The post-translational processing of chromogranin A in the pancreatic islet: involvement of the eukaryote subtilisin PC2

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The post-translational processing of chromogranin A (CGA) and the nature of the enzyme(s) involved were investigated in rat pancreatic islet and insulinoma tissue. Pulse–chase radiolabelling experiments using sequence-specific antisera showed that the 98 kDa (determined by SDS/PAGE) precursor was processed to an N-terminal 21 kDa peptide, a C-terminal 14 kDa peptide and a 45 kDa centrally located peptide with a rapid time course ($t_{\frac{1}{2}}$ approx. 30 min) after an initial delay of 30–60 min. The 45 kDa peptide was, in turn, converted partially into a 5 kDa peptide with pancreastatin immunoreactivity and a 3 kDa peptide with WE-14 immunoreactivity over a longer time period. Incubation of bovine CGA with rat insulinoma secretory-granule lysate produced peptides of 18, 16 and 40 kDa via intermediates of 65 and 55 kDa. N-terminal sequence analysis indicated that cleavage occurred at the conserved paired basic sites Lys^{114}-Arg^{115} and Lys^{330}-Arg^{331}, suggesting that cleavage of the equivalent sites (Lys^{129}-Arg^{130} and Lys^{357}-Arg^{358}) in the rat molecule produced the initial post-translational products observed in intact pancreatic β-cells. The enzyme activity responsible for the cleavage of bovine CGA co-chromatographed on DEAE-cellulose with the type-2 proinsulin endopeptidase and with PC2 immunoreactivity. The type-1 enzyme (PC1/3) appeared inactive towards CGA. The requirement for Ca^{2+} ions and an acidic pH for conversion was consistent with the involvement of a member of the eukaryote subtilisin family, and the composition of the released peptides in pulse–chase and secretion studies suggested that conversion occurred in the secretory-granule compartment. The overall catalytic rate as well as the relative susceptibilities of the Lys^{114}-Arg^{115} and Lys^{330}-Arg^{331} sites to cleavage were affected by pH, suggesting that the ionic environment of the processing compartment may play a role in the differential processing of CGA which is evident in various neuroendocrine cells.

INTRODUCTION

Chromogranin A (CGA) is an acidic glycoprotein of molecular mass 48 kDa (70–100 kDa by SDS/PAGE) found in the dense-core secretory vesicles of many neuroendocrine cells [1,2]. Along with other members of the granin family (CGB and secretogranin II), it has served as an important marker for studies of secretory cell biology and neuroendocrine tissue pathology [3]. A number of functions have been proposed for the intact molecule. These include amine binding [4], osmoregulation [5], Ca^{2+} binding [6] and sorting of secretory proteins [2]. There is growing support, however, for its role as a prohormone. Its primary amino acid sequence contains several conserved paired basic residues [7,8] which constitute potential sites of cleavage. In rat pancreatic β-cells, CGA undergoes extensive post-translational processing in parallel with proinsulin to release the N-terminal peptide β-granin [9]. Inhibitor and ion-dependency studies have shown that the processing activities were indistinguishable from those involved in proinsulin conversion, with the most likely cleavage site being Lys^{129}-Arg^{130} [10] (see Figure 1). Studies using an ileal carcinoid tumour have shown that CGA is cleaved at two other Lys-Arg sites (Lys^{341}-Arg^{342} and Lys^{357}-Arg^{358} in rat CGA) to liberate a tetradecapeptide, WE-14, which is conserved between the human, rat, bovine, mouse and pig CGA sequences [11]. CGA also contains the sequence for pancreastatin (PST) which has been shown to be a potent inhibitor of pancreatic islet [12], parietal cell [13] and parathyroid cell [14] secretion. In contrast with the cleavages that give rise to β-granin and WE-14, PST is probably generated by excision of single basic residues flanking the sequence followed by the action of a peptide-amidating mono-oxygenase at the C-terminus to produce the observed amidated structure. Other CGA-derived peptides have been reported including chromostatin (residues 124–143, bovine CGA), an inhibitor of chromaffin cell secretion [15], and the vaso-inhibitory peptide vasostatin (residues 1–76, bovine CGA) [16].

CGA, like pro-opiomelanocortin (POMC) and proglucagon, appears to be processed in a tissue-specific manner, and within particular tissues there is considerable heterogeneity in the immunostaining pattern suggestive of differential processing [17]. It has been postulated that such differences reflect the distribution of the endopeptidases responsible for the initial cleavage of the precursor. This issue has been addressed here by investigating the relationship between the CGA-converting activity and the type-1 and type-2 proinsulin-processing enzymes which correspond to the subtilisin-related proteases PC1 and PC2 respectively [18,19]. These enzymes show sequence-specific cleavage, differential regulation by pH and Ca^{2+} [20] and differential tissue distribution [38,39], which make them strong candidates as regulatory elements in a proteolytic cascade.

EXPERIMENTAL

Materials

Unless indicated, all chemicals were purchased from either Sigma (Poole, Dorset, U.K.) or BDH (Poole, Dorset, U.K.).

