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Kinetic aspects of compartmental storage and secretion of insulin and zinc

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Introduction

In the normal animal, the endocrine pancreas stores enough insulin for several hours of stimulated secretion (with a turnover time of approximately 16 h)[82]. In man, with his typical fasting-feeding patterns, stored insulin represents a 5-day supply. Several compounds necessary for the formation, maturation and condensed storage of this hormone are co-stored in secretory vesicles. Some are also co-secreted with insulin and, for a short period of time, may modify the efficacy of insulin at its target organs. Therefore, subtle alterations of storage and secretion of hormone in the B-cell may have profound affects on glucose homeostasis.

Kinetics of insulin secretion

Beta cells, as well as other secretory cells, respond to constant stimulation by secretion of their hormone in multiphasic patterns[94]. Kinetics of these patterns have been studied both to evaluate the underlying biochemical mechanisms regulating minute-to-minute secretion and to establish their possible relevance for total body homeostasis.

Several characteristics of insulin secretion from the perfused rat pancreas are shown in figure 1. When glucose is presented as a rapid onset, constant infusion, there is an immediate burst of insulin for 2–5 min followed by a brief nadir. This is succeeded, in turn, with a second ascending secretion which reaches equilibration with time. Transient negative spikes of insulin release can be produced by suddenly reducing the glucose to a lower stimulating level[49, 94]. Thus, insulin secretion is sensitive to the rate of change as well as the static concentration of a secretagogue.

In addition, prolonged stimulation with glucose produces a time-dependent potentiation of the B-cells, resulting in hypersensitization to a subsequent stimulus (fig. 1). Potentiation is time and dose dependent on the initial glucose stimulation and has a half-time 'memory' of approximately 30 min[16, 46, 47, 51]. Therefore, although the removal of glucose causes an immediate cessation of insulin secretion, metabolic components in the B cell remain, for a limited period, in an activated state. A metabolite of glucose is probably involved since glyceraldehyde is active, whereas non-metabolizable galactose is not[47]. Other fuel substrates (e.g. alpha-ketoisocaproic acid) are effective time-dependent potentiators. Insulin secretion during the priming period is not essential, glucose remaining effective when secretion is blocked by somatostatin and, in some reports, calcium deprivation[47]. The nature of the intracellular factors causing priming are unknown. They probably are similar to those causing the second rising phase of insulin secretion[94], and probably do not involve cyclic-AMP[47]. Other insulin secretagogues such as the depolarizing agents, sulfonylurea or potassium, cause first phase insulin secretion with only a small, sustained second phase; in the presence of low glucose, a more typical multiphasic release results[48, 50, 52, 61].

A number of mathematical models have been described to account for multiphasic insulin secretion (reviewed in Landahl and Grodsky[79] and O'Connor et al.[94]). These models incorporate different structures to provide sensitivity to rate of change and concentration of secretagogue and fall in two general categories. First are 'storage-limited models' in which insulin is presumed to be stored heterogeneously in compartments or pools

with different labilities to secretagogues. First-phase secretion and rate sensitivity are the result of rapid release and depletion of a small labile compartment. Second are 'signal-limited models' in which a negative feedback or an exciter-inhibitor (metabolic or ionic) phenomenon is invoked to create the transient first-phase secretion. An added, but different phenomenon, which can be identical in both models, accounts for second phase release and probably for time-dependent potentiation. The limitations and capabilities of these models are reviewed[94] and a combination of the two was shown to approximate numerous stimulation-secretion patterns for insulin secretion[79]. Although these models do not identify the metabolic nature of their components, they provide insight as to the quantitative and temporal interrelationships to be expected of those components, once identified.

Recent attempts to elucidate the metabolic components responsible for the kinetics of insulin secretion have focused on ion fluxes, particularly those of potassium and calcium[5, 13, 60, 61, 124]). A variety of techniques have shown that calcium is localized in the cytosol and organelle compartments of the B-cell and that distribution changes rapidly during the different phases of stimulated insulin secretion[4, 32, 33, 74, 125, 127, 128]. Shifts of calcium between cytosol, secretory vesicles and the plasma membrane suggest these cellular compartments are of particular importance for regulation of kinetic secretion whereas endoplasmic reticulum, and possibly mitochondria, serve primarily as calcium storage sites[60, 124]. Several models for dynamic insulin secretion have been proposed with calcium in the central role[5, 107]. Most are still in developmental stages and are complicated by the current incomplete knowledge of calcium intracellular distribution, changes between bound and ionic forms[128] and possible overemphasis of cytosolic calcium as the direct determinant for secretion.

