

RESEARCH ARTICLE

Bulk-like endocytosis plays an important role in the recycling of insulin granules in pancreatic beta cells

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ABSTRACT

Although bulk endocytosis has been found in a number of neuronal and endocrine cells, the molecular mechanism and physiological function of bulk endocytosis remain elusive. In pancreatic beta cells, we have observed bulk-like endocytosis evoked both by flash photolysis and trains of depolarization. Bulk-like endocytosis is a clathrin-independent process that is facilitated by enhanced extracellular Ca^{2+} entry and suppressed by the inhibition of dynamin function. Moreover, defects in bulk-like endocytosis are accompanied by hyperinsulinemia in primary beta cells dissociated from diabetic KKAy mice, which suggests that bulk-like endocytosis plays an important role in maintaining the exo-endocytosis balance and beta cell secretory capability.

KEYWORDS bulk-like endocytosis, clathrin-independent endocytosis, dynamin, diabetic KKAy mice

INTRODUCTION

After secretory vesicles fuse with the plasma membrane, membrane retrieval must occur to maintain a constant cell size and to facilitate the reuse of vesicular membrane components. In the past three decades, at least three types of vesicle recycling processes have been found in excitable cells, such as neuronal and endocrine cells. These processes include clathrin-dependent endocytosis and two types of clathrin-independent endocytosis: kiss and run endocytosis and bulk endocytosis (Royle and Lagnado, 2003). Among

these clathrin-independent types of membrane retrieval, bulk endocytosis is a relatively new concept, which was first proposed by Takei et al. in 1996 (Clayton and Cousin, 2009). Using electron microscopy to observe nerve terminals, Takei et al. observed stimulation-evoked internalization of larger areas of plasma membrane as endosomes from which multiple vesicles could subsequently bud off. Unlike clathrin-mediated endocytosis, bulk retrieval is often triggered by high-frequency membrane depolarization (Clayton et al., 2008), and its formation does not directly involve clathrin (Royle and Lagnado, 2003). Bulk endocytosis has been reported in various neuronal systems, including hippocampal neurons (Takei et al., 1996), retinal bipolar cells (Paillart et al., 2003), neuromuscular junctions (Teng et al., 1999; Richards et al., 2000), and the calyx of Held (Wu and Wu, 2007). Despite the widespread existence of bulk endocytosis cell types, the functional significance of bulk endocytosis in specific physiological processes has not been demonstrated.

In pancreatic beta cells, stimulated insulin secretion has been found to be coupled with enhanced vesicle recycling processes (Orci et al., 1973). Recently, we have shown that elevated $[Ca^{2+}]_i$ triggers both clathrin-dependent slow endocytosis and clathrin-independent fast endocytosis in pancreatic beta cells (He et al., 2008). However, whether clathrin-independent endocytosis plays a role in the recycling of insulin vesicles under physiologically relevant stimulation remains to be determined.

In the present study, we show that both flash photolysis and depolarization pulses evoke bulk-like endocytosis in primary pancreatic beta cells. The frequency but not the amplitude of the bulk-like retrieval events is increased by an

increase in the intracellular Ca^{2+} concentration and inhibited by dynasore (dynamin blocker). In addition, we found significant inhibition of depolarization-induced bulk-like endocytosis in hyperinsulinemic beta cells isolated from diabetic KKAY mice compared with age-matched C57 controls, which suggested a role of bulk-like endocytosis in maintaining beta cell function during the development of diabetes.

RESULTS

Flash photolysis and depolarization can elicit clathrin-independent bulk-like endocytosis

Similar to a previous report from our group (He et al., 2008), homogeneously elevating $[\text{Ca}^{2+}]_i$ in primary beta cells via controlled UV photolysis ("uncaging") of 1-(4,5-dimethoxy-2-nitrophenyl)-1,2-diaminoethane-N,N,-N',N'-tetraacetic acid (DMNP-EDTA) evoked excessive membrane retrieval, which contains a fast and a slow component (Fig. 1A). By magnifying the capacitance trace in the time scale, step-like decreases in capacitance were frequently observed in the rapid declining phase (Fig. 1A). These downward capacitance shifts were not accompanied by the alteration of membrane conductance (Gm, Fig. 1A) and may occur concomitantly with a transient decrease in series conductance (Gs, Fig. 1A). The electrophysiological characteristics shown in Fig. 1 have been defined as bulk-like endocytosis (Wu and Wu, 2007).

Because similar responses were also obtained in insulin-secreting INS-1 cells (Fig. 1B), we performed molecular manipulations in INS-1 cells. An average step-like capacitance decline of 19 ± 0.7 fF was recorded in cells with levels of $[\text{Ca}^{2+}]_i$ elevated to approximately 20 $\mu\text{mol/L}$, which corresponded to the scission of a vacuole of approximately 780 nm in diameter (assuming 1 fF/ μm^2 as the specific membrane capacitance). When we knocked down clathrin in INS-1 cells (He et al., 2008), the capacitance drops persisted with similar amplitude (19 ± 1 fF) and frequency (clathrin shRNA, 0.54 ± 0.11 event/s, $n = 15$; control, 0.51 ± 0.04 event/s, $n = 40$), which confirmed their association with clathrin-independent endocytosis (Fig. 1D). This clathrin-independent endocytosis is obviously different from the "cavcapture" model, in which similar increases and decreases in capacitance are observed during large fusion pore flickering (MacDonald et al., 2006).

