Biosynthesis of betagranin in pancreatic β -cells

Identification of a chromogranin A-like precursor and its parallel processing with proinsulin

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The biosynthesis of insulin and betagranin, a 20–21 kDa co-secreted chromogranin A-related protein, were investigated in isolated insulinoma cells and islets. The insulinoma tissue processed proinsulin to insulin with kinetics similar to those reported in islet tissue. Unlike islets, however, the insulinoma released almost one-quarter of the newly synthesized proinsulin into the medium 10–40 min after its formation. Betagranin was initially immunoprecipitated as a 100 kDa precursor form, which was indistinguishable from chromogranin A in size and immunoreactivity and by peptide mapping. After an initial lag of 10–20 min, the precursor was converted progressively into betagranin, which appeared to be a stable end product. Formation of betagranin and insulin from their respective precursors followed a parallel course and could likewise be inhibited by NH_4^+ , chloroquine and monensin, added either before labelling or at any point of time up to 15 min after labelling. As with proinsulin, approximately one-quarter of the betagranin precursor was released 10–40 min after synthesis. It is concluded that betagranin is produced by limited proteolysis from a chromogranin A precursor in pancreatic β -cells by a cellular pathway indistinguishable from that of insulin from proinsulin. Chromogranin A is highly conserved in the *N*-terminal region represented by betagranin, further suggesting that the biological activity of chromogranin A may reside in a derived peptide rather than in the parent molecule.

INTRODUCTION

Betagranin, a series of closely related 20 kDa co-secreted proteins of pancreatic β -cells, share a common *N*-terminus with chromogranin A, a major 72 kDa (by SDS/polyacrylamide-gel electrophoresis) co-secreted protein of the bovine adrenal medulla (Sopwith *et al.*, 1984; Hutton *et al.*, 1985). Antisera raised to rat insulinoma betagranin recognize adrenal-medullary chromogranin A and peptides of a wide M_r range in a number of tissues of the diffuse neuroendocrine system (Hutton *et al.*, 1987). Betagranin itself is limited in distribution, being most prominent in pancreatic islets, anterior pituitary and to a lesser extent posterior pituitary.

Many bioactive peptides and most polypeptide hormones are synthesized as higher- M_r inactive precursor molecules, which are proteolytically processed to active forms at sites in the molecule defined by pairs of basic amino acids (see Docherty & Steiner, 1982). Within any single precursor not all such sites may be attacked, thus permitting the differential expression of biological activities contained within the same sequence. For insulin biosynthesis from proinsulin, the endopeptidase(s) responsible for initial cleavage appear to be activated soon after the passage of the precursor through *trans* elements of the Golgi apparatus to the nascent secretory granule. These continue their activity in the maturing granule (Orci, 1982).

We report here the results of an investigation of betagranin biosynthesis in cells obtained from a trans-

plantable rat insulinoma (Chick *et al.*, 1977) and in pancreatic islets. It was of particular interest to establish whether a precursor-product relationship exists between chromogranin A and betagranin, to define the cellular localization of any proteolytic event involved and to investigate its relationship to the biosynthesis and segregation of proinsulin.

MATERIALS AND METHODS

Preparations of insulinoma cells

Insulinoma tissue was propagated in New England Deaconess Hospital (NEDH) rats as previously described (Hutton *et al.*, 1981). One to three tumours (1–3 g wet wt.) were transferred to ice-cold Hanks saline (Hanks & Wallace, 1949), the fibrous capsule was removed and the tumour minced into 1 mm³ pieces, and rinsed twice with Hanks saline and twice with incubation medium, composed of Krebs bicarbonate buffer (115 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 1 mM-CaCl₂ and 24 mM-NaHCO₃) containing 1 mg of bovine serum albumin (Sigma)/ml, 16.7 mM-glucose and 10 mM-Hepes buffer, pH 7.4. The tumour pieces were then pushed through a plastic tea strainer (0.5 mm mesh) with a rubber-capped syringe plunger (30 ml: Plastipak; Becton Dickinson, Dun Laoghaire, Ireland), resuspended in 25 ml of the incubation medium and then forced with a syringe through three layers of 1 mm × 0.5 mm nylon mesh held in a 2.5 cm-diam. Swinney filter holder (Millipore Corp., Bedford, MA, U.S.A.). The filtrate was