Electrophysiological studies show depolarization results in voltage-dependent calcium uptake into B-cells (a positive signal for secretion), but that intracellular calcium can also activate a calcium-sensitive potassium channel causing hyperpolarization, (a negative signal for secretion[5]). This is a possible explanation of an ionic-feedback inhibition predicted in the signal-limited model described above. On the other hand, the storage-limited model becomes particularly attractive if one assumes the compartments do not represent insulin but, instead, pools of calcium. The glucose-sensitive, labile compartment would represent cytosolic calcium. However, cytosolic calcium would not be the direct determinant for secretion but the reservoir from which glucose mobilizes calcium to the secretory site (secretory granules? plasma membrane?), and to which glucose can also subsequently add calcium from calcium-storing organelles or by uptake of extracellular calcium. Thus, the first phase of insulin secretion could involve a decrease in cytosolic calcium whereas the second phase could reflect provision to and replenishment of that compartment. A possible primary action of glucose to mobilize cytosolic calcium has been suggested from electrophysiological experiments[6], is consistent with some of the histological observations of calcium shifts induced by glucose[127, 128] and is supported by recent observations that glucose can cause a decrease as well as an increase in cytosolic calcium[100, 125].

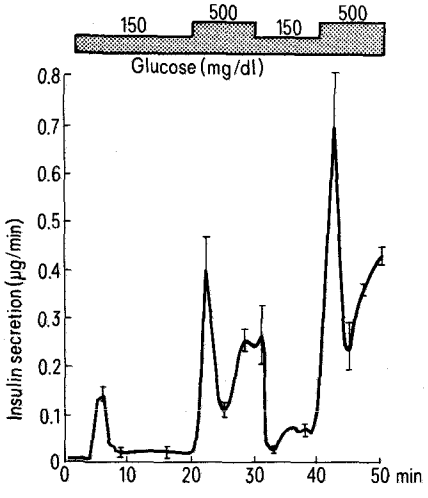

Figure 1. Typical patterns of insulin secretion from the perfused rat pancreas during various step stimulations with glucose. Modified from O'Connor et al.[94]. Reproduced with permission from publisher.

Observations showing that a combination of sulfonylurea or calcium ionophore with the tumor-promoting phorbol ester, 12-O-tetradecanylphorbol-13-acetate (TPA), approximates glucose-induced diphasic insulin release suggest that both protein kinase C and calmodulin-regulated kinases represent dual target sites (the final calcium compartments?) for calcium action[107, 129].

Phasic and rate-sensitive release of insulin from the B-cell may play an important role in total body homeostasis. In the Type II diabetic, loss of first-phase-insulin release is characteristic[17, 109]. Studies using the closed-loop artificial pancreas demonstrate that the ability of normal B-cells to respond transiently and rapidly to changes in glucose reduces the total insulin required for proper regulation and minimizes the development of subsequent hypoglycemia[3, 14]. Therefore, in a closed-loop endocrine system, positive rate sensitivity may provide a priming dose at the active site of the hormone. This would initiate rapid regulation, with relatively little secreted hormone, before glucose levels become excessive. At the same time, a negative rate sensitivity would prevent hypersecretion of insulin, when falling glucose levels, if considered only as static regulators, are still in the stimulatory range. After ingestion, glucose is absorbed into the circulation at a rate insufficiently fast to cause a detectable diphasic insulin secretion, leading to the assumption that rate sensitivity may not be necessary for glucose control in diabetes. However, it is emphasized that subtle differences in initial insulin secretion between subjects with rate sensitivity intact and those without may prove significant, and needs consideration when closed-loop pumps become available clinically.

Rapid oscillations of basal and stimulated secretion are seen both in vitro[112] and in vivo[42], suggesting such oscillations are initiated at the B-cell. Recent observations that oscillating insulin levels may provide better regulation at the insulin receptor sites indicate an important role of this kinetic aspect of insulin secretion[90].

Evidence for nonrandom release of newly synthesized vs older stored insulin

Early studies with the perfused pancreas showed that acute stimulation of insulin secretion is only mildly inhibited when insulin synthesis is blocked[50]. Thus, for short periods, release of stored insulin represents an immediate and abundant supply of hormone. Despite this, several laboratories using pulse-labeling experiments with radioactive amino acids have shown that the newly synthesized, rather than older, stored insulin is preferentially mobilized for secretion during continuous glucose stimulation[18, 35, 38, 39, 40, 56, 57, 65, 104, 105, 106, 120]. This conclusion is based on two different types of data: 1) the specific activity of secreted insulin usually exceeds the specific activity of the average cellular insulin (measurements independent of recovery of insulin); and 2) the cells secrete radioactive insulin at fractional rates that are higher than those of immunoreactive insulin (measurements dependent on comparable recoveries of secreted and cellular insulins). Thus, cellular storage of insulin is not uniform but rather is heterogenous and compartmental.

In addition, these observations underline the importance of considering insulin biosynthesis and storage as integrated processes which together can effect complex patterns of regulated insulin secretion.