We also tested whether bulk-like endocytosis occurred under physiological-relevant stimulation. To record bulk-like endocytosis, we used trains of 200 ms depolarization (1 Hz) from -70 mV to 0 mV to repetitively depolarize primary pancreatic beta cells (using an interval of 10 s after each sweep), thereby simulating the membrane potential oscillation of the beta cells during blood glucose elevation. Indeed, bulk-like endocytosis was observed in 39.3% of the beta cells (12/28 cells) under the standard whole-cell configuration (an example is shown in Fig. 2). The time required for bulk-like endocytosis to occur was variable, and bulk-like retrieval events can be observed during

both the depolarization sweep (synchronized) and the interval between different sweeps (asynchronized). We also directly tested whether physiological stimulation induced bulk-like endocytosis *in vivo* using electron microscopy. Occasionally, large invaginations connected to the plasma membrane could be observed after intact islets were stimulated with KCl (Fig. 2D). More frequently, fused chain of insulin granules were observed in the primary beta cells (Fig. 2A, arrow), similar to previous reports (Kwan and Gaisano, 2005; Hoppa et al., 2012). They collapse into the plasma membrane and release insulin in a multiple-granular manner (Fig. 2C) and can be retrieved back as a whole unit (Fig. 2B, arrowhead), which may suggest a type of bulk-like membrane retrieval.

Depolarization-induced bulk-like endocytosis is Ca^{2+} and dynamin dependent

Wu et al. demonstrated that Ca^{2+} initiates bulk-like endocytosis during depolarization at calyx nerve terminals (Wu et al., 2009). Similarly, we attempted to determine whether the bulk-like endocytosis in primary beta cells depends on the amount of calcium that enters the cells. A depolarization train evokes an approximately 20% greater capacitance in a 10 mmol/L Ca^{2+} bath solution compared with a 2.6 mmol/L Ca^{2+} bath solution (Fig. 3A and 3B). In contrast, depolarization in a 10 mmol/L Ca^{2+} bath solution evoked an approximately 2.5-fold increase in the frequency but not the average amplitude of bulk-like endocytosis (the mean frequency was 0.010 ± 0.003 Hz in 2.6 mmol/L Ca^{2+} compared with 0.025 ± 0.005 Hz in 10 mmol/L Ca^{2+} , $P < 0.05$) (Fig. 3C and 3D). The increased number of bulk-like events was primarily due to an increase in the bulk retrieval during the intervals between the depolarization trains ($P = 0.08$, Fig. 3E), which suggested that bulk-like endocytosis is a delayed mechanism that is triggered by prolonged Ca^{2+} elevation.

Dynamin is a large GTPase that is principally involved in the scission of newly formed vesicles from the membrane (Cao et al., 1998). Dephosphorylation of dynamin I has been demonstrated to participate in activity dependent bulk endocytosis in neurons. To verify the role of dynamin in bulk-like endocytosis in pancreatic beta cells, we preincubated beta cells in RPMI 1640 with 80 $\mu\text{mol/L}$ dynasore for 30 min, which potentially inhibits dynamin function (Macia et al., 2006; Newton et al., 2006). Thereafter, cells were transferred to a recording chamber containing 10 mmol/L Ca^{2+} and 80 $\mu\text{mol/L}$ dynasore (to prevent a washout effect). In contrast to previous reports (Tsai et al., 2009; Wu et al., 2009), short-term pretreatment with dynasore did not inhibit exocytosis. Instead, depolarization evoked an approximately 2-fold increase in capacitance in the 10 mmol/L Ca^{2+} solution (Fig. 3A and 3B) and significantly reduced the frequency of the delayed bulk-like event (Fig. 3D and 3E). Given that knocking-down of clathrin did not affect bulk-like endocytosis (Fig. 1), dynamin may play a clathrin-independent facilitatory role in stimulation-activated bulk-like endocytosis in primary beta cells.

