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Heterogeneity of chromogranin A-derived peptides in bovine gut, pancreas and adrenal medulla

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Chromogranin A is produced in many endocrine cell types, and is widely used as a marker in endocrine-cell pathology and secretory-cell biology. There is some evidence that it may be proteolytically processed to yield the putative pancreatic regulatory peptide, pancreastatin, and, in order to characterize the relevant pathways in gastrointestinal and pancreatic endocrine cells, we have used, in radioimmunoassay, site-directed antibodies to pancreastatin itself (L331) and to a sequence of chromogranin A immediately *C*-terminal to pancreastatin (L300). The latter antibody revealed three major forms of immunoreactivity of 8 kDa and five peptides of approx. 3 kDa in bovine pancreas and gut extracts. The 8 kDa peptides were characterized as chromogranin A-(248–313)-peptides, i.e. *C*-terminally extended forms of pancreastatin; two of the 8 kDa variants differed in two positions, confirming a polymorphism predicted from cDNA sequencing. One of the 3 kDa peptides was characterized as chromogranin A-(297–313)-peptide, i.e. the *C*-terminal heptadecapeptide of the 8 kDa peptide that would be liberated after cleavage to yield pancreastatin. On the basis of chromatographic studies, immunohistochemistry and the stoichiometry of different immunoreactive peptides, three different pathways of chromogranin A processing were identified: in adrenal chromaffin cells chromogranin A existed mainly as the unmodified intact protein, in pancreatic islet and gastric antral endocrine cells pancreastatin and the 3 kDa peptides were major products, but in small intestine and gastric corpus endocrine cells there was little nor no pancreastatin and the 8 kDa cleavage product predominated. There are therefore important differences in the distribution of chromogranin A-derived peptides between quite closely related populations of endocrine cells that are attributable not only to variable post-translational cleavage but also to the expression of different primary sequences. It seems possible that in different cell types chromogranin A-derived peptides might subserve a variety of different functions.

INTRODUCTION

The three members of the chromogranin family (chromogranin A, chromogranin B and secretogranin II) are secretory proteins found in a wide variety of endocrine cells [1,2]. As a group they provide important markers for studies of endocrine-cell pathology and secretory-cell biology. Even so, their normal functions remain poorly understood. It has often been thought that the chromogranins might in some way act as packaging proteins within the core of secretory granules [3–5]. There are, however, reasons for thinking that at least one of them, chromogranin A, might have other functions. In particular the primary amino acid sequence of chromogranin A includes several pairs of basic residues, which by analogy with other endocrine-cell secretory peptides constitute potential sites of proteolytic cleavage involved in the production of smaller biologically active products [6–11]. In this context it is therefore significant that the pancreatic islet cells post-translationally process chromogranin A to give the *N*-terminal peptide β-granin [12,13] and also the putative regulatory peptide pancreastatin [14]. However, in bovine adrenal chromaffin cells, which are perhaps the best-studied cells expressing the chromogranin A gene, there appears to be limited cleavage [15,16]. It is not yet clear whether other chromogranin A-producing cells possess the capacity to complete the repertoire of processing events giving rise to pancreastatin. The resolution of this question is of interest in seeking to understand the functional significance of work in which chromogranin A has been used as a marker for endocrine-cell activity. Chromogranin A occurs in a wide variety of gut endocrine cells [17–22], which together provide a model system that allows detailed examination of cell-specific patterns of processing. In the present work we have studied how the pancreastatin region of chromogranin A is processed in the adrenal, pancreas and endocrine cells in three regions of the gut. The results indicate that adrenal chromaffin cells are atypical in executing a rather limited cleavage of chromogranin A; similarly the pancreatic-islet processing pathway is not representative of that in many enteric endocrine cells. The differences appear to be attributable to differential processing of two closely related chromogranin A gene products. As a whole, the data raise the idea that chromogranin A is a plurifunctional secretory protein that subserves different roles in different cell types.

METHODS

Peptides

YLSKEWEDA was synthesized by a standard solid-phase method using L-amino acids with t-butyloxycarbonyl amino-protecting groups which were coupled using *NN'*-dicyclohexylcarbodi-imide in dichloromethane [23]. HF was used to cleave the peptide from the resin and remove the protecting groups. The crude synthetic peptide was purified by reverse-phase h.p.l.c. on a Waters Z-module with a C_{18} μBondapak cartridge eluted with acetonitrile containing 0.1 % trifluoroacetic acid. Amino acid analysis of the final product demonstrated that the peptide contained the amino acid residues tyrosine (Y), leucine (L), serine (S), lysine (K), glutamic acid (E), aspartic acid (D) and alanine (A) in the proportions 1:1:1:1:2:1:1, the

Abbreviation used: ir, immunoreactivity.

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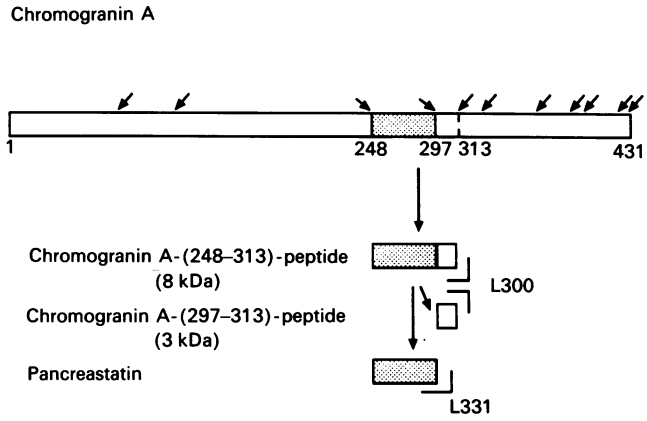

Fig. 1. Schematic representation of bovine chromogranin A showing chromogranin A-(248–313)-peptide, chromogranin A-(297–313)-peptide and pancreastatin-(1–47)-peptide (stippled)

The diagram shows a proposed processing pathway for the synthesis of pancreastatin. The small arrows indicate the potential proteolytic cleavage sites and the L-shaped bars represent the epitopes of antisera L300 and L331.

