Introduction

Glucose-stimulated insulin release displays a biphasic pattern in both in vitro and in vivo systems (Curry et al., 1968; Rorsman et al., 2000). This pattern consists of a rapidly initiated and transient first phase preceding a sustained second phase. The ability of glucose to evoke first-phase release is shared by other stimuli (such as high KCl stimulation), resulting in membrane depolarization followed by increased cytosolic Ca\(^{2+}\), whereas only fuel secretagogues are able to initiate second-phase insulin release (Henquin, 2000). Electrophysiological experiments in single β cells have shown that first-phase release reflects Ca\(^{2+}\)-dependent exocytosis of primed granules in a readily releasable pool of granules, whereas second-phase release involves an ATP-dependent release of granules that may be located further from the release site in a reserve pool (Rorsman et al., 2000; Rorsman and Renstrom, 2003). These results suggest that the two phases of release subject insulin granules to nonsynonymous regulatory mechanisms.

Fundamental components of secretory machinery, such as SNARE, required for the docking and fusion of vesicles in neuronal cells (Südhof, 2004), are expressed in pancreatic β cells and play an important role in insulin exocytosis (Nagamatsu et al., 1996; Wheeler et al., 1996; Nagamatsu et al., 1999). Although the function of SNAREs in docking and fusion during exocytosis is already established (Jahn et al., 2003; Südhof, 2004), the distinct role of SNAREs in the individual phases of insulin release remains unclear.

Interestingly, the expression of t-SNARE, syntaxin 1A/HPC-1 (Synt1A; Bennett et al., 1992; Inoue et al., 1992), and its cognate SNARE partners, synaptosome-associated protein of 25 kD (SNAP-25) and vesicle-associated membrane protein 2 (VAMP2), reportedly decreased in islets of the Goto-Kakizaki rat, an animal model for human type 2 diabetes (Nagamatsu et al., 1999; Gaisano et al., 2002; Zhang et al., 2002), and in type 2 diabetic patients (Ostenson et al., 2006). Because type 2 diabetes is associated with disturbances in the release pattern manifested as the selective loss of first-phase release (Ward et al., 1984; O’Rahilly et al., 1986; Cerasi, 1994), SNAREs may have...
a specialized role in phasic insulin exocytosis. In the present study, we used Synt1A−/− mice and total internal reflection fluorescence (TIRF) imaging to investigate a potential role for Synt1A in first-phase insulin release. Synt1A−/− pancreatic β cells displayed no fusion from previously docked granules in first-phase release, whereas fusion from newcomers, which are responsible for second-phase release, was still preserved. Thus, we propose a new model for biphasic insulin release wherein docking and fusion of insulin granules is Synt1A dependent during the first phase but Synt1A independent during the second phase.

**Results**

**Interaction between insulin granules and Synt1A during biphasic insulin release**

We initially analyzed the dynamic interaction between insulin granules and Synt1A in control mouse β cells using dual-color TIRF microscopy (TIRFM). Expression of GFP-tagged insulin allowed insulin granule observation, and Synt1A was detected by a TAT-conjugated Cy3-tagged mAb. Here, we chose not to use a conventional overexpression approach, such as Synt1A tagged with GFP or RFP, because overexpression of syntaxin disturbs the function of endogenous syntaxin molecules (Nagamatsu et al., 1996). Therefore, to analyze the interaction between insulin granules and Synt1A clusters during biphasic insulin release, we labeled the endogenous Synt1A clusters with TAT antibody. As previously reported (Ohara-Imaizumi et al., 2004a), TAT-conjugated Cy3-labeled anti-Synt1A antibody was rapidly transduced into living β cells (unpublished data). We ensured that TAT-conjugated Cy3-labeled anti-Synt1A antibody specifically labeled endogenous Synt1A clusters in the plasma membrane. Cells treated with TAT-conjugated Cy3 anti-Synt1A mAb for 50 min were fixed and immunostained with anti-Synt1A pAb. As shown in Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200608132/DC1), there was overlapping of Synt1A clusters labeled with TAT-conjugated Cy3 anti-Synt1A mAb (red) and those stained with anti-Synt1A pAb (green). In addition, it should be noted that most endogenous Synt1A was labeled with TAT antibody.