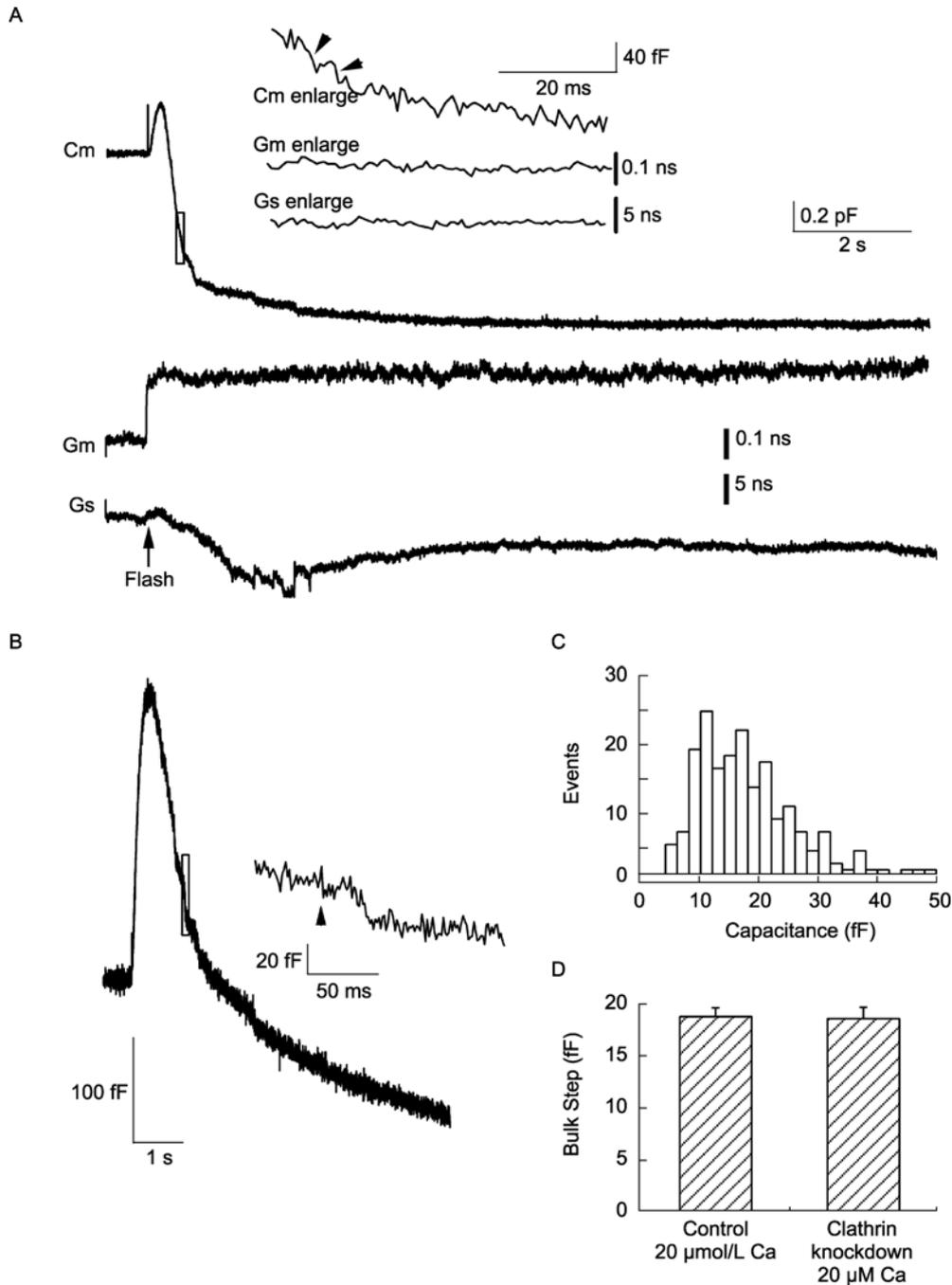


Figure 1. Flash photolysis can elicit clathrin-independent bulk-like endocytosis. (A and B) An example of UV photolysis-induced fast decay in the capacitance of primary beta cells and INS-1 cells. The trace enclosed in the red box is enlarged in the inset, which shows a single step-like decrease in capacitance. (C) The size histogram of the step-like capacitance declines in INS-1 cells after a homogenous increase in $[Ca^{2+}]_i$ to approximately 20 $\mu\text{mol/L}$. (D) A summary of the average step-like capacitance decreases in control INS-1 cells ($[Ca^{2+}]_i$ approximately 20 $\mu\text{mol/L}$, $n = 203$) and in INS-1 cells where clathrin expression was knocked down ($[Ca^{2+}]_i$ approximately 20 $\mu\text{mol/L}$, $n = 81$).

Impairment of the bulk-like endocytic mechanism in beta cells from diabetic KKAY mice

Insulin secretion from pancreatic beta cells plays a crucial

role in the regulation of blood glucose homeostasis. Therefore, it is well accepted that type II diabetes involves a perturbation of the exocytotic machinery of pancreatic beta cells (Eliasson et al., 2008). However, whether changes in the