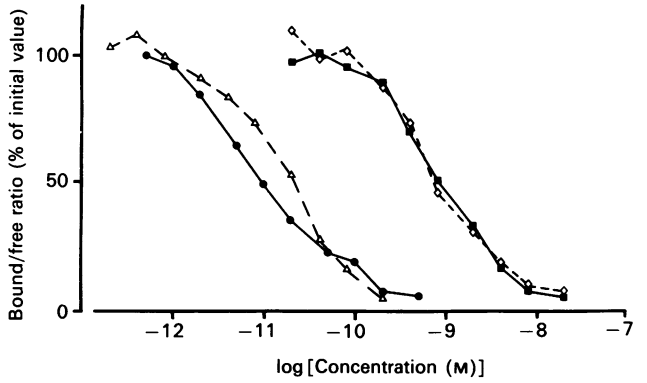

Fig. 2. Characterization of antiserum L300

Chromogranin A was purified from bovine adrenal medulla, as described in the Methods section, and incubated with 10 μg of trypsin for 3 h at 37 °C followed by 0.2 μg of carboxypeptidase B for 1 h at 37 °C. At each step the immunoreactivity was determined by radioimmunoassay with antiserum L300 and the results were compared with the YLSKEWEDA standard curve. ●, YLSKEWEDA; △, chromogranin A + trypsin + carboxypeptidase B; ◇, chromogranin A + trypsin; ■, chromogranin A.

tryptophan (W) residue having been destroyed during acid hydrolysis of the peptide. The *C*-terminal nonapeptide of bovine pancreastatin, i.e. residues 39–47 with tyrosine in the first position (YRAPQVLFRGamide), was purchased from Multiple Peptide Systems (San Diego, CA, U.S.A.) and the sequence was verified by Edman degradation sequencing.

Antibodies

YLSKEWEDA was coupled to thyroglobulin with glutaraldehyde as follows. Peptide (305 nmol) was mixed with thyroglobulin (2 mg) in 1.2 ml of 0.1 M-phosphate buffer, pH 7.3; 50 μl of 5% glutaraldehyde was added and the mixture was left for 70 min. The mixture was then dialysed against distilled water (4 litres for 24 h at 4 °C). The incorporation of peptide was 75%, based on recovery of a trace amount of labelled peptide. Rabbits ($n = 4$) were immunized with the equivalent of 35 nmol of YLSKEWEDA emulsified in Freund's complete adjuvant and boosted with 25 nmol at 6-week intervals. The highest-titre antibody (antiserum L300) was used for radioimmunoassay. Antibodies to the *C*-terminus of pancreastatin (antiserum L331) were raised by using a similar conjugation protocol except that 1 μmol of peptide was conjugated to 2 mg of thyroglobulin. The incorporation was 64%. The immunization protocol was identical with that for YLSKEWEDA except that the equivalent of 50 nmol of peptide was used for the initial immunization.

Radioimmunoassay

LSKEWEDA immunoreactivity (ir) was determined by radioimmunoassay with antiserum L300 at a dilution of 1:1000000. Incubations were performed in 1 ml of 0.02 M-phosphate buffer, pH 7.4, containing 0.5% (v/v) Bovumin (Ortho Diagnostics, Raritan, NJ, U.S.A.) and 0.05% (w/v) NaN_3 at 4 °C for 24 h. Total LSKEWEDA ir, which was used as a measure of chromogranin A and high-molecular-mass variants, was determined after digestion of samples with 10 μg of Tos-Phe-CH_2Cl-treated trypsin (Worthington Corp., Freehold, NJ, U.S.A.) for 3 h at 37 °C in 200 μl of 0.05 M-NH_4HCO_3 followed by digestion with 0.2 μg of carboxypeptidase B for 1 h at 37 °C. After each step the samples were boiled, and the product was finally freeze-dried [24]. Incubation of synthetic YLSKEWEDA with excess trypsin (100 μg) for 24 h at 37 °C did not affect the immunoreactivity of the peptide. Pancreastatin ir was determined with antiserum L331 at a dilution of 1:100000 in 1 ml of 0.02 M-phosphate buffer, pH 7.4, containing 0.14 M-NaCl, 1% (v/v) Bovumin and 0.05% (w/v) NaN_3 for 2 days at 4 °C. Radiolabelled synthetic YLSKEWEDA and YRAPQVLFRGamide were produced by the chloramine-T method and purified by reverse-phase h.p.l.c. Quantification of unknown samples was made by reference to standards of synthetic YLSKEWEDA or YRAPQVLFRG-amide. Antibody-bound and free radiolabelled peptides were separated by centrifugation with a suspension (100 μl) of charcoal/dextran/non-fat milk powder/water in the proportions 10:1:1:100, (w/w/w/v) for antiserum L300 and 10:1:0.5:100 for antiserum L331.

Tissue extraction

Chromogranin A-derived peptides were recovered from boiling-water extracts of bovine ileum (0.1 g/ml) by using Whatman DE-23 DEAE-cellulose resin as previously described [25]. The extracted acidic peptides were further purified by fractionation on a Sephadex G-50 (fine grade) column (5 cm × 100 cm) eluted with 0.05 M-NH_4HCO_3 containing 0.05% (w/v) NaN_3 at 4 °C; the relevant fractions were loaded on to a Whatman DE-52 DEAE-cellulose column (1 cm × 10 cm) and eluted with a gradient of 0.05–0.5 M-ammonium acetate buffer, pH 6.5, at 4 °C.

Small samples of bovine tissues, which had been frozen on solid CO_2 at the abattoir, were extracted by boiling in water (0.1 g/ml) for 5–10 min followed by homogenization. The extracts were centrifuged at 20000 *g* for 30 min, and the pellet was re-extracted with 3% (v/v) acetic acid (0.1 g/ml) and re-centrifuged.

Bovine chromogranin A, used to characterize the antisera, was isolated from boiling-water extracts of bovine adrenal medulla by using concanavalin A–Sepharose chromatography, Whatman DE-52 DEAE-cellulose anion-exchange chromatography and SDS/PAGE with a 12% polyacrylamide gel [26], from which the 75 kDa chromogranin A band was excised and eluted. Quantification was by the Bradford protein assay [27].