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then passed through a 50 μm -mesh filter (Pharmacia, Stockholm, Sweden) in the same apparatus. During the latter operation, the filters were replaced whenever the back pressure on the syringe rose significantly, thus avoiding compaction of the cells on the mesh. Portions (5 ml) of the cell suspension were then layered on to a step density gradient, composed of 1 ml of 60% (v/v) Percoll (Pharmacia) in incubation medium underlying 4 ml of 42% Percoll in incubation medium. The tubes containing the gradients (120 mm $\times$ 16 mm conical polystyrene centrifuge tubes; Sterilin, Feltham, Middx., U.K.) were centrifuged at room temperature for 15 min at 3500 rev./min ($r_{\text{max.}} = 12.5 \text{ cm}$) in a MSE bench-top centrifuge. In the resultant gradient, red blood cells sedimented to the bottom of the tube, viable cells collected at the 42%–60%–Percoll interface, whereas non-viable cells, large clumps of cells and debris accumulated at the interface between the medium and 42% Percoll. The material at the 42%/60%–Percoll interface was diluted in incubation medium and recovered by centrifugation at room temperature for 5 min at 800 g . Two further washes were performed, the cells being recovered each time by centrifugation for 5 min at 800 g . The cell number was determined with a haemocytometer, and a final suspension was made at 2×10^7 cells/ml in ice-cold incubation medium. A typical preparation yielded 3×10^7 cells, their viability by Trypan Blue exclusion being greater than 90%.

Isolated islets of Langerhans were prepared by collagenase digestion of pancreases of NEDH rats by the method of Lacy & Kostianovsky (1967).

Radioisotopic labelling

Tissue (2×10^6 insulinoma cells/1.0 ml; 200 islets/0.5 ml) were preincubated in incubation media for 20–45 min at 37 °C under O_2/CO_2 (19:1) in 45 mm $\times$ 12.5 mm polypropylene tubes (lymphocyte tubes; GIBCO, Uxbridge, Middx., U.K.), recovered by centrifugation at room temperature for 5 min at 800 g and then resuspended in 1.5 ml of prewarmed (37 °C) incubation medium containing 300 μCi of [^{35}S]methionine (800 Ci/mmol; SJ235; Amersham International, Amersham, Bucks., U.K.) and other additions where noted. The incubations were normally terminated after 10 min by addition of 1.5 ml of ice-cold incubation medium. The samples were immediately centrifuged for 30 s at 3300 g (MSE Microcentaur micro-centrifuge) to separate cells from the medium. The cells were sonicated for 15 s in 1 ml of a lysis buffer consisting of 25 mM- $\text{Na}_2\text{B}_4\text{O}_7$ (pH 9), 3% (w/v) bovine serum albumin, 1% Tween 20, 1 mM-phenylmethanesulphonyl fluoride (Sigma), 0.1 mM-E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (Sigma), 1 mM-EDTA, 20 μM -leupeptin (Sigma) and 0.1% NaN_3 . The sonicated material was centrifuged for 5 min at 13000 g and the supernatant retained.

In pulse-chase experiments, an initial 10 min labelling was performed, cells were recovered by centrifugation for 5 min at 800 g and then resuspended in 1.5 ml of prewarmed incubation medium without radioisotope but containing 3 mM-L-methionine. These incubations were terminated at various time points and treated identically with the 10 min samples above. Where release of radioactivity into the media was monitored, the medium was diluted with an equal volume of lysis buffer before immunoprecipitation.

Immunoprecipitation

Chromogranin-related peptides. Extracted samples (1.0×10^6 – 2.5×10^6 d.p.m. of trichloroacetic acid-precipitable material in 200 μl of lysis buffer) contained in 1.5 ml-capacity micro-centrifuge tubes were mixed with 20 μl of a suspension of Cowan-strain *Staphylococcus aureus* cells (Sigma; 100 mg/ml) for 2 h at room temperature, then centrifuged for 5 min at 13000 g . The supernatant was transferred to a new tube and incubated with 10 μl of whole antiserum overnight at 4 °C. A suspension (300 μl) of 10% (v/v) preswollen Protein A–Sepharose (Pharmacia) was then added and a further incubation performed for 2 h at room temperature with end-over-end mixing. The protein A–Sepharose was recovered by centrifugation (13000 g for 1 min) and subsequently washed four times with lysis buffer, twice in 50 mM-Tris/HCl, pH 7.5, containing 150 mM-NaCl, 5 mM-EDTA, 0.1% SDS, 1% Triton X-100 and 1% sodium deoxycholate, and then twice in distilled water. Specifically bound radioactivity was recovered by two successive elutions with 35 μl of 20 mM-HCl, and the pooled eluate was freeze-dried.