The cellular mechanism for preferential secretion of newly synthesized insulin has not been fully elucidated, but is not unique to the B-cell. Preferential secretion of newly synthesized material also has been reported for placental lactogen[116], prolactin[117], parathyroid hormone[86], salivary amylase[108], pancreatic zymogens[99], pancreatic amylase[110], gonadotropin[62], vasopressin[103], thyroglobulin[87], actylcholine[19] and renin[73]. Heterogeneous secretion could represent compartments of cells in a different functional state as suggested for pituitary cells[93], or in different storage sites within the same cell. For insulin, it also could represent an artifact created by anoxic cells since it has been suggested that the innermost B-cells of large islets may be anoxic and inactive[81]. In a pulse-labeling experiment this would contribute a fixed store of cellular, unlabeled insulin and result in an apparent preferential release of new hormone from the active fraction of B-cells. However, newly synthesized insulin is preferentially released from both large and small islets in an identical and consistent manner[38] and heterogeneous secretion occurs both for pancreatic zymogens[99] and prolactin[117] in vivo and for dispersed parathyroid cells in vitro[92]. Therefore, artifacts due to poor permeation of nutrients into thick tissues are unlikely. The ventral and dorsal pancreas contain islets with different cellular and hormonal compositions[7] and with different sensitivities to secretagogues[121]. However, since newly synthesized insulin is preferentially released from both ventral and dorsal islets in an identical manner[38], regional differences in islets probably are also not the explanation for heterogeneous insulin secretion. Recent evidence from labeling experiments, in which the pulse times were sufficiently brief to dissect the kinetics of cellular transport and hormone processing in the chase period, suggests that: 1) compartments are located within the B-cell; and 2) preferential secretion of newly synthesized insulin is a regulated process[35]. Regulation or 'marking' occurs during cellular transport of secretory proteins to, or through the Golgi apparatus during Golgi formation of new secretory vesicles. Marking is a cellular mechanism by which new protein is selected either for immediate release (during periods of high metabolic demand) or for subsequent, longer-term storage (during periods of low metabolic demand); evidence that marking represents a novel regulatory mechanism is discussed in detail below.

Evidence that marking is a regulated process

The cellular route from insulin biosynthesis to storage and secretion is shown schematically in figure 2 and is essentially the same as that initially elucidated for the exocrine pancreas[71]. Experiments employing cell fractionation[75] and radioautographic analysis[95] of pulse-labeled islets have shown that biosynthesis occurs on the rough endoplasmic reticululm in a manner consistent with the signal hypothesis[9]. As found by Steiner et al.[114, 115], and as shown in figure 3, all the labeled hormone for approximately 20 min after a pulse-labeling is the same size as proinsulin; labeled preproinsulin is usually[115], but not always[2, 97], too transitory to detect. Delay before onset of conversion of proinsulin to mature hormone corresponds to the time for energy-dependent vesicular transport of secretory proteins from the rough endoplasmic reticulum to the Golgi apparatus and secretory vesicles where conversion occurs[37, 64]. In normal islets, once onset of conversion of proinsulin to insulin begins, it follows pseudo-first-order kinetics with a $t_{½}$ of approximately 1 h (fig. 3)[38, 104, 115]. Transport time to the Golgi and kinetics of conversion of labeled proinsulin to insulin are identical in high and low glucose[35]. Substrate specificity of insulin-converting enzymes in the B-cell is similar to that of ACTH-converting activity for when the foreign insulin gene is inserted into cultured pituitary cells, proinsulin is both synthesized and cleaved to a protein the size of insulin by pituitary-cell converting enzymes[91]. However, the endogenous activity maturing glucagon in isolated secretory vesicles from fish A-cells does not convert exogenous mammalian proinsulin to insulin[29]. Shortly after sequestration into secretory vesicles, protein secretion can begin. Total

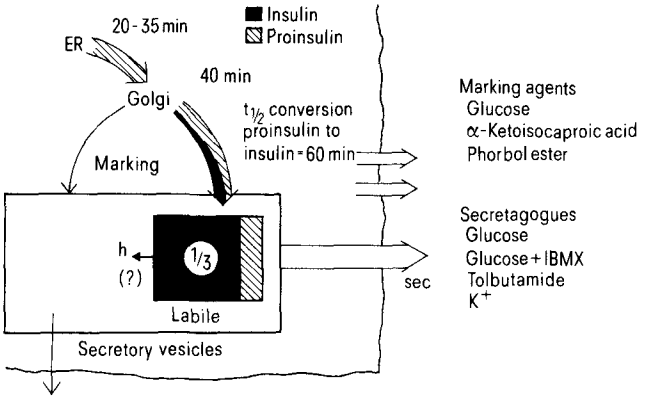

Figure 2. Schematic representation of the biosynthesis and compartmental storage of insulin within B-cells of islets from untreated rats.

