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Structural characterization of a high-molecular-mass form of calcitonin [procalcitonin-(60–116)-peptide] and its corresponding *N*-terminal flanking peptide [procalcitonin-(1–57)-peptide] in a human medullary thyroid carcinoma

J. Michael CONLON,*§ Lars GRIMELIUS† and Lars THIM‡

*Klinische Arbeitsgruppe für Gastrointestinale Endokrinologie der Max-Planck-Gesellschaft, Universität Göttingen, Gosslerstrasse 10D, D-3400 Göttingen, Federal Republic of Germany, †Department of Pathology, University of Uppsala, S-75185 Uppsala, Sweden, and ‡Novo Research Institute, DK-2880 Bagsvaerd, Denmark

Four peptides derived from procalcitonin were isolated in high yield from an extract of a human medullary thyroid carcinoma. The peptides were identified as procalcitonin-(1–57)-peptide, procalcitonin-(60–91)-peptide (calcitonin), procalcitonin-(60–116)-peptide and procalcitonin-(96–116)-peptide (katacalcin). Determination of the amino acid sequence of procalcitonin-(1–57)-peptide has demonstrated that the Ala^{25}–Ala^{26} bond in preprocalcitonin is the site of cleavage of the signal peptide. Procalcitonin-(60–116)-peptide represents calcitonin extended from its *C*-terminus by the sequence Gly-Lys-Lys-Arg-katacalcin, and its formation is indicative of an aberrant pathway of procalcitonin processing in the tumour cells.

INTRODUCTION

The primary structure of the biosynthetic precursor of human calcitonin (preprocalcitonin) may be deduced from the nucleotide sequence of cDNA clones isolated from medullary thyroid carcinoma tissue (Craig *et al.*, 1982; Le Moullec *et al.*, 1984) and from a bronchial carcinoma cell line (Riley *et al.*, 1986). Human preprocalcitonin is a protein comprising 141 amino acid residues in which the calcitonin sequence is flanked at its *C*-terminus by a 21-residue peptide termed katacalcin or PDN-21. The gene directing the synthesis of human calcitonin (*CALC-I*) comprises six exons (Edbrooke *et al.*, 1985; Steenbergh *et al.*, 1986) and gives rise to two mRNAs by an alternative RNA-processing mechanism (Steenbergh *et al.*, 1984; Jonas *et al.*, 1985). Preprocalcitonin mRNA contains the products of transcription of exons 1–4, and preprocalcitonin-gene-related peptide (preproCGRP) mRNA contains the products of transcription of exons 1–3, 5 and 6. Both preprocalcitonin mRNA and preproCGRP mRNA are expressed in a variety of healthy tissues including thyroid (Tschopp *et al.*, 1984) and in tumours of the thyroid (Morris *et al.*, 1984) and lung (Edbrooke *et al.*, 1985). The human genome contains a second calcitonin/CGRP gene (*CALC-II*) that is structurally similar to the *CALC-I*) gene (Steenbergh *et al.*, 1985). The *CALC-II* RNA transcripts do not appear to be differentially processed, as only preproCGRP-II mRNA and not preprocalcitonin-II mRNA is detected in tissues that express the *CALC-I* gene (Alevizaki *et al.*, 1986; Steenbergh *et al.*, 1986). Previous studies have shown that the calcitonin-like immunoreactivity in extracts of human tumours of the thyroid (Desplan *et al.*, 1980; Dermody *et al.*, 1981), lung (Lumsden *et al.*, 1980; Zajac *et al.*, 1985) and pancreas (Galmiche *et al.*, 1980; Tobler *et al.*, 1983) is heterogeneous. High-molecular-mass forms of calcitonin have been identified by gel-permeation chromatography and polyacrylamide-gel electrophoresis but have not been characterized structurally. The present study describes the isolation and characterization of four peptides derived from procalcitonin in an extract of human medullary thyroid carcinoma.

