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Proteolytic processing of chromogranin A in purified insulin granules

Formation of a 20 kDa *N*-terminal fragment (betagranin) by the concerted action of a Ca^{2+}-dependent endopeptidase and carboxypeptidase H (EC 3.4.17.10)

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The nature and subcellular localization of the enzymic activities responsible for the production of the 20 kDa protein betagranin from its 100 kDa chromogranin-A-like precursor was investigated in transplantable insulinoma tissue. [^{35}S]Methionine-labelled precursor was converted by lysed insulin-secretory granules into betagranin and one or more proteins of 47 kDa, via intermediates in the 60–65 kDa range. Lysosome-enriched fractions also processed the precursor, but not into the peptides found *in vivo*; other fractions, including those enriched in Golgi, were inactive. Conversion of the precursor by granules was quantitative and the products were stable. Inhibitor studies showed that processing occurred by initial endoproteolytic cleavage at sites marked by pairs of basic amino acids, followed by removal of these by carboxypeptidase H. The endopeptidase activity appeared to be a novel metalloenzyme, with a markedly acidic pH optimum (4.8–5). It was inhibited by alanyl-L-lysyl-L-arginyl chloromethane ($K_{0.5} = 1.3\ \mu$M), but to a much lesser extent by inhibitor analogues of processing sites defined by single or unpaired basic amino acid residues, e.g. alanyl-L-norleucyl-L-arginylchloromethane ($K_{0.5} > 100\ \mu$M), leupeptin ($K_{0.5} = 150\ \mu$M) and antipain ($K_{0.5} = 40\ \mu$M). *p*-Chloromercuribenzoate ($K_{0.5} = 13\ \mu$M), Hg^{2+} ($K_{0.5} = 16\ \mu$M), Zn^{2+} ($K_{0.5} = 0.8$ mM) and vanadate ($K_{0.5} = 7\ \mu$M) also abolished activity, as did various anions ($SCN^- > I^- > Cl^- > SO_4^{2-}$). Group-specific inhibitors of serine, thiol and acidic endopeptidases were without effect. EDTA and CDTA (1,2-cyclohexanediaminetetra-acetic acid), but not 1,10-phenanthroline, abolished endoproteolytic activity. Several bivalent cations could restore activity after EDTA or CDTA inhibition, including Ca^{2+}, Zn^{2+}, Mn^{2+} and Sr^{2+}; however, the ion of physiological importance appeared to be Ca^{2+} ($K_{0.5} = 8\ \mu$M). The properties of the granule endopeptidase and its subcellular localization suggested that it is of importance in processing chromogranin A in the pancreatic β-cell.

INTRODUCTION

Betagranin, a series of 20–21 kDa proteins found in the insulin-secretory granule, appears to be structurally equivalent to the *N*-terminus of chromogranin A, the major co-secreted protein of the adrenal medulla (Hutton *et al.*, 1985, 1987*b*).

Pulse–chase-labelling experiments in insulinoma tissue and pancreatic islets reported in the preceding paper (Hutton *et al.*, 1987*a*) have shown that betagranin is initially synthesized as a 100 kDa precursor which is indistinguishable from chromogranin A, and that the cellular pathway of its production and subsequent secretion follows that of insulin.

We report here the characterization of proteolytic activities involved in the processing of this precursor, using subcellular fractions obtained from a transplantable insulinoma. It was of particular interest to establish the nature of the reactions involved and the relationship of the enzymes responsible to those involved in proinsulin processing in the pancreatic β-cell.

MATERIALS AND METHODS

Tissues

Insulinoma tissue propagated in New England Deaconess Hospital strain rats (Chick *et al.*, 1977) was used to prepare isolated viable cell suspensions (Hutton *et al.*, 1987*a*). Homogenates of the insulinoma prepared in 0.27 M-sucrose/10 mM-Mes (pH 6.5)/1 mM-EGTA were fractionated by Percoll-density-gradient centrifugation as described previously (Hutton *et al.*, 1982; Docherty & Hutton, 1983). This yielded a lysosomal fraction [fraction 1, 27% (w/v) Percoll gradient], a mixed lysosome/secretory-granule fraction A_2, secretory-granule fraction B, fraction enriched in mitochondria, endoplasmic reticulum and Golgi (E), endoplasmic

Abbreviations used: Tos-Phe-CH_2Cl, tosyl-L-phenylalanylchloromethane ('TPCK'); Tos-Lys-CH_2Cl, tosyl-L-lysylchloromethane ('TLCK'); Ala-Nle-Arg-CH_2Cl, alanyl-L-norleucyl-L-arginylchloromethane ('ANACK'); Ala-Lys-Arg-CH_2Cl, alanyl-L-lysyl-L-arginylchloromethane ('ALACK'); GEMSA, guanidinoethylmercaptosuccinic acid; CDTA, 1,2-cyclohexanediaminetetra-acetic acid; PAGE, polyacrylamide-gel electrophoresis.

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reticulum and plasma-membrane (F), and a soluble protein component (G). In the lysosomal and granule fractions the cross-contamination, as evaluated by marker enzymes, was 10–20%.

Immunoabsorbent preparation

Antiserum was raised to 21 kDa betagranin purified from the insulinoma as previously described (Hutton *et al.*, 1987*b*). An immunoglobulin fraction was prepared by Protein A–Sepharose affinity chromatography and subsequently bound to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden; 7 mg of IgG/ml of swollen gel) by published procedures (Axen *et al.*, 1967).

