe-Glu-His-Leu-Leu-Gln-Arg-Ile-Ala, which represents *N*-tyrosylated human β -preprotachykinin-(48–56)-peptide, was synthesized by Cambridge Research Biochemicals (Harston, Cambridge, U.K.) and its identity was confirmed by amino acid analysis and fast-atom-bombardment mass spectrometry.

Patient details

The patient was a 22-year-old woman with a 3-month history of nausea, tachycardia and episodes of moderate hypertension. A large tumour (approx. 8 cm in diameter) was detected in the right adrenal gland by ultrasonography and computerized tomography. Total resection of the tumour was carried out, and histopathological analysis indicated a benign pheochromocytoma. Urinary output of vanillylmandelic acid was 3-fold elevated before the operation, but was in the healthy range after resection of the tumour.

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Antiserum

N-Tyrosylated β -preprotachykinin-(48–56)-peptide (5 mg) was coupled to keyhole-limpet haemocyanin (5 mg) in 0.05 M-sodium phosphate buffer, pH 7.4, with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (50 mg). The reaction proceeded for 6 h at room temperature and was terminated by dialysis. New Zealand White rabbits ($n = 6$) were injected at multiple subcutaneous sites with an emulsion (2 ml/animal) formed from equal volumes of Freund's adjuvant (complete for the primary immunization and incomplete for subsequent injections) and 0.05 M-sodium phosphate buffer, pH 7.4, containing the conjugate (160 nmol for the primary and 80 nmol for booster injections). Animals were injected at monthly intervals and blood (10 ml) was taken from an ear vein 10 days after injection.

Radioiodination

N-Tyrosylated β -preprotachykinin-(48–56)-peptide (10 nmol) was radiolabelled using the Iodogen method as described by McGregor *et al.* (1988). The iodinated peptide was purified by reversed-phase h.p.l.c. using a Nova-Pak column (0.46 cm $\times$ 15 cm) (Waters Associates, Milford, MA, U.S.A.) eluted at a flow rate of 1.5 ml/min with a linear gradient (90 ml) formed from 0.1% (v/v) trifluoroacetic acid and acetonitrile/water/trifluoroacetic acid (700:299:1, by vol). Fractions (1.5 ml) were collected, and portions of the radiolabelled-peptide peak were stored in portions at -20°C .

Radioimmunoassays

Antiserum NPPT-48 (100 μl ; final dilution 1:12500) and *N*-tyrosylated β -preprotachykinin-(48–56)-peptide (3–1572 fmol/tube) or test sample (100 μl) were incubated with 0.05 M-sodium phosphate buffer, pH 7.4, containing 0.2% (w/v) bovine serum albumin (final vol. 400 μl). After 24 h, radiolabelled tracer (approx. 6000 c.p.m.; 100 μl) was added, and after a further 24 h incubation, antibody-bound radioactivity was precipitated by addition of bovine γ -globulin (100 μl ; 10 mg/ml) and poly(ethylene glycol) 6000 (1 ml; 20%, w/v). Radioactivity was counted in an LKB 1274 γ -radiation counter, and peptide concentrations were calculated by using an LKB spline function program.

Substance P-like immunoreactivity was measured by using an antiserum directed against the *N*-terminal region of substance P, which shows no reactivity with other tachykinins (Deacon & Conlon, 1987). Neurokinin A-like immunoreactivity was measured by using an antiserum directed against the *C*-terminal region of neurokinin A, which shows full cross-reactivity with neuropeptide K but only 0.5% cross-reactivity with substance P (Conlon *et al.*, 1986). All concentrations are expressed relative to synthetic standards from Peninsula Laboratories (Europe) Ltd. (St. Helens, Lancs., U.K.).

Extraction of tumour tissue

Tumour tissue (8.76 g) was placed in liquid N_2 immediately after resection and stored at -70°C until the time of extraction. The tissue was cut into small pieces (0.5 cm $\times$ 0.5 cm $\times$ 0.5 cm) while still frozen and boiled for 10 min in water (70 ml). The tissue was homogenized with an Ultra-Turrax blender, and the homogenate was stirred at 4°C for 16 h. The homogenate was centrifuged (20000 *g* for 1 h at 4°C). The pellet was

rehomogenized with 1 M-acetic acid (50 ml), and a second supernatant was obtained by centrifugation as described above. The supernatant from the water extraction was pumped at a flow rate of 1 ml/min through four Sep-pak C_{18} cartridges (Waters Associates, Milford, MA, U.S.A.) connected in series. Bound material was eluted with 70% (v/v) acetonitrile and freeze-dried.

