Review article

The mechanism of insulin secretion*

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There had been a number of attempts before 1889 to assess the effects of pancreatectomy on blood glucose regulation, but the results had been controversial, probably because of the difficulty of removing the whole organ. Those who had seen effects on urine volume and glucose production following removal of the pancreas variously ascribed them to the absence of pancreatic juice in the intestine, or to the fact that the pancreas acted as a sink or depository for glycogen which, following its removal, spilled over as glucose into the blood and urine. After completion of a number of different experiments, including, of course, total pancreatectomy in dogs, Von Mering and Minkowski [1] were able to eliminate both of these theories and concluded that: "... diabetes as observed after complete removal of the pancreas is exclusively to be attributed to a stopping of the function of that organ which is necessary for sugar metabolism. We have here to deal with a function of the pancreas hitherto completely unknown." Minkowski published a further paper on the subject in 1893 [2], by which time his basic observations had been confirmed by a number of others, but he never became fully engaged in the hunt for the factor which was produced by the pancreas. He published extensively in many fields of experimental and clinical medicine before his death in 1931, aged 74 years.

Studies involving the removal of the pancreas have of course continued to this day, and were given a particular impetus by the discovery [3–5] that collagenase digestion of the pancreas caused its selective breakdown and the subsequent release of large numbers of isolated islets of Langerhans, which are presumably resistant to the action of collagenase. These islets were identified and handled according to procedures first described by Hellerström [7] for use with micro-dissected islets. Availability of this material, which still consists of only 70% of β cells, allowed for the first time the application

to insulin-producing tissue of many modern techniques for biochemical analysis. This resulted in an explosion of publications using these procedures, amounting to at least 1250 original papers in the period from 1967 to 1982 (Fig. 1). The present discussion deals with two aspects of these studies which have been of particular interest to the author during this period: the structure and function of the insulin storage granule (β granule), and the role of cytoskeletal proteins in the mechanism of insulin secretion.

The insulin storage granule

The structure of the insulin storage granule has been described in at least 30 species from 6 phyla [8]. There is a remarkable uniformity of size of the granule in most of these species, along with an equally remarkable diversity of fine structure of the granule core. Given the highly conserved nature of the insulin molecule, which appears to be markedly divergent only in the South American hystrichomorph rodents (guinea-pig, coypu), it

Fig. 1. Citation by year of some established procedures for collagenase isolation of islets of Langerhans [4–6]. The totals underestimate the true figures since they do not include papers in which variants of these procedures are used, or those in which the original procedures are not cited. Work involving microdissected islets is also excluded

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Fig. 2. Ultrastructure of β granules of **A** human and **B** dog islets of Langerhans. Glutaraldehyde fixation; osmium post-fixation; staining in uranylacetate. ($\times 60,000$)

Table 1. Contents of the insulin storage granule [8]

1. 200,000 molecules of insulin stored in (para)-crystalline form
2. Connecting peptide in equimolar amounts
3. Zinc co-ordinated to insulin through B ₁₀ histidines (except guinea-pig, coypu, hag fish, where this residue is absent)
4. Biogenic amines
5. Calcium in a slowly exchangeable pool
6. Adenine nucleotides, inorganic phosphate [22]
7. Enzymes: Proinsulin converting enzymes
Ca ²⁺ and Mg ²⁺ dependent ATPase
Acid phosphatase
Protein kinase

Table 2. Structure of human and dog connecting peptides

	1	2	3	4	5	6	7	8	9	10	11
Human NH ₃ ⁺	Glu	Ala	<i>Glu</i>	Asp	Leu	Glu	Val	Gly	Gln	Val	Glu
Dog NH ₃ ⁺	Asp	Val	<i>Glu</i>	—	—	—	—	—	—	—	—
	12	13	14	15	16	17	18	19	20	21	22
	<i>Leu</i>	Gly	<i>Gly</i>	Gly	Pro	Gly	Ala	Gly	Ser	<i>Leu</i>	Gln
	<i>Leu</i>	Ala	<i>Gly</i>	Ala	Pro	Gly	Glu	Gly	Gly	<i>Leu</i>	Gln
	23	24	25	26	27	28	29	30	31		
	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	<i>Gln</i>	COO ⁻	
	Pro	Leu	Ala	Leu	Glu	Gly	Ala	Leu	<i>Gln</i>	COO ⁻	

Residues in italics are constant in all species examined [10]

seems unlikely that this variation in fine structure of the granule (e.g. dog versus human β granules, Fig. 2) could be attributed to variation in insulin primary structure which differ by only a single amino-acid (B 30) in dog and man [9]. This leads us to consider other components of the granule and their possible influence on granule structure. Some of these other components are listed in

Table 1. In quantitative terms the major protein component, apart from insulin, is the excised connecting peptide which is present in equimolar amounts with insulin. There is certainly more variation in connecting peptide primary structure than that of insulin (Table 2) [10], but it is unclear to what extent its presence will affect the crystallization process.