EXPERIMENTAL

Preparation of tumour material

Details of the patient and a description of the histopathology of the tumour have been provided in a previous paper (Conlon *et al.*, 1988). Tumour tissue (2.81 g) from a metastasis of a medullary thyroid carcinoma located in the mediastinum was placed in liquid N_2 immediately after resection and stored at −70 °C until time of extraction. The tissue was homogenized while still frozen with ethanol/0.7 M-HCl (3:1, v/v; 40 ml) in an Ultra-Turrax blender. The homogenate was stirred at 4 °C for 16 h, then centrifuged (1600 *g* for 1 h at 4 °C), and ethanol was removed from the supernatant under reduced pressure. After a further centrifugation (20000 *g* for 1 h at 4 °C), the supernatant was pumped at a flow rate of 1 ml/min through two Sep-Pak 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 peptides

The tumour extract, 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 × 25 cm) (Supelco, Bellefonte, PA, U.S.A.) equilibrated with

Abbreviation used: CGRP, calcitonin-gene-related peptide.

§ To whom correspondence and requests for reprints should be addressed.

0.1% (v/v) trifluoroacetic acid. The column was eluted at 30 °C and at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 49% (v/v) over 80 min with linear gradients. Absorbance was measured at 214 nm and 280 nm. Peptides derived from procalcitonin (peaks 1–4 in Fig. 1) were purified to apparent homogeneity by rechromatography on a Vydac 218TP54 reversed-phase C_{18} h.p.l.c. column (0.46 cm × 25 cm) (Separations Group, Hesperia, CA, U.S.A.). The column was eluted at 30 °C and at a flow rate of 1.5 ml/min. The following linear gradients of acetonitrile were used for elution: peak 1 (katacalcin), from 0% to 21% (v/v) over 30 min; peak 2 [procalcitonin-(60–116)-peptide], from 28% (v/v) to 39% (v/v) over 30 min; peak 3 (calcitonin), from 28% (v/v) to 42% (v/v) over 40 min; peak 4 [procalcitonin-(1–57)-peptide], from 35% (v/v) to 49% (v/v) over 40 min. All solvents contained 0.1% (v/v) trifluoracetic acid. Cysteine residues in procalcitonin-(60–116)-peptide were alkylated by using 4-vinylpyridine by the method of Andrews & Dixon (1981). The pyridylethylated peptide was isolated by h.p.l.c. under the same conditions of chromatography used for the purification of the unmodified peptide.

Digestion with CNBr

Procalcitonin-(1–57)-peptide (approx. 10 nmol) was incubated with a small crystal of CNBr for 16 h at room temperature in 70% (v/v) formic acid (100 μl). The reaction mixture was freeze-dried. Peptide fragments were isolated by chromatography on a Vydac 218TP54 column equilibrated with 0.1% (v/v) trifluoroacetic acid at 30 °C and at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 49% (v/v) over 60 min.

Structural analysis

Amino acid compositions were determined by using a Durrum automatic analyser as described previously (Conlon *et al.*, 1987). Cysteine and tryptophan residues were not measured. Amino acid sequence analysis was carried out by automated Edman degradation in an Applied Biosystems model 470 A gas-phase sequencer (Thim *et al.*, 1987). The detection limit for amino acid phenylthiohydantoin derivatives was 0.5 pmol.

Radioimmunoassay methods

Calcitonin-like immunoreactivity was measured by the use of an antiserum raised against human calcitonin supplied by Calbiochem–Behring (La Jolla, CA, U.S.A.). The radioimmunoassay was carried out according to the supplier's instructions, and human calcitonin from Peninsula Laboratories (Europe) Ltd. (St. Helens, Lancs., U.K.) was used as standard. The detection limit for the assay was 23 fmol/ml. Calcitonin-gene-related peptide (CGRP) was measured by the use of an antiserum supplied by Peninsula Laboratories (Europe) Ltd. that shows equal reactivity with human CGRP-I and CGRP-II. Human CGRP-I from Peninsula Laboratories (Europe) Ltd. was used as standard. The detection limit for the assay was 33 fmol/ml.