Purification of the peptide

The boiling-water extract of the tumour, after partial purification on Sep-pak cartridges, was redissolved in 0.1% (v/v) trifluoroacetic acid (2 ml) and injected on to a Supelcosil LC-18-DB reversed-phase C_{18} h.p.l.c. column (1 cm $\times$ 25 cm) (Supelco, Bellefonte, PA, U.S.A.) equilibrated at 30°C and at a flow rate of 2 ml/min with 0.1% (v/v) trifluoroacetic acid. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 49% (v/v) over 70 min by using linear gradients. Absorbance was measured at 280 nm and 214 nm, and prominent peaks were collected manually. Protachykinin-(1–37)-peptide was purified further by chromatography on a Supelcosil LC-3DP column (diphenylmethylsilylsilica) (0.46 cm $\times$ 25 cm) equilibrated at 30°C and at a flow rate of 1.5 ml/min with acetonitrile/water/trifluoroacetic acid (250:749:1, by vol.). The concentration of acetonitrile in the solvent was raised to 45% (v/v) over 50 min by using a linear gradient. The peptide was purified to apparent homogeneity by chromatography on a Vydac 218TP54 column (0.46 cm $\times$ 25 cm) (Separations Group, Hesperia, CA, U.S.A.) eluted at 30°C and at a flow rate of 1.5 ml/min with a linear gradient (60 ml) formed from acetonitrile/water/trifluoroacetic acid (290:709:1, by vol.) and acetonitrile/water/trifluoroacetic acid (430:569:1, by vol.).

Structural analysis

Amino acid composition was determined by using a Durrum D-500 automatic analyser as described previously (Conlon *et al.*, 1987). Hydrolysis was carried out by using approx. 800 pmol of peptide; the contents of cysteine and tryptophan were not measured. Amino acid sequence analysis was carried out by automated Edman degradation on an Applied Biosystems model-470A gas-phase sequencer (Thim *et al.*, 1987). The detection limit for amino acid phenylthiohydantoin derivatives was 0.5 pmol.

RESULTS

Radioimmunoassay

Trace radioiodination of *N*-tyrosylated β -preprotachykinin-(48–56)-peptide produced a single major peak of radioactivity that was eluted from the h.p.l.c. column at a concentration of approx. 28% (v/v) acetonitrile. The specific radioactivity of the tracer was estimated to be $> 50 \text{ TBq/mmol}$. Within 3 months, two out of six rabbits had produced antisera to the synthetic peptide that were of sufficiently high titre ($1 > 5000$ dilution) to be of use in radioimmunoassay. Serum from animal NPPT-48 had the highest affinity constant ($1.6 \times 10^{10} \text{ litre/mol}$) and gave the most sensitive standard curve (Fig. 1). By using *N*-tyrosylated β -preprotachykinin-(48–56)-peptide as standard, the mid-range (IC_{50} , the concentration causing half-maximal inhibition) of the assay was 95 fmol/tube, and the minimum detectable

Fig. 1. Inhibition of binding of ^{125}I -labelled *N*-tyrosylated β -preprotachykinin-(48-56)-peptide [$^{125}\text{I}\beta\text{PPT}(48-56)$] to antibody NPPT-48 (NPPT-48) by synthetic *N*-tyrosylated β -preprotachykinin-(48-56)-peptide [$\text{S}\beta\text{PPT}(48-56)$] standards

The diagram at the top shows a schematic representation of β -preprotachykinin and an indication of the specificity of antiserum NPPT-48. Other abbreviations: SP, substance P, NKA, neurokinin A, NPK, neuropeptide K; RRRK, Arg-Arg-Arg-Lys.

