Regulation of Insulin Exocytosis by Munc13-1*

Laura Sheu‡§, Eva A. Pasyk‡§, Junzhi Ji‡§, Xiaohang Huang‡§, Xiaodong Gao‡, Frederique Varoqueaux‡§, Nils Brose‡, and Herbert Y. Gaisano‡§

From the Departments of §Medicine and ¶Physiology, University of Toronto, Toronto, Ontario MSS 1A8, Canada and the ¶Max Planck Institute for Experimental Medicine, Department of Molecular Neurobiology, and Center for Molecular Physiology of the Brain, Hermann-Rein-Strasse 3, D-37075 Göttingen, Germany

The slower kinetics of insulin release from pancreatic islet beta cells, as compared with other regulated secretory processes such as chromaffin granule secretion, can in part be explained by the small number of the insulin granules that are docked to the plasma membrane and readily releasable. In type-2 diabetes, the kinetics of insulin secretion become grossly distorted and, to therapeutically correct this, it is imperative to elucidate the mechanisms that regulate priming and secretion of insulin secretory granules. Munc13-1, a synaptic protein that regulates SNARE complex assembly, is the major protein determining the priming of synaptic vesicles. Here, we demonstrate the presence of Munc13-1 in human, rat, and mouse pancreatic islet beta cells. Expression of Munc13-1, along with its cognate partners, syntaxin 1a and Munc18a, is reduced in the pancreatic islets of type-2 diabetes non-obese Goto-Kakizaki and obese Zucker fatty rats. In insulinoma cells, overexpressed Munc13-1-enhanced green fluorescent protein is translocated to the plasma membrane in a temperature-dependent manner. This, in turn, greatly amplifies insulin exocytosis as determined by patch clamp capacitance measurements and radioimmunoassay of the insulin released. The potentiation of exocytosis by Munc13-1 is dependent on endogenously produced diacylglycerol acting on the overexpressed Munc13-1 because it is blocked by a phospholipase C inhibitor (U73122) and abrogated when the diacylglycerol binding-deficient Munc13-1H567K mutant is expressed instead of the wild type protein. Our data demonstrate that Munc13-mediated vesicle priming is not restricted to neurotransmitter release but is also functional in insulin secretion, where it is subject to regulation by the diacylglycerol second messenger pathway. In view of our findings, Munc13-1 is a potential drug target for therapeutic optimization of insulin secretion in diabetes.
Munc13-1 expression levels are reduced in rat models of type-2 diabetes. We demonstrate that overexpression of Munc13-1 in insulinoma HIT-15 and INS-1 cells causes a potentiation of glucose-, phorbol ester-, and membrane depolarization-evoked insulin secretion. Our data demonstrate that Munc13 proteins are functionally relevant potent priming proteins for regulated secretion from non-neural/non-neuroendocrine cells.

EXPERIMENTAL PROCEDURES

Reagents—The male Goto-Kakizaki ( GK ) rat pancreatic islets were isolated from the Stockholm colony as a gift from Claes-Goran Ostenson (Karolinska Institute, Stockholm, Sweden). Zucker fa/fa rats, its normal, lean counter part Zucker rats, and Wistar rats were from Charles River Laboratories (St. Constant, Quebec, Canada). The normal human pancreatic islets were kindly provided by J. Lakey (Clinical Islet Isolation Laboratory, Edmonton, University of Alberta, Edmonton, Alberta, Canada). The hamster HIT-T15 cell line was a gift from Paul Robertson (Pacific Northwest Research Foundation, Seattle, WA), and the mouse INS-1 cell line was from American Type Culture Collection. Mouse monoclonal (Synaptic Systems) and rabbit polyclonal antibodies were raised against amino acids 1399–1736 and 1–305 of Munc13-1, respectively. Both antibodies specifically recognized Munc13-1 (25, 26). Guinea pig anti-insulin antibody was a gift from R. Pederson (University of British Columbia, British Columbia, Canada).

Insulinoblasts—Pancreatic islets from Zucker fa/fa rats, and lean and obese Zucker fa/fa rats, and CD-1 mice (Charles River Laboratories) were isolated by collagenase digestion as described previously (30, 31). These islets and insulinoma cell lines (HIT-T15, INS-1) were solubilized in sample buffer (with 2% SDS), and the indicated amount of protein of each sample was loaded and separated on polyacrylamide gels (Munc13-1, 15 μg of protein/lane, 6% PAGE; nSec1 and syntaxin 1a, 5 μg of protein/lane, 14% PAGE). The proteins were then transferred onto nitrocellulose membranes and incubated with the following rabbit polyclonal primary antibodies for 1.5–2 h at room temperature: anti-Munc13-1, 1:5000; anti-syntaxin 1A, 1:1000 (Calbiochem, San Diego, CA); anti-nSec1/Munc18a, 1:1000 (Transduction Laboratories, Lexington, KY). The antibodies were detected with peroxidase-labeled goat anti-rabbit secondary antibodies. Positive reactions were visualized by chemiluminescence (Pierce) and exposure of the membranes to Kodak BMR film (Eastman Kodak, Rochester, NY) for 1 to 10 min.