Abbreviations used: CGA, chromogranin A; PST, pancreastatin; POMC, pro-opiomelanocortin; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

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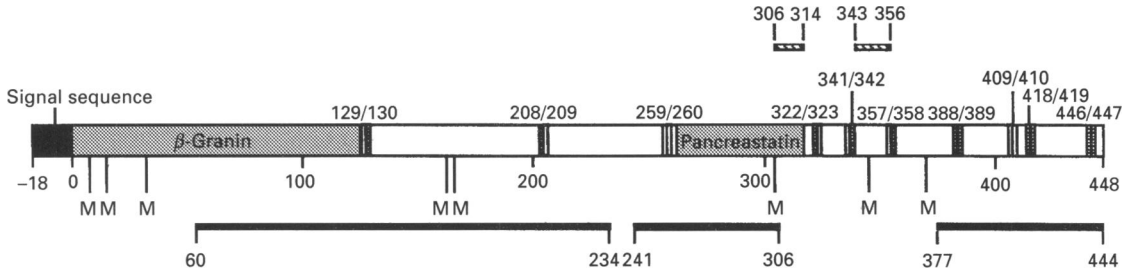

Figure 1 Schematic representation of rat CGA

Paired basic amino acid sites are indicated by combinations of open (Lys) and hatched (Arg) bars. Methionine residues are indicated by the single-letter code M. Antisera were produced in rabbits immunized with either β-galactosidase fusion proteins (■) or synthetic peptides (▒) incorporating the indicated sequences.

Tissues

Insulinoma tissue was propagated in New England Deaconess Hospital (NEDH) rats and the tumour cells were purified by Percoll-density-gradient centrifugation as described previously [9]. Secretory granules were prepared from rat insulinoma homogenates by Nycodenz density-gradient centrifugation by the method of Hutton et al. [21]. Granules were lysed by sonication for 15 s at an amplitude of 3 μm (MSE sonifier) in 1 ml of 25 mM sodium acetate (pH 5.5) containing 1% (v/v) Triton X-114, 10 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), 1 mM phenylmethanesulphonyl fluoride and 10 μM pepstatin A, and then centrifuged for 30 min at 48000 *g* to remove insoluble material. The supernatant was incubated for 3 min at 30 °C and centrifuged for 3 min at 13000 *g* to separate the detergent and aqueous phases. The aqueous phase was retained and stored at −70 °C before use. Islets of Langerhans were isolated from NEDH rat pancreata by a collagenase-digestion technique [22].

Antisera

β-Galactosidase fusion proteins incorporating amino acids 241–306 and 377–444 of rat CGA (see Figure 1) were produced by cloning *Hae*III and *Sau*3A–*Pau*II restriction fragments respectively into the *Sma*I site of the bacterial expression vectors pUEX 3 and 1 [23]. Inclusion-body preparations were subjected to SDS/PAGE and fusion proteins were visualized by u.v. shadowing, excised, homogenized using a glass tube homogenizer (Jencons Scientific, Leighton Buzzard, Beds., U.K.) and injected into rabbits for antisera production. An antiserum against a β-galactosidase fusion protein incorporating residues 60–234 of CGA (see Figure 1) was raised in guinea pigs as described previously [17]. Rabbit antisera were produced against synthetic peptide–BSA conjugates corresponding to amino acids 306–314 (C-terminally amidated) and 343–356 of rat CGA (see Figure 1) as described by Curry et al. [24]. Antisera were raised in rabbits against glutathione S-transferase fusion proteins incorporating residues 111–137 and 158–391 of rat PC1 and PC2 respectively, which were produced using the bacterial expression vector pGEX-3X essentially as described [19].

Purification of CGA

Bovine CGA, partially purified from chromaffin granules, was a gift from Dr. D. K. Apps (University of Edinburgh, U.K.). The protein was purified further by h.p.l.c. on a μBondapak C18 (Waters Associates Milford, MA, U.S.A.) column (0.5 cm × 25 cm) eluted at 1 ml/min using a curvilinear 16–43% (v/v) acetonitrile gradient in 0.1% trifluoroacetic acid. The purification procedure was monitored by SDS/PAGE and Coomassie Brilliant Blue R-250 staining for proteins.

Pulse–chase radiolabelling

Batches of 1×10^6 insulinoma cells or 200 islets were subjected to pulse–chase radiolabelling using [^{35}S]methionine (1 mCi/ml; 1175 Ci/mmol; NEN–Du Pont) for the indicated times, and cell lysates prepared essentially as described in Hutton et al. [21]. Lysates were heated for 5 min at 100 °C in the presence of 0.5% deoxycholate and 2 mM dithiothreitol before the addition of region-specific antisera, and the final immunoprecipitates were subjected to SDS/PAGE using the method of Laemmli [25] or a modification of the method of Schagger and Jagow [21] (for low-molecular-mass peptides) and fluorography was performed as described previously [22].

Endoglycosidase treatment

Endoglycosidase H and *N*-glycanase (NBS Biologicals, Hatfield, Herts, U.K.) treatment of ^{35}S-labelled islet lysates was performed as described [26] followed by immunoprecipitation of CGA-related peptides as detailed above.

CGA-processing assays

CGA-conversion assays were performed at 30 °C overnight in 50 μl (final volume) of 100 mM sodium acetate (pH 4.8) containing 5 mM $CaCl_2$, 0.1% Triton X-100, 10 μM E64, 10 μM pepstatin A, 0.1 mM Tos-Phe-CH_2Cl ('TPCK'), 1 mM phenylmethanesulphonyl fluoride and 4 μg of bovine CGA. Reactions were initiated by the addition of fractionated insulin granule proteins (20 μl) or 2 μg of insulinoma granule extract and terminated by addition of 50 μl of 0.25 M Tris/HCl (pH 6.8) containing 4% (w/v) SDS, 12% (w/v) sucrose, 20 mM EDTA, 130 mM dithiothreitol and 0.01% Bromophenol Blue, followed by heating for 5 min at 100 °C. The samples were subjected to SDS/PAGE followed by Coomassie Blue staining, electrophoretic transfer to Immobilon-P membranes for N-terminal sequencing [27] or electrophoretic transfer to nitrocellulose paper for immunoblot analysis [28].

