Reviews

The insulin secretory granule*

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Summary. The insulin secretory granule of the pancreatic B cell is a complex intracellular organelle comprised of a many proteins with different catalytic activities and messenger functions. With the advent of tumour models of the B cells and the application of immunological and molecular cloning techniques considerable progress has been made in recent years towards the elucidation of the structure and function of these granule proteins. A number of examples are selected here for review. Particular emphasis given to how the activities of quite different granule proteins are interdependent and how this contributes to the co-ordination and integration of the organelle's biological functions.

Key words: Insulin, granule, vesicle, proton pump, prohormone conversion, autocrine, chromogranin A.

1989 marks the centenary of the publication of the seminal work by Oskar Minkowski and Joseph von Mering which showed that total pancreatectomy in the dog produced symptoms indistinguishable from Type I (insulin-dependent) diabetes in Man [1]. The experiment, which was a remarkable surgical feat at the time, was actually initially designed to resolve a dispute between the authors as to whether the pancreas was involved in lipid digestion [2]. Minkowski, the junior member of the team, then in his early thirties, noticed that the house-trained animal could no longer control its bladder. This led him to make the crucial determination of urinary sugar.

These investigations opened the way to the study of diabetes in experimental animals and shifted the focus away from diabetes as a disease of the organism to a disease affecting a particular organ. The link between the metabolic function of the pancreas identified by von Mering and Minkowski and the islets of Langerhans, which had been discovered some twenty years previously in 1869, was finally established by the work of Opie on human diabetic pancreases at the beginning of the twentieth century [3]. Progress towards understanding the secretion of insulin at the level of the islet however was impeded until techniques for the isolation of islets from experimental animals were introduced in the middle sixties by Hellerström [4], Moskalewski [5] and Lacy et al. [6].

Our own investigations have aimed to take us a step further, to define the process of secretion at the subcellular level, and to relate the complex intracellular events which accompany insulin release to the properties of the individual molecules which constitute the secretory pathway. This is a formidable task, but one which now can be realized as a result of the introduction of transplantable insulinomas as an abundant source of pancreatic B cells, refinements in morphological techniques particularly with the use of antibodies, and the introduction of immunoaffinity purification, molecular cloning and patch-clamping techniques to allow the isolation, manipulation and study of the behaviour of individual cellular proteins.

This review will discuss just one component in the secretory mechanism, the insulin storage granule. These subcellular organelles have a deceptively single morphology and are characterized in electron micrographs as spheres of around 200–300 nm in diameter comprised of a crystalline core of insulin surrounded by a mantle of less dense material and enveloped by a simple phospholipid bilayer. The granule, however, is far more than just a repository of the hormone in the cell. It is the site of proteolytic activation of the hormone. It is involved in intercellular communication through the secretion of other biologically active molecules. Its mem-
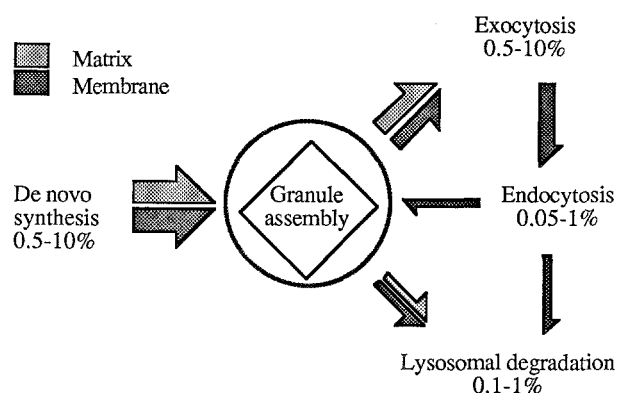
Fig. 1. A model for insulin granule biogenesis. Exocytosis, expressed as a percentage of the granule protein pool lost per hour is balanced by *de novo* synthesis of most of the granule matrix proteins and at least some of the membrane constituents. Granule membrane proteins which constitute approximately a tenth of the granule mass are recovered by endocytosis and then delivered either to lysosomal compartment for degradation or returned to the site of granule assembly; the fate of individual proteins vary greatly at this step. Lysosomal degradation of entire secretory granules by crinophagy constitute another pathway of granule disposal and one which may be regulated by secretagogues [8]

brane contains a variety of proteins which are key components of the machinery which controls secretion. It effectively constitutes a link between internal membrane systems and the cell surface, and in this guise possibly acts as a courier for plasma membrane transport and receptor molecules. It may influence other cellular functions by active modulation of the ionic composition of the cytoplasm.

What is not immediately apparent from electron micrographs is that the insulin granule, perhaps more than any other organelle in the B cell, is a dynamic structure (Fig. 1). Around 10% of the granule population turns over every hour during active secretion. Under such conditions the insertion and retrieval of the granule membrane from the sites of exocytosis in the cell periphery is equivalent to a turnover of half of the total area of the plasma membrane per hour. Granule biogenesis and turnover are clearly very precisely controlled processes since the granule population and cell morphology remain relatively stable under different physiological states. At the molecular level this involves the regulation of the expression of multiple genes. Some of these are encode proteins which are used only once during the exocytotic cycle, others, proteins which are recycled. Newly synthesized proteins are sorted at the level of the Golgi apparatus and incorporated in the correct proportions into the nascent granule. The endocytic events which recover granule membrane constituents from the plasma membrane are similarly tightly controlled as probably are the sorting events that determine whether a retrieved protein is to be incorporated into new granules or degraded by lysosomes. Morphological studies [7] show that the insulin granule buds off from specialized regions of the trans Golgi network which are

characterized by the partial coating of the cytosolic surface of the adjacent membrane with clathrin-like proteins and the presence within the lumen of condensing secretory material. Subsequent maturation involves further condensation of the matrix constituents, reduction in granule diameter and the dissociation of the coat proteins. The fact that granule surface area decreases during maturation suggests that membrane lipid is being removed, presumably by a microvesicle-mediated process. Whether the converse process occurs or whether proteins can be imported selectively into the granule once the secretory material has been enveloped by membrane warrants further study.