Preparation of endogenously labelled 100 kDa precursor

Batches of insulinoma cells (2×10^7) were incubated for 10 min at 37 °C in 2.5 ml of Krebs bicarbonate buffer containing 10 mM-Hepes (pH 7.4), 16.7 mM-glucose, 1% (w/v) bovine serum albumin (fraction V; Sigma) and 600 μCi of [^{35}S]methionine (800 Ci/mmol; Amersham International, Amersham, Bucks., U.K.) under O_2/CO_2 (19:1) in a 25 ml-capacity Erlenmeyer flask. Ice-cold incubation media without radioisotope was added to stop radioisotope incorporation, and the cells were recovered by centrifugation at 800 *g* for 5 min at 4 °C. The pelleted cells were immediately sonicated for 30s (MSE Sonifier) in 1 ml of lysis buffer, composed of 25 mM-$Na_2B_4O_7$ (pH 9), containing 3% bovine serum albumin, 1% Tween 20, 1 mM-phenylmethanesulphonyl fluoride, 1 mM-EDTA, 0.1 mM-E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane], 20 μM-leupeptin and 0.1% NaN_3.

The cell lysate was centrifuged at room temperature for 5 min at 9000 *g* (MSE Microcentaur), and 50 μl portions of the supernatant were made to a final volume volume of 450 μl with a suspension of immunoadsorbent (equivalent to 80 μl of swollen gel) and mixed end-over-end at 4 °C overnight. The immunoabsorbent was washed with 4 × 1 ml of lysis buffer, once with 1 ml of 50 mM-Tris/HCl (pH 7.5) containing 150 mM-NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 5 mM-EDTA, then twice with distilled water, before being finally eluted with 2 × 80 μl of 20 mM-HCl. The eluates pooled from several such incubations were freeze-dried and finally resuspended in water at approx. 1×10^6 d.p.m./ml.

Proteolytic processing assay

Assays were usually conducted in a 50 μl final volume in 1.5 ml-capacity micro-centrifuge tubes (Alpha Laboratories, Eastleigh, Hants., U.K.) by using a reaction mixture comprising 10 mM-sodium acetate, pH 5, 0.1% Brij 35, 2.8 μM-E-64, 1 μg of pepstatin A/ml and 0.1 mM-Tos-Phe-CH_2Cl (final concns.). The buffer (5 × conc.) and substrate (8000–10000 d.p.m./assay) were mixed together, any further additions were made, the volume was adjusted to 40 μl and the reaction initiated by addition of 10 μl of secretory-granule suspension (10–20 μg of protein in 10 mM-Mes/NaOH, pH 6.5). The reaction was terminated after 20 min at 37 °C by the addition of 25 μl of 375 mM-Tris/HCl (pH 6.8) containing 6% (w/v) SDS, 15 mM-EDTA, 0.75 M-sucrose and 0.003% Bromophenol Blue and immediately heating for 5 min at 100 °C. Samples were cooled to room temperature and electrophoresed on SDS/polyacrylamide gels polymerized from 15% (w/v) acrylamide and 0.08% *NN'*-methylenebisacrylamide, by using the discontinuous buffer system of Laemmli (1970). Gels were subjected to fluorography as previously described (Hutton *et al.*, 1987*a*). The resulting fluorographs were quantified by scanning densitometry (Chromoscan III; Joyce–Loebl, Gateshead, U.K.) and results expressed in terms of percentage changes in the absorbance of the 100 kDa substrate or the 20 kDa product. M_r calibration was performed with a mixture of ^{14}C-labelled proteins (BRL, Gaithersburg, MD, U.S.A.), which included myosin heavy chain, phosphorylase *b*, bovine serum albumin, ovalbumin, chymotrypsinogen, β-lactoglobulin and lysozyme.

Other assays and reagents

Protein was determined by a dye-binding procedure (Bradford, 1976), with bovine serum albumin (Sigma) as standard. Insulin-secretory-granule carboxypeptidase H was purified from the insulinoma as described elsewhere (Davidson & Hutton, 1987). Ala-Nle-Arg-CH_2Cl and Ala-Lys-Arg-CH_2Cl were kindly provided by Dr. E. Shaw, Friedrich Miescher Institut, Basel, Switzerland. Biosynthetic human proinsulin and insulin were gifts from Dr. B. Frank, Eli Lilly, Indianapolis, IN, U.S.A.

Calculation of free ion concentrations

The free ion concentrations of various bivalent metals in mixtures containing chelating agents were determined by using published metal stability constants (Chaberek & Martell, 1959) and an IBM 3081D computer program modified from Storer & Cornish-Bowden (1976). Endogenous ion concentrations in granule preparations were determined by atomic-absorption spectrometry (Hutton *et al.*, 1983).

RESULTS

The substrate used to investigate proteolytic processing of chromogranin A in insulinoma subcellular fractions consisted of the 100 kDa immunoprecipitable form of betagranin obtained after a 10 min labelling of isolated insulinoma cells. This was eluted from an immunoabsorbent produced by covalent coupling of immunoglobulins derived from immune sera to Sepharose 4B, using CNBr, rather than a Protein A adsorbent as used in pulse–chase-labelling experiments (Hutton *et al.*, 1987*a*). This proved an essential modification, as the presence of antibody in the Protein A eluate otherwise inhibited the processing reactions *in vitro*.

Subcellular localization of processing activity

Incubation of the precursor with a secretory-granule fraction obtained by Percoll-density-gradient centrifugation of an insulinoma homogenate resulted in the emergence of radioactive proteins of 60–65, 47 and 20 kDa (Fig. 1). The last of these was indistinguishable from the major betagranin form recognized on immunoblots of insulinoma and islet tissue (Hutton *et al.*, 1987*b*) and the major radioactive product after a 2 h chase period in labelled cells. Immunoprecipitation of the products of the incubation *in vitro* resulted in recovery of the 20 kDa product and residual precursor, but loss of the 47 and 60–65 kDa components.

