

Munc13-1 is required for the sustained release of insulin from pancreatic β cells

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Summary

Munc13-1 is a presynaptic protein that is essential for synaptic vesicle priming. Deletion of Munc13-1/unc13 causes total arrest of synaptic transmission due to a complete loss of fusion-competent synaptic vesicles. The requirement of Munc13-1 for large dense-core vesicles (LDCVs), however, has not been established. In the present study, we use Munc13-1 knockout (KO) and diacylglycerol (DAG) binding-deficient Munc13-1^{H567K} mutant knockin (KI) mice to determine the role of Munc13-1 in the secretion of insulin-containing LDCVs from primary cultured pancreatic β cells. We show that Munc13-1 is required for the sustained insulin release upon prolonged stimulation. The sustained release involves signaling of DAG second messenger, since it is also reduced in KI mice. Insulin secretion in response to glucose stimulation is characterized by a biphasic time course. Our data show that Munc13-1 plays an essential role in the development of the second phase of insulin secretion by priming insulin-containing LDCVs.

Introduction

Insulin release from pancreatic β cells in response to glucose is characterized by a biphasic time course (Curry et al., 1968). Interestingly, diabetes is associated with disturbance in the biphasic pattern of insulin secretion, resulting in a blunted first phase and a diminished second phase (Kahn, 2001). Thus, it is of great importance to understand the mechanisms whereby the biphasic insulin response is generated and regulated. It has been suggested that the biphasic nature of insulin secretion reflects the release of spatially and functionally distinct insulin-containing LDCVs. It is postulated that the first phase of insulin secretion is attributable to the release of fusion competent LDCVs from a so-called readily releasable pool (RRP), and the second phase involves the recruitment of LDCVs to the RRP (Olofsson et al., 2002; Rorsman and Renström, 2003). However, as the generation of biphasic insulin release probably involves concerted action of multiple factors, the significance of different functional LDCVs in the contribution of biphasic insulin release under glucose stimulation remains to be clarified.

Munc13 proteins constitute a family of four mammalian homologs of *C. elegans* Unc-13 and *Drosophila* Dunc-13 (Koch et al., 2000). Genetic deletion of Munc13-1/unc13 causes total arrest of synaptic transmission due to a complete loss of fusion-competent synaptic vesicles (Augustin et al., 1999; Richmond et al., 1999). Munc13 proteins contain C2 domain and a DAG binding C1 domain. The binding of DAG/phorbol-ester to C1 domain leads to enhanced priming activity of Munc13-1 (Betz et al., 1998; Rhee et al., 2002). The requirement of Munc13s for LDCVs, however, has not been established. Although overexpression of Munc13-1 has been demonstrated to facilitate the release of LDCVs in chromaffin cells (Ashery et al., 2000) and pancreatic

β cells (Sheu et al., 2003), Munc13-1-deficient chromaffin cells are found to have normal secretion (Stevens et al., 2005). Munc13-1 expression level is considerably low in chromaffin cells (Ashery et al., 2000) but is abundant in β cells (Sheu et al., 2003). Recently, it has been reported that the levels of Munc13-1 are severely reduced in pancreatic islets from type 2 diabetic rats (Sheu et al., 2003) as well as in diabetic human islets (Ostenson et al., 2006). This study is aimed at defining the exact role of Munc13-1 in insulin secretion.

Results

Isolation of functional pancreatic β cells from newborn mice

Munc13-1-deficient mice do not feed and can be distinguished from wild-type (WT) animals shortly after birth by a lack of milk in their stomachs, weakness, and reduced breathing rate (Augustin et al., 1999). Mutant mice die within a few hours of birth. Hence, we had to isolate β cells from newborn mice. Islets isolated from pancreas of newborn mice have an average diameter of $72 \pm 3 \mu\text{m}$ ($n = 6$, see Figure S1 in the Supplemental Data available with this article online), generally much smaller than those from adult mice (diameter, $150 \pm 12 \mu\text{m}$, $n = 7$). The number of islets per pancreas is also smaller in newborn mice. As a consequence, the yield of pancreatic β cells is consistently low for newborn mice. It has been noticed that freshly isolated embryonic or newborn islets usually don't respond to glucose and that full glucose-dependent insulin release appears only during the second day after birth (Bliss and Sharp, 1994; Meneghel-Rozzo et al., 2004). A recent study has demonstrated that β cells in islet slices from newborn mice progressively gained the ability to respond to secretagogues after 2 days in culture

