SERCA3 Ablation Does Not Impair Insulin Secretion but Suggests Distinct Roles of Different Sarcoendoplasmic Reticulum Ca^{2+} Pumps for Ca^{2+} Homeostasis in Pancreatic β-cells

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Two sarcoendoplasmic reticulum Ca^{2+}-ATPases, SERCA3 and SERCA2b, are expressed in pancreatic islets. Immunocytochemistry showed that SERCA3 is restricted to β-cells in the mouse pancreas. Control and SERCA3-deficient mice were used to evaluate the role of SERCA3 in β-cell cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_c$) regulation, insulin secretion, and glucose homeostasis. Basal $[Ca^{2+}]_c$ was not increased by SERCA3 ablation. Stimulation with glucose induced a transient drop in basal $[Ca^{2+}]_c$ that was suppressed by inhibition of all SERCAs with thapsigargin (TG) but unaffected by selective SERCA3 ablation. Ca^{2+} mobilization by acetylcholine was normal in SERCA3-deficient β-cells. In contrast, $[Ca^{2+}]_c$ oscillations resulting from intermittent glucose-stimulated Ca^{2+} influx and $[Ca^{2+}]_c$ transients induced by pulses of high K^+ were similarly affected by SERCA3 ablation or TG pretreatment of control islets; their amplitude was increased and their slow descending phase suppressed. This suggests that, during the decay of each oscillation, the endoplasmic reticulum releases Ca^{2+} that was pumped by SERCA3 during the upstroke phase. SERCA3 ablation increased the insulin response of islets to 15 mmol/l glucose. However, basal and postprandial plasma glucose and insulin concentrations in SERCA3-deficient mice were normal. In conclusion, SERCA2b, but not SERCA3, is involved in basal $[Ca^{2+}]_c$ regulation in β-cells. SERCA3 becomes operative when $[Ca^{2+}]_c$ rises and is required for normal $[Ca^{2+}]_c$ oscillations in response to glucose. However, a lack of SERCA3 is insufficient in itself to alter glucose homeostasis or impair insulin secretion in mice. *Diabetes* 51:3245–3253, 2002

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ACh, acetylcholine; $[Ca^{2+}]_c$, cytosolic-free Ca^{2+} concentration; ER, endoplasmic reticulum; IP_3, $Ins(1,4,5)P_3$; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; TG, thapsigargin.

The sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) transfers Ca^{2+} from the cytosol to the lumen of the reticulum. Three SERCA genes (SERCA1–3) have been identified, all of which can generate several alternatively spliced isoforms (1–4). Pancreatic islets express SERCA2b and the three isoforms of SERCA3 (a–c) (1,5). SERCA2b is nearly ubiquitous and considered essential for viability (6–8). SERCA3 has a restricted tissue distribution (4,9,10) and shows lower Ca^{2+} affinity, higher sensitivity to vanadate, and higher pH optimum than SERCA1 or SERCA2 (11). The evidence for distinct physiological roles of the various SERCAs in cells is scant (3,12,13) and difficult to obtain because it requires experiments with genetically modified mouse or cell models or analysis of spontaneously occurring genetic diseases (8).

Glucose-stimulated β-cells display oscillations of the membrane potential that cause intermittent openings of voltage-dependent Ca^{2+} channels (14) and induce synchronous cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_c$) oscillations (15,16). The endoplasmic reticulum (ER) plays complex roles in β-cell Ca^{2+} homeostasis. Upon stimulation by glucose, it sequesters Ca^{2+} (17–19), leading to a lowering of $[Ca^{2+}]_c$ if Ca^{2+} influx is prevented. Upon stimulation by phospholipase C–activating agonists, such as acetylcholine (ACh) (17,20), it massively releases Ca^{2+}. During $[Ca^{2+}]_c$ oscillations induced by periodic influx of Ca^{2+}, such as those triggered by glucose, the ER takes up Ca^{2+} during the upstroke of the $[Ca^{2+}]_c$ rise and releases it slowly when Ca^{2+} influx stops (21).

An impaired $[Ca^{2+}]_c$ response to glucose is observed in β-cells of animal models of type 2 diabetes (22,23). Several studies have suggested that this dysregulation results from an impaired control of $[Ca^{2+}]_c$ by the SERCAs, in particular SERCA3. Thus, a decreased Ca^{2+}-ATPase activity has been reported in islets of rats made diabetic by neonatal injection of streptozotocin (24). The activity of all SERCAs was also found to be reduced in spontaneously diabetic *db/db* mice (25). Other studies have suggested that a deficiency of SERCA3 specifically might be involved. Indeed, a major decrease of the expression of SERCA3 but not of SERCA2b was observed in Goto-Kakizaki rats (5)

and in 90% pancreatectomized rats (26). Several missense mutations of SERCA3 have been detected in type 2 diabetic patients (27). Overexpression of insulin receptor substrate-1, which occurs after prolonged exposure of βTC6 cells to insulin (a phenomenon that might occur in hyperinsulinemic patients), inhibited SERCA3 gene expression without affecting that of SERCA2b (28). All of these data therefore suggest that a decreased expression or activity of SERCA3 could contribute to the impaired β-cell $[Ca^{2+}]_c$ and insulin responses observed in type 2 diabetes.

In the present study, we studied by immunocytochemistry the specific cell localization of SERCA3 in islets. Then, we addressed the role of SERCA3 in β-cell Ca^{2+} signaling by using the recently generated SERCA3 knockout mice (SERCA3 −/−) (29) and their wild-type littermates (SERCA3 +/+). The $[Ca^{2+}]_c$ response to various agents was compared in β-cells expressing SERCA2b and SERCA3 (SERCA3 +/+ mice), in cells expressing only SERCA2b (SERCA3 −/− mice), and in cells treated by thapsigargin (TG) to block all SERCA activity (30).

RESEARCH DESIGN AND METHODS

Immunodetection of SERCA3 and islet hormones. The pancreas of adult NMRI mice was fixed with 4% paraformaldehyde and embedded in paraffin. Immunodetection (peroxidase-diaminobenzidine technique) of insulin or SERCA3 was performed on serial sections, pretreated (SERCA3) or not (insulin) with microwaves, using a monoclonal anti-insulin antiserum (Novo Biolabs, Temecula, Denmark) or the rabbit anti-SERCA3 N89 antibody directed against a sequence corresponding to amino acid 29–39 of the human SERCA3 (31). For double-fluorescent immunodetections of non–β-cells and SERCA3, the sections were incubated with a cocktail of monoclonal anti-glucagon (Novo Biolabs), monoclonal anti-somatostatin (Novo Biolabs), and rabbit anti-SERCA3 antisera. Detection of SERCA3 by a red immunofluorescent color (Texas Red) required a tyramine amplification step, as described elsewhere (32). Non–β-cells were immunolabeled in green fluorescent color (fluorescein isothiocyanate). Control experiments without primary antibody never showed any labeling.