A purified lysosomal fraction prepared from the insulinoma degraded the 100 kDa precursor, but failed to produce any of the immunoreactive forms of

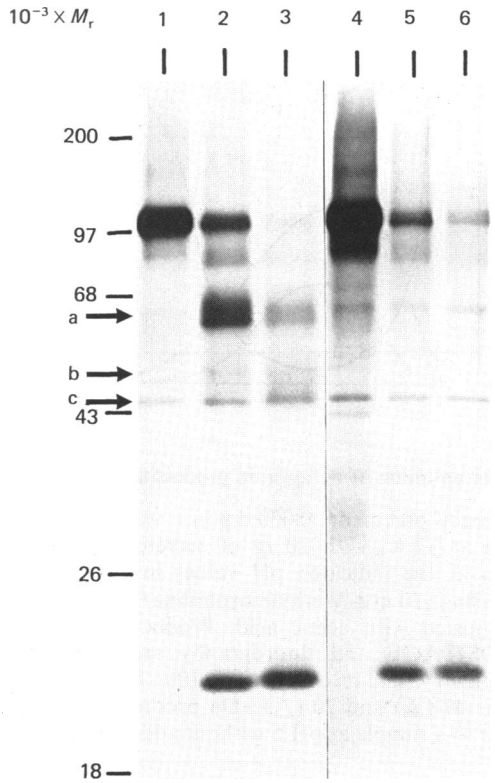

Fig. 1. Processing of the betagranin precursor and immunoprecipitation of the products of proteolysis

Betagranin precursor (8000 d.p.m.) was incubated for 0, 20 or 90 min with 20 μg of secretory-granule-lysate protein at 37 °C and either analysed directly by SDS/PAGE and fluorography (lanes 1, 2 and 3 respectively) or subjected to immunoprecipitation with immobilized anti-betagranin antibody before analysis (lanes 4, 5 and 6 respectively). The positions of the 60–65 (a) and 47 kDa (b) proteins and contaminating actin (c) are indicated by arrows: positions of M_r ($\times 10^{-3}$) markers are also indicated (and in subsequent gel photographs).

betagranin recognized in pulse–chase-labelling experiments (Fig. 2). A subcellular fraction enriched in both lysosomes and secretory granules (fraction A_2; Hutton *et al.*, 1982) produced significant quantities of the 60–65 and 20 kDa components, but only when a mixture of inhibitors of lysosomal enzymes was included in the assay. The purified granule fractions (fractions B and C) produced the same products irrespective of the addition of the inhibitor mixture. Fractions enriched in mitochondria (fraction D), endoplasmic reticulum and Golgi (fraction E), endoplasmic reticulum and plasma membrane (fraction F) or soluble proteins (fraction G) failed to degrade the precursor molecule.

The subcellular distribution of the activity producing the 20 kDa component correlated with the insulin content of the fractions (Hutton *et al.*, 1982), as well as their contents of carboxypeptidase H activity as determined by hydrolysis of benzyl-glycyl-arginine (Docherty & Hutton, 1983). This again suggested a predominantly granular localization of activity. All subsequent studies were performed with fraction B, the most highly enriched granule preparation.

Time course and concentration-dependence

Examination of the time course of conversion (Fig. 3*a*) suggested that the 20 and 47 kDa proteins were stable end products of conversion and that the 60–65 kDa components, which appeared transiently, may have been intermediates in the process. Neither the 47 nor the 60–65 kDa proteins appeared as condensed bands on electrophoretic separation at any time point, suggesting that they may have consisted of multiple components. This heterogeneity did not appear to be related to their glycosylation, since no alteration in migration or definition of the bands was achieved by deglycosylation by either trifluoromethanesulphonic acid hydrolysis or treatment with endoglycosidase F or H (H. W. Davidson & J. C. Hutton, unpublished work).

The recovery of radioactive proteins derived from the 100 kDa precursor as determined by scanning densitometry was 70–90%. The 20 kDa component at the 120 min time point constituted $53 \pm 3\%$ ($n = 4$) of the initial radioactivity and was not further degraded during an overnight incubation.

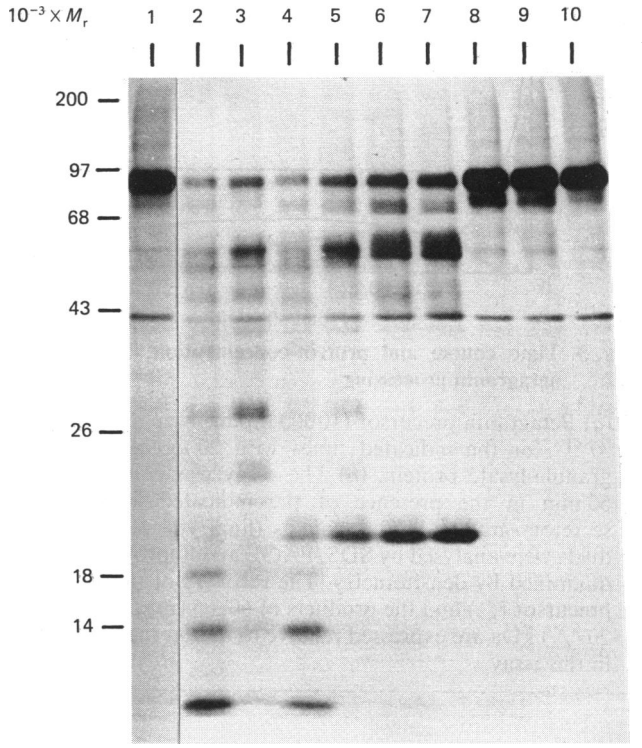

Fig. 2. Subcellular localization of processing activity

Betagranin precursor (8000 d.p.m.) was incubated for 20 min at 37 °C with 20 μg of protein derived from various subcellular fractions prepared by Percoll-density-gradient centrifugation of insulinoma homogenates (Hutton *et al.*, 1982). The following additions were made: 1, none; 2, purified lysosomes; 3, purified lysosomes and inhibitor mixture (Tos-Phe-CH_2Cl, E-64 and pepstatin A); 4, mixed lysosome/granule fraction (fraction A_2); 5, mixed lysosome/granule fraction and inhibitor mixture; 6, purified secretory granules (fraction B); 7, purified secretory granules plus inhibitor mixture; 8, mitochondria, endoplasmic reticulum and Golgi-enriched fraction (fraction E); 9, plasma-membrane-enriched fraction (fraction F); 10, soluble proteins (fraction G).