Pancreatic β cells that expressed insulin-GFP (Fig. S1, green) and were treated with TAT-conjugated Cy3 anti-Synt1A antibody (red) were stimulated by 22 mM glucose. Dual-color TIRF images were obtained every 300 ms (Fig. 1 A). Approximately 75% of insulin granule fusion during the first phase (<4 min after stimulation) involved previously docked rather

![Figure 1. Dual-color TIRFM of dynamic interaction between docking and fusing GFP-tagged insulin granules and Cy3-labeled Synt1A clusters in glucose-induced insulin release in control mouse β cells.](image-url)
than newcomer granules (Fig. 1B). We observed that most fusion events involving previously docked granules occurred at the site of Synt1A clusters (Fig. 1, B and C), whereas fusion from newcomers occurred at sites distinct from the Synt1A clusters. There was no significant difference in the number of fusion events between control (see Fig. 3 B) and TAT-conjugated Cy3 anti-Synt1A mAb-treated β cells (Fig. 1 A): the total number of fusion events from previously docked granules in wild-type (WT) versus TAT-treated cells was 18.2 ± 1.8 versus 14.9 ± 3.1 in 0–4 min (P = NS; n > 5 cells), suggesting that the introduction of TAT-conjugated Cy3 anti-Synt1A mAb into β cells does not affect insulin exocytosis. These results suggest that first-phase release heavily involves a Synt1A-based SNARE complex, whereas second-phase release is chiefly independent of a Synt1A-based SNARE complex.

Morphometric analysis of insulin granules in Synt1A−/− mice

If Synt1A is essential for docking and fusing insulin granules specifically during the first phase, the deletion of Synt1A may cause reduction in first-phase but not second-phase insulin release. To examine this hypothesis, we used β cells from Synt1A−/− mice (Fujiiwar et al., 2006) as a context for analyzing docking and fusion of insulin granules by TIRFM. We first investigated Synt1A protein levels in Synt1A−/− versus WT mouse pancreatic islets. Fig. S2 (available at http://www.jcb.org/cgi/content/full/jcb.200608132/DC1) shows the lack of Synt1A protein expression in Synt1A−/− islets. Expression of Synt1B was not observed in either Synt1A−/− or WT islets, in accord with the report that Synt1B is expressed at very low levels in control β cells (Nagamatsu et al., 1996), although the brain abundantly expresses Synt1B (Bennett et al., 1992). We found no difference between WT and Synt1A−/− islets in expression levels of other plasma membrane proteins, such as Synct3, Synct4, the other SNAREs, and related proteins SNAP-25, VAMP2, and Munc18. We then examined the pancreatic islets morphologically (Fig. S3). We found that paraffin-embedded pancreatic tissue sections showed insulin immunofluorescence patterns typical for β cells with no notable difference between the Synt1A−/− and WT islets (Fig. S3, A and B). EM of pancreatic β cells also revealed that cell size, total number of granules per section, and mean granule diameter were similar between WT and Synt1A−/− β cells (Fig. S3, C–F). Thus, Synt1A−/− β cells displayed specific Synt1A protein depletion but were similar to WT cells in these other traits assayed.

Docking status of insulin granules in Synt1A−/− β cells

We examined the docking status of insulin granules in Synt1A−/− β cells using TIRFM with immunostaining for insulin (Fig. 2 A). Because evanescent field illumination reaches a <100-nm-thick layer immediately adjacent to the cover glass under our TIRF conditions, TIRFIM illuminates only the plasma membrane with its associated organelles, such as synaptic vesicles (Zenisek et al., 2000), secretory granules (Parsons et al., 1995), and glucose transporter 4 (GLUT4) vesicles (Lizunov et al., 2005), where a cell adheres tightly to the cover glass. We interpret the individual fluorescent spots shown in the TIRF image in Fig. 2 A to be equivalent to morphologically docked granules (see Materials and methods). We rarely observed morphologically docked granules in Synt1A−/− β cells (number of docked granules: 253.3 ± 10.2 vs. 12.3 ± 2.2 granules per 200 μm2 in WT and Synt1A−/− β cells, respectively; n = 12 cells; P < 0.0001). Plasma membrane staining with a lipophilic dye ensured that the Synt1A−/− β cells adhered tightly to the cover glass (unpublished data).