The freeze-dried samples were reconstituted in 60 μl of 125 mM-Tris/HCl, pH 6.8, containing 2% SDS, 65 mM-dithiothreitol, 0.25 M-sucrose, 5 mM-EDTA and 0.001% Bromophenol Blue, then heated at 100 °C for 5 min. The samples were then electrophoresed on slab gels (150 mm $\times$ 150 mm $\times$ 1.5 mm) polymerized from 15% (w/v) acrylamide and 0.08% *NN'*-methylenebisacrylamide, by using the discontinuous buffer system of Laemmli (1970). Gels were subsequently shaken for 15 min in two changes of acetic acid, for 3 h with 22% (w/v) 2,5-diphenyloxazole in acetic acid, washed in running tap water for 30 min before vacuum drying and exposure at –70 °C for 1–5 days to preflashed Cronex 4 X-ray film (Dupont, Stevenage, Herts., U.K.). Fluorographs were analysed by scanning densitometry (Chromoscan III; Vickers, Gateshead, U.K.) or by liquid-scintillation counting of excised gel pieces equilibrated overnight with a Triton X-100-based fluor (Fisofluor; Fisons, Loughborough, Leics., U.K.). Molecular-size calibration was achieved with ^{14}C -labelled lysozyme, β -lactoglobulin, α -chymotrypsinogen, ovalbumin, bovine serum albumin, phosphorylase *b* and myosin heavy chain (BRL, Paisley, Scotland, U.K.).

The primary antisera used in these studies were raised in guinea pigs to betagranin purified from a transplantable rat insulinoma (Hutton *et al.*, 1987) or in rabbits to electrophoretically purified bovine chromogranin A. The latter was a gift from Dr. D. T. O'Connor, University of California, San Diego, CA, U.S.A. Control immunoprecipitations were performed with non-immune sera from the same species.

Insulin. A mouse monoclonal antibody (3B7) raised to human proinsulin, which recognizes epitopes within the insulin structure (Gray *et al.*, 1987), was partially purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and coupled to diazotized cellulose by established procedures (Hales & Woodhead, 1980). Extracted samples (1×10^6 d.p.m. of trichloroacetic acid-precipitable radioactivity in 200 μl of lysis buffer) were incubated for 2 h at room temperature with 100 μl of immunoabsorbent suspension (10 mg of cellulose/ml, binding capacity 2.5 i.u. of insulin/ml) in lysis buffer. Washing steps were performed as for

betagranin immunoprecipitates and the bound proteins were eluted with 0.5 ml of 3.5 M-acetic acid. Samples were freeze-dried and reconstituted in 100 μ l of 2.5 mM-Tris/HCl, pH 8.6, containing 6 M-urea and 0.001% Bromophenol Blue. Electrophoresis was performed on slab gels (150 mm $\times$ 150 mm $\times$ 1.5 mm) polymerized from 7.5% (w/v) acrylamide and 0.2% *NN'*-methylene-bisacrylamide containing 8 M-urea, 12.5 mM-Tris and 80 mM-glycine, pH 8.6. Gels were pre-run in 12.5 mM-Tris/80 mM-glycine, pH 8.6, for 600 V $\cdot$ h, the upper tank buffer was then replaced, samples were loaded and electrophoresis was performed for a further 1000 V $\cdot$ h (1.6 gel lengths). Fluorography was performed as detailed above. Control incubations used an immobilized monoclonal antibody to human insulin receptor.