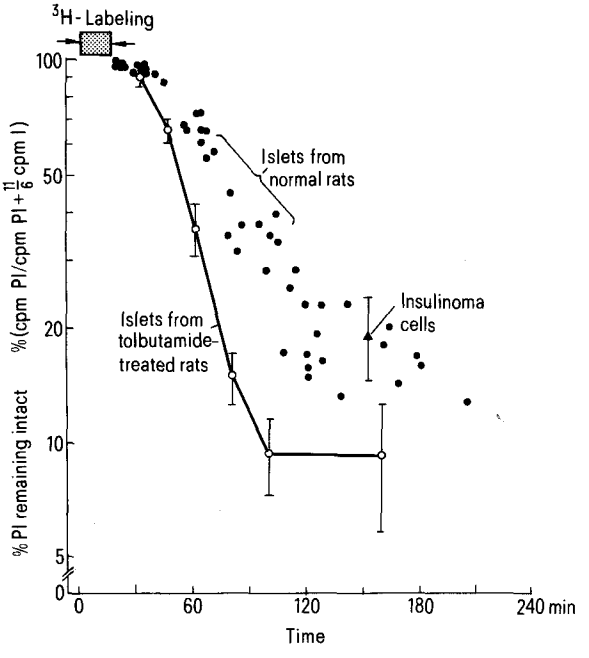

Figure 3. Time course of the conversion of labeled proinsulin to labeled insulin for islets from untreated rats, tolbutamide-treated rats and for dispersed cells from a transplantable rat insulinoma. Radioactivity eluting from Biogel P-30 in the insulin region is multiplied by 11/6 to correct for loss of ^{3}H-leucine-labeled C-peptide during preparation. Solid circles represent individual experiments with islets from untreated rats; open circles represent M ± SE for three experiments with islets from tolbutamide-treated rats and the triangle represents M ± SE for 16 experiments with dispersed cells from a transplantable rat insulinoma.

conversion of proinsulin to insulin need not precede secretion. The proportion of proinsulin secreted depends on conversion rate and elapsed time before secretion is stimulated[34]. In this regard, the secretory vesicle appears blind to the proinsulin content. As further evidence of secretory vesicle insensitivity to its proinsulin content, biosynthesized proinsulin, modified by incorporation of amino acid analogs so it could not be clevaed to insulin by cellular converting enzymes, was still secreted in a normal, regulated manner[58].

Neither newly labeled proinsulin nor newly labeled insulin is secreted at the same constant fractional rate as is immunoreactive insulin[38]. Hence, as is illustrated in figure 2, insulin storage is represented as compartmental and heterogeneous. Although insulin secretion may occur by release into the cellular cytosol, by vesicles budding off from the endoplasmic reticulum, or by a direct channel from the endoplasmic reticulum to the exterior of the cell[23], there is not yet compelling evidence supporting the existence of these as major routes in any regulated, protein-secreting cell. Unique secretory pathways have been reported for endocrine cells in culture including; a short, accelerated route in addition to a longer, regulated pathway for prolactin secretion from cells from the anterior pituitary[96]; and a constitutive route for viral proteins and ACTH precursors in addition to a regulated pathway for mature ACTH in virally-transformed pituitary cells[55]. In normal islets, however, both labeled proinsulin and labeled insulin appear to be secreted by a regulated pathway, which begins only after labeled secretory proteins accumulate in newly forming secretory vesicles[38]. Therefore, both proinsulin and insulin are likely transported from site of synthesis to site of release primarily if not exclusively, via the orderly sequence of subcellular vesicular compartments previously outlined.

At a critical time after biosynthesis and during cellular transport of secretory proteins, the concentration of glucose has a significant effect on subsequent preferential secretion of newly synthesized insulin (fig. 4). As shown in figure 2, this period corresponds to the time newly synthesized insulin is approaching and transiting the Golgi and the formation of new secretory vesicles. With high glucose present during the critical marking period, the ratio of specific activities of secreted to stored insulin in a subsequent test, the stimulation period was always greater than unity (fig. 4). This high specific activity ratio near 3.0 indicates that newly synthesized insulin is directly and preferentially secreted without first intermixing with the majority of stored cellular insulin (whereas a ratio near 1.0 would indicate random storage and mobilization). The secretagogue or the secretory rates in the subsequent test period had little effect; specific activity ratios remained elevated whether secretion was induced by high glucose (as shown here) or by low glucose ± tolbutamide, low glucose +50 mM potassium or high glucose +IBMX[35]. With low glucose present during the marking period, new hormone was sorted for random storage and mobilization. Again, the nature of the secretagogue and resulting secretory rate in the test period were relatively unimportant.

It is emphasized that with both concentrations of glucose, labeled proteins were well established in the secretory vesicles of the B-cell before the test periods and that preferential secretion of new insulin was not simply a consequence of exposing islets to elevated concentrations of glucose. Regulation occurred only with exposure to glucose during the critical marking period and extended periods of exposure either before or after this period were not effective. Thus, since marking depends on the temporal site of newly synthesized hormone rather than the total time of exposure to a marking agent, secretory compartments are in the same cell rather than in different cells.