Fig. 2. Reversed-phase h.p.l.c. on Supelcosil LC-18-DB column of a boiling-water extract of adrenal-medullary pheochromocytoma tissue

Details of the elution conditions are given in the text. The broken line shows the concentration of acetonitrile in the eluting solvent. The peak shown by the arrow was the only peak in the chromatogram that was immunoreactive towards antiserum against preprotachykinin-(48-56)-peptide.

Fig. 3. Purification of protachykinin-(1-37)-peptide on a reversed-phase Supelcosil LC-3DP phenyl h.p.l.c. column

The concentration of acetonitrile in the eluting solvent is shown by the broken line. The peak containing protachykinin-(1-37)-peptide is shown by the arrow and was the only peak in the chromatogram that was immunoreactive towards the antiserum against preprotachykinin-(48-56)-peptide.

concentration (causing a fall of 2 s.d. from B_0 , the binding in the absence of unlabelled peptide, was 4 fmol/tube. The intra- and inter-assay coefficients of variation were 4 and 11 % respectively. The antiserum showed no cross-reactivity with substance P, neurokinin A, neuropeptide K or human β -preprotachykinin-(117-126)-peptide at concentrations up to 1 $\mu\text{g}/\text{ml}$.

Concentrations of protachykinin-derived peptides in the tumour extract

The concentration of immunoreactive material measured with the antiserum to *N*-tyrosylated β -preprotachykinin-(48-56)-peptide in the boiling-water extract of the tumour was 381 pmol/g wet weight. The corresponding concentration in the acetic acid extract was 2 pmol/g. The total concentration of substance P-like immunoreactivity in the tumour extract (water and acid extracts combined) was 421 pmol/g, and the total concentration of neurokinin A-like immunoreactivity was 445 pmol/g.

Purification of protachykinin-(1-37)-peptide

The elution profile on a C_{18} reverse-phase h.p.l.c. column of the boiling-water extract of the tumour extract, after concentration on Sep-pak cartridges, is shown in Fig. 2. The prominent peak shown by the arrow was the only peak in the chromatogram that was reactive towards the antiserum to preprotachykinin-(48-56)-peptide. The peak was selected for further purification by rechromatography on an analytical diphenylmethylsilyl-silica column (Fig. 3). The peak denoted by the arrow possessed a very strong absorbance at 280 nm and was purified to apparent homogeneity on an analytical C_{18} h.p.l.c. column (Fig. 4). The peak denoted by the arrow contained protachykinin-(1-37)-peptide. The final yield of pure peptide was 1600 pmol, which represents 48 % of the immunoreactive material in the initial tumour extract.

Fig. 4. Purification to apparent homogeneity of protachykinin-(1-37)-peptide on an analytical Supelcosil LC-18-DB h.p.l.c. column

The concentration of acetonitrile in the eluting solvent is shown by the broken line, and the final yield of pure peptide, shown by the arrow, was 1600 pmol.

Table 1. Amino acid composition of preprotachykinin-(20-56)-peptide isolated from a human neuroendocrine tumour

Values in parentheses are those predicted from the nucleotide sequence of a cDNA clone complementary to human β -preprotachykinin mRNA.

Amino acid	Relative amount (residues/molecule)
Asx	7.1 (7)
Ser	2.2 (2)
Glx	8.9 (8)
Pro	1.8 (2)
Gly	1.4 (1)
Ala	2.1 (2)
Ile	2.6 (3)
Leu	3.8 (4)
Tyr	2.0 (2)
Phe	1.2 (1)
His	1.0 (1)
Lys	1.2 (1)
Arg	1.0 (1)

Characterization of protachykinin-(1-37)-peptide

The amino acid composition of the peptide (Table 1) indicated a total of 35 amino acids, excluding possible tryptophan and cysteine residues. With the exception of a slightly high value for the relative amount of glutamic acid/glutamine, the observed composition was in good agreement with the composition of human preprotachykinin-(20-56)-peptide predicted from the nucleotide sequence of a cDNA (Harmar *et al.*, 1986). The peptide was subjected to eleven cycles of automated Edman degradation (Table 2). The *N*-terminal sequence (Glu-Glu-Ile-Gly-Ala-Asn-Asp-Asp-Leu-Asn-Tyr) corresponds to residues 20-30 of human preprotachykinin. Thus the Ala¹⁹-Glu²⁰ bond is identified as the site of

