Islet Secretory Defect in Insulin Receptor Substrate 1 Null Mice Is Linked With Reduced Calcium Signaling and Expression of Sarco(endo)plasmic Reticulum Ca^{2+}-ATPase (SERCA)-2b and -3

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Mice with deletion of insulin receptor substrate (IRS)-1 (IRS-1 knockout [KO] mice) show mild insulin resistance and defective glucose-stimulated insulin secretion and reduced insulin synthesis. To further define the role of IRS-1 in islet function, we examined the insulin secretory defect in the knockouts using freshly isolated islets and primary β-cells. IRS-1 KO β-cells exhibited a significantly shorter increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) than controls when briefly stimulated with glucose or glyceraldehyde and when L-arginine was used to potentiate the stimulatory effect of glucose. These changes were paralleled by a lower number of exocytotic events in the KO β-cells in response to the same secretagogues, indicating reduced insulin secretion. Furthermore, the normal oscillations in intracellular Ca^{2+} and O_2 consumption after glucose stimulation were dampened in freshly isolated KO islets. Semiquantitative RT-PCR showed a dramatically reduced islet expression of sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA)-2b and -3 in the mutants. These data provide evidence that IRS-1 modulation of insulin secretion is associated with Ca^{2+} signaling and expression of SERCA-2b and -3 genes in pancreatic islets and provides a direct link between insulin resistance and defective insulin secretion. *Diabetes* 53:1517–1525, 2004

The insulin/IGF-1 receptor signaling pathway plays a significant role in the regulation of both insulin secretion and synthesis (1–5). We and other laboratories have shown that the insulin receptor substrate (IRS)-1/phosphatidylinositol (PI) 3-kinase pathway is involved in the growth and function of islets (6–9). Further evidence for a role for this substrate in islet function is derived from IRS-1 knockout (KO) mice that exhibit hyperplastic islets but show a reduced insulin content, decreased insulin mRNA, and decreased secretion in response to glucose and L-arginine (8,10,11). Overexpression of the arginine-to-glycine polymorphism at position 972 in the IRS-1 gene in β-cells leads to apoptosis (12) and a reduced insulin secretory response (13), whereas, conversely, overexpression of IRS-1 in β-cells has been shown to increase insulin secretion (14).

Cytoplasmic calcium is a ubiquitous messenger that is essential for physiological responses in most mammalian cells including pancreatic β-cells. Glucose and several other insulin secretagogues depolarize β-cells, leading to activation of voltage-sensitive Ca^{2+} channels and an influx of Ca^{2+} ions across the plasma membrane. This increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers exocytosis of insulin (15). The Ca^{2+} store in the endoplasmic reticulum is an important source that can also trigger insulin secretion (16). In either case, the increase in $[Ca^{2+}]_i$, observed just before insulin secretion, is followed by rapid lowering of intracellular Ca^{2+} concentrations either by extrusion and/or by uptake into the endoplasmic reticulum against a concentration gradient. In islet β-cells, similar to other eukaryotic cells, these active movements of Ca^{2+} ions are mediated by Ca^{2+} pumps (17). The sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) is one such intrinsic membrane protein with a single polypeptide chain of 110–115 kDa. Among the SERCA isoforms that have been identified so far, SERCA-2a, -2b, and -3 are also expressed in pancreatic β-cells (18,19). The SERCA proteins have been shown to lower intracellular Ca^{2+} levels in β-cells by sequestering the ions into the endoplasmic reticulum (20,21).

Abnormalities in Ca^{2+} signaling have been related to the development of some forms of type 2 diabetes in several rodent models and in humans (20,22). In the *db/db* mouse (20) and in Goto-Kakizaki rats (19), a loss of SERCA activity is associated with defects in the patterns of glucose-stimulated changes in $[Ca^{2+}]_i$ and potentially in insulin secretion. Furthermore, in humans with type 2 diabetes, alteration in the normal pulsatility of insulin secretion has been recognized as a significant islet secretory defect (23,24). Although the mechanism underlying normal oscillations in insulin secretion has not been

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5-HT, 5-hydroxytryptamine; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; IRS, insulin receptor substrate; KRB, Krebs-Ringer buffer; PI, phosphatidylinositol; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase.

precisely defined, defects in glucose metabolism leading to altered glycolysis and the ATP/ADP ratio (25–27), altered mitochondrial priming (28), defects in mechanisms that maintain normal oscillations in $[Ca^{2+}]i$ (29), and a feedback effect of insulin itself (30) have all been implicated as factors that can potentially disrupt the normal pulsatile pattern of insulin release (31).

