Stimulation of Insulin Release by Glucose Is Associated With an Increase in the Number of Docked Granules in the β-Cells of Rat Pancreatic Islets

Susanne G. Straub, Geetha Shanmugam, and Geoffrey W.G. Sharp

Electron microscopy and quantitative stereological techniques were used to study the dynamics of the docked granule pool in the rat pancreatic β-cell. The mean number of granules per β-cell was 11,136. After equilibration in RPMI containing 5.6 mmol/l glucose, 6.4% of the granules (~700) were docked at the plasma membrane (also measured as [means ± SE] 4.3 ± 0.6 docked granules per 10 μm of plasma membrane at the perimeter of the cell sections). After a 40-min exposure to 16.7 mmol/l glucose, 10.2% of the granules (~1,060) were docked (6.4 ± 0.8 granules per 10 μm of plasma membrane). Thus, the docked pool increased by 50% during stimulation with glucose. Islets were also exposed to 16.7 mmol/l glucose in the absence or presence of 10 μmol/l nitrendipine. In the absence and presence of nitrendipine, there were 6.1 ± 0.7 and 6.3 ± 0.6 granules per 10 μm of membrane, respectively. Thus, glucose increased granule docking independently of increased [Ca^{2+}]i and exocytosis. The data suggest a limit to the number of docking sites. As the rate of docking exceeded the rate of exocytosis, docking is not rate limiting for insulin release. Only with extremely high release rates, glucose stimulation after a 4-h incubation with a high concentration of fatty acid–free BSA, was the docked granule pool reduced in size. *Diabetes* 53:3179–3183, 2004

In glucose-stimulated biphasic insulin secretion, the first phase is due to the ATP-sensitive K^+ channel–dependent pathway of glucose signaling, depolarization of the cell, increased Ca^{2+} influx, and exocytosis of an "immediately releasable" pool of docked granules (1–4). After the first phase, in rat and human, the second phase is characterized by an increasing rate of secretion to a plateau. It has been suggested that the second phase is due to time-dependent potentiating signals (5–7) generated by the ATP-sensitive K^+ channel–independent or –amplifying pathway (7). The mechanisms underlying this pathway and the second phase of release are unknown (1,7–9), although it has been suggested that its action is at the level of the docked granules to increase the rate at which they enter the immediately releasable pool or state (7). Second-phase secretion over a lengthy period requires that the β-cell replace the released docked granules. For this to happen, a flow of granules from the large reserve pool to the plasma membrane followed by docking and subsequent priming and preparation for release has to occur. Little is known about this movement or about the dynamics of the docked granules. The aim of this research was to determine whether the increased rate of insulin secretion during the second phase was associated with any change in the number of granules docked at the β-cell plasma membrane.

From the from the Department of Molecular Medicine, College of Veterinary Medicine, Cornell University Ithaca, New York.

Address correspondence and reprint requests to Dr. Geoffrey W.G. Sharp, Department of Molecular Medicine, College of Veterinary Medicine, Cornell University Ithaca, NY 14853-6401. E-mail: gws2@cornell.edu.

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RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats weighing 250–350 g were used for the study and fed ad libitum. They were killed by CO_2 asphyxiation and the pancreata removed. Pancreatic islets were isolated by collagenase digestion (10), as previously described using a Krebs-Ringer bicarbonate HEPES buffer of the following composition (in mmol/l): 129 NaCl, 5 $NaHCO_3$, 4.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, and 10 HEPES at pH 7.4 (9). The buffer was supplemented with 0.1% BSA and 2.8 mmol/l glucose.