Peptide isolation and amino acid sequence analysis

Homogeneous peptides were obtained by using four systems of reverse-phase h.p.l.c.: a μBondapack C_{18} Z-module cartridge

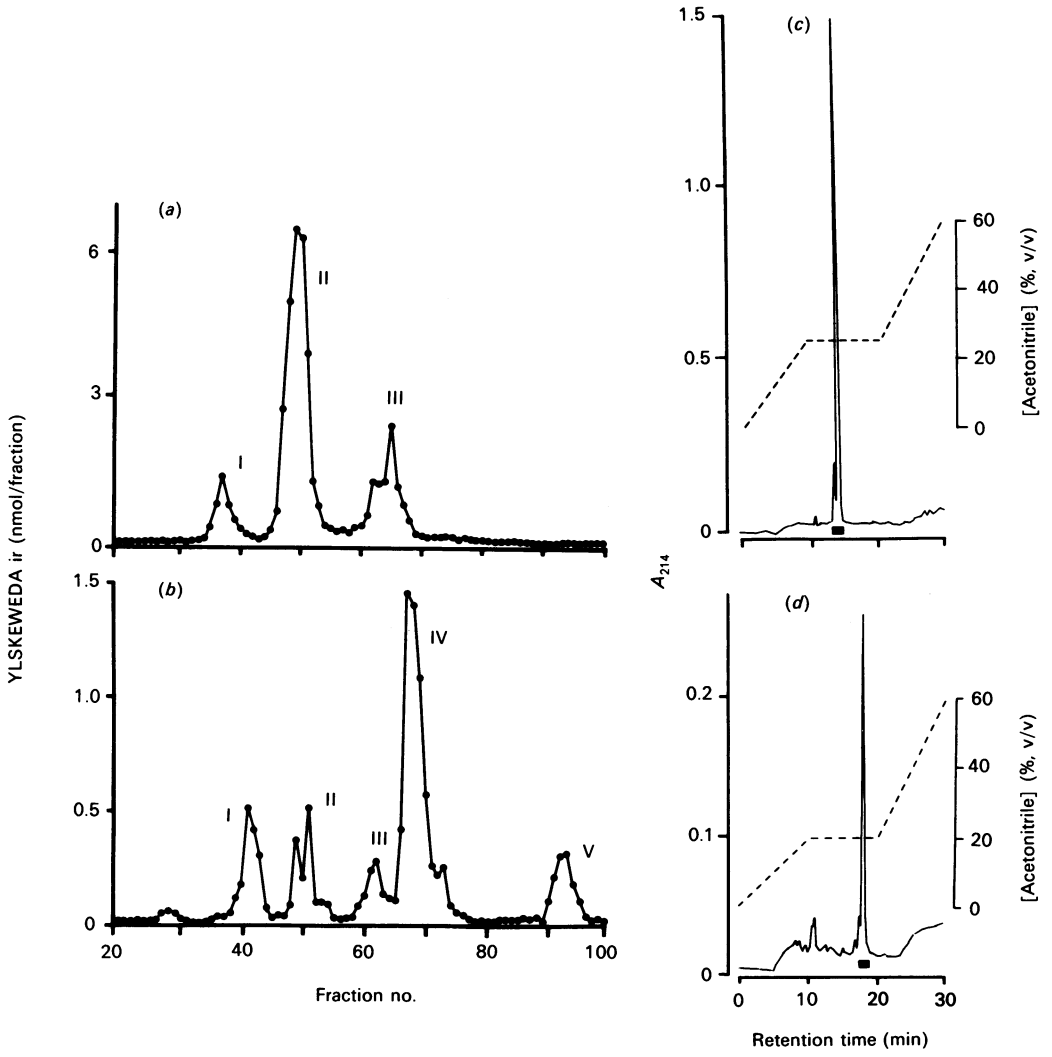

Fig. 3. Purification of the 8 kDa and 3 kDa LSKEWEDA ir peaks from bovine ileum

The 8 kDa and 3 kDa LSKEWEDA ir peaks from Sephadex G-50 gel filtration were separately fractionated by Whatman DE-52 DEAE-cellulose anion-exchange chromatography. (*a*) Immunoreactivity profile of the 8 kDa material; the three peaks are numbered with increasing acidity (I–III). (*b*) Immunoreactivity profile of the 3 kDa material; the variants are numbered similarly (I–V). Further purification to homogeneity was performed by reverse-phase h.p.l.c. (*c*) Final purification of 8 kDa peak II on a Vydac C_{18} column (4.6 cm × 250 cm) eluted with acetonitrile containing 0.1 % trifluoroacetic acid at a flow rate of 1 ml/min. All three 8 kDa peptides were eluted with similar retention times with isocratic elution conditions of 25 % acetonitrile. The 3 kDa peak IV (*d*) was eluted with isocratic elution conditions of 21 % acetonitrile on the same column. The bars in (*c*) and (*d*) indicate the fractions containing LSKEWEDA ir.

(Waters Associates) eluted with 0.1 % trifluoroacetic acid and acetonitrile containing 0.1 % trifluoroacetic acid; a Zorbax C8 PEP RP/1 column (DuPont) eluted with 50 mM-triethylamine phosphate, pH 3.5, and acetonitrile; a PLRP-S C_{18} column (Polymer Laboratories) with a buffer system of 0.05 M-NH_4HCO_3 and acetonitrile; final purification was made with a Vydac 'Protein & peptide' C_{18} column eluted with 0.1 % trifluoroacetic acid and acetonitrile containing 0.1 % trifluoroacetic acid. H.p.l.c. eluates in acid buffers were immediately neutralized with saturated NH_4HCO_3 to prevent the loss of peptides, which were acid-labile. Before amino acid sequence analysis, the peptides were desalted by Sephadex G-10 gel filtration.

Edman degradation sequence analysis was performed with an Applied Biosystems gas-phase sequencer. With the larger peptides the complete sequence was derived from peptide fragments after treatment with 1 % (w/v) CNBr (Pierce Chemical Co., Rockford, IL, U.S.A.) in 2 ml of 0.1 M-HCl for 5 h at room temperature. The reaction was terminated by fractionation on a Vydac C_{18} reverse-phase h.p.l.c. column and the fragments were eluted with a gradient of 0–30 % acetonitrile containing 0.1 % trifluoroacetic acid.