Transfection and Insulin Secretion—HIT-T15 and INS-1 cells were transfected with expression vectors encoding Munc13-1 or Munc13-1H567K in frame with EGFP (pEGFP-Munc13-1 and pEGFP-Munc13-1H567K, 25), or pcDNA3-EGFP as control, as described previously (32–34). Briefly, cells were grown in a 5% CO2 atmosphere in RPMI 1640 medium containing 2 g/liter (11 mM) glucose and supplemented with 10% fetal calf serum (Cansera, Rexdale, Ontario, Canada). Cells were trypsinized and resuspended in serum-free RPMI 1640. A total of 1.5–2 × 105 cells in 1 ml of medium was then collected from each well, and its insulin content was determined by radioimmunoassay according to the instructions from the manufacturer. Data are presented as mean ± S.E. Data were compared by unpaired Student’s t test for single comparisons and by analysis of variance for multiple comparisons. p < 0.05 was considered to be statistically significant.

RESULTS

Munc13-1 Is Present in Human and Rat Pancreatic Islet and Insulinoma Beta Cells—We determined the expression levels of Munc13-1 in human, mouse and Wistar rat islets and in the insulinoma cell lines HIT (from hamster) and INS-1 (from mouse) by Western blotting using a rabbit antibody against the N-terminal amino acids 1–305 of Munc13-1 (26). Like rat brain, human, mouse, and rat islets, as well as insulinoma cell lines HIT-T15 and INS-1, contain Munc13-1 (Fig. 1A). Islets contain not only insulin-secreting beta cells but also glucagon-secreting alpha and somatostatin-secreting delta cells. To determine which cell type expresses Munc13-1, we performed laser confocal microscopy on rat and human islets after double labeling for insulin and Munc13-1 (Fig. 1B). In these experiments, Munc13-1 was detected using a commercially available antibody that preferentially interacts with Munc13-1 (Synaptic Systems, Göttingen, Germany; Ref. 25). We found that, in both rat and human islets, Munc13-1 immunofluorescence was strongest in insulin-positive beta cells (indicated by arrows) but also detectable in other cell types, indicating that Munc13-1 is most abundant in beta cells. In all positive cells, Munc13-1 immunofluorescence was detectable in the cytosol and on the plasma membrane.

Munc13-1 Levels in Islets Are Reduced in Type-2 Diabetes Rat Islets—Patients with type-2 diabetes exhibit abnormalities in glycemic control and lipid metabolism (35). These aspects of...
the disease have been well studied in obese and non-obese diabetic rodent models, such as the non-obese GK rats (14, 36, 37). In Zucker diabetic fatty rats, the islets accumulate excessive fat (38) and exhibit a defective response to fatty acid stimulation (39). Interestingly, lowering of the islet fat by troglitazone treatment restores islet secretory function (38). Long-chain acyl-CoA, the intracellular form of free fatty acids, as well as other fatty acids, including palmitate and myristate, acutely stimulate insulin secretion even in the absence of ATP or extracellular Ca\(^{2+}\) (40, 41). It was suggested that free fatty acids act directly on the exocytotic machinery (41), for example by increasing the size of the docked pool of insulin granules that regulate the first phase of insulin secretion in a SNARE-dependent manner (42). In addition, DAG levels within the islet beta cells are altered by changes in the cellular free fatty acid metabolism (38–40). We therefore examined whether Munc13-1, a cellular DAG receptor, might be an indirect target of free fatty acids in the regulation of insulin secretion and whether Munc13-1 levels are altered in models of type-2 diabetes.