Insulin secretion. Two series of experiments were performed. In the experiment shown in Fig. 2, isolated islets were equilibrated in RPMI medium containing 5.6 mmol/l glucose in the presence of 0.068% fatty acid–free BSA for 4 h before perifusion. Then, after loading the islets into 70-μl chambers they were perifused at a flow rate of 1 ml/min with Krebs-Ringer bicarbonate HEPES buffer containing 0.068% fatty acid–free BSA. After 20 min at 2.8 mmol/l glucose, sample collection was started (still at 2.8 mmol/l glucose). The glucose concentration was changed to 16.7 mmol/l after an additional 10 min and the perifusion continued for a further 40 min.

In the experiments shown in Fig. 3, isolated islets were equilibrated in RPMI medium containing 5.6 mmol/l glucose in the presence of either 0.068 or 0.68% fatty acid–free BSA for 4 h before perifusion. BSA of 0.68% (100 μmol/l BSA) is a concentration that has been used frequently to buffer fatty acids to known "free" fatty acid concentrations. This is a high concentration relative to the BSA concentration used in routine islet studies but appears to have no adverse effects. Therefore, it was used to trap fatty acids exiting the islets. We arbitrarily used one-tenth (0.068%) as the low BSA control. This is close to the 0.1% BSA that we use normally.

The perifusions were performed as described for the first series of experiments, except that the islets were exposed to 16.7 mmol/l glucose in the absence and presence of 10 μmol/l nitrendipine. At the end of the experiments, samples were radioimmunoassayed for insulin using a charcoal separation technique (11).

Electron microscopy. Two series of experiments were performed. In the first series of experiments, islets were equilibrated in RPMI (with 5.6 mmol/l glucose and 0.068% fatty acid–free BSA) for 4 h and then either fixed under control conditions or after stimulation with 16.7 mmol/l glucose for 40 min. Immediately before fixing, the islets were washed twice with PBS. In the second series of experiments, islets were equilibrated in RPMI (5.6 mmol/l glucose and either 0.068 or 0.68% fatty acid–free BSA) for 4 h and then treated similarly to the islets used for the perifusion experiments in Fig. 3. They were transferred to a Krebs-Ringer bicarbonate HEPES buffer containing 2.8 mmol/l glucose for 30 min and then exposed to 16.7 mmol/l glucose in the absence or presence of 10 μmol/l nitrendipine for 40 min. The islets were fixed with 2.5% glutaraldehyde in sodium cacodylate buffer at pH 7.4 and subsequently treated

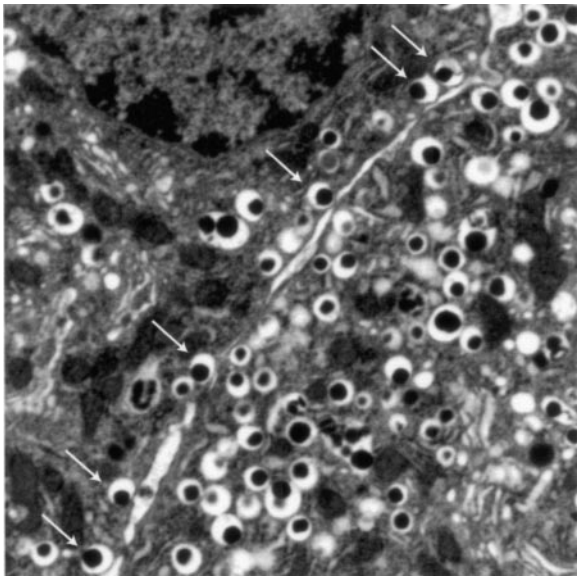

FIG. 1. Docked granules in pancreatic β-cells. The electron micrograph is of two adjacent β-cells. These two were chosen because of the distinct intercellular space that makes the illustration of docked granules easier. Six docked granules are indicated by white arrows in the cell in the left and upper portion of the picture.

with 2% osmium tetroxide. After dehydration, the islets were infiltrated and embedded with epon araldite. Sections that were 70 nm thick were prepared from the islets. The electron microscopists, who were unaware of the experimental conditions, coded the samples before analysis. The β-cells (easily identified by the characteristic appearance of the granules) were selected randomly. Electron micrographs were prepared at a magnification of ×10,000.