RESULTS

Concentrations of calcitonin and CGRP in the tumour extract

The concentration of calcitonin-like immunoreactivity in the tumour extract was 32.6 nmol/g wet wt. The antiserum used in the radioimmunoassay showed only very low reactivity with procalcitonin-(60–116)-peptide (approx. 1%). The concentration of CGRP-like immuno-

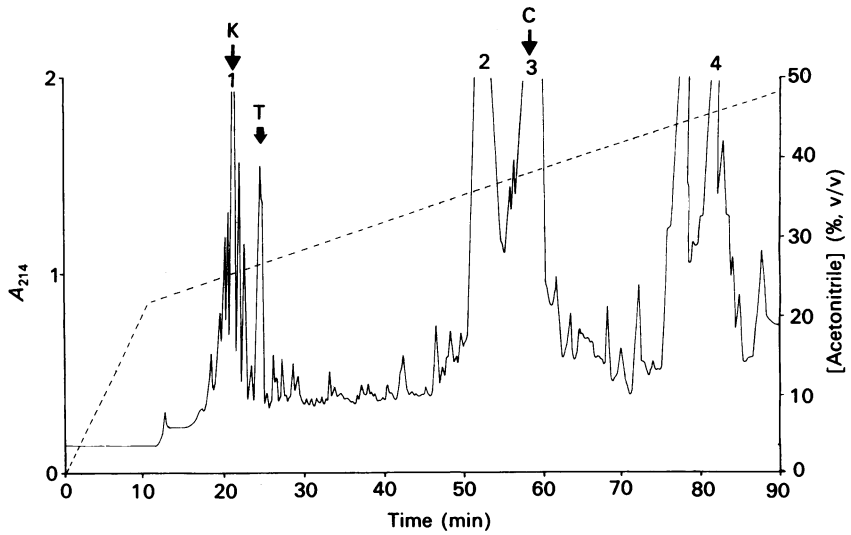

Fig. 1. Reversed-phase h.p.l.c. on a Supelcosil LC-18-DB column of an extract of a human medullary thyroid carcinoma after partial purification on Sep-Pak cartridges

Details of the elution conditions are given in the text. The broken line shows the concentration of acetonitrile in the eluting solvent. The arrows show the retention times of human katacalcin (K), calcitonin (C) and thymosin β_4 (T). Peaks 1–4 were purified further.

reactivity in the tumour extract was 5.0 nmol/g wet wt.

Purification of the procalcitonin-derived peptides

The elution profile on reversed-phase h.p.l.c. of the tumour extract, after concentration on Sep-Pak cartridges, is shown in Fig. 1. The retention time of peak 1 suggested identity with katacalcin [procalcitonin-(96–116)-peptide] and the retention time of peak 3 suggested identity with calcitonin. Tumour katacalcin and calcitonin were purified to apparent homogeneity on a Vydac C_{18} h.p.l.c. column (chromatograms not shown). Mixtures of tumour katacalcin and synthetic katacalcin and tumour calcitonin and synthetic calcitonin were eluted from the h.p.l.c. column under isocractic elution conditions as sharp symmetrical peaks. Purification of peak 2 material [procalcitonin-(60–116)-peptide] is shown in Fig. 2, and the purification of peak 4 material [procalcitonin-(1–57)-peptide] is shown in Fig. 3. The approximate final yields of purified peptides were: procalcitonin-(1–57)-peptide, 22 nmol; procalcitonin-(60–91)-peptide (calcitonin), 55 nmol; procalcitonin-(60–116)-peptide,

