Post-translational processing of chromogranin A: differential distribution of phosphorylated variants of pancreastatin and fragments 248–313 and 297–313 in bovine pancreas and ileum

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Chromogranin A is a secretory protein expressed widely in neuroendocrine cells. It is known to be phosphorylated but the precise sites of phosphorylation are not known. We have isolated, from bovine pancreas and ileum, chromogranin A fragments corresponding to a region giving rise to a biologically active product, pancreastatin. Phosphorylation patterns were determined by fast atom bombardment mass spectrometry and alkaline phosphatase digestion followed by ion-exchange chromatography and radioimmunoassay. In the pancreas, there were unmodified, mono- and di-phosphorylated forms of the fragment chromogranin A(248–313) with Arg and Glu at positions 293 and 301 respectively; in addition, there were small amounts of monophosphorylated peptide with an alternative primary sequence of His and Lys at 293 and 301 respectively. Two products of cleavage, pancreastatin and the fragment 297–313, were also found in unmodified and monophosphorylated forms. In the ileum, peptides with both alternative primary sequences were found, pancreastatin was absent, and phosphorylation was generally less than in the pancreas. Chromogranin A-derived peptides therefore exhibit tissue-specific patterns of phosphorylation and cleavage, and at least two phosphorylation sites occur in the region giving rise to a biologically active product.

INTRODUCTION

Peptides of the chromogranin family are widely distributed in neuroendocrine cells [1,2]. The chromogranins are potentially important in the packaging of protein in secretory granules [3,4], but in addition they may also be the precursors of biologically active peptides [5]. The first member of the family to be characterized, chromogranin A (CGA), has been cloned and sequenced from bovine adrenal medulla [6–8]. The primary amino acid sequence of CGA includes several pairs of basic amino acids which constitute well-known sites of endopeptidase cleavage in the generation of small active peptides. One active fragment of CGA, pancreastatin [CGA(248–249)amide], has been shown to have inhibitory actions on endocrine and exocrine pancreatic secretion and on gastric parietal cells [9–12].

We recently showed that the region of CGA giving rise to pancreastatin is subject to tissue-specific patterns of processing in adrenal, pancreas and gastrointestinal endocrine cells [13]. In adrenal, there was relatively little cleavage of CGA; in endocrine cells of the gut and pancreas, most CGA was cleaved at pairs of basic residues in positions 246/247 and 314/315: in the ileum and acid-secreting part of the stomach, the product CGA(248–313) predominated, but in pancreatic and pyloric antral endocrine cells the major products of processing in this region were pancreastatin and a peptide corresponding to CGA(297–313) (Figure 1). All three peptides found in the gut and pancreas occurred in a variety of forms which were separated by ion-exchange chromatography. We were able to characterize some of these variants as corresponding to the products of two different cDNA sequences which have previously been reported [6–8]. It is known that, during biosynthesis, CGA is also modified by phosphorylation, sulphation and glycosylation [2,14–18]. However, the precise sites at which these modifications occur are not known. Because there are several putative phosphorylation sites in the sequence CGA(248–313), it seems possible that the variants found earlier might differ in phosphorylation state. We have now examined this possibility in more details.

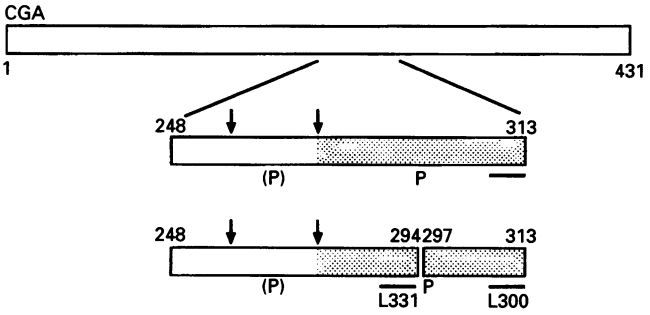

Figure 1 Schematic representation of the structure of bovine CGA

The specificity of antibodies used in the present study is shown: antibody L300 reacts with peptides having an exposed C-terminus corresponding to CGA(306–313); antibody L331 reacts with the amidated C-terminus of bovine pancreastatin. Peptides 248–313, 297–313 and pancreastatin have been identified by isolation and sequencing; the stippled regions correspond to the C-terminal CNBr fragments used for f.a.b.-m.s. studies. Arrows indicate CNBr-cleavage sites. Phosphorylation sites are shown as P.