In contrast with storage in normal islets, only homogeneous insulin storage was seen with rat insulinoma cells that both secrete and store insulin[34]. In these cells, glucose concentration had little effect on either the fractional rate of insulin secretion[34,111] (which was continuously higher than in maximally glucose-stimulated islets) or the near-random secretion of newly synthesized vs older stored insulin. Insulin in the tumor cells is mobilized as if all stored hormone is uniformly marked for rapid release and in rapid flux (shown schematically in fig. 5). Thus, marking is a novel explanation for the shortened periods of hormone storage in tumors. The rate of labeled proinsulin-to-insulin conversion was within the normal range (fig. 3). This coupled with the high fractional secretion induced by the increased marking leads to excessive secretion of prohormone. Excessive proinsulin secretion is characteristic of human insulinomas[18], particularly those with B-cells that stain poorly for insulin[8], and store newly synthesized hor-

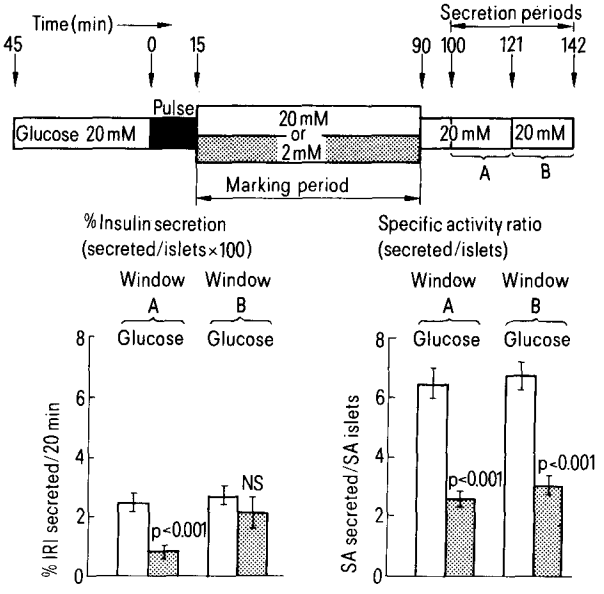

Figure 4. Effects of glucose concentration during the marking period on the secretory rate of immunoreactive insulin and the specific activity ratio between secreted and stored islet insulins. Noncumulative samples of secreted insulins were collected during two consecutive 20-min test periods (windows A and B). Secreted and islet insulins were purified separately without carrier insulin for determination of specific activities[38]. Reproduced with permission from publisher.

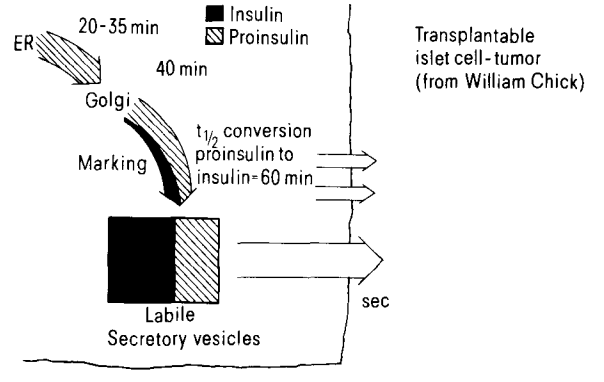

Figure 5. Schematic representation of the biosynthesis and storage of insulin within dispersed B-cells of a transplantable rat insulinoma.

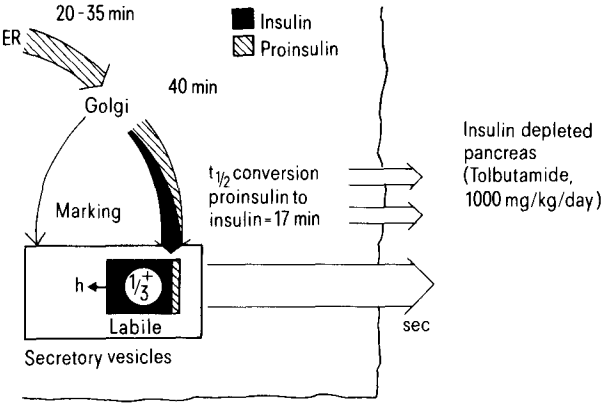

Figure 6. Schematic representation of the biosynthesis and compartmental storage of insulin within B-cells of insulin-depleted islets from tolbutamide-treated rats.

mone only for short periods of time before the secretory event.