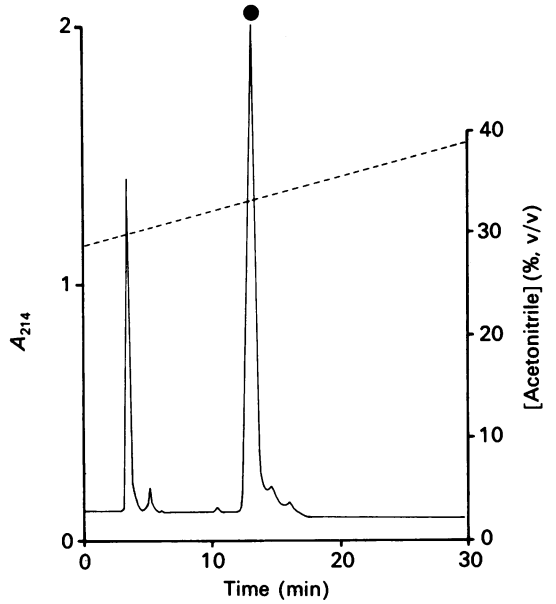

Fig. 2. Purification of procalcitonin-(60–116)-peptide on a reversed-phase Vydac 218TP54 column

The concentration of acetonitrile in the eluting solvent is shown by the broken line, and procalcitonin-(60–116)-peptide (approx. 16 nmol) is denoted by the dot (●).

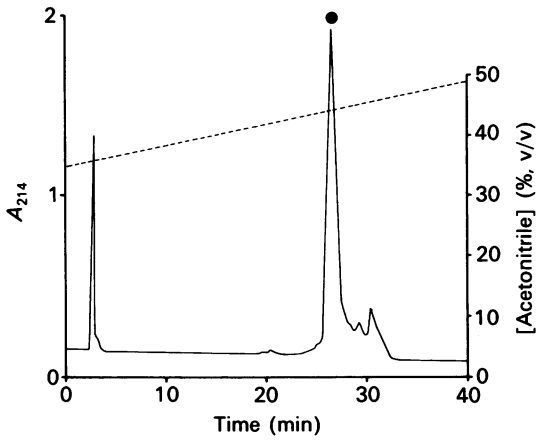

Fig. 3. Purification of procalcitonin-(1–57)-peptide on a reversed-phase Vydac 218TP54 column

The concentration of acetonitrile in the eluting solvent is shown by the broken line, and procalcitonin-(1–57)-peptide (aprox. 22 nmol) is denoted by the dot (●).

Table 1. Amino acid composition of peptides derived from procalcitonin isolated from a medullary thyroid carcinoma

Determinations were carried out in duplicate and the values in parentheses represent the numbers of residues expected from the predicted amino acid sequence of procalcitonin. Procalcitonin-(1–36)-peptide and procalcitonin-(37–57)-peptide were generated from procalcitonin-(1–57)-peptide by digestion with CNBr.

Amino acid	Procalcitonin peptide ...	Amino acid composition (mol of residue/mol)					
		(1–57)	(1–36)	(37–57)	(60–91)	(60–116)	(96–116)
Asx		3.9 (4)	2.0 (3)	1.0 (1)	3.1 (3)	8.1 (8)	5.1 (5)
Thr		0.9 (1)	0.9 (1)		4.7 (5)	4.7 (5)	
Ser		8.6 (9)	3.7 (4)	4.7 (5)	1.2 (1)	4.2 (4)	3.0 (3)
Glx		12.0 (12)	5.4 (5)	7.4 (7)	2.3 (2)	5.2 (4)	2.4 (2)
Pro		4.3 (4)	2.6 (3)	0.7 (1)	2.1 (2)	4.2 (4)	2.1 (2)
Gly		1.0 (1)		1.0 (1)	4.2 (4)	5.3 (5)	
Ala		7.6 (8)	6.9 (7)	1.0 (1)	1.8 (2)	3.1 (3)	1.0 (1)
Val		1.8 (2)	2.0 (2)		0.9 (1)	1.9 (2)	1.0 (1)
Met		1.1 (1)			1.0 (1)	2.6 (3)	2.0 (2)
Ile					1.0 (1)	1.0 (1)	
Leu		7.7 (8)	6.1 (6)	1.9 (2)	2.1 (2)	2.6 (3)	1.0 (1)
Tyr		0.9 (1)	0.9 (1)		1.0 (1)	1.2 (1)	
Phe		0.9 (1)	0.9 (1)		3.0 (3)	3.0 (3)	
His					1.1 (1)	2.7 (3)	2.0 (2)
Lys		1.0 (1)		1.0 (1)	1.2 (1)	2.9 (3)	
Arg		3.6 (4)	1.9 (2)	1.9 (2)		3.0 (3)	2.0 (2)