Immunohistochemistry

Bovine tissues were fixed by immersion in 4 % (w/v) paraformaldehyde in phosphate-buffered saline (0.14 M-NaCl in 0.1 M-sodium phosphate buffer, pH 7.3) or in 0.4 % parabenzoquinone in 0.1 M-sodium cacodylate buffer, pH 7.3, washed in 0.1 M-sodium phosphate buffer, pH 7.4, containing 30 % (w/v) sucrose and left overnight at 4 °C. Sections (8 μm) were incubated with L300 (1:100) and L331 (1:400) in phosphate-buffered saline containing 1 % BSA, 5 % thyroglobulin and 0.4 % sodium ethylamine tetra-acetate for 15 h at 4 °C, washed with 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.5 M-NaCl and then incubated with goat or pig anti-(rabbit IgG) serum conjugated to fluorescein isothiocyanate (1:40) for 1 h at room temperature and mounted in glycerol/phosphate-buffered

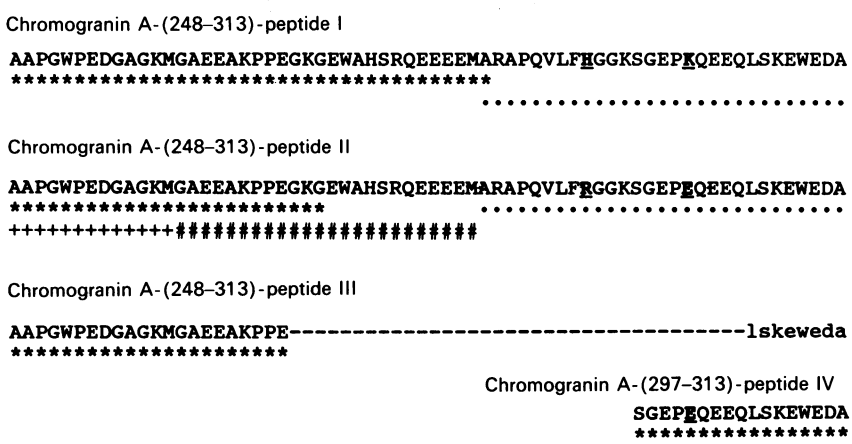

Fig. 4. Amino acid sequencing of 8 kDa peaks I, II and III and 3 kDa peak IV

Homogeneous samples of 8 kDa peaks I, II and III and 3 kDa peak IV from bovine ileum were subjected to Edman degradation sequence analysis. The complete sequence of 17 cycles was obtained for 3 kDa peak IV, and sequence data for 38, 25 and 22 cycles were initially obtained for 8 kDa peaks I, II and III respectively, the derived amino acids being denoted by ★. CNBr treatment of 8 kDa peaks I and II each produced three fragments (i–iii); fragment iii of 8 kDa peak I and all three fragments of 8 kDa peak II were sequenced to determine the complete 66-residue sequence of both peptides. The CNBr-cleavage fragments are designated by symbols: #, i; +, ii; ●, iii. Chromogranin A-(248–313)-peptide peaks I and II differed in the assignment of two amino acids (underlined), namely His-293 and Lys-301 in peak I and Arg-293 and Glu-301 in peak II. The complete amino acid sequence of chromogranin A-(248–313)-peptide III was deduced from both *N*-terminal sequencing (★) and the *C*-terminal immunoreactivity (denoted by the use of lower case).

saline (9:1, v/v) [28,29]. The sections were examined with a Leitz fluorescence microscope.

RESULTS

Radioimmunoassay

The concentration of YLSKEWEDA required for 50% inhibition of binding of label to antiserum L300 (Fig. 1) was 8.3 ± 0.7 pM ($n = 16$). When the LSKEWEDA sequence is enclosed within the bovine chromogranin A molecule this immunoreactivity was markedly reduced (0.01% relative to synthetic YLSKEWEDA). Tryptic digestion of chromogranin A, which liberates the LSKEWEDA sequence extended at the *C*-terminus by lysylarginine or lysine, had no effect on immunoreactivity (Fig. 2). However, further digestion with carboxypeptidase B, to remove the *C*-terminal basic residues and expose the free *C*-terminal LSKEWEDA sequence, sharply increased immunoreactivity; the immunochemical potency of the product of trypsin and carboxypeptidase B digestions was similar to that of synthetic YLSKEWEDA (Fig. 2). Digestion with trypsin or carboxypeptidase B had no effect upon synthetic YLSKEWEDA. To investigate the effect of *C*-terminal deletions upon immunoreactivity, synthetic YLSKEWEDA was digested with carboxypeptidase A (10 units for 1 h at 37 °C); the product showed a 45% reduction in immunoreactivity. The synthetic peptide YERLSREWED, which is the rat equivalent of YLSKEWEDA, and lacks the *C*-terminal alanine residue, cross-reacted poorly with antiserum L300 (< 0.001% compared with synthetic YLSKEWEDA). Thus antiserum L300 is specific for the *C*-terminus of the LSKEWEDA sequence, and deletions or extensions to the *C*-terminus markedly reduce immunoreactivity. Antiserum L300 did not cross-react with pig pancreastatin-(1–49)-peptide (1 μM) or with the synthetic fragment of bovine pancreastatin, YRAPQVLFRGamide (1 μM).

Synthetic YRAPQVLFRGamide, which corresponds to the *C*-terminus of bovine pancreastatin (Fig. 1), produced 50% inhibition of binding of label to antibody L331 at 30.8 ± 2.7 pM ($n = 16$). The antiserum appeared to be species-specific, since it cross-reacted poorly with synthetic pig pancreastatin-(1–49)-peptide (< 0.001% relative to synthetic YRAPQVLFRGamide) and was unable to detect any immunoreactive material in pancreatic extracts from rat or guinea pig. Treatment with carboxypeptidase A (10 units for 1 h at 37 °C) reduced immunoreactivity by 29%, demonstrating a partial requirement of the *C*-terminal glycine amide for binding. Chromogranin A and YLSKEWEDA did not cross-react with antiserum L331 (< 0.001% relative to synthetic YRAPQVLFRGamide); furthermore trypsin digestion of chromogranin A, alone or coupled with carboxypeptidase B digestion, did not affect immunoreactivity.