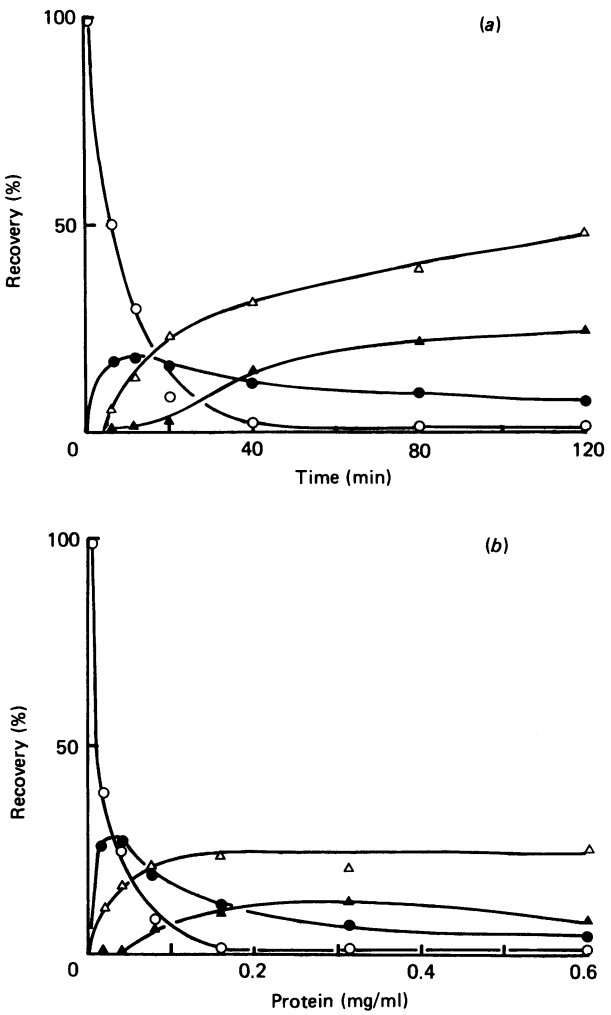

Fig. 3. Time course and protein-concentration dependence of betagranin processing

(*a*) Betagranin precursor (10000 d.p.m.) was incubated at 37 °C for the indicated times with 20 μg of secretory-granule-lysate protein. (*b*) The assay was conducted for 60 min in the presence of the indicated amounts of secretory-granule-lysate proteins (final vol. 100 μl). Products were analysed by SDS/PAGE and fluorography and quantified by densitometry. The recovery of the 100 kDa precursor (○) and the products of 60–65 (●), 47 (▲) and 20 (△) kDa are expressed relative to that at the zero point in the assay.

Examination of the dependence of conversion on the protein concentration (Fig. 3*b*) was consistent with the precursor–product relationship deduced from time-course experiments. From consideration of both sets of data, it was decided to evaluate the effects of potential modulators on the reaction under conditions where the rate of production of the 20 kDa form was essentially linear and where 50–80% of the precursor form had undergone conversion.

pH optimum

Converting activity assessed by either the loss of the precursor or the appearance of the 20 and 47 kDa products was optimal at pH 4.8–5.0 (Fig. 4). Very little activity was observed at pH 4 or below, or at pH 6 and above. One of the enzymes involved in these conversions, carboxypeptidase H (see below), also exhibits an acidic pH optimum, but is active over a wider pH range than the overall conversion process determined here. Since the same optimum of conversion was observed when carboxypeptidase H was inhibited by GEMSA (J. C. Hutton & M. Peshavaria, unpublished work), the data shown appear to reflect the pH optimum of endoproteolytic cleavage.

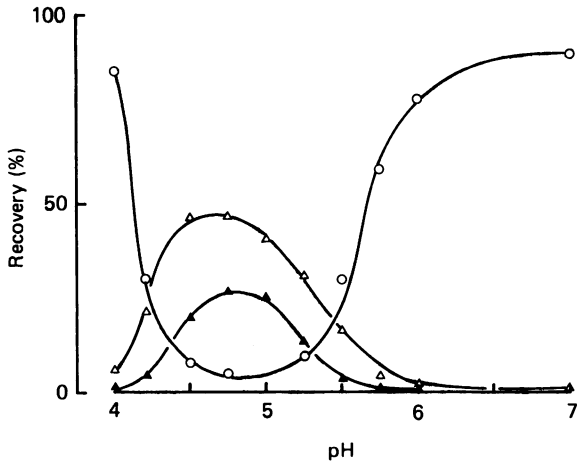

Fig. 4. pH optimum of betagranin processing

Betagranin precursor (8000 d.p.m.) was incubated for 30 min at 37 °C with 20 μg of secretory-granule-lysate protein at the indicated pH values in a buffer system comprising 20 mM-*N*-ethylmorpholine/20 mM-Mes with pH adjusted with acetic acid. Products were analysed by SDS/PAGE and fluorography and quantified by densitometry. The recovery of the 100 kDa precursor (○) and the 47 (▲) and 20 (△) kDa products are expressed relative to a sample at pH 5 without added granule lysate.