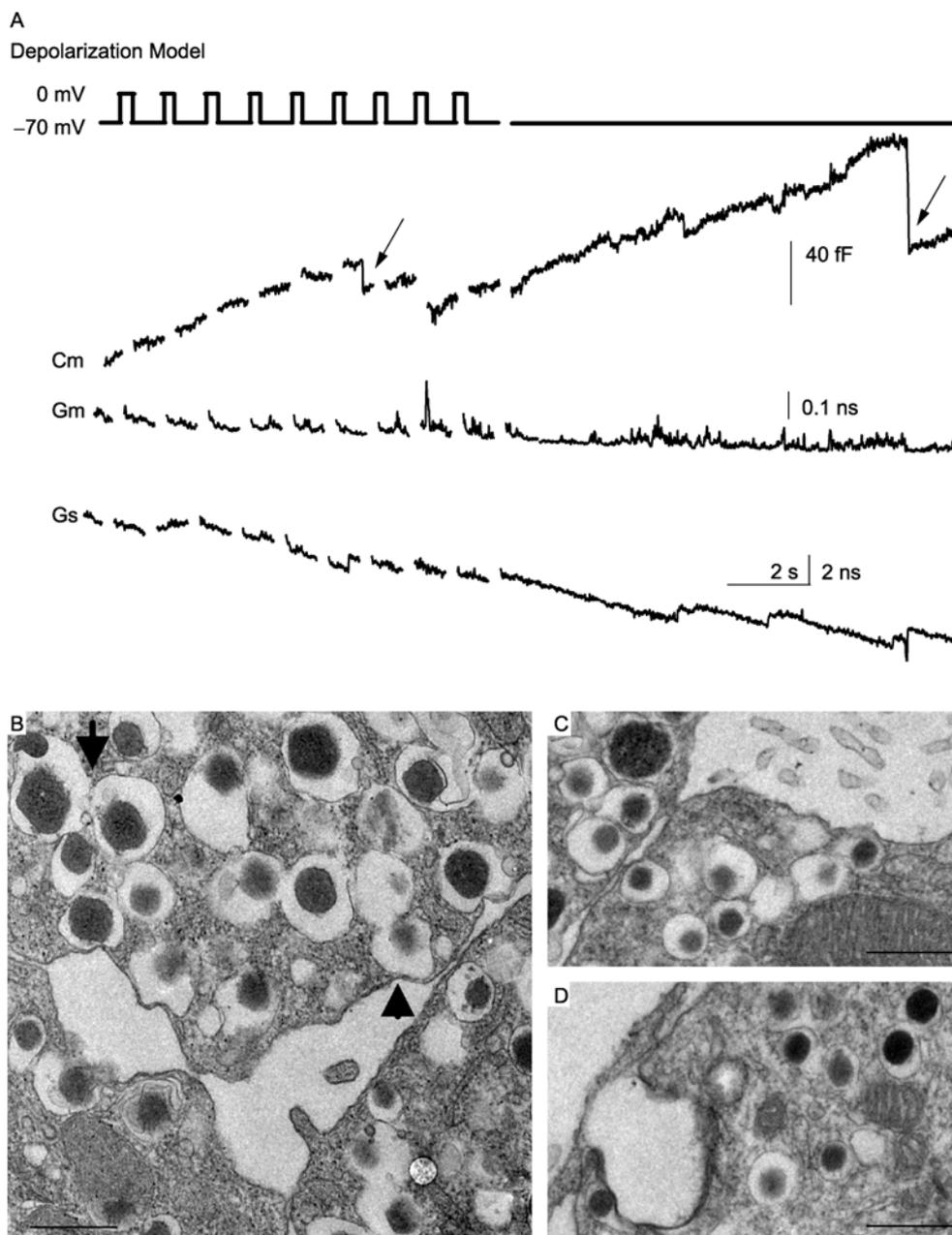


Figure 2. Depolarization elicit bulk-like endocytosis in pancreatic β cells. (A) Trains of ten 200-ms pulses (frequency 1 Hz) induced a capacitance jump (C_m), except for abrupt bulk endocytosis (arrows). The measured values of G_m and G_s are shown below the figure. (B) After 5 min of 20 mmol/L glucose stimulation, two insulin granules are contacted and fused with each other (indicated by arrow) and three fused granules are retrieval together after the release of the contents (indicated by arrow head). (C) Secretory granules fuse together (also partially emptied) after 70 mmol/L KCl stimulation for 5 min. (D) Large invagination connected to the plasma membrane after 70 mmol/L KCl stimulation. Scale bar: 500nm in all EM figures.

membrane recycling process participate in such pathological processes has not been investigated. We used yellow-coated KKAY mice that exhibit spontaneous hyperglycemia and glucose intolerance at 8 weeks of age (Srinivasan and Ramarao, 2007). Consistent with a previous report (Srinivasan and Ramarao, 2007), KKAY mice had greater body weight than

age-mated C57Bl/6 controls (Fig. 4A) and exhibited impaired glucose tolerance and pronounced insulin resistance at 10 weeks (Fig. 4B and 4C). Compared with the control group, depolarization evoked a greater than two-fold increase in capacitance in KKAY beta cells in both 2.6 mmol/L and 10 mmol/L Ca^{2+} bath solutions (Fig. 4D and 4E), which is