Isolation of insulin secretory granules

Early attempts [9–12] to study the biochemical properties of insulin secretory granules in isolation were hampered by the relatively small amounts of material available from pancreatic islets. The granules were of heterogeneous composition by virtue of the cellular heterogeneity of the starting material, contamination with other cellular organelles was a problem and many of the techniques used appeared to either disrupt the granule or render it osmotically unstable. The introduction of a X-ray induced transplantable rat insulinoma by Chick and colleagues in 1977 [13] changed things dramatically in this respect for it became possible to obtain from a single transplanted animal a quantity of B cells equivalent to islets from a 1000 rats.

Such tumours can be passaged by subcutaneous injection of tumour cell suspension into inbred New England Deaconess Hospital-strain rats. They develop in 4–6 weeks to around 0.2–1 g wet weight, at which point the host animal is profoundly hypoglycaemic. Electron microscopy shows that the major endocrine cell type has an abundance of cytoplasmic granules with a typical B-cell morphology (Fig. 2). The rough endoplasmic reticulum is highly developed and there are conspicuous Golgi elements consistent with a cell actively engaged in the synthesis of proteins for export.

Although in many ways an excellent model of B-cell function, one must be cautious in extrapolating biochemical studies in these cells to normal islet tissue. The stimulus-secretion coupling mechanism in rat insulinoma cells resembles that in islets in so far as it depends upon cellular energy, an intact microtubular system and changes in cytosolic Ca^{2+} and cyclic nucleotides. Nevertheless, the responsiveness to glucose and methylxanthines differs from that in islets [15]. The transplantable tumours and derived cell lines tend to lose their differentiated character or change their phenotype after extensive passage [16]. Cell lines adapted to tissue culture usually contain around 1% of the insulin content of tumours developed in animal hosts, probably a combined result of defective granule biogenesis and hormone storage. Quantitative differences in the cellular
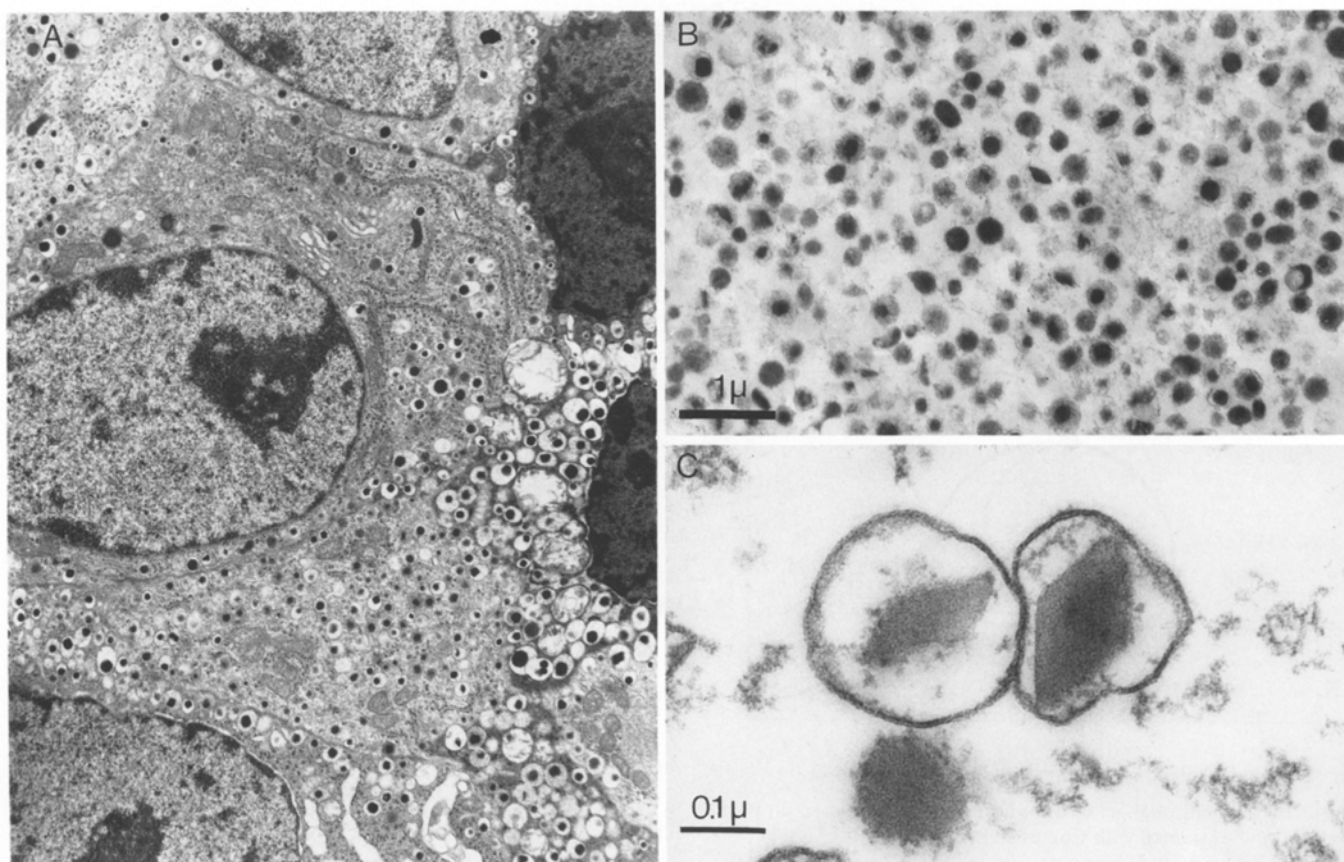
Fig. 2. A Transplantable islet cell tumour showing characteristic B-cell morphology B and C Secretory granules isolated from such tumours by density gradient centrifugation for the most part have an identical morphology to those in the parent tissue though a minor proportion appear to be devoid of their membrane envelope

composition of secretory granule proteins are also observed between tumour tissue and normal rat islets [14, 17].

Our current methods of isolation of insulinoma granules (Fig. 3) rely on homogenization of the tissue in isotonic sucrose, centrifugation to remove cell debris and nuclei, followed by density-gradient centrifugation in two different isotonic media with different separation characteristics. This is followed by a series of washes to remove the gradient material. The majority of granules have a typical B cell morphology though a small percentage seem to lose their membrane during the isolation procedure (Fig. 2 Band C). Using the same centrifugation techniques subcellular fractions enriched in endoplasmic reticulum, mitochondria, plasma and Golgi membrane and lysosomal elements can also be obtained.

