The Cell Physiology of Biphasic Insulin Secretion

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Glucose-stimulated insulin secretion consists of a transient first phase followed by a sustained second phase. Diabetes (type II) is associated with abnormalities in this release pattern. Here we review the evidence that biphasic insulin secretion reflects exocytosis of two functional subsets of secretory granules and the implications for diabetes.

Insulin plays a central role in the control of the body's metabolism. It does so by accelerating glucose uptake into a number of tissues while simultaneously suppressing glucose production and lipolysis. Lack of insulin leads to the serious metabolic disturbances that characterize diabetes mellitus. Insulin is produced and secreted by the β-cells of the islets of Langerhans, the endocrine part of the pancreas, so named after their discoverer, Paul Langerhans. In his thesis, which he published at the age of 22 in 1869, Langerhans described the cells within the pancreatic islets as “small irregular structures with homogenous contents and with diameters ranging between 10 and 12 micrometers.” He also remarked that the cells associate to form clusters with a diameter of 0.1–0.24 mm and that they are distributed throughout the glandular parenchyma. As to the function of the islets, Langerhans wrote “...I frankly acknowledge that I have no explanation [for the function of these structures].” It was to take another 20 years until Oscar Minkowski discovered their relationship to the pancreas and 50 years before the discovery of insulin was made.

Insulin secretion is controlled by β-cell electrical activity

As in neurons and many other endocrine cells, electrical signals play a central role in the regulation of secretion. In 1968, Dean and Matthews demonstrated that the β-cell is electrically excitable (2). When the extracellular glucose concentration is elevated from the basal level of 5 mM (at which little insulin secretion is observed) to the insulin-releasing concentration of 10 mM, the β-cell undergoes a slow depolarization from the resting potential (−70 mV) up to a threshold from which regenerative electrical activity is elicited. This electrical activity (Fig. 1B) consists of oscillations in the membrane potential between depolarized plateaus, from which Ca2+-dependent action potentials originate, which are separated by electrically silent (repolarized) intervals. The induction of electrical activity is a central part of the cascade of events that leads to the initiation of insulin secretion. Very recently, it has been possible to demonstrate that the periods of electrical activity coincide with pulsatile release of insulin (3).

Patch-clamp experiments during the last 15 years have revealed that two classes of ion channel are particularly important in the generation of β-cell electrical activity: ATP-regulated K+ channels (KATP channels) and voltage-gated L-type (dihydropyridine-sensitive) Ca2+ channels (2). A model for the stimulus-secretion coupling of the β-cell is summarized in Fig. 1A. In the absence of glucose, the cytoplasmic ATP/ADP ratio is low and the KATP channels are open. The constant outflow of positively charged K+ through the open KATP channels accounts for the negative membrane potential of the β-cell in the absence of glucose. When the extracellular glucose concentration is elevated, the rapid uptake of glucose (via GLUT2 transporters) and subsequent metabolic degradation of the sugar result in an elevated cytoplasmatic ATP/ADP ratio. This entrains the closure of the KATP channels, membrane depolarization, opening of voltage-gated Ca2+ channels, an increase in the cytoplasmatic Ca2+ concentration, and, ultimately, the exocytosis of the insulin-containing secretory granules.

From a pathophysiological point of view, it is of great interest that the hypoglycemic sulphonylureas, which have been used clinically in the treatment of diabetes for more than 40 years, lead to the closure of the KATP channels in a way that is independent of the cell's metabolic state (2). The effect is mediated by binding to a sulphonylurea receptor (SUR), which, together with the inwardly rectifying K+ channel protein Kir6.2, form functional KATP channels (8). Sulphonylureas thus produce membrane depolarization, the opening of the Ca2+ channels, and initiation of insulin secretion as outlined above.