Reduced storage and increased fractional secretion does not necessarily reflect either a highly marked homogeneous insulin-storage compartment or lead to secretion of higher-than-normal amounts of proinsulin. As an example, islets from rats treated on the three previous days with multiple high doses of tolbutamide synthesize about normal amounts of proinsulin, but store only 20% as much insulin as normal[36]. Although actual secretory rates are low, fractional rates of insulin secretion are higher-than-normal during glucose stimulation. As indicated schematically in figure 6, after tolbutamide treatment, conversion rates of proinsulin to insulin were 2–3 fold more rapid (see fig. 2). A rapid conversion rate also is noted for insulin-depleted islets from rats infused with glucose[72] suggesting that depletion of insulin, rather than either glucose or tolbutamide treatment per se, is probably responsible for accelerated conversion rates. Thus, normal, but not transformed, B-cells have a mechanism for keeping the percentage of secreted proinsulin low during periods when cellular stores of hormone are limited and the secretory process must draw more heavily on available active hormone to maintain glucose homeostasis.

Marking newly synthesized insulin for direct, preferential secretion is neither unique for the intact glucose molecule nor does it depend on hexose metabolism. As shown in table 1, alpha-ketoisocaproic acid is an effective marking agent. Although, like glucose, this leucine metabolite is an excellent secretagogue, not all islet secretagogues are marking agents, as the triose, D-glyceraldehyde stimulates secretion but does not mark. On the other hand, inhibition of protein secretion or cellular traffic of secretory proteins can, but need not, affect the marking process. For example, glucose-stimulated marking was unchanged by monensin, a sodium-proton ionophore[39] which at high concentrations may block protein traffic through the Golgi apparatus (as reported for unregulated protein-secreting cells)[118, 119]. Low concentrations (10^{-10} to 10^{-6} M) inhibit secretion of both newly synthesized and older stored insulin to a comparable degree at all concentrations. Prohormone-to-hormone conversion is also inhibited and inhibition is correlated in a 1.1 relationship with inhibition of insulin secretion. The inhibition patterns suggest that these low concentrations may specifically disrupt the acidic environment in secretory vesicles of the B-cell which, though not affecting marking, produce concerted inhibition of proton-dependent processes, such as secretion and conversion of proinsulin to insulin[75]. The buffer,

Table 1. Effect of secretagogues and inhibitors on the marking process

Agent and concentration	Effect on preferential insulin secretion
Glucose (20 mM)	Marks
Glucose-Ca	No effect
α-KIC (25 mM)	Marks
TPA (100 nm)	Marks
D-Glyceraldehyde (10 mM)	No effect
L-Glyceraldehyde	No effect
Glucose (2 mM)	No effect
Tunicamycin	No effect
Monensin	No effect

TRIS, may have specificity for inhibiting the marking process because, unlike antimycin A[113], and colchicine[89], which block protein transport to the Golgi, TRIS blocks cellular protein transport out of the Golgi apparatus[59].

Nature of the marking process

Since marking appears to occur in or around the Golgi, some changes in the vesicular structures in this organelle may be involved. Coated membranes and coated vesicles are observed in the Golgi of the B-cell[95]. These are similar to those implicated both in selective delivery of specific ligands from the plasma membranes to intracellular addresses[15,41] and in Golgi-related sorting processes in other tissues[102].

Recently a population of secretory granules with similar coating have been described at, and adjacent to, the trans region of the B-cell Golgi[95]. These are probably immature or maturing granules since their protein core is less dense than that of the mature granule. Furthermore, in pulse labeling experiments using tritiated leucine, label appears in these organelles before it appears in the characteristic mature granule[95]. The fact that glucose rapidly increases the number of these coated granules at a time coinciding with the marking period, makes this special granule population an attractive site for the regulation of preferential secretion of newly synthesized insulin.

Geographical location of secretory vesicles within the cytosol may be another important determinant of preferential secretion of new insulin; recent evidence indicates that B-cells are polarized with secretion occurring only from an apical surface on the plasma membrane[11]. Based on biochemical evidence and computer modeling, the size of the preferential releasable compartment of insulin is as much as 33% of the total stored islet insulin[38]. Despite the fact that, at any one time, they normally contain little secretory protein and represent a very small fraction of the cellular volume, coated granules could still be active in cellular transport and the target site for marking. In cultured cells infected with vesicular stomatitis virus, coated vesicles were postulated to account for all cellular transport of viral protein G in two successive waves – one from the rough endoplasmic reticulum to the Golgi apparatus and the other from the Golgi to the plasma membrane[101]. Beta cells have sufficient potential to form membranous vesicles required for a high flux through a small active cellular compartment, since more than 1000 vesicles can be formed per hour[21]. It is also possible that the coating may 'decorate', and thereby chemically mark, secretory vesicles, and that when the coating is removed, these vesicles may retain for a substantial period, a biochemical mark identifiable by the secretory process.