Animals, solutions, and preparations. The SERCA3 gene contains 22 exons. SERCA3 knockout mice (SERCA3 −/−) were generated previously by disrupting exon 5 (29). Age- (4–8 months) and sex-matched homozygous wild-type (SERCA3 +/+) and SERCA3 knockout mice (SERCA3 −/−) were used. The mutant mice (25 ± 1 g) were indistinguishable from their wild-type littermates (26 ± 1 g) in their gross phenotype and behavior. To evaluate glucose homeostasis of the mice, blood glucose was measured (glucometer; Bayer, Zurich) after 24 h of food deprivation and again 1 and 3 h after ad libitum refeeding. One week later, the same animals were fasted again, and blood glucose was measured after 24, 25, and 27 h of fasting. Plasma insulin was measured as described (33).

The medium used for the isolation of pancreatic islets and for $[Ca^{2+}]_c$ and insulin secretion experiments was a bicarbonate-buffered solution (21). When the concentration of KCl was increased, that of NaCl was decreased accordingly. Ca^{2+}-free solutions were prepared by substituting $MgCl_2$ for $CaCl_2$ and were supplemented with 2 mmol/l EGTA.

Islets were obtained by collagenase digestion of the pancreas, and clusters were prepared by dispersion of islet cells in a Ca^{2+}-free medium (34). Islets were cultered for 1 or 2 days, and cell clusters for 2 days in RPMI 1640 medium containing 10 mmol/l glucose.

Semiquantitative radioactive RT-PCR analysis of SERCA2 and SERCA3 mRNA. Islet total RNA was extracted, quantified, and reverse transcribed in cDNA as described (26). The sense (5′-GTC ATC ATG CTA ATC CTT GTG GC-3′) and antisense (5′-GGG GGT CTG GAA TGG CA-3′) primers for amplification of mouse SERCA3 mRNA (nucleotides 277–592 of the cDNA, Genbank accession nos. U49393 and U49394) were chosen on opposite sides of the 84-bp region deleted in SERCA3 −/− mice (29), giving products of 316 and 232 bp in wild-type and knockout mice, respectively. The sense (5′-GGC TTT TAC AGG GCG AGA GT-3′) and antisense (5′-ACC AGA TTG ACC CAG AGT AAC TG-3′) primers for mouse SERCA2 were chosen in a region common to both a and b isoforms (from nucleotides 531 in exon 2 to nucleotide 949 in exon 4 of SERCA2 mRNA, Genbank accession no. AJ131870), giving rise to a product of 441 bp. SERCA3 cDNA was amplified in duplex with cyclophilin as internal control gene (for primers, see ref. 26). Semiquantitative radioactive PCRs were performed as previously described (26), except that the thermal cycle profile was a 10-min denaturing step at 94°C followed by 22 cycles (1-min steps at 94°C, 60°C, and 72°C each) and a final extension step of 10 min at 72°C.

Measurements of $[Ca^{2+}]_c$ and insulin secretion. $[Ca^{2+}]_c$ was measured at 37°C in cultured islets or clusters of cells loaded with fura-PE3/AM (Teflabs, Austin, TX) as previously described (21). When needed, 1 μmol/l TG was added to the loading solution. Insulin secretion was measured in a perifusion system using batches of 15 islets (35).

Presentation of the results. The experiments are illustrated by traces that are means ± SE or by representative traces of results obtained with the indicated number of islets or clusters of islet cells from at least three different cultures, or with the indicated number of mice. The statistical significance of differences between means was assessed by unpaired or paired Student's *t* test as appropriate. Differences between means were considered significant at $P < 0.05$.

RESULTS

Immunolocalization of SERCA3. Immunodetection of SERCA3 revealed a strong labeling in islets, but no staining in the exocrine pancreas (Fig. 1*A*). The close similarity of the immunostaining pattern of serial sections for SERCA3 (Fig. 1*A*) and insulin (Fig. 1*B*) indicated that SERCA3 is present in β-cells. A minority of islet cells, often localized at the periphery and hence thought to be non–β-cells, did not display SERCA3 immunoreactivity (arrows in Fig. 1*A* and *B*). This was confirmed by double immunodetection of non–β-cells and SERCA3, which unequivocally revealed that glucagon- or somatostatin-secreting cells were not immunoreactive for SERCA3 (Fig. 1*C*– *H*). SERCA3 expression is thus limited to β-cells.

RT-PCR experiments. The expression of SERCA3 and SERCA2 in islet cells was assessed by RT-PCR on total RNA extracted from SERCA3 +/+ and SERCA3 −/− islets (Fig. 2). As expected, the size of SERCA3 amplicon from SERCA3 −/− mice was smaller than that of SERCA3 +/+ mice (Fig. 2*B*). This difference in size results from the excision of the last 84 nucleotides in exon 5, which impairs the pump activity of SERCA3 (29). Under semiquantitative PCR conditions, the levels of the mutated SERCA3 mRNA of SERCA3 −/− islets were much lower than those of the wild-type SERCA3 mRNA of SERCA3 +/+ islets (Fig. 2*A*). However, the expression level of SERCA2 mRNA (presumably SERCA2b) relative to the control mRNA cyclophilin in SERCA3 −/− islets was 98.7% of that observed in SERCA3 +/+ islets ($n = 2$). This suggests that the absence of SERCA3 is not compensated for by an overexpression of SERCA2 in islets.

Evaluation of the role of SERCA3 at basal $[Ca^{2+}]_c$. During perifusion of the islets with a glucose-free medium or a medium containing glucose and the ATP-sensitive K^+ channel opener diazoxide, $[Ca^{2+}]_c$ was low and stable. There was no significant difference between basal $[Ca^{2+}]_c$ in SERCA3 +/+ and SERCA3 −/− islets in all experimental series (pooled data of G0, G3, G0 plus diazoxide, G10 plus diazoxide, and G20 plus diazoxide: 99 ± 3 nmol/l, $n =$ 38, in SERCA3 +/+ islets vs. 94 ± 3 nmol/l, $n = 38$, in SERCA3 −/− islets). Pretreatment of the islets with TG did not increase basal $[Ca^{2+}]_c$ in SERCA3 +/+ (106 ± 7 nmol/l, $n = 8$) and SERCA3 −/− islets (78 ± 6 nmol/l, $n =$ 7). This suggests that basal $[Ca^{2+}]_c$ is mainly set by mechanisms other than the SERCAs.