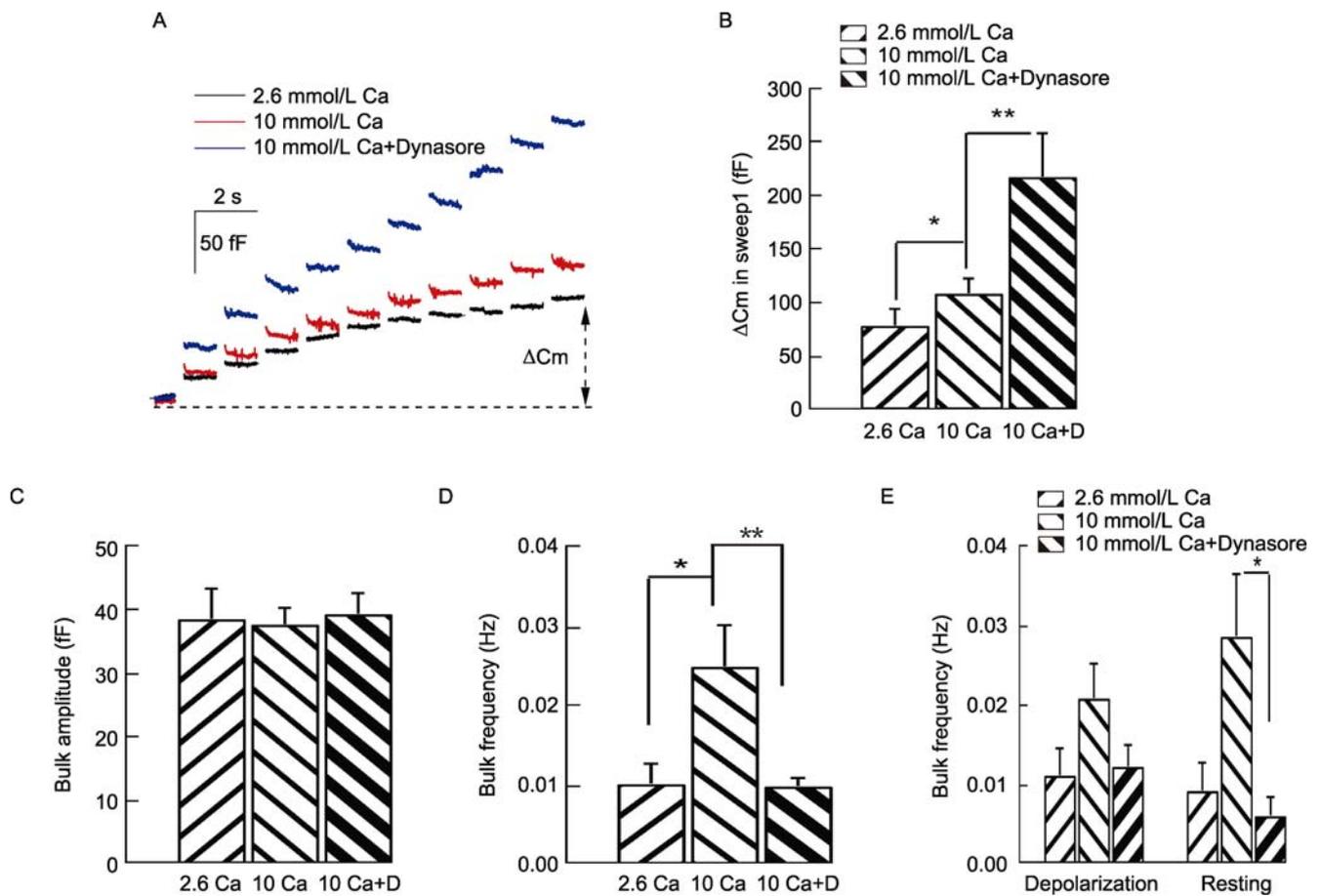


Figure 3. Depolarization-induced bulk-like endocytosis is Ca^{2+} and dynamin dependent. (A) The average C_m traces induced by ten 200-ms pulses (frequency 1 Hz) in 2.6 mmol/L Ca^{2+} (black), 10 mmol/L Ca^{2+} (red) or 10 mmol/L Ca^{2+} plus 80 $\mu\text{mol/L}$ dynasore (blue) (2.6 mmol/L Ca^{2+} , $n = 28$; 10 mmol/L Ca^{2+} , $n = 31$; 10 mmol/L Ca^{2+} plus 80 $\mu\text{mol/L}$ dynasore, $n = 17$). (B) The average capacitance jump (ΔC_m) in the first sweep. (C and D) The average bulk amplitude and frequency under the three conditions (* $P < 0.05$, ** $P < 0.01$). (E) The average bulk frequency during the depolarization time and the resting time (* $P < 0.05$).

consistent with the beta cell hyperinsulinemia reported at an earlier stage in diabetic animals or patients (Homo-Delarche, 1997; Liang et al., 2011). Although the mean amplitude of bulk-like endocytosis was not changed, markedly fewer bulk-like retrieval events were observed in the diabetic beta cells stimulated in either 2.6 mmol/L or 10 mmol/L Ca^{2+} bath solutions (Fig. 4F and 4G). Taken together, these data suggest that bulk-like endocytosis is severely impaired in pancreatic beta cells isolated from diabetic KKAy mice.