N-terminal sequence analysis

Sections of Coomassie Blue-stained Immobilon-P membranes were processed for N-terminal amino acid sequence analysis of

the bound protein using a 730A Sequenator (Applied Biosystems, Foster City, CA, U.S.A.) equipped with an on-line phenylthiohydantoin amino acid analyser.

Ion-exchange chromatography

Approx. 10 mg of Triton X-100-solubilized granule proteins were fractionated by anion-exchange chromatography on DEAE-cellulose to separate the type-1 and type-2 endopeptidase activities as described previously [20].

RESULTS AND DISCUSSION

Immunoprecipitation of biosynthetically labelled CGA-related peptides

Pulse–chase radiolabelling studies were conducted initially using rat insulinoma cells rather than pancreatic islets, as the amount of tissue available facilitated detection of minor cleavage products of CGA conversion. After a 15 min pulse-labelling of insulinoma cells with [^{35}S]methionine, antiserum raised against amino acids 60–234 of rat CGA (Figure 1) immunoprecipitated major proteins with apparent molecular masses of 98 and 80 kDa (Figure 2). Processing commenced in the interval 30–60 min after the beginning of the labelling period and was marked by the disappearance of the 98 and 80 kDa forms and the concomitant appearance of a major 21 kDa peptide and a minor 45 kDa peptide. The 21 kDa peptide co-migrated with the N-terminal CGA fragment β-granin purified from rat insulinoma tissue and detected by immunoblotting [29].

Antiserum raised to a central region of CGA (amino acids 241–306) also immunoprecipitated the 98 and 80 kDa peptides by the end of the pulse-labelling period, and these forms decreased progressively during the chase incubation with the appearance by 45 min of peptides of 65 and 45 kDa (Figure 2). Disappearance of the 65 kDa peptide was observed by 75 min chase incubation, whereas the 45 kDa peptide appeared to be a stable end product.

Immunoprecipitation using antiserum to the C-terminal residues 377–444 resulted in the appearance of the precursor forms after 15 min pulse-labelling followed by a progressive disappearance during the chase incubation. Additional peptides of 65 and 14 kDa appeared transiently by 45 min chase incubation.

Pancreatic islets were radiolabelled with [^{35}S]methionine to provide a comparison of CGA processing between the insulinoma and normal tissue. A 4 h labelling period was used to provide sufficient incorporation of [^{35}S]methionine into protein and analysis was performed using the gel system for separation of low-molecular-mass peptides (Figure 3). As with insulinoma cells, antisera raised against amino acids 60–234, 241–306 and 377–444 immunoprecipitated the 98 kDa precursor form along with the 21 kDa, 45 kDa and 14 kDa peptides respectively (Figure 3, lanes 1–3). Pancreatic islet β-granin in these experiments was resolved clearly into two bands whereas it appeared as a single band in insulinoma tissue. This latter finding parallels the differences found between islet and insulinoma tissue in previous biosynthetic and immunoblot studies [9].

Immunoprecipitation of the 4 h radiolabelled islet lysates with an antiserum raised against a C-terminally amidated nonapeptide corresponding to the PST C-terminus (CGA residues 306–314) resulted in isolation of the precursor form of CGA and the 45 kDa peptide along with an additional component of approx. 5 kDa (Figure 3, lane 4). An antiserum raised against rat CGA residues 343–356 (WE-14) also recognized the CGA precursor

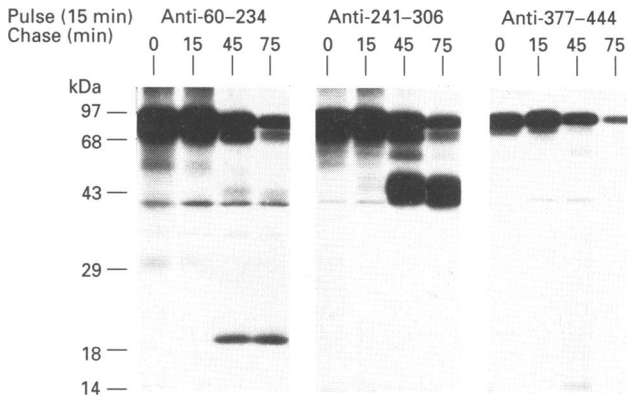

Figure 2 Immunoprecipitation of ^{35}S-labelled insulinoma lysates

Antisera were raised against amino acids 60–234, 241–306 and 377–444 of rat CGA. Cells were pulse–chase labelled for the indicated times, immunoprecipitated and then analysed by SDS/PAGE and fluorography as described in the Experimental section.

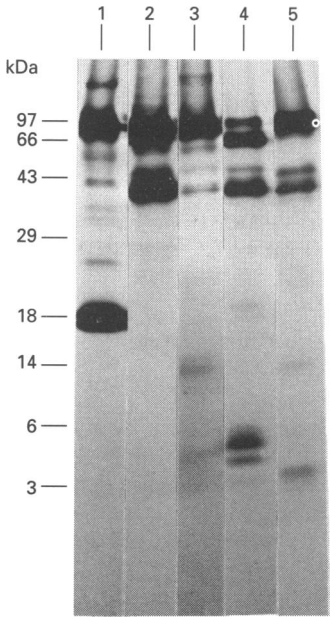

Figure 3 Immunoprecipitation of rat islets radiolabelled for 4 h

Antisera were raised against amino acids 60–234 (lane 1), 241–306 (lane 2), 377–444 (lane 3), 306–314 (lane 4) and 343–356 (lane 5). Isolated rat islets were incubated for 4 h in the presence of [^{35}S]methionine. The cellular forms of labelled CGA were immunoprecipitated and analysed by SDS/PAGE, using the gel system for resolution of low-molecular-mass peptides, and fluorography.

form, the 45 kDa peptide and, in this case, a minor peptide of approx. 3 kDa (Figure 3, lane 5).