The ion composition of such isolated granules differs markedly from the cell as a whole and from the surrounding cytoplasm [18]. High concentrations of Zn (approx. 20 mmol/l intragranular water) are present,

for the major part associated with insulin. High total Ca (120 mmol/l) and Mg (70 mmol/l) contents are also found, these cations being for the major part complexed to the phosphate anion (70 mmol/l) and adenine nucleotides (10 mmol/l). Another major anion of importance is C-peptide (40 mmol/l) which bears 5 negative charges human which are not balanced by positive charges within the same molecule. The apparent increase in granule Ca content that is observed following glucose stimulation of islets [19–21] suggests that the organelle may have modulatory effects on cytosolic free Ca^{2+} concentrations. How Ca and inorganic ions get into the granule interior, however, is presently unknown.

Around 80% of the protein in the granule is accounted for by insulin and C-peptide, a further 10% appears to be in the granule core and its surrounding mantle and a further 10% in the membrane [14]. Two dimensional electrophoresis shows that the protein composition of isolated granules is extremely complex (Fig. 4). The insulin-related molecules account for just a handful of the proteins seen. Among the remainder, some of the complexity is accounted for by the presence of different amounts of carbohydrate on the same protein as indicated by strings of spots on the electrophoretograms increasing in charge and molecular weight. Nevertheless, it would appear that upwards of 100 different polypeptides may be present. Further subfrac-
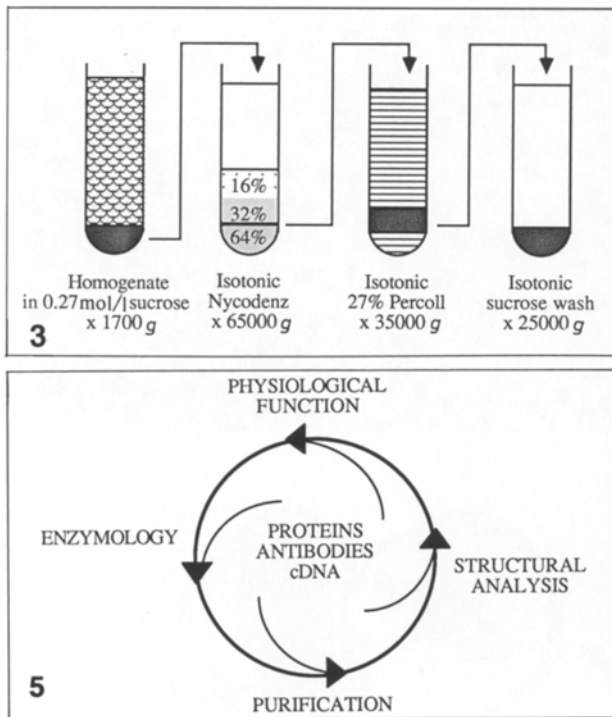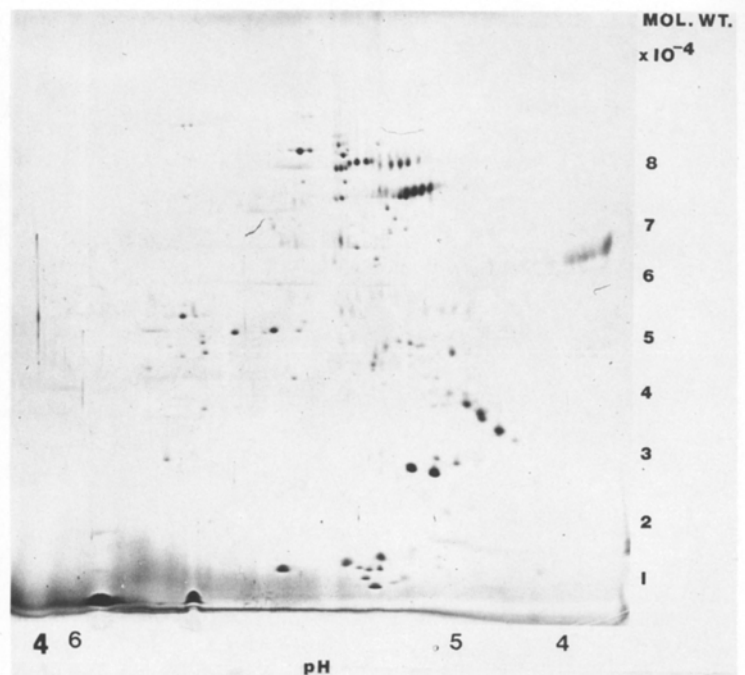
Fig. 3. Granule isolation by density gradient centrifugation

Fig. 4. Two dimensional gel electrophoretogram of isolated rat insulinoma granules stained with Comassie Blue for proteins

Fig. 5. Approaches to the investigation of the structure and function of insulin granule proteins

tionation indicates that much of the diversity originates from the multiplicity of proteins in the secretory granule membrane.

What we have done over the last six or so years is to examine a number of these insulin granule proteins in detail and try to relate their biochemical properties to their function within the granule and the B cell. Insulinoma granules have invariably been used as a source of proteins, however functional studies have been performed with isolated islets wherever possible.

Although the approach to the study of each protein has varied, there seems to be a wheel of fortune to which our efforts have been tied (Fig. 5) which I hope to illustrate with three examples. The first is the proton-translocating ATPase of the granule membrane. Here we started with the description of a physiological function and then went in search of an enzyme. The second example concerns the enzymes involved in the conversion of proinsulin to insulin where we started with an enzymic activity. The third example is a protein which started life as a secreted peptide defined only by its molecular weight for which we deduced a function through structural analysis. Antibodies generated to defined granule components occupy a central position in the hub of the wheel and provide important tools for assessing the *in vivo* functions of the granule constituents. In the instance of the secretory granule membrane, which has a complex protein composition and where individual proteins are present in tiny amounts, the use of monoclonal antibodies raised at random to secretory granule membranes has provided another means of obtaining specific molecular probes and an affinity-based approach to the purification and elucidation of structure of granule proteins [22, 23].