IRS-1 has been reported to be a mediator between the tyrosine phosphorylation cascade and calcium signaling in skeletal muscle by interacting with SERCA-1 and SERCA-2 (32). We hypothesized that the modulation of SERCA proteins by IRS-1 could contribute to the altered stimulus-secretion coupling in β-cells in insulin-resistant states. Supporting this idea, it has recently been shown that IRS-1 and SERCA-3b are colocalized and coimmunoprecipitated in β-cells (33). To test this hypothesis, we examined glucose-stimulated Ca^{2+} flux and insulin exocytosis in primary β-cells and the oscillations in Ca^{2+} and O_2 consumption in freshly isolated islets of wild-type and IRS-1 KO mice. Furthermore, we studied changes in SERCA expression in KO islets as a possible mechanism underlying the secretory defect.

RESEARCH DESIGN AND METHODS

Animals, islet isolation, and in vitro culture of β-cells. IRS-1 KO mice (10,34) derived by breeding IRS-1 heterozygous mice were maintained on the original 129Sv/C57Bl hybrid background at Taconic Farms (Germantown, NY). Three- to 4-month-old male mice were shipped to the Joslin Animal Facility or to the University of Florida and kept on a 12-h light/12-h dark cycle with ad libitum access to water and food. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and the University of Florida.

For islet isolation, mice were anesthetized with sodium amytal (200 mg/kg body wt; Eli Lilly, Indianapolis, IN), and the islets were obtained by the intraductal collagenase method (35). Islets were handpicked under a stereomicroscope (Nikon Stereozoom GZ7) and used for either RNA extraction or for amperometry and oscillation experiments. For experiments on β-cells, islets were dispersed into single cells by shaking in trypsin/EDTA (0.025%) as described previously (8). Cells were cultured in RPMI 1640 (with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) and used on days 2–4 after isolation.

Amperometric detection of exocytosis. To gain an estimate of insulin secretion from single β-cells, exocytotic events were recorded by amperometry as described previously (3). β-Cells were identified by their relatively larger size compared with non–β-cells (37) and by their ability to exocytose in response to stimulation with glucose. For amperometry experiments, β-cells were incubated in RPMI 1640 media with 1 mmol/l 5-hydroxytryptamine (5-HT) for 16 h before secretion measurements to allow for accumulation of 5-HT into secretory vesicles. 5-HT is co-released with insulin secretory vesicles (38–40) and can be directly detected by amperometry. Secretion measurements were made by positioning C-fiber microelectrodes poised at 0.60 V versus a sodium-saturated calomel electrode ~1 μm away from a cell. Stimulants were applied from a micropipette placed ~30 μm away from the cell. During recordings, cells were incubated at 37°C in a modified Krebs-Ringer buffer (KRB). Stimulant solutions (18 mmol/l glucose, 18 mmol/l glyceraldehydes, 18 mmol/l L-arginine with 3 or 10 mmol/l glucose) were prepared by diluting appropriate stock solutions into KRB. All stimulants were applied for 40 s. Insulin secretion was estimated as the number of exocytotic events evoked by the application of secretagogue (38,40).

Oscillatory Ca^{2+} and O_2 consumption measurements. Islets or single β-cells on coverslips were incubated for 30 min with 2 μmol/l Fura-2 acetoxymethyl ester (Molecular Probes) at 37°C in RPMI 1640 media. Coverslips containing the fura-2 loaded islets or cells were placed in a coverslip holder with a capacity of 300 μl, and experiments were performed in KRB. Excitation of fura-2 was accomplished using an Xe lamp with sequential excitation at 340 and 380 nm. Fluorescence emission was collected and stored using DM3000M data acquisition software (Instruments SA) for quantification of Ca^{2+} concentration (41).