(Meneghel-Rozzo et al., 2004). Consistent with this finding, we found that isolated β cells from newborn mice apparently developed typical β cell characteristics including Na^+ channel inactivation and response to glucose (see Figure S1) after 3 days in culture. These results thus open up the possibility for future functional studies of insulin secretion employing lethal knockout (KO) mice.

Munc13-1 ablation impairs the sustained component of exocytosis

To avoid the complications of Ca^{2+} microdomains associated with Ca^{2+} influx and Ca^{2+} mobilization, exocytosis was elicited by flash photolysis of caged Ca^{2+} (Neher, 1998). Exocytosis was monitored as an increase in the whole-cell membrane capacitance (Cm). Cm increase in β cells has been mostly ascribed to the contribution of exocytosis from LDCVs (Braun et al., 2004). As shown in Figure 1A, the Cm increase in response to step-like $[\text{Ca}^{2+}]_i$ elevation starts with an exocytotic burst component (within 1 s after flash) followed by a linear sustained release in newborn mouse β cells. It is generally held that the exocytotic burst represents the release from RRP, and the sustained phase of secretion is thought to reflect the rate-limiting step of priming for fusion competence (Sørensen, 2004; Xu et al., 1998). Detailed examination of the exocytotic burst revealed a double exponential time course (inset of Figure 1A), indicating the presence of two releasable vesicle pools with distinct release kinetics (Sørensen, 2004). The exocytotic burst can be best described by a sum of two exponential components: a fast burst component (162.2 ± 9.8 fF, $n = 24$) with a time constant of ~ 50 ms, and a slow burst component (220.9 ± 20.5 fF) with a time constant of ~ 500 ms at around ~ 20 μM $[\text{Ca}^{2+}]_i$. The sustained component has a slope of 18.9 fF/s, corresponding to a supplying rate of 6 granules/s if we assume ~ 3 fF for one insulin-containing LDCV (Olofsson et al., 2002). The characteristics of these exocytotic components were not significantly different from previous studies on β cells from adult mice (Olofsson et al., 2002; Takahashi et al., 1997).

Similar $[\text{Ca}^{2+}]_i$ stimulation protocols were applied to β cells from Munc13-1-deficient mice, which were later confirmed by PCR genotyping (Augustin et al., 1999). In contrast to the complete loss of fusion-competent synaptic vesicles in Munc13-1 deletion mutants, surprisingly, we found that the burst component persists in Munc13-1-deficient β cells. The amplitude of the fast burst component averages 168.5 ± 15.7 fF ($n = 17$), which is indistinguishable from that of WT cells. The slow burst component has an average size of 159.0 ± 33.0 fF and is slightly smaller than that of WT cells. Importantly, we found that the sustained component was almost abolished in the absence of Munc13-1, resulting in an 81% inhibition to an average release rate of 3.6 ± 1.4 fF/s. This result indicates that Munc13-1 is essential for the sustained exocytosis but not required for the maintenance of the releasable LDCVs.