To confirm the TIRFM data, we used EM to examine insulin granules that were morphologically docked to the plasma membrane. Using EM, granules at their shortest distance of <10 nm from the plasma membrane qualified as morphologically docked granules (Parsons et al., 1995; Fig. 2 B). The number of morphologically docked granules observed by EM was significantly reduced in Synt1A−/− β cells (9.6 ± 1.5 vs. 0.8 ± 0.2 granules per 10 μm of plasma membrane in WT and Synt1A−/− cells, respectively; n = 12 cells; P < 0.0001). Along with the results of the morphometric analysis, these data suggest that Synt1A deficiency specifically impairs the docking of insulin granules to the plasma membrane.

**Figure 2.** Synt1A deficiency impairs docking of insulin granules to the plasma membrane in pancreatic β cells. [A] TIRF of insulin granules morphologically docked to the plasma membrane. (left) Typical TIRF images of docked insulin granules in WT or Synt1A−/− β cells. The surrounding lines represent the outline of cells that attached to the cover glass. Bar, 5 μm. Pancreatic β cells were prepared from WT and Synt1A−/− mice, fixed, and immunostained for insulin. (right) Number of insulin granules docked to the plasma membrane. Individual fluorescent spots shown in TIRF images were manually counted per 200 μm2; n = 15 cells. (B) Electron micrograph of β cell sections. ([top] Typical EM images of the plasma membrane area facing the blood capillary of WT and Synt1A−/− β cells (B). Bar, 500 nm. (bottom) Number of morphologically docked insulin granules per 10 μm of plasma membrane. Granules at their shortest distance of <10 nm from the plasma membrane were qualified as morphologically docked granules (red arrowheads). Results are provided as the mean ± SEM.
Effects of Synt1A deficiency on insulin exocytosis

We explored the effects of Synt1A deficiency on the dynamic motion of single insulin granules. In agreement with what has been reported for rat β cells (Ohara-Imaizumi et al., 2004b), we found that in WT mouse β cells, fusion of insulin granules with the plasma membrane during first-phase release mainly involved previously docked granules (Fig. 3, A and B; and Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200608132/DC1). In contrast, because Synt1A−/− β cells have fewer docked granules, TIRF analysis in these cells showed that the fusion from previously docked granules was severely abolished (Fig. 3, C and D; and Video 4). Despite an appreciable number of fusion events from previously docked granules in WT β cells, there was no fusion from previously docked granules in Synt1A−/− β cells (18.2 ± 1.8 vs.0 in 0–4 min, WT vs. Synt1A−/−; Fig. 3, B and D). However, some fusion from newcomer granules was observed during the first phase even in Synt1A−/− β cells. During second-phase release (>4 min), there was no significant difference in the total number of newcomer fusion events between WT and Synt1A−/− β cells (WT, 43.1 ± 5.0, and Synt1A−/−, 49.8 ± 3.7, during 4–17 min; P = NS; n = 10 cells; Fig. 3, B and D). ELISA data evaluating endogenous insulin release from perfused WT and Synt1A−/− β cells (Fig. 3 E) were compatible with the TIRFM data. The small peak of first-phase release from Synt1A−/− β cells shown in perfusion analysis is inferred to be composed of fusion from newcomers. Both the amplitude and time course of the glucose-induced rise in intracellular Ca2+ concentration ([Ca2+]i) measured using Fura-2 AM. Time 0 indicates when the high glucose was added. The fluorescence ratio (340/360) at time 0 was taken as 1. Results are provided as the mean ± SEM.