We next evaluated the role of SERCA3 in glucose-stimulated Ca^{2+} uptake by the ER. Switching from a

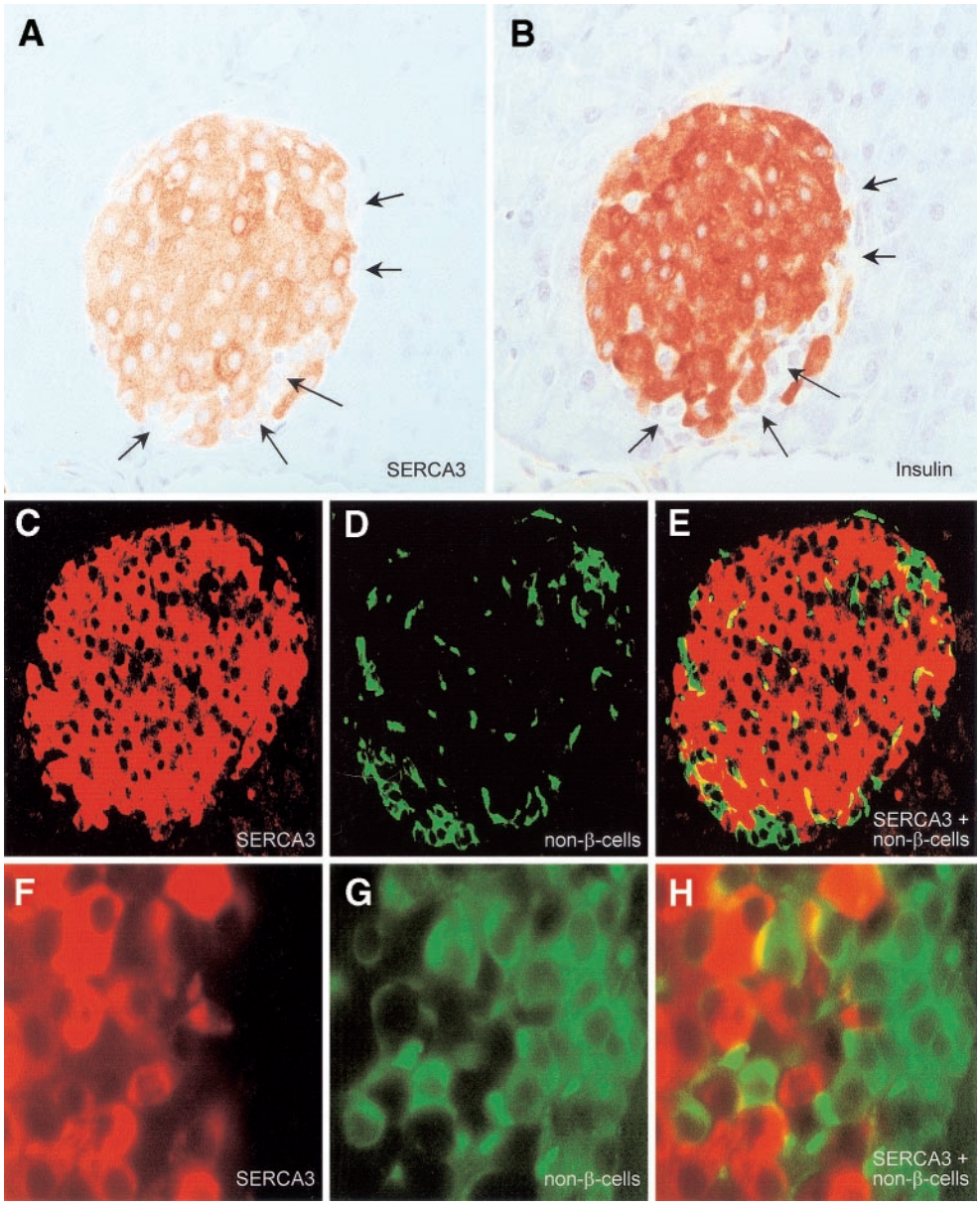

FIG. 1. Immunolocalization of SERCA3 in the mouse pancreas. *A* and *B*: Immunodetection of SERCA3 (*A*) and insulin (*B*) on serial sections suggested that SERCA3 and insulin immunoreactivity have a similar localization within the islet. Some peripheral islet cells (arrows) and the exocrine tissue were not stained. (*C*–*H*) Double immunodetections of SERCA3 in red and glucagon plus somatostatin in green (non–β-cells) on the same section. Red and green colors were digitally separated in *C*, *D*, *F*, and *G*, whereas they were digitally combined in *E* and *H*. In *E* and *H*, a yellow color was seen in restricted areas, where the cytoplasms of red- and green-labeled cells slightly overlapped due to the thickness of the section. However, no overlapping of the two colors was ever observed in whole cells, indicating that non–β-cells of the islet do not express SERCA3. Original magnifications: *A* and *B*: ×150; *C*–*E*: ×500; *F*–*H*: ×1,000.

glucose-free medium to a medium containing 15 mmol/l glucose induced an initial decrease in $[Ca^{2+}]_c$ that preceded a $[Ca^{2+}]_c$ rise in SERCA3 +/+ islets (Fig. 3*A*). This drop is attributed to Ca^{2+} uptake by the ER because it was completely prevented by TG. Glucose also transiently lowered $[Ca^{2+}]_c$ in SERCA3 −/− islets (Fig. 3*B*). The amplitude of this initial fall was similar in SERCA3 +/+ (19 ± 2 nmol/l, $n = 9$) and SERCA3 −/− islets (15 ± 1 nmol/l, $n = 9$). Similar observations were made when the experiments were performed in the presence of diazoxide (not shown).

The ER comprises Ins(1,4,5)P_3 (IP_3)-sensitive and -insensitive Ca^{2+} stores that are both refilled by SERCAs during exposure to glucose (19,36,37). ACh mobilizes Ca^{2+} from IP_3-sensitive stores, whereas TG mobilizes Ca^{2+} from both stores (19,36). To evaluate the role of SERCA3 in the replenishment of these Ca^{2+} stores and the maintenance of their Ca^{2+} content at basal $[Ca^{2+}]_c$, islets and clusters of cells were initially perifused with a medium containing Ca^{2+}, diazoxide, and 10 or 20 mmol/l glucose, which maximally stimulates Ca^{2+} uptake by the ER (19,38). They were then perifused for 5 (islets) or 8 min (clusters of cells) with a Ca^{2+}-free medium supplemented with 2 mmol/l EGTA to prevent Ca^{2+} influx. Subsequent addition of ACh triggered a large transient rise in $[Ca^{2+}]_c$ in islets (Fig. 4*A*) and clusters (Fig. 4*B*). In clusters, addition of TG 8 min after removal of ACh induced a small $[Ca^{2+}]_c$ elevation that reflects release of residual Ca^{2+}, not mobi-

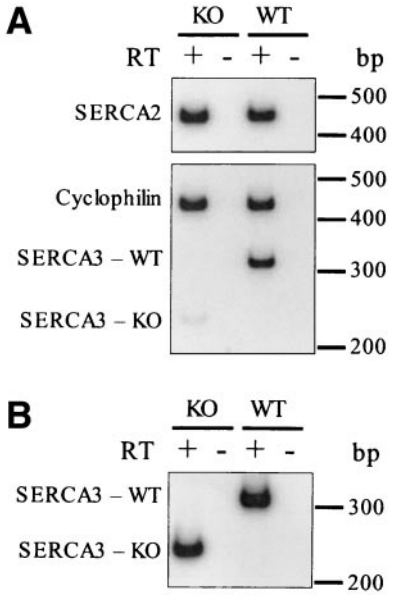

FIG. 2. Comparison of SERCA2 and SERCA3 mRNA levels by RT-PCR in isolated islets from SERCA3 +/+ (WT) and SERCA3 −/− (KO) mice. *A*: After reverse transcription (RT+) of islet total RNA, SERCA3 and SERCA2 cDNAs were amplified for 22 cycles under semiquantitative conditions. Cyclophilin was used as an internal control gene and amplified in duplex with SERCA3. SERCA2 mRNA expression was not affected by SERCA3 ablation. Compared with the 316-bp product in islets from wild-type mice, the shorter SERCA3 amplicon in SERCA3 −/− islets only gave a faint band under these conditions. *B*: SERCA3 cDNA was amplified for 40 cycles to confirm the presence and size of the shorter SERCA3 amplicon in islets from knockout mice. No PCR products were amplified when reverse transcriptase was omitted from the reverse transcription reaction (RT−). The results of both panels are representative of experiments performed on islets from two mice.