The arrangement of zinc insulin hexamers within the crystals or paracrystals which make up the electron opaque granule core have been studied in a number of species, and they seem likely to form a rhombohedral [8] or rhombic dodecahedral configuration [10]. Again the factors which influence this packaging are not well understood; the absence of calcium, as well as the absence of zinc, may induce the cell to produce abnormally large and non-crystalline granules [11]. Zinc is well known to facilitate insulin crystallization; it is not clear whether the change produced by calcium represents a direct requirement for calcium in the packaging, or whether it merely reflects the reduced activity of a calcium-dependent enzyme in the granule formation or proinsulin conversion processes.

Calcium appears to be bound in a rather poorly exchangeable pool within the granule sac or on its membrane [12]. This calcium pool does not appear to be the one which is released during exocytosis since at least five conditions have been identified in which calcium efflux from islet cells can be dissociated from insulin release [13]. Similarly, the role of biogenic amines, although their existence within the granules is well documented [14], is far from clear.

The size of the individual insulin storage granule (0.2–0.3 μ m) is very small in comparison to that of insu-

Fig. 3. A and B Ultrastructural localization of cationic ferritin on the surface of β cells; this material is regarded as a marker for anionic sites on the plasma membrane. At the points (arrows) where granule membrane and plasma membrane have fused during exocytosis no ferritin labelling is evident, consistent with a reduction of negative charge in the granule membrane. Reproduced with permission [16] ($\times 75,000$)

Table 3. Some calculations of granule numbers

No. of insulin molecules/granule	$\approx 200,000$
No. of granules/cell	$\approx 13,000$
No. of β cells/islet	$\approx 4,000$
No. of islets/human pancreas	$\approx 250,000$
No. of insulin granules/pancreas	1.3×10^{13}
Assume 5% of insulin content secreted/h	6.5×10^{11}
Exocytoses/s	1.8×10^8

lin crystals grown in vitro, but is consistent with the majority of other endocrine storage granules (e.g. growth hormone $0.3 \mu\text{m}$, corticotropin $0.2 \mu\text{m}$, thyrotropin $0.125 \mu\text{m}$). Nevertheless, their small size necessitates the storage of very large numbers of granules – 13,000 in each β cell [15]. It is thus possible to calculate the number of granules stored in the pancreas, and the number which must be secreted per hour during rapid insulin secretion in man (Table 3); it will be seen that exocytosis of β granules is a rather frequent event.

The question of whether the β granule merely plays a passive role as a storage granule for insulin, or whether the granule itself plays an active part in its own extrusion remains to be resolved. It certainly seems likely that changes in charge on the granule membrane might contribute to the final fusion of granule and plasma membrane in exocytosis, as shown in experiments in which cationic ferritin was used to locate anionic sites on the granule and plasma membranes [16]. It is clear that the granule membrane itself carries a net negative charge and that this may be reduced on stimulation of secretion, possibly as a result of a local change in calcium concentration in the area immediately beneath the plasma membrane (Fig. 3). In addition, it has been suggested that the granule might contribute to its own extrusion as a result of increasing osmotic pressure and ul-

timately bursting, following uptake of anions through activity of a magnesium-dependent proton pump ATPase. This is termed the chemiosmotic hypothesis of granule/membrane fusion [17]. Evidence in its favour is indirect but includes the inhibition of insulin secretion following substitution of chloride in incubation media by the impermeable anion isethionate, or by increasing the osmotic pressure of the medium by addition of sucrose [18]. Yaseen et al., in experiments using permeabilized islets in which the ions have direct access to the site of exocytosis, have suggested recently that uptake of cations rather than anions might be important in promoting this type of mechanism of exocytosis [19]. This process is seen as quite distinct from the mechanisms involved in the intracellular movement of granules from the cytoplasmic pool to the granule membrane, to a distance presumably determined by van der Waals' forces and electrostatic repulsion [20].

Finally, if (as is the case with chromaffin granule membranes [21]), the β granule membrane should contain even small amounts of myosin, which is known to be present within the β cells, then this would certainly allow the granule to contribute to its own movement through the cell before its extrusion (see below). A magnesium-dependent ATPase has been identified recently in granule membranes of an insulin secreting tumour [22] which might conceivably be related to myosin-ATPase.

