

## Regulation of resident and newcomer insulin granules by calcium and SNARE proteins

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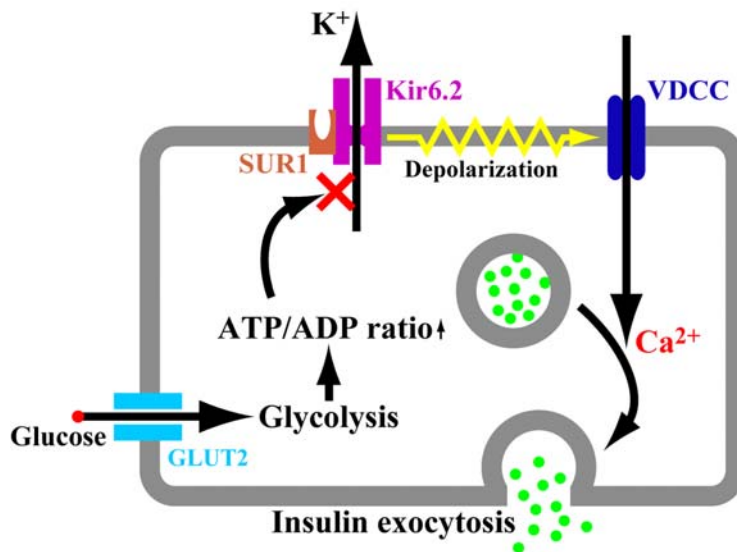
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## 1. ABSTRACT

Insulin, stored in large dense core granules, is biphasically exocytosed by glucose stimulation in pancreatic beta-cells. Several molecules, such as SNARE proteins, and  $Ca^{2+}$  ion are involved in the regulation of insulin exocytosis. Indeed, studies using gene targeting mice revealed critical roles of SNARE proteins and their accessory proteins, which may be associated with diabetes mellitus. In particular, the total internal reflection fluorescent (TIRF) imaging technique shed new light on the molecular mechanism of the insulin exocytotic process. In this review we discuss the mechanism of insulin exocytosis mainly from a point of view of imaging techniques.

## 2. INTRODUCTION

Insulin, a peptide hormone, is released from pancreatic beta-cells of the islets of Langerhans. Secreted insulin accelerates glucose uptake into target tissues and also suppresses glucose output from the liver. Therefore, insulin negatively regulates the concentration of the blood glucose. Because a dysfunction or insufficiency of the insulin secretory response is commonly observed in various forms of type 1 and type 2 diabetes mellitus (1-3), elucidation of the molecular mechanism underlying insulin secretion is quite important. The regulated exocytosis consists of several elementary stages (4-6). Understanding the insulin exocytotic process has recently much



**Figure 1.** Model of glucose-induced insulin secretion. Glucose metabolism increases the ATP/ADP ratio, which closes K<sup>+</sup> channels, resulting in membrane depolarization and influx of Ca<sup>2+</sup>. The elevation of [Ca<sup>2+</sup>]<sub>i</sub> triggers insulin exocytosis.

progressed, though the molecular mechanism of insulin exocytosis is still unclear. Here, we discuss the molecular mechanism of insulin secretion in pancreatic beta-cells and recent results mainly using TIRF microscopy.

### 3. OVERVIEW OF INSULIN SECRETION

#### 3.1. Functional coupling between glucose stimulation and insulin secretion

Insulin secretion is coupled to the electrical activity elicited by the elevations of the blood glucose level (Figure 1). When the concentration of extracellular glucose is increased, glucose enters the beta-cell through high-capacity glucose transporter type 2 (GLUT2) in rodents (7) and subsequent metabolic breakdown of the sugar results in an elevation of the cytosolic ATP/ADP ratio (8, 9). This increased ATP/ADP ratio in turn induces the closure of ATP-sensitive K<sup>+</sup> channels (K<sub>ATP</sub> channels), leading to the depolarization of the membrane potential (10). The membrane depolarization induces the opening of voltage-gated Ca<sup>2+</sup> channels to increase the intracellular calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) (11). In response to the [Ca<sup>2+</sup>]<sub>i</sub> elevation, insulin-containing granules (insulin granules) fuse with the plasma membrane resulting in the release of insulin into the extracellular space. A rise in [Ca<sup>2+</sup>]<sub>i</sub>, however, is not likely to be the only regulator of exocytosis (12). G protein (13, 14), long-chain fatty acyl moieties (15, 16), and protein kinases (17, 18) also contribute to the induction of exocytosis. Because of space limitation, we do not discuss the mechanism of insulin exocytosis induced by those factors.