The proton-translocating ATPase

Evidence for the presence of a proton-translocating ATPase in the insulin secretory granule membrane has come from studies of the ATPase activity in freshly-isolated osmotically-intact insulin granules combined with determination of the pH gradient across the secretory granule membrane, measured by the space of distribution of a radiolabelled permeant base, methylamine, and the transmembrane voltage difference, determined from the distribution of a radiolabelled anion, thiocyanate [24–26]. The results of such experiments are readily interpreted within a chemiosmotic model which postulates firstly, that there is a catalytic activity in the membrane which transports hydrogen ions vectorially from the cytosol into the granule interior at the expense of ATP hydrolysis, and secondly that the passive conductivity of the membrane to protons is low. ATP hydrolysis generates a pH gradient of around 1–1.5 units and establishes a voltage potential across the membrane of around 45 mV, positive inside. In terms of its substrate specificity, ionic requirement and inhibitor profile, the insulin granule proton pump is similar to the multimeric protein isolated from chromaffin granules of adrenal medullary cells, to proton translocases found

in elements of the endocytic pathway and indeed to similar enzymic activities found in intracellular vesicles in distantly related organisms [27, 28]. Antibodies raised to major peptide constituents of the proton pump of beetroot tonoplast membranes recognize peptides of similar molecular size in both chromaffin and insulin granules (Fig. 6), further indicating the wide distribution and highly-conserved nature of this catalytic activity.

The function of the proton pump in granules within living cells has been examined using permeant bases to determine the pH of their interior relative to the cytoplasm. Determinations have been made, either directly with fluorescent probes at the light microscopic level [29], or at the ultrastructural level using antibodies raised to permeant dinitrophenol congeners [7]. Interestingly, newly-formed clathrin-coated granules appear to be relatively neutral whereas the non-coated granules, which are derived from them, have a markedly acidic internal environment. These granule sub-types can be distinguished also in that the former are rich in proinsulin whereas the latter contain predominantly insulin. This suggests that intragranular acidification occurs at a relatively late stage in secretory granule biogenesis and that this is instrumental in the initiation of prohormone conversion. Whether such acidification occurs as a result of the insertion of the proton translocase into the granule membrane, by the activation of pre-existing proton pumps or by the removal or inactivation of other ionic conductances (e.g. a H^+/cation antiporter) from the granule membrane, remains to be resolved.

Functions of the proton pump

It would be wrong to try to ascribe a single function to the insulin granule proton pump. In the most general sense it serves to energise the secretory granule membrane and provide the driving force for many transmembrane energy-requiring transport processes (Fig. 7). In the mitochondria, redox reactions are coupled to proton movements and the chemiosmotic gradients so produced are coupled to ATP generation via ATP synthase, a proton translocating ATPase which is similar to, though distinguishable from, the granule proton pump. In the granule, the energy stored in ATP can be converted back into a chemiosmotic gradient of protons which may then be coupled through other proteins to perform useful work in many different ways. A number of the consequences of proton translocation in the granule are illustrated in Figure 8.

Acidification of the granule interior to around pH 5.5 as a result of proton translocation will promote the crystallization of insulin and affect intragranular enzymes with a strong pH dependence. It will induce the dissociation of metal ion complexes within the granule. This may be of particular relevance to the associ-
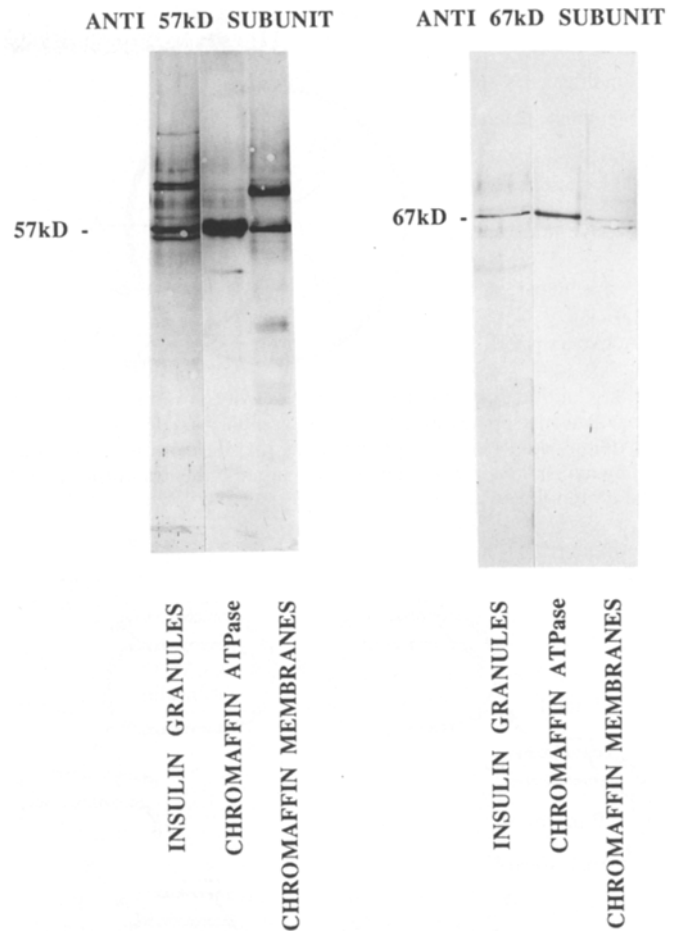
Fig. 6. Western blot analysis of isolated insulin secretory granules, isolated chromaffin granules and chromaffin granule membranes using antibodies raised either to the 57 kD or 67 kD subunits of the beetroot tonoplast proton pump
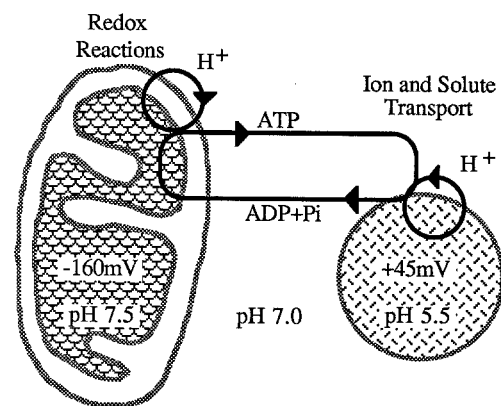
Fig. 7. Coupling of mitochondrial redox reactions to ion and solute transport processes in the granule mediated by proton translocases