Munc13-1 deletion retards the recovery of RRP from depletion

To complement our flash experiments, we further applied paired trains of depolarization (1 Hz, 10 pulses from -70 to 0 mV) to elicit exocytosis. As shown in Figure 2, a train of depolarization elicits a Cm increase of ~ 150 fF, similar to what has been described in pancreatic β cells (Meneghel-Rozzo et al., 2004; Kanno et al., 2004). Interestingly, when the cumulative increase in Cm was

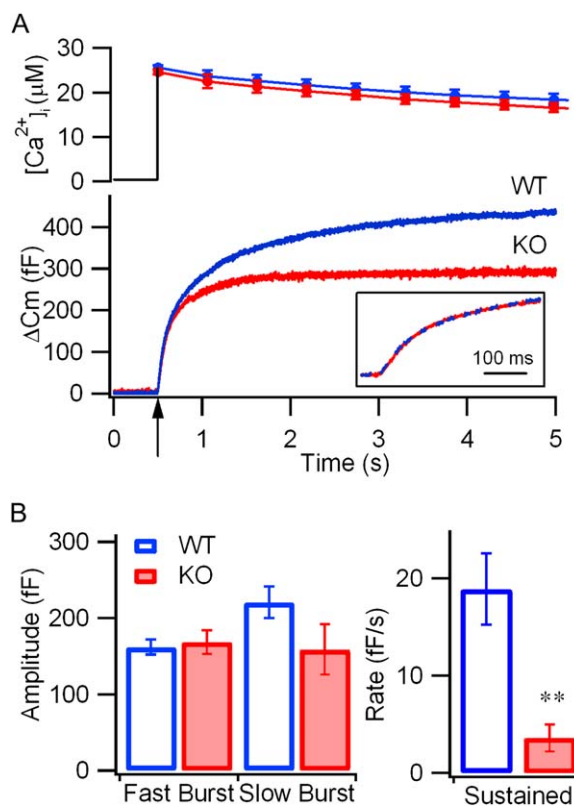


Figure 1. Munc13-1 knockout abolishes the sustained release from pancreatic β cells

A) Averaged $[\text{Ca}^{2+}]_i$ and Cm responses from WT ($n = 24$) and Munc13-1 knockout (KO, $n = 17$) cells. Inset compares the time course of the exocytotic burst in expanded time scale.

B) Averaged amplitudes of the exocytotic burst and sustained components for WT and KO β cells. The Cm response was fitted by triple exponentials and the amplitudes of the two fast components were taken as the size of the fast burst and slow burst component, respectively. The release rate of the sustained component was quantified by a line fit to the 2–5 s of the Cm trace. Values represent the mean \pm SEM. Asterisks denote significant differences (t test, $**p < 0.01$) compared with WT.

compared between WT and KO cells (Figure 2B), we found that Cm increment during the first two pulses was almost identical, whereas the responses to the later pulses were significantly reduced in the KO cells. We reasoned that the Cm increment during the first two pulses represents exocytosis from a subset of RRP close to the Ca^{2+} channels (Kanno et al., 2004), whereas the later responses may also include refilling process to the RRP. The sum of the first two Cm increment recovered after depletion with a τ of 24.8 s (Figure 2C), very similar to that reported previously (Gromada et al., 1999). Munc13-1 deletion significantly retarded this recovery in β cells as shown in Figure 2C.

DAG signaling is involved in the development of the sustained component

Glucose is known to activate intracellular DAG signaling among many other targets (Nesher et al., 2002). Munc13-1 contains a DAG/phorbol-ester binding C1 domain and is a potential target of DAG signaling (Betz et al., 1998). In order to examine the significance of the Munc13-1 C1 domain in insulin secretion, we further examined LDCV exocytosis in genetically modified mice that express a mutant Munc13-1^{H567K} variant. The H567K mutation in