Restoration of Synt1A expression in β cells of Synt1A−/− mice

We performed rescue experiments to confirm Synt1A function in the docking and fusing of granules during first-phase release.
We restored Synt1A protein expression to Synt1A−/− β cells by infecting them with an adenovirus encoding Synt1A, Adex1CA Synt1A (Ax-Synt1A; Fig. 4 A). The number of Synt1A clusters was considerably restored, although to still subnormal levels (270.8 ± 13.0 vs. 212.4 ± 15.7, WT vs. Ax-Synt1A–infected Synt1A−/− cells; P < 0.05). In accordance with restored Synt1A cluster levels, the number of docked insulin granules in Ax-Synt1A–infected Synt1A−/− cells was restored (261.1 ± 13.6 vs. 230.0 ± 12.0, WT vs. Ax-Synt1A–infected Synt1A−/− cells; P = NS). Infection of Adex1CA Synt1A did not alter the number of SNAP25 clusters (Fig. 4 B) that interact with Synt1 clusters (Lang et al., 2002). We then performed TIRFM analysis of

Figure 4. Rescue of the number of docked insulin granules and fusion events by restoring Synt1A clusters to normal levels in Synt1A−/− β cells. [A–C] TIRF images and the quantitation of Synt1A clusters (A), SNAP-25 clusters (B), and docked insulin granules (C) on the plasma membrane in WT or Synt1A−/− β cells. Synt1A−/− cells were infected with empty virus Adex1w (Ax-Cont) or with Adex1CA Synt1A (Ax-Synt1A). Cells were fixed and immunostained for Synt1A (A), SNAP-25 (B), and insulin (C). (top) Typical TIRF images. Surrounding lines represent the outline of cells attached to the cover glass. Bars, 5 μm. (bottom) Number of Synt1A (A) and SNAP-25 (B) clusters and docked insulin granules (C) on the plasma membrane. Individual fluorescent spots in TIRF images were manually counted per 200 μm². Data are mean ± SEM (*, P < 0.05; **, P < 0.0001; n = 15 cells). (D) Rescue of fusion events in Synt1A−/− β cells. Synt1A−/− cells were infected with Adex1CA insulin GFP and then Ax-Cont or Ax-Synt1A. The histogram shows the number of fusion events (per 200 μm²) at 60-s intervals after high glucose stimulation. A marked increase in fusion events from previously docked granules was observed in the Synt1A−/− cells infected with Ax-Synt1A relative to Ax-Cont–infected cells. (E) Dual-color TIRFM of docking and fusing GFP-tagged insulin granules (green) and Synt1A clusters labeled with TAT-conjugated Cy3-labeled anti-Synt1A antibody (red) in glucose-induced release from Ax-Synt1A–infected Synt1A−/− cells. Most fusion from previously docked granules occurred at Synt1A clusters (76% of all fusion from previously docked granules); fusion from newcomers occurred external to Synt1A clusters (81% of all fusion from newcomers) during the first phase. Data are mean ± SEM (n = 5 cells). Solid boxes (1 μm × 1 μm) represent the sites of fusion events at the Synt1A clusters. Dashed boxes indicate the sites of fusion events external to the Synt1A clusters. (F) Sites of insulin granule fusion during second-phase insulin release (>4 min after glucose stimulation). Fusion events during second-phase release occurred mostly from newcomers and at sites distinct from Synt1A clusters (82% of all fusion from newcomers; n = 5 cells). Data are mean ± SEM. Solid and dashed boxes are as described above.
the docking and fusion of insulin granules stimulated by 22 mM glucose in Ax-Synt1A–infected Synt1A−/− β cells. This analysis showed a substantial increase in fusion events from previously docked granules (Fig. 4 D and Video 5, available at http://www.jcb.org/cgi/content/full/jcb.200608132/DC1). The total number of fusion events from previously docked granules during the first phase in Ax-Synt1A–infected Synt1A−/− cells was restored (18.2 ± 1.8 vs. 12.7 ± 3.3 in 0–4 min, WT vs. Ax-Synt1A–infected Synt1A−/− cells; P = NS). Synt1A restoration did not affect fusion events from newcomers during the second phase. In addition, we examined the interaction between insulin granules and Synt1A clusters labeled with TAT-conjugated Cy3 anti-Synt1A mAb in Ax-Synt1A–infected Synt1A−/− cells. Dual-color TIRFM showed that previously docked granules fused at the site of the Synt1A clusters during the first phase; during the second phase, newcomer granules fused external to the Synt1A clusters (Fig. 4, E and F). This was also observed in WT β cells. These data support a model where Synt1A clusters are required for previously docked granules to dock and fuse during the first phase but dispensable for newcomers to dock and fuse during the second phase.