ation of Ca with phosphates and proteins [18]. Acidification will result in the uptake of endogenous permeant bases and can certainly account for the accumulation of 5-hydroxytryptamine and dopamine in the granule [30] and the subsequent co-secretion of these bioactive amines with insulin. It is notable that the specific amine
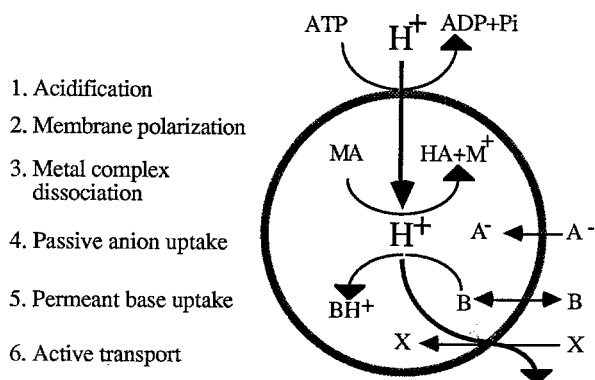
Fig. 8. Coupling of proton translocase activity to intragranular and transmembrane processes in the insulin granule. MA designates metal ion/anion complexes; A^-, permeant anions; B, permeant bases and, X, any molecule transported in either charged or neutral form by specific proton-linked carrier

1. Acidification
2. Membrane polarization
3. Metal complex dissociation
4. Passive anion uptake
5. Permeant base uptake
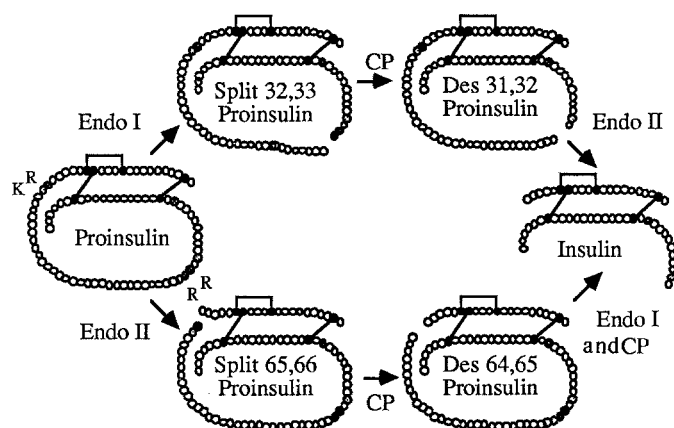6. Active transport

Fig. 9. Pathway of conversion of proinsulin to insulin

transporter present in the chromaffin granule membrane has a very low activity in the insulin granule and thus the insulin granule concentrates these amines only 50-fold relative to the surrounding media as opposed to the several thousand-fold accumulation seen in amine storage granules.

The establishment of a membrane potential of 45 mV across a 9 nm wide membrane corresponds to an electrical field of around 50000 V/cm. This may affect the rigidity of the membrane and the conformation of membrane proteins and thus may be of regulatory importance. The membrane potential will promote the inward movement of permeant anions and there is evidence for the existence of an anion transporter within the granule membrane. A possible consequence of such activity could be the intragranular accumulation of Cl^-. In other vacuolar systems proton translocation has been shown to be coupled to the active transport of diverse molecular species through specific carriers including amino acids, Ca, nucleotides, nucleotide sugars and proteins [27, 28]. A role for the proton pump in similar activites in the insulin granule warrants further investigation.

Proteolytic conversion of proinsulin

Proinsulin is converted to insulin by excision of the Arg Arg sequence at positions 31,32 and the Lys Arg sequence at positions 64,65 in the molecule (Fig. 9). Conversion appears to involve initial attack by endoproteases on the C-terminal side of these dibasic sequences followed by the action of an exopeptidase specific for C-terminal basic amino acids to remove sequentially the exposed basic residues [31]. Such a mechanism is as initially predicted some 20 years ago by Steiner and his colleagues. However, only recently has progress been made in the isolation and characterization of the enzymes involved in the conversion process. This is in part related to the finding that the endopeptidases are very fastidious in regard to their ionic and pH requirements and are rapidly inactivated by conditions previously used to extract tissue. Also, in most preparations used to date, their activity is obscured by the presence of non-specific proteases of lysosomal origin. This is where, again, the availability of highly purified subcellular fractions from insulinoma tissue has proved invaluable.

Assays for processing activity in subcellular fractions prepared from insulinoma tissue have demonstrated that the secretory granule compartment is a major intracellular site of concentration of the converting enzymes [32] (Fig. 10). Reaction products include both insulin and the intermediates des 31,32 proinsulin and des 64,65 proinsulin which are formed by cleavage at one or other of the basic sites followed by carboxypeptidase H action (Fig. 9). These intermediates are also produced in intact B cells and correspond to the major circulating forms of proinsulin immunoreactivity [33].

Further analysis reveals that at least three different catalytic activities are involved in the conversion process. These are designated type I endopeptidase, type II endopeptidase and carboxypeptidase H [34, 35] (Table 1). One endopeptidase (type I) cleaves exclusively after the Arg 31, Arg 32 sequence at the B-C chain junction, the other (type II) cleaves preferentially after the Lys 64, Arg 65 sequence at the A-C chain junction though it will also attack the B-C chain junction at around a tenth to a fifth of the rate. Carboxypeptidase H works equally well with substrates extended C-terminally by Lys or Arg residues albeit at different rates [34]. The primary amino acid sequence of the processing site appears to be a major determinant of type I and II endopeptidase specificity. This was suggested by the finding that the same specificity is exhibited towards dibasic sequences in other proprotein substrates (proalbumin and chromogranin A), and that tripeptides which bear a dibasic sequence preceded by an Ala residue on the N-terminal side and followed by a reactive sulphonium group inhibited each enzyme in accordance with its preferred site of cleavage in proinsulin [36]. The pH optimum of all the enzymes is 5.5 and thus corresponds to the pH of the mature secretory granule

[25]. The pH profiles in each case are sharp suggesting that modulation in intragranular pH in the range of 4–7 could provide a means of regulating their activities. There is a subtle difference between the two endopeptidases, however, in this regard; whereas the type I activity (Arg Arg-specific) is virtually abolished at pH 7, the type II enzyme (Lys Arg-preferred) retains about 30% of its activity. The carboxypeptidase enzyme is markedly reduced at neutral pH, however, even at 5% of its maximal activity, it still vastly exceeds the maximal rate of endoproteolytic cleavage.