MATERIALS AND METHODS

Peptides

A synthetic analogue of the C-terminal nonapeptide of pancreastatin, Y^{0}CGA(286–294) [i.e. YRAOQVLFRGamide or pancreastatin (39–47)] was obtained from Multiple Peptide

Abbreviations used: CGA, chromogranin A; f.a.b. m.s., fast atom bombardment m.s.
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Systems (San Diego, CA, U.S.A.). Synthetic Y^{0}CGA(306–313) (i.e. YLSKEWEDA), which corresponds to a fragment of the region to the immediate C-terminus of the pancreastatin sequence, was prepared as previously described [13].

Radioimmunoassay

Peptides with the C-terminal sequence of pancreastatin were detected by radioimmunoassay using antibody L331 raised to the C-terminal nonapeptide fragment of pancreastatin as described by Watkinson et al. [13] (Figure 1). Peptides with the C-terminal sequence corresponding to CGA(306–313) were detected by radioimmunoassay using antibody L300 as described previously [13] (Figure 1).

Tissue extracts

The extraction of CGA-related peptides from the pancreas and gut was carried out as previously described [13]. Briefly, tissues were obtained from a local abattoir and transported to the laboratory on solid CO_2. Peptides were extracted by adding deeply frozen tissue directly to boiling water, followed by homogenization and centrifugation; CGA-related peptides were then concentrated on Whatman DE-23 and further purified on Sephadex G-50 fine (5 cm × 100 cm) eluted with 0.05 M NH_4HCO_3 containing 0.05% (w/v) NaN_3 at 4 °C. Immunoreactive peptides in the column eluates were further purified by ion-exchange chromatography on Whatman DE-52 (1 cm × 10 cm) or f.p.l.c. Mono Q with a gradient of 0.05–0.5 M ammonium acetate, pH 6.5, at 4 °C or (in the case of pancreastatin) 0.02–0.2 M ammonium acetate, pH 7.7.

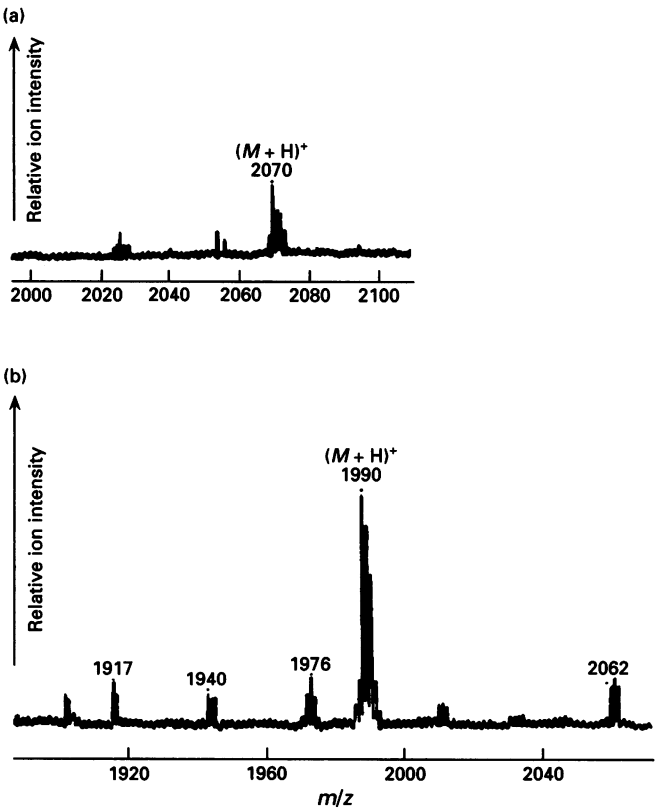

Figure 2 F.a.b. m.s. analysis of bovine pancreatic CGA(297–313) peptides

Two peptides were separated by ion-exchange chromatography and detected in radioimmunoassays using antibody L300. The molecular mass of the more acidic peptide (**a**) corresponded to phosphorylated CGA(297–313) and that of the less acidic variant (**b**) to unphosphorylated CGA(297–313).

Reversed-phase h.p.l.c.

Further purification of peptides was obtained using three systems: μBondapak C18 z-module cartridge (Waters Associates, Milford, NJ, U.S.A.) eluted with 0.1% trifluoroacetic acid and acetonitrile; PLRP-S C18 (Polymer Laboratories, Church Stretton, Shropshire, U.K.) eluted with 50 mM NH_4HCO_3 and acetonitrile, and Techsil 5μ C18 (4 mm × 250 mm) eluted with 0.1% trifluoroacetic acid and acetonitrile.