Peptide characterization

On Sephadex G-50 gel filtration (results not shown) two peaks of LSKEWEDA ir were separated in extracts of ileum with apparent molecular masses of 8 and 3 kDa. Whatman DE-52 DEAE-cellulose anion-exchange chromatography further separated the 8 kDa peak into three peaks (Fig. 3*a*, I–III in order of elution) and the 3 kDa peak into five peaks (Fig. 3*b*, I–V). All of the 8 kDa peptides (I, II and III) and the 3 kDa variant IV were purified by reverse-phase h.p.l.c. (Figs. 3*c* and 3*d*) and subjected to *N*-terminal amino acid sequence analysis.

The 3 kDa material consisted of a 17-residue peptide corresponding to chromogranin A-(297–313)-peptide (Fig. 4). Sequencing of this material also revealed minor amounts of additional amino acid phenylthiohydantoin derivatives in the initial cycles, indicating the presence of peptides with similar sequences to the major species, but with one or more residues missing from the *N*-terminus.

The *N*-terminal sequences of the 8 kDa peptides I, II and III were determined for their first 38, 25 and 22 residues respectively (Fig. 4); where they overlapped, the sequences were identical. CNBr treatment of 8 kDa peaks I and II each yielded three fragments (i–iii) with similar retention times on reverse-phase h.p.l.c. All three fragments of 8 kDa peak II and fragment iii of 8 kDa peak I were sequenced. Peptide iii reacted with antiserum L300, and so corresponded to the *C*-terminal fragment. Alignment of the sequences obtained for CNBr-cleavage fragments and for the intact 8 kDa peak II with the sequence deduced from cDNA structure [6,8] indicated that this peptide corresponded to chromogranin A-(248–313)-peptide (8 kDa). Similarly, align-

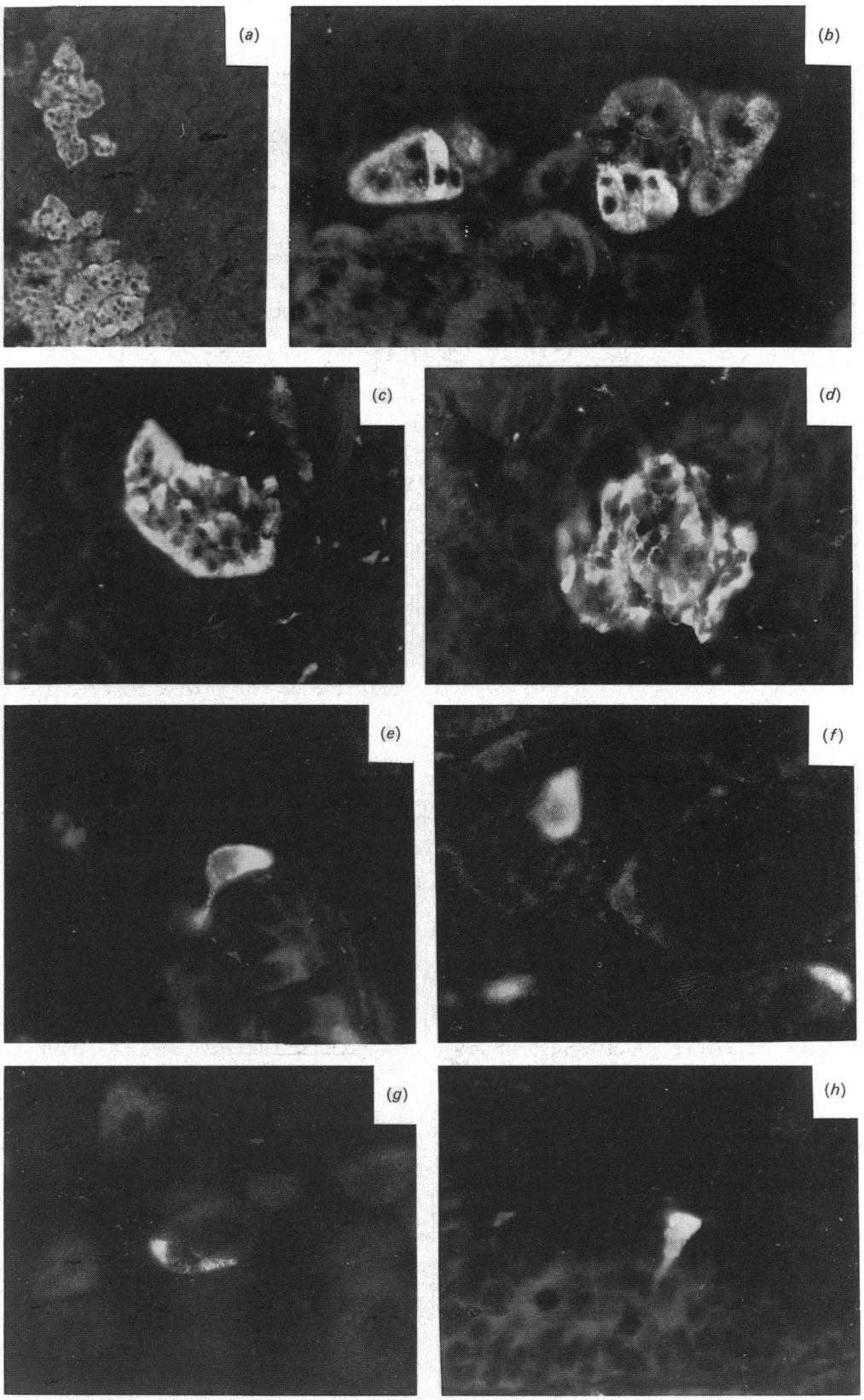

Fig. 5. Immunohistochemical detection of immunoreactivity with antisera L331 and L300 in endocrine cells of the adrenal medulla, pancreas and gut

Tissue sections (8 μm) were treated with antisera L300 and L331 as described in the Methods section. The photomicrographs show adrenal medulla with antiserum L300 (× 180) (*a*) and (× 280) (*b*); pancreas with antiserum L300 (× 280) (*c*) and with antiserum L331 (× 280) (*d*); antral mucosa with antiserum L300 (× 565) (*e*) and with antiserum L331 (× 565) (*f*); corpus mucosa with antiserum L300 (× 565) (*g*); ileum with antiserum L300 (× 565) (*h*).