lized by ACh (right side of Fig. 4*B*). The magnitude of the $[Ca^{2+}]_c$ rises induced by ACh and TG was similar in SERCA3 +/+ and SERCA3 −/− islet cells. The effect of 100 μmol/l ACh on $[Ca^{2+}]_c$ was also tested in islets perifused with a medium containing Ca^{2+}, 20 mmol/l glucose, and 250 μmol/l diazoxide. The amplitude of the mobilization of Ca^{2+} was again similar in SERCA3 +/+ (189 ± 12 nmol/l, $n = 3$) and SERCA3 −/− islets (202 ± 28 nmol/l, $n = 3$). These results suggest that the IP_3-sensitive and -insensitive Ca^{2+} pools of the ER do not require

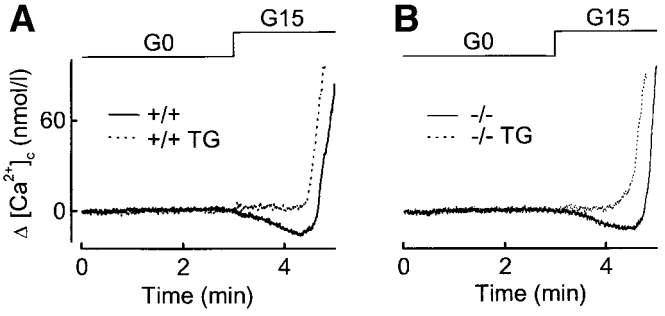

FIG. 3. SERCA3 does not mediate the initial $[Ca^{2+}]_c$ decrease induced by glucose. Isolated islets from SERCA3 +/+ (*A*) or SERCA3 −/− (*B*) mice were pretreated (dotted line) or not (solid line) with 1 μmol/l TG during loading with fura-PE3. After an initial perifusion without glucose (G0), they were perifused with a medium containing 15 mmol/l glucose (G15), as indicated on the top of the panels. The traces show mean changes (Δ) around basal $[Ca^{2+}]_c$ in the absence of glucose. Basal levels were 104 ± 9 nmol/l, $n = 9$ (*A*, solid line); 111 ± 13 nmol/l, $n = 3$ (*A*, dotted line); 80 ± 7 nmol/l, $n = 9$ (*B*, solid line); and 70 ± 11 nmol/l, $n = 3$ (*B*, dotted line).

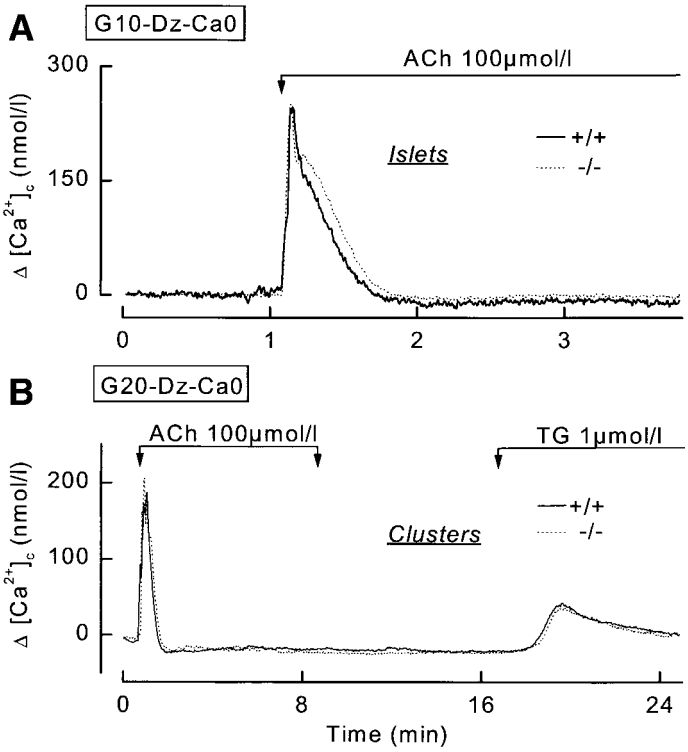

FIG. 4. SERCA3 is not required for refilling IP_3-sensitive and -insensitive Ca^{2+} pools in mouse islets. Before the recording, islets (*A*) or clusters of islet cells (*B*) were bathed in a medium containing 2.5 mmol/l Ca^{2+}, 100 μmol/l diazoxide (Dz), and 10 (G10) or 20 mmol/l glucose (G20), respectively. They were then perifused with a Ca^{2+}-free medium supplemented with 2 mmol/l EGTA for 5 (islets) or 8 min (clusters of cells) before addition of 100 μmol/l ACh. In clusters only, 1 μmol/l TG was added as indicated. The traces show mean changes (Δ) around basal $[Ca^{2+}]_c$ levels at the beginning of the recording. Basal levels were 117 ± 8 nmol/l, $n = 5$ (*A*, solid line); 98 ± 7 nmol/l, $n = 6$ (*A*, dotted line); 41 ± 6 nmol/l, $n = 4$ (*B*, solid line); and 36 ± 9 nmol/l, $n = 4$ (*B*, dotted line).

SERCA3 activity to be filled with Ca^{2+} during glucose stimulation and to retain Ca^{2+} when $[Ca^{2+}]_c$ is low.