V8-proteinase peptide mapping. Samples of the radio-labelled chromogranin A-related proteins eluted from immunoaffinity supports (2000 d.p.m., containing approx. 30 μ g of immunoglobulin) were freeze-dried, reconstituted in 60 μ l of 125 mM-Tris/HCl, pH 6.8, containing 2% SDS, heated at 100 $^{\circ}$ C for 2 min, cooled and then mixed with 5 μ l of 250 mM-ammonium acetate containing 6–600 μ g of *Staph. aureus* V8 proteinase (Miles, Slough, U.K.)/ml and incubated for 40 min at 37 $^{\circ}$ C. Then 5 μ l of 1 M-dithiothreitol was added, and the samples were heated for 5 min at 100 $^{\circ}$ C and then electrophoresed on SDS/polyacrylamide gels polymerized from 18% (w/v) acrylamide and 0.08% *NN'*-methylenebisacrylamide. Fluorography was performed as above.

Other assays

Protein was determined by the method of Bradford (1976) with bovine serum albumin as standard. Radioactivity incorporated into proteins was determined after precipitation with ice-cold 8.3% (w/v) trichloroacetic acid. The precipitated material was collected by centrifugation for 5 min at 13000 *g* (MSE Microcentaur), and the pellet redissolved in 100 μ l of 1 M-methylbenzethonium hydroxide in methanol (Sigma) and then mixed with 5 ml of 0.5% 2,5-diphenyloxazole/0.02% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene for liquid-scintillation counting of radioactivity. Unless otherwise specified, all reagents used were of the highest grade available and obtained from BDH, Poole, Dorset, U.K. The identification of intermediates generated during the processing of proinsulin was made on the basis of the electrophoretic migration of intermediates generated from human proinsulin by using a combination of trypsin and carboxypeptidase B. Confirmation of the identity of such standards was obtained by h.p.l.c. separation and amino acid analysis.

RESULTS

Quantitative aspects of betagranin and insulin biosynthesis and release

The incorporation of [35 S]methionine into insulinoma proteins under the standard incubation conditions was essentially linear with time for 10 min, at which point $18.9 \pm 3.3\%$ (S.E.M.; $n = 7$) of the radioactivity from the initial media was incorporated into trichloroacetic acid-precipitable material in the cell. Incubation for a further 20 min resulted in a further doubling of

incorporation, whereafter little further increase was observed. The incorporation of radioactivity into the proteins of specific interest, betagranin and insulin, followed a similar time course to that for the trichloroacetic acid-precipitable material, and represented at the 10 min time point $0.30 \pm 0.06\%$ ($n = 7$) and $1.67 \pm 0.27\%$ ($n = 7$) respectively of the total trichloroacetic acid-precipitable radioactivity.

With insulinoma cells, changes in the glucose concentration between 2.8 and 28 mM, or the addition of secretagogues such as 10 mM-L-leucine, 10 mM-theophylline or 10 mM-alanine, did not enhance the incorporation of [35 S]methionine into trichloroacetic acid- or immuno-precipitable material. All experiments were thus performed by the standard protocol, with 16.7 mM-glucose present.

During a chase incubation with unlabelled methionine, the cellular content of trichloroacetic acid-precipitable radioactivity declined progressively, following a time course described by a simple exponential ($t_{1/2} = 150$ min). By contrast, cellular betagranin and insulin immuno-precipitable radioactivity declined more rapidly, especi-

Fig. 1. Secretion of labelled insulin- and betagranin-immunoreactive peptides by insulinoma cells

Cells were labelled for 10 min and then resuspended ($t = 0$) in non-radioactive medium containing 3 mM-methionine. The medium was again replaced after 10, 20 and 40 min. Radioactivity immunoprecipitated with either anti-insulin (a) or anti-betagranin (b) antibodies is expressed relative to the initial cellular content of the respective immunoreactive protein at the end of the 10 min labelling period as determined in parallel incubations. Each result is the mean of determinations in two separate experiments.

ally during the first 40 min of chase incubation. By 40 min the cellular contents represented $46.1 \pm 5.6\%$ and $48.3 \pm 4.4\%$ ($n = 6$) of the initial contents respectively. The subsequent decline (40–120 min) in immunoprecipitable radioactivity in both cases followed a similar exponential time course to that for the total trichloroacetic acid-precipitable radioactivity.