**Other plasma membrane syntaxins assayed are not involved in second-phase release**

As shown in Fig. 3 D, the fusion of newcomers during the second phase was well preserved in the absence of Synt1A. Yet the question remained of whether other syntaxin isoforms might be functioning in second-phase release, as pancreatic β cells do express detectable levels of plasma membrane–localized syntaxin isoforms, such as Synt3 and 4 (Jacobsson et al., 1994; Wheeler et al., 1996). To investigate whether these membrane syntaxins Synt3 and 4 are involved in the second phase, we used TAT fusion proteins that encode the Synt3-H3 (TAT-Synt3-H3) and Synt4-H3 (TAT-Synt4-H3) domains. We previously reported that the recombinant Synt1A SNAP motif (H3 domain) fused to TAT (TAT-Synt1A-H3) rapidly transduced into MIN6 β cells, inhibiting insulin release (Ohara-Imaizumi et al., 2002b). Because the syntaxin H3 domain contributes to one of the four α-helical bundles in the SNARE core complex (Jahn and Südhof, 1999), a large molar excess of the Synt1A-H3 domain fused to TAT interrupted the formation of functional SNARE complexes (Ohara-Imaizumi et al., 2002b), as previously reported in other systems (Zhong et al., 1997; O’Connor et al., 1997). We therefore used TAT-H3 of each syntaxin isoform to perform dominant-negative type experiments.

We first produced TAT fusion proteins encoding the Synt3-H3 (TAT-Synt3-H3) and Synt4-H3 (TAT-Synt4-H3) domains. In addition, we produced TAT fusion proteins that encoded the Synt1A-H3 (TAT-Synt1A-H3) and Synt1B-H3 (TAT-Synt1B-H3) domains. A non–coiled-coil domain of ELKS, which has no effect on insulin exocytosis, composed the peptide fusion in our TAT-Control (Ohara-Imaizumi et al., 2005). As shown in Fig. 5 (C and D), the transduction of TAT-Synt3-H3 and TAT-Synt4-H3 into WT β cells reduced the number of fusion events from

![Figure 5. TIRFM of fusion of GFP-tagged insulin granules in biphasic insulin release from WT β cells treated with TAT-syntaxin-H3.](image_url)
previously docked granules during the first phase to ~58 and ~59% that of control levels, respectively. Second-phase release, which consisted mostly of newcomers, was unaffected by the Synt3-H3 and Synt4-H3 constructs expressed. TAT-Control treatment had no effect on either phase (Fig. 5 A). However, TAT-Synt1A-H3 treatment strongly reduced the total number of fusion events from previously docked granules during the first phase to ~23% that of control levels, while showing no effect on second-phase release (Fig. 5 B). These data are consistent with our results from Synt1A−/− β cells. Synt1B does not express in β cells, but TAT-Synt1B-H3 treatment showed results similar to those in the TAT-Synt1A-H3 treatment, reducing the total number of fusion events from previously docked granules during the first phase to ~28% that of control levels (Fig. 5 E). This may be a reflection of a higher homology of Synt1B-H3 to Synt1A-H3. Overall, these findings suggest that these other syntaxin family members are not involved in second-phase release.

**Synt1A ablation results in impaired glucose tolerance**

Because Synt1A−/− β cells exhibit reduced first-phase insulin release, these mice would be expected to develop diabetes. The Goto-Kakizaki rat model for human type 2 diabetes is known to be defectice in first-phase insulin release and displays hyperglycemia (Ostenson et al., 1993). In contrast, we found that Synt1A−/− mice did not show any significant hyperglycemia; fasting blood glucose levels of Synt1A−/− mice were not different from those of WT mice (Synt1A+/−, 63.9 ± 4.3 mg/dl [n = 7], vs. WT, 65.1 ± 3.3 mg/dl [n = 11]; P = NS). However, the oral glucose tolerance test did show impaired glucose tolerance in Synt1A−/− mice (Fig. 6 A). 30 min after challenge, blood glucose levels in Synt1A−/− mice were significantly higher than in WT mice (Synt1A+/−, 385.0 ± 14.1 mg/dl [n = 7], vs. WT, 286.3 ± 10.4 mg/dl [n = 11]; P < 0.0001). In agreement with these data, we found serum insulin levels to be lower in Synt1A−/− than in WT mice at 30 min after challenge (Fig. 6 B). Thus, Synt1A−/− mice displayed an impaired glucose tolerance but not marked hyperglycemia.