#### 3.2. Biphasic insulin secretion

A physiological feature of insulin secretion is that fuel secretagogue, glucose, induces a biphasic pattern of insulin secretion. Dr. Grodsky clearly demonstrated the characteristics of the glucose-induced insulin secretion

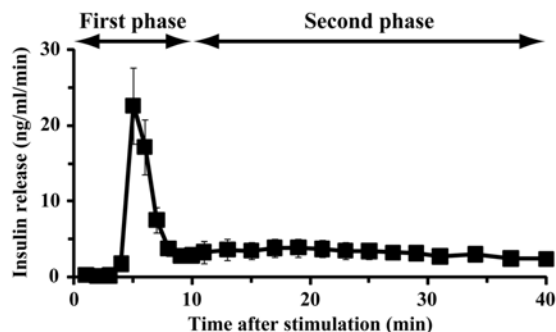
by *in vitro* perfusion of rat pancreas (19) as follows (Figure 2): 1) the initial response is a transient but rapid elevation of insulin release (first phase), which terminates in 5 to 10 min; 2) this is followed by a progressively increasing second phase (prominent in rat, but low or flat in mice) (20). However, interestingly, non-metabolizable stimulation, such as high KCl, evoked transient insulin secretion very similar to the glucose-induced first phase, but cannot induce sustained second phase (12). The different secretory patterns evoked by metabolizable and non-metabolizable stimuli suggest that distinct mechanisms underlie the first and second phase secretion.

#### 3.3. Exocytotic process and SNARE functions

In neurons and neuroendocrine cells, neurotransmitters and hormones are secreted by a sequential process, exocytosis. A large number of studies using these secretory cells revealed that the process leading to Ca<sup>2+</sup>-dependent secretion consists of some elementary steps: 1) translocation of secretory vesicles from inside the cell to the plasma membrane region, 2) docking of vesicles to specific sites on the plasma membrane, 3) ATP- and temperature-dependent priming of vesicles to acquire the competency for the subsequent fusion step, and 4) fusing of the vesicular and plasma membranes to release the vesicular contents into the extracellular space (4-6). It is widely accepted that Ca<sup>2+</sup>-dependent secretion is accomplished by conducting this series of elementary steps on the plasma membrane.

This Ca<sup>2+</sup>-dependent exocytosis is mediated by a group of proteins, known as “Soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor (SNARE) proteins” such as syntaxin, synaptosomal-associated protein of 25 kDa (SNAP-25), and vesicle associated membrane protein-2 (VAMP2)/synaptobrevin. Syntaxin and SNAP-25 localized in the plasma membrane and VAMP2 on secretory vesicles associate to form a

## Imaging analysis of insulin secretion



**Figure 2.** Glucose-stimulated biphasic insulin secretion in mouse pancreas. Mouse pancreas was perfused with Krebs-Ringer buffer (KRB) containing 2.8 mM glucose for 20 min to establish the basal insulin secretion, then stimulated with 16.8 mM glucose for 40 min. The perfusates were analyzed for insulin by ELISA. Glucose stimulation evoked a rapidly developed but transient insulin secretion, which peaked at 5 min after the onset of stimulation (first phase), then declined rapidly to a plateau. The sustained insulin secretion lasted until the end of this experiment (second phase).

ternary core complex, and the formation of this SNARE complex is thought to force the secretory vesicle membrane and the plasma membrane into close proximity, initiating membrane fusion (4, 5, 21). Indeed, SNARE proteins reconstituted into distinct artificial liposomes were demonstrated to mediate membrane fusion (22). The functional importance of SNARE proteins on  $\text{Ca}^{2+}$ -dependent exocytosis has been established using tetanus and botulinum neurotoxins. These neurotoxins specifically cleave SNARE proteins and severely suppress the  $\text{Ca}^{2+}$ -dependent neurotransmitter release (23). Furthermore, the genetic ablation of SNARE proteins abolished electrical depolarization-induced exocytosis in neurons and chromaffin cells (24-27).

The mechanism of insulin secretion in beta-cells shows great similarity to that of  $\text{Ca}^{2+}$ -dependent secretion in neurons and neuroendocrine cells. In beta-cells, a subset of insulin granules is morphologically docked to the plasma membrane (28, 29) and ATP- and temperature-dependent steps prior to  $\text{Ca}^{2+}$ -dependent fusion are shown to be essential for electrical depolarization-induced insulin secretion (30-32). The expression of SNARE proteins in beta-cells has also been reported (30, 33-38). In permeabilized insulin-secreting cells, tetanus and botulinum neurotoxins severely inhibited  $\text{Ca}^{2+}$ -dependent insulin secretion (30, 34, 35), and inhibition of the SNARE function by specific antibodies or synthetic peptides also markedly suppressed  $\text{Ca}^{2+}$ -dependent exocytosis (39, 40). In addition, the expression of syntaxin and SNAP-25 were reduced in diabetic Goto-Kakizaki rat in which glucose-induced insulin secretion was severely impaired. The insufficiency of insulin secretion in response to glucose stimulation was significantly recovered by exogenously expressed syntaxin and SNAP-25 (41-43), indicating the

functional linkage between insulin secretion and SNARE proteins.

