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Insulin Exocytotic Mechanism by Imaging Technique

Mica Ohara-Imaizumi* and Shinya Nagamatsu
Department of Biochemistry, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611

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Insulin is stored in pancreatic β cell granules, and released biphasically by the exocytotic mechanism induced by nutrient glucose. Insulin exocytosis must be critically regulated to finely control body glucose homeostasis because insulin is the only hormone that can promptly reduce the blood glucose level. Recent advanced techniques in molecular biology and electrophysiology revealed the molecular mechanism of insulin release in the process from glucose entry to increased $[Ca^{2+}]_i$. However, the insulin exocytotic process such as translocation, docking and fusion of insulin granules was largely unknown. In order to reveal the molecular mechanism of this process, we utilized a newly innovated imaging technique, TIRF imaging system. Here we review recent results of our studies into docking and fusion of insulin granules analyzed by TIRF system.

Key words: exocytosis, insulin release, pancreatic β cell, TIRF.

Insulin release consists of a multistage process: (1) biosynthesis of preproinsulin in the endoplasmic reticulum, (2) processing from proinsulin to mature insulin in insulin secretory granules, (3) translocation of granules from the cytoplasm to the specific region of the plasma membrane, and (4) exocytosis of insulin *via* the process of granule docking to the final fusion. Although pancreatic β cells have a large number of storage granules (~13,000 per cell) (*1*, *2*), only a fraction of them is released. In quantitatively important route for the release of insulin is regulated by Ca^{2+} dependent exocytosis of the secretory granules (*3*, *4*). Insulin released into the blood promotes glucose uptake by tissues such as fat and muscle at the same time that it inhibits glucose output by the liver, thereby, rising insulin levels depress the concentration of blood glucose. Recently, multiple new techniques have been applied to study insulin secretion, such as capacitance measurements, amperometry, confocal microscopy, and two-photon microscopy (*5–10*). Among those, imaging techniques are powerful tools for studying insulin release and they have provided significant advances in understanding the mechanism of exocytosis (*11–13*). In particular, total internal reflection fluorescence microscopy (TIRFM: also called evanescent wave microscopy), which allows fluorescence excitation with 100 nm from the plasma membrane (*14*, *15*), has permitted us to observe single insulin granules undergoing exocytosis in live β cells, thereby we are able to evaluate the fusion of single insulin granules quantitatively (*16*). This review focuses on the mechanism of biphasic insulin exocytosis analyzed by TIRF imaging system.

Proposed model of glucose-induced insulin release

Glucose-stimulated insulin release from β cells is unique in that the signals that stimulate insulin release are derived from the intracellular metabolism of glucose, rather than being generated by a ligand–receptor interaction (*17*, *18*). The model for the sequence of events in the activation of the secretory responses of β cell is as follows (Fig. 1). Glucose enters the β cell through the high-capacity glucose transporter-type 2 (GLUT-2), which allows for rapid equilibration of glucose across the β cell plasma membrane (*19*). Glucose is then phosphorylated by glucokinase (GK), the first enzyme in the glycolytic pathway in β cells. The generation of ATP from glycolysis increases the intracellular ratio of ATP/ADP (*20*, *21*). ATP binds K_{ATP} channels on the β cell membrane, probably concomitant with phosphorylation of K_{ATP} channel, closes the channels, resulting in depolarization of the β cell (*22*, *23*). When the β cell membrane potential reaches –30 to –40 mV, the voltage-sensitive calcium channels (Ca^{2+}-channels) open, leading to an influx of calcium ions (*24*). The increase of intracellular calcium ion triggers the exocytosis of insulin granules. In addition, increased $[Ca^{2+}]_i$ then activates (opens) voltage-sensitive potassium channels, thereby restoring the resting potential to –70 to QJ;–60 mV (see review *25*). Thus, although the detailed studies concerning the steps prior to $[Ca^{2+}]_i$ elevation, few studies of exocytotic process exist.

Our laboratory has focused on the process of insulin exocytosis after $[Ca^{2+}]_i$ elevation. There are many proteins involved in docking and fusion of synaptic vesicle with the plasma membrane in neuronal cells (*26*, *27*), and those proteins are found to play a critical role in insulin exocytosis as well (*28–30*). We tried to use confocal microscopy and TIRF imaging techniques in order to detect the dynamic motion of insulin granules from docking to fusion.