During the 10 min labelling period, the media contained little radioactivity associated with either immunoreactive betagranin or insulin (Fig. 1). During the first 40 min of chase incubation, however, substantial quantities of immunoprecipitable betagranin and insulin radioactivity appeared in the media, accounting for 27 and 21% of the initial cellular immunoprecipitable radioactivity respectively. Release declined thereafter, so that by 120 min 32 and 28% of each protein had been released respectively ($n = 3$). Mono[^{125}I]iodinated proinsulin added to media under identical incubation conditions was degraded by less than 10% over 120 min, suggesting that neither re-uptake of released proteins nor their proteolytic degradation in the media contributed to this transient phenomenon.

Molecular forms of labelled immunoreactive insulin

Proinsulin was the major cellular form of immunoprecipitable insulin radioactivity by completion of the initial 10 min labelling period (approx. 90%), but within 40 min of the chase period two new forms appeared (Fig. 2). One of these co-migrated with proinsulin cleaved at either of the sites marked by dibasic residues from which the newly exposed basic amino acids had been removed (i.e. des-Lys⁶⁴,Arg⁶⁵-proinsulin or des-Arg³¹,Arg³²-proinsulin). This intermediate form appeared within 10 min of the chase incubation and reached a steady state by about 40 min (Fig. 2). Insulin was first observed in the immunoprecipitates between 20 and 40 min, and increased progressively. By the end of a 2 h chase it contributed more than 50% of the total immunoprecipitable radioactivity.

The insulin immunoreactivity which appeared in the incubation medium during the first 40 min of the chase period reflected the composition of that present in the cells, initially comprising proinsulin alone, then increasing amounts of the intermediate form. During the 40–120 min chase interval insulin was also present (Fig. 2).

Calculation of the half-time of conversion of proinsulin into insulin was complicated by the fall in radioactivity of cellular proteins, reflected in the decrease in cellular trichloroacetic acid-precipitable radioactivity and by the release of proinsulin and intermediate into the media. Correction for these gave a $t_{1/2}$ of conversion of 27 min. The recovery of radioactivity in the insulin molecule in the cells at the longest time point examined (120 min) was $15 \pm 3\%$ ($n = 4$) of the radioactivity originally incorporated into proinsulin.

Molecular forms of labelled immunoreactive betagranin

At the completion of the 10 min labelling period, the majority of radioactivity immunoprecipitated by anti-betagranin antisera appeared in a protein of 100 kDa (Fig. 3). Minor amounts of 92 kDa component were also present, which ranged from 2 to 10% of the 100 kDa band.

During the chase period the 100 and 92 kDa proteins decreased rapidly and in parallel after a lag of about

Fig. 2. Molecular composition of labelled insulin immunoprecipitates in pulse-chase-labelled insulinoma cells

Cells were labelled for 10 min, then resuspended in non-radioactive medium containing 3 mM-methionine. The cellular forms of labelled insulin were isolated upon termination of the incubation at the indicated times (min). The forms in the medium were determined on a separate batch of cells by using the protocol outlined in Fig. 1 legend. Alkaline-urea/polyacrylamide-gel electrophoresis and fluorography showed the presence of proinsulin (Pro), insulin (Ins) and an intermediate (Int) identified as des-Arg³¹,Arg³²- and/or des-Lys⁶⁴,Arg⁶⁵-proinsulin.

20 min. From 20 min onwards a 20 kDa component appeared, which increased progressively to reach, by 120 min, approx. 50% of the radioactivity immunoprecipitated.

The betagranin immunoreactivity which appeared in the incubation medium during the first 40 min of chase period was predominantly in the 92 and 100 kDa forms. In the subsequent 40–120 min chase interval, a significant proportion of the 20 kDa protein was evident.

After correction for release of radioactivity into the media, the apparent half-time of intracellular conversion of the 100 kDa protein was 23 min during the 10–40 min chase period and 30 min in the 40–120 min interval. The recovery of radioactivity in the 20 kDa protein at 120 min was approx. 7% of that originally incorporated into the 100 kDa protein.