**Discussion**

Our dual-color TIRFM approach has shown that during first-phase release insulin granules fuse at the site of Synt1A clusters, but during second-phase release the granules fuse external to Synt1A clusters. We previously found that granules fusing during the first phase originated mostly from morphologically previously docked granules, whereas granules fusing during the second phase arose from newcomers that were originally stored intracellularly (Ohara-Imaizumi et al., 2004b). We also reported that previously docked insulin granules were colocalized with Synt1A clusters in the plasma membrane of MIN6 β cells (Ohara-Imaizumi et al., 2004a). Collectively, these findings suggested that Synt1A is probably essential for docking and fusing insulin granules during the first phase; however, no direct evidence existed to verify this. Recently, it was reported that other isoforms of the syntaxin family might be associated with biphasic insulin release (Saito et al., 2003; Spurr and Thurmond, 2006). We therefore used Synt1A−/− mice to directly address how Synt1A functions in granule docking and fusing in biphasic insulin exocytosis.

First, we examined the docking status of insulin granules in Synt1A−/− β cells. TIRFM and EM analysis in Synt1A−/− β cells documented a marked reduction of the number of granules docked onto the plasma membrane. Because granules fused during the first phase originated from docked granules, as expected, TIRFM revealed that there was no fusion from docked granules during the first phase in knockout cells. However, fusion from newcomers was still preserved in Synt1A−/− β cells under glucose stimulation. Consistent with these data, perfusion analysis of Synt1A−/− β cells showed a marked reduction in first-phase insulin release but no change in second-phase release. Furthermore, restoration of Synt1A to subnormal levels via the adenoviral vector in Synt1A−/− β cells restored the insulin granules docked onto the plasma membrane, accompanied by an appreciable number of fusion events from these granules. Thus, our data provide direct evidence that Synt1A is essential for docking and fusion of insulin granules during first-phase release. The docking status of synaptic vesicles in the brain hippocampus showed no difference between WT and Synt1A−/− mice (Fujiwara et al., 2006). The reason for this discrepancy between brain and pancreatic β cells is unknown, but it may be due to the expression of Synt1B, which is highly homologous to Synt1A and is abundant in brain cells (Bennett et al., 1992) but not in pancreatic β cells (Nagamatsu et al., 1996). Although the function of Synt1B may not be equal to that of Synt1A in pancreatic β cells (Nagamatsu et al., 1996), the brain may have either a tremendous safety network or a different system from pancreatic β cells that permits Synt1B or other homologues to compensate for the lack of Synt1A in brain tissue.

Although our data specify a requirement for Synt1A during first-phase release, we still do not know whether other isoforms of the syntaxin family participate in the first phase. WT β cells transduced with TAT-Synt3-H3 and TAT-Synt4-H3, which function in a dominant-negative manner to the corresponding syntaxin isoforms, showed reduction to some extent in the fusion events from previously docked granules during the first phase (Fig. 5). Yet, as no docked insulin granules were seen on
SNARE complex. Indeed, granule behavior between the first and second phases of release is quite different. As previously reported, upon reaching the plasma membrane, newcomers dock on the plasma membrane from the intracellular store and then fuse with the plasma membrane without any interaction with Synt1A clusters.

During first-phase insulin release, granules dock to Synt1A clusters and fuse at the site of Synt1A clusters. During second-phase release, granules move to the plasma membrane from the intracellular store and then fuse with the plasma membrane without any interaction with Synt1A clusters.

the plasma membrane in Synt1A−/− mice, it is difficult to conclude that both Synt3 and -4 are associated with first-phase exocytosis. Rather, we assume that the reduction of fusion events during the first phase by TAT-Synt3-H3 and TAT-Synt4-H3 treatment may reflect the homology of their amino acid sequence to Synt1A-H3. Nevertheless, it remains to be empirically determined if, and how, the other plasma membrane syntaxins contribute to the first phase.