Role of SERCA3 at elevated $[Ca^{2+}]_c$. The role of SERCA3 was next evaluated under conditions where $[Ca^{2+}]_c$ was transiently elevated by depolarizing the plasma membrane with 45 mmol/l K^+ to stimulate Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Diazoxide was present throughout to prevent glucose-induced $[Ca^{2+}]_c$ oscillations (15) and to impose similar membrane potentials in the presence and absence of TG (21). Depolarizing SERCA3 +/+ islets for 30 s induced a large and rapid $[Ca^{2+}]_c$ rise followed by a slightly ascending plateau and a descending phase characterized by an initial fast component and a secondary slow decline (Fig. 5*A*, solid line). As previously reported (21), TG pretreatment increased the rising phase to the peak ($P < 0.05$), gave the plateau a slowly descending shape, and suppressed the secondary slow component of the recovery phase (Fig. 5*A*, dotted line). These changes have been interpreted as evidence that the ER takes up Ca^{2+} during the upstroke of the $[Ca^{2+}]_c$ rise and releases it slowly when Ca^{2+} influx stops (21). Strikingly, the $[Ca^{2+}]_c$ changes induced by the high K^+ pulse in SERCA3 −/− islets had a similar shape to that observed in SERCA3 +/+ islets after TG pretreatment (compare Fig. 5*A* dotted line with Fig. 5*B* dotted line). In contrast, TG pretreatment of SERCA3 −/− islets did not

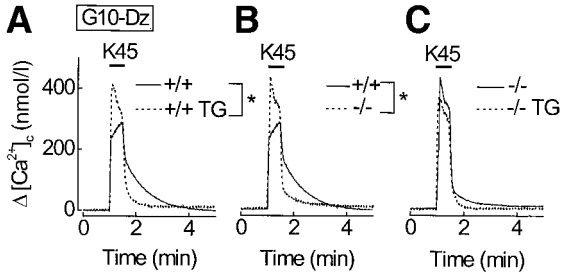

FIG. 5. SERCA3 influences $[Ca^{2+}]_c$ changes induced by a 30-s pulse of 45 mmol/l K^+ in mouse islets. Isolated islets from SERCA3 +/+ or SERCA3 −/− mice were pretreated or not with 1 μmol/l TG during loading with fura-PE3. They were then perifused throughout with a medium containing 10 mmol/l glucose (G10) and 100 μmol/l diazoxide (Dz). The K^+ concentration was increased from 4.8 to 45 mmol/l when indicated by the horizontal bar. The traces show mean changes (Δ) around basal $[Ca^{2+}]_c$ levels before the pulse of high K^+. Basal levels were 122 ± 7 nmol/l, $n = 7$ (*A* and *B*, solid line); 104 ± 7 nmol/l, $n = 6$ (*B*, dotted line and *C*, solid line); 104 ± 9 nmol/l, $n = 5$ (*A*, dotted line); and 84 ± 7 nmol/l, $n = 4$ (*C*, dotted line). *$P < 0.05$ for comparisons of the mean amplitude of the high K^+-induced elevation in $[Ca^{2+}]_c$ between groups shown by dotted and solid lines.

significantly change the characteristics of the $[Ca^{2+}]_c$ rise and decrease induced by the K^+ pulse (Fig. 5*C*). These observations indicate that the difference ($P < 0.05$) in $[Ca^{2+}]_c$ peak amplitude between SERCA3 +/+ and SERCA3 −/− islets, illustrated in Fig. 5*B*, is due to a buffering action of the ER that is mainly mediated by SERCA3 activity. They also show that a major component of the slow recovery phase observed in SERCA3 +/+ islets corresponds to the release of Ca^{2+} from ER Ca^{2+} pools that were replenished by SERCA3. A small contribution of SERCA2b is suggested by the minor residual slow recovery $[Ca^{2+}]_c$ phase in SERCA3 −/− islets (Fig. 5*C*).

Influence of SERCA3 on glucose-induced $[Ca^{2+}]_c$ oscillations. Increasing the glucose concentration from 3 to 7 mmol/l triggered a transient rise in $[Ca^{2+}]_c$ in all islets. Thereafter, $[Ca^{2+}]_c$ started to oscillate in 12/14 SERCA3 +/+ and 7/13 SERCA3 −/− islets and returned to basal levels in the others (Fig. 6*A* and *B*). In 10 mmol/l glucose, $[Ca^{2+}]_c$ oscillations occurred in all islets. In 20 mmol/l glucose, $[Ca^{2+}]_c$ was steadily elevated in 12/14 SERCA3 +/+ and 9/13 SERCA3 −/− islets, whereas it continued to oscillate in the others. Average $[Ca^{2+}]_c$, integrated over the last 5 min at each glucose concentration, was similar in both types of islets (Fig. 6*C*). However, several features of $[Ca^{2+}]_c$ oscillations were different. The nadir between oscillations was more elevated in SERCA3 +/+ than SERCA3 −/− islets, in both 7 mmol/l glucose (120 ± 4 nmol/l, $n = 12$ vs. 98 ± 6 nmol/l, $n = 7$) and 10 mmol/l glucose (156 ± 5 nmol/l, $n = 14$ vs. 119 ± 6 nmol/l, $n = 13$). Therefore, the difference between nadir and basal $[Ca^{2+}]_c$ at 3 mmol/l glucose was significantly larger in SERCA3 +/+ than SERCA3 −/− islets (Δ1 and Δ2 in Fig. 6*A*, *B*, and *D*). In contrast, when 20 mmol/l glucose induced a sustained elevation of $[Ca^{2+}]_c$, there was no difference between SERCA3 +/+ (233 ± 8 nmol/l, $n = 12$) and SERCA3 −/− islets (225 ± 13 nmol/l, $n = 9$); therefore, Δ3 with basal $[Ca^{2+}]_c$ was similar (141 ± 7 vs. 138 ± 13 nmol/l). The differences between nadirs reflect differences in the amplitude of $[Ca^{2+}]_c$ oscillations, which was smaller in SERCA3 +/+ than SERCA3 −/− islets (Fig. 6*E*). The frequency of $[Ca^{2+}]_c$ oscillations was similar in the two types of islets during the last 5 min of perifusion with 7