It is of note that a substantial amount of insulin was released even when the SNARE function was inhibited in beta-cells. Furthermore, in non-permeabilized living beta-cells, inhibition of the SNARE functions caused only mild suppression of  $\text{Ca}^{2+}$ -dependent insulin secretion (44, 45), and transient expression of botulinum neurotoxin almost completely inhibited KCl-evoked insulin secretion but a large amount of glucose-induced insulin secretion was preserved (45). These results suggest the possibility that pancreatic beta-cells have an alternative secretory pathway that is botulinum neurotoxin-insensitive and involved in the second phase secretion. Although numerous findings on the insulin secretory mechanism have been drawn from a large number of studies based on the similarity of the secretory response of pancreatic beta-cells to that of other secretory systems, it should be important to elucidate the mechanism specific for the characteristic biphasic insulin secretion in beta-cells.

### 3.4. Current model for biphasic insulin secretion

As mentioned above, SNARE proteins appear to play a key role in insulin exocytosis, but, it is unknown how these proteins function in biphasic insulin release. Thus, the mechanism underlying the glucose-induced biphasic insulin release is still largely unclear. Rorsman and his colleagues proposed a model for the biphasic insulin secretion in which insulin granules can be divided into two functionally distinct pools with different releasability (46, 47). Membrane capacitance measurements revealed that the elevation of  $[\text{Ca}^{2+}]_i$  induced a burst of the exocytotic response, and subsequently the rate of exocytosis gradually dropped and eventually reached a lower steady-state level (31, 48, 49). It is currently accepted that the initial drop of the exocytotic rate reflects the depletion of insulin granules which can immediately undergo exocytosis in response to the  $[\text{Ca}^{2+}]_i$  elevation, and the pool of these release-competent insulin granules is referred to as the “readily releasable pool (RRP)”. A series of electrophysiological techniques revealed that the RRP consists of 20 – 100 granules in single beta-cells (31, 49, 50, 51). Given that a single beta-cell contains ~10,000 insulin granules (28, 29), most insulin granules are incompetent for  $\text{Ca}^{2+}$ -dependent exocytosis and consequently are assumed to reside in a “reserve pool”. Because the number of insulin granules in the RRP is not sufficient to support a sustained exocytotic response, the RRP should be refilled, and the reserve pool is thought to be the source of insulin granules that are replenished into the RRP. The ATP and temperature-dependent reactions were shown to be required for the sustained exocytotic response evoked by electrical depolarization (31, 32), indicating that insulin granules in the pre-primed and fusion-incompetent state(s) should undergo priming steps for replenishing the RRP. Therefore, the steady-state level of exocytosis detected by capacitance measurements would reflect the rate at which the RRP is replenished by insulin granules recruited from the reserve pool.

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The observation of the biphasic exocytotic response detected by capacitance measurements was extended to glucose-induced insulin secretion by Rorsman's group (46, 47, 52, 53). They proposed that the insulin granules in the RRP would be responsible for the glucose-induced first phase and that the sustained insulin secretion during the second phase would correspond to the rate of replenishment of insulin granules from the reserve pool to the RRP. The estimated number of granules in the RRP was very close to that of insulin granules released during the glucose-induced first phase (47, 54), suggesting that the insulin granules in the RRP would be responsible for the first phase insulin secretion. Meanwhile, although there is no experimental evidence showing that ATP- and temperature-dependent priming steps are required for the glucose-induced second phase insulin secretion in beta-cells, there is a consensus that recruitment of insulin granules from the reserve pool to the RRP should be required for the second phase insulin secretion because the number of insulin granules in the RRP in the non-stimulated condition cannot support the biphasic insulin secretion lasting for a few hours (55). Although the model provided by Rorsman's group seems to fit very well with the feature of biphasic insulin secretion, this model is entirely based on the hypothesis that all insulin granules should be released from the RRP. However, several independent studies on neurons recently raised a question about this hypothesis by demonstrating that not only the RRP but also another pool provided secretory vesicles for exocytosis (56-58). Indeed, there had been no direct observation of real-time insulin granule motion in glucose-stimulated beta-cells to ascertain whether all insulin granules exhibited the same dynamic behavior prior to fusion. However, a recent advance in technology, total internal reflection fluorescent (TIRF) imaging system, allowed us to directly observe the dynamic motion of single insulin granules undergoing insulin exocytosis.

## 4. IMAGING ANALYSIS OF INSULIN SECRETION

### 4.1. TIRF microscopy

TIRF microscopy is a technique that specifically illuminates fluorophores within a closely restricted layer just adjacent to the interface at which total internal reflection occurs (59, 60). When angled light strikes the interface and the light undergoes total internal reflection, some of the light penetrates the interface as an electromagnetic field called "evanescent wave". Because the evanescent wave vanishes exponentially with distance from the interface, the penetration depth of the evanescent field is usually less than 100 nm. Therefore, when cells are grown on a coverslip, a very thin layer adjacent to the coverslip can be specifically illuminated by the evanescent wave formed by the total internal reflection occurring at the interface between the coverslip and extracellular medium. In pioneering work by Daniel Axelrod, this technique was first applied to the study of live cells, primarily to investigate cell surface adhesions (61). Then, several studies of protein dynamics, topography of cell-substrate contacts, endocytosis and exocytosis using this technique were reported (62-67).