Although insulin is not a glycoprotein, glycoproteins in the plasma membrane or the bristle coating could be involved in the selection or identification of vesicles involved in the marking process. However, tunicamycin, which inhibits completion of the carbohydrate moieties of glycoproteins, does not interfere with marking in the B-cell[36]. Also, as reported for ACTH and other secretory proteins, tunicamycin does not interfere with exocytosis of insulin. In contrast, phosphoproteins may be important in the marking process. TPA is a known secretagogue of insulin[88,123], and also is an effective marking agent (table 2). Since TPA activates protein kinase C, a lipid- and calcium-activated kinase that catalyzes the synthesis of specific phosphoproteins, this enzyme may be an important regulator of both the secretory and the marking processes in the B-cell.

Table 2. Effect of phorbol ester on marking of islets for preferential secretion (data presented as ratio of specific activities of secreted vs stored insulin)

	Marking agent during transit through Golgi	
Experiment	Phorbol ester (100 nM)	Control (2 mM glucose)
1	2.51	1.12
2	2.45	1.01
3	2.37	0.94
Mean	2.44	1.02

Co-secretion of other granular components

As noted above, conversion of proinsulin to insulin occurs in the maturing secretory vesicles of the B-cell. The cathepsin-like, thiol enzyme responsible for proteolysis is bound to the inner surface of the plasma membrane[22,44] and is probably not co-secreted with insulin. As conversion of proinsulin to insulin proceeds, insulin rapidly forms an insoluble aggregate, usually with crystalline structure[45]. Formation of insulin crystals is favored because: 1) insulin is highly concentrated within the secretory vesicle (calculated from a secretory vesicle volume of 10–12% of B-cell volume[20], insulin concentration in the vesicle is about 10^{-2} M or 60 mg/ml[37]); 2) zinc (which is present in the secretory vesicles of most species) has a high avidity for both proinsulin and insulin[31] and forms insoluble crystals with insulin but not proinsulin[43]; and 3) the pH of secretory vesicles, estimated at approximately 6.0[1,67] is close to the isoelectric point of insulin.

This acidic pH may improve integrity of the secretion vesicles, which are unstable at neutral pH in vitro[66,78]. Furthermore, the low pH of the secretory vesicle and the neutral cytosol of the B-cell implies formation of a proton gradient across the vesicular membrane. A magnesium-ATPase in the secretory vesicle membrane is probably responsible for the inward pumping of protons needed to maintain the gradient[80]. Several related nucleotides including ATP, ADP, AMP, and c-AMP have been identified in the B-cell secretory vesicle and co-secrete, more or less in phase, with insulin[80,122].

Aggregation and crystal formation of insulin accounts for the appearance of the mature secretory vesicle characterized by a membrane enclosed, dense, crystalline core[45] surrounded by a clear halo[95]. Proinsulin, consisting of about 5–10% of the total hormone in a mature vesicle is co-secreted during exocytosis, but, as discussed above, the ratio of co-secretion of proinsulin and insulin is highly dependent on amount of converting enzymes and rate of mobilization for secretion of newly formed secretion vesicles[18,34]. Thus, during hyperglycemia in diabetes or rapid unregulated secretion from islet cell tumors, both secretory and circulating levels of proinsulin are proportionally high[18,84].

The other products of proinsulin processing, amino

acids, and C-peptide, are retained within the halo of the secretory vesicle in the soluble fraction[95]. C-peptide is co-secreted with insulin in equal molar concentrations during exocytosis[76,98]. It is, therefore, measured as an index of insulin secretion in insulin-treated diabetics where circulating antibody makes an insulin radioimmunoassay unreliable[98]. Because C-peptide is cleared from the blood (primarily by the kidneys) at a rate different from that of insulin (primarily by the liver), levels of C-peptide in blood or urine must be interpreted with caution, particularly in the more severe diabetic with renal abnormalities[77].

The role of zinc in insulin processing, storage, and secretion has been recently reviewed in detail[24,37,63]. In most species, islets concentrate zinc more than 50-times blood levels[27]; uptake is carrier mediated or occurs by diffusion depending on extracellular zinc concentration[83]. Recent measurement of zinc distribution in normal islets and in islet cell tumors have shown that only ⅓ of the islet zinc is intragranular[28,69]. Since zinc and insulin are in roughly molar equivalence in the B-cell, the ⅓ fraction in the secretory vesicle is just adequate for insulin storage as a 2-zinc hexamer. Large amounts of zinc can be bound to insulin under specific in vitro conditions[24] but, the limited zinc content of the secretion vesicle precludes existence of these complexes in the normal B-cell. Cytosolic zinc equilibrates slowly with granular zinc suggesting that this ion does not exchange rapidly across formed granular membranes, but probably enters the secretion vesicle during their initial formation[27]. This is consistent with the hypothesis that non-crystalizing, zinc-proinsulin is formed prior to its conversion to zinc-insulin.