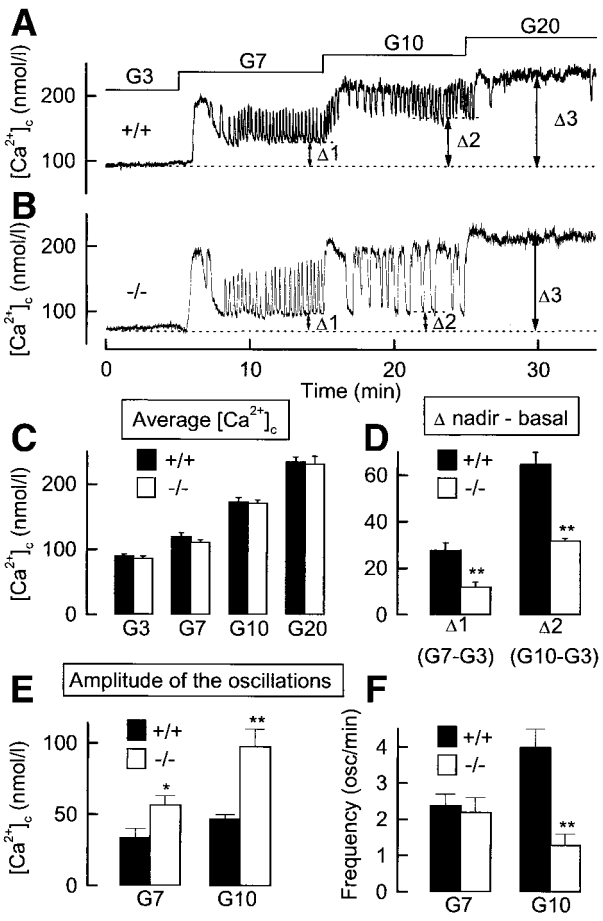

FIG. 6. SERCA3 influences glucose-induced $[Ca^{2+}]_c$ oscillations in mouse islets. *A* and *B*: Isolated islets from SERCA3 +/+ or SERCA3 −/− mice were perifused with a medium containing 3, 7, 10, and 20 mmol/l glucose (G) as indicated. Δ shows the difference between basal $[Ca^{2+}]_c$ at 3 mmol/l glucose and the nadir of $[Ca^{2+}]_c$ oscillations at 7 (Δ1) or 10 mmol/l glucose (Δ2) or the sustained $[Ca^{2+}]_c$ elevation at 20 mmol/l glucose (Δ3). The traces are representative of results obtained in six (*A*) and five (*B*) islets. *C*: Average $[Ca^{2+}]_c$ calculated by integrating $[Ca^{2+}]_c$ over the last 5 min of stimulation with each glucose concentration in all experiments similar to those illustrated in *A* and *B*. *D*: Difference between basal $[Ca^{2+}]_c$ at 3 mmol/l glucose and the nadir of $[Ca^{2+}]_c$ oscillations at 7 (Δ1) or 10 mmol/l glucose (Δ2). Amplitude (*E*) and frequency (*F*) of $[Ca^{2+}]_c$ oscillations calculated during the last 5 min of perifusion with 7 or 10 mmol/l glucose. *C–F*: Data are means ± SE. *$P < 0.05$, ** $P < 0.01$ for differences between SERCA3 +/+ and SERCA3 −/− islets.

mmol/l glucose but was much higher in SERCA3 +/+ than SERCA3 −/− islets in 10 mmol/l glucose (Fig. 6*F*). The rate of the $[Ca^{2+}]_c$ changes during the oscillations was also different. The increase and decrease in $[Ca^{2+}]_c$ during the oscillations were clearly slower in SERCA3 +/+ than SERCA3 −/− islets (Fig. 7*A* and *B*).

Similar features were observed in clusters of islet cells in which glucose-induced $[Ca^{2+}]_c$ oscillations are slower and often more regular than in islets (Fig. 7*C* and *D*). Average $[Ca^{2+}]_c$ integrated over 15 min of perifusion with 20 mmol/l glucose was similar in SERCA3 +/+ (287 ± 28 nmol/l, $n = 8$) and in SERCA3 −/− cells (286 ± 24 nmol/l, $n = 10$). The nadir between $[Ca^{2+}]_c$ oscillations was higher ($P < 0.01$) in SERCA3 +/+ than SERCA3 −/− cells (60 ± 8 vs. 35 ± 4 nmol/l above basal $[Ca^{2+}]_c$ in a Ca^{2+}-free medium, respectively, not shown in Fig. 7*C* and *D*). The amplitude of $[Ca^{2+}]_c$ oscillations was smaller in SERCA3 +/+ than SERCA3 −/− clusters of cells (306 ± 33 vs.

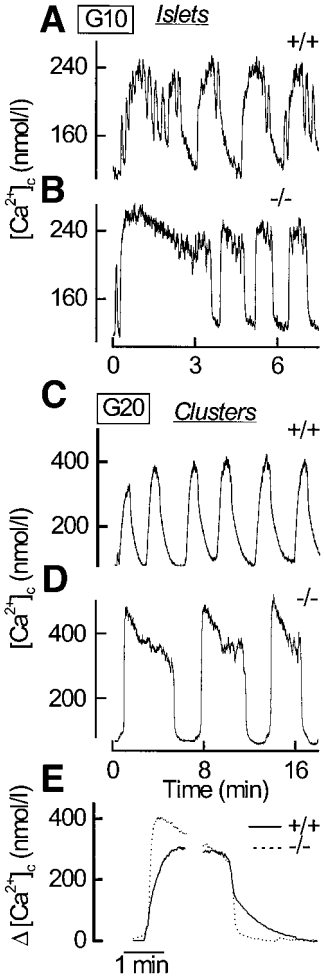

FIG. 7. SERCA3 influences glucose-induced $[Ca^{2+}]_c$ oscillations in mouse islets and clusters of islet cells. Isolated islets (*A* and *B*) or clusters of islet cells (*C*–*E*) from SERCA3 +/+ or SERCA3 −/− mice were perifused with a medium containing 10 (G10) or 20 mmol/l glucose (G20) as indicated. Traces *A*–*D* are representative of results obtained in six (*A*) and five (*B*) islets and in 8 (*C*) and 10 (*D*) clusters. *E*: Average of 8 or 10 $[Ca^{2+}]_c$ oscillations (1 oscillation taken per each experiment) induced by 20 mmol/l glucose in clusters of islets cells from SERCA3 +/+ and SERCA3 −/− mice, respectively. Simple averaging could be misleading because the oscillations have different durations. Averaging was thus performed at the onset and end of all oscillations to show the differences in mean rates of $[Ca^{2+}]_c$ rise and decline in the two groups.

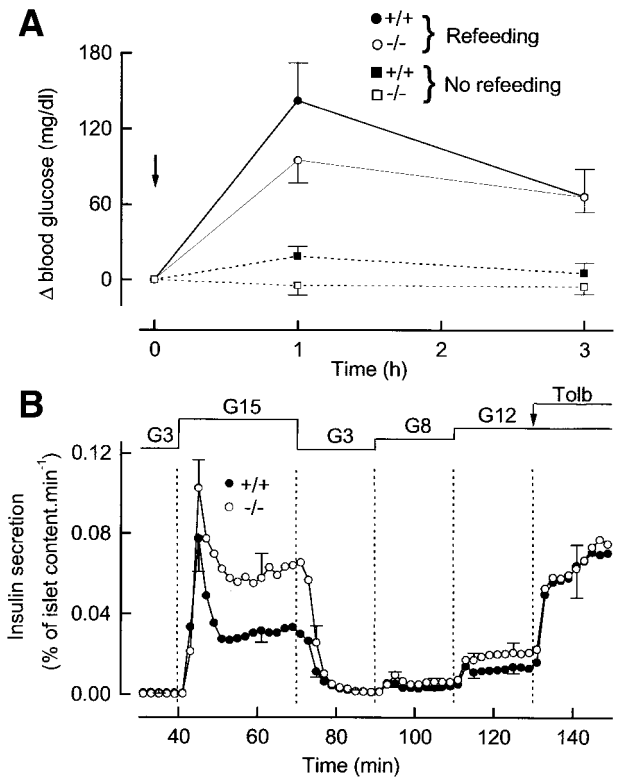

FIG. 8. SERCA3 ablation does not impair glucose tolerance and glucose-induced insulin secretion. *A*: SERCA3 +/+ (● and ■) and SERCA3 −/− mice (○ and □) were fasted for 24 h. After a first measurement of blood glucose (time zero), the animals were refed ad libitum (● and ○) and blood glucose was again measured 1 and 3 h later. One week later, the same animals (■ and □) were fasted again, and blood glucose was measured after 24 (time zero), 25, and 27 h of fasting. Results are expressed as differences (Δ) from blood glucose at time zero (arrow) for each animal (90 ± 10 mg/dl in SERCA3 +/+ and 89 ± 9 mg/dl in SERCA3 −/− mice). Values are means ± SE of results obtained with seven mice for each group. *B*: Batches of 15 islets from SERCA3 +/+ (●) and SERCA3 −/− mice (○) were perifused with a medium containing 3, 8, 12, or 15 mmol/l glucose (G) or 100 μmol/l tolbutamide (Tolb), as indicated. Insulin secretion is expressed as a percentage of islet insulin content. Values are means ± SE of seven experiments.