Treatment of islet lysates with *N*-glycanase, which cleaves high-mannose, complex and hybrid N-linked oligosaccharides, followed by immunoprecipitation with a combination of anti-60–234 and anti-241–306 resulted in a downwards shift of the 45 kDa band by 2 kDa (Figure 4). Treatment with endoglycosidase H, which cleaves only high-mannose N-linked oligosaccharides, had no effect (Figure 4).

The finding that antiserum to amino acids 60–234 immunoprecipitated a 21 kDa peptide which co-migrated with the N-terminal fragment of CGA, β-granin, implicates cleavage of CGA at Lys^{129}-Arg^{130}. Only the 45 kDa peptide contained N-

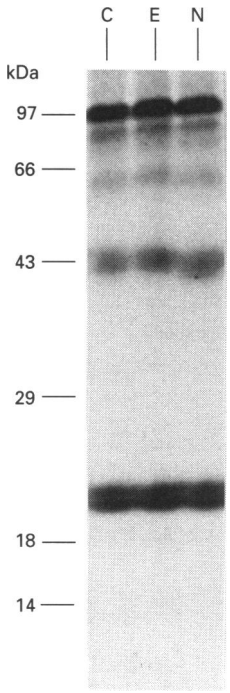

Figure 4 *N*-Glycanase and endoglycosidase H treatment of radiolabelled CGA peptides

Islets were labelled for 2 h in the presence of [^{35}S]methionine, treated with *N*-glycanase (N) or endoglycosidase H (E) as described in the Experimental section and immunoprecipitated with a mixture of 60–234, 241–306 and 377–444 antisera. The control incubation is shown in lane C.

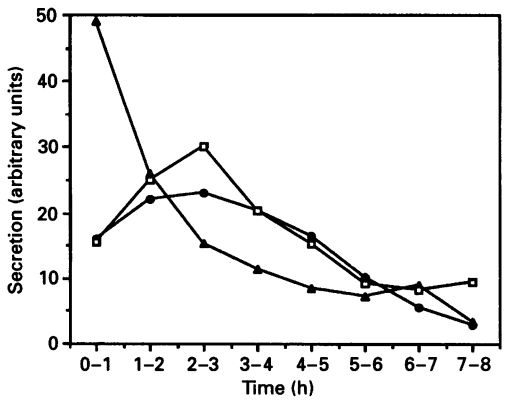

Figure 5 Secretion of CGA-related peptides from pulse–chase-labelled islets

Islets were incubated with [^{35}S]methionine for 1 h in Krebs medium containing 16.7 mM glucose. The medium was replaced with Dulbecco's modified Eagle's medium containing 16.7 mM glucose and 10% (v/v) fetal calf serum at 1 h intervals for up to 8 h of chase incubation. The molecular forms of CGA [98 kDa (▲), 45 kDa (●) and 21 kDa (□)] were immunoprecipitated with a mixture of antisera against amino acids 60–234, 241–306 and 377–444 and analysed by SDS/PAGE and fluorography followed by scanning densitometry of the bands.

linked carbohydrate, as shown by a 2 kDa decrease in its molecular mass after treatment with *N*-glycanase (Figure 4). This finding provides additional evidence that the cleavage that gives rise to the 21 kDa and 45 kDa peptides occurs at Lys^{129}-Arg^{130}, the only paired basic sequence proximal to the glycosylated residue.

The 65 kDa peptide is recognized by both anti-241–306 and anti-377–444 (Figure 2), indicating that it incorporates all or part of the 45 kDa and 14 kDa sequences. The 65 kDa peptide appeared only transiently, suggesting that it is an intermediate in the conversion process. The observation that anti-241–306 also immunoprecipitated a stable 45 kDa peptide suggests that it may be produced by cleavage of the 65 kDa intermediate.

The 45 kDa peptide is recognized weakly by anti-60–234 but not at all by anti-377–444, suggesting that it spans a central region of CGA commencing at amino acid 131 and terminating before amino acid 377. The 45 kDa peptide is also immunoprecipitated by antisera to the C-terminus of PST (amino acids 306–314) and to WE-14 (amino acids 343–356), suggesting that its sequence runs at least to amino acid 356. Antisera to PST and WE-14 also immunoprecipitate 5 kDa (the mass predicted for rat PST) and 3 kDa peptides respectively, suggesting further cleavage of the 45 kDa peptide. The 3 kDa fragment most likely comprises the tetradecapeptide, WE-14, which has been shown to be released from human CGA in a midgut carcinoid tumour after cleavage at two Lys-Arg sites (corresponding to Lys^{341}-Arg^{342} and Lys^{357}-Arg^{358} in rat CGA) flanking the sequence [11].

The C-terminal antiserum (anti-377–444) immunoprecipitated a 14 kDa peptide, which disappeared at longer chase intervals, possibly as the result of further processing. The further analysis of such cleavage products by pulse–chase labelling was impeded by the fact that there is only one methionine residue in the region C-terminal to the Lys^{357}-Arg^{358} cleavage site as compared with four methionine residues within the 45 kDa peptide sequence and three within the 21 kDa peptide sequence. Western-blotting analyses using anti-377–444 (as well as anti-241–306) which might have shed further light on the nature of the final products were precluded by the poor reactivity of the antibodies in such studies.