Fusion from newcomer granules was not altered at all regardless of Synt1A deletion, indicating that this type of fusion may occur via some mechanism other than the Synt1A-based SNARE complex. Indeed, granule behavior between the first and second phases of release is quite different. As previously reported, upon reaching the plasma membrane, newcomers fused immediately (<50 ms), whereas granules previously docked on the plasma membrane stayed at the same place for a relatively long time (O’Rahilly et al., 2004b).

2nd phase (Synt1A−/−-independent) Reserve pool

1st phase (Synt1A-dependent) docking

Figure 7. Schematic drawing of the role of Synt1A in biphasic insulin release. During first-phase insulin release, granules dock to Synt1A clusters and fuse at the site of Synt1A clusters. During second-phase release, granules move to the plasma membrane from the intracellular store and then fuse with the plasma membrane without any interaction with Synt1A clusters.

Materials and methods

Generation of Synt1A−/− mice

We generated Synt1A−/− mice as previously described (Fujiwara et al., 2006). The genotyping of mice was performed by PCR. Mice were backcrossed with strain C57BL/6 over at least five generations and were used at the age of 10–14 wk. Animal experiments were approved by the Kyorin University Animal Care Committee.

Cells

Pancreatic islets of Langerhans were isolated from male WT and Synt1A−/− mice by collagenase digestion as described previously (O’Rahilly et al., 2004b). Isolated islets were dispensed in calcium-free Krebs-Ringer buffer (KRB) containing 1 mM EGTA and cultured on fibronectin-coated (KOKEN Co.), high refractive index cover glass (Olympus) in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 200 U/ml penicillin, and 200 μg/ml streptomycin at 37°C in an atmosphere of 5% CO2.

Immunoblotting

Proteins were extracted from mouse whole brain or mouse pancreatic islets and immunoblotted as previously described (O’Rahilly et al., 2005). Anti-Synt1A mAb and anti-Synt1B pAb were obtained also as previously
His6 tag at the N terminus. The resulting products were confirmed by an pPROEX HTa bacterial expression vector (Invitrogen) with an additional (Ohara-Imaizumi et al., 2002b). PCR products were subcloned into a Adex1CA insulin-GFP–infected cells were treated with body was separated from the free TAT PTD peptide by gel filtration eluted (QIAGEN) before washing, and stepwise removal of urea was performed (Car-gille laboratories) was used to make contact between the objective lens and the high refractive index cover glass. Light propagates through the cover glass at an angle measured as 65° and undergoes total internal reflection at the glass–cell interface. The refractive indices for glass (n = 1.8 at 488 nm) and cells (n = 1.37) predict an evanescent field declining e-fold within 44 nm from the interface and to ~10% within 100 nm. A granule 100 nm from the interface would be illuminated too dimly to be visible under these conditions. Thus, we could barely 100 nm into the car after having comparable to the thickness of ultrathin sections cut for EM (Zenisek et al., 2000). In an evanescent field declining e-fold within 44 nm, a granule at 80% brightness would have a vertical distance of 9.6 nm from the plasma membrane and qualify as a morphologically docked granule (granule distance from plasma membrane <10 nm in EM studies; Parsons et al., 1995). Images were projected onto a CCD camera (DVB8TD5CAV, Andor) operated with MetaMorph version 6.3. Images were acquired at 300-ms intervals. For real-time images of GFP-tagged insulin granule motion by TIRFM, treated β cells were placed on the high refractive index glass, mounted in an open chamber, and incubated for 30 min at 37°C in KRB containing 110 mM NaCl, 4.4 mM KCl, 1.45 mM KH2PO4, 1.2 mM MgSO4, 2.3 mM calcium chloride, 4.8 mM NaHCO3, 2.2 mM glucose, 10 mM Hepes, pH 7.4, and 0.3% bovine serum albumin. Cells were transferred to the thermostat-controlled stage (37°C) of TIRFM, and stimulation with glucose was achieved by the addition of 52 mM glucose–KRB into the chamber for a final concentration of 22 mM glucose. Most analyses, including tracking [single projection of different images] and area calculations were brought into superposition by shifting one image using MetaMorph software. To analyze the data, fusion events were manually selected, and the mean fluorescence intensity of individual granules in a 1 μm × 1 μm square placed over the granule center was calculated. The number of fusion events was manually counted while looping ~5,000 frame time lapses. To observe the fluorescence of GFP and Cy3 simultaneously, we used the 488-nm laser line for excitation and an image splitter (Optical Insight) that divided the green and red components of the images with a 565-nm dichroic mirror (Q565; Chroma Technology Corp.), passing the green component through a 530-nm bandpass filter (HQ530/30 m; Chroma Technology Corp.) and the red component through a 630 nm ± 25 nm bandpass filter (HQ630/50 m [Chroma Technology Corp.]; Ohara-Imaizumi et al., 2005). Images were then projected side by side onto a CCD camera. The two images were brought into focus in the same plane by adding weak lenses to one channel, and they were brought into register by careful adjustment of the mirrors in the image splitter. Before each experimental session, we took an alignment image that showed density by means of scattered 90 nm TetraSpeck fluorescent beads (Invitrogen). They were visible in both the green and red channels, and thus provided markers in the x-y plane. Beads in the two images were brought into superposition by shifting one image using MetaMorph software.