### 4.2. Application of TIRF microscopy to imaging analysis of the dynamics of secretory granules

Because the exocytotic process occurs in the vicinity of the plasma membrane, TIRF microscopy is a powerful tool to analyze the exocytotic process of individual insulin granules in living pancreatic beta-cells. To observe the insulin secretory process under TIRF microscopy, insulin granules should be labeled with fluorescent probes. Previously, a weak base fluorescent dye, acridine orange, was used for labeling granules because the inside of most vesicles is acidic (68). However, acridine orange accumulates not only in insulin granules but also other secretory vesicles such as GABA-containing small synaptic-like vesicles. Thus, the expression of GFP-tagged insulin would be best to specifically analyze the motion of insulin granules. GFP-tagged neuropeptide Y and phogrin were also used to label insulin granules (69-72), but their expression patterns were not completely identical (71). Therefore, we made a recombinant adenovirus encoding human preproinsulin tagged with GFP, which is localized in insulin secretory granules (73), allowing us to analyze the real-time motion of insulin granules.

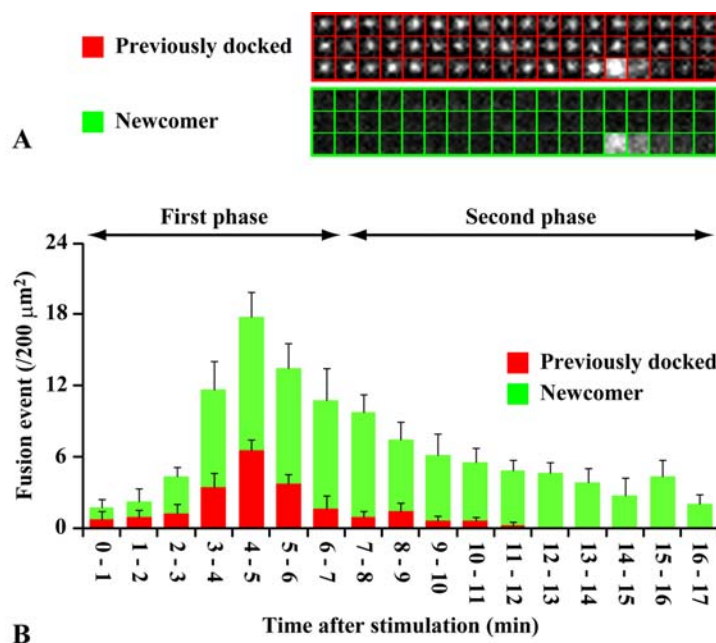
### 4.3. Docked granules detected by electron and TIRF microscopies

When pancreatic beta-cells expressing insulin-GFP are imaged by TIRF microscopy, punctate fluorescent spots can be observed (74, 75). These punctate signals most likely correspond to docked insulin granules because of the following supporting evidence. First, the intensity of the evanescent field exponentially declines with the distance from the coverslip. Thus, the intensity of excitation light at 100 nm away from the coverslip declines by 89% in our TIRF microscopy setting (73-75). Second, the number of fluorescent spots observed under TIRF microscopy is similar to that of insulin granules morphologically docked on the plasma membrane detected by electron microscopy. Under TIRF microscopy,  $253.3 \pm 10.2$  per 200  $\mu\text{m}^2$  punctate signals were detected (75). Using the morphometric data obtained by electron microscopy (29), this value can be converted to 692 fluorescent spots per single beta-cell. Because two studies using electron microscopy reported that 600 – 700 insulin granules morphologically docked on the plasma membrane (28, 29), our result obtained by TIRF microscopy is very similar to those from electron microscopy. Finally, in pancreatic beta-cells prepared from syntaxin1A<sup>-/-</sup> mice or diabetic Goto-Kakizaki rats, the number of punctate signals detected by TIRF microscopy was severely reduced. The electron microscopic analyses of these animals also revealed the dramatic reduction of morphologically docked insulin granules (41, 74, 75). Taken together, insulin GFP signals observed under the TIRF microscopy are highly likely to correspond to insulin granules morphologically docked on the plasma membrane detected by electron microscopy.

### 4.4. Analysis of biphasic insulin secretion by TIRF microscopy

TIRF microscopy revealed that glucose stimulation induced interesting exocytotic responses that

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**Figure 3.** Analysis of single GFP-labeled insulin granule motion in mouse primary beta-cells during glucose stimulation. (A) Sequential images (1  $\mu\text{m} \times 1 \mu\text{m}$  per 300 ms intervals) of a granule docking and fusion with the plasma membrane are shown during 22 mM glucose-stimulation. (B) Histogram showing the number of fusion events at 60-s intervals after stimulation. The red column shows fusions from previously docked granules and the green column shows those from newcomer granules.

originated from two distinct types of insulin granules with completely different behaviors prior to fusion; “previously docked granules” that are visible before the onset of stimulation under TIRF microscopy, and “newcomer granules” that cannot be detected by TIRF microscopy or are dimly visible before stimulation (Figure 3). It should be noted that in primary cultured pancreatic beta-cells, newcomer granules fuse immediately after they reach the plasma membrane. The time from landing to fusion is less than 50 ms (74, 75). More interestingly, these two types of fusion events reveal different temporal patterns. Fusions originating from previously docked granules are detected preferentially during the first phase, whereas fusion events during the second phase mostly arose from newcomer granules (41, 74, 75).