424 ± 41 nmol/l, $P < 0.05$). In addition, differences in the rate of $[Ca^{2+}]_c$ changes during the oscillations can be seen in the representative experiments shown in Fig. 7*C* and *D* and become even more evident when single oscillations from all experiments are pooled (Fig. 7*E*). At the onset of the oscillation, the $[Ca^{2+}]_c$ rise is faster and larger in SERCA3 −/− than SERCA3 +/+ clusters. At the end of the oscillation, the return to basal $[Ca^{2+}]_c$ is also faster with no slow component. These characteristics of the glucose-induced $[Ca^{2+}]_c$ oscillations are similar to those of the $[Ca^{2+}]_c$ changes induced by pulses of high K^+ (Fig. 5), which highlights the important contribution of SERCA3 in $[Ca^{2+}]_c$ oscillations resulting from periodic Ca^{2+} influx.

SERCA3 ablation does not impair glucose tolerance and glucose-induced insulin secretion. Blood glucose concentrations were similar in fed SERCA3 +/+ (120 ± 3 mg/dl, $n = 17$) and SERCA3 −/− mice (111 ± 4 mg/dl, $n = 17$). There was also no difference after 24 h fasting (90 ± 10 mg/dl, $n = 14$ and 89 ± 9 mg/dl, $n = 14$, two samplings in each mouse). The increase in blood glucose occurring after refeeding was not different between the two groups (Fig. 8*A*), suggesting that SERCA3 ablation does not impair glucose homeostasis. Plasma insulin was also similar in SERCA3 +/+ (2.2 ± 0.5 ng/ml, $n = 11$) and SERCA3 −/− fed mice (2.0 ± 0.4 ng/ml, $n = 11$).

Glucose-induced insulin secretion was evaluated in vitro with perifused islets. Stimulation with 15 mmol/l glucose, after 40 min of perifusion with 3 mmol/l glucose, induced biphasic insulin release that was larger in SERCA3 −/− than SERCA3 +/+ islets (Fig. 8*B*). Thus, the percentage of insulin secreted over the 30 min of stimulation relative to the islet insulin content was 1.8 ± 0.3 vs. 1.0 ± 0.2%, $P < 0.05$. After a 20-min period rest at 3 mmol/l glucose, the islets were stimulated by stepwise increases of the glucose concentration to 8 and 12 mmol/l and, eventually, by 100 μmol/l tolbutamide (Fig. 8*B*). During these three periods of stimulation, fractional insulin secretion was not different between SERCA3 +/+ and SERCA3 −/− islets.

DISCUSSION

The present study shows that in the mouse pancreas, SERCA3 is present only in insulin-secreting β-cells. By using the recently generated SERCA3 knockout mice (29), we also demonstrate that SERCA3 does not contribute to glucose-induced Ca^{2+} uptake by the ER at basal $[Ca^{2+}]_c$ but has a specific role in the modulation of $[Ca^{2+}]_c$ oscillations induced by periodic Ca^{2+} influx in β-cells. Finally, SERCA3 expression is dispensable for normal insulin secretion and glucose homeostasis in mice maintained under normal conditions.

The model. Pancreatic islets express SERCA2b and the three SERCA3 isoforms (SERCA3a–c) (1,39). The SERCA3 −/− mouse model permits investigation of the role of all SERCA3 isoforms together, but not that of individual isoforms, because excision of part of exon 5 during processing of the primary transcript blocks the pump activity of all three alternatively spliced isoforms, which are the product of the same gene but differ by no, partial, or total retention of exon 21 (1). RT-PCR did no reveal any obvious difference in the expression level of SERCA2 mRNA (presumably SERCA2b) between SERCA3 +/+ and SERCA3 −/− islets, suggesting that the loss of SERCA3 activity is not compensated for by an overexpression of SERCA2 in islets of SERCA3-deficient mice.

Immunolocalization of SERCA3 and islet hormones showed that within the islet, SERCA3 expression is restricted to β-cells. This makes the interpretation of $[Ca^{2+}]_c$ measurements easier because any difference in $[Ca^{2+}]_c$ changes between SERCA3 +/+ and SERCA3 −/− islets can be attributed specifically to β-cells. The lack of SERCA3 immunoreactivity in pancreatic acini has been documented previously (12).

Role of SERCA3 when $[Ca^{2+}]_c$ is low in β-cells. Voltage-dependent Ca^{2+} channels are the main Ca^{2+} entry pathway in the electrically excitable β-cell (40). A small capacitative Ca^{2+} entry (through voltage-independent Ca^{2+} channels) can also be evoked when TG, a SERCA inhibitor, or IP_3-producing agents deplete the Ca^{2+} content of the ER (41,42). However, because this entry mechanism inactivates rapidly in a Ca^{2+}-dependent manner (43), it is best detected upon readdition of Ca^{2+} to a Ca^{2+}-free medium. Moreover, Ca^{2+} influx through this pathway is small and barely increases $[Ca^{2+}]_c$ in the steady state (42). This probably explains why no significant differences in basal $[Ca^{2+}]_c$ levels were observed here between islets that were or were not pretreated with TG. This lack of difference also indicates that basal $[Ca^{2+}]_c$ in β-cells is mainly set by Ca^{2+} extrusion systems other than the SERCAs.

Stimulation of β-cells with glucose induces an initial TG-inhibitable drop in $[Ca^{2+}]_c$ that precedes the $[Ca^{2+}]_c$ rise and is explained by Ca^{2+} being pumped into the ER (18,42). As this drop was of similar amplitude in SERCA3 −/− and SERCA3 +/+ mice, we can rule out the intervention of SERCA3 in this phenomenon. Likewise, SERCA3 does not seem to be involved in the glucose-induced replenishment of the IP_3-sensitive and -insensitive Ca^{2+} pools of the ER, nor in the maintenance of their Ca^{2+} content at basal $[Ca^{2+}]_c$ over time. Indeed, ACh and TG elicited similar Ca^{2+} mobilizations in SERCA3 +/+ and SERCA3 −/− β-cells preexposed to glucose. Glucose-induced Ca^{2+} uptake by the ER at basal $[Ca^{2+}]_c$ is thus probably mediated by SERCA2b, the other SERCA isoform expressed in β-cells, which is compatible with its much higher affinity for Ca^{2+} ($K_{1/2}$ of ~0.2 μmol/l) relative to that of SERCA3 ($K_{1/2}$ of ~2 μmol/l) (1,10,11,44). A recent study suggested that decreased expression of SERCA2, but not SERCA3, by injection of specific antisense oligonucleotides reduced the Ca^{2+} content of the ER at basal $[Ca^{2+}]_c$ (13).