DISCUSSION

Eliasson et al. was the first author to report the induction of endocytosis by transient elevation of cytosolic calcium in mouse pancreatic beta cells, including large amplitude capacitance fluctuations that resemble bulk endocytosis (Eliasson et al., 1996). Our data corroborate their findings by demonstrating bulk-like endocytosis evoked by both flash

photolysis and whole-cell depolarization in primary beta cells. Moreover, we show that bulk-like endocytosis is not altered when endogenous clathrin is knocked down with shRNA, which has been demonstrated to inhibit both transferrin internalization and clathrin-mediated slow endocytosis in a previous study by our group (He et al., 2008). Although clathrin-coated pits have been shown to bud from large infoldings on the plasma membrane (Lenzi et al., 2002), we believe that bulk-like endocytosis in beta cells is a clathrin-independent process. Interestingly, studies have proposed that calcium activates bulk-like endocytosis in various types of neurons (Clayton et al., 2009; Wu et al., 2009). Qualitatively, our data fit with the hypothesis that bulk-like endocytosis is a Ca^{2+} -dependent process because enhanced Ca^{2+} influx doubled the frequency of bulk endocytosis, whereas only 30% increase in exocytosis was observed. However, high levels of Ca^{2+} in the bath solution did not enhance the mean amplitude of bulk-like endocytic events and failed to affect the