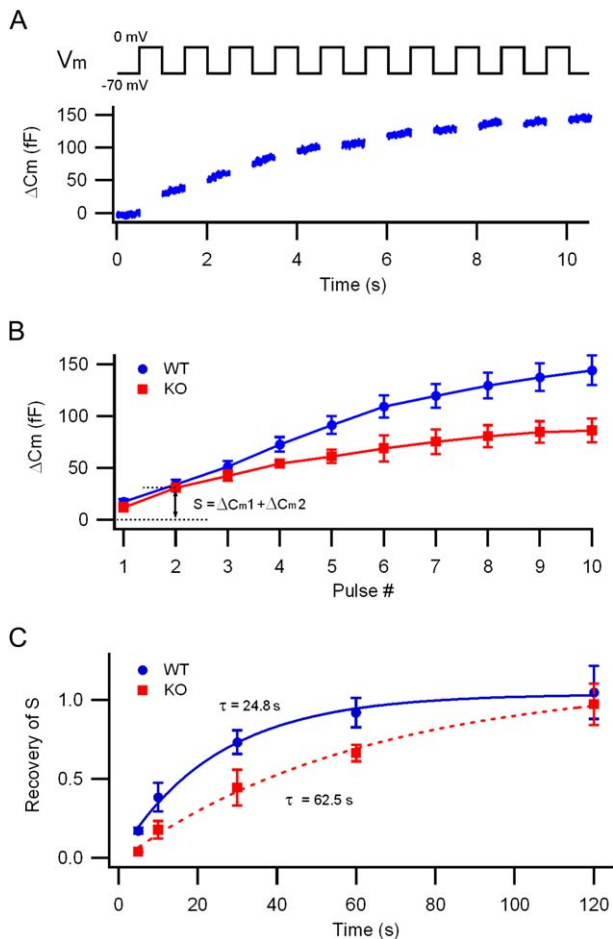


Figure 2. The recovery of RRP after depletion is retarded in KO cells

A) Example Cm increase elicited by a train of ten 500 ms depolarization pulses from -70 to 0 mV applied at 1 Hz.

B) Averaged cumulative Cm increments in response to trains of depolarization pulses from WT ($n = 41$) and Munc13-1 KO cells ($n = 32$).

C) Comparison of the recovery of S values between WT and KO cells. Two trains of depolarization pulses separated by different intervals from 5 – 120 s were applied to the same cell. S equals to the Cm increment during the first two pulses as depicted in **(B)**. The S value from the second train was normalized to that of the first train and plotted versus time intervals. The time constants of recovery are indicated.

C1 domain abolishes the binding of DAG/phorbol-ester (Betz et al., 1998). Like Munc13-1 deletion mutants, the homozygous Munc13-1^{H567K} mutant mice deteriorate rapidly and die within several hours after birth (Rhee et al., 2002). We found that Munc13-1^{H567K} knockin (KI) also resulted in a significant blockade of the sustained exocytosis from β cells (sustained release rate, 8.6 ± 2.0 fF/s, $n = 18$, Figure 3), an effect similar to but less severe than what we observed for the KO mice. Likewise, the exocytotic burst from KI β cells (fast burst, 153.4 ± 14.4 fF; slow burst, 156.5 ± 35.8 fF) was not statistically different from WT values. Phorbol esters have been shown to augment insulin secretion from pancreatic β cells (Zawalich et al., 1983; Ämmälä et al., 1994). However, the targets of phorbol ester action remain to be clarified. We examined the consequences of the Munc13-1^{H567K} mutation on phorbol-12-myristate-13-acetate- (PMA)-mediated augmentation of exocytosis. We found that application of 100 nM PMA augmented the Ca^{2+} -stimulated exocytosis in WT but not KI β cells (Figure 3). We thus conclude that DAG