Some recent studies demonstrated that glucose stimulation evoked pulsatile insulin secretion not only in isolated pancreatic islets but also at the single beta-cell level (76-78). However, many groups could not always detect the pulsatile response (75, 79-83). Chow and his colleagues observed the glucose-induced pulsatile insulin secretion under TIRF microscopy but they did not address the granule behavior prior to the fusion (77). Thus, which types of insulin granules are involved in the pulsatile secretion currently remains unclear.

### 4.5. Relationship between SNARE proteins and biphasic insulin release

We examined the relationship between syntaxin and the biphasic insulin release using TIRF microscopy. Because syntaxin was shown to play a crucial role in secretory granule docking and subsequent fusion steps in

the exocytotic process (84-86), we performed image analysis to reveal the relationship between syntaxin localization on the plasma membrane and the dynamic motion of insulin granules using dual-color TIRF microscopy. It was reported that syntaxin was not homogeneously distributed but was enriched in distinct sites and formed numerous clusters in an artificially isolated plasma membrane (87-89). The clustered distribution of syntaxin together with its functional importance in docking and the subsequent exocytotic process strongly implicated the dynamic interaction between syntaxin clusters and the exocytotic sites of insulin granules. Using the protein transduction domain of HIV-1 TAT protein, which has been shown to efficiently cross biological membranes (90-91), syntaxin clusters in living beta-cells and insulinoma MIN6 cells were visualized by fluorescently labeled antibodies (75, 93). Syntaxin clusters colocalized with a substantial number of docked insulin granules detected by TIRF microscopy, and high KCl stimulation evoked the exocytosis of insulin granules docked on the syntaxin clusters in living MIN6 cells (93). Glucose stimulation also induced the exocytosis of insulin granules docked on the syntaxin clusters in living pancreatic beta-cells (75). We also found that ELKS, a protein localized in presynaptic active zones (94, 95) and involved in membrane trafficking (95, 96), also exhibited the clustered localization in the plasma membrane, and a substantial fraction of the exocytotic responses was observed on the ELKS clusters (97). These results prompted us to further explore the physiological role of SNARE proteins using syntaxin1A<sup>-/-</sup> pancreatic beta-cells and beta-cells treated with interleukin-1 $\beta$  to reduce the amount of SNAP-25 (75, 98).

## Imaging analysis of insulin secretion

TIRF microscopy revealed that genetic ablation of syntaxin1A severely reduced the number of docked insulin granules and also that a reduction of SNAP-25 by interleukin-1beta diminished the docked insulin granules (75, 98). These results clearly indicate that syntaxin and SNAP-25 are key components for the docking of insulin granules. In line with these results, a series of recent studies in chromaffin cells demonstrated that syntaxin-SNAP-25 complex is essential for the docking of secretory granules (84, 86). Specific cleavage of syntaxin by botulinum neurotoxin resulted in the loss of docked secretory granules (85), and an equivalent docking defect was observed in SNAP-25<sup>-/-</sup> chromaffin cells (84). Therefore, these reports based on electron microscopic analysis strongly support our TIRF microscopy results. Furthermore, concomitantly with the reduced number of docked insulin granules, genetic ablation of syntaxin1A and reduction of the amount of SNAP-25 by interleukin-1beta treatment selectively disturbed the glucose-induced exocytotic responses originating from previously docked granules during the first phase (75, 98). These results clearly demonstrated the functional linkage between docked insulin granules and the first phase insulin secretion.

Several lines of evidences also support the idea that docked insulin granules would be involved in the first phase insulin secretion. According to the model for biphasic insulin secretion proposed by Rorsman's group, the RRP should consist of a subset of docked insulin granules that have experienced priming steps. Therefore, part of the docked insulin granules should be responsible for the first phase insulin secretion. In good agreement with this model, an ultrastructural study revealed that 600 – 700 insulin granules are morphologically docked at the plasma membrane in single beta-cell (28, 29), and the first phase insulin secretion mimicked by high KCl stimulation was accompanied by a significant reduction of the number of docked insulin granules (28). Second, Sharp and his colleagues used the molecular interaction between VAMP2 and syntaxin to estimate the amount of docked insulin granules (99). The amount of syntaxin-VAMP2 complex was decreased during the first 10 min after the onset of glucose stimulation (corresponding to the first phase), suggesting that molecularly docked insulin granules were responsible for the first phase insulin secretion. Third, electrophysiological experiments showed that ~50 insulin granules were estimated to functionally interact with L-type voltage-gated Ca<sup>2+</sup> channels to constitute the RRP (50). These independent results obtained from ultrastructural, molecular, electrophysiological, and biochemical approaches clearly demonstrated that a subset of docked insulin granules constitute the RRP and is, at least partly, responsible for the glucose-stimulated first phase insulin secretion.