Visualization of insulin exocytosis by confocal laser microscopy

At first, we tried to visualize the process of insulin exocytosis using confocal laser-scanning microscopy. In order to specifically label the insulin secretory granules, we generated an expression construct in which GFP is

*To whom correspondence should be addressed. Tel: +81-422-47-5511(Ext. 3476), Fax: +81-422-47-5538, E-mail: mimaizu@kyorin-u.ac.jp

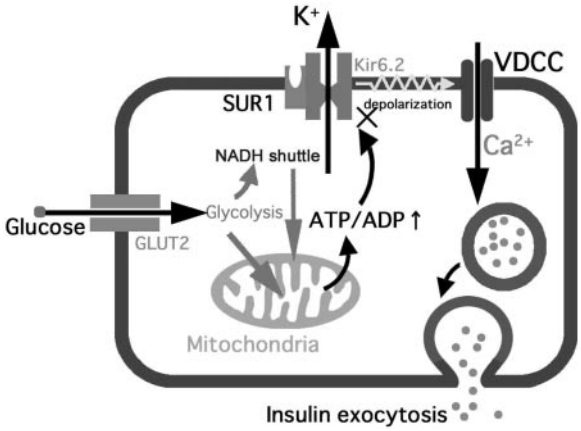

Fig. 1. **Model of glucose-induced insulin release.** Glucose metabolism increases the ATP/ADP ratio, which closes K_{ATP} channels, resulting in membrane depolarization, influx of Ca^{2+}, thereby an increase in $[Ca^{2+}]_i$ that triggers insulin exocytosis.

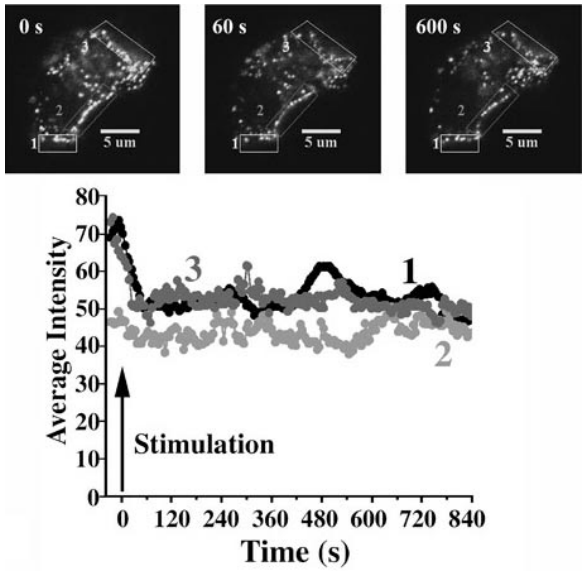

Fig. 2. **Confocal images of 22 mM glucose-induced exocytosis of insulin-GFP.** MIN6 cells expressing insulin-GFP were stimulated by 22 mM glucose. Fluorescent images were obtained by confocal laser microscopy, and then changes of the average fluorescent intensities near the peripheral region of cells (1, 2 and 3) were plotted with time. Stimulation started at 0 s, and images were acquired every 5 s.

located at the C terminus of preproinsulin (insulin-GFP). MIN6 β cells harboring insulin-GFP showed the punctuate pattern of fluorescence of GFP. Imnunohistochemistry with anti-IAPP antibody showed that the intense punctuate fluorescence of GFP was localized to IAPP containing granules, indicating that the insulin-GFP fusion protein was correctly delivered to the insulin secretory granules (*16*). In order to examine the motion of insulin granule by glucose-stimulation, MIN6 cells harboring insulin-GFP were stimulated by high-glucose, then the movement of insulin granules labeled with GFP was monitored by confocal laser microscopy (Fig. 2). Although the confocal microscopy could detect the random movement of single granule on the peripheral region of MIN6 cells, it was difficult to define the direction of granule movement and whether they are fused or not, because of the limitation of the resolution of confocal microscopy. Therefore, in order to more finely detect the granule movement near the plasma membrane, we decided to use TIRF imaging technique.