We reported that the expression of the SNARE proteins syntaxin-1A, VAMP-2, and SNAP-25 is down-regulated in the islets of fa/fa Zucker (30) and, more recently, also in the islets of GK rats (37). Because syntaxin 1A is a substrate of Munc13-1 and down-regulated in Munc13-1-deficient neurons (19), we tested whether Munc13-1 expression is also dysregulated in type-2 diabetes. For this purpose, we examined the levels of Munc13-1, nSec1/Munc18–1, and syntaxin-1A in the islets of two diabetic models, the obese fa/fa Zucker rats and the non-obese GK rats. As controls for these diabetic rats, we used the respective lean Zucker and Wistar rats. The male GK rats (2–3 months, 300–350 g) used here were from a larger batch of rats of the Stockholm colony that we had also used in a recent reported study (37), along with age-matched Wistar rats (300–350 g). The 9 a.m. serum glucose for this batch of GK rats was 19.7 ± 2.0, whereas the Wistar rats was 7.0 ± 1.1 mm. The male Zucker fa/fa (13–15 weeks old, 650 g) and the age-matched lean Zucker controls (370 g) were from the same commercial source (Charles River Laboratories) as we had previously reported (30). The Zucker rats were sacrificed shortly after arrival to extract the islets. Normal rat chow was fed to all of these rats prior to sacrifice. We found that Munc13-1 as well as nSec1/Munc18–1 and syntaxin-1A were present in the control and the corresponding type-2 diabetes rat models. However, the expression levels of all three proteins were reduced in the diabetic rats as compared with their normal controls (Fig. 2).

Munc13-1 Translocates to the Plasma Membrane of Insulinoma Cells in a Temperature-dependent and DAG-dependent Manner—We showed previously that, in chromaffin cells and fibroblasts, phorbol esters induce a PKC-independent translocation of heterologously expressed Munc13-1-EGFP fusion proteins to the plasma membrane (22, 25). This translocation is mediated by the phorbol ester binding C1 domain of Munc13-1 and abolished by perturbation of the C1 domain structure by a single point mutation (H567K). Moreover, the enhancing effect of wild type Munc13-1-EGFP on secretion from chromaffin cells was reduced in the Munc13-1H567K-EGFP mutant (22). These data indicated that Munc13-1-EGFP is a fully functional priming protein and that the intact C1 domain is necessary for optimal function. Indeed, we recently demonstrated that the intact Munc13-1 C1 domain is essential for survival because it mediates activity-dependent increases of synaptic vesicle priming during phases of strong presynaptic activity (26).

Given the functional importance of the Munc13-1 C1 domain, we examined whether application of the phorbol ester TPA (100 nM) and glucose (10 mM) induces the translocation of Munc13-1 to the plasma membrane in HIT-T15 cells. For that purpose, we transfected HIT-T15 (passages 75–80) with a vector encoding Munc13-1-EGFP (n = 14 cells from three experiments). We first found that gradually raising the temperature from 23 to 37 °C was sufficient to cause Munc13-1-EGFP translocation to the plasma membrane (Fig. 3A). The plasma membrane fluorescence signal (reflecting membrane-associated Munc13-1-EGFP) increased when the temperature was raised from the ambient 23 to 28 °C, and increased maximally when the temperature was further raised to 37 °C. Interestingly, when these cells were then exposed 100 mM TPA or to high glucose (10 mM

**Fig. 1.** Munc13-1 is present in human and rat pancreatic islet beta cells and insulinoma cells. A, the presence of Munc13-1 was investigated by Western blots of homogenates from the indicated tissues or cell lines. Rat brain homogenate (2 μg of protein) was used as a positive control. B, cellular localization of endogenous Munc13-1 in rat and human pancreatic islets as determined by immunofluorescence labeling and confocal microscopy. Double labeling for Munc13-1 and insulin shows the abundance of Munc13-1 in beta cells (arrows). Scale bar, 25 μm.

**Fig. 2.** Munc13-1 levels are reduced in type-2 diabetes GK and fa/fa Zucker rat islets. Immunoblots of pancreatic islets homogenates (10 μg of protein) isolated from respective pairs of Wistar (control) versus GK type-2 diabetes non-obese rats (left panel), and from a pair of Zucker (ZK) lean (control) versus Zucker fa/fa obese rats (right panel) show a dramatic decrease of the amount of Munc13-1, as well as of nSec1 and syntaxin-1A, in the diabetes rat models.
Fig. 3. Temperature-dependent translocation of Munc13-1-EGFP to the plasma membrane in HIT-T15 cells. HIT-T15 cells were transfected with Munc13-1-EGFP (A and B) or Munc13-1H567K-EGFP (C) and subsequently exposed to increasing temperatures, from 23 °C (RT) to 28 and 37 °C. 100 nM TPA was then added followed by 10 mM glucose (glu). A single confocal image was taken along the same plane 3–5 min after each temperature or agonist change. Imaging parameters remained unchanged. A shows one of the Munc13-1-EGFP experiments. Some of the Munc13-1-EGFP-transfected cells are indicated by the arrows. The images obtained after treatments with TPA and glucose are not shown because there were no further changes observed in these images when compared with the 23 °C image. In B, regions of interest were drawn along the entire plasma membrane of these randomly chosen Munc13-1-EGFP-transfected cells, and the resulting fluorescence pixel intensities obtained are summarized in a bar graph (three experiments, n = 14 cells), and are expressed in relation to the 23 °C fluorescent signal. The observed temperature-dependent increase of the fluorescence indicates a translocation of Munc13-1-EGFP to the membrane. An identical study was performed on Munc13-1H567K-EGFP-transfected HIT-T15 cells, and analyzed as described in B. C is a bar graph summary of this analysis (two experiments, n = 10 cells), which shows no significant translocation of Munc13-1H567K-EGFP to the membrane occurred. In B, * indicates p < 0.01 when compared with the 23 °C fluorescent signal, and ** indicates no significant difference when compared with 37 °C fluorescent signal. In C, *** indicates no significant difference when compared with the 23 °C fluorescent signal. Scale bar, 25 μm.