Table 2. Determination of the primary structures of procalcitonin-(1–36)-peptide, procalcitonin-(37–57)-peptide and procalcitonin-(60–116)-peptide by automated Edman degradation

PE-Cys refers to the vinylpyridine derivative of cysteine. Residue 36 in procalcitonin-(1–36)-peptide was homoserine lactone, which was not quantified.

	Procalcitonin-(1–36)		Procalcitonin-(37–57)		Procalcitonin-(60–116)			Procalcitonin-(1–36)		Procalcitonin-(60–116)	
Cycle No.	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (nmol)	Cycle no.	Amino acid	Yield (pmol)	Amino acid	Yield (nmol)
1	Ala	1943	Lys	591	PE-Cys	6.7	29	Leu	93	Val	1.6
2	Pro	1767	Ala	873	Gly	7.8	30	Val	73	Gly	1.4
3	Phe	1877	Ser	279	Asn	10.0	31	Gln	52	Ala	1.6
4	Arg	200	Glu	581	Leu	10.5	32	Asp	31	Pro	1.4
5	Ser	607	Leu	610	Ser	3.5	33	Tyr	41	Gly	1.2
6	Ala	1275	Glu	622	Thr	1.8	34	Val	47	Lys	1.8
7	Leu	1207	Gln	412	PE-Cys	4.7	35	Gln	30	Lys	2.4
8	Glu	981	Glu	548	Met	7.0	36			Arg	1.1
9	Ser	356	Gln	378	Leu	6.6	37			Asp	1.1
10	Ser	351	Glu	441	Gly	4.5	38			Met	1.0
11	Pro	509	Arg	204	Thr	1.2	39			Ser	0.4
12	Ala	612	Glu	387	Tyr	4.9	40			Ser	0.4
13	Asp	390	Gly	256	Thr	1.4	41			Asp	0.6
14	Pro	403	Ser	121	Gln	4.3	42			Leu	0.5
15	Ala	409	Ser	131	Asp	4.6	43			Glu	0.4
16	Thr	84	Leu	163	Phe	4.8	44			Arg	0.4
17	Leu	314	Asp	187	Asn	3.4	45			Asp	0.5
18	Ser	105	Ser	61	Lys	5.0	46			His	0.2
19	Glu	206	Pro	99	Phe	3.9	47			Arg	0.4
20	Asp	137	Arg	66	His	2.2	48			Pro	0.3
21	Glu	145	Ser	33	Thr	0.8	49			His	0.3
22	Ala	180			Phe	2.7	50			Val	0.2
23	Arg	92			Pro	2.1	51			Ser	0.1
24	Leu	118			Gln	2.1	52			Met	0.2
25	Leu	225			Thr	0.6	53			Pro	0.2
26	Leu	135			Ala	1.9	54			Gln	0.2
27	Ala	96			Ile	1.6	55			Asn	0.03
28	Ala	123			Gly	1.4	56			Ala	0.1
							57			Asn	0.03

16 nmol; procalcitonin-(96–116)-peptide (katacalcin), 28 nmol. Yields are determined by amino acid analysis, but should be interpreted in the light of the probability that efficiencies of extraction and recoveries during chromatography are different for the four peptides. The fact that only the central portions of peaks 1–4 (Fig. 1) were used for further purification will also contribute to the variabilities in yield.