Inhibition studies

Group-specific inhibitors of serine, thiol and acidic proteinases failed to affect either the decrease in precursor or the generation of the 20 kDa product of conversion (Fig. 5, Table 1). The metalloproteinase inhibitors EDTA and CDTA decreased processing by over 80%. This property was not shared by EGTA; however, this proved to be a poor metal chelator at the pH used (pH 5). 1,10-Phenanthroline, another bivalent-cation chelator with a preference for transition elements, only slightly decreased the conversion of precursor, but markedly decreased the production of the 20 kDa protein. In its place, however, a protein approx. 1 kDa larger in apparent molecular size was observed, and the sum of the 20 and 21 kDa peptides represented 86% of the control in the absence of inhibitor. GEMSA, an active-site-directed inhibitor of carboxypeptidase H (Fricker *et al.*, 1983), had a similar effect on the products of conversion, thus suggesting that the changes induced by 1,10-phenanthroline were attributable to its inhibition of granule carboxypeptidase H activity.

Active-site-directed inhibitors of the lysosomal thiol proteinases, Tos-Lys-CH_2Cl and Tos-Phe-CH_2Cl, did not affect processing significantly. In contrast, Ala-Lys-Arg-CH_2Cl, a chloromethane with a dibasic amino sequence, was markedly inhibitory, with half-maximal inhibition of both precursor loss and the generation of

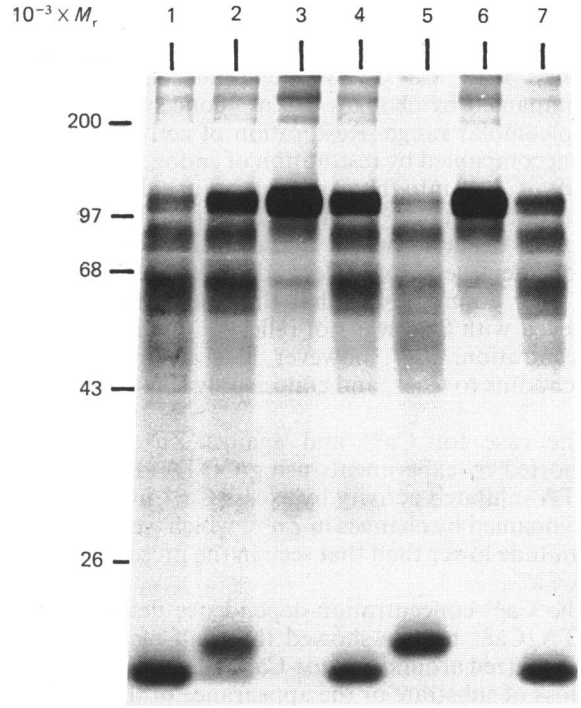

Fig. 5. Inhibitors of betagranin processing

Betagranin precursor (8000 d.p.m.) was incubated for 20 min at 37 °C with 20 μg of secretory-granule-lysate protein alone (lane 1) or in the presence of 1 mM-1,10-phenanthroline (lane 2), 100 μM-Ala-Lys-Arg-CH_2Cl (lane 3), 100 μM-Ala-Nle-Arg-CH_2Cl (lane 4), 30 μM-GEMSA (lane 5), 1 mM-EDTA (lane 6) and 1 mM-EDTA plus 1.2 mM-$CaCl_2$ (lane 7). Products were analysed by SDS/PAGE and fluorography.

the 20 kDa product occurring in the low-micromolar range ($K_{0.5}$ = 1.3 μM). Ala-Nle-Arg-CH_2Cl, a monobasic analogue, produced only slight inhibition at 100 μM concentration. Similarly leupeptin ($K_{0.5}$ = 150 μM) and antipain ($K_{0.5}$ = 40 μM), which are analogues of sites defined by single basic amino acids, were inhibitory, but only at concentrations far exceeding those in which maximally inhibitory effects are seen, for example on lysosomal cathepsins B and L (Barrett, 1973; Mason *et al.*, 1984).

p-Hydroxymercuribenzoate and Hg^{2+} both proved to be potent inhibitors of the converting activity, with half-maximal effects registered at 13 μM and 16 μM respectively. Cu^{2+} (1 mM), Co^{2+} (5 mM) and Zn^{2+} (5 mM) also abolished processing, Zn^{2+} showing a half-maximal effect at 0.8 mM. Sodium orthovanadate inhibited both precursor loss and production of 20 kDa peptide ($K_{0.5}$ = 7 μM), whereas sodium molybdate was without effect at concentrations up to 1 mM.

The endopeptidase activity was also sensitive to a number of anions, including the halide series, SO_4^{2-} and SCN^-. Examination of the effects of these ions at fixed concentrations of 30 and 100 mM indicated that the sensitivity followed the Hofmeister chaotropic series, i.e. $SCN^- > I^- > Cl^- > SO_4^{2-}$. The $K_{0.5}$ for Cl^- inhibition was 30 mM irrespective of whether the K^+, Na^+ or NH_4^+ salt was used. The effects of Cl^- were irreversible, as indicated by the failure to restore endoproteolytic activity after preincubation at 37 °C for 30 min in 200 mM-NaCl and dilution of the reaction mixture to give a final NaCl concentration of 20 mM in the assay.

Table 1. Effect of proteinase inhibitors on betagranin-precursor processing

Betagranin precursor (10000 d.p.m.) was incubated for 20 min at 37 °C with 20 μg of secretory-granule-lysate protein in the presence of the indicated substances. Products were analysed by SDS/PAGE and quantified by densitometry. Results are means of three separate experiments and are expressed as a percentage of the radioactivity incorporated into the 20–21 kDa proteins observed in a control incubation in which no additions were made. In this control 81% of the precursor radioactivity was consumed, of which 44% appeared in the 20–21 kDa product.