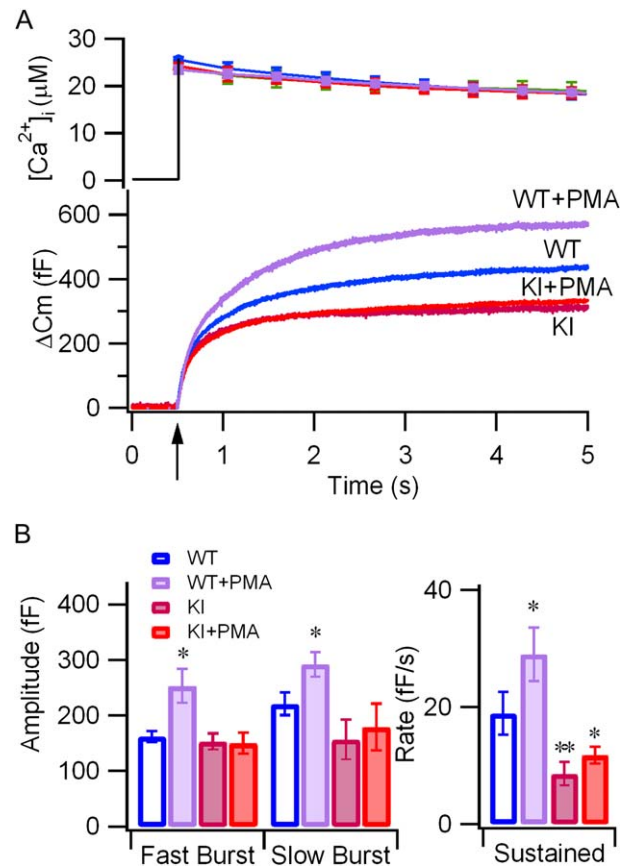


Figure 3. DAG/phorbol-ester binding C1 domain of Munc13-1 is involved in the sustained release from pancreatic β cells

A) Averaged $[Ca^{2+}]_i$ and Cm responses from WT ($n = 24$) and KI cells ($n = 18$), in the absence and presence of 100 nM PMA (WT + PMA, $n = 14$; KI + PMA, $n = 15$).

B) Comparison of the averaged amplitudes of the exocytotic burst and sustained components, which were calculated as explained in Figure 1. Values represent the mean \pm SEM. Asterisks denote significant differences (paired t test, ** $p < 0.01$, * $p < 0.05$) compared with WT.

signaling is involved in the development of the sustained component and one of the main targets of PMA in augmenting insulin secretion is Munc13-1.

Munc13-1 acts upstream of the Ca^{2+} -triggering step

To test whether Munc13-1 acts downstream of priming, we analyzed the kinetics of the exocytotic burst from β cells. As shown in the inset of Figure 1A, the normalized exocytotic burst from KO mice is almost identical to that of WT. We further plotted the individual rate constants of the burst component versus the postflash $[Ca^{2+}]_i$ levels in Figure S2. Clearly, the rate constants and their dependence on $[Ca^{2+}]_i$ were indistinguishable between WT, KO, and KI mice. The steep $[Ca^{2+}]_i$ dependence of the rate constants agrees with previous studies in β cells (Takahashi et al., 1997). These results indicate that Munc13-1 acts upstream of the Ca^{2+} -triggered fusion step.

Munc13-1 is required for the second phase of insulin release induced by glucose

To verify whether the phenotype we saw in the Cm signal correlates indeed with insulin secretion, we directly measured the time course of insulin secretion from isolated islets. The isolated

islets were first bathed in KRBB solution for 1 hr to record the basal release rate, and then were subjected to 30 min stimulation with glucose (17.5 mM). As shown in Figure 4, glucose stimulation elicited a biphasic insulin secretion typical for mouse pancreatic islets (Zawalich and Zawalich, 2001). The insulin release rate per islet is lower than that reported for adult mice (Zawalich and Zawalich, 2001), perhaps reflecting the fact that the islets from newborn mice are smaller. Indeed, the insulin content was measured to be 1.07 ng/islet for newborn mice and 130.9 ng/islet for adult mice. Interestingly, lack of Munc13-1 preferentially inhibited the sustained phase of insulin release by 71%, in contrast to a 34% inhibition in the peak release rate 2 min after glucose stimulation (Figure 4). It should be noted that the peak release rate at 2 min should include the combination of release from both the RRP as well as some of the refilling LDCVs. As for KI mice, the second phase of insulin release was impaired to a lesser extent. Consistent with our Cm measurements, PMA treatment augmented the biphasic insulin release from WT but not KI mice.