The enzymes involved in proinsulin processing require metal ions for activity. The carboxypeptidase is a classic Zn metalloenzyme, while the endopeptidases are activated by Ca. Again, there are differences between the endopeptidase subtypes; the type I enzyme requires Ca in the millimolar range for half-maximal activation, the type II a 25-fold lower concentration. The free Ca content of secretory granules is probably in the millimolar range [18] and would thus support maximal activity. However, in the compartments through which proinsulin passes prior to reaching the granule it is unlikely to reach levels that would support type I activity. This is because, firstly, the overall content of Ca is lower and secondly, more Ca will be in a complexed form due to the more alkaline pH of these compartments. This dual control exerted by Ca and pH ensures that final conversion of the single chain precursor does not occur until it reaches the granule compartment. This is important since insulin is considerably less soluble than proinsulin [37].

Another unusual property of the endopeptidases, particularly the type II enzyme, is that they are irreversibly inhibited by monovalent anions. Since the insulin granule may accumulate anions as a result of the proton translocase establishing a transmembrane potential difference, such anion sensitivity might provide the basis for the inactivation of the processing activity. This would limit conversion to a particular time window following maturation of the granule and thereby prevent non-specific proteolysis of other key granule components.

The activity of the endopeptidases is probably the rate-limiting step in the overall process of conversion and, thus, the regulatory effects of pH, Ca and anions documented *in vitro* may have relevance in the intact cell. Pulse-chase labelling experiments in which insulin-related peptides are first immunoprecipitated and then analysed by HPLC (Fig. 11) show that the principal product of type I endopeptidase cleavage, des 31,32 proinsulin, appears in parallel with insulin after a 30 min delay [35]. This coincides with the transfer from the newly-formed coated granule to the uncoated granule compartment and its acidification (Fig. 12) [7]. The des 64,65 intermediate, however, appears earlier, at a point in time when the precursor is distributed within the trans Golgi network and in the newly-formed coated vesicles. This is consistent with the finding that
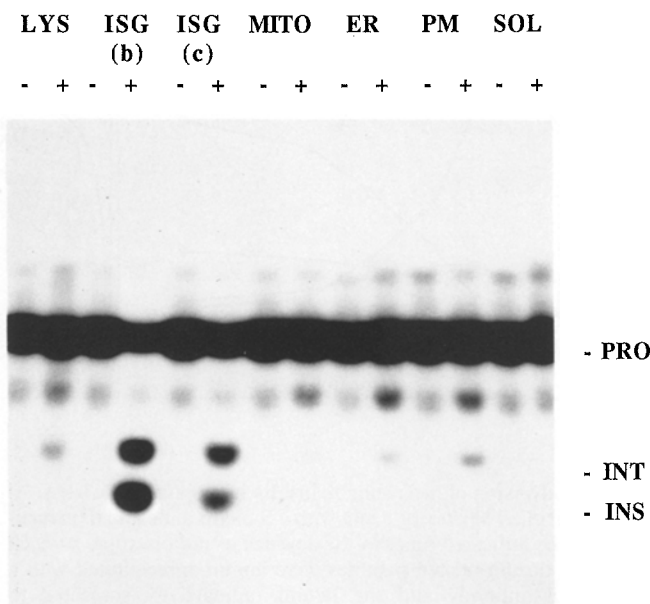

Fig. 10. Conversion of ^{125}I proinsulin by various subcellular fractions of rat insulinoma tissue including fractions enriched in lysosomes (LYS), secretory granules (ISG), mitochondria (MITO), endoplasmic reticulum and Golgi (ER), plasma membrane (PM) and cytosolic proteins (SOL). Reaction products were separated by alkaline urea gel electrophoresis and analysed by autoradiography. The positions of migration of proinsulin (PRO), the des 31,32 proinsulin and des 64,65 proinsulin intermediates (INT) and insulin (INS) are indicated

the type II enzyme activity can operate at a more neutral pH and at lower Ca concentrations than the type I.

We know that the proteases involved in proinsulin processing are capable of processing other proproteins such as proalbumin and that similar enzymes are found in other mammalian tissues [38]. It may be that the type II enzyme plays an additional role in the processing of other proteins in the B cell that are not segregated to the secretory granule, such as proteins that are transferred from the Golgi to the plasma membrane or other intracellular vacuolar compartments. Also, having two enzymes with differing sensitivities to pH, Ca and anion concentration may provide the mechanism by which a single prohormone can be processed differently in different tissue or differentially processed within the same tissue under different physiological condi-