The cytoskeleton and insulin secretion

The concept of the cytoskeleton as a framework for the structure of cells and for some of their intracellular movements is well established. Elements of the cytoskeleton include microtubules, microfilaments and a vari-

Table 4. Effects of various agents on microtubules and insulin secretion

Agent	Action	Effect on secretion	Phase affected
Colchicine	Depolymerization	Inhibition	Second [26]
Vinblastine	Paracrystal formation	Inhibition	First and second [27]
Nocodazole	Depolymerization	Inhibition	First and second [28]
Deuterium oxide	Polymerization	Inhibition	First and second [29]
Taxol	Polymerization	Inhibition	First and second [28]

Fig. 4. High resolution scanning electron micrograph of part of an islet cell prepared by the method of Tanaka [23]. Secretory granules (G) joined by microtubules or microfilaments (arrows) are clearly seen. ($\times 100,000$)

ety of other types of intermediate filaments. Operationally, however, the term cytoskeleton has frequently been used to describe that fraction of the cell contents which is resistant to mild extraction by Triton-X-100. This normally includes microfilaments, but not microtubules. We have examined recently the application of these cytoskeletal extraction procedures to isolated rat islets of Langerhans and found that as in other tissues, islet nuclei and microfilaments, but not microtubules, are resistant to extraction by detergent. It proved possible to separate these organelles by filtration through polycarbonate filters, which allow the microfilaments through but retain the nuclei (SL Howell and M Tyhurst, unpublished observations). The constituents of the cytoskeleton separated in this way, and their properties, are currently under investigation.

Unfortunately, the insulin storage granules do not

survive the Triton extraction procedure outlined above, so that it is impossible to examine granule-cytoskeleton interactions in this system. A further drawback is the lack of survival of the microtubules, which represent an integral part of both cytoskeleton and granule-translocation systems. Therefore, we have sought alternative means of extraction of the cytoplasm to reveal the organization of the cytoskeleton in a three-dimensional way, and have used for this purpose the osmium-dimethylsulphoxide-osmium (O-D-O) technique of Tanaka [23], combined with high resolution scanning electron microscopy. By this means, it should be possible to reconstruct the three dimensional relationships between granule and cytoskeletal elements in some detail. Examples of the relationship between β granules and tubular elements, identified by their diameter as microtubules, are shown in Figure 4. Evidence for the role of microtubules and microfilaments in the mechanism of insulin secretion has been reviewed recently [24]. An overview of this work, together with some speculations about the nature of their involvement in the secretory process, are presented here.

The role of microtubules

The involvement of the microtubular/microfilamentous system in the intracellular transport of granules to the plasma membrane was originally suggested by Lacy et al. [25] on the basis of effects of colchicine in inhibiting insulin secretion from isolated rat islets of Langerhans. Over the years the concept had been extended by the use of several other agents which interfere with microtubule function (Table 4). It is clear from this table that agents which increase polymerization (taxol, deuterium oxide), and those which produce depolymerization both inhibit first and second phases of secretion. It appears that the microtubules do not act simply as a 'railway' system, with the presence of an increased number of microtubules producing greater secretion rates, but rather that the dynamic regulation of polymerization is more important in the secretory process. Accordingly, the extent of polymerization of tubulin to microtubules in different conditions has been studied, affording results which suggest that tubulin polymerization is increased in situations in which cyclic AMP concentrations in the islets are raised [30]. The regulation of polymerization is often supposed to be achieved by phosphorylation not of the microtubules themselves, but of microtubule associated proteins – τ proteins and microtubule-associated protein-2 [31]. However, very recently Colca et al. [32] have been able to demonstrate phosphorylation of tubulin subunits themselves in rat islets, by a calcium-calmodulin-dependent protein kinase. The extent of this phosphorylation was increased on exposing islets to glucose stimulation of secretion, but in the 54k subunit alone and at some time points only, in particular 3 min after stimulation [32].

The role of microfilaments

Perhaps the most interesting feature of microfilaments, whose constituent protein actin is known to comprise 1%–2% of the total islet protein (c.f. 10% for insulin), is that less than 50% of the actin is present in the polymerized (filamentous or F-actin) form, despite the fact that the conditions inside the cell (high salt concentrations) appear to favour its complete polymerization. Much of this microfilamentous material may be present in the cell web [33]. The degree of polymerization of actin is enhanced from about 30%–50% of the total [34, 35], on stimulation of insulin secretion by 20 mmol/l glucose, and in general the extent of its polymerization parallels the rates of insulin secretion. Absence of extracellular calcium does not affect actin polymerization, yet markedly inhibits insulin secretion while lowering of intracellular ATP levels by the uncoupling agent DNP inhibits both insulin secretion and actin polymerization [34].