TIRF

TIRF microscopy is a technique that specifically illuminates fluorophores in a thin optical section above the interface between two media with different diffractive indices (*15*, *31*). To achieve this, laser light is directed obliquely at the interface between two media from a high to a low diffractive index with an incident angle greater than the critical angle of total internal reflection. Under these conditions, laser light is totally internally reflected at the interface. Even so, some of the light penetrates the medium of lower diffractive index as an electromagnetic field called the "evanescent wave." The evanescent wave vanishes exponentially with distances. Evanescent field penetration depths (the distance from the interface) is usually less than 100 nm, resulting in images with very low background fluorescence, virtually no out-of-focus fluorescence, and minimal exposure of cells to light at any other planes in the sample. This offers a great advantage over the optical resolution achievable with confocal microscopy (≈500 nm). The cells are grown on the glass coverslip, imaging of fluorophores immediately below the plasma membrane can be achieved in live cells. In pioneering work by Daniel Axelrod, this technique was first applied to the study of live cells, primarily to investigate cell-surface adhesions (*14*), then several studies of protein dynamics, topography of cell-substrate contacts, endocytosis or exocytosis using this technique were reported (*32*–*37*).

Application of TIRF to pancreatic β cells

Because Insulin secretory granules (diameter ≅ 300 nm) approach the plasma membrane and dock to the plasma membrane with subsequent fusion, TIRF technique appeared attractive for step-wise analysis of insulin exocytosis from β cells. TIRF observation of insulin granule motion in β cells was first reported by Rutter's group in 2000 (*38*). In previous studies, granules were labeled with the weak base fluorescent dye, such as acridine orange, which accumulates in acidic compartments in cells, although acridine orange labels not only secretory granules but also other acidic compartments in cells. In order to overcome this problem and directly label the insulin secretory granules, we generated a construct of expression vector in which GFP is located at the C-terminus of preproinsulin (insulin-GFP) as mentioned above. We later produced the recombinant adenovirus using our insulin-GFP construct in order to label the insulin secretory granules in primary pancreatic β cells prepared from rat pancreas. To acquire the real-time images of insulin granules with high-sensitive resolution during glucose-stimulation, TIRFM was equipped with a cooled change-coupled device (CCD) camera, operated with metamorph software. Using this TIRF system, we were able to acquire the images every 50 ms and track the individual granules in rat primary β cells (*39*).

Biphasic insulin exocytosis analyzed by TIRF system

Pancreatic β cells release insulin by glucose-stimulation with a biphasic pattern which consists of a rapidly initiated and transient first phase followed by a sustained second phase. More than 30 years ago, Grodsky proposed an insulin storage-limited mathematical model, so called "two compartmental model" (*40–42*). Recently, Rorsman's group performed elegant studies using combination of capacitance measurements and electronmicroscopy (*43–46*), and proposed a model for biphasic insulin release (*45, 46*). This model divides secretory vesicles into two distinct functional pools. One pool, the readily releasable pool (RRP) where granules gather near the plasma membrane, is release-competent and can undergo exocytosis. A second reserve pool of granules exists in the intracellular space to recruit granules to RRP for replenishment. RRP is estimated to contain 20 to 100 granules (0.2–1.0%) and a substantial part of first-phase insulin release could be attributable to exocytosis of RRP-granules (the 1st phase). Once this pool of granules has been emptied, exocytosis proceeds at a lower rate, reflecting the low rate at which new granules are supplied for release by priming of reserve granules (the 2nd phase). Although this seems to be a perfect model that is completely in agreement with two compartment model, their models are based on indirect evidences such as electrophysiology, capacitance measurement, *etc.*, but not direct observation of real-time insulin granule motion. Because of technical limitation, it was impossible to monitor the dynamic motion of insulin granules in living β cells. However, new technology of TIRF imaging system allowed us to directly observe the dynamic motion of single insulin granules undergoing insulin exocytosis. We took advantage of using the TIRF system, where we analyzed docking and fusion of insulin granules with the plasma membrane during biphasic insulin release using living pancreatic β cells.