Glucose, 30 mM KCl, there was no significant further increase in membrane fluorescence, and it is therefore not shown. The lack of a TPA effect at 37 °C is in contrast to our studies in chromaffin cells and fibroblasts, where TPA-induced Munc13-1-EGFP translocation was observed even at 37 °C. Fig. 3B shows a quantification of the translocation data. We quantified the pixel intensity of the plasma membrane Munc13-1-EGFP fluorescence by drawing regions of interest encompassing the most of the plasma membrane (without the cytosol) of randomly selected cells (n = 14 cells) from three separate experiments, and expressed these values relative to the fluorescence intensity at 23 °C. At 28 °C, there was only a slight increase in Munc13-1-EGFP fluorescence over control levels (1.24 ± 0.08; range, 1.03–2.28), but we noted a rapid increase in plasma membrane EGFP fluorescence when the temperature was raised above 30 °C. At 37 °C, the plasma membrane EGFP fluorescence was 2.83 ± 0.39 times higher (range, 1.14–5.98) than under control conditions. The values after additional exposure to 100 nM TPA and to 10 mM glucose were 2.9 ± 0.37 (range, 1.21–5.98) and 2.70 ± 0.31 (range, 1.42–5.2), respectively, which were not significantly different from the 37 °C fluorescent signal (p = 0.87 and 0.79, respectively), and therefore indicate no further increase in Munc13-1 translocation to the plasma membrane. As the intensity gain in these studies was not altered between image acquisitions, the slight reduction in membrane Munc13-1-EGFP fluorescence we sometimes observed during the glucose or TPA exposure was likely caused by photobleaching resulting from the repeated laser exposure of the same optical section.

If the DAG-mediated translocation of Munc13-1 to the plasma membrane is indeed dependent on its C1 domain, then the H567K mutant of Munc13-1, which is unable to bind DAG and phorbol esters, would be expected not to translocate the plasma membrane in response to endogenous DAG or exogenous phorbol esters (22, 25). We therefore transfected Munc13-1H567K-EGFP into HIT-T15 cells and performed identical studies as with Munc13-1-EGFP (Fig. 3C). In two separate experiments (n = 10 cells), we indeed did not notice a significant increase in plasma membrane fluorescence of Munc13-1H567K-EGFP when the temperature was raised from 23 °C to 37 °C (p > 0.4). After the addition of 100 nM TPA, and the subsequent addition of 10 mM glucose, there was no additional increase in the membrane fluorescence (p > 0.5 when compared with the 23 °C fluorescent signal, and p > 0.9 when compared with 37 °C fluorescent signal).

Munc13-1 Overexpression in Insulinoma Cells Potentiates Glucose- and Phorbol Ester-mediated Insulin Secretion—We next examined the effects of the overexpression of Munc13-1-EGFP in HIT-T15 cells on glucose- and phorbol ester-evoked insulin secretion. The passages used were 75–85, which was the passage range we noted to retain secretory competence. The HIT-T15 cells, cultured in RPM1 1640 (11 mM glucose), were washed with a low glucose (0.5 mM glucose) media for at least 1 h, prior to commencing the study. In control insulin secretory studies with untransfected HIT-T15 cells, EC50 was 2 mM glucose and maximal secretion was at 10 mM glucose, and therefore, we have used 10–15 mM glucose for maximal stimulation. Transfection efficiency of the Munc13-1-EGFP was 30–40% as determined by the fraction of EGFP fluorescent cells (32–34).