This regulation of polymerization of actin suggests the possibility of the existence of one or more specific actin-binding proteins which may associate with actin in its globular form and dissociate from it when increased polymerization of actin is required during stimulation of secretion. Actin-binding proteins have been observed in other tissues, and their existence provides a potential regulatory step for $G \rightleftharpoons F$ actin conversion (Fig. 5), and ultimately for intracellular granule movement; they include actinogelin [36], gelsolin [37] fragmin [38], villin [39] and an F-actin binding protein related to erythrocyte spectrin, termed fodrin [40]. Some of these proteins induce gelation of actin in vitro in a calcium-dependent way. Of particular interest is an F-actin binding protein caldesmon, which is sensitive to calcium, and which has been identified in aorta, uterus and platelets [41]. Binding of caldesmon to F-actin is enhanced at low calcium concentrations; at high calcium concentrations ($< 1 \mu\text{mol/l}$) caldesmon-actin binding is reduced and F-actin is released. Thus a further potential mechanism is available for the regulation of the activity of F-actin, but the existence of such a protein has yet to be demonstrated in islets.

Islet cytosol preparations have been shown to contain factors which can prevent the polymerization of rabbit skeletal muscle actin in vitro [42]. Each was calcium dependent, and the two factors on separation had molecular weights of 200,000 and 40,000 daltons. However, the effect of activation of these binding proteins would be to inhibit polymerization of actin in the presence of the increased calcium concentrations seen on stimulation of secretion by glucose, an action which is inconsistent with the observed increase in overall polymerization.

Myosin has been demonstrated in islet extracts and to exist as heavy and light chains as in other tissues [43]. Its overall concentration appears to be 0.5%–1% of total islet protein. The enzyme myosin light chain kinase has

Fig. 5. A possible role for actin-binding proteins in the regulation of microfilament (F-actin) polymerization. The tight binding of the protein to G-actin is released on stimulation of secretion by elevation of calcium concentrations

Fig. 6. Model showing the relative size of myosin molecule and β granule, drawn to scale. In an artificial system [54] the presence of 25 myosin molecules was sufficient to propel a bead of diameter 0.7 μm along an actomyosin contractile system

also been identified in islet [44, 45] and insulin secreting tumour [46] extracts, and shown to phosphorylate exogenous myosin light chains.

Granule interactions with cytoskeletal proteins

There is some evidence for direct interactions of β granules with microtubules from bovine brain, provided that microtubule-associated proteins are added to the system [47]. The association is further promoted by the addition of cyclic AMP, but it is not clear whether this is a result of phosphorylation following activation of a cyclic AMP-dependent protein kinase.

It is clear that granules can interact also with actin filaments and that this effect is specific at least to the extent that it cannot be mimicked by the use of collagen fibres or Ficoll solutions of comparable viscosity [48]. However, actomyosin will interact with isolated granules at far lower concentrations, suggesting that the actomyosin rather than actin might be more likely to be the force-generating component of the intracellular granule movement mechanism [49]. It would be fairly easy to imagine how actomyosin could produce a force-generating system, either with the granule membrane attached by some means to the filaments, or alternatively with myosin as a component of the granule membrane (Fig. 6). The fact that endogenous myosin (in actomyosin) is required for optimal granule membrane interaction perhaps suggests that any myosin in the granule

Fig. 7. The outline of a speculative model for the possible interactions of tubulin, actin and myosin in the mechanism of insulin secretion

Fig. 8. The same speculative model for the possible interactions of tubulin, actin and myosin as in Figure 7, showing details of its possible regulation. MAP = microtubule-associated proteins

membrane after isolation is in a dephosphorylated form which cannot interact with actin alone.

The relationship between elevation of cytosolic calcium concentrations and the activation of insulin secretion by exocytosis is of central importance for the understanding of insulin secretion. There is evidence that at least some of the effects of calcium are mediated through calmodulin, since glucose-stimulated [50, 51] (and possibly cyclic AMP stimulated) secretion is inhibited by the calmodulin inhibitor trifluoperazine. Unless trifluoperazine has other non-specific effects on islet metabolism [52], this suggests that the cyclic AMP-stimulated pathway may be relatively calmodulin independent, and a possible scheme which allows for synergistic contributions from the cyclic AMP and calcium-

stimulated components of the secretory mechanism is outlined in Figures 7 and 8. Calmodulin-binding proteins which could act as interfaces between calmodulin and the proteins of the contractile system (Fig. 9) include myosin light chain kinase (to actomyosin), τ proteins and microtubule-associated proteins (to microtubules) and a whole series of actin-binding proteins (Table 5).