Role of SERCA3 in $[Ca^{2+}]_c$ oscillations induced by periodic Ca^{2+} influx in β-cells. We previously demonstrated that the ER shapes $[Ca^{2+}]_c$ oscillations induced by periodic Ca^{2+} influx in β-cells (21). It limits the amplitude of the oscillation by rapidly taking up Ca^{2+} during the upstroke and prolongs the duration of the oscillation by slowly releasing Ca^{2+} into the cytosol after Ca^{2+} influx has stopped. These findings are confirmed here by the observation that high K^+-induced transient $[Ca^{2+}]_c$ elevations were larger and devoid of a slow recovery phase in islets whose SERCAs were blocked by TG. Importantly, our present results also demonstrate that this effect of the ER can largely be attributed to the activity of SERCA3. Thus, high K^+-induced transient $[Ca^{2+}]_c$ elevations in SERCA3 −/− islets were similar to those in SERCA3 +/+ islets pretreated with TG and were virtually unaffected by TG pretreatment.

The present results further show that SERCA3 also plays a role during $[Ca^{2+}]_c$ oscillations induced by glucose. The amplitude of these oscillations was indeed larger and their nadir lower in SERCA3 −/− than SERCA3 +/+ islets. As for high K^+-induced transient $[Ca^{2+}]_c$ elevations, the larger amplitude of glucose-induced $[Ca^{2+}]_c$ oscillations likely results from reduced Ca^{2+} uptake by the ER in SERCA3 −/− β-cells. As a consequence, less or no Ca^{2+} is slowly released by the ER during the intervals, and the return of $[Ca^{2+}]_c$ to basal levels is not impeded (the nadir is lower) before the next $[Ca^{2+}]_c$ oscillation starts. Blockade of all SERCAs by TG is known to augment glucose-induced $[Ca^{2+}]_c$ increase by activating a depolarizing store-operated current, which then facilitates the opening of voltage-dependent Ca^{2+} channels (18,42,45,46). However, selective loss of SERCA3 did not increase average $[Ca^{2+}]_c$ compared with control islets, probably because the activity of SERCA2 prevented activation of a store-operated current with a similar amplitude to that induced by TG.

A distinct subcellular distribution of various SERCA isoforms has been reported in acinar cells (12) but not in Jurkat T or HEK-293 cells expressing SERCA2b and SERCA3 (44). Spatial separation of SERCAs with distinct Ca^{2+} affinities might explain the existence of subcompartments of the ER with high and low Ca^{2+} concentrations (47). Whether the high Ca^{2+}-affinity SERCA2b, operating mainly at low $[Ca^{2+}]_c$, and the low Ca^{2+}-affinity SERCA3, operating when $[Ca^{2+}]_c$ increases are localized at distinct sites of the ER best fitting their specific roles, remains to be investigated in β-cells.

SERCA3 ablation does not impair glucose tolerance and insulin secretion in mice. Glucose-induced $[Ca^{2+}]_c$ responses are altered in islets from animal models of type 2 diabetes (22,23), and several studies have incriminated a reduced activity of Ca^{2+}-ATPase (24), all SERCAs (25), or

more specifically SERCA3 (5,26,27). The present experiments with SERCA3 −/− mice do not support this suggestion. Indeed, SERCA3 −/− mice were normoglycemic, had a normal insulinemia and body weight, and showed essentially the same response to a refeeding test as SERCA3 +/+ mice. Moreover, perifused islets from SERCA3 −/− mice displayed a similar or better insulin secretory response to glucose than did islets from their wild-type littermates. The reasons for this are unclear because average $[Ca^{2+}]_c$ was similar between the two types of islets. Differences in the kinetics of $[Ca^{2+}]_c$ changes during the oscillations might allow $[Ca^{2+}]_c$ to stay above the threshold for exocytosis for longer periods of time in SERCA3 −/− than SERCA3 +/+ β-cells. Localized $[Ca^{2+}]_c$ in zones of exocytosis might also be higher after ablation of the buffering effect of SERCA3.

The essentially normal glucose tolerance and insulin secretion of SERCA3 −/− mice suggest that the mutations or polymorphisms of the SERCA3 gene reported in type 2 diabetic patients (27) are not causally involved in the development of diabetes. The reduced expression or activity of SERCA3 observed in type 2 diabetes could result partly from the associated hyperglycemia. Indeed, culturing rat islets in high glucose concentrations decreased their Ca^{2+}-ATPase activity (24). Moreover, the large reduction of SERCA3 expression that was observed in rats rendered hyperglycemic by 90% pancreatectomy was completely reversed after normalization of the glycemia by phlorizin treatment (26). Another possibility is that SERCA3 knockout mice have developed compensatory mechanisms that type 2 diabetic patients have not. Indeed, a recent study using mice lacking one copy of the SERCA2 gene indicated that Ca^{2+} signaling mechanisms exhibit a high degree of plasticity (48). Further studies will be needed to determine whether alteration in SERCA3 activity could increase susceptibility to diabetes development in a predisposing environment.

Our data demonstrate that within the pancreas, SERCA3 is expressed in β-cells only. They also suggest that Ca^{2+} uptake by the ER at basal $[Ca^{2+}]_c$ is attributable to SERCA2b rather than SERCA3. In contrast, the contribution of SERCA3 becomes important when $[Ca^{2+}]_c$ increases. The distinct roles of the two SERCA isoforms are compatible with their respective affinities for Ca^{2+} (1,10,11,44). Activation of SERCA3 by a rise in $[Ca^{2+}]_c$ has an impact on $[Ca^{2+}]_c$ oscillations induced by periodic Ca^{2+} influx. Indeed, SERCA3 allows the ER to rapidly buffer Ca^{2+} during the upstroke of each oscillation, thereby limiting the amplitude of $[Ca^{2+}]_c$ oscillations. Once Ca^{2+} influx stops, $[Ca^{2+}]_c$ decreases, but Ca^{2+} accumulated in the ER is slowly released into the cytosol, thereby prolonging the $[Ca^{2+}]_c$ oscillation. These effects of SERCA3 do not seem to have a major impact on insulin secretion. Although differences of the pulsatility of secretion may occur in the absence of SERCA3, no diabetes develops in these mice.

Even if SERCA3 seems dispensable for normal insulin response, it might play other roles in β-cells. The observation that $[Ca^{2+}]_c$ oscillations were different in SERCA3 +/+ and SERCA3 −/− islets corroborates the previous proposal that the membrane potential of β-cells is modulated by the ER (21) and suggests that this control involves SERCA3. Several studies reported that the Ca^{2+} content of the ER is important for synthesis, modifications, and folding of proteins (49,50). It is possible that cyclic elevations of the Ca^{2+} concentration within the ER during $[Ca^{2+}]_c$ oscillations play a role in this process (49).

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