Morphometry. Classical quantitative morphometry using stereological techniques was applied (12–14). All measurements and counts were performed without knowledge of the experimental conditions.

To estimate the mean diameter of the β-cell granules, the diameters of 2,664 granules observed in randomly selected sections of β-cells were measured. The diameters were placed in a frequency distribution plot with 40-nm ranges from 1 to 40 nm to 561 to 600 nm, and the mean diameter was calculated from the following equation (14):

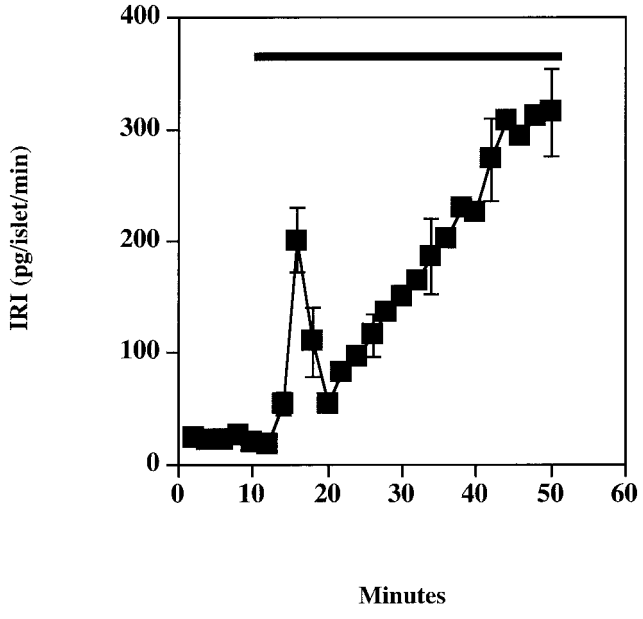

FIG. 2. Insulin secretion under basal (2.8 mmol/l glucose) and stimulated (16.7 mmol/l glucose) conditions. The bar at the top of the figure indicates the time of exposure to 16.7 mmol/l glucose. A characteristic biphasic response to glucose stimulation is shown.

TABLE 1
The percentage of docked granules, the number of docked granules per cell, and the number of docked granules per 10 μm of plasma membrane in the β-cell sections

Conditions	Percentage of docked granules	No. docked per cell	No. docked per 10 μm of plasma membrane
5.6 mmol/l glucose	6.4	712	4.3 ± 0.6
16.7 mmol/l glucose	10.2*	1,060*†	6.4 ± 0.8‡

Morphometric cell and granule measurements under basal conditions. The rat pancreatic β-cell has an average diameter of 13.2 μm and a volume of 1,202 μm^3. It contains 11,136 granules with a mean diameter of 348 nm and a volume of 0.022 μ^3. The two conditions (similar to those in Fig. 2) are paired rat pancreatic islets preincubated for 4 h in RPMI with 5.6 mmol/l glucose and 0.068% fatty acid–free BSA and subsequently exposed for 40 min to either 5.6 mmol/l glucose or 16.7 mmol/l glucose. *$P < 0.05$ vs. 5.6 mmol/l glucose control; †calculated on the percentage of docked granules (10.2%) and the estimated number of granules remaining in the cell after stimulation with 16.7 mmol/l glucose for 40 min; ‡$P < 0.02$ vs. 5.6 mmol/l glucose control.

$$D = \frac{\pi}{2} \times \frac{N}{n_1/d_1 + n_2/d_2 \ldots n_n/d_n} \quad (1)$$

where D = the mean diameter in nm, N = the total number of granules measured, n = the number in a size group, and d = the mean diameter of the size group in nm.

The mean diameter of the β-cell granules was 348 nm, a value that compares well with previous measurements in mouse islets of 289 (15) and 357 nm (3) and 304 nm for rabbit islets (16). Assuming a spherical shape and a diameter of 348 nm, the mean granule volume is 0.022 μ^3.