Histologic data demonstrates that zinc in the B-cell is concentrated in the protein core of the secretory vesicles[26,126]. That this zinc is firmly bound (presumably to the insulin as a 2-zinc-hexamer) has been recently established. When granules from ^{65}Zn loaded islets were sonicated in the presence of Chelex-100 to obtain instantaneous measurement of free (Chelex-bound) zinc, most of the granular zinc was in the bound form[53]. In other studies, isolated aggregated core protein from secretory vesicles of B-cell tumors retained the major portion of secretory vesicle zinc[69].

Since zinc is distributed in granular and nongranular compartments of the B-cell, co-secretion of zinc with insulin as well as independent zinc release, can contribute to total zinc release. In ^{65}Zn-loaded islets glucose-IBMX caused a rapid co-secretion of ^{65}Zn and insulin with similar kinetics[30]. After correcting for basal, nongranular release, zinc and insulin co-secreted in a 1 to 3 molar relationship indicating that insulin-bound zinc was quantitatively released during exocytosis at the expected ratio if orginating from a 2-zinc-insulin hexamer. In contrast, basal zinc efflux was much greater than that expected if zinc was released only proportionally with basal insulin[30]. Other secretagogues such as leucine cause similar co-secretion of zinc and insulin but also stimulated zinc efflux from the nongranular compartments. Furthermore, both D_2O and decreased temperature-inhibited basal zinc efflux. Thus, zinc flux from compartments unrelated directly to insulin storage is highly sensitive to biochemical modification[30].

Table 3. Distribution of ^{65}Zn in perifusate from rat islets collected and analyzed on C^{18} Sep-Paks within 1 min after secretion

	Exp. No.	Buffer eluate (albumin) %	Methanol eluate (insulin-Zn) %	Sep-Pak residual (free Zn) %
Experiment	1	56	35	9
(with insulin	2	72	20	8
secretion)	3	77	12	11
Mean SEM		68 ± 6	22 ± 7	9 ± 1
Control	1	51	42	7
(no insulin	2	62	27	11
secretion)	3	70	19	11
Mean SEM		61 ± 6	29 ± 7	10 ± 1

Conditions: Aliquots were collected during glucose-IBMX stimulation of perifused islets preloaded with ^{65}Zn as described[30]. Efflux of zinc and insulin over baseline were at ratios of 1:3 respectively, consistent with co-secretion from a 2 zinc-insulin hexamer. Control: Aliquots were collected during Zn–^{65}Zn exchange of similarly ^{65}Zn loaded islets. ^{65}Zn efflux was at the same level as during glucose-IBMX stimulation but without co-secretion of insulin. Albumin-bound ^{65}Zn passes through the Sep-Pak (Waters Associates, Inc., Milford, Mass). Insulin (+ bound ^{65}Zn) is retained but can be eluted with 80% ethanol. Free ^{65}Zn is retained but only partially eluted with alcohol. Conclusion: Zinc and insulin are not bound together immediately after cosecretion (Grodsky, G.M., and Formby-Schmid, F., unpublished).

Secreted forms of insulin

Although insulin is stored in aggregate form and secreted as such by exocytosis, it is unlikely that insulin polymers exist in the portal blood sufficiently long to reach the liver located a few seconds down stream. Secreted insulin complexes are diluted approximately 10^7-fold during transfer from vesicles to portal vein blood. At this dilution, soluble zinc-insulin complexes dissociate within seconds[28]. Furthermore, factors in the blood, including phosphate ion, zinc-binding proteins and bicarbonate could favor dissociation. As noted above, zinc and insulin are co-secreted. However, rapid chemical analysis of the efflux from perifused islets utilizing C-18 chromatography (Sep-Pak) shows that insulin, less than 60 sec after secretion, is no longer associated with the zinc ion (table 3). Thus insulin, once secreted, probably exists in the portal and peripheral blood as its non-zinc-binding, monomeric form.

The above components of the secretory vesicle are by no means complete, but merely represent those known to affect insulin storage and secretion. In addition to calcium, potassium and phosphate are biologically important ions found at high concentrations in secretory vesicles[10,12,63,124]. Biogenic amines[25,70,85] and perhaps opioid peptides[54] are co-stored and co-secreted with insulin. Furthermore, there are more than 150 proteins in preparations of secretory vesicles[68]. If families of these proteins are concentrated in the bristle-coating, microtubules or other components of the secretory apparatus, or if they are co-secreted (ubiquitous peptides like chromagranin are candidates) is not yet established but remain questions for future research.

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Islet cell interactions with pancreatic B-cells

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Introduction

The exquisite glucose-sensitivity of the pancreatic B-cell plays a key role in the hormonal control of glucose homeostasis. The B-cell response is characterized by a rapid discharge of the hormone and by a precise titration of the amount to be released. Its secretory activity is adjusted to other hormonal regulators, so that metabolic demands are quickly met without disturbing other insulin-dependent processes.

A well developed vascular and neural network is thought to rapidly inform the pancreatic B-cells about