Table 1. Determination of total LSKEWEDA ir and pancreastatin ir in extracts of bovine adrenal medulla, pancreas, antral mucosa, corpus mucosa and ileum

The tissues were extracted in water followed by 3% (v/v) acetic acid, as described in the Methods section, and the extracts were assayed by radioimmunoassay for LSKEWEDA ir (antiserum L300) and pancreastatin ir (antiserum L331). The values represent means ± S.E.M. ($n = 5$) for all determinations. Less than 5% of total immunoreactivity was found in the acid extracts, and the values for acid and boiling-water extracts are therefore not given separately. Extracts were also treated with 10 μg of trypsin for 3 h at 37 °C followed by 0.2 μg of carboxypeptidase B for 1 h at 37 °C, and assayed by radioimmunoassay with antiserum L300 to give the total LSKEWEDA ir; the ratios of pancreastatin ir to LSKEWEDA ir and of free to total LSKEWEDA ir are shown.

	LSKEWEDA ir (pmol/g)	Pancreastatin ir (pmol/g)	Pancreastatin ir/ LSKEWEDA ir	LSKEWEDA ir (free/total)
Adrenal medulla	3397±1110	142.2±37.0	0.04±0.005	0.11±0.02
Pancreas	639.2±51.7	229.3±22.5	0.36±0.087	1.09±0.20
Antral mucosa	264.4±87.4	135.3±18.0	0.51±0.35	0.78±0.17
Corpus mucosa	187.4±18.1	3.0±0.4	0.02±0.001	0.76±0.13
Ileum	59.2±11.4	5.0±0.6	0.09±0.012	0.52±0.05

ment of the sequences obtained for 8 kDa peak I fragment iii, and for intact 8 kDa peak I, with the sequence predicted from cDNA [7] indicated that 8 kDa peak I also corresponded to chromogranin A-(248–313)-peptide. There were, however, two amino acid residues that differed in 8 kDa peaks I and II; in 8 kDa peak I the residue corresponding to position 293 of chromogranin A was histidine instead of arginine, and at position 301 there was lysine instead of glutamic acid. The differences at these positions are compatible with alternative cDNA sequences previously published [6–8].

Distribution

In immunohistochemistry, antiserum L300 revealed endocrine cells in all regions of the gastrointestinal tract examined (Figs. 5*e*, 5*g* and 5*h*). In contrast, few L331-immunoreactive cells were detected in the gut, although an exception to this was the antral mucosa, where numerous endocrine cells were seen in the deeper parts of the glands (Fig. 5*f*). In pancreatic islets, cells that tended to occur around the islet periphery were similarly stained with both antiserum L300 and antiserum L331 (Figs. 5*c* and 5*d*). Antiserum L300 also revealed chromaffin cells in the adrenal medulla; in contrast, there was no staining with antiserum L331. The specificity of localization was indicated by the fact that staining with antiserum L331 was abolished by YRAPQVLFRG-amide (1 μM) but not by bovine chromogranin A, pig pancreastatin-(1–49)-peptide or YLSKEWEDA (all 1 μM). Staining with L300 was abolished by YLSKEWEDA and bovine chromogranin A (both 1 μM).

To obtain an estimation of the total amount of chromogranin A and chromogranin A-derived proteins in the boiling-water extracts, portions were treated with trypsin and carboxypeptidase B, to release the LSKEWEDA epitope from *C*-terminally extended forms. The resultant ratio of free LSKEWEDA ir to 'total LSKEWEDA ir' gave an estimate of the extent of chromogranin A cleavage in each tissue (Table 1). In adrenal medulla the ratio was 0.11, indicating that a relatively low proportion of chromogranin A was cleaved. In contrast, in the gut there was extensive cleavage, with ratios of 0.76, 0.78 and 0.52 for corpus mucosa, antral mucosa and ileum respectively, and in pancreas the ratio was 1.09 indicating that all chromogranin A was probably cleaved.

To study the processing of chromogranin A further the stoichometric relationships of LSKEWEDA ir and pancreastatin ir were examined in extracts of bovine adrenal medulla, pancreas and gastrointestinal tissues (Table 1). High concentrations of LSKEWEDA ir were found in all five tissues extracted. In contrast, pancreastatin ir was found in relatively high concentrations in pancreas and pyloric antral mucosa, but in low concentrations in gastric corpus mucosa and the ileum (Table 1). The molar ratios of pancreastatin ir to LSKEWEDA ir in antrum and pancreas were 0.51 and 0.36 respectively, compared with less than 0.1 in corpus, ileum or adrenal.

The molecular forms of free LSKEWEDA ir and pancreastatin ir in different tissues were investigated by using Sephadex G-50 gel filtration. The peptides of 8 kDa ($K_{av.}$ 0.2) and 3 kDa ($K_{av.}$ 0.47) were present in all extracts examined, although the ratio of the two forms differed between tissues. In the ileum and corpus mucosa there was a 2-fold excess of 8 kDa over 3 kDa material (Figs. 6*d* and 6*e* and Table 2), whereas 3 kDa material predominated over 8 kDa material in the pancreas and antral mucosa by about 2-fold (Figs. 6*b* and 6*c* and Table 2). In the adrenal medulla the 3 kDa and 8 kDa peptides combined represented < 15% of the free LSKEWEDA ir, and the remainder of the immunoreactivity was high-molecular-mass material found in the void region ($K_{av.}$ 0) (Fig. 6*a* and Table 2); in extracts of corpus mucosa and ileum 20–40% of LSKEWEDA emerged in the void region, but in the pancreas and antral mucosa this material was less than 10%.

Two peaks of pancreastatin ir were detected in each tissue. One had high molecular mass and emerged in the void volume ($K_{av.}$ 0) and the second had an apparent molecular mass of 5 kDa ($K_{av.}$ 0.36) and emerged in a similar position to that of synthetic pig pancreastatin ($K_{av.}$ 0.31). In the pancreas and antral mucosa the pancreastatin-(1–47)-peptide peak predominated and was quantitatively similar to the 3 kDa peak (Figs. 6*b* and 6*c* and Table 2). In the adrenal medulla pancreastatin ir emerged mainly in the void volume (Fig. 6*a* and Table 2).