### 4.6. Second phase insulin release and fusions from newcomer granules

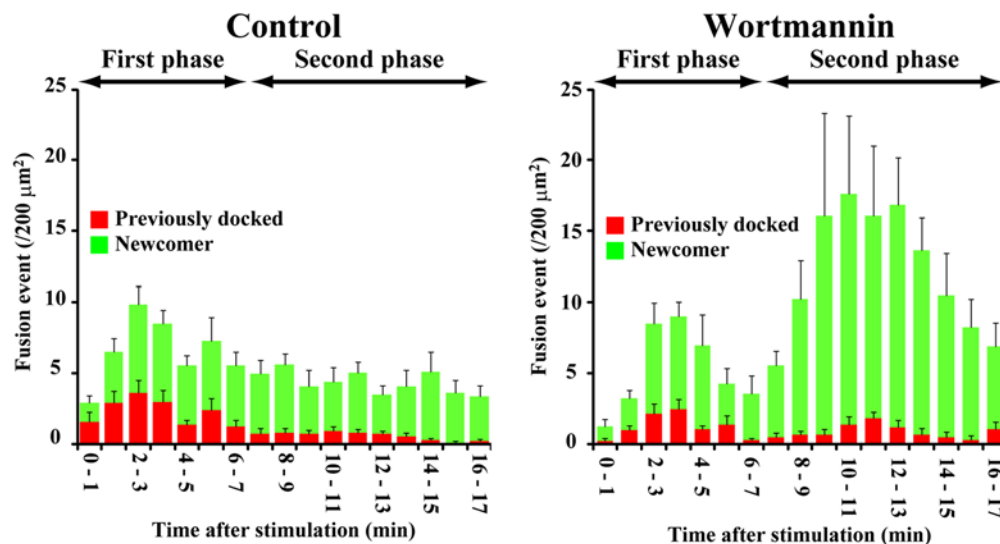
Although new technologies revealed that SNARE proteins are involved in the fusion from previously docked granules during the first phase insulin release, the molecules related to the exocytosis of newcomer granules

during the second phase are not well understood yet. Interestingly, we found several differences between fusions from newcomers and those from previously docked granules.

First, fusions arising from newcomer granules were detectable in syntaxin1A<sup>-/-</sup> beta-cells. This was consistent with the perfusion experiments showing that genetic ablation of syntaxin1A impaired the first phase but not the second phase insulin secretion. This result indicates that fusions from newcomer granules are preserved in syntaxin1A<sup>-/-</sup> cells, because the second phase insulin release mostly consisted of fusions from newcomer granules. In addition, fusions from newcomer granules were detected outside of syntaxin1 clusters on the plasma membrane (75). These results suggest that fusions from newcomer granules were 1) independent of syntaxin1A and 2) involved in the second phase insulin secretion. Consistent with these results, a previous study showed that botulinum neurotoxin, which specifically cleaves several syntaxin isoforms, inhibited 95% of KCl-induced insulin secretion, but only 25% of glucose-induced secretion (45). In addition, the reduction of syntaxin1 and SNAP-25 in cultured beta-cells prepared from diabetic GK rats affected the fusions originating from previously docked granules but not those from newcomer granules. Furthermore, newcomer fusions were not sensitive to the reduction of SNAP-25 and VAMP2 by interleukin-1beta treatment. Thus, syntaxin1A, SNAP-25 and VAMP2 may not be involved in newcomer fusion. Indeed, SNARE isoform-independent exocytosis has been reported (25, 27). In VAMP2 or SNAP25 deleted neurons, evoked neurotransmitter release was completely abolished but spontaneous release was preserved. In addition, several independent studies in neurons recently demonstrated that secretory vesicles involved in evoked neurotransmitter release and spontaneous release belonged to the distinct vesicle pools (56-58). If this is the case, what molecules are involved in newcomer fusion? What mechanisms work in the fusion process of newcomers? The mechanism of exocytosis for previously docked granules appears to be different from that of newcomer granules. Thus, it would be possible that different SNARE protein isoforms are involved in these two pathways. Alternatively, the conformation of proteins on these granules might be different because the acidification of the inside of granules was reported to be important for the sustained exocytotic response in electrophysiologically stimulated beta-cells (100), and the granular acidification may affect the conformation of proteins on the granules (101). Further studies are required to identify the molecules regulating the dynamics of newcomer granules.