Addition	Rate (%)
1 mM-Phenylmethanesulphonyl fluoride	114
0.4 mM-3,4-Dichloroisocoumarin	105
1 mM-2,2′-Dipyridyl disulphide	97
1 mM-Iodoacetate	103
1 mM-*N*-Ethylmaleimide	105
1 mM-Dithiothreitol	99
0.5 mM-Pepstatin A	92
1 mM-EDTA	2
1 mM-CDTA	8
1 mM-1,10-Phenanthroline	77
1 mM-Tos-Phe-CH_2Cl	104
1 mM-Tos-Lys-CH_2Cl	104
0.1 mM-Ala-Lys-Arg-CH_2Cl	0
0.1 mM-Ala-Nle-Arg-CH_2Cl	58
1 mM-Leupeptin	8
1 mM-Antipain	0
1 mM-E-64	68
30 μM-GEMSA	88
0.1 mM-$HgCl_2$	0
0.1 mM-*p*-Hydroxymercuribenzoate	0
5 mM-$ZnCl_2$	2
75 mM-NaCl	27
75 mM-NaI	14
75 mM-NH_4SCN	14
30 mM-Na_2SO_4	48

Bivalent-cation-dependence of conversion

The suggestion that the endopeptidase activity was a metalloenzyme was further examined in a series of metal-ion replacement experiments using the chelating agents CDTA and EDTA (Table 2). The addition of Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Sr^{2+} or Ba^{2+} alone at concentrations of 0.2 mM did not markedly affect activity. When these ions were added at 1.2 mM in the presence of 1 mM-EDTA, Ca^{2+}, Mn^{2+}, Zn^{2+} and to a lesser extent Sr^{2+} restored activity; Mg^{2+} and Ba^{2+} were without effect.

Calculation of the free ion concentrations of Ca^{2+}, Mg^{2+} and Zn^{2+} in these incubations after EDTA addition indicated that at the prevailing pH (pH 5) the endogenous Mg^{2+} was poorly chelated, but that Ca^{2+} and Zn^{2+} concentrations were substantially decreased. Restoration of activity by Zn^{2+} addition was accompanied not only by an elevation of Zn^{2+}

Table 2. Effect of bivalent-metal-ion chelation and ion reconstitution

Assays were performed for 20 min at 37 °C with 10 μg of granule protein. The reaction mixture contained endogenous Ca^{2+} and Zn^{2+}, determined by atomic-absorption spectroscopy to be 60 and 10 μM respectively. Addition of metal ions were at 0.2 mM when used alone or at 1.2 mM when combined with either 1 mM-EDTA or 1 mM-CDTA. Products were analysed by SDS/PAGE and fluorography, and quantified by densitometry. Results are means of three separate experiments and are expressed as a percentage of the radioactivity incorporated into the 20–21 kDa protein observed in the control incubation in which no additions were made. In this control 87% of the precursor radioactivity was consumed, of which 48% appeared as the 20–21 kDa product.

Addition	$[Ca^{2+}]$ (μM)	$[Zn^{2+}]$ (μM)	Rate (%)
–	60	10	100
Ca^{2+}	260	10	104
Mg^{2+}	60	10	96
Mn^{2+}	60	10	92
Ba^{2+}	60	10	90
Sr^{2+}	60	10	90
Zn^{2+}	60	210	69
EDTA	4	2×10^{-6}	18
EDTA + Ca^{2+}	200	24×10^{-6}	98
EDTA + Mg^{2+}	5	2×10^{-6}	38
EDTA + Mn^{2+}	60	60×10^{-6}	101
EDTA + Ba^{2+}	4	2×10^{-6}	46
EDTA + Sr^{2+}	5	2×10^{-6}	78
EDTA + Zn^{2+}	60	200	96
CDTA	1	2×10^{-7}	19
CDTA + Ca^{2+}	200	7×10^{-7}	99
CDTA + Zn^{2+}	60	200	96

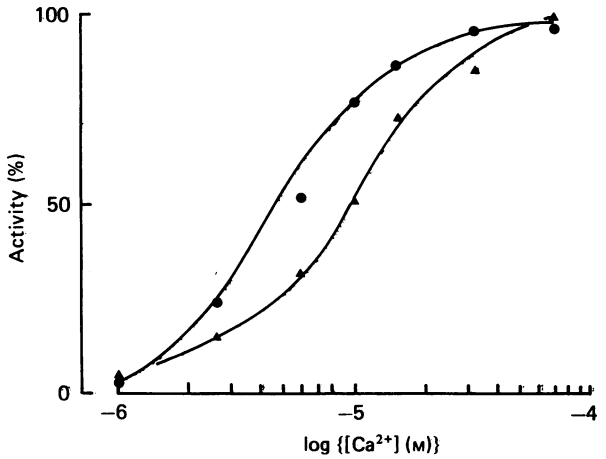

Fig. 6. Ca^{2+}-concentration-dependence of betagranin processing

Betagranin precursor (10000 d.p.m.) was incubated for 20 min at 37 °C with 20 μg of secretory-granule-lysate protein at pH 5 in the presence of 1 mM-CDTA and various concentrations of $CaCl_2$ to give the indicated free Ca^{2+} concentrations. Products were analysed by SDS/PAGE and fluorography and quantified by densitometry. Activity is expressed either in terms of the loss of precursor (●) or generation of the 20–21 kDa product (▲).

concentration but also by restoration of the Ca^{2+} concentration to the endogenous value. Restoration of activity with Ca^{2+}, on the other hand, was not accompanied by changes in Zn^{2+} concentration outside the picomolar range. Restoration of activity with Mn^{2+} was accompanied by restoration of endogenous Ca^{2+} and a shift of Zn^{2+} into the nanomolar range. Mg^{2+} and Ba^{2+} did not affect Ca^{2+} and Zn^{2+} concentrations. The simplest explanation of these results was that the endoproteinase activity depended on Ca^{2+} for activity. The only anomaly was that the restoration of activity achieved with Sr^{2+} was not reflected in changes in Ca^{2+} concentration. Sr^{2+}, however, has a similar hydrated ionic radius to Ca^{2+}, and conceivably substitutes for this ion.