Table 2. Determination of the *N*-terminal sequence of preprotachykinin-(20-56)-peptide by automated Edman degradation

Cycle no.	Amino acid phenylthiohydantoin derivative	Yield (pmol)
1	Glu	70
2	Glu	72
3	Ile	44
4	Gly	35
5	Ala	38
6	Asn	31
7	Asp	26
8	Asp	39
9	Leu	32
10	Asn	28
11	Tyr	21

cleavage of the signal peptide. In view of the fact that approx. 800 pmol of pure peptide was loaded on to the sequencer disc, the yields of phenylthiohydantoin amino acids are very low. It is probable, therefore, that the α -amino group at the *N*-terminus of the peptide is partially blocked, possibly by formation of a pyroglutamic acid residue. This reaction may represent an authentic post-translational modification occurring in the tumour tissue or it may have arisen during the extraction and isolation procedure. Loss of material may also have occurred by the irreversible binding of the hydrophobic peptide to the walls of the plastic tube containing the highly purified material.

DISCUSSION

Phaeochromocytomas are generally associated with the production of vasoconstrictor substances such as catecholamines and neuropeptide Y (Corder *et al.*, 1984), which results in hypertension of variable severity in the patient. The synthesis of the vasodilator peptides, substance P and neurokinin A, by a phaeochromocytoma is relatively rare, and it may be significant that hypertension in the patient was episodic rather than continuous, despite evidence that circulating catecholamine concentrations were elevated. Substance P has, however, been identified in the non-tumorous adrenal medulla from a variety of species, including man (Bucsis *et al.*, 1981), and the peptide may play a role in inhibiting acetylcholine-stimulated catecholamine secretion (Boksa & Livett, 1984). The fact that the tumour extract contained approximately equimolar concentrations of substance P and neurokinin A is indicative of the synthesis of β - and/or γ -preprotachykinin rather than α -preprotachykinin. The pathways of post-translational processing of the human preprotachykinins are incompletely understood. The present paper describes the use of an antiserum directed against a sequence within the common *N*-terminal region of α , β - and γ -protachykinins to isolate a 37-amino-acid peptide that flanks the substance P sequence in the precursors. Determination of the amino acid sequence at the *N*-terminus of the peptide has shown that the Ala¹⁹-Glu²⁰ bond in preprotachykinin is the site of cleavage of the signal peptide. The antiserum recognized only one molecular component in the tumour

extract, and this component was isolated in pure form in relatively high yield. It is likely, therefore, that cleavage at this bond represents the exclusive site of cleavage of the signal peptide. It is also improbable that the peptide isolated in the present study is derived from a larger peptide by the action of an aminopeptidase, as cleavage of a signal peptide at the C-terminal side of the Phe¹⁸ or Leu¹⁷ residues in preprotachykinin would be extremely unusual (von Heijne, 1983).

The substance P sequence in human β -preprotachykinin (Harmer *et al.*, 1986) is preceded by a single arginine residue (Arg⁵⁷), which acts as a recognition site for a processing enzyme (Schwartz, 1986). The amino acid composition of the N-terminal protachykinin fragment (Table 1) showed that only one arginine residue was present. As there is an arginine residue at position 54 in preprotachykinin, this result demonstrates that the Arg⁵⁷ residue has probably been removed from the C-terminus of the fragment after cleavage of the Arg⁵⁷-Arg⁵⁸ bond. There was insufficient material for an independent determination of the amino acid sequence at the C-terminus of human protachykinin-(1-37)-peptide. However, the analogous peptide has been isolated from bovine corpus striatum (R. Kage & J. M. Conlon, unpublished work), and this peptide also contains only a single arginine residue. The isolation of a peptide that flanks the substance P sequence in preprotachykinin at its N-terminal side complements previous studies from our group that have led to the isolation of neuropeptide K [β -preprotachykinin (72-107)-peptide], which flanks substance P at its C-terminal side (Conlon *et al.*, 1986) and β -preprotachykinin (111-126)-peptide, which flanks neuropeptide K at its C-terminal side (McGregor *et al.* 1988).

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