Fig. 4A shows that the overexpressed Munc13-1-EGFP had no effect on basal insulin secretion (0 glucose, 4.8 mM KCl, 37 °C), with basal release of 1.1 ± 0.21 ng/well in the control GFP-transfected cells, and 1.1 ± 0.06 ng/well in the Munc13-1-GFP-transfected cells. This is in contrast to Fig. 3, where at basal glucose levels (0.5 mM), just raising the temperature to

![Image](http://www.jbc.org/Downloaded from http://www.jbc.org)
37 °C was sufficient to cause maximal Munc13-1-EGFP translocation to the plasma membrane. This would suggest that the DAG-mediated translocation of Munc13-1 translocation to the plasma membrane would increase the efficiency of priming of the insulin granules but would not affect exocytosis per se, and hence there was no effect on basal insulin secretion. To affect insulin secretion would still require secretagogues, such as glucose (Fig. 4A) or the phorbol ester (TPA, Fig. 4B).

Indeed, Fig. 4A also shows the effect of maximal stimulation with 15 mM glucose compared with the basal levels (0 glucose, 4.8 mM KCl). In control cells transfected with EGFP, 15 mM glucose caused an increase in insulin secretion from a basal level of 1.1 ± 0.21 ng/well to 2.58 ± 0.54 ng/well (13 wells from four experiments). In cells transfected with Munc13-1-EGFP, insulin secretion increased from basal levels of 1.1 ± 0.06 ng/well to 3.42 ± 0.55 ng/well (13 wells from four experiments), which was significantly larger than in control cells (p < 0.01).

When the net glucose-induced secretion (as determined by subtracting basal insulin release from secretion evoked by 15 mM glucose) was compared, Munc13-1-EGFP overexpression (0.76 ng/well) caused a ∼56% (p = 0.15) increase in insulin secretion over the control cells.

In additional experiments, we examined the effects of Munc13-1-EGFP on insulin secretion stimulated by 100 nM TPA. At basal levels (0.5 mM glucose, 4.8 mM KCl), Munc13-1-EGFP overexpression (0.76 ± 0.14 ng/well) had no effect compared with control cells (0.87 ± 0.18 ng/well). Exposure of Munc13-1-EGFP-transfected cells to 100 nM TPA caused an increase to 2.89 ± 0.54 ng/well (18 wells from six experiments) compared with 1.67 ± 0.25 ng/well in control cells, indicating a statistically significant facilitating effect of Munc13-1-EGFP on TPA-enhanced insulin secretion (p = 0.03). When the net TPA-enhanced secretion (as determined by subtracting the basal release from that in the presence of 100 nM TPA) was compared, the Munc13-1-EGFP overexpression caused a ∼160% increase in TPA-enhanced insulin secretion over the control cells.

Because the limited transfection efficiency in these biochemical experiments led to an underestimation of Munc13-1-mediated effects on secretion (as was the case with the glucose-mediated insulin secretion) and made the accurate quantitative analysis of functionally deficient Munc13-1 mutant constructs (i.e. Munc13-1H567K) very difficult, we used patch clamp capacitance measurements of single cells as a more direct measure of exocytotic activity upon overexpression of wild type and mutant Munc13-1-EGFP constructs. The advantage of this approach, as shown in Fig. 5, is that transfected cells can be identified by their EGFP fluorescence and studied in isolation without contaminating wild type contributions.

In these experiments, we used INS-1 cells for these patch clamp capacitance experiments because INS-1 cells very reliably exhibit an increase in membrane capacitance (Cm) in response to stimulation, as we had previously reported (33, 34). In Fig. 5, we examined Ca2+ -evoked exocytosis by membrane depolarization to 0 mV, a voltage that we previously showed to evoke maximal Ca2+ influx current in this cell line (33, 34). We first performed the Cm measurements at room temperature (23 °C) (data not shown) and found that the Cm increases induced by membrane depolarization in cells expressing Munc13-1-EGFP (middle panel) was similar to those seen in EGFP-expressing control cells (left panel). At room temperature, the presence of 100 nM TPA did not alter the Cm increases induced by depolarization in Munc13-1-EGFP-expressing or control cells (data not shown).