### 4.7. Different characteristics of Ca<sup>2+</sup> sensing in previously docked and newcomer granules

Recently, we reported that the Ca<sup>2+</sup> sensitivities of fusions originating from previously docked and newcomer granules are different (102). A rapid and marked elevation in the subplasma membrane Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>PM</sub>) caused fusions mostly from previously docked granules, whereas a slow and sustained elevation in [Ca<sup>2+</sup>]<sub>PM</sub> induced fusions from newcomer



**Figure 4.** Wortmannin treatment enhances fusions from newcomer granules during second phase in MIN6 cells. Histograms of the numbers of exocytotic events from control ( $n = 22$ ) and 100 nM wortmannin treated ( $n = 6$ ) cells. MIN6 cells expressing insulin-GFP were stimulated with 22 mM glucose at time 0 and exocytotic responses (per 200  $\mu\text{m}^2$ ) detected within every 1-min were manually counted. The red columns shows the fusions from previously docked granules and the green column shows those from newcomer granules.

granules. These results were in good agreement with a recent kinetic model study, describing that the  $\text{Ca}^{2+}$  concentrations determine the fusion events of previously docked granules and those of newcomer granules (103). In addition, L-type voltage gated  $\text{Ca}^{2+}$  channels were tightly associated with rapid exocytosis and the first phase insulin secretion (104), whereas R-type  $\text{Ca}^{2+}$  channels regulated the second phase insulin secretion (105). These studies suggested that the  $\text{Ca}^{2+}$  ion has different impacts on the first and second phase secretion. Therefore, it is conceivable that there are different  $\text{Ca}^{2+}$ -sensing mechanisms controlling fusions from previously docked versus newcomer granules. Synaptotagmins are candidates for  $\text{Ca}^{2+}$  sensors of membrane fusion, in concert with SNARE complex. In primary beta-cells, synaptotagmin 5, 7, and 9 are expressed (106-109), but it still remains unclear whether these synaptotagmins regulate the glucose-induced first or second phase insulin secretion. It is also possible that  $\text{Ca}^{2+}$  may control the recruitment of insulin granules during the second phase via myosin Va (110, 111).

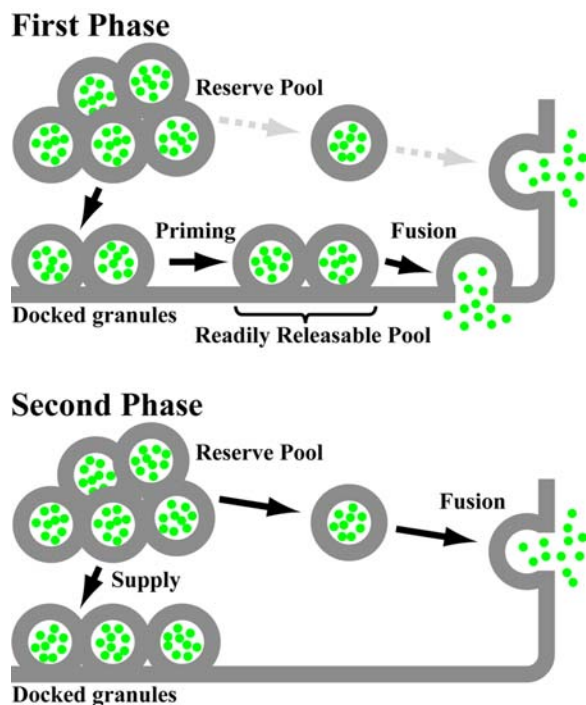
#### 4.8. Regulation of second phase insulin release by phosphatidylinositol 3-kinase

Phosphatidylinositol 3-kinase (PI3K), a key component that transmits the insulin signal to downstream effectors (112, 113), is reported to negatively regulate the glucose-induced second phase insulin secretion (114-117). Although class 1B PI3K, a minor component of PI3K in pancreatic beta-cells, was reported to positively modulate the KCl-stimulated insulin secretion in beta-cells (118), its involvement in the glucose-induced biphasic insulin secretion is unknown. On the other hand, the genetic ablation of p85 $\alpha$ , a major regulatory subunit of PI3K in beta-cells, resulted in the enhancement of insulin secretion preferentially during the second phase (114). A highly

selective inhibitor for PI3K, wortmannin, was also well-known to enhance the insulin secretion (115, 116) without affecting the glucose-induced first phase release (117). Because PI3K regulates the trafficking of intracellular vesicles and exocytosis in many cell types (119, 120), PI3K and its downstream effectors should be involved in the regulation of the dynamics and exocytosis of newcomer granules, and consequently the second phase insulin secretion.

The experiments shown in Figure 4 were performed to examine whether the insulin-PI3K pathway could regulate fusions from newcomer granules. Here, insulin granules in MIN6 cells were labeled by insulin-GFP and observed under TIRF microscopy. Stimulation with 22 mM glucose applied at 0 min induced the biphasic exocytotic responses as we reported previously (75). When MIN6 cells were pre-treated with 0.1  $\mu\text{M}$  wortmannin for 15 min, the number of exocytotic responses from newcomer granules during the second phase but not that of the first phase was markedly increased (Figure 4). The effect of wortmannin on newcomer granules during the second phase was specific because over-expression of a dominant negative form of p85 $\alpha$  induced the same effect on newcomer granule fusion (unpublished data). These results, together with previous reports, clearly indicate that downstream effector(s) of PI3K would regulate the dynamics of newcomer granules and the second phase insulin secretion. Although more than 100 molecules are reported to be activated downstream of PI3K, Akt, a Ser/Thr kinase activated downstream of PI3K, would be of particular interest because Akt phosphorylates and regulates several molecules that are involved in insulin secretion including Rac1 (121), Pak1 (122), cystein string protein (CSP) (123), and AS160 (124, 125)





**Figure 5.** Proposed model for biphasic insulin release. Some of the previously docked granules are primed and constitute the RRP. During the first phase, the elevation of  $[Ca^{2+}]_i$  evokes the fusion events from granules in the RRP. During the second phase, newcomer granules jump directly from the reserve pool (inner pool of granules) to the fusion site on the plasma membrane without approaching the RRP and are quickly fused. Simultaneously, depleted RRP is refilled with granules from the reserve pool.