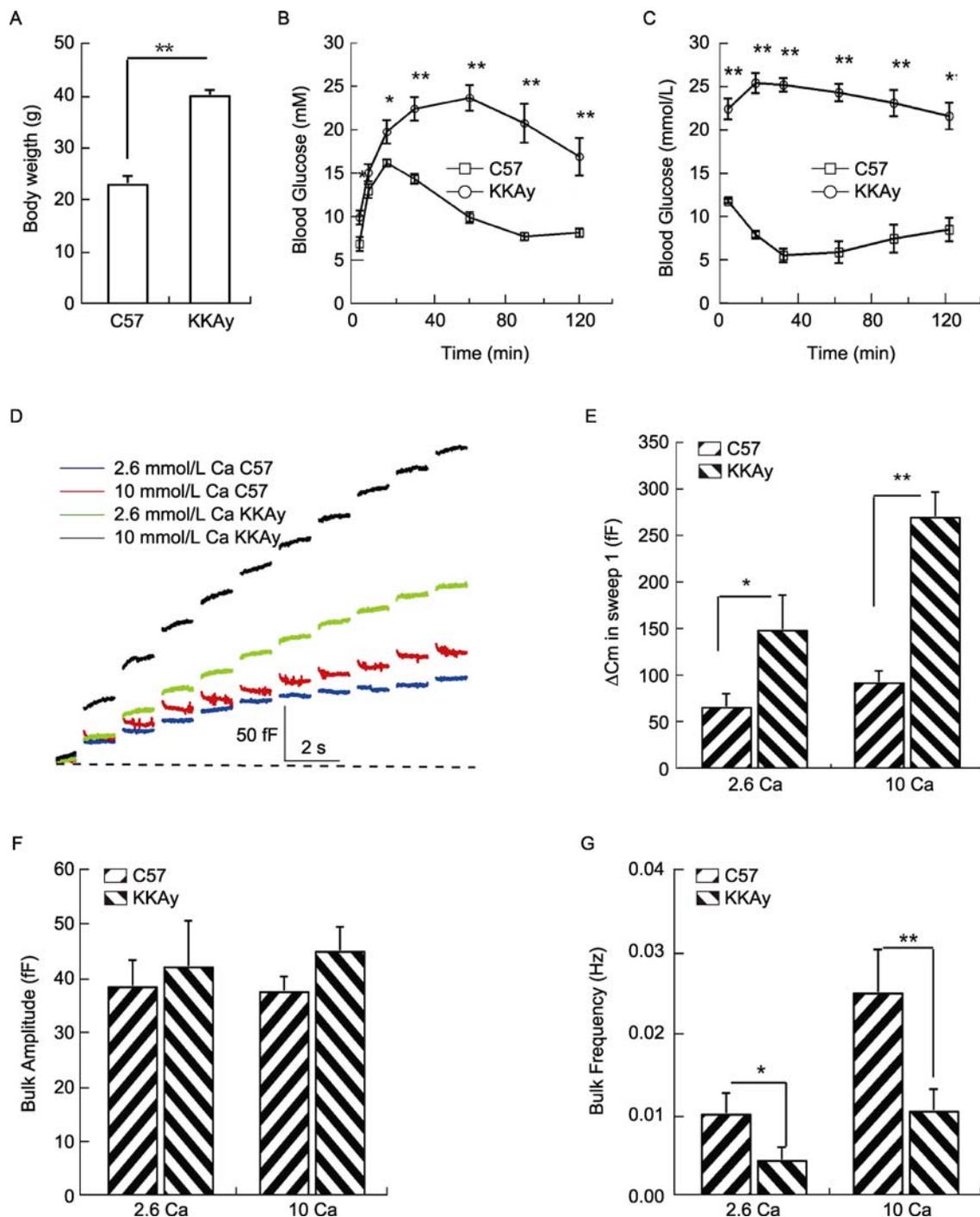


Figure 4. Impairment of the bulk-like endocytic mechanism in beta cells from diabetic KKAY mice. Comparison of (A) body weight, (B) blood glucose during GTT (i.p. administration of 2 g/kg body weight glucose), and (C) blood glucose during ITT (i.p. administration of 0.5 U/kg body weight insulin) between C57BI/6 and KKAY mice. (D) The average Cm traces induced by ten 200-ms pulses (frequency 1 Hz) in C57 and KKAY beta cells (blue, 2.6 mmol/L Ca²⁺, C57, *n* = 28; green, 2.6 mmol/L Ca²⁺, KKAY, *n* = 25; red, 10 mmol/L Ca²⁺, C57, *n* = 31; black, 10 mmol/L Ca²⁺, KKAY, *n* = 15). Beta cells from C57 and KKAY mice are of similar size in resting condition (C57 beta cells, $C_{slow} = 5.0 \pm 0.1$ pF; KKAY $C_{slow} = 4.9 \pm 0.3$ pF). (E) The average capacitance jump (ΔC_m) in the first sweep. (F and G) The average bulk amplitude and frequency under 2.6 mmol/L Ca and 10 mmol/L Ca conditions (* $P < 0.05$, ** $P < 0.01$).

frequency of bulk retrieval during the depolarization (Fig. 3A and 3C). This suggests that the stimulatory effect of Ca^{2+} on bulk-like endocytosis is facilitatory rather than obligatory.

Although inhibition of dynamin function with dynasore in primary beta cells failed to inhibit exocytosis, it abolished the stimulatory effects of the high bath concentration of Ca^{2+} on bulk-like endocytosis, which is consistent with the function of Ca^{2+} and dynamin in the same pathway in bulk-like endocytosis. Bulk-like endocytosis is activated by strong stimulation in neurons (Clayton et al., 2008). In parallel, elevated Ca^{2+} activates the calcium/calmodulin-dependent protein phosphatase calcineurin, which leads to the dephosphorylation of dynamin I and its interaction with the F-BAR protein, syndapin I (Anggono et al., 2006; Clayton et al., 2009). Therefore, our data appear to agree with the specific role of dynamin I rephosphorylation at SER-774 in bulk-like endocytosis in neurons (Clayton et al., 2010). Interestingly, bulk-like endocytosis persisted during depolarization in cells pretreated with dynasore, which argues against the mandatory role of dynamin in bulk-like endocytosis. Similarly, many more endosome-like structures have been observed in dynamin I KO synapses after acute strong stimulation compared with control synapses (Hayashi et al., 2008), which indicates the existence of a bulk retrieval mechanism that is independent of dynamin I. Therefore, we postulate that neither Ca^{2+} nor dynamin is required for the initiation of clathrin-independent bulk-like endocytosis; however, they facilitate bulk-like endocytosis in an activity-dependent manner. In addition, extremely high increases in the intracellular calcium concentration evoked by flash photolysis may temporally coordinate different bulk-like endocytic events that contribute to the rapid clathrin-independent phase of endocytosis that has been observed in primary beta cells and corticotrophs (Eliasson et al., 1996; Lee and Tse, 2001; He et al., 2008).

Although bulk-like endocytosis has been found in a number of neurons and endocrine cells, its mechanism and physiological significance remain elusive. Bulk-like endocytosis has been proposed to be a retrieval mechanism evoked by conditions under which classical endocytosis cannot match pace with exocytosis (e.g., dynamin I knockout or clathrin-inactivation during high-frequency stimulation) (Hayashi et al., 2008; Heerssen et al., 2008). Moreover, the intermediate organelle generated by bulk-like endocytosis is proposed to be incapable of efficiently generating fusion-competent synaptic vesicles (Hayashi et al., 2008; Heerssen et al., 2008). However, compound exocytosis of prefused insulin granules was reported in rat pancreatic beta cells in 2005 (Kwan and Gaisano, 2005). Recently, compound exocytosis has been further demonstrated to play an important role under conditions associated with the global elevation of cytosolic Ca^{2+} (Hoppa et al., 2012), which is the condition that augments the bulk-like endocytosis found in the present study. Our electron microscope data also show examples of compound fusion of several granules to the plasma membrane (Fig. 2C) and retrieval of three inter-connected

nearly empty vacuoles (Fig. 2B, arrowhead). Therefore, bulk-like endocytosis may be a result of compound or sequential exocytosis evoked by physiological stimulation in pancreatic beta cells. Most importantly, we demonstrate the selected reduction of bulk-like endocytosis in beta cells isolated from mice at earlier stage of diabetes. This contrasts with the enhanced insulin secretion from the beta cells compared with the control cells. Because bulk-like endocytosis may play an important role in the recycling of insulin granules, the combination of defective recycling and enhanced secretion will lead to the run-down and down-regulation of beta cell secretory ability as diabetes progresses, which has been observed in human diabetic patients (Ostenson et al., 2006). Therefore, elucidating the molecular mechanism of bulk-like endocytosis and discovering reagents that upregulate the endocytic pathway may prolong the preservation of primary beta cell function and postpone the onset of type II diabetes.