In control cells transfected with EGFP (left panel), at room temperature (23 °C), the depolarization pulse evoked a Cm increase of 3.2 ± 1.1 fF (n = 6). When the temperature was increased to 30–32 °C, the depolarization-induced Cm change in these cells was 12.5 ± 1.7 (p < 0.05). Further addition of 100 nM TPA to these cells resulted in a depolarization-induced Cm change of 19.2 ± 3.4 fF (p < 0.05), which is a 53% increase over that without TPA. In Munc13-1-EGFP-expressing INS-1 cells (middle panel) at 23 °C, the depolarization evoked Cm increase was 5.1 ± 1.6 fF (n = 6 cells). When the temperature was raised to 30–32 °C, a depolarizing pulse caused a Cm increase in the same cells to 51.7 ± 12.5 fF (p < 0.05). Thus, raised tempera-
Temperature-sensitive potentiation of insulin exocytosis is mediated by endogenous diacylglycerol action on Munc13-1. Transfected INS-1 cells were stimulated by a voltage pulse depolarization from −70 to 0 mV successively at room temperature (23 °C), at 30–32 °C (30 °C) in the absence and then presence of TPA (TPA) for GFP and Munc13-1EGFP-expressing cells or U73122 (U7) followed by TPA (for Munc13-1EGFP-expressing cells). Three- to 5-min rest interval between depolarization pulse protocols ensured refilling of the releasable pools. The membrane capacitance \(C_m\) increase was taken as a measure of exocytosis. Data shown are the mean ± S.E. of \(n\) experiments. * indicates a \(p < 0.05\) significance of the indicated values being compared. ** indicates no significant difference between the indicated values. Munc13-1 overexpression enhances the potentiating effect of high temperature and TPA on depolarization-induced secretion from INS-1 cells. This effect is not seen with the DAG- and phorbol ester-insensitive Munc13-1-HE67K mutant.

The Munc13-1-EGFP-transfected cells (middle panel), contiguously exposed to a temperature of 30–32 °C, were then subjected to additional depolarization protocols in the presence of U73122 and, subsequently, of TPA (see below). There was a 3–5-min rest between each of these depolarization pulses to allow the releasable pool to refill. It was difficult to maintain a seal when the temperature was raised over 32 °C, and therefore the high temperature studies were performed at 30–32 °C. It was at this temperature that we had also noted the major Munc13-EGFP translocation to the plasma membrane shown in Fig. 3. These studies were done exclusively on passages 60–65 of the INS-1 cells to reduce the variability in the insulin granule content between cell line passages.

In a separate set of experiments performed on INS-1 cells from earlier passages (passages 55–60), depolarization-induced \(C_m\) changes in Munc13-1-EGFP-transfected cells (30–32 °C) before and after the addition of 100 nm TPA were 127 ± 12 fF (\(n = 5\)) and 183 ± 27 fF (\(n = 5\)), respectively, which constitutes a 44% increase as a result of TPA (data not shown). This effect of TPA is similar to that seen in EGFP-transfected control cells (Fig. 5, left panel).

High Temperature-induced Increases in Exocytosis from Munc13-1-expressing INS-1 Cells Depends on Endogenous DAG Production—Given that Munc13-1 is a DAG receptor (25, 26) and that glucose is known to generate DAG in islet beta cells (43, 44), it is possible that the temperature sensitivity of the effect of Munc13-1-EGFP overexpression on exocytosis is a result of a DAG-mediated mechanism. Even at ambient glucose concentrations (0.5 mM), INS-1 cells (and beta cells) could be metabolically active such that phospholipase C isozymes are activated to generate higher DAG levels at higher cellular temperatures, which would then act on the expressed Munc13-1-EGFP to cause its translocation to the plasma membrane, as was shown in the HIT-T15 cell study in Fig. 3, which is required for Munc13-1 to exert its priming action (22). We examined this possibility using two strategies.

First, we blocked a possible high temperature-mediated increase in endogenous DAG production using the specific phospholipase C inhibitor, U73122 (Fig. 5, middle panel). To allow a direct comparison, we used the same Munc13-1-overexpressing cells as described above, where the \(C_m\) increase at 30–32 °C was 51.7 ± 12.5 fF. Cells (at 30–32 °C) were blocked with a specific phospholipase C inhibitor, U73122 (10 μM), for 5 min, which was also sufficient time to replenish the releasable pool. A depolarization pulse applied to these cells evoked a \(C_m\) increase of 7.3 ± 5.3 fF, a level similar to those obtained at room temperature (23 °C), indicating that endogenous DAG is required for the potentiating effect of Munc13-1-EGFP on exocytosis. We then added 100 nm TPA to these cells in the continued presence of U73122 and still at 32 °C, and applied another depolarization pulse. This evoked a \(C_m\) increase of 50.5 ± 16.2 fF, similar to that observed prior to the addition of U73122. This indicates that the Munc13-1-EGFP remains functionally intact to respond fully to an exogenously added phorbol ester. The fact that depolarization induced secretion at high temperature alone and in the presence of a combination of U73122 and TPA supports the conclusion that the overexpressed Munc13-1-EGFP indeed enhances the exocytic response at high temperatures by responding to endogenously generated DAG.