We infected rat primary pancreatic β cells with recombinant adenovirus encoding insulin-GFP in order to label the insulin secretory granules, and then examined the dynamic motion of granules near the plasma membrane (*39*). Figure 3A shows the TIRF image of single β cells where fluorescence spots represent the insulin granules docked to the plasma membrane. The cells were stimulated by 22 mM glucose, then the sequential images of single granules were acquired every 50 ms (Fig. 3A). The data revealed the marked difference in exocytotic pathways between the 1st phase and the 2nd phase. As shown in Fig. 3B, during the 1st phase, within the first 4 min after the addition of 22 mM glucose, the fusing granules originated mostly from previously docked granules that were visible before glucose stimulation. The fluorescent spot suddenly brightened and vanished within 300 ms. On the other hand, during the 2nd phase, the fusing granules arose from "newcomers," which had been absent or only dimly visible before stimulation. It should be noted that the newcomer is found immediately after it reaches the plasma membrane. We plotted the number of fusion events *vs.* time after stimulation. The histogram clearly shows that during the 1st phase, fusion occurs mostly from previously docked granules, in contrast, in the 2nd phase, the fusing granules originate mostly from newcomers (Fig. 3B). Thus, direct imaging of granule motion in primary β cells by our TIRF system confirmed that Rorsman's hypothesis (*45, 46*) is basically correct; however, concerning the 2nd phase some correction is required. During the 2nd phase, newcomers directly fuse with the plasma membrane without approaching the RRP. Although depleted RRP is refilled with new granules coming from the inner pool, they do not fuse. Thus, it is likely

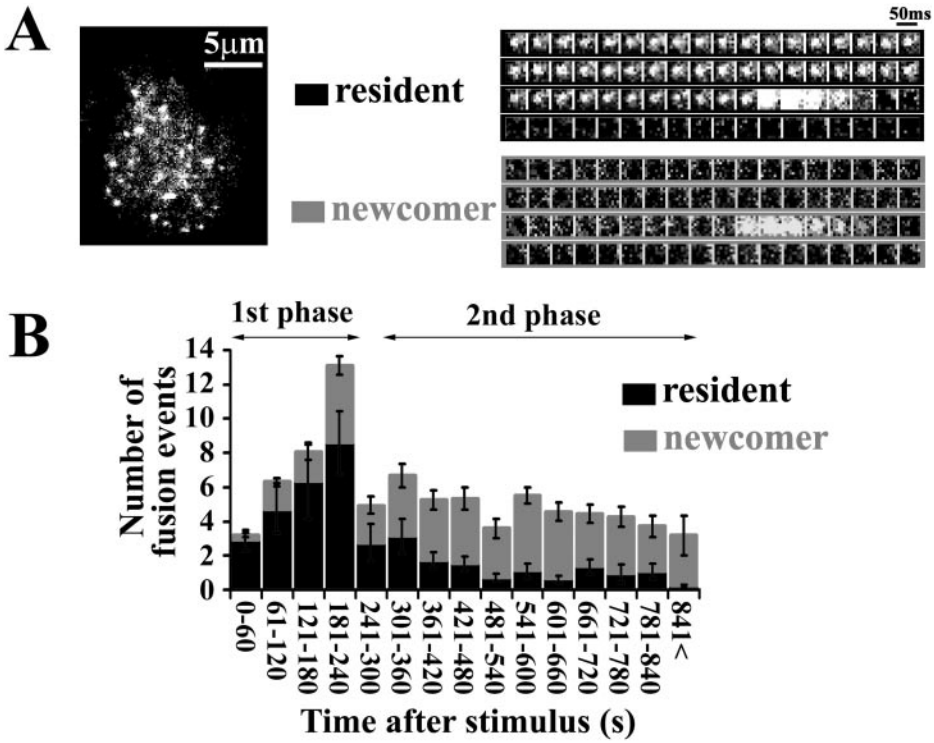

Fig. 3. **TIRF images and analysis of single GFP-labeled insulin granule motion in rat primary β cells during glucose-stimulation (*39*).** (A) Left panel; The real-time motion of GFP-labeled insulin granules was imaged. This is TIRF image of single cell harboring insulin-GFP, showing that many insulin granules docked to the plasma membrane. Right panel; Sequential images (1 μm x 1 μm per 50 ms intervals) of a granule docking and fusing with the plasma membrane were shown during 22 mM glucose-stimulation. (B) Histogram showing the number of fusion events at 60-s intervals after stimulation. The black column shows the fusion from residents (previously docked granules) and the grey column shows that from newcomers. Reproduced with permission, from Mica Ohara-Imaizumi, Chiyono Nishiwaki, Toshiteru Kikuta, Shintaro Nagai, Yoko Nakamichi and Shinya Nagamatsu (2004) *Biochem. J.* **381**, 13–18.

