Insulin secretion in mammals is a tightly regulated process designed to ensure near constant blood glucose levels in the face of increases in supply during meals and varying rates of utilization of the sugar by tissues. Loss of insulin-secreting pancreatic islet \( \beta \)-cells thus underlies the pathology of Type 1 diabetes (20), whereas cellular dysfunction (25) perhaps compounded by a decrease in total cell mass (7) contributes to Type 2 diabetes (56, 69).

At the cellular and molecular levels, the release of insulin through exocytosis shares many similarities with that of other peptide hormones and neurotransmitters, in each case involving the Ca\(^{2+}\)-regulated fusion of a secretory vesicle at the plasma membrane. However, there are several aspects of the upstream regulation of insulin release that are essentially unique to the \( \beta \)-cell and are designed to couple changes in nutrient (principally glucose) concentration to exocytosis. Although in vivo there is further regulation by neuronal and hormonal inputs, we will focus in this review on the intracellular signalling mechanisms by which glucose itself triggers insulin release. We will then discuss recent insights into the characteristics of the exocytotic event. Pathological aspects of this topic are discussed in a separate review (59).

**Glucose Sensing by the \( \beta \)-Cell**

In healthy individuals, the rate of insulin release varies over a range of at least two orders of magnitude and is prompted in two phases (first- and second-phase release) by relatively small changes in blood glucose concentration (4–8 mM) (22). The mechanisms underlying the activation of insulin release are increasingly well understood (58) and are generally thought to involve metabolic coupling mechanisms. Thus uptake of glucose via a glucose transporter (Glut2 in rodents) is followed by glycolytic generation of pyruvate, whose oxidation by mitochondria is favored over conversion to lactate by remarkably low levels of lactate dehydrogenase (65) and plasma membrane lactate transport activity (31, 98) in the \( \beta \)-cell. Oxidation of pyruvate leads to increases in intracellular ATP-to-ADP ratio (3, 34) and closure of ATP-sensitive K\(^+\) channels (K\(_{ATP}\) channel) (2) followed by cell depolarization. Opening of voltage-gated Ca\(^{2+}\) channels (62) then leads to Ca\(^{2+}\) influx and ultimately to the exocytosis of preformed insulin-containing granules at the plasma membrane (38).

The importance of K\(_{ATP}\) channel-dependent mechanisms is reflected by the appearance of diabetes in patients with mutations in either channel subunit (25) and by the efficacy of sulphonylurea drugs, which act on the regulatory subunit (“sulphonylurea receptor” or SUR) to enhance insulin release in Type 2 diabetes (38). Nevertheless, glucose is still able to stimulate secretion from isolated islets to some extent even when K\(_{ATP}\) channels are bypassed (26) or deleted at the genetic level (64), implicating other metabolic sensors in the so-called “potentiating” (26) effects of the sugar. Other primary metabolic sensors, notably including AMP-activated protein kinase (12, 13, 60, 63), are thus also likely to be involved in glucose sensing minute to minute through mechanisms that will be discussed in more detail in the following section.

**Recruitment of Secretory Vesicles to the Cell Surface**

Rodent \( \beta \)-cells contain between 9 and 13,000 (mouse) (14, 52) or 10 and 12,000 (rat) (5, 68) dense-core secretory vesicles of which only a small proportion (~7% of total) are docked at the plasma membrane, whereas ~20% are localized to within ~300 nm of the cell sur-
active, extended conformation (90) before vesicle movement (FIGURE 1B). Nevertheless, results from this laboratory (13) have shown that an activated form of AMPK blocks glucose-stimulated insulin secretion as well as the recruitment of dense-core vesicles to the cell surface (80). Thus loss of an AMPK-dependent phosphorylation of either the heavy or light chains of kinesin-1 (FIGURE 1B) may conceivably be involved in regulated vesicle movement, especially since consensus phosphorylation sites for AMPK \( [\Omega]xxx(st)xxx[\Omega] \), where \( \Omega \) represents a large hydrophobic residue (23) exist in both kinesin heavy and light chains (FIGURE 2A). Whether the activation of vesicle movement at elevated glucose concentrations involves the activation of an already extant microtubule-kinesin-1-vesicle complex (FIGURE 2B) or recruitment of components of this complex to individual vesicles “selected” for movement remains to be established. At present, the molecular identity of the protein(s) and other factors involved in linking vesicles to the microtubule network are not yet fully defined, although roles for kinectin (6) and the cJun-NH\(_2\)-terminal binding proteins JIP1-3 (89) have been proposed (FIGURE 2B).