Second, we examined whether the endogenous DAG generated by raising the temperature is acting on Munc13-1 and specifically on its C1 domain. For that purpose, we used the H567K mutant of Munc13-1, which is unable to bind DAG and phorbol esters (25, 26) (Fig. 5, right panel). In the Munc13-1H567K-EGFP-transfected INS-1 cells (passages 60–65) at room temperature (23 °C), a depolarization pulse evoked a \(C_m\) increase of 4.5 ± 1.1 fF (\(n = 6\) cells), which was similar to that in Munc13-1-EGFP and control EGFP-expressing cells. After raising the temperature to 30–32 °C, another depolarization pulse applied to the same cells evoked a \(C_m\) increase of 17.4 ± 2.3 fF (\(n < 0.05\)). Upon further addition of 100 nm TPA to these cells, the evoked \(C_m\) increase was 27.4 ± 4.6 fF (\(p < 0.05\)). Thus, the effects of high temperature and TPA on secretion from INS-1 cells overexpressing Munc13-1H567K-EGFP are only slightly larger than the ones observed in control EGFP-expressing cells (Fig. 5, left panel), but much smaller than those observed in cells overexpressing wild type Munc13-1-EGFP, indicating that DAG binding to the C1 domain is involved in the potentiating effect of Munc13-1 on insulin secretion, but is not an absolute requirement for Munc13-1 action.

DISCUSSION

The first phase of insulin secretion depends on assembled SNARE complexes formed by docked and primed insulin granules in the releasable pool (42), and the second phase of insulin secretion relies on the subsequent recruitment of granules to this pool (10, 11). Munc13-1 is a secretory vesicle priming factor (19, 22, 25) that is thought to initiate SNARE complex formation by stabilizing the open conformation of syntaxin 1A, thereby allowing its assembly with cognate SNAP-25 and VAMP-2 (24, 45). Munc13-1 function may also involve an interaction with Munc18–1 (24, 46, 47), but the functional significance of this interaction is unclear and the evolutionary conservation of a functional Munc13-1/Munc18–1 interaction is unlikely. a A. Betz and N. Brose, unpublished observations.
Based on its essential role in secretory vesicle priming, Munc13-1 would be predicted to regulate both the initial size of the releasable insulin granule pool and the recruitment of secretory granules to this pool, as is the case in the regulation of chromaffin granule secretion by Munc13-1 (22). Indeed, Munc13-1 is expressed in beta cells (Fig. 1), and overexpression of Munc13-1 in insulin secreting cells leads to an increased releasable granule pool and increased insulin secretion (Figs. 4 and 5), indicating that Munc13-1 acts as a priming protein in insulin granule exocytosis.

In type-2 diabetes, there is an early loss of the first phase of insulin secretion (35). Our present results show that Munc13-1 is present in beta cells of both human and rat islets (Fig. 1), and, more importantly, that in the islets of type-2 diabetes Zucker fa/fa and GK rat models, Munc13-1 levels, along with syntaxin 1A and Munc18–1 levels, are greatly reduced. It is likely that the reduced levels of these proteins lead to the perturbed fine tuning of the various steps in the two-phase insulin granule secretion (docking, priming, and exocytotic fusion) observed in these diabetes models. Interestingly, Munc13-1 is more abundant in the beta cells of islets than in the non-beta cells, indicating that Munc13-1-mediated priming of secretory granules may be more important in beta cells than in non-beta cells. This would allow beta cells to respond dynamically to postprandial glycemic demands. Compared with chromaffin cells, islet beta cells possess a very small morphologically docked pool of secretory granules, and within this pool, an even smaller primed readily releasable granule pool (9–11). The secretory function of the islet beta cell would therefore be particularly sensitive to any reduction in Munc13-1 levels as observed here in two different diabetes models. Moreover, an increase in the priming of insulin granules to more effectively replenish the readily releasable pool by boosting Munc13-1 function (e.g. via gene therapeutic overexpression or pharmacological activation) would be of great therapeutic benefit in diabetes as it would effectively overcome the relative insulin secretory deficiency to the glycemic demand (35). As a proof of principle, we show here that overexpression of Munc13-1 in HIT and INS-1 cells leads to increased glucose-stimulated and depolarization-evoked insulin exocytosis (Figs. 4 and 5).