Taken together, the results indicate that initial processing of rat CGA at Lys^{129}-Arg^{130} and Lys^{357}-Arg^{358} liberates 21 kDa, 45 kDa and 14 kDa peptides. Further cleavage of the 45 kDa peptide giving rise to smaller peptides containing the PST and WE-14 sequences appears to occur at a low rate. The 14 kDa peptide may also undergo further processing.

Immunoprecipitation of CGA-related peptides using a mixture of anti-60–234, anti-241–306 and anti-377–444 from media collected from islets labelled for 1 h and chased at 1 h intervals for up to 8 h revealed secretion of precursor CGA, the 45 kDa and 21 kDa peptides (Figure 5). Secretion of the precursor decreased throughout the time course whereas that of the 45 kDa and 21 kDa peptide products followed a parallel course peaking after 2–3 h chase incubation. Release of the 21 kDa and 45 kDa peptides occurred by the regulated pathway of secretion, as the above peptides were not detected in media from islets incubated in the presence of 2.8 mM glucose (results not shown).

Proteolytic conversion *in vitro*

The bovine adrenal chromaffin granule form of CGA was used in *in vitro* conversion assays as it facilitated structural analysis of the cleavage products. Biosynthetically labelled rat insulinoma CGA was shown to undergo a similar pattern of proteolysis in previous studies [10]. The rat molecule exhibits a high degree of homology with the bovine sequence CGA [23], and, of the ten dibasic sites in the rat molecule (Figure 1), seven are conserved in bovine CGA. One notable difference between the two species

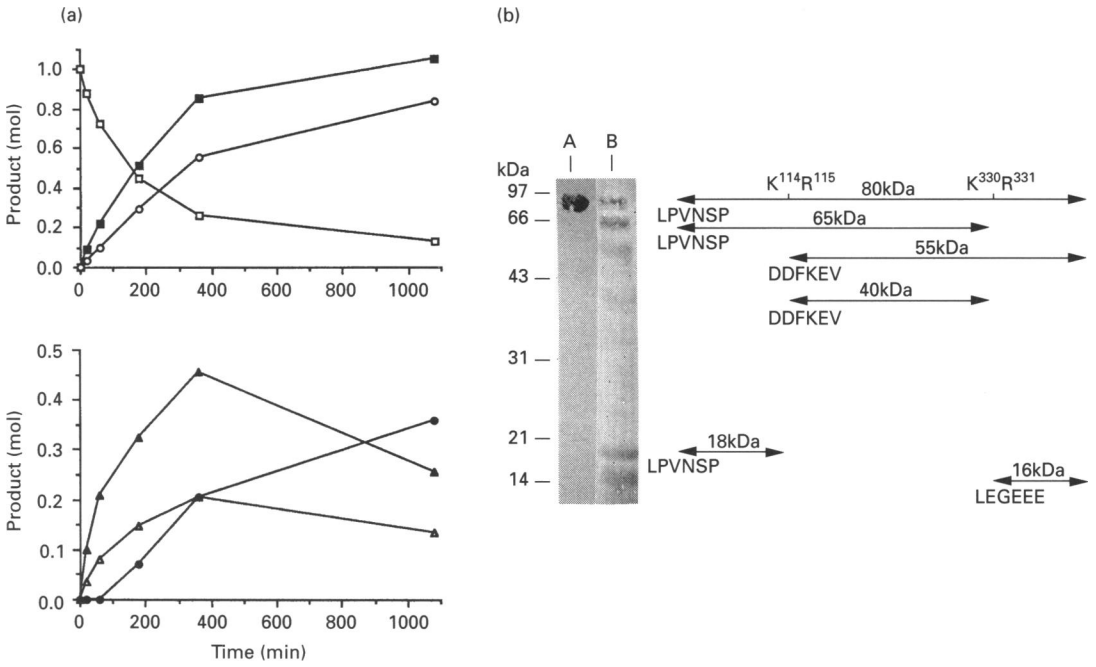

Figure 6 Proteolytic conversion of bovine CGA by lysed insulinoma granules

(**a**) CGA was incubated with lysed insulinoma granules for the indicated times and the products were analysed by SDS/PAGE followed by Coomassie Blue staining and densitometry. The levels of precursor (□) and products of 18 kDa (■), 16 kDa (○), 65 kDa (△), 55 kDa (▲) and 40 kDa (●) are expressed in mol. (**b**) CGA was incubated in the absence (lane A) or presence (lane B) of lysed rat insulinoma granules and the products were analysed by SDS/PAGE followed by electrophoretic transfer to Immobilon-P membranes and Coomassie Blue staining as described in the Experimental section. Product sizes (kDa), N-terminal amino acid sequences (single-letter code) and Lys-Arg cleavage sites are indicated.

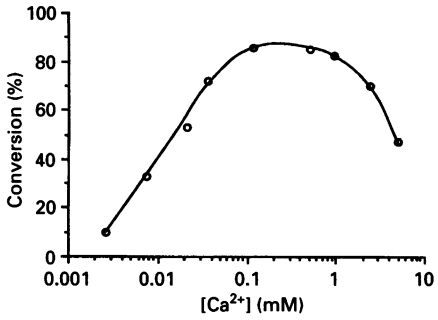

Figure 7 Ca^{2+}-concentration-dependence of CGA processing

Bovine CGA was incubated with insulin secretory granules for 5 h at 30 °C at pH 5.5 in the presence of 2 mM EDTA and different concentrations of $CaCl_2$ to give the indicated free Ca^{2+} concentrations. Products were analysed by SDS/PAGE and Coomassie Blue staining and quantified by densitometry. Conversion is expressed in terms of percentage loss of precursor.