Dissection of the cellular and molecular mechanisms of insulin secretion

The objective of β-cell electrical activity is to generate the signal that initiates insulin secretion, i.e., the increase in intracellular Ca2+ concentration ([Ca2+]i). The recent development of techniques that allow the detection of secretion in single cells [capacitance measurements and carbon fiber amperometry (1, 3)], combined with the tools of molecular biology, have resulted in a dramatic increase in the knowledge of the processes involved. Many of the proteins that are involved in the regulation of neurotransmitter release have recently been identified also in the pancreatic β-cells. These...
include the SNARE proteins synaptobrevin/VAMP, SNAP-25, syntaxin, α-SNAP, and the putative Ca\(^{2+}\)-sensing proteins synaptotagmin I and II (for review, see Ref. 15). In this review, we focus on the characterization of the functional properties of exocytosis in the β-cells. The experiments we describe were conducted by a combination of the whole cell configuration of the patch-clamp technique with capacitance measurements as a single-cell indicator of insulin secretion (1, 2). This experimental approach enables us to study the properties of secretion in single voltage-clamped β-cells. Exocytosis can thereby be determined independently of any spontaneous changes of the membrane potential, which would (via modulation of voltage-dependent Ca\(^{2+}\) influx) influence the rate of Ca\(^{2+}\)-induced secretion. Additional advantages of capacitance measurements over more traditional approaches to detecting insulin secretion include 1) that the measurements can be conducted on individual cells and 2) the high (1–10 ms) temporal resolution.

**Nutrients induce a biphasic stimulation of insulin secretion**

Glucose-stimulated insulin secretion in vivo typically follows a biphasic time course (Fig. 1C) (3–5, 10). Shortly after elevation of the glucose concentration, a transient stimulation of insulin secretion is observed, referred to as “first phase secretion,” which at later times is followed by a gradually developing secondary stimulation, “second phase secretion.” Only fuel secretagogues are capable of eliciting the second phase, and, when insulin secretion is evoked by nonmetabolizable stimuli, only the first phase is observed. This suggests that second phase insulin secretion is an energy-dependent process. Interestingly, type II diabetes [non-insulin-dependent diabetes mellitus (NIDDM)] is associated with disturbances in the release pattern manifested as the selective loss of first phase secretion, which precedes other manifestations of the disease (4).

Biochemical and electrophysiological experiments in other endocrine cells (11) have suggested that the secretory granules exist in different pools, which vary with regard to releasability (Fig. 1D). Most of the granules (>95%) belong to the reserve pool and need to be chemically modified, or even physically translocated, to become immediately available for release. The latter subset of granules is referred to as the readily releasable pool (RRP) and characteristically contains <5% of the total granule number. The process in which the granules proceed from the reserve pool into the RRP has been termed mobilization and involves one or several ATP-dependent reactions (7, 11).

**Exocytosis triggered by train of voltage-clamp depolarizations**

Insulin secretion is elicited by bursts of Ca\(^{2+}\)-dependent action potentials. Experimentally, it is more convenient to trigger exocytosis by application of voltage-clamp depolarization.
Figure 2A shows an experiment in which a train of 14 depolarizations was applied to a β-cell under control conditions (A, top) and 6 min after addition of 2 μM forskolin (to activate PKA; B, top). Note larger exocytotic response in presence of forskolin. In this particular cell, maximum increase in cell capacitance amounted to 125 fF under control conditions and 500 fF in presence of forskolin. A, bottom: schematic representation of situation before (left) and after (right) depletion of RRP. Ca2+ channels continue to open after RRP depletion, but no granules remain to be released, resulting in cessation of exocytosis. B, bottom: fourfold increase in size of RRP in presence of PKA. Compare with A (bottom left). For technical reasons, it is not possible to monitor cell capacitance during depolarizations. Step increase in cell capacitance following each depolarization reflects insertion of granular membranes that occurred during voltage pulse. With a conversion factor of 2 fF/granule, capacitance changes can be converted into exocytosis of granules. An increase in cell capacitance of 50 fF accordingly corresponds to exocytosis of 25 secretory granules.

Why does exocytosis cease during the train? One possibility is that the cessation of exocytosis reflects the depletion of RRP. Ultrastructural measurements indicate that a single β-cell contains 13,000 secretory granules (7). The maximum increase in cell capacitance elicited by the train of depolarizations (up to 125 fF) corresponds to the discharge of 60 granules using a conversion factor of 2 fF/granule (1). This is only 0.3% of the total granule number. Interestingly, agents that activate protein kinase A (PKA), such as forskolin and glucagon-like peptide-1 (GLP-1), increase the exocytotic capacity of the β-cells by increasing the total number of granules that are available for release (Fig. 2B and Ref. 12). The maximum increase in cell capacitance that can be elicited by a train of depolarization averages 300 fF (150 granules) in the presence of the PKA activator, a fourfold increase relative to control conditions (Fig. 2B). In general, agents that stimulate exocytosis act by both increasing the size of RRP and accelerating the mobilization of new granules from the reserve pool (12). Collectively, these effects give rise to a >10-fold increase in the rate at which granules are supplied for release.