As vesicles approach to within a few hundred nanometers of the plasma membrane, movement along the cortical network of microfilaments, driven by actin-based motors including myosin Va (32, 88), appears to play the predominant role. The mechanisms through which vesicle movement immediately beneath the plasma membrane are regulated are again uncertain. Once again, the nature of the physical interaction between MyoVa and the vesicle is not resolved, although roles for the small GTPase Rab27a (33) and Slac2c/MyRIP have been proposed (93), and islets from Rab27a–/– mice display abnormal insulin secretion (33). Interestingly, despite the above requirement of microfilament-dependent motors for sustained exocytosis, the cortical actin network appears also to play a role as a barrier to exocytosis. Thus its disruption with latrunculin (80) or after overexpression of Clostridium botulinum toxin C2 to depolymerise F-actin (41) enhances insulin release, whereas jasplakinolide, which prevents actin depolymerization, suppresses Ca\(^{2+}\)-induced insulin secretion (48, 76, 80). Thus the cortical actin network orchestrates temporally (and perhaps spatially) different processes involved in the arrival and eventually docking of vesicles at the plasma membrane.

**Exocytosis: What Happens to the Dense-Core Vesicle at the Plasma Membrane?**

Fusion of secretory vesicles at the cell surface has been the subject of extensive study over many years. Seminal work by Rothman (57) and others (70) has identified a “minimal machinery” for exocytosis com-
prising soluble NH$_2$-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) (67). Whereas “Q-SNAREs” such as syntaxin are localized principally on the plasma membrane with SNAP25 (synaptosome-associated protein of 25 kDa), synaptobrevin [vesicle-associated membrane protein (VAMP)] is concentrated on the vesicle membrane. Interaction of the coiled coil domains of these proteins to form a SNARE bundle or “SNAREpin” appears to provide both membrane selectivity and the thermodynamic driving force for membrane fusion. Ca$^{2+}$ sensitivity is conferred on the process by additional proteins such as members of the synaptotagmin family (55), which are able to interact with the SNARE pair. Synaptotagmins possess a single transmembrane domain and a Ca$^{2+}$ and phospholipid binding C2A and C2B domains. In the β-cell, synaptotagmin V (11) [also called synaptotagmin IX (19): note that a distinct isoform also exists that is also, confusingly, called synaptotagmin V (40)] has been implicated in regulated exocytosis (30) despite biophysical evidence that Syt V/IX is unable to mediate “fast” exocytosis in neurons (66). CAPS (Ca$^{2+}$-dependent activator protein for secretion) may also contribute to Ca$^{2+}$ sensitivity, although the importance of the latter has recently been questioned by studies in knockout mice (71, 92).

Recent studies (97) suggest that two pools of vesicles, one of these a “high Ca$^{2+}$ sensitivity” pool, may exist in β-cells as a result of the phosphorylation of one or more of the above synaptotagmin isoforms. Thus it is proposed that an increase in the proportion of the phosphorylated isoform results in an overall increase in the number of granules with “high Ca$^{2+}$ sensitivity” and thus is able to detect changes in Ca$^{2+}$ ion concentration over a lower range. This model may thus explain the existence in β-cells of vesicles able to fuse with the membrane at lower “global” Ca$^{2+}$ levels (~1 µM or less).

Other important regulators include small G-proteins of the Rab family such as Rab3a (96), Rab27 (33), and ARF6 (39), as well as piccolo (18). Piccolo possibly serves to integrate input from changes in intracellular cAMP concentration via cAMP-dependent GTP exchange factor cAMP-GEFII (29). Roles for the GTP-binding protein cdc42 have also been proposed (48, 76). The mechanisms through which Rab27a links vesicles to the actin network is uncertain, although, by analogy with the situation in melanosomes, it is likely that an intermediary molecule such as MyRIP (93) is involved in forming a bridge (FIGURE 3). Recently, Noc2, a further potential binding partner and downstream effector of GTPases, has also been implicated in regulating insulin secretion (10, 45) and may serve to recruit vesicles to the readily releasable pool at the plasma membrane.