Characterization of the procalcitonin-derived peptides

The amino acid compositions of the purified peptides from peaks 1–4 are shown in Table 1. The data confirm that peak 1 is katacalcin and that peak 3 is calcitonin, and suggest that peak 2 is procalcitonin-(1–57)-peptide [preprocalcitonin-(26–82)-peptide] and that peak 4 is procalcitonin-(60–116)-peptide [preprocalcitonin-(85–141)-peptide]. The presence of a *C*-terminal amidated amino acid residue in the tumour calcitonin was not confirmed, but its presence was suggested by the identical chromatographic properties of the tumour and synthetic peptides. Peak 4 material was weakly immunoreactive towards the antiserum to human calcitonin, such that approx. 1 nmol of pure peptide was equivalent to 10 pmol of the human calcitonin standard. Procalcitonin-(1–57)-peptide contains a methionine residue at position 36 (Le Moullec *et al.*, 1984). Digestion of peak 2 material with CNBr generated two major peptide fragments whose amino acid compositions (Table 1) indicated identity with the expected procalcitonin-(1–36)-peptide and procalcitonin-(37–57)-peptide. The homoserine lactone residue at the *C*-terminus of procalcitonin-(1–36)-peptide was not quantified. The primary structures of the procalcitonin-derived peptides were confirmed by automated Edman degradation (Table 2). The complete amino acid sequences of procalcitonin-(1–36)-peptide, procalcitonin-(37–57)-peptide and procalcitonin-(60–116)-peptide were obtained with the exception that no amino acid phenylthiohydantoin derivative corresponding to homoserine lactone was observed at cycle 36 of the Edman degradation of procalcitonin-(1–36)-peptide. The primary structures of the peptides are identical with the structures predicted from the nucleotide sequences of cloned cDNAs from human tumours of the thyroid (Le Moullec *et al.*, 1984) and lung (Riley *et al.*, 1986). The average repetitive yields during operation of the sequencer were: procalcitonin-(1–36)-peptide, 87.7%; procalcitonin-(37–57)-peptide, 88.5%; procalcitonin-(60–116)-peptide, 91.6%. Elucidation of the primary structure of procalcitonin-(1–36)-peptide establishes the

site of cleavage of the signal peptide as the Ala^{25}–Ala^{26} bond in preprocalcitonin.

DISCUSSION

The high level of expression of the calcitonin (*CALC-I*) gene in a human medullary thyroid carcinoma has enabled the isolation of peptides that together comprise the full sequence of procalcitonin. The very high concentration of calcitonin-like immunoreactivity in the tumour extract estimated by radioimmunoassay was consistent with the observation that nearly all tumour cells were immunostained by the use of an antiserum to calcitonin (Conlon *et al.*, 1988). A previous study with the same tissue extract has led to the isolation of the immunoregulatory peptide, thymosin β_4 (Conlon *et al.*, 1988). A comparison of the predicted amino acid sequences of preprocalcitonin and preproCGRP indicates that the precursors share a common *N*-terminal sequence of 75 amino acid residues (Le Moullec *et al.*, 1984; Edbrooke *et al.*, 1985). The *N*-terminal flanking sequence to calcitonin in preprocalcitonin terminates in Ser^{76}-Leu^{78}-Asp^{79}-Ser^{80}-Pro^{81}-Arg^{82}-Ser^{83}, whereas the corresponding sequence in proCGRP is Arg^{76}-Ile^{77}-Ile^{78}-Ala^{79}-Gln^{80}. Structural characterization of procalcitonin-(1–57)-peptide confirms that this component is derived from preprocalcitonin and establishes the site of cleavage of the signal peptide as the Ala^{25}–Ala^{26} bond. This conclusion is in agreement with the results of partial *N*-terminal microsequencing of radiolabelled procalcitonin synthesized by a human medullary thyroid carcinoma cell line (Gkonos *et al.*, 1986). The site of cleavage of the signal peptide in rat preprocalcitonin is the corresponding Ala^{25}–Val^{26} bond (Birnbaum *et al.*, 1984).