Betagranin formation in pancreatic islets

Islets labelled for 10 min in the presence of [^{35}S]methionine and then subjected to a 45 min chase incubation in the absence of radioisotope were found to have incorporated radioactivity into 92 and 100 kDa components which were indistinguishable from the chromogranin-A like protein observed in tumour cells together with proteins of 20 and 21 kDa (Fig. 3b). Whereas in insulinoma cells the labelled 20 kDa betagranin appeared as either a single band or two poorly separated components, the equivalent proteins in

Fig. 3. Molecular composition of labelled betagranin immunoprecipitates in pulse-chase-labelled insulinoma cells and islets

(a) Insulinoma cells were labelled for 10 min, then resuspended in non-radioactive medium containing 3 mM-methionine. The cellular forms of labelled betagranin were isolated upon termination of the incubation at the indicated times (min). The forms in the medium were determined on a separate batch of cells, by using the protocol outlined in Fig. 1. (a) SDS/polyacrylamide-gel electrophoresis and fluorography showed the presence of 100, 92 and 20 kDa peptides as the major forms. (b) Groups of 200 islets were labelled for 20 min and then incubated for a further 45 min in non-radioactive medium containing 3 mM-methionine. The glucose concentration of the medium was maintained throughout at 2.8 or 16.7 mM as indicated. Immunoprecipitates prepared with anti-betagrnanin antisera were analysed by SDS/polyacrylamide-gel electrophoresis. Positions of M_r ($\times 10^{-3}$) markers are indicated.

islets were more clearly resolved into two bands. This paralleled the differences which are seen between islet and insulinoma tissue by immunoblotting analysis (Hutton *et al.*, 1987). The incorporation of radioactivity into betagranin immunoprecipitates was markedly stimulated by increasing the glucose concentration in the medium.

Relationship of the 100 kDa protein to chromogranin A

When cells were labelled for 10 min and immunoprecipitated with either anti-(chromogranin A) or anti-betagrnanin antisera, equivalent amounts of radioactivity were recovered. In both cases it appeared in the forms of the 92 and 100 kDa protein. Immunoprecipitation was virtually quantitative, since a further cycle of immunoprecipitation of the unbound fraction with the same sera resulted in little further recovery of radioactivity. When immunoprecipitation with anti-(chromogranin A) antiserum was followed by anti-betagrnanin antiserum, and vice versa, no further binding was observed in the second cycle.

Immunoprecipitation performed at the end of the 120 min chase was again virtually quantitative, and both anti-(chromogranin A) and anti-betagrnanin antisera precipitated equivalent amounts of the residual 100 kDa form. Anti-(chromogranin A) antiserum, however, failed to immunoprecipitate the 20 kDa form recognized by anti-betagrnanin antiserum. The most probable explanation for these results was that anti-(chromogranin A) antiserum failed to recognize the *N*-terminal region of chromogranin A comprising betagranin (Hutton *et al.*, 1987). The possibility that the different antisera recognized different precursor molecules was eliminated by

the observation that the molecular forms immunoprecipitated after a 10 min labelling period when subsequently treated with *Staph. aureus* V8 proteinase yielded an identical pattern of proteolytic fragments at three different proteinase/substrate ratios.

Effects of chloroquine

The addition of 0.2 mM-chloroquine to the incubation media during the 10 min labelling period did not significantly affect the incorporation of radioactivity into trichloroacetic acid-precipitable material, proinsulin, or the betagrnanin 100 kDa precursor (Fig. 4). The inclusion of chloroquine throughout the labelling and chase incubation period, however, prevented the formation of insulin and insulin intermediate and the formation of betagranin. The release of proinsulin and the 100 kDa protein occurring 10–40 min after labelling was also completely blocked by chloroquine addition during the labelling and chase incubations. Addition of chloroquine at the end of the labelling period or 10–15 min after the initiation of the chase period arrested processing at the extent that had been achieved at that time. The composition of cellular immunoprecipitates at the end of the 120 min chase period was thus comparable with that attained at the time of the introduction of these agents into the media. Identical results were obtained in experiments using 5 mM- NH_4^+ or 50 nM-monensin.