A step increase in [Ca2+]i elicits a biphasic stimulation of exocytosis

It could be argued that an increase in [Ca2+]i, resulting from Ca2+ entry through the voltage-gated Ca2+ channels only accesses a fraction of the total number of granules that are immediately releasable and that the trains of depolarizations underestimate the true size of the RRP (Fig. 3D). A way to
circuit this problem is to use photorelease of caged Ca\(^{2+}\). In these experiments, Ca\(^{2+}\) is provided as a complex with \(\beta\)-nitrophenyl EGTA (NP-EGTA), a photolabile form of the Ca\(^{2+}\) chelator EGTA (7). Once loading has been completed, Ca\(^{2+}\) is liberated by a flash of ultraviolet light. This cleaves the photosensitive bonds within the NP-EGTA molecule and results in a 10,000-fold decrease in the affinity for Ca\(^{2+}\), which consequently is released to exert its biological actions. In this way, an instant and uniform increase of [Ca\(^{2+}\)], is obtained and the spatial arrangement in terms of relationship between the Ca\(^{2+}\) channels, the secretory granules, and the release sites does not interfere with the measurements (Fig. 3E). Another important feature of these measurements is that Ca\(^{2+}\) has not been present at exocytotic levels before it was released. This means that any granules that resided in RRP at the time the whole cell configuration was established remain there until Ca\(^{2+}\) subsequently is elevated to exocytotic levels.

The observed increase in cell capacitance represents the summation of the exocytotic events. To visualize how exocytosis varies with time, we approximated a double exponential to the data points of the capacitance trace. Figure 3C shows the time derivative of this function. By these maneuvers it becomes quite clear that that the exocytotic response consists of an initial rapid phase followed by a sustained slower phase. During the first 400 ms, a total of 40–50 granules undergo exocytosis in an ATP-independent way (7). At later times (>500 ms after the flash), exocytosis proceeds in an ATP-dependent fashion (7) but at a much lower rate (1–10 fF/s, or 0.5–5 granules, depending on the experimental conditions; cf. Ref. 11). It seems likely that the initial rapid component is attributable to the release of granules from RRP, whereas the slower component reflects the time-dependent mobilization of the secretory granules from the reserve pool. This pattern is obviously reminiscent of the biphasic nature of glucose-stimulated insulin secretion (Fig. 1), and it is naturally tempting to speculate that it represents the single-cell equivalent. This raises the question of whether it is possible to compare the two components of capacitance increase with the different phases of insulin secretion in a quantitative manner.

First phase insulin secretion typically lasts 10 min and reaches a peak rate of secretion of 80 pg·min\(^{-1}\)·islet\(^{-1}\) (Fig. 1). If we approximate first phase insulin secretion to a triangle, we can calculate that the first phase insulin secretion corresponds to a total of 400 pg/islet. A secretory granule contains up to 2 fg of insulin (7). Thus, during the entire first phase of insulin secretion, the number of granules released can be estimated to be 200,000 per islet, which corresponds to ~100 granules per \(\beta\)-cell in an islet containing 2,000 cells. This value is not vastly different from the size of the RRP estimated from the depletion experiments (60 granules; Fig 2A). This argues that a substantial
fraction of first phase insulin secretion is attributable to the release of granules belonging to the RRP. However, it must be acknowledged that the two processes take place over vastly different time periods: a few hundred milliseconds compared with a few minutes. Several explanations can be put forward to account for this discrepancy. They include lack of synchronization of exocytosis in the individual cells within the islets and the intensity of stimulation. It also seems likely that the stimulus (several tens of micromoles of $[Ca^{2+}]_i$) is far stronger than anything experienced by the $\beta$-cells in vivo.

In this scenario, the second phase reflects the time- and ATP-dependent mobilization of granules from the reserve pool into the RRP. The value for the mobilization we observe under control conditions ($>0.5$ granule $\cdot$ s$^{-1} \cdot$ $\beta$-cell$^{-1}$) corresponds to 120 pg/islet-min. This is not too different from the rates of second phase insulin secretion being observed experimentally [30 pg/islet-min in mouse $\beta$-cells (Fig. 1) and 140 pg/islet-min in rat $\beta$-cells (10)].