**Vesicle Docking: the Exocyst Complex**

The processes whereby vesicles located remotely finally interact with the plasma membrane seem certain to be complex and involve several steps (88) with varying dependency on MgATP and phospholipids including phosphatidylinositol 4,5 bisphosphate (PIP$_2$) (44). It has recently emerged that the mammalian homolog (78) of the yeast “exocyst” complex (42), involved in “constitutive” secretion in that organism (49), may be involved in the docking of regulated secretory vesicles in β-cells (83) as well as in neurons (83) (FIGURE 4). Strikingly, and in contrast to the situation that may exist in yeast, three of the key components of this complex structure display quite distinct subcellular localization before vesicle fusion (FIGURE 4, “Docking”), presumably coming together to form a unit only at the plasma membrane (83). Whether the formation of this complex is regulated or not, and how,
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is unclear, but its importance in the replenishment of the pool of “docked” vesicles has been demonstrated by the effects of dominant-negative interfering mutants (83).

**Transient Versus Sustained Fusion Pore Opening**

The large negative free energy of SNARE pair formation binding would seem at first to suggest that vesicle fusion is likely to be an irreversible process. This, in turn, would imply that vesicle fusion, and the ultimate recapture of the vesicle membrane from the plasma membrane, must be spatially and temporally distinct events. However, experiments in neuromuscular junction preparations more than 30 yr ago (9) demonstrated that, after their fusion and neurotransmitter release, synaptic vesicles are probably reformed rapidly at the cell surface. In other words, these studies raised the possibility that an individual vesicle may remain essentially intact during a transient or “kiss-and-run” exocytotic event without a full merger of the vesicle and plasma membranes (9).

Could such a form of transient exocytosis operate for dense-core vesicles in the β-cell, and what would be its purpose? Early electron microscopy studies implied that the vesicle cargo was probably released on mass through a large and expanding pore (or “Ω structure”) that eventually flattened into the membrane (53). Indeed, it seems quite likely that such events do occur on strong stimulation, such as during repetitive membrane depolarizations under patch clamp, where progressive increases in capacitance are likely to reflect an increase in total plasma membrane area as vesicles fuse and integrate (2). Moreover, experiments using multiphoton microscopy of the fluorescent dye sulphorhodamine B, a cell-impermeant dye, have been proposed to demonstrate dye entry into an Ω structure during individual events, followed by dye expulsion (seen as a disappearance of a fluorescent spot) when the vesicle flattens (72). Similar findings were also reported by Ma et al. (43). However, the interpretation of none of the experiments is straightforward. First, work with single fluorescent reporters may be criticized since 1) the reported events are difficult to ascribe unambiguously as exocytotic, rather than endocytotic, processes in which acidification and fluorescence quenching may contribute to the loss of the dye signal; and 2) the reporters may “miss” events in which exocytosis occurs under a “positive pressure,” i.e., where the vesicle cargo is effectively “flushed” from the vesicle, for example, as a result of Cl– channel opening and flow of water molecules into the vesicle interior: under these circumstances, the vesicle “ghost” will remain unlabeled and thus events missed. Similar criticisms may also be made of experiments using lipophilic fluorescent dyes such as FM-143 in an attempt to explore changes in vesicle structure during exocytosis (37).

As an approach to visualize the vesicle membrane and cargo simultaneously, and thus to provide a means of monitoring the behavior of the vesicle unambiguously during exocytotic events, we and others have combined the use of multiple recombinant fluorescent probe with two-color total internal reflection of fluorescence (TIRF) microscopy (84). Importantly, this allows the behavior of the vesicle membrane proteins (or lipids) to be monitored simultaneously with the release of vesicle cargo at the single-vesicle level. This approach provided the unexpected finding that the vesicle membrane resident protein phogrin (94), also called IA-2β or tissue plasminogen activator (tPA), remains associated with the vesicle during exo-/endocytosis, whereas neuropeptide Y Venus (where Venus is an enhanced form of yellow fluorescent dye such as FM-143 in an attempt to explore changes in vesicle structure during exocytosis (37).

![FIGURE 3. Putative myosin V-vesicle docking complexes in melanosomes and β-cells](http://physiologyonline.physiology.org/) MyRIP is likely to serve in a bridging complex between vesicles and MyoVa (93). Note that granuphilin (21) and Noc2 (10), other effectors of Rab27a, are likely to act as docking molecules for vesicles at the plasma membrane, possibly interacting with a syntaxin-1a-Munc18-1 complex.
integrate into the dense crystalline core (46). By contrast, fusion of insulin directly to GFP tends to lead to mistargeting of the resulting chimera in clonal β-cells (54), although this problem is less severe in primary β-cells (61, 82).