Discussion

In the present study, we determined the role of Munc13-1 in insulin secretion from pancreatic β cells using Munc13-1-deficient mice and DAG binding-deficient Munc13-1^{H567K} mutant KI mice. Cm measurements combined with flash photolysis, which has the advantage of monitoring secretion from the whole population of vesicles with millisecond time resolution, was employed to assess the exocytosis from different pools of LDCVs. We found that the sustained component of exocytosis was almost completely abolished in Munc13-1-deficient β cells, and to a lesser extent in KI cells. To our surprise, the exocytotic burst, which represents the releasable vesicles, was not significantly influenced. One concern of our Cm data is that the reduction in the sustained component could be due to an increase in endocytosis. However, this possibility is quite unlikely because: 1) we have directly measured the insulin release and found the sustained release is indeed reduced in KO/KI cells (Figure 4); 2) the recovery of RRP after depletion, which is unlikely to be contaminated by endocytosis, is also significantly retarded in KO cells (Figure 2). We thus conclude that Munc13-1 is required for the second phase of insulin secretion in response to glucose stimulation.

Although Munc13-1 is absolutely necessary for neurotransmitter release from synaptic vesicles (Augustin et al., 1999; Richmond et al., 1999), the requirement of Munc13-1 for LDCV exocytosis has not been demonstrated. A recent study in chromaffin cells, which serves as a perfect model for studying LDCV exocytosis, has revealed that the endogenous Munc13-1 is very low, and Munc13-1-deficient chromaffin cells have normal LDCV exocytosis (Stevens et al., 2005). We now show that Munc13-1 is required for sustained insulin secretion from LDCV in pancreatic β cells. In contrast to the complete loss of fusion-competent synaptic vesicles in synapses (Augustin et al., 1999), however, we found that the releasable LDCVs persist in the Munc13-1-deficient β cells. This discrepancy can be explained by a different mechanism in the maintenance of primed vesicles, such that LDCVs are more stable in the primed state whereas synaptic vesicles turnover rapidly in the absence of Munc13-1. It is conceivable that Munc13-1 is required for accelerating (or catalyzing) the refilling of LDCVs in response to demanding stimulation but not

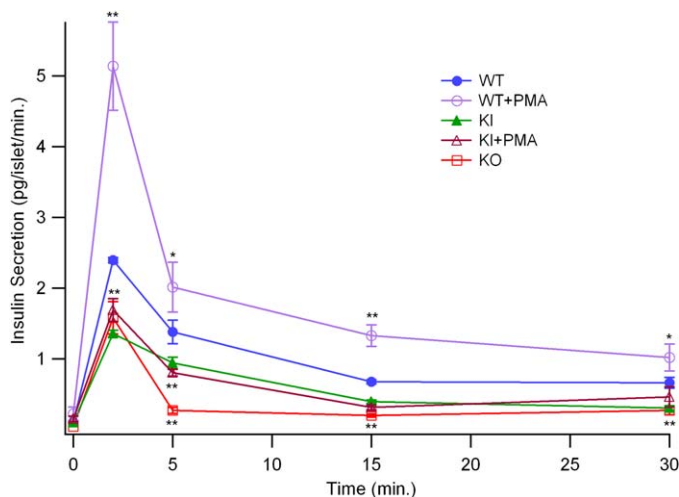


Figure 4. Biphasic insulin response to glucose stimulation from isolated islets. Insulin release was assayed by ELISA and expressed as pg/islet/min. 20 islets from newborn WT (n = 4), KO (n = 4), and KI (n = 4) mice were manually selected, incubated in KRBB solution for 1 hr, followed by glucose (17.5 mM) stimulation for 30 min. PMA (200 nM) was added 30 min prior to the stimulation and remained throughout stimulation. Data points were sampled before stimulation (0 min), and at 2, 5, 15, and 30 min after stimulation. Values represent the mean \pm SEM. Asterisks denote significant differences (paired t test, *p < 0.05; **p < 0.01) compared with WT.

required for the slow refilling at resting conditions. Alternatively, other Munc13 isoforms, such as Munc13-2, may compensate the lack of Munc13-1, although we cannot reliably detect Munc13-2 expression in pancreatic islets (data not shown).