DISCUSSION

The present findings indicate that chromogranin A is processed by several different pathways in different populations of endocrine cell. In bovine adrenal medulla intact chromogranin A is the major storage product, whereas in pancreatic islets the *C*-terminally amidated fragment, pancreastatin, is a major product. Neither product is well represented in intestinal and gastric corpus endocrine cells; instead *C*-terminally extended forms of pancreastatin predominate. The data imply that in different cells chromogranin A and its products may subserve different functional roles. In part the present findings can be ascribed to cell-specific patterns of post-translational processing pathways. But this may not be the sole mechanism responsible for the distribution of different molecular forms. Thus the sequencing and chromatographic data, taken together with previous cDNA

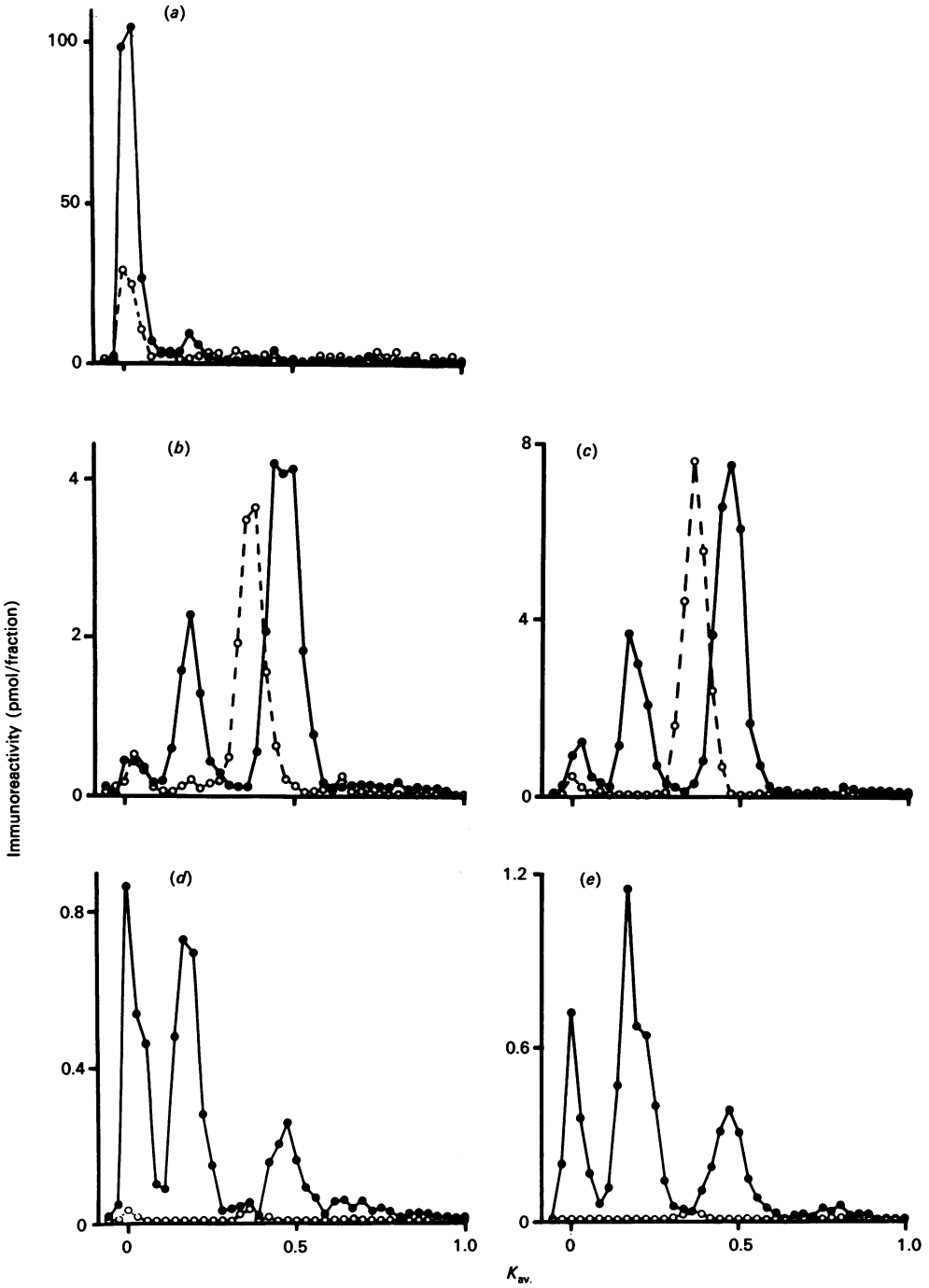

Fig. 6. Analytical Sephadex G-50 gel filtration of bovine adrenal medulla, pancreas and gut extracts

Tissue extracts were applied to a Sephadex G-50 (fine grade) gel-filtration column (1 cm × 100 cm) and eluted with 0.05 M-NH_4HCO_3 containing 0.05% NaN_3 at 4 °C. Fractions (1 ml) were collected and assayed by radioimmunoassay for LSKEWEDA ir (●) and pancreastatin ir (○). The void and bed volumes were determined by Dextran Blue and $Na^{125}I$ respectively. The extracts are: (*a*) adrenal medulla; (*b*) antral mucosa; (*c*) pancreas; (*d*) corpus mucosa; (*e*) ileum.

sequence information, suggest that there are two alternative primary sequences of chromogranin A-derived peptides and that these may be differentially expressed in endocrine cells of the intestine and pancreas.