To determine the average volume of the β-cell and the number of granules contained in it, point-counting and volume-density measurements were utilized. For this, a 3-mm square grid was placed over the electron micrographs, and the number of intersecting points within each of 45 cells was determined. These numbers, representing the areas of the 45 cell sections were placed in a frequency distribution plot in 100-point ranges from 300 to 2,200 points. The mean number of intersecting points was calculated from equation 2:

$$P = \frac{\pi}{2} \times \frac{N}{n_1/p_1 + n_2/p_2 \ldots n_n/p_n} \quad (2)$$

where P = the mean number of intersecting points, N = the total number of cells counted, n = the number of cells in a particular range, and p = the mean number of a particular range.

The average β-cell volume was then calculated from the mean number of intersecting points, each of which embraces an area of 9 mm^2, the magnification of the electron micrographs, and the assumption that the β-cell is spherical.

The mean β-cell volume was 1,202 μ^3 compared with 1,260 μ^3 found in rabbit (16) and 1,434 μ^3 in mouse (15).

To determine the average number of granules in the β-cell, point counting of 11 cells and of the granules within the cells was performed. The total number of intersecting points in the cells was 11,611. The total number of points intersecting with the granules was 2,374. Therefore, the granules occupied 20.4% of the cell volume so that with a cell volume of 1,202 μ^3, the granules occupy 245 μ^3. As the volume of a single granule is 0.022 μ^3, the number of granules per β-cell is 11,136. This value lies between previous estimates for the mouse β-cell of 13,000 (14) and 9,000 (15) and 9,200 for the rabbit (16).

To determine the number and percentage of granules docked at the plasma membrane, all the granules in each section and that were in contact with the plasma membrane were counted. We took a conservative view in that granules were considered docked only if they were touching the membrane. Those that were close to the membrane and might have been in contact above or below the section under examination were not counted. While this slightly underestimates the number of docked granules, the underestimate should be constant for each experimental situation. Examples of docked granules are shown in Fig. 1.

An additional method used to compare the number of docked granules under different experimental conditions was as follows. The length of the

TABLE 2
The number of docked granules per 10 μm of plasma membrane in the β-cell sections

Conditions	Treatment	No. docked per 10 μm of plasma membrane
0.068% BSA	16.7 mmol/l glucose	6.1 ± 0.7
0.068% BSA	16.7 mmol/l glucose + 10 μmol/l nitrendipine	6.3 ± 0.6
0.68% BSA	16.7 mmol/l glucose	1.9 ± 0.2*
0.68% BSA	16.7 mmol/l glucose + 10 μmol/l nitrendipine	6.5 ± 0.5

Data are means ± SE. The four conditions (similar to those in Fig. 3) are paired rat pancreatic islets preincubated for 4 h in RPMI with 5.6 mmol/l glucose and either 0.068% or 0.68% fatty acid–free BSA and subsequently exposed for 40 min to either 16.7 mmol/l glucose or 16.7 mmol/l glucose with 10 μmol/l nitrendipine. *$P < 0.001$ vs. 16.7 mmol/l glucose control (0.068% BSA) and vs. 16.7 mmol/l glucose with 10 μmol/l nitrendipine (0.68% BSA).

perimeter of each cell section was measured with a cartographer's micrometer wheel. The docked granules at the perimeters were identified, counted, and expressed as the number of granules per 10 μm of membrane. For all studies at least 10 cells were analyzed for each condition.

RESULTS

Using quantitative morphometric techniques, it was determined that the average β-cell has a diameter of 13.2 μm and a volume of 1,202 μm^3. It contains 11,136 granules, with a mean diameter of 348 nm.

Shown in Fig. 2 is a typical biphasic insulin secretion response of rat islets to stimulation by glucose. Following the equilibration period in the presence of 2.8 mmol/l glucose, the islets were challenged with 16.7 mmol/l glucose at 10 min. After a short delay in which glucose metabolism in the cell is accelerated, there follows the first phase a nadir at the 20-min point and the rising second phase to a plateau at maximum release rates.