Alkaline phosphatase digestion

Purified peptides were incubated with 2 units of alkaline phosphatase (*Escherichia coli*, 60 units/mg, type III, Sigma) in 100 μl of 0.05 M NH_4HCO_3 for 5 h at 37 °C. The products were separated by f.p.l.c. on a mono Q anion-exchange column (Pharmacia, Uppsala, Sweden) eluted with a gradient from 0.05 M to 1 M ammonium acetate, pH 6.5.

Fast atom bombardment m.s. (f.a.b. m.s.)

Fast atom bombardment analysis was carried out using a VG Analytical ZAB 2SE high-field mass spectrometer operating at $V_{acc.}$ = 8 kV. A caesium ion gun was used to generate ions for the acquired mass spectra which were recorded using either a PDP 11-250J data system or chart paper. Mass calibration was performed using caesium iodide. Samples were dissolved in 5% (v/v) acetic acid and analysed using a matrix of glycerol/thioglycerol (1:1, v/v).

RESULTS

Isolation and characterization of CGA-derived peptides

Assays using antibody L300

Two groups of CGA-derived peptides were identified in extracts of pancreas fractionated by Sephadex G-50 and detected by radioimmunoassays using antibody L300; the two groups have previously been shown to correspond to peptides with the sequence CGA(297–313) and CGA(248–313) [13]. When the CGA(297–313) fraction was separated by f.p.l.c., two peaks were resolved, which were purified to homogeneity by h.p.l.c. Analysis of the first peak by f.a.b. m.s. produced an ion of 1990 Da $(M+H)^+$ which is compatible with the predicted mass off SGEPEQEEQLSKEWEDA, i.e. CGA(297–313) (Figure 2). The second peak had a molecular mass of 2070 Da $(M+H)^+$; the difference in mass is attributable to serine phosphorylation.

Peptides of the CGA(248–313) fraction were resolved by ion-exchange chromatography into four peaks (1–4; Figure 3). In extracts of pancreas, peak 1 was a minor component which was only visualized adequately after prior concentration. The major peak (3, in order of elution) was purified to homogeneity by h.p.l.c., cleaved by CNBr, and the products were separated by reversed-phase h.p.l.c. The C-terminal CNBr fragment was identified by radioimmunoassay using antibody L300, and analysed by f.a.b. m.s. Ions with masses of 3353 Da $(M+H)^+$ and 3375 Da $(M+Na)^+$ were identified (Figure 4); these are compatible with the predicted mass of monophosphorylated CGA(285–313) (i.e. the C-terminal CNBr fragment of 248–313: ARAPQVLFRGGKSGEPEQEEQLSKEWEDA). The initial

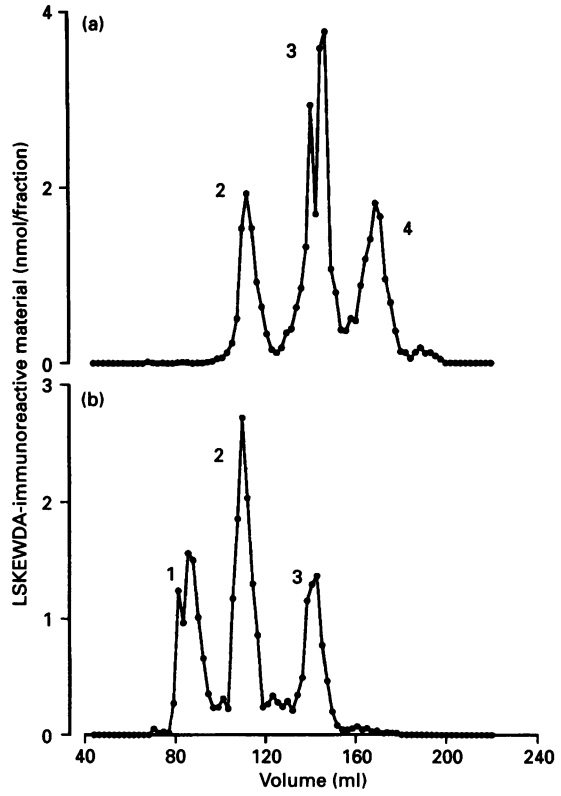

Figure 3 Separation by ion-exchange chromatography of CGA(248–313) immunoreactive material from pancreas (a) and ileum (b)

Note four peaks of activity: the first three (1–3) occur in the ileum, but in the pancreas peak 1 is poorly represented and peaks 2–4 predominate. Samples were fractionated on DE-52 (1 cm × 10 cm) using a gradient from 0.05 to 0.5 M sodium acetate (pH 6.5).

decapeptide in this sequence is also shared with pancreastatin; there are, however, two possible structures for this region of CGA based on predictions from cDNA sequencing [6–8,13,19]. The polymorphism may arise from different alleles, one encoding arginine and the other histidine in position 9 of this sequence. Peak 3 is evidently the monophosphorylated Arg^9 variant.