MATERIALS AND METHODS

Islets and single beta cell preparation

C57BL/6 male mice were purchased from the Vital River Experimental Animal Company, Beijing, China, and age-mated KKAy mice were purchased from the Institute of Laboratory Animal Science at the Chinese Academy of Medical Sciences, Beijing, China. All of the mice were maintained under specific pathogen-free conditions. The handling of the mice and the experimental procedures were conducted in accordance with the Committee of the Use of Live Animals in Teaching and Research at the Institute of Biophysics at the Chinese Academy of Sciences. The mice were sacrificed by cervical dislocation, and primary islets and beta cells were isolated as previously described. The isolated cells were plated on coverslips precoated with poly-L-lysine and maintained in a 37°C, 5% CO_2 incubator for 24–48 h in modified RPMI 1640 medium. We selected cells with diameters >11 $\mu\text{mol/L}$ with the expectation that more than 80%–90% of the cells would be beta cells (Rorsman and Trube, 1986) more than.

Patch capacitance measurements

Electrophysiological experiments were conducted with standard whole-cell recordings using an EPC-10 patch-clamp amplifier (HEKA). The bath solution contained (mmol/L) 138 NaCl, 5.6 KCl, 1.2 MgCl_2 , 2.6 CaCl_2 , 5 glucose, and 10 HEPES (pH 7.4, adjusted with NaOH). For the flash experiments, the intracellular solution contained (mmol/L) 105 Cs-Glutamate, 2 MgATP, 0.3 GTP, 33 HEPES, 0.4 Mag-Fura-2, 0.4 Fura-4F, 4 CaCl_2 , and 5 NP-EGTA (pH 7.2, adjusted with NaOH or HCl). For the depolarization experiments, the pipette solution contained (mmol/L) 125 Cs-Glutamate, 2 Mg-ATP, 0.3 $\text{Na}_2\text{-GTP}$, 1 MgCl_2 , and 0.1 EGTA (pH 7.2, adjusted with CsOH). In addition, 10 mmol/L TEA-Cl and 2 mmol/L forskolin were added to the bath solution. The extracellular calcium concentration was 2.6 mmol/L unless otherwise indicated. Capacitance measurements were performed using the 'sine + DC' mode of the software lock-in extension of Pulse software. An 800-Hz, 20-mV peak-to-peak sinu-

soidal voltage stimulus was applied at a DC holding potential of -70 mV. Ten trains of depolarization (from -70 mV to 0 mV for 200 ms) were applied to single beta cells to evoke an increase in capacitance, which was indicative of insulin secretion. Flash photolysis and increases in the membrane capacitance were performed as previously described (Duman et al., 2006).

Transmission electron microscopy

The mouse islets were grown in culture medium for one day before being harvested. On the day of experiments, islets were stimulated with either 70 mmol/L KCl or 20 mmol/L glucose for 5 minute before fixed in 2.5% glutaraldehyde in PBS (pH 7.4) at room temperature for 30 min. The islets were then postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate for one hour. Following dehydration in a graded acetone series, the samples were embedded in Embed 812 and sliced into 70-nm sections using a Leica ultramicrotome EM UC6 (Leica, Germany). After being contrasted with uranyl acetate and lead citrate, the sections were observed under a Spirit transmission electron microscope (FEI, the Netherlands).

Intraperitoneal glucose tolerance and insulin resistance tests

For the intraperitoneal glucose tolerance test (IGTT), the mice were starved overnight and then given an i.p. glucose injection (2 g/kg body weight). Venous blood was drawn from the tail vein, and the blood glucose level was measured using a glucometer (ACCU-CHEK Active, Roche). For the insulin tolerance test (ITT), the mice were starved for 4–5 hours prior to an i.p. injection of 0.5 U/kg body weight of human regular insulin (Sigma-Aldrich, St. Louis, MO, USA). Blood glucose levels were measured using a glucometer.

Data analysis

All of the data were analyzed using Igor Pro software (Wavemetrics, Lake Oswego, OR). The results are presented as the mean value \pm SEM. Statistical significance was evaluated using either Student's *t* test for single Gaussian distributed datasets or the Mann-Whitney rank sum test for non-single Gaussian distributed datasets. The symbols * and ** denote statistical significance with *P* values of less than 0.05 and 0.01, respectively.

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