Under nonstimulated conditions, after equilibration of the islets in RPMI containing 5.6 mmol/l glucose, 6.4% of the granules in the β-cell (~700) were docked at the plasma membrane. Using another method of measurement and expression of the number of docked granules, it was found that there were 4.3 ± 0.6 docked granules per 10 μm of plasma membrane at the perimeter of the cells. Under stimulated conditions, after 40 min in the presence of 16.7 mmol/l glucose, 10.2% of the granules were docked ($P < 0.05$ vs. controls). Even allowing for the granules released over the 40-min stimulation, this indicates a 50% increase in the size of the docked granule pool. This glucose-induced increase in the number of docked granules was also documented as 6.4 ± 0.8 granules per 10 μm of plasma membrane vs. 4.3 ± 0.6 docked granules per 10 μm under nonstimulated conditions ($P < 0.02$). As the size of the docked granule pool increased at a time when the rate of granule release was stimulated, the rate of granule docking exceeded the rate of release.

In a separate series of experiments, the number of docked granules after exposure of islets to 16.7 mmol/l glucose for 40 min was 6.1 ± 0.7 per 10 μm of membrane (Table 2). Under the same conditions but in the presence of 10 μmol/l nitrendipine to block Ca^{2+} influx and to prevent any stimulation of insulin secretion, the number of docked granules was 6.3 ± 0.6 per 10 μm of plasma membrane, which is slightly but not significantly increased.

Treatment of islets for 4 h with 0.68% fatty acid–free BSA is known to cause a massive enhancement of glucose-stimulated insulin release (17). This high concentration of BSA was chosen to maximize the trapping of fatty acids exiting the islets. As shown by the results in Fig. 3, after a 40-min exposure to 16.7 mmol/l glucose, the rate of release from islets exposed to 0.68% fatty acid–free BSA was over 1,000 pg · $islet^{-1}$ · min^{-1}. This rate was five times greater than that of the paired islets treated with 0.068% fatty