The case for Ca^{2+} and against Zn^{2+} was further supported by experiments using CDTA. Restoration of CDTA-inhibited activity by excess Ca^{2+} in this case was accompanied by changes in Zn^{2+}, which were an order of magnitude lower than that seen in the presence of EDTA plus Ca^{2+}.

The Ca^{2+}-concentration-dependence determined with CDTA/Ca^{2+} buffers showed that half-maximal activation occurred around 5–8 μM-Ca^{2+} depending on whether the loss of substrate or the appearance of the 20–21 kDa proteins was determined (Fig. 6).

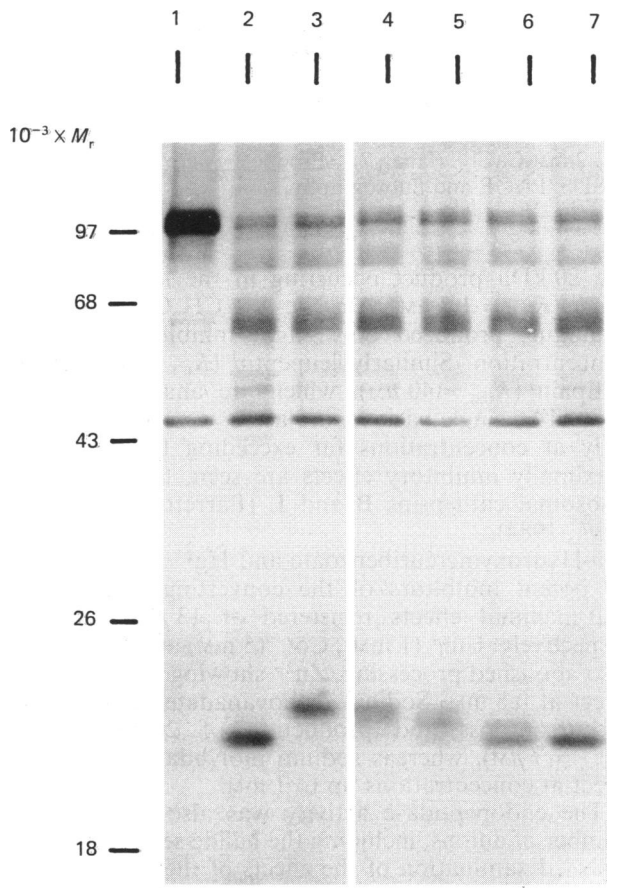

Fig. 7. GEMSA inhibition of betagranin processing

Betagranin precursor (lane 1) (8000 d.p.m.) was incubated for 20 min at 37 °C with 10 μg of secretory-granule-lysate protein either alone (lane 2) or with 1 mM-, 0.1 mM-, 10 μM-, 1 μM- or 0.1 μM-GEMSA (lanes 3–7 respectively). Products were analysed by SDS/PAGE and fluorography.

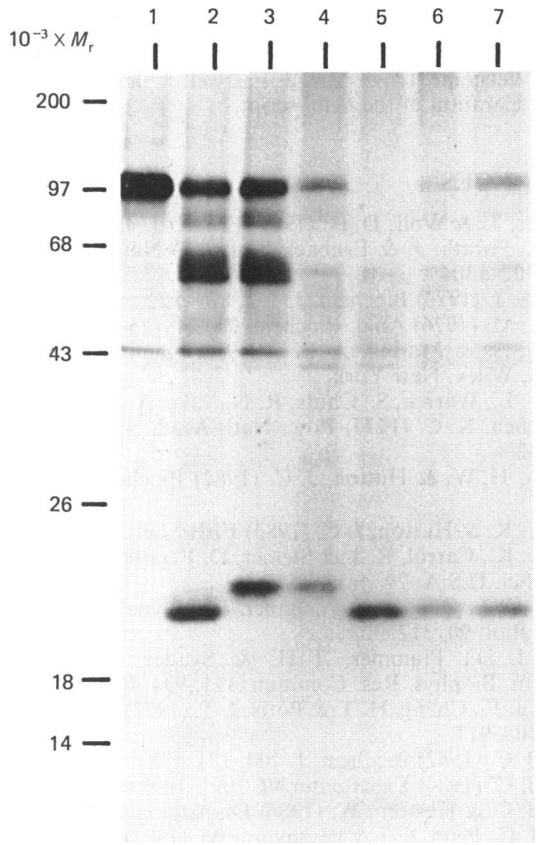

Fig. 8. Conversion of the GEMSA-inhibited 21 kDa protein into betagranin

Samples of betagranin precursor (lane 1; 8000 d.p.m.) were incubated for 20 min at 37 °C with 20 μg of secretory-granule-lysate protein either alone (lane 2) or with 30 μM-GEMSA (lane 3). The products of the reaction in the presence of GEMSA were immunoprecipitated (lane 4) and then subsequently incubated for 60 min at 37 °C with 10 μg of secretory-granule-lysate protein (lane 5) or with 2 μg of pig pancreatic carboxypeptidase B (Sigma; 20 units/mg) (lane 6) or 2 μg of insulinoma carboxypeptidase H (15.5 units/mg). Products were analysed by SDS/PAGE and fluorography.

The role of carboxypeptidase H

The above-mentioned inhibitor studies using 1,10-phenanthroline and GEMSA indicated that, under conditions where the insulin-granule carboxypeptidase H is inhibited, chromogranin A is converted quantitatively into a molecular form approx. 1 kDa larger than 20 kDa betagranin (Fig. 5). At a concentration of GEMSA that was approximately half-maximally effective, a product which was intermediate in molecular size was observed (Fig. 7), which suggested that the carboxypeptidase H was catalysing a two-step reaction.