Pancreastatin: assays using antibody L331

Sephadex G-50 gel filtration of pancreatic extracts separated a major and a minor peak of material detected with the antibody specific for the C-terminus of pancreastatin, L331 (Figure 5). The major peak was co-eluted with synthetic pancreastatin. On ion-exchange chromatography, it was further resolved into major and minor immunoreactive components (pancreastatins 1 and 2), which were purified to homogeneity on h.p.l.c. After CNBr cleavage, each yielded three fragments with no apparent differences between pancreastatins 1 and 2. The two C-terminal fragments were identified by immunoreactivity with L331; on analysis by f.a.b. m.s., they both had a molecular mass of 1114 Da $(M+H)^+$ which corresponded to that predicted for the C-terminal CNBr-cleavage product of pancreastatin, i.e. ARAPQVFRGamide ($+H^+$, 1113.7 Da) (Figure 6).

Alkaline phosphatase digestion

Information on phosphorylation patterns was obtained by incubation of extracts with alkaline phosphatase and subsequent Mono Q f.p.l.c. Treatment of pancreastatin 2 changed its retention time from 13 min to 6 min which coincided with the retention time of pancreastatin 1 (Figure 7). The latter was resistant to alkaline phosphatase.

Alkaline phosphatase digestion also converted the more acidic of the two CGA(297–313) peptides into material that had a

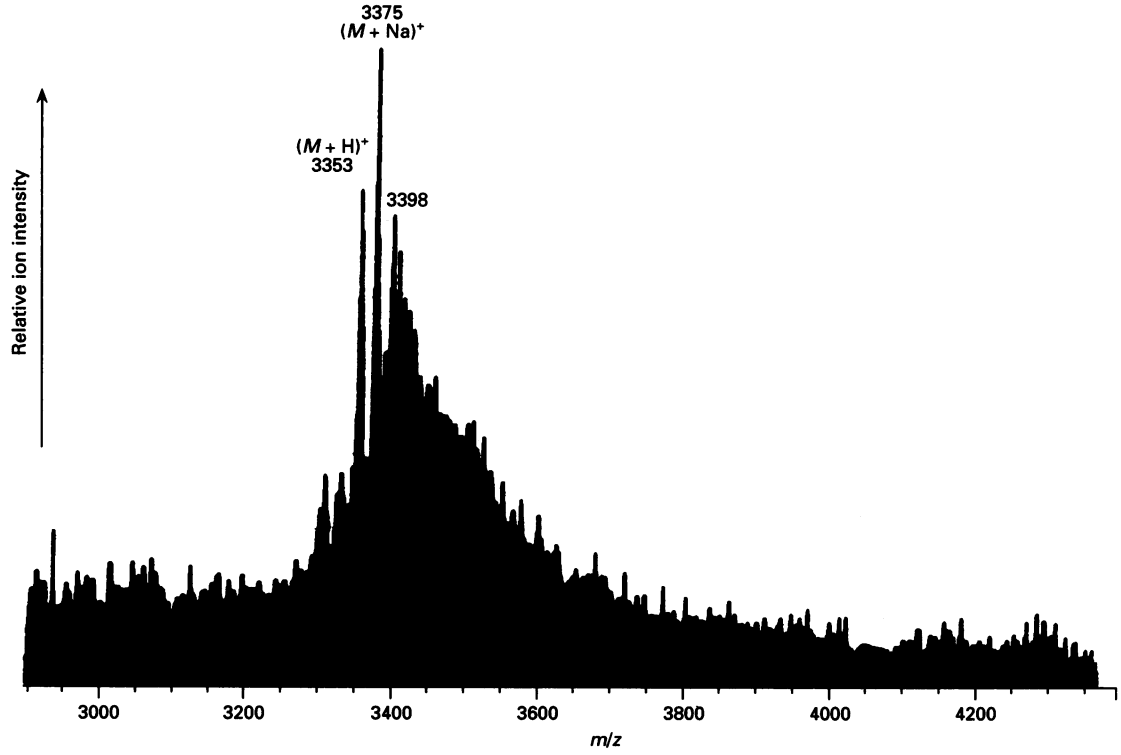

Figure 4 F.a.b. m.s. analysis of the C-terminal CNBr-cleavage fragment of the major form of CGA(248–313) in bovine pancreas

Note molecular ions corresponding to the monophosphorylated C-terminal CNBr fragment of CGA(248–313) ($M+H^+$, 3353; $M+Na^+$, 3375).