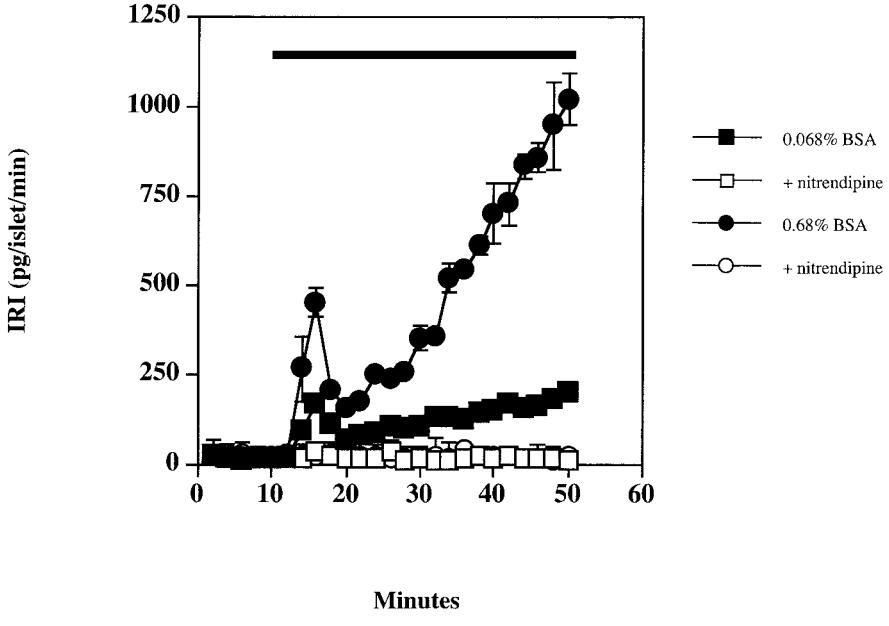

FIG. 3. Insulin secretion by islets exposed to 2.8 and 16.7 mmol/l glucose after treatment of the islets for 4 h with either 0.068 or 0.68% fatty acid–free BSA. The bar at the top of the figure indicates the time during which all the islets were exposed to 16.7 mmol/l glucose in the absence or presence of 10 μmol/l nitrendipine. In control islets treated with 0.068% BSA, the characteristic biphasic response to glucose stimulation is shown (note the change of scale on the ordinate compared with Fig. 2). In islets treated with 0.68% BSA, a massive enhancement of the biphasic response to glucose stimulation is seen. The stimulation of insulin release under both conditions was completely blocked by 10 μmol/l nitrendipine. Under control conditions (0.068% BSA), the number of docked granules in β-cells exposed to 16.7 mmol/l glucose for 40 min was 6.1 ± 0.7 per 10 μm of membrane. In the presence of nitrendipine, the number of docked granules was not significantly different at 6.3 ± 0.6 per 10 μm of plasma membrane. Under the test conditions (0.68% BSA), the number of docked granules in β-cells exposed to 16.7 mmol/l glucose for 40 min decreased to 1.9 ± 0.2 per 10 μm of membrane. In the presence of nitrendipine, the number of docked granules was 6.5 ± 0.5 per 10 μm of plasma membrane.

acid–free BSA for 4 h. Under these conditions, the number of docked granules present at the start of the stimulation would be sufficient to supply only the first 15 min of the greatly stimulated insulin release (data not shown). Despite this high rate of secretion and the need for granule translocation and docking to resupply the docked pools for exocytosis, the biphasicity was present and the timing of the onset, the peak of the first phase, the nadir, and the rising second phase was similar to that of the control islets. The docked granule pool of 1.9 granules per 10 μm of plasma membrane (see below) would be sufficient to maintain exocytosis for only 5 min or so at the very high rate of second-phase secretion under these conditions. Also shown in Fig. 3 is the complete abolition of stimulated insulin release by 10 μmol/l nitrendipine.

Under these conditions (a 4-h exposure to 0.68% fatty acid–free BSA), stimulation with 16.7 mmol/l glucose for 40 min decreased the size of the docked granule pool (1.9 ± 0.2 docked granules per 10 μm of membrane compared with 6.1 ± 0.7 docked granules per 10 μm of membrane for the controls, $P < 0.001$). Therefore, the rate of docking failed to keep up with the extraordinarily high rate of exocytosis under these conditions. However, under the same conditions but in the presence of 10 μmol/l nitrendipine (which blocked stimulated insulin release through inhibition of voltage-dependent L-type Ca^{2+} channels), the docked granule pool was increased to the same level as in control islets also exposed to 16.7 mmol/l glucose in the presence of nitrendipine (6.5 ± 0.5 docked granules per 10 μm of membrane compared with 6.3 ± 0.6 granules per 10 μm of membrane in the controls). The similarity in the size of the docked granule pool under different conditions of glucose challenge and different rates of insulin release suggests that the number of available docking sites is limited. Importantly, the increase in the number of docked granules induced by 16.7 mmol/l glucose in the presence of nitrendipine points to the fact that glucose stimulation of granule docking occurs independently of a rise in $[Ca^{2+}]_i$ and of stimulated exocytosis. The morphometric data are summarized in Tables 1 and 2.