The 21 kDa form produced in the presence of a maximally effective GEMSA concentration was quantitatively immunoprecipitated by anti-betagranin antisera (Fig. 8). When the immunoprecipitate was subsequently incubated with lysed secretory granules, with carboxypeptidase H purified from the insulinoma or with di-isopropyl fluorophosphate-treated pig pancreatic carboxypeptidase B, a product identical in size with betagranin was obtained.

DISCUSSION

The vast majority of polypeptide hormones and many other secreted proteins are derived from higher-M_r precursors which are cleaved at sites marked by pairs of basic amino acids (Loh *et al.*, 1984). In spite of the apparent evolutionary conservation of this mechanism, the endopeptidase involved in initial cleavage seems not to be conserved. All classes of proteinases have been implicated in one circumstance or another. This apparent diversity may have arisen from the need to generate specific cleavage where precursor molecules have multiple potential sites of processing or where a variety of potential precursors are localized in the same compartment of the cell. A physiological role for many of the postulated enzymes, however, is unproven; of the five or more endopeptidases variously proposed to process proinsulin in the pancreatic β-cell, the subcellular localization or the kinetic properties have generally not been reported, and none have been studied in a purified form. Also, in many instances the products of reaction have not been unequivocally identified, nor has the specificity and yield of the conversion process been assessed (see Hutton, 1984). The carboxypeptidase H activity, which can remove *C*-terminal basic amino acid exposed by endoproteolytic cleavage, by contrast, appears to be similar in all tissues studied to date (Loh *et al.*, 1984). The pancreatic β-cell enzyme is characteristically a Co^{2+}-stimulated metalloproteinase with an acidic pH optimum (Docherty & Hutton, 1983).

Against this background we report here the presence within insulin secretory granules of enzymic activities capable of converting chromogranin A *in vitro* in quantitative yield to produce the *N*-terminal protein betagranin, which previous studies have shown is co-packaged and co-secreted with insulin (Sopwith *et al.*, 1984).

Proteolytic processing of chromogranin A was found in subcellular fractions of insulinoma tissue enriched in either lysosomal or secretory-granule marker proteins. The 20 kDa protein betagranin identifiable on the basis of size and immunoprecipitability, however, was only generated by secretory granules. The localization of processing activity in this compartment was consistent with the results of pulse–chase-labelling experiments in pancreatic islets and insulinoma tissue (Hutton *et al.*, 1987*a*) which suggested that conversion paralleled that of proinsulin and was initiated at or soon after passage of the precursor through the Golgi and entry into the granule compartment.

Within chromogranin A, the residues Lys^{114} and Arg^{115} have been deduced as being the most probable site of cleavage (Hutton *et al.*, 1987*a*). Accordingly, processing *in vitro* was markedly inhibited by the dibasic amino acid chloromethane derivative Ala-Lys-Arg-CH_2Cl, but not by the monobasic analogue. Inhibition of carboxypeptidase H, whether by GEMSA or 1,10-phenanthroline, resulted in the generation of an *N*-terminal protein approx. 1 kDa larger than the normal 20 kDa product. This in turn could be converted into the normal product by carboxypeptidase B or H in what appeared to be a two-step reaction. These data suggested

that cleavage of the precursor immediately *C*-terminal to the basic amino acid pair was initially catalysed by an endopeptidase, giving rise to an intermediate which was transformed to the final product by two cycles of carboxypeptidase H activity.

The inhibitor profile for the endoproteolytic activity indicated the participation of a novel Ca^{2+}-dependent enzyme with a markedly acidic optimum. Enzyme activities which process proparathyroid hormone and proinsulin have been reported to be inhibited by EDTA (Kemmler *et al.*, 1973; Habener *et al.*, 1977), but these have not been extensively characterized. A Ca^{2+}-dependent thiol endopeptidase, the *kex*-2 gene product (Julius *et al.*, 1984), has been reported to process pro-alpha mating factor in yeast; however, this enzyme has a neutral pH optimum, is more sensitive than the present activity to inhibition by Zn^{2+} and is inhibited by dithiothreitol and not by Co^{2+} (Achstetter & Wolf, 1985).

Hg^{2+} and *p*-hydroxymercuribenzoate were powerful inhibitors of the present converting activity, which suggested the importance of thiol groups to the integrity of the enzyme. Processing was not affected, however, by dithiothreitol or alkylating agents, which distinguishes the endopeptidase from secretory-granule thiol proteinases which have previously been postulated to be involved in proinsulin processing (Fletcher *et al.*, 1981; Docherty *et al.*, 1982). The inhibition of the enzyme by vanadate is shared by lysosomal cathepsin D and a mitochondrial-matrix ATP-sensitive proteinase. However, unlike these enzymes, processing was not affected by pepstatin A (Pillai & Zull, 1985; Watabe & Kimura, 1985). This would also distinguish the endopeptidase from one reported to process pro-opiomelanocortin (Loh *et al.*, 1985).

The properties of the chromogranin A-processing activity suggested that the enzyme involved would be maximally active under the ionic and pH conditions prevailing in the granule interior (Hutton, 1982; Hutton *et al.*, 1983). At the neutral pH values found in the Golgi apparatus of the β-cell (Orci, 1985), little activity would be expected. This would account for the observation that proteolytic processing of chromogranin A does not commence until 20 min or more after its biosynthesis, when it might be expected that entry into the granule compartment had occurred. It similarly accords with the observation that agents which disrupt chemiosmotic gradients across insulin-granule membranes (NH_4^+, monensin and chloroquine) inhibit processing (Hutton *et al.*, 1987*a*).

The specificity of cleavage, the stability of products, the subcellular localization of the enzyme(s) involved and the compatibility with the intragranular environment all point to the conclusion that we have identified the activity involved in chromogranin A processing *in vivo*.

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