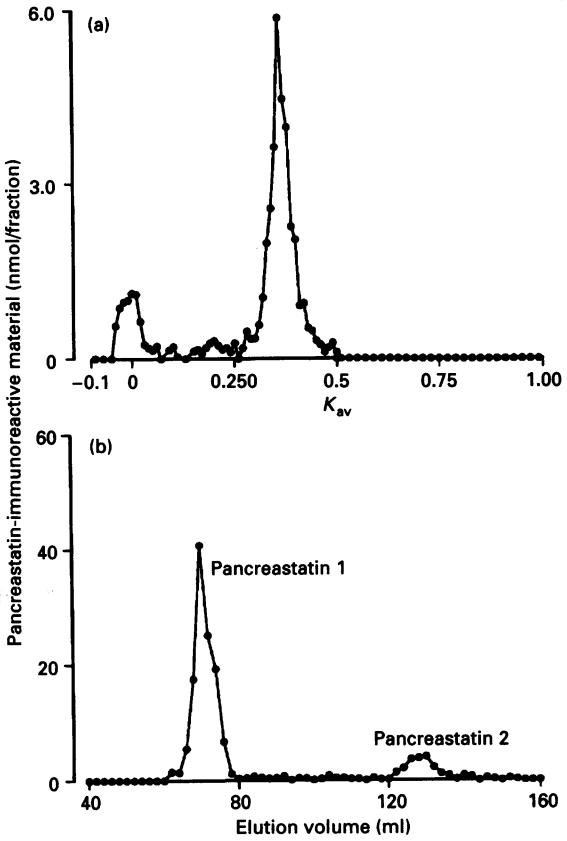

Figure 5 Purification of pancreastatin from bovine pancreas

(**a**) Separation on Sephadex G-50 fine (5 cm × 100 cm, in 50 mM sodium acetate) of bovine pancreas. Column eluates were assayed with antibody L331 which is specific for the C-terminus of pancreastatin. Fractions of 12 ml were collected. (**b**) The major peak in Sephadex eluates was applied to DE-52 (1 cm × 10 cm, eluted with a gradient from 0.02 to 0.2 M sodium acetate, pH 7.7) when two immunoreactive forms (pancreastatins 1 and 2) were separated.

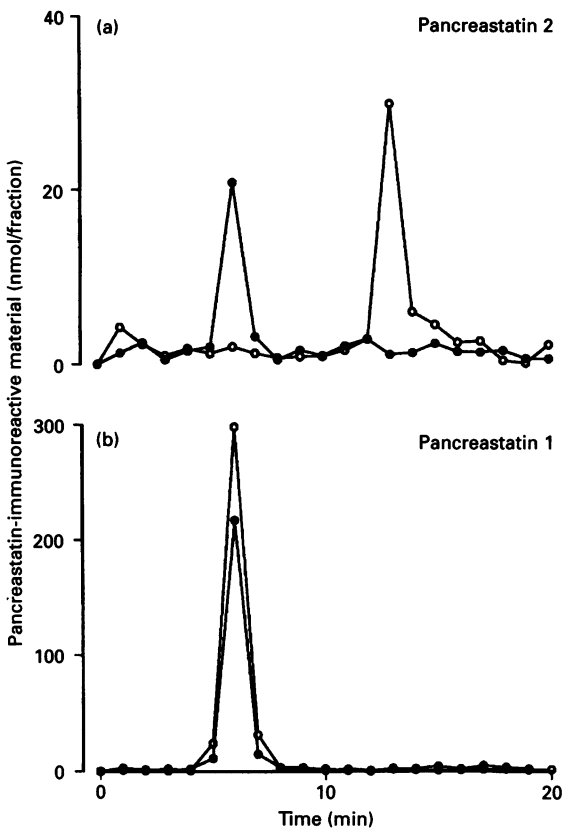

Figure 7 Digestion of the two peaks of pancreastatin immunoreactivity with alkaline phosphatase

(**a**) After alkaline phosphatase digestion and separation by ion-exchange chromatography on Mono Q, pancreastatin 2 (see Figure 5b) was converted into material with the retention time of pancreastatin 1. (**b**) The elution position of pancreastatin 1 taken from DE-52 eluates (Figure 5) was not altered by alkaline phosphatase digestion. ○, Control; ●, alkaline phosphatase.