The mechanism underlying the generation of the biphasic insulin response has been a matter of debate. It has been suggested that the first phase of insulin secretion is attributable to the release of vesicles from the RRP, while the second phase involves the mobilization and priming of granules from a reserve pool (Rorsman and Renström, 2003). This view, however, has been questioned because the biphasic response to glucose occurs over a lengthy period of time in minutes (Nesher et al., 2002), whereas the RRP and sustained release happen within seconds (Olofsson et al., 2002; this study). The generation of biphasic insulin release probably involves a concerted action of multiple factors, i.e., the time course of glucose input, the waveform of $[Ca^{2+}]_i$ signals, the generation of second messengers, the availability of releasable granules and recruitment of granules, the diffusion of insulin after exocytosis, etc. It is thus very important to evaluate the relative contribution of different factors in the production of the biphasic insulin release by specific interference such as genetic approach (Berggren et al., 2004; Jing et al., 2005). We now show that genetic ablation of Munc13-1 preferentially abolishes the second phase of insulin response to glucose as well as the sustained component of exocytosis measured by Cm increase, providing direct evidence that the priming of LDCVs from an unprimed pool constitutes a rate-limiting step in the second phase of insulin release.

Nonmetabolic stimuli (e.g., arginine, which is a membrane-depolarizing agent that promotes Ca^{2+} influx) elicit only first-phase insulin response with minimal or no second-phase response. Given in combination with a submaximal concentration of glucose (10 mM), arginine stimulation can produce a biphasic response (Nesher et al., 2002). Moreover, carbamyl choline

(CCh) produces a biphasic insulin response at substimulatory glucose levels but no response at all in the absence of glucose (Nesher et al., 2002). CCh has been shown to recruit Ca^{2+} via activating IP3 and accelerates granule recruitment (Gromada et al., 1999). These results suggest that metabolic signals (e.g., DAG) other than the membrane-depolarizing signal are required for the production (or amplification) of the second phase. Our results showing that the sustained insulin release is impaired in DAG binding-deficient Munc13-1^{H567K} KI mice suggest that glucose-activated DAG signaling is involved in the second-phase insulin response to glucose and that Munc13-1 acts as a key target of DAG/phorbol-ester. It is interesting that the effect of PMA is completely abolished in the Munc13-1 KI mice, seemingly to suggest that PKC activation by PMA plays no role in the control of exocytosis in mouse β cells. However, an alternative explanation could be that PKC acts upstream of Munc13-1 (Arkhammar et al., 1994). Also, it should be noted that significant differences exist between rodent species regarding the responses to glucose and the role of PKC in insulin secretion (Zawalich and Zawalich, 2001). For example, PKC inhibitors staurosporine or Gö 6976 significantly impaired insulin release in response to glucose from rat but not mouse islets (Zawalich and Zawalich, 2001). The conclusive role of PKC in insulin secretion will have to wait for further detailed studies through combined efforts from genetic manipulation to integrated physiological analysis.

Experimental procedures

Preparation of newborn mouse pancreatic β Cells and solutions

Munc13-1-deficient and Munc13-1^{H567K} mutant mice were obtained by interbreeding of animals heterozygous for the Munc13-1 deletion and Munc13-1^{H567K} mutation, respectively. Mice were routinely genotyped by PCR as previously described (Augustin et al., 1999; Rhee et al., 2002). Pancreas was dissected from newborn mice and placed in filtered KRBB solution (in mM, 136 NaCl, 4.7 KCl, 1.2 KH_2PO_4 , 5 NaHCO_3 , 2.5 CaCl_2 , 1.2 MgSO_4 , 3 glucose, 10 HEPES, 1 mg/ml BSA, [pH 7.2]). To isolate islets, the gland was incubated in 4 ml of 1 mg/ml Collagenase V (Sigma C-9263) solution at 37°C for 5 min, followed by centrifugation after addition of 8 ml KRBB solution. Pancreatic islets were collected by hand and dissociated to single cells by digestion with Dispase II (Roche #165859, Mannheim, Germany) and DNase I (Sigma DN25). Dispersed β cells were kept in DMEM supplemented with 25 mM HEPES, 2 g/L NaHCO_3 , 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 10% fetal calf serum (Gibco, Grand Island, NY) and used within 3–4 days after plating.