#### 4.9. Akt substrates, possible regulator of second phase insulin secretion

Cdc42, a small GTPase protein, was recently reported to selectively and positively regulate the second phase insulin secretion (126). Because the positive effect of Cdc42 on insulin secretion was dependent on its downstream effectors, Pak1 and Rac1, it is reasonable to assume that the second phase insulin secretion would be regulated by Pak1 and Rac1. Although the role of Rac1 and Pak1 in the biphasic insulin secretion is still unclear, Li *et al.* reported that expression of a dominant-negative form of Rac1 caused the inhibition of glucose-induced, but not KCl-induced, insulin secretion indicating the involvement of Rac1 in the second phase release (127). In addition, the phosphorylation at Ser71 of Rac1 by Akt was reported to inhibit the activation of Rac1 (121) suggesting that the inhibition of PI3K should activate Rac1 and enhance the second phase insulin secretion. Akt also phosphorylates and modestly activates Pak1, a Ser/Thr kinase, but the role of the activated Pak1 on the biphasic insulin secretion still remains to be elucidated.

CSP is a presynaptic protein involved in neurotransmitter release. Several studies revealed that CSP is associated with the membrane of secretory vesicles and regulated various aspects of exocytosis:  $Ca^{2+}$ -influx,

docking, and  $Ca^{2+}$ -dependent fusion steps (128, 129). The expression of CSP in beta-cells was reported, and the involvement of CSP in  $Ca^{2+}$ -dependent insulin exocytosis was demonstrated (130, 131). Because CSP was shown to interact with syntaxin and synaptotagmin through its N-terminal region, the regulation of exocytosis by CSP was at least partially mediated by these molecular interactions (132, 133). Interestingly, Akt and PKA phosphorylate the same residue, Ser10 (123, 134), and this phosphorylation reduces the binding affinity of CSP to syntaxin and synaptotagmin (123, 132). Thus, the inhibition of PI3K would be expected to reinforce the interaction of CSP with syntaxin and synaptotagmin resulting in the enhancement of insulin secretion.

AS160 was initially identified as an Akt substrate in 3T3-L1 cells (124). Insulin stimulation induced the phosphorylation of five residues (Ser318, Ser570, Ser588, Thr642, Thr751) in AS160, and the phosphorylation of these residues was essential for insulin-dependent exocytosis of GLUT4-containing granules in 3T3-L1 cells (125). Recently, Bouzakri *et al.* reported that AS160 regulated glucose-stimulated insulin secretion in beta-cells (135). They showed that AS160 was also phosphorylated by Akt in response to PI3K activation, and depletion of AS160 by siRNA suppressed the glucose-induced insulin secretion. Interestingly, in contrast to the glucose-induced insulin secretion, the basal insulin release was enhanced in AS160 depleted MIN6 cells and beta-cells. Because fusion events can be spontaneously observed in a non-stimulated condition and TIRF microscopy revealed that most spontaneous fusion events arose from newcomer granules (75), it might be interesting to hypothesize that AS160 has dual effects on the exocytosis of insulin granules: a positive effect for fusions from previously docked granules but a negative effect on fusions from newcomer granules.

#### 5. PROPOSED MODEL FOR BIPHASIC INSULIN SECRETION

From our observations and supporting evidences described above, we propose a model for the dynamics of insulin granules during the glucose-induced biphasic secretion, in which two distinct pathways for exocytosis are involved (Figure 5). The glucose-induced first phase insulin secretion consists of fusions mostly originating from previously docked granules, which is also supplemented by those from newcomer granules. The proportion of newcomer fusions during the first phase seems to depend on the experimental conditions (41, 75, 94, 136). On the other hand, in our model, mainly based on evidence from direct imaging techniques, the second phase secretion is mostly derived from newcomer granules which directly fuse with the plasma membrane without passing through the RRP. Besides fusions originating from newcomer granules during the second phase, insulin granules are newly recruited to the docked granule pool. However, only a few of the newly recruited insulin granules are released (74) probably because they are not primed.

To validate our model, the molecular mechanism underlying the dynamics of newcomer granules should be



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elucidated to identify the specific molecules involved in the second phase insulin secretion. As discussed above, newcomer granules translocate from the inside pool to the plasma membrane in response to stimulation, but we did not have any result concerning the translocation process of newcomer granules; what molecule regulates the dynamic motion of newcomer granules and triggers the translocation, and how do they translocate to the plasma membrane? To answer these questions, the analysis of intracellular insulin granules is becoming more important to shed new light on the molecular mechanisms of the second phase insulin secretion.

## 6. ACKNOWLEDGEMENT

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