Table 1. Enzymes involved in proinsulin conversion

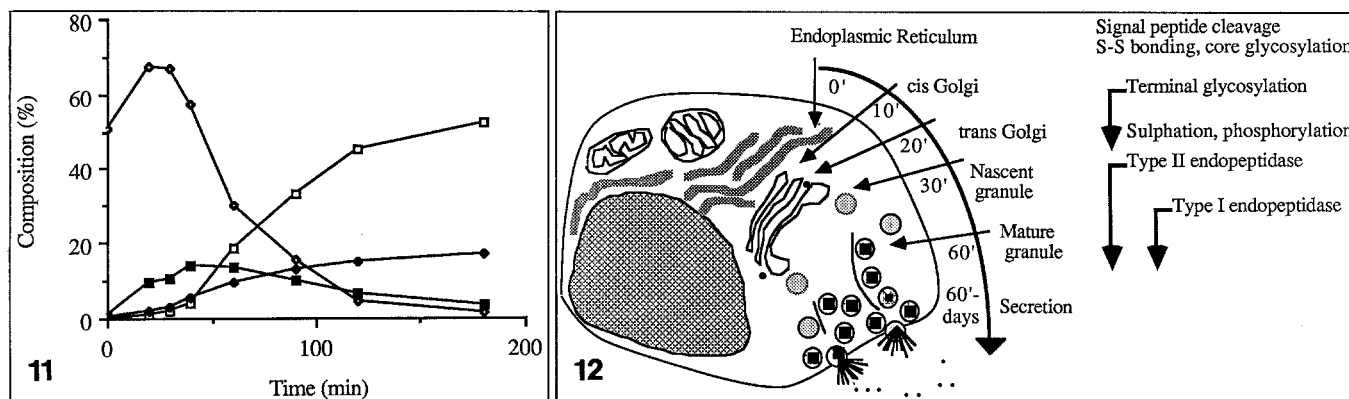|	Endopeptidase		CBZ-H
	Type I	Type II	
Specificity	X-R-R▼X	X-(K/R)-R▼X	X▼R or X▼K
Granule Activity (nmol·min⁻¹·mg⁻¹)	0.68	0.46	100
pH optimum	5.5	5.5	5.5
Activity @ pH7	2%	30%	5%
Cation (Ka)	Ca (2.5 mmol/l)	Ca (0.1 mmol/l)	Zn (10 nmol/l)
Anion (Ki)	Cl (160 mmol/l)	Cl (18 mmol/l)	

Fig. 11. Conversion of proinsulin to insulin in rat pancreatic islets. Islets were labelled *in vitro* for 5 min with ^{35}S-methionine and then incubated for the indicated times in the absence of radioisotope. At each time point insulin-related peptides were immunoprecipitated with a monoclonal antibody and the bound radioactivity subjected to HPLC. Incorporation of radioactivity into proinsulin (◇), des 31,32 proinsulin (□), des 64,65 proinsulin (■) and insulin (◆) is expressed as a percentage of the maximal incorporation observed (20 min point)

Fig. 12. Post-translational events in insulin secretory granule biogenesis

tions. The differential processing of proglucagon in the gut and in pancreatic endocrine tissue is a good example in this respect [39].

Cosecreted peptides

The third example of a secretory granule protein is involved in communication between the cells which constitute the pancreatic islet. The major islet hormones clearly play an important role in this area [40], however, what concerns us here are the minor granule constituents that are co-secreted with the hormone and which potentially serve as local regulators. Because of the small amounts of these peptides which are secreted their targets are most likely to be either the cell which releases them or adjacent cells including those of the microvasculature. Such peptides were investigated initially by SDS gel electrophoretic analysis of the proteins which were co-secreted with insulin from islets or insulinoma cells after extensive labelling with radioactive amino acids [41]. Twelve peptides, ranging in size from 5-60 kD, were observed whose release paralleled that of insulin. As predicted by the exocytotic model of secretion these were found in significant concentrations in insulinoma granules.

One of the more abundant of these, a 21 kD protein termed betagranin has been purified [42]. Using antibodies raised to the purified protein, it was shown by immunofluorescence to be restricted to cells of the neuroendocrine system, and hence appeared in the islet, pi-

tuitary, adrenal and isolated cells in the gut [16]. In the islet it was present in most pancreatic endocrine cells but unevenly distributed being concentrated in subpopulations of A and B cells. N-terminal amino acid sequence analysis yielded the surprising finding that this small protein was homologous to the much larger co-secreted protein of the adrenal chromaffin cell, chromogranin A. By pulse-chase labelling experiments it was demonstrated that the protein was originally synthesized as a larger precursor of the same size as rat adrenal chromogranin A and that this was converted in time to peptides identical in size to the native 21 kD protein [43]. The conversion paralleled that of proinsulin to insulin suggesting that it was subjected to similar post-translational proteolytic events. The precursor form was prepared by biosynthetic radiolabelling and immunoprecipitation of insulinoma cells and used in *in vitro* conversion assays similar to those performed with proinsulin. Secretory granule lysates converted the labelled protein into a 21 kD fragment whereas other subcellular fractions, either did not affect it, or in the case of lysosomes, cleaved it into fragments of a different molecular size [44]. Inhibitor and ion-dependency studies demonstrated that the conversion required a type II-like endopeptidase activity and carboxypeptidase H thus suggesting that cleavage was directed at a Lys Arg sequence.

To obtain further sequence information, an insulinoma expression vector cDNA library constructed in lambda phage gt 11 was screened with the antisera to the native 21 kD protein. This produced a number of short clones, fragments of which were used to screen a second cDNA library prepared from rat islets. From this a further three clones were obtained which together with the original clone covered 1696 bp of DNA and encoded the complete sequence of the precursor protein [45] (Fig. 13). The authenticity of the clones was confirmed by a series of antibody controls and gasphase sequencing of tryptic fragments of the original protein.

The protein of 448 aa and 51 kD had some rather unusual features including a stretch of 20 glutamine residues and long stretches of acidic residues. Second-

ary structure predictions indicated extensive regions of random coil sequence. A total of 10 different dibasic sites were present in the molecule, one of which, a Lys Arg sequence, appeared just beyond the most C-terminal peptide fragment that had been sequenced from betagranin. This fitted with the prediction that the cleavage was Lys Arg-directed based on the enzymology of conversion of the precursor *in vitro*. Two concensus sequences for N-linked glycosylation were observed consistent with the apparent glycosylation of the betagranin molecule. The sequence Arg Gly Asp also appeared towards the C-terminus. This is found in a number of cell adhesion molecules such as fibronectin and vitronectin and constitutes the region of these molecules which interact with cell membranes [46]. The precursor cDNA was homologous to bovine and human chromogranin A and apart from one minor difference in the 3' untranslated region, was identical to chromogranin A of the rat adrenal [47]. The strongest homology at the amino acid level occurred in the N- and C-termini, notably in the parts of the molecule which might produce small peptides as a result of proteolytic cleavage at pairs of basic amino acids. Seven of the 10 dibasic sites in the rat molecule are conserved in bovine and human chromogranin A.