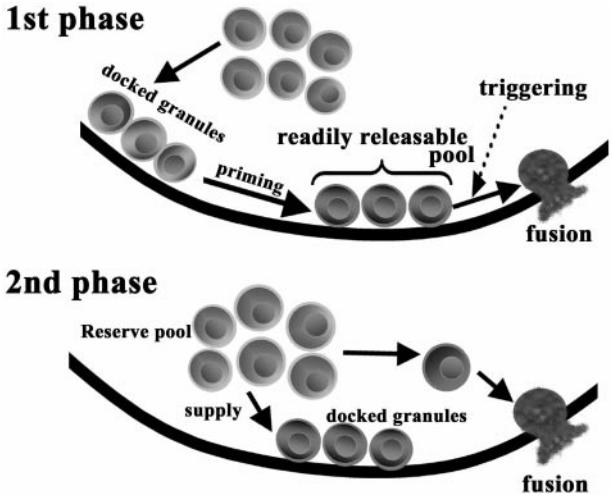

Fig. 4. **Proposed model for biphasic insulin exocytotic mechanism (*39*).** Some of the previously docked granules (residents) are primed and form the RRP. During the first phase, the rise in $[Ca^{2+}]_i$ evokes the fusion events from granules in such a pool. During the second phase, the granules, the so called 'newcomers' 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 supplied from and refilled with granules from the reserve pool. Reproduced with permission, from Mica Ohara-Imaizumi, Chiyono Nishiwaki, Toshiteru Kikuta, Shintaro Nagai, Yoko Nakamichi and Shinya Nagamatsu (2004) *Biochem. J.* **381**, 13–18. © The Biochemical Society.

that there is mechanistic difference between the 1st and the 2nd phases of insulin release. We propose the model for biphasic insulin exocytotic mechanism as shown in Fig. 4.

Developing the new technology to analyze the translocation of insulin granules

Present model of our TIRF system can analyze the docking and fusion of insulin granules near the plasma membrane, however, it is impossible to observe the translocation process of insulin granules from the inside pool to the plasma membrane. In order to overcome this problem, we are currently developing the variable TIRFM (v-TIRFM) collaborating with S. Terakawa (Hamamatsu photo medical center). A significant advantage of v-TIRF is that it is able to visualize the intracellular events more than 100 nm apart from the plasma membrane. V-TIRF modulates the penetration depth of the evanescent wave. Because the penetration depth depends on the relative refractive index and incident angle, TIRF with a variable angle of incident light makes it possible to acquire the information along the *z*-axis. Although the basic methods were already reported as variable-angle TIRFM (*47–49*), we modified and established a simpler method of variable-angle TIRFM by combining the ultra high NA objective with a galvanomirror. Although data are not shown here, movement of single insulin granule from the inside pool, located about 500 nm from the plasma membrane, to the plasma membrane was observed, which eventually fused with the plasma membrane (observed as flashing). Thus, v-TIRF is an useful method to analyze the movement of insulin granules in the cytoplasm, and thereby we might be able to explore the mechanism of 2nd phase insulin release that is mostly consisted of newcomer fusion.

At present, the molecular mechanism concerning the 2nd phase insulin release is far more obscure than that of the 1st phase. There is no doubt that the L-type Ca^{2+} channel (high voltage activated channel; Cav 1.2/1.3) triggers the fusion of previously docked granules during the 1st phase, however, what triggers the fusion of newcomers during 2nd phase? Even for fusion of newcomers during the 2nd phase, the increased $[Ca^{2+}]_i$ must be essential, however the pattern of increased $[Ca^{2+}]_i$ during the 2nd phase may be different from that in the 1st phase. Recently, Jing et. al. reported that the 2nd phase release was suppressed in R-type Cav 2.3 channel null mice whereas the 1st phase release was unaffected (*50*), indicating that Cav 2.3 channel plays an important role in intracellular translocation of insulin granules.

Thus, analysis of intracellular insulin granules movement by v-TIRF is becoming more important to shed light on the molecular mechanisms of the 2nd phase insulin release.

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