There has been considerable interest recently in the possibility that calmodulin may lead to activation of myosin light chain kinase in islets, and indeed, phosphorylation of exogenous myosin light chains by the islet enzyme in the presence of calcium is readily observed [44–46]. Much more difficult to demonstrate has been the phosphorylation of endogenous myosin light

Fig. 9. Possible role of calcium-binding proteins in mediating the effects of activation of calcium-calmodulin on the cytoskeleton. Specific examples of such proteins are shown in Table 5

Table 5. Some calmodulin binding proteins

Protein	Molecular weight (daltons)	Site of action
Myosin light chain kinase	135,000	Myosin light chain phosphorylation
τ proteins and microtubule-associated proteins	55–60,000	Tubulin
Caldesmon	150,000	Actin
Fodrin	240,000	
Actinogelin	100,000	
Gelsolin	160,000	
Fragmin	50,000	
Villin	95,000	

chains in intact cells during stimulation of insulin secretion; this could be a result either of rapid dephosphorylation of myosin in the intact cells, or of the masking of any changes by rapid alteration in the specific activity of endogenously ^{32}P -labelled ATP. Alternatively, it may be that it does not occur, especially in insulin secreting tumours which are not responsive to glucose, and in which an intermediate filament protein, vimentin may be phosphorylated on stimulation of secretion [53]. We are currently examining the possible existence of alternative calmodulin-binding proteins in normal islets which might provide linkages between calmodulin and activation of the insulin secretory mechanism.

There are some clues from model systems about the way in which such a granule-actomyosin system might operate. Of particular interest is the recent demonstration of a model system in which plastic beads 0.7 μm in diameter are coated in heavy mero-myosin – the active head region of the myosin molecule, and are then observed to be transported along actin filaments present on the surface of an algal cell [54]. The movement was unidirectional, ATP-dependent and required a functional myosin ATPase activity, but was not altered by variation of calcium concentration. It was calculated that a single myosin molecule attached to such a bead could provide enough force to move it at 5 $\mu\text{m/s}$, and that 25 myosin molecules could provide continuous movement at this rate. This type of model is of course extremely attractive in the context of the postulated as-

sociation of myosin with β granules shown in Figure 6.

In regard to tubulin-actin associations, again only indirect evidence is available and this relies on the demonstration [55] of microtubule-actin interactions in vitro and subsequent work suggesting that microtubule-associated proteins play a crucial role in regulating this association. Thus phosphorylation of microtubule-associated proteins [56], or the addition of ATP [57] has been shown to reduce the interaction of their associated microtubules with actin.

Future developments

The work of the last 15 years has been based on two particular propositions; firstly, that rat islets of Langerhans provide an adequate model for human islets of metabolism and secretory characteristics and, secondly, that the metabolism of the isolated islets adequately represents the 70% of B cells within the islets, while the contributions of the residual 30% of A and D cells can be ignored. We hope in the foreseeable future to confront these two assumptions directly. The first requires that human islets should be obtainable in adequate and reproducible numbers. There have been some recent suggestions for improvement of the existing collagenase techniques to make them more useful for isolation of islets from pancreas of large mammals, which has hitherto been impossible. In particular the 'Velcro' procedure of Lacy et al. [58] seems to be very effective for canine pancreas; there seems no reason why it should not be useful for human pancreas as well.

In regard to separation and purification of the B cell population, it has been shown that fluorescence activated cell sorting systems may provide an effective way of separating the individual cell types to give 95% purity for B cells, and considerable enrichment of A and D cells [59]. Unfortunately the cost and complexity of this equipment renders it unlikely to be a practical procedure for routine use in many laboratories. We are currently examining the possibility of using an immunological system for cell separation, based on the specific binding of A, B or D cells to specific antibodies, which can in turn be coupled to a suitable matrix. The procedure is analogous to 'panning' to achieve separation of lymphocyte subsets, which is a well established procedure. We have been able to show that rat adult B cells (but not A or D cells) bind to a monoclonal antibody A₂B₅ and it seems likely that antisera, either monoclonal or from recently diagnosed diabetic patients [60], will be available which can bind specifically to A or D cells. Production of an affinity column of the appropriate antibody therefore provides the potential for the initial elution of the unbound cells, and subsequent recovery of those which have been specifically bound. This method, coupled with the possibility of isolation of large numbers of human islets, provides potential for the use of human B cells in future studies. Detailed examina-

tion of the metabolism of these cells in pancreas obtained from patients with non-insulin dependent diabetes might be of special interest.

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