Importantly, phogrin.EGFP migrated away from the site of exocytosis when the function of the GTPase dynamin-1, implicated in retrieval of endocytic vesicles in other settings (27), was blocked using a dominant-negative form of the latter (81), consistent with the complete collapse of the vesicle under these “artificial” conditions. The observation that dynamin-1.EGFP was recruited to sites of exocytosis simultaneously with the loss of the vesicle cargo (NPY.mRFP) is also consistent with the notion that dynamin-1 recruitment leads to the closure of the fusion pore and recapture of a vesicle without its complete fusion with the plasma membrane.

These findings in β-cells are also in line with results of similar experiments in other neuroendocrine cells. Thus anti-dynamin-1 antibodies blocked the “rapid phase” of exocytosis in chromaffin cells, a process apparent in capacitance measurements when cells are exposed to a relatively brief train of depolarizing pulses (1). Correspondingly, “rapid endocytosis” in chromaffin cells (1) and NPY.mRFP release in MIN6 β-cells (81) do not involve the formation of clathrin-coated structures or the engagement of other mediators of classical endocytosis (dynamin-2, epsin, amphiphysin, for example). By contrast, blockade of dynamin-1 function had no effect on the slower endocytosis that followed more sustained episodes of depolarization, which are likely to saturate the machinery of rapid endocytosis (i.e., dynamin-1) and thus lead to “full fusion” of vesicles with the plasma membrane (1). Moreover, in a study (28) in which uptake of extracellular markers (sulphorhodaamine B and horseradish peroxidase) was demonstrated into transiently fused dense-core vesicles in PC12 cell plasma membrane sheets, recruitment of dynamin-1 to sites of NPY.GFP release was clearly observed. Similar findings to the above have been reported in intact PC12 cells (74). Interestingly, the latter study also demonstrated that the vesicle remained largely intact after exocytotic events. A later study from the same group (73) also demonstrated the ready release of the preloaded membrane marker FM1-43 from the vesicle, indicating that lipid components of the vesicle membrane are likely to be able to exchange rapidly with the plasma membrane.

The mechanisms through which dynamin-1 is recruited at the precise site of exocytosis are still unknown, although the local generation of PIP2 (81), to which dynamin-1 may be more tightly bound than mediators of the slow phase of endocytosis (e.g., amphiphysin and epsin), or possibly the prior recruitment of members of the sorting nexin (SNX) family (8) capable of serving as coincidence detectors for both membrane curvature and PIP2, are intriguing possibilities.

A number of studies have indicated that the uptake of large (kDa) species, including anti-phogrin (4) antibodies and other dyes (Alexa568) (50) can occur from immediately adjacent membrane. In this case, the vesicle, after fusion, releases its cargo directly into the cytosol, where it is available to be retrieved by the endocytosis machinery.

FIGURE 4. An exocytotic-endocytotic cycle in β-cells?

Clockwise from left: the approach of vesicles to the membrane requires the formation of a Sec6-Sec8 (exocyst) complex (DOCKING). Fusion of docked vesicles requires an elevation of Ca2+ concentration and a Ca2+ sensor such as synaptotagmin V/IX. Fusion is usually incomplete, releasing only a proportion of the vesicle’s soluble cargo and of vesicle membrane-associated SNAREs such as synaptobrevin (brown) (84). Insulin is released as dimers with Zn2+ through a fusion pore likely to be less than ~4 nm across, whereas low molecular mass species such as ATP (50) and lipid components of the vesicle membrane are likely to rapidly associate with plasma membrane lipids (not shown) (74). Such “cavity recapture” (cavicapture) events are terminated by the recruitment of dynamin-1 (RECAPTURE). Vesicles that have passed through a single round of exocytosis, but still retain adequate numbers of SNAREs, may then undergo further fusion events. Alternatively, vesicles may fuse with the endosomal network for destruction or may exchange membrane proteins and/or soluble cargo with maturing vesicles (EXCHANGE). The relative importance in the delivery to mature vesicles of membrane proteins (SNAREs, Sec6, phogrin, etc.) of de novo delivery at the TGN, and later delivery by the recycling mechanisms above, remains to be established. The vesicle’s dense core of crystalline insulin is represented in grey.
the extracellular space during individual “exocytotic” events. Two important studies have sought quantitatively to assess the degree of opening (i.e., fusion pore size) during exocytosis in β-cells. Thus Takahashi and workers (72) demonstrated using fluorescent dextrans of defined diameters that a fusion pore formed initially of 1.8 nm. Importantly, this study demonstrated that the fusion pore was semistable (i.e., resealable) when the pore diameter was between 1.4 and 4 nm, the latter value being close to the likely diameter of molecular insulin. Similarly, analysis of capacitance flickers suggested a pore diameter of ~2 nm (17). Thus, if we assume that the insulin crystal is able to partially dissolve when the fusion pore forms, then the release of insulin monomers or dimers during these transient kiss-and-run or “cavicapture” (74) events is perfectly conceivable. The mechanisms (passive or active?) involved in the rapid dissolution of the insulin crystal before and during exocytosis, as well as the process itself, provide an important challenge for future study.