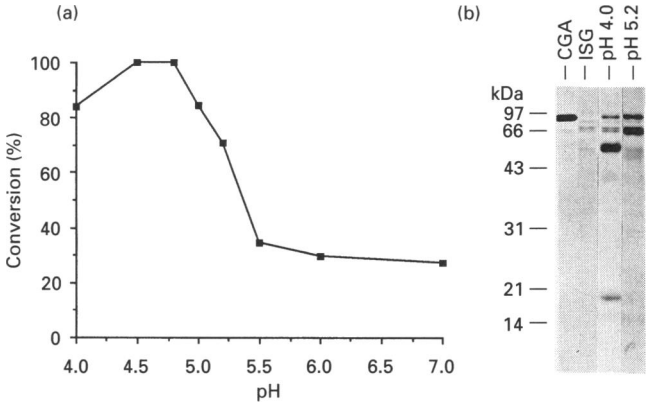

Figure 8 pH optimum of CGA processing

Bovine CGA was incubated alone or with insulin-secretory granules (ISG) for 5 h at 30 °C at the indicated pH values. (**a**) Products were analysed as described in the legend to Figure 5. Conversion is expressed in terms of percentage loss of precursor. (**b**) Products were analysed by SDS/PAGE and Coomassie Blue staining.

occurs in the N-terminal region in which the sequence of 20 consecutive glutamine residues found in rat CGA is absent from the bovine molecule.

Incubation of purified bovine CGA with lysed rat insulinoma secretory granules resulted in conversion of the 80 kDa precursor into peptides of 65, 55, 40, 18 and 16 kDa as revealed by fractionation of products on SDS/PAGE followed by Coomassie Blue staining and densitometry (Figure 6a). Production of the 65 kDa and 55 kDa peptides decreased after 360 min incubation, whereas that of the 18, 16 and 40 kDa peptides continued to increase throughout the time course, indicating that the 65 and 55 kDa peptides may be intermediates in the conversion of CGA into 18, 16 and 40 kDa peptide products.

N-terminal sequence analysis of the peptides produced from a 5 h incubation of CGA with lysed insulinoma granules showed that the 18 and 65 kDa peptides had the same N-terminal sequence, which corresponds to residues 1–6 of CGA (Leu-Pro-Val-Asn-Ser-Pro) (Figure 6b). The same analysis showed that the 55 and 40 kDa forms had amino acid sequences commencing at