Munc13-1 function in insulin-secreting cells appears to be subject to complex regulation by the DAG second messenger system. In insulinoma cells, Munc13-1 translocates to the plasma membrane in a temperature-dependent manner (Fig. 3). Similarly, increased temperatures led to more pronounced facilitatory effects of Munc13-1 overexpression on insulin exocytosis from INS-1 cells (Fig. 5). This effect appears to be dependent on increased levels of DAG synthesized at high temperature because U73122, a specific phospholipase C inhibitor, blocked this temperature-dependent effect of Munc13-1 overexpression in a TPA-sensitive manner, and because the DAG-insensitive Munc13-1H567K mutant showed no such temperature dependence (Figs. 3 and 5). The overexpressed Munc13-1H567K-EGFP, although defective in its C domain and therefore insensitive to DAG regulation, is a functionally somewhat compromised but still active priming protein (25, 26), which explains the higher exocytotic response observed in cells overexpressing this mutant Munc13-1 as compared with EGFP-transfected control cells. We had observed similar effects with chromaffin cells overexpressing Munc13-1H567K-EGFP (22) and knock-in mutant nerve cells expressing Munc13-1H567K-EGFP (26). In islet beta cells, glucose leads to the generation of several fuels, including ATP, which activates KATP channels, as well as of DAG and long chain acyl-CoA, which may act directly on the exocytotic machinery (41). In diabetes, the derangement of glucose and lipid metabolism has adverse effects (gluco-lipotoxicity) on islet secretory function (13, 14, 38, 39), some of which may indeed involve abnormal expression or regulation and function of Munc13-1. This view would be supported by our finding that GK and Zucker diabetic rat models, which exhibit such derangement in glucose and lipid metabolism, show a reduction of Munc13-1 levels in their islets.

Our study demonstrates that Munc13-1 acts as a secretory granule priming protein in insulin exocytosis and that Munc13-1 may act as a key target of the DAG second messenger system in insulin-secreting cells. Of course, these findings do not exclude the possibility that PKC isoforms play important roles as DAG targets within islet beta cells by potentiating insulin secretion. Pharmacological approaches of activating PKCs using phorbol esters have demonstrated effects on not only insulin exocytosis (48) but also islet beta cell membrane ion channels, including Ca2+ and K+ channels (49, 50). A number of PKC variants, including phorbol ester-sensitive classical isoforms (α, β) were shown to translocate to the islet beta cell plasma membrane upon phorbol ester stimulation (51–53). However, these pharmacological data do not allow one to draw conclusions about the relative contribution of PKCs and Munc13s to DAG and phorbol ester effects in insulin secreting cells. In this context, genetic approaches may represent a promising way to resolve this issue. In fact, we have used genetically modified mice that express a DAG/phorbol ester binding-deficient Munc13-1H567K variant instead of the wild type protein to show that Munc13s are the main DAG/phorbol ester receptors in the regulation of transmitter release from hippocampal nerve cells (26). Similar studies in insulin-secreting cells, possibly also involving mutations in PKC genes, are likely to clarify the relative importance of PKCs and Munc13s for the regulation of insulin secretion.

Munc13 proteins interact with a number of synaptic proteins, including RIMs (23, 54). In RIM1-deficient neurons, Munc13-1 levels (and not those of other synaptic proteins) are reduced (54, 55), indicating that RIM1 may not only influence Munc13-1 function also act to maintain the stability of Munc13-1. RIMs are Rab3 effector proteins that were originally found to enhance neurotransmitter release (56). RIM1 and RIM2 are also expressed in the beta cells where they regulate insulin secretion (57, 58). RIM1 knock-out mice (55, 59) and in particular RIM-deficient C. elegans (60) exhibit reductions in vesicle priming and release probabilities that are similar to but milder than those observed in Munc13-1/Uncl3-deficient neurons. In addition, RIM1-deficient neurons were found to be deficient in mossy fiber long term synaptic plasticity, which involves a CAM/PKA-dependent signaling pathway, indicating that RIM1 may act as a PKA substrate in PKA-mediated mossy fiber long-term plasticity (59). In this context, it is interesting to note that RIMs also bind to CAMP-GEFII, a guanylic nucleotide exchange factor that is also essential for long term synaptic plasticity (58) and involved in the regulation of insulin exocytosis (61). It thus appears that islet beta cells contain a set of proteins involved in secretory vesicle priming that is also present and functional at nerve cell synapses. It will be important to examine whether additional as yet unidentified dys-regulations in the expression of or interactions between these priming proteins contribute to the abnormal insulin secretion in diabetic islets, and whether a therapeutic up-regulation of the expression or action of Munc13-1 or of its partners can improve insulin secretion from normal and diabetic islets.

Acknowledgments—We thank Yong Song for stimulating discussion, Jonathan Lakey and Mark Cattral for the human islets, Claes-Goran Ostenson for the GK rat islets, and Youhou Kang and Na He for technical assistance.
Regulation of Insulin Exocytosis by Munc13-1
Laura Sheu, Eva A. Pasyk, Junzhi Ji, Xiaohang Huang, Xiaodong Gao, Frederique Varoqueaux, Nils Brose and Herbert Y. Gaisano

J. Biol. Chem. 2003, 278:27556-27563.
doi: 10.1074/jbc.M303203200 originally published online May 13, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303203200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 61 references, 23 of which can be accessed free at http://www.jbc.org/content/278/30/27556.full.html#ref-list-1