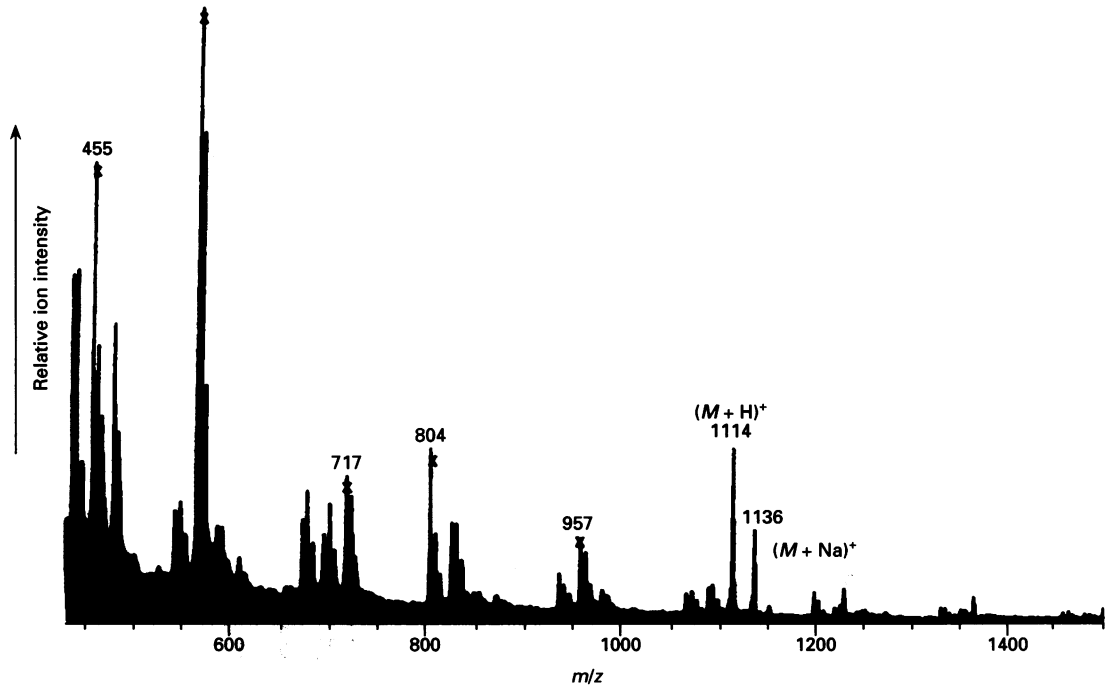

Figure 6 F.a.b. m.s. analysis of the C-terminal CNBr-cleavage fragment of pancreastatin 1

The products of CNBr digestion were fractionated by h.p.l.c., and the C-terminus was identified by radioimmunoassay using antibody L331. Note molecular mass of 1114 Da which corresponds to that predicted. Similar results were obtained for pancreastatin 2.