Further support of the idea that release of distinct subsets of secretory granules underlies the biphasic insulin release pattern comes from the observation that first and second phase glucose-induced insulin secretion are stimulated four- and sixfold in the presence of forskolin, respectively (5). These effects closely echo the increase in the size of the RRP of granules (Fig. 2) and the acceleration of granule mobilization obtained in response to activation of PKA as determined from measurements of cell capacitance (11).

What is meant by mobilization?

The possibility that the different phases of insulin secretion reflect the release of distinct subsets of the secretory granules raises the question of whether it is possible to distinguish the RRP and reserve pool by ultrastructural means. Electron microscopy on $\beta$-cells within intact islets suggest that as many as 10% of the granules are docked to the plasma membrane and up to 30% of the granules are situated within one granule diameter from the plasma membrane (L. Eliasson, unpublished observations). In a cell with 13,000 secretory granules, these values correspond to 1,300 and 4,000 granules, respectively. If these granules undergo exocytosis they would give rise to a capacitance increase of 2–8 pF. These values are close to the increases in cell capacitance that can be elicited by infusion of $Ca^{2+}$ through the recording electrode (Fig. 4C), a condition associated with strong stimulation of granule mobilization. These considerations indicate that only a fraction of the docked granules functionally belongs to the RRP and that the mobilization of new granules for release does not require extensive physical translocation within the $\beta$-cells. From these considerations, we conclude that the RRP does not correspond to an ultrastructurally definable subset of granules. This is consistent with recent optical measurements in chromaffin cells using evanescent wave microscopy, showing that the pool of docked granules turns over with a much slower time course than the functional replenishment of the RRP (6 min vs. 10 s) (13).
Significance to diabetes?

The selective loss of first phase insulin secretion is an early feature of type II diabetes (Fig. 4A and Ref. 4). The possible relationship between first and second phase insulin secretion and distinct, functionally defined populations of secretory granules makes it tempting to speculate about the cell physiological mechanisms that underlie the secretory defect of type II diabetes. Studies of glucose metabolism in diabetic patients and animals have revealed that the secretory disturbance develops in parallel with the appearance of substrate cycles of glucose metabolism (Ref. 9 and Fig. 4B). In a diabetic β-cell, a significant fraction of glucose-6-phosphate entering glycolysis is converted back to glucose instead of proceeding through glycolysis. The cycle is futile since there is no change of the participating reactants except that one molecule of ADP has been generated at the expense of one molecule of ATP. The resultant lowering of the cytoplasmic ATP/ADP ratio impairs the ability of glucose to inhibit the K_{ATP} channels (14). However, lowering the ATP/ADP ratio also interferes with the refilling of the RRP, and this may contribute to the secretory defect of type II diabetes. Thus inclusion of 5 mM ADP in the pipette solution in the continued presence of 3 mM ATP (to mimic the consequence of the metabolic disturbance in type II diabetes) leads to complete suppression of granule mobilization (Fig. 4C and Ref. 7). As discussed above, maneuvers that modulate the size and/or refilling of the RRP influence the subsequent secretory capacity. Conversely, conditions that interfere with the refilling of the RRP (such as ADP) can be expected to reduce the size of this pool and thus the magnitude of first phase insulin secretion. The metabolic defect would also explain why the second phase is reduced. Because of the metabolic disturbance, the increase in the cytoplasmic ATP/ADP ratio, which influences the mobilization of granules, is smaller than in the nondiabetic β-cells (Fig. 4D). Consequently, the mobilization of granules from the reserve pool into the RRP proceeds at a lower speed than would otherwise have been the case. Researchers should therefore consider whether the secretory defect seen in type II diabetes results from both incomplete closure of the K_{ATP} channels and interference with the mobilization of granules into the RRP. The recent observation that the hypoglycemic sulphonylureas are insulinotropic both by closure of the K_{ATP} channels and via a more distal mechanism (6) would be consistent with this concept. Future experiments are required to determine to what extent the interaction of the sulphonylureas with the exocytotic machinery can be exploited therapeutically.

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References