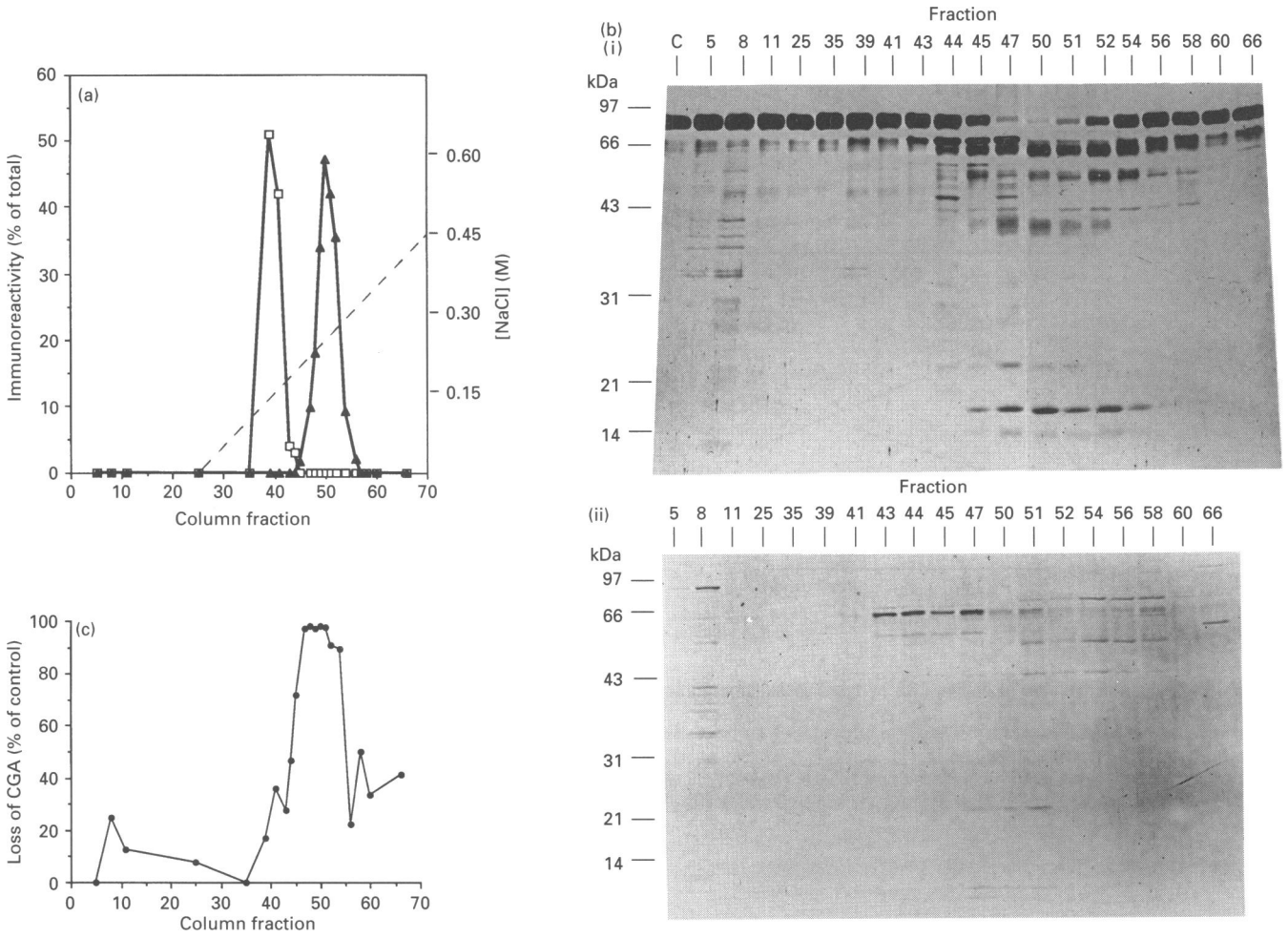

Figure 9 CGA processing by insulin granule ion-exchange chromatography fractions

(**a**) PC1 (□) and PC2 (▲) immunoreactivity in each ion-exchange fraction was determined by reflective densitometric scanning of stained proteins after immunoblot analysis and is expressed in arbitrary units. The NaCl gradient is indicated by a dashed line. (**b**) (i) Bovine CGA was incubated alone (C) or with insulinoma granule ion-exchange chromatography fractions, and the reaction products were analysed by SDS/PAGE and Coomassie Blue staining. (ii) The endogenous proteins in the ion-exchange fractions were analysed by SDS/PAGE and Coomassie Blue staining for proteins. (**c**) The disappearance of CGA was quantified by densitometry and expressed relative to the control incubation.

CGA residue 116 (Asp-Asp-Phe-Lys-Glu-Val), indicating that they were generated by cleavage at the conserved site Lys^{114}-Arg^{115}, and the 16 kDa form began at residue 332 (Leu-Glu-Gly-Glu-Glu-Glu), thus implicating cleavage at the conserved Lys^{330}-Arg^{331} sequence (Figure 6b).

The above results reveal that cleavage of both rat and bovine CGA by a rat insulin secretory-granule endoprotease occurs at corresponding Lys-Arg sites to produce peptides of similar electrophoretic mobility. The difference in molecular mass between the rat and bovine N-terminal peptide (β-granin) can be explained by the absence of the sequence of glutamine residues from bovine CGA.

Ca^{2+}-dependence and pH optimum of conversion

Maximal conversion of CGA occurred in the presence of Ca^{2+} concentrations of between 0.12 and 1 mM (Figure 7).

Incubation of bovine CGA with lysed secretory granules at a range of pH values showed an acidic pH optimum of conversion (pH 4.5–4.8) (Figure 8a). At pH values between 5.5 and 7.0, conversion was less than 35% of the total.

Comparison of CGA processing at pH 4 and pH 5.2 showed that a larger proportion of the precursor was converted into 55 kDa, 40 kDa and 18 kDa peptides at pH 4, whereas at pH 5.2 more of the 65 kDa and 16 kDa peptides were apparent (Figure 8b). The 55 kDa and 18 kDa peptides are produced by cleavage of CGA at Lys^{114}-Arg^{115}; the 65 kDa and 16 kDa peptides are produced by cleavage at Lys^{330}-Arg^{331} (see Figure 6b). The 40 kDa peptide can be produced only by cleavage at both of the Lys-Arg sites (Figure 6b). Scanning densitometry of bands in Figure 8b showed that, at pH 4, 9.8% of CGA cleavage occurred at Lys^{330}-Arg^{331}, 71.8% at Lys^{114}-Arg^{115} and 18.4% at both sites. At pH 5.2, 77% of cleavage occurred at Lys^{330}-Arg^{331}, 19.2% at Lys^{114}-Arg^{115} and 3.8% at both sites.

Identification of the CGA-converting endopeptidase

Fractionation of solubilized insulinoma granule proteins by anion-exchange chromatography resulted in separation of the type-1 and type-2 proinsulin endopeptidases as previously shown [20]. Western-blot analysis (Figure 9a) using antisera raised to the eukaryotic subtilisins PC1/3 and PC2 confirmed the pre-

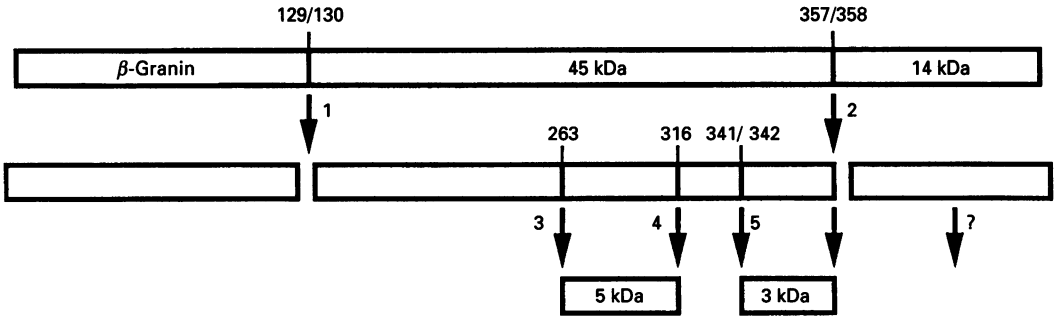

Figure 10 Summary of CGA processing

Rat CGA-cleavage sites are represented by the numbers 1–5.

viously demonstrated correlation between type-1 activity with PC1/3 and type-2 with PC2 [18,19]. Incubation of bovine CGA with the ion-exchange fractions, followed by separation of the products on SDS/PAGE showed conversion of the 80 kDa precursor into peptides of 65, 55, 40, 18 and 16 kDa by fractions 45–56 (Figures 9b and c), i.e. those fractions containing PC2 immunoreactivity. In contrast, little conversion was seen in the presence of the type-1 (PC1/3)-containing fractions.