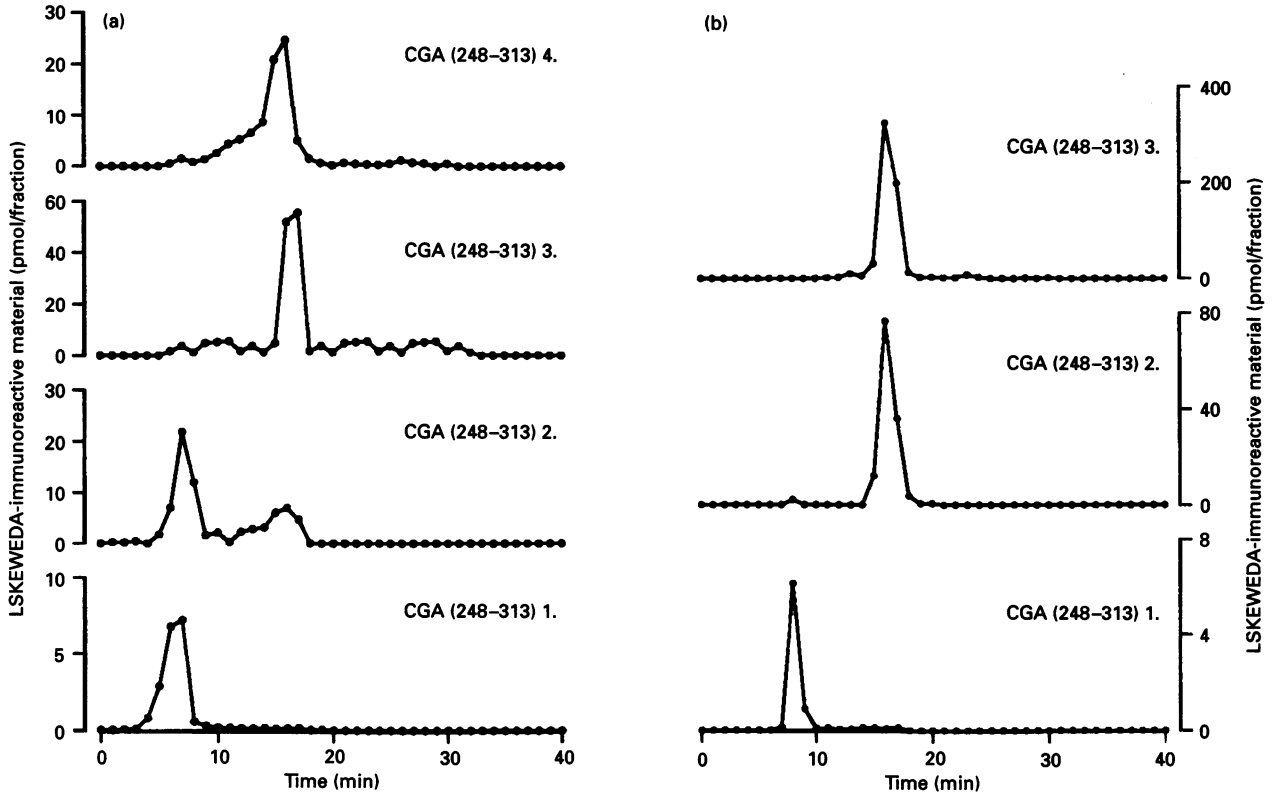

Figure 8 Alkaline phosphatase digestion of CGA(248–313) peptides from bovine pancreas and ileum

(**a**) Pancreatic peptides: note that after ion-exchange chromatography, alkaline phosphatase digests of peak 2 (see Figure 3) were separated into two products; the major one had a retention time identical with that of peak 1, and the minor one had the retention time of the starting material. Peak 1 (see Figure 3) was resistant to alkaline phosphatase. Digestion of peaks 3 and 4 (see Figure 3) produced material that was co-eluted with the original peak 2 material. (**b**) Ileal peptides: note that peak 1 had a similar retention time to peak 1 from pancreas and was resistant to alkaline phosphatase digestion. Unlike the corresponding material from the pancreas, ileal peak 2 was also resistant to alkaline phosphatase. Digestion of ileal peak 3 gave material that was co-eluted with peak 2, and so resembled the corresponding pancreatic material.

retention time (22 min) identical with that of the less acidic peptide. The less acidic peptide was resistant to alkaline phosphatase. Of the four CGA(248–313) peptides found in the pancreas, the least acidic peptide [CGA(248–313)1] was unaffected by alkaline phosphatase (Figure 8). The two most acidic peptides (3 and 4 in order of elution) were both converted into material that was co-eluted with peak 2. Interestingly, when native peak-2 material was digested with alkaline phosphatase, two products were separated: one had its original retention time and was presumably unaltered, the other was co-eluted with peak 1.

Comparison of pancreatic and ileal peptides

Pancreastatin-like immunoreactive peptides were virtually undetectable in the bovine ileum, whereas both CGA(248–313) and CGA(297–313) peptides were present, indicating tissue-specific patterns in post-translational processing of the C-terminus of pancreastatin. In the ileum, as in the pancreas, two forms of CGA(297–313) were separated by ion-exchange chromatography, and the more acidic of them was converted by alkaline phosphatase into material with a retention time identical with the less acidic peptide (not shown).

As previously noted [13], only three peaks of CGA(248–313) were separated in ileal extracts after ion-exchange chromatography. These corresponded to peaks 1–3 in pancreatic extracts (Figure 3). Digestion of the ileal peptides with alkaline phosphatase converted peak 3 into material with the retention time of peak 2 (Figure 8). However, when the ileal peaks 1 and 2 were digested with alkaline phosphatase, there was no change in their elution positions (Figure 8).

DISCUSSION

CGA is known to be phosphorylated at multiple sites [14–16]. In the present study we have identified one of these sites as the region giving rise to the active peptide pancreastatin and another in the sequence immediately C-terminal to pancreastatin. The patterns of phosphorylation differ between bovine pancreas and ileum, and between two alternative primary sequences of CGA expressed in bovine ileum. It is of interest that, in the same tissues, there are differences in the patterns of cleavage and amidation in peptides derived from this region of CGA [13].