It should be noted that the above approaches provide no clear evidence for “compound” exocytosis. The latter, in which a second vesicle may fuse with another vesicle just before or during the fusion of the first vesicle, has been proposed to be an important mechanism in pancreatic acinar cells (75). Recently, Kwan and Gaisano (37) proposed that a similar mechanism may be involved in β-cells based on the appearance of incremental increases in FM1-43 fluorescence (see above). An alternative explanation of these data is that the stepwise increase in the fluorescence of this lipophilic dye is due to repeated interactions of the same vesicle at the same site on the plasma membrane. Of note, a similar mechanism might also underlie the appearance of so-called hot spots proposed to be similar to neuronal “active zones.” Thus, using evanescent wave microscopy, Ohara-Imaizumi and coworkers (51) reported that the same region of the plasma membrane, which was enriched in ELK, a protein structurally related to cytomatrix active zone proteins such as CAST, which define this region in neurons, appeared to serve as the site of docking and fusion for multiple vesicles.

What May Be the Role of “Partial” Exocytotic Events?

By showing that release of SNARE complexes into the plasma membrane is relatively slow (compared with that of “soluble” vesicle cargoes) (84), our findings suggest that vesicles may, at least in theory, be capable of repeated rounds of exocytosis (see Transient Versus Sustained Fusion Pore Opening). This may, for example, permit an initial event to release low Mr species such as ATP (50) or a small amount of insulin (see below and FIGURE 4), whereas subsequent visits to the plasma membrane might release the remaining protein cargo. Evidence for such “repeat events” does, in fact, come from experiments using single dyes, although it is usually interpreted very differently (see discussion above). On the other hand, our capacity to track the fate of the same single vesicle in the β-cell has so far not allowed us to make similar observations by two-color TIRF if subsequent fusion events occur at a remote site (since the vesicle is essentially lost from the field between the events), so really overwhelming evidence for such processes is still lacking.

One might reasonably speculate that, once denuded of cargo, vesicles are likely to fuse with endosomes to be targeted for destruction, given that incorporation of peptide cargo seems highly unlikely to occur other than through synthesis in the ER and concentration in the TGN and maturing vesicle. Correspondingly, experiments monitoring the fate of internalized antibodies to the luminal domain of phogrin antibodies have provided evidence for limited return of fused vesicles to the endosomal system (91). Intriguingly, however, a significant proportion (35%) of the internalized anti-phogrin antibodies become colocalized with insulin-containing structures, suggesting an exchange of vesicle markers (e.g., phogrin) and membrane with maturing dense-core vesicles. By contrast, co-uptake of anti-phogrin and anti-insulin antibodies was not apparent in this study, suggesting that the fusion pore dilation sufficient to allow antibody incorporation is associated with complete release of the cargo insulin. This finding (91) contrasts with observations in neuroendocrine PC12 cells (4), where antibodies to the dense-core resident protein chromogranin were incorporated into apparently recycled vesicles and also with our own observations using NPY:mRFP as an insulin surrogate.

Conclusions

The findings described above allow us to assemble a scheme showing some of the key elements involved in the docking at the plasma membrane and final fusion of insulin-secreting vesicles (FIGURE 4). The extent to which these processes may become defective in some forms of Type 2 diabetes, as recently described after culture of β-cells at high glucose concentrations (82), is likely to form an exciting avenue of new research. ■

This review was supported by grants from the Wellcome Trust, MRC, and the Juvenile Diabetes Research Fund. G. A. Rutter is a Wellcome Trust Research Leave Fellow.

References