DISCUSSION

These experiments were performed mostly in isolated transplantable insulinoma cells rather than pancreatic islets, since the available amounts of tissue facilitated the

Fig. 4. Effect of chloroquine on the conversion of proinsulin and the betagranin precursor in pulse-chase-labelled insulinoma cells

Cells were labelled for 10 min, then subjected to the following treatments: 1, immediate termination; 2, chase incubation of 10 min; 3, chase incubation of 80 min; 4, chloroquine during labelling and immediate termination; 5, chase incubation of 80 min with chloroquine throughout chase; 6, chase incubation of 80 min, chloroquine added after 10 min of chase. (a) Insulin immunoprecipitates were analysed by alkaline-urea/polyacrylamide-gel electrophoresis, and (b) betagranin immunoprecipitates by SDS/polyacrylamide-gel electrophoresis. For abbreviations see Fig. 2. Positions of M_r ($\times 10^{-3}$) markers are indicated.

analysis of the biosynthesis of minor cellular proteins such as betagranin. This also avoided the problem that, within islets, cells other than the β -cell contain betagranin (Hutton *et al.*, 1987). Normal rat pancreatic islets were shown to synthesize both the 100 kDa and 20 kDa forms of betagranin referred to in these experiments.

Synthesis and storage of insulin in transplantable insulinoma cells

The present investigation supports previous studies which have suggested that the rat transplantable insulinoma synthesizes and stores insulin, with similar kinetics to pancreatic islets (Gold *et al.*, 1984a). Unlike islets, however, it was evident that approximately one-third of the proinsulin was released into the media 10–40 min after it had been synthesized. The point in time at which release occurred and the limited processing that was evident suggested a constitutive release process emanating from elements of the Golgi apparatus. Unlike constitutive release defined in other systems, e.g. AtT 20 cells (Moore *et al.*, 1983), it was abolished by the addition of chloroquine to the media during the labelling period. This rapid release of newly synthesized proinsulin may explain the high basal secretion of 'insulin' immunoreactivity found in these cells *in vitro* (Sopwith *et al.*, 1981) and the high circulating amounts of proinsulin immunoreactivity found in the serum of animals harbouring these tumours (N. Fleming, J. P. Luzio & J. C. Hutton, unpublished work). It is notable that hyperproinsulinaemia is also frequently manifest in patients bearing pancreatic tumours (Creutzfeldt *et al.*, 1973).

Although the observed delay of 20–30 min for insulin formation is consistent with previous studies in insulinomas (Gold *et al.*, 1984a) and islet tissue (Steiner *et al.*, 1974), it was surprising to find that a processing intermediate(s) appeared much earlier than this. The intermediate involved (des-Arg³¹, Arg³²- and/or des-Lys⁶⁴, Arg⁶⁵-proinsulin) would not have been detected in most studies performed to date, since analyses have usually relied on gel filtration, in which these intermediate forms are indistinguishable from uncleaved proinsulin.

The appearance of the intermediate form at a point 10–20 min after synthesis may be consistent with the initial processing occurring in coated granules or soon after their uncoating, as postulated by Orci *et al.* (1985). However, our results do not support the conclusion reached by those authors, that the coated granule is the only, or major, processing compartment, since the formation of insulin proceeds more slowly than that of the intermediate. It is noteworthy that the technique used by Orci *et al.* (1985) detects the loss of proinsulin antigenicity associated with the cleavage of the B–C-chain junction (Madsen *et al.*, 1984) rather than the complete conversion process. Des-Arg³¹, Arg³²-proinsulin is the major proinsulin processing intermediate found in the circulation (de Haen *et al.*, 1978), which suggests that, for a single-compartment model, the cleavage at the B–C junction occurs faster than cleavage of the A–C junction. We conclude therefore that processing, although initiated soon after segregation of proinsulin into coated granules, continues for several hours after the uncoating of the granule.

The generation of both the intermediate form of insulin and insulin itself in insulinoma tissue depended on the

establishment of ionic gradients across the processing compartment(s), as evidenced by the abolition of processing by chloroquine, NH_4^+ and monensin. Inhibition of processing occurred whether additions were made during preincubation, labelling, or in the chase period up to 15 min after labelling was completed. The simplest explanation, based on the chemiosmotic properties of isolated secretory granules (Hutton, 1982) and studies in intact cells with monensin (Smith *et al.*, 1984; Gold *et al.*, 1984b), is that these agents inhibit processing by preventing the acidification of the interior of the nascent secretory granule. The enzymes thought to be involved in proinsulin processing typically have acidic pH optima and are inactive at neutral pH (Docherty & Hutton, 1983; Hutton *et al.*, 1986).