DISCUSSION

Previous data estimated the number of insulin-containing granules in the mouse β-cell at 13,000 (15) and 9,000 (3) and the number of docked granules as 650 or ~7% of the total (3). Our estimates for the rat β-cell are similar in that there are ~11,000 granules, of which some 700 are docked. Within the pool of docked granules is a small subpool prepared for immediate release during the first phase in response to glucose. This pool has been estimated to consist of ~50–100 granules (2,4,18). Thus, the pool of docked granules is heterogeneous. In accord with the functional aspects of insulin secretion from β-cells and with other cell types that have similar heterogeneous docked pools and biphasic release patterns (19,20), the docked granules can be defined as present in either an immediately or readily releasable pool (1,2,4,7,18). Our results show that the percentage of morphologically docked granules in the rat β-cell under nonstimulated conditions is 6.4% of the total number of granules in the cell. More importantly, the percentage of docked granules increased to 10.2% during 40 min of stimulation with glucose. It is clear, therefore, that during glucose-stimulated insulin release, the rate of granule docking exceeds the rate of release. As there is a surfeit of docked granules at all times throughout the stimulation, granule translocation and docking at the plasma membrane appears not to be rate limiting for glucose-stimulated insulin release. This implies that the rate-limiting step that controls the second phase of insulin release must be the rate at which the docked granules are prepared for release (7).

In the experiments in which the islets were stimulated with glucose in the presence of nitrendipine so that the release of docked granules was blocked, no significant increase in the size of the docked pool occurred relative to the situation where release was taking place. This strongly suggests that there is a limit to the number of granules that can be docked at the membrane. Glucose can move and dock granules at the membrane at a rate that exceeds the normal rate of exocytosis, but in the absence of release, docking does not exceed a certain ceiling. Despite this, the pool may be dynamic and increase or decrease under other conditions that we have not yet tested. It also points to the fact that the stimulation of granule flow and docking at the membrane does not require exocytosis to take place nor does it require an increase in $[Ca^{2+}]_i$. This is in accord with the report that glucose-stimulated granule movement is independent of an increase in $[Ca^{2+}]_i$ (21).

Our data demonstrate that during glucose-stimulated insulin release, the rates of granule flow to the membrane and docking exceed the rate of exocytosis. Thus, during the second phase of glucose-stimulated insulin release, the granules that are available for exocytosis are those that were docked before and those that docked during the stimulation. Furthermore, as ATP-dependent priming is so rapid (3), granules could be available for release within 400 ms after docking. This implies that granules from the reserve pool could dock at the plasma membrane early in the second phase and that they could be released before those that were already docked, i.e., the granules are not necessarily released in the order that they are docked. This would be in accord with early findings that glucose "marks" β-cells (22) such that newly synthesized insulin is preferentially released (22–24) and recent evidence for the release of recently docked granules. The latter came from studies (25) using evanescent wave microscopy in MIN6 cells. Stimulation of the cells with 22 mmol/l glucose released insulin from previously docked granules for the first 2 min of stimulation. Over the next 10 min, however, newly recruited granules that progressively accumulated at the membrane were also released. Furthermore, the progressive accumulation over this period resulted in a 40% increase in the number of docked granules.

One of the many questions to be answered concerning granule dynamics is what determines the releasability of a granule? The equally important converse of this question is why, when there are >700 docked granules in the rat β-cell, are so few of them released per minute under stimulated conditions? What is it that determines whether a granule is releasable or not? Suggestions include a close association with specific exocytotic sites or Ca^{2+} channels and changes in Ca^{2+} sensitivity so that exocytosis occurs at lower $[Ca^{2+}]_i$ (3,4,17,26,27).

With respect to the mechanisms underlying the rising

portion of the second phase of glucose-stimulated insulin release and the subsequent high plateau, it is possible that the increase in the number of docked granules is a contributory factor to the increased rate of secretion. In other words, the more granules that are docked and available for release, the more likely it is that they will be released. Similarly, the increase in docked granules may be associated with the phenomenon of time-dependent potentiation, whereby exposure to a stimulatory glucose concentration results in the enhancement of a subsequent response (28). It will be interesting to determine whether glucose stimulation increases the size of the docked granule pool in the mouse β-cell that does not manifest a rising second phase (29–31) and does not exhibit time-dependent potentiation (32,33).

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