The heterogeneity of the calcitonin-like immunoreactivity in tissue extracts and plasma arises from dimerization and oxidation of the calcitonin monomer and from the presence of incompletely processed biosynthetic intermediates of higher molecular mass (Dermody *et al.*, 1981; Tobler *et al.*, 1983). The presence of glycosylated forms of human procalcitonin in tumour extracts has been claimed (Baylin *et al.*, 1981), but no evidence for such components was obtained by other workers (Goltzman & Tischler, 1978; Cate *et al.*, 1986). The calcitonin sequence in procalcitonin is separated from the katacalcin sequence by the tetrapeptide Gly-Lys-Lys-Arg. After proteolytic cleavage at the site of the basic residues, the glycine residue functions as donor to the α-amidated *C*-terminal proline residue of calcitonin. In healthy man, calcitonin and katacalcin circulate in approximately equimolar concentrations, suggesting that processing of procalcitonin at this site is virtually complete (Hillyard *et al.*, 1983). The isolation of procalcitonin-(60–116)-peptide in relatively high yield has identified an aberrant pathway of post-translational processing in the tumour cells in which the site is not utilized by the cleavage enzyme(s). The proposed pathway is illustrated schematically in Fig. 4. A biologically active peptide with the same elution properties on reverse-phase h.p.l.c. as procalcitonin-(60–116)-peptide was identified in an extract of a benign human pancreatic tumour, but structural characterization was not attempted (Tobler *et al.*, 1983).

In addition to post-translational processing at the site of pairs or groups of basic amino acid residues (Steiner *et al.*, 1974), proteolytic cleavage of prohormones at the site of single arginine residues has been described for several systems (Schwartz, 1986; Benoit *et al.*, 1987). Similarly, cleavage at the site of a Leu–Leu bond has been observed in prosomatostatin (Schmidt *et al.*, 1985) and is involved in the formation of angiotensin I. The *N*-terminal flanking sequence of human procalcitonin (residues 1–57) contains four potential single arginine cleavage sites and two potential Leu–Leu cleavage sites. The amino acid compositions of the peptides in all the major peaks shown in Fig. 1 have been determined, and no evidence for the presence of peptides arising from cleavage at the Arg^4, Arg^{23}, Arg^{47} or Arg^{56} residues or from cleavage of the Leu^{24}–Leu^{25} or Leu^{25}–Leu^{26} bonds was obtained.

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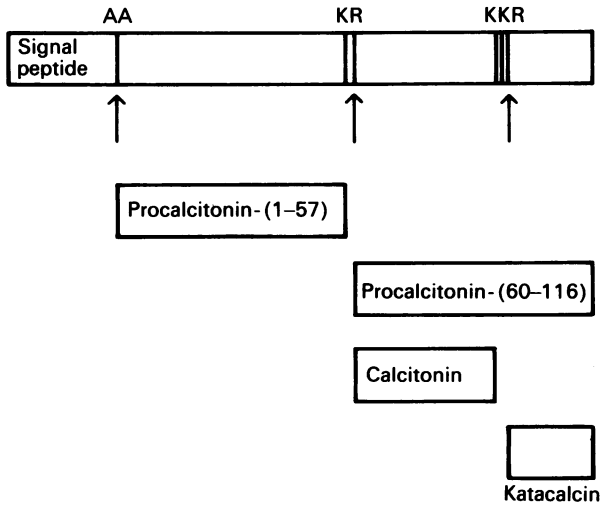

Fig. 4. Schematic representation of the products of post-translational processing of procalcitonin isolated from a human medullary thyroid carcinoma

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