The amino acid sequence of chromogranin A has been deduced from the nucleotide sequence of cDNA clones; the three cDNA sequences reported for bovine chromogranin A differ in the assignment of seven amino acid residues spread throughout the molecule. Two of these amino acid assignments fall within the sequence for the 8 kDa peptide, chromogranin A-(248–313)-peptides. We have found that in 8 kDa peak I residue 46 (position 293 of chromogranin A) was histidine and residue 54 (position 301 of chromogranin A) was lysine, and this corresponds to one of the reported cDNA sequences [7]. In 8 kDa peak II the residues in these two positions were arginine and glutamic acid respectively, and these assignments correspond to the cDNA sequences reported in two other studies [6,8]. Preliminary studies using digestion with alkaline phosphatase

Table 2. Relative proportions of molecular forms of LSKEWEDA ir and pancreastatin ir in extracts of adrenal medulla, pancreas, antral mucosa, corpus mucosa and ileum

The extracts were fractionated by analytical Sephadex G-50 gel filtration, as described in the Methods section, and the immunoreactivity of each peak was determined. The values represent means ± S.E.M. ($n = 5$) for all determinations. In addition the ratios of 8 kDa to 3 kDa peaks and of pancreastatin-(1–47)-peptide to 3 kDa peaks were determined.

	Molecular forms of LSKEWEDA ir (%)				Molecular forms of pancreastatin ir (%)		
	Void	8 kDa	3 kDa	8 kDa/3 kDa	Void	Pancreastatin-(1–47)-peptide	Pancreastatin-(1–47)-peptide/3 kDa
Adrenal medulla	86±4	12±1	2±1	4.52±2.56	81±3	19±3	0.19±0.4
Pancreas	7±1	29±2	64±2	0.45±0.03	5±1	95±1	0.99±0.22
Antral mucosa	6±1	27±1	67±1	0.42±0.03	13±2	61±3	0.59±0.10
Corpus mucosa	42±2	39±3	19±1	2.28±0.27	39±3	61±3	0.14±0.03
Ileum	2±1	57±2	21±2	2.75±0.46	13±3	87±4	0.26±0.12

indicate that the 8 kDa peak III from ileum is a phosphorylated variant of 8 kDa peak II (A. Watkinson & G. J. Dockray, unpublished work). The alternative cDNA sequences are unlikely therefore to be artifacts of nucleotide sequencing. One possibility is that there has been duplication of the bovine chromogranin A gene [30]; this would explain the polymorphism in the chromogranin A-(248–313)-peptides, as we have isolated both products from the ileum of a single animal. Bovine pancreastatin has been isolated from pancreatic extracts with the use of a chemical method to detect the *C*-terminal glycine amide [31]. Despite there being two possible variants of bovine pancreastatin, only a single 47-residue form was identified and sequenced; the sequence corresponded unambiguously to that of the *N*-terminal 47 amino acid residues of 8 kDa peak II. The 3 kDa material that we have characterized corresponds to the *C*-terminal fragment of 8 kDa peak II, i.e. the co-product of cleavage yielding pancreastatin-(1–47)-peptide (Fig. 1). The identity of other 3 kDa products is unknown; however, since there was sequence information to suggest *N*-terminal trimming of 3 kDa material, it may be that these peptides are readily degraded, and that the less acidic forms are partial degradation products. The latter are in any case usually minority molecular species. The present results raise the interesting possibility that one chromogranin A sequence can be processed to pancreastatin but the other can only yield the 8 kDa peptide, and is not processed further. The two alternative cDNA sequences were both derived from clones of adrenal medulla and hence presumably both genes are expressed in this tissue as well as in the ileum. Direct support for the idea of multiple cloning-related chromogranin A genes that are differentially expressed has recently been provided by Abood & Eberwine [32].

Our immunohistochemical data show differential localization of LSKEWEDA ir and pancreastatin ir and so clearly demonstrate the cell-specific nature of chromogranin A processing. It was also noticeable that both pancreastatin ir and LSKEWEDA ir were found only in endocrine cells. Chromogranin A has been reported to occur in adrenergic neurons [19,33], but our antisera did not reveal nerve fibres, suggesting that proteolytic cleavage of chromogranin A is an endocrine-cell characteristic. Taken together, the immunohistochemical, sequence and distribution data suggest that there are three alternative chromogranin A-processing pathways: an adrenal medulla-type pathway, a pancreatic and antral mucosal-type pathway and an ileal and corpus mucosal-type pathway. The adrenal medulla is exceptionally rich in chromogranin A and large chromogranin A-derived fragments [2,15,16,33] and proteolytic cleavage is the least extensive, so that high-molecular-mass forms predominate. The high-molecular-mass LSKEWEDA-ir material in adrenal medulla had an apparent molecular mass of 51 kDa on SDS/PAGE (results not shown), which, owing to its size and immunoreactivity, is probably chromogranin A-(1–313)-peptide. The partial processing of the peptide in adrenal chromaffin cells is reminiscent of that of the opioid precursor proenkephalin, which also occurs in high-molecular-mass forms in the adrenal [34]. Evidently the cleavage mechanisms of the bovine chromaffin cell are poorly developed.

The biosynthetic pathway in the pancreas and antral mucosa leads to the production of the putative active product pancreastatin-(1–47)-peptide and its major co-product chromogranin A-(297–313)-peptide (i.e. 3 kDa peptide) (Fig. 1). In the ileum and corpus mucosa there is extensive cleavage of chromogranin A but there is minimal conversion into pancreastatin. Instead, chromogranin A-(248–313)-peptide is a major product, although significant amounts of the high-molecular-mass material also occur. The physiological relevance of this pathway remains to be established, and in particular it will be interesting to see whether *C*-terminally extended forms of pancreastatin, such as chromogranin A-(248–313)-peptide, retain biological activity. The major actions of pancreastatin include inhibition of insulin (and possibly glucagon and somatostatin) release from pancreatic islets [14,35], the inhibition of pancreatic exocrine secretion and the inhibition of histamine- or carbachol-stimulated gastric acid secretion [36–38]. Since *C*-terminally extended forms of pancreastatin are well represented in the gastric corpus mucosa, it would seem that gastric functions deserve special consideration.

It is well recognized that multiple patterns of processing are commonly found in endocrine peptide precursors. The chromogranins appear unique, however, in that (*a*) they are widely distributed, (*b*) there are extensive differences in the distribution of different forms and (*c*) the different naturally occurring fragments are derived from variation in the processing of more than one related primary sequence. The processing mechanisms themselves would appear to be governed by rules that are applicable to other endocrine cell peptides, e.g. cleavage at pairs of basic residues, which encourages the idea that the multiple products produced in different cell types subserve different biological functions.

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