Synthesis and storage of betagranin

The 100 kDa protein immunoprecipitated with anti-betagranin antisera after a 10 min labelling of insulinoma cells with [^{35}S]methionine coincided in electrophoretic mobility with proteins detected by immunoblotting of adrenal-medullary tissue and pituitary with either anti-betagranin or anti-(chromogranin A) antisera (Hutton *et al.*, 1987). Its identification as chromogranin A was further supported in the present studies by the immunoprecipitation, with anti-(chromogranin A) antisera, of a labelled protein of identical size and V8-proteinase map. Insulinoma and islet tissue however, clearly differ from the adrenal medulla in that the 100 kDa chromogranin A form is not a stable cellular constituent. Immunoblotting analyses show at best only minor amounts of this protein compared with lower- M_r immunoreactive forms.

Within bovine chromogranin A, eight potential sites for limited proteolysis marked by pairs of dibasic residues have been deduced from the cDNA sequence of the molecule (Iacangelo *et al.*, 1986; Benedum *et al.*, 1986). One of these, at amino acids 114/115, is conserved in a rat betagranin cDNA clone which has been sequenced in our laboratory (Hutton & Kastern, 1986) and occurs two amino acids C-terminally of the C-terminus of betagranin as determined by amino acid sequencing (J. C. Hutton & E. Nielsen, unpublished work). Processing at this site would produce N-terminal proteins comparable in size with betagranin.

The reciprocal disappearance of the 100 kDa form with the appearance of the 20 kDa peptide suggested that betagranin was a product of chromogranin A proteolysis. No other forms of intermediate molecular size were identified, indicating that the site that gave rise to betagranin was either attacked preferentially or at a higher rate than other sites. Betagranin, with two methionine residues/molecule (Hutton *et al.*, 1985), may be expected to contain around one-third to one-half the methionine present in rat chromogranin A. Thus the observed conversion of 6.5% of the initial immunoprecipitable 100 kDa form into the 20 kDa peptide by 120 min would correspond to 14–20% conversion on a molar basis. This approximated to the observed molar conversion of proinsulin (15%) at this time, thus suggesting that the betagranin stored in insulin-secretory granules is the major and stable end product of chromogranin A proteolysis.

The pathway of synthesis and storage of betagranin in these studies was indistinguishable from that of insulin. The time course of disappearance of the chromogranin

A precursor form from cells paralleled that of proinsulin and, as with proinsulin, was attributable to at least two processes, namely release occurring 10–40 min after synthesis and a slower proteolytic event lasting several hours. Betagranin in the cell appeared with a similar time course to the intermediate in proinsulin processing, and likewise its processing was blocked by addition of chloroquine, NH_4^+ and monensin, which disrupt ionic gradients across intracellular membranes. These observations, plus the finding that insulin and betagranin on final storage (5 h after synthesis) are released in parallel in response to insulinotropic stimuli (Sopwith *et al.*, 1984), suggest that these molecules share a common intracellular pathway which they traverse at a similar rate.

The biological function of chromogranin A has, in spite of many years of investigation, remained elusive. It has variously been postulated as an amine-binding protein (Sharp & Richards, 1977), a Ca^{2+} -binding protein (Felicita & Gratzl, 1986), an osmoregulatory protein (Helle *et al.*, 1985), or a sorting element involved in segregation of proteins into the secretory pathway (Benedum *et al.*, 1986). However, no consensus has been reached as to any of these roles. The observation that it is proteolytically degraded in cells has been made previously (Settleman *et al.*, 1985); however the possibility that its cellular function may reside in derived peptides such as betagranin has largely been overlooked. This in part is attributable to the emphasis that has been placed on the role of chromogranin A in the adrenal medulla. In this tissue, unlike the pancreatic β -cell, chromogranin A is only partially processed, and at a very slow rate (Falkensammer *et al.*, 1985).

One could argue that, as a randomly coiled protein (Smith & Winkler, 1967), chromogranin A is unlikely to adopt a stable conformation necessary for a specific biological function. This may not be true, however, of peptides derived from the protein. The fact that the N-terminal sequence which encodes betagranin is highly conserved between the cow and the rat (around 90% for the first 30 amino acids; J. C. Hutton & E. Nielsen, unpublished work) suggests evolutionary conservation of structure related to a specific biological function in the cell.

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