Insulin release assay
β cells were housed in a small chamber (~5 × 105 cells/chamber) and perfused with KRB (2.2 mM glucose) for 60 min at a flow rate of 0.5 ml/min at 37°C before collecting fractions. Insulin release was stimulated by 22 mM glucose. Fractions were collected at 1-min intervals. Insulin release in aliquots of media was measured by an insulin EUSA kit (Morinaga).
Online supplemental material

Fig. S1 shows a TIRF image of Synt1A clusters in the plasma membrane labeled with TAT-conjugated Cy3-labeled anti-Synt1A mAb and stained with anti-Synt1A pAb. Fig. S2 shows an immunoblot analysis of Synt1A and other SNARE proteins in the brain and pancreatic islets from WT and Synt1A−/− mice. Fig. S3 shows microscopic examination of pancreatic islets in WT and Synt1A−/− mice. Video 1 displays dual-color TIRF images of GFP-tagged insulin granules and Cy3-labeled Synt1A clusters during first-phase insulin release (0–4 min after glucose stimulation). Video 2 shows dual-color TIRF of GFp-tagged insulin granules and Cy3-labeled Synt1A clusters during second-phase insulin release (>4 min after glucose stimulation). Video 3 shows TIRF of GFp-tagged insulin granule motion in the WT mouse β cell under 22 mM glucose stimulation. Video 4 shows TIRF of GFp-tagged insulin granule motion in the Synt1A−/− mouse β cell under 22 mM glucose stimulation. Video 5 shows TIRF of GFp-tagged insulin granule motion in the Synt1A−/− mouse β cell infected with Ax-Synt1A under 22 mM glucose stimulation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200608132/DC1.

We thank Dr. Eckhard Lammert for his critical reading of the manuscript and C. Nishiwaki for technical assistance.

This work was supported by grants-in-aid for scientific research (B) [15390108; to S. Nagamatsu], scientific research (C) [17590277; to M. Ohara-Imaizumi], and scientific research on priority areas [18050033; to M. Ohara-Imaizumi] from the Japanese Ministry of Education, Culture, Sports, Science and Technology. Additional support includes a grant-in-aid from Kyorin University School of Medicine, Collaboration Project 2006, Kyorin University School of Medicine (to S. Nagamatsu), and a grant-in-aid from Kyorin University School of Medicine, Kyorin Medical Research Award 2006 (to M. Ohara-Imaizumi).

Submitted: 22 August 2006
Accepted: 18 April 2007

References


We thank Dr. Eckhard Lammert for his critical reading of the manuscript and C. Nishiwaki for technical assistance.


Downloaded on June 27, 2017