Standard bath solution for the experiments contained (in mM): 138 NaCl, 5.6 KCl, 1.2 MgCl_2 , 2.6 CaCl_2 , 3 Glucose, 5 Hepes (pH 7.2, 310 mosm). Standard intracellular solution consisted of (in mM): 110 Cs-Glutamate, 2 MgATP, 0.3 GTP, 35 Hepes. For the flash experiments, 5 mM DM-nitrophen (80% loaded with Ca^{2+}) and 0.2 mM fura-6F were added ([pH 7.2], 300 mosm). The basal $[\text{Ca}^{2+}]_i$ was measured to be around 200 nM by fura-2. DM-nitrophen, fura-2, fura-2 AM, and fura-6F were purchased from Molecular Probes (Eugene, Oregon). Unless otherwise stated, all chemicals were from Sigma-Aldrich (St. Louis, Missouri).

Cm measurement

We selected cells with diameters > 11 μm for study, so that >80%–90% of the cells were expected to be β cells (Rorsman and Trube, 1986). Occasionally, the selected cells were validated by their $[\text{Ca}^{2+}]_i$ response to glucose (see Figure S1). Conventional whole-cell recordings were performed at 30°C using silyard-coated 3–4 M Ω pipettes. Series resistances ranged from 4–12 M Ω were included in the analysis. Cm was measured using an EPC-10 patch-clamp amplifier (HEKA, Lambrecht, Germany) controlled by the lock-in module of PULSE software, as previously described (Bai et al., 2006). The capacitance traces were imported to Igor Pro (WaveMetrics, Lake Oswego, Oregon) and were analyzed with triple exponential fitting (Bai et al., 2006). The sustained component of release was measured as

the slope of Cm increase between 2 and 5 s after each flash. Cm traces with fast endocytosis (Heinemann et al., 1994) were not included in the analysis.

Measurements of $[\text{Ca}^{2+}]_i$ and Photolysis of Caged Ca^{2+}

Flashes of ultraviolet light and fluorescence-excitation light were generated as described previously (Xu et al., 1998). In the flash experiments, exocytosis was elicited by photorelease of caged Ca^{2+} preloaded into the cell via the patch pipette. $[\text{Ca}^{2+}]_i$ was measured with the Ca^{2+} indicator dyes fura-2 or fura-6F. $[\text{Ca}^{2+}]_i$ was determined from the ratio (R) of the fluorescence signals excited at the two wavelengths (340/380 nm), following the equation (Grynkiewicz et al., 1985): $[\text{Ca}^{2+}]_i = K_{\text{eff}} \cdot (R - R_{\text{min}}) / (R_{\text{max}} - R)$, where K_{eff} , R_{min} , and R_{max} are constants obtained from intracellular calibration as previously described (Xu et al., 1998).

ELISA insulin secretion

For assay of insulin release, 20 islets from one animal were preincubated for 60 min at 30°C in 200 μl KRBB solution (see above). Then a portion of the solution (70 μl) was withdrawn for measuring the basal insulin release. The rest solution was added to a total volume of 300–400 μl KRBB solution at a final glucose concentration of 17.5 mM. At each measuring time point (2, 5, 15, and 30 min.), 70 μl incubation medium was withdrawn for insulin measurement after gentle agitation and was replaced by 70 μl fresh KRBB solution supplemented with 17.5 mM glucose. All the operation was conducted under a dissection microscope to avoid damaging islets. Insulin was quantified by ELISA with commercially available kits according to the manufacturer's specifications (Rat/Mouse Insulin ELISA Kit; Linco Research, St. Charles, Missouri).

Supplemental data

Supplemental data include two figures and <http://www.cellmetabolism.org/cgi/content/full/3/6/463/DC1/>.

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