Phosphorylation is a relatively common modification of secretory peptides. It is found in peptides released by both the constitutive, e.g. casein and vitellogenin [20,21], and the regulated e.g., prolactin, adenocorticotropin, gastrin and enkephalin [22–25], routes of secretion. It seems to be a relatively late post-translational modification and probably occurs in the *trans*-Golgi or in the *trans*-Golgi network. In several instances, e.g. casein, progastrin and proenkephalin, serine-phosphorylation sites are followed by one or several acidic residues at positions two or more to the C-terminus and are potential substrates for physiological casein kinase. The significance of phosphorylation

of secretory peptides is still largely unknown, but, in some peptides secreted by the regulated pathway, it is clear that phosphorylation sites are either immediately adjacent or relatively close to important sites of cleavage, e.g. in progastrin, proenkephalin and adrenocorticotropin. The putative phosphorylation sites in CGA are at serine residues in the same general sequence as that defined above and, in the case of the 297–313 fragment, are close to a cleavage site important for generating a biologically active product.

There are two alternative sequences in the 248–313 region of bovine CGA: Arg^{293} and Glu^{301} compared with His^{293} and Lys^{301} [13]. The Arg/Glu variant has a net additional negative charge compared with the His/Lys variant and so the two can be readily separated by ion-exchange chromatography; they correspond to peaks 2 and 1 respectively after alkaline phosphatase digestion of the CGA(248–313) fraction. When the His/Lys variant is phosphorylated, the extra negative charge causes it to be co-eluted on ion-exchange chromatography with the unphosphorylated Arg/Glu variant, so that alkaline phosphatase digestion is needed to assay the two forms separately.

In the pancreas, the His/Lys variant appears to be largely monophosphorylated, whereas in the ileum it is largely unphosphorylated. The Arg/Glu variant predominates in the pancreas, and, unlike the His/Lys variant, occurs in phosphorylated form in the ileum. Moreover, the Arg/Glu variant occurs in both mono- (Figure 3, peak 3) and di- (Figure 3, peak 4) phosphorylated forms in the pancreas but only in the monophosphorylated form in the ileum. It is interesting that substitution of Lys for Glu in position 301 would remove a positive charge four positions C-terminal to serine which is the putative phosphorylation site. One consequence could be that this site is less favoured as a substrate for Golgi-resident kinases. In any event it appears that there are differences between tissues in the phosphorylation of peptides derived from the 248–313 region of CGA, and that these differences include the extent to which the two alternative primary sequences are modified. Although it is clear that CGA is expressed in pancreatic islet cells and gut endocrine cells, it is not known whether the two primary sequences are expressed in a single population of pancreatic and ileal cells, and indeed whether they have similar functions. A recent report on the genomic sequence of bovine CGA gave a predicted sequence corresponding to Arg^{293}/Glu^{301} and suggested that alternative sequences were cloning or sequencing artifacts [19]. Our previous finding that both primary structures predicted from cDNA cloning can be found by amino acid sequencing [13] makes this explanation unlikely. Further work is needed to provide a molecular basis for our understanding of the significance of the alternative sequence.

The biosynthesis of pancreastatin in bovine pancreas evidently proceeds via cleavage of CGA at pairs of basic residues in positions 246/247 and 314/315 to generate the fragment 248–313 which is then further cleaved at Lys^{296}. Our data indicate that both cleavage products, i.e. pancreastatin and CGA(297–313), occur in phosphorylated and unphosphorylated forms. We therefore interpret the data as indicating that CGA(248–313) may be phosphorylated at either or both of two sites on each side of Lys^{296}; cleavage at this site can therefore give rise to both phosphorylated and unphosphorylated pancreastatin and CGA(297–313). The relative proportions of phosphorylated and unphosphorylated CGA(297–313) were similar in the ileum and pancreas. However, CGA(297–313) was relatively much more abundant (compared with CGA(248–313) in the pancreas than the ileum [13]. Moreover, in bovine ileum, pancreastatin is virtually undetectable, suggesting that, although there might be some cleavage at Lys^{296}, there is no further processing to the amidated C-terminus of pancreastatin. It is of interest that mono- but not di-phosphorylated CGA(248–313) was detected in the ileum and that, as a proportion of total immunoreactivity, the phosphorylated peptide accounted for about 25% of total in the ileum compared with 75% in the pancreas. As found elsewhere [26] therefore, there is an approximation between the degree of phosphorylation and the degree of cleavage and amidation. The basis for this is not known, but it seems possible that for at least some peptides delivered to the regulated pathway of secretion, phosphorylation may provide a marker for the extent of further processing to which the precursor is subjected.

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