During the course of these investigations the peptide sequence was published of a 49 amino acid peptide from porcine pancreas, named pancreastatin, which inhibited insulin secretion from the perfused rat pancreas [48]. It was immediately apparent that this was homologous (56%) to amino acids 267–314 of the rat chromogranin A sequence. The production of pancreastatin, however, would seem to involve cleavage, not at a dibasic site, but at a single lysine residue at position 316. Subsequent removal of the exposed lysine by carboxypeptidase H would generate a C-terminal glycine. This could then act as the amide donor to the preceding glycine residue to produce the observed C-terminal amidated structure. Such a mechanism suggests the participation of an endopeptidase which can recognize single basic residues and also of a peptide amidating mono-oxygenase (PAM) activity. Such enzymes clearly occur in other secretory granule types and, in the case of PAM, has been reported in neonatal islets [49].

Pancreastatin immunoreactivity was demonstrated in the B cell by specific antisera raised to the porcine molecule [50]. It was also found in D cells but no A-cell immunoreactivity was observed, which is in contrast to the islet distribution of betagranin immunoreactivity [17]. This might be due to a species difference, however it raises the possibility that chromogranin A is processed to different end products in different islet cells. It will be interesting to discover what other bioactive peptides reside in the chromogranin A molecule and whether the regulation of intragranular proteolytic processing by pH and Ca can determine the differential generation of this and other autocrine and paracrine regulators in the endocrine pancreas.
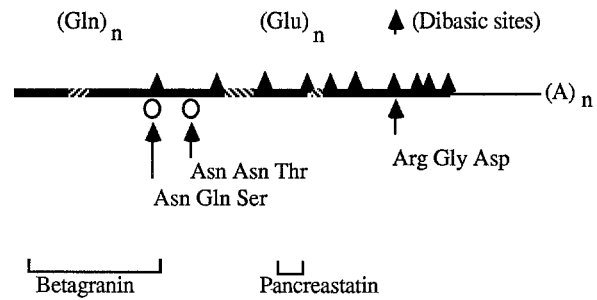

Fig. 13. Structural outline of the precursor of betagranin from the endocrine pancreas. The cloned sequence was comprised of an open reading frame of 1360 bp and 336 bp of 3' untranslated sequence incorporating a concensus sequence for poly A addition 23 bp from the 3'terminus (A_n). The encoded protein exhibited a typical signal peptide followed by the 448 aa's of rat chromogranin A. Ten pairs of basic residues are indicated as are the 2 concensus sequences for N-linked glycosylation (Asn X Ser/Thr) and the tripeptide equivalent to the fibronectin receptor binding site (Arg Gly Asp). Also to note are the polyglutamine sequence (aa's 74–93) encoded principally by a CAG repeat and the extensive tracts of glutamyl residues between aa's 213–231 and 326–332. The β-granin sequence corresponds to amino acids 1–128 (up to the first dibasic site) and pancreastatin amino acids 267–314
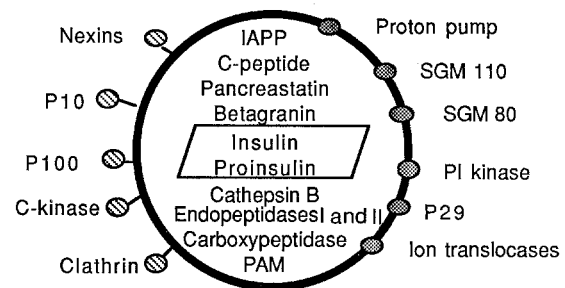

Fig. 14. Insulin secretory granule proteins: a current view. Four major sites of protein localization are defined; the granule core, the mantle and/or the inner surface of the granule membrane, intrinsic membrane proteins with cytosolic domains and loosely- or transiently-associated membrane constituents. The individual components include those mentioned above, substrates (P10, P29 and P100) and enzymes (C-kinase) involved in granule membrane protein phosphorylation [51, 52], enzymes of phosphatidylinositol metabolism [53], cathepsin-B related proteins [54], Ca^{2+}-binding proteins [55] and membrane antigens (SGM 80 and SGM 110) [22, 23]

Conclusions

One message that emerges quite emphatically from studies such as these is the extent to which granule functions are interrelated and how the granule operates as an organelle in the true sense of the word. Most of its constituent proteins seem to be either substrates for other granule components or at least regulated by the activity of other granule proteins. The proton translocase, for example, plays a major role in determining the ionic composition of the granule interior and thus the activity of the intragranular proteases and the physical state of the stored hormone. It also regulates the intragranular accumulation of bioactive amines and gener-

ation of autocrine factors. In this way it will have an impact on how the B cell senses stimuli and how it monitors its own activity.

None of the proteins considered here appear to be confined exclusively to the B cell; indeed of the twenty or so other accessory proteins we have now identified in the insulin granule (Fig. 14) most are found in one or other endocrine secretory granule types. That is not to say, that apart from the presence of the principal secretory product, the composition of all secretory granules is uniform. Indeed the quantitative differences in protein composition between individual polypeptide hormone-secreting granules are sufficiently great as to effectively mean that any one granule type is almost as unique as its principal secreted protein.

Granule constituents are under complex regulatory control and are in a constant state of flux as a consequence of the exocytotic/endocytotic cycle. In addition, proteins on the inner aspect of the granule membrane are periodically exposed to the extracellular media. As a consequence one might anticipate that the insulin granule would be a likely origin of pathogenic changes in diabetes. Indeed, there are several indications that disturbances in secretory granule function do occur. Many recently-diagnosed untreated Type 1 diabetic patients have high titres of insulin autoantibodies. There are increased circulating concentrations of proinsulin and processing intermediates in Type 2 diabetic patients and in discordant identical twins of Type 1 diabetic patients [56, 57]. The intracellular and extracellular accumulation of a secretory granule protein that is homologous to calcitonin gene-related-product occurs in the islets of Type 2 diabetic patients [58, 59]. These disturbances could arise from alterations in the process of segregation of proteins to the granule, changes in the biosynthesis or activation of the processing enzymes or increases in the mean transit time of granules between the Golgi and plasma membrane during secretion. It is clear that there is considerable scope for looking at insulin granule function in the diabetic and prediabetic state. Further insight may be gained from experimental studies in which individual secretory granule proteins are genetically manipulated or where the secretory granule protein composition is deliberately altered. Such investigations will become increasingly easy with time as specific molecular probes in the form of antibodies and cDNA's to secretory granule components become available as a result of efforts to gain an understanding of the secretory process at the molecular level.

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