Conclusions

It is a feature of many proproteins that they contain, within their sequence, multiple peptides which are only active once excised [30]. POMC, for example, is processed to adrenocorticotropin, γ-lipotropin and β-endorphin-(1–31) in anterior pituitary corticotropes, whereas in intermediate pituitary melanotropes it undergoes additional cleavages to produce γ-melanocyte-stimulating hormone and β-endorphin-(1–27) [31]. Similarly, proglucagon is processed in pancreatic α-cells to glucagon and the major proglucagon fragment, whereas in peptidergic cells of the intestinal tract the major products are glicentin and the glucagon-like peptides 1 and 2 [32]. The present studies support the concept that CGA is also subjected to limited proteolytic cleavage.

CGA exhibits striking differences in extent of processing and distribution of derived peptides throughout cells of the neuroendocrine system. Unprocessed CGA appears to be the major storage product in secretory vesicles of adrenal medulla, hypothalamus and anterior pituitary [33]. CGA processing is more extensive, however, in endocrine cells of the gut and pancreas where the putative regulatory peptides PST and β-granin appear to be the major conversion products. In small intestine and gastric corpus endocrine cells, conversion into PST is incomplete, with the majority occurring as a C-terminally extended form, whereas in gastric antral endocrine cells and pancreatic islets, mature C-terminally amidated PST predominates [34]. In islets, PST immunoreactivity appears localized to the insulin-containing β-cells and somatostatin-containing δ-cells [35].

In order to provide a better understanding of the pathway of CGA conversion in the endocrine pancreas we have used a panel of antisera raised against different regions of the molecule. Immunoprecipitation of radiolabelled islet and insulinoma proteins using these antisera provided evidence for extensive endoproteolytic processing of CGA at basic residues, as outlined in Figure 10. The precursor undergoes initial cleavage at two highly conserved Lys-Arg sites (positions 1 and 2) to liberate the 21 kDa, 45 kDa and 14 kDa peptides. Cleavage at these sites was supported by sequence analysis of the analogous products derived from bovine CGA processing. The 45 kDa peptide sequence was subject to additional cleavages, most likely at conserved single basic sites (positions 3 and 4) and another Lys-Arg sequence (position 5), resulting in generation of smaller peptides containing the PST and WE-14 sequences. Production of PST probably involves initial cleavage at two single lysine residues located at amino acids 263 and 316 in rat CGA [8]. Removal of Lys^{316} by carboxypeptidase H would expose a C-terminal glycine which could then act as an amide donor to the preceding glycine residue to produce the C-terminally amidated structure of PST. This process would require an endopeptidase which recognizes single basic residues along with a peptide-amidating mono-oxygenase activity. Production of the WE-14 sequence requires excision of two highly conserved Lys-Arg sequences (beginning at residues 341 and 357 in rat CGA) as described recently by Curry et al. [11].

Under conditions in which 72% CGA is converted into β-granin, the apparent conversion into PST and WE-14 is 49.4% and 17.8% respectively, on a molar basis (determined by scanning densitometry of immunoprecipitated islet proteins from Figure 3). A possible explanation for the differences in extent of conversion is due to alternative pathways of CGA processing in the different islet endocrine cell types and indicated by immunohistochemical analyses discussed above. An alternative explanation is suggested by the finding that the rate of conversion of the 45 kDa protein was relatively low compared with the time taken for proteins to transit the secretory pathway, and that, under conditions in which the cell is actively secreting, conversion is not completed before exocytosis occurs. Secretion studies showed that the 45 kDa form was released in response to glucose stimulation which is indicative of its generation in the β-cell. The low abundance of radiolabelled PST and WE-14 precluded similar analyses being performed for these molecules. Release of unprocessed chromogranin A was also observed, but this did not respond to glucose, suggesting that either it was released via the constitutive pathway or active secretion from the A-cell at low glucose was occurring.

The mammalian Kex2 homologue PC2 appeared to be the enzyme responsible for cleavage of CGA at the conserved Lys-Arg sites (positions 1 and 2; see Figure 10) which gives rise to the 21 kDa, 45 kDa and 14 kDa peptides. This was shown by correlation of the CGA-converting activity with PC2 immunoreactivity in insulin granule ion-exchange fractions. The finding that the insulin granule PC2 was able to process bovine adrenal medullary CGA is at odds with the observation that chromaffin-granule membranes contain PC2 [36] yet process CGA at a very

low rate [37]. This anomaly may in part be explained by the relatively high levels of PC2 mRNA in pancreatic β-cells compared with adrenal chromaffin cells [38,39], although the possibility that PC2 is regulated differently in the two cell types cannot be precluded. Processing of chromogranin A *in vitro* showed an absolute dependence on the presence of an acidic environment and the presence of Ca^{2+}, as previously observed with proinsulin and POMC as substrates [20,40]. This is consistent with the processing in the secretory-granule compartment, a proposal that was supported by the finding that the β-granin and 45 kDa peptides were released from stimulated islets in the same relative proportions as they were generated *in vitro*. More subtle effects, however, were in evidence in as much as the extent to which the two major sites in the CGA precursor were processed appeared to be pH-dependent. This points to the possibility that regulation of compartmental pH, whether brought about by differences in the numbers of active proton translocases or coupled ion conductances, is potentially an important mechanism in the differential processing of proproteins.

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