O_2 levels inside islets were measured using a platinum microsensor held at −0.60 V versus a sodium-saturated calomel reference electrode, and detection of oxygen was accomplished by measuring the current due to reduction of O_2 at the platinum surface (42). Current was amplified using a Keithley 428 current amplifier, and data were collected with an IBM-compatible personal computer. Islets were placed in modified Nunclon dishes with a capacity of 100 μl. Islets were perfused at a rate of 1–2 ml/min with KRB heated to 37°C.

Semiquantitative RT-PCR. Islet RNA was isolated using Trizol (Gibco BRL). Contaminating genomic DNA was removed using 1 μl RNase-free DNase-I (Boehringer) per 5 μg RNA. Conditions used for reverse PCR followed the method of Wilson and Melton (43) and were performed as described earlier (44). Primer sequences are available on request.

Statistical analysis. All data unless otherwise indicated are presented as means ± SE. The number (n) of experiments performed is shown in parentheses. Cells and islets were obtained from at least three different mice of each genotype. Data were analyzed using an unpaired Student's t test, and the null hypothesis was rejected at 0.05.

RESULTS

Altered $[Ca^{2+}]_i$ in IRS-1 KO β-cells in response to insulin secretagogues. To evaluate the effect of different insulin secretagogues on changes in intracellular calcium ($[Ca^{2+}]_i$), we stimulated wild-type and IRS-1 KO primary β-cells with glucose or glyceraldehyde and examined the ability of L-arginine to potentiate the secretory response to glucose in these cells.

Stimulation of both wild-type and KO cells with 18 mmol/l glucose produced an increase in $[Ca^{2+}]_i$. The rise in $[Ca^{2+}]_i$ was evident even before the application of the stimulus was completed in the wild-type cells, whereas the latency was longer in the IRS-1 KO cells ($n = 7$ each; $P < 0.001$; Fig. 1*A* and *D*). Although both the wild-type and KO cells showed a similar peak response, the total duration of the $[Ca^{2+}]_i$ response above basal was significantly less in the KO cells ($P < 0.001$, Fig. 1*A* and *E*). A similar pattern was observed in response to glyceraldehyde with a reduction in the total duration of $[Ca^{2+}]_i$ response ($n = 6$ wild-type, $n = 8$ KO; $P < 0.02$) (Fig. 1*B* and *E*). The latency of response was also longer in the knockouts but did not reach statistical significance compared with controls. When L-arginine was used as a stimulant in the presence of 3 mmol/l glucose, no significant differences in the changes in $[Ca^{2+}]_i$ were observed between the groups (data not shown; $n = 6$ wild-type, $n = 7$ KO). However, in the presence of a higher glucose concentration (18 mmol/l), a longer latency ($P < 0.05$; Fig. 1*C* and *D*) and a reduction in the total duration of the rise in $[Ca^{2+}]_i$ was observed in the KO cells ($n = 4$ wild-type, $n = 7$ KO; $P < 0.01$, Fig. 1*C* and *E*), similar to the changes observed in response to glucose alone (Fig. 1*A* and *E*). These data indicate that β-cells lacking IRS-1 exhibit altered Ca^{2+} flux when they are stimulated with common secretagogues.

IRS-1 KO β-cells exhibit diminished exocytotic events in response to insulin and insulin secretagogues. Insulin secretion in single β-cells measured by recording current spikes due to 5-HT has been shown to correlate well with the actual amount of hormone secretion when measured as number of exocytotic events (38–40,45). Application of insulin (100 nmol/l) to primary wild-type β-cells showed a series of current spikes on an amperometric trace (Fig. 2*A*). This indicates a stimulation of secretory events and is similar to the pattern of insulin secretion induced by glucose that was observed in our previous study with insulin (8). Virtually no current spikes were evident when IRS-1 KO β-cells were stimulated with insulin ($n = 6$), even though all these cells responded to glucose stimulation, confirming that IRS-1 is pivotal in the insulin-stimulated secretory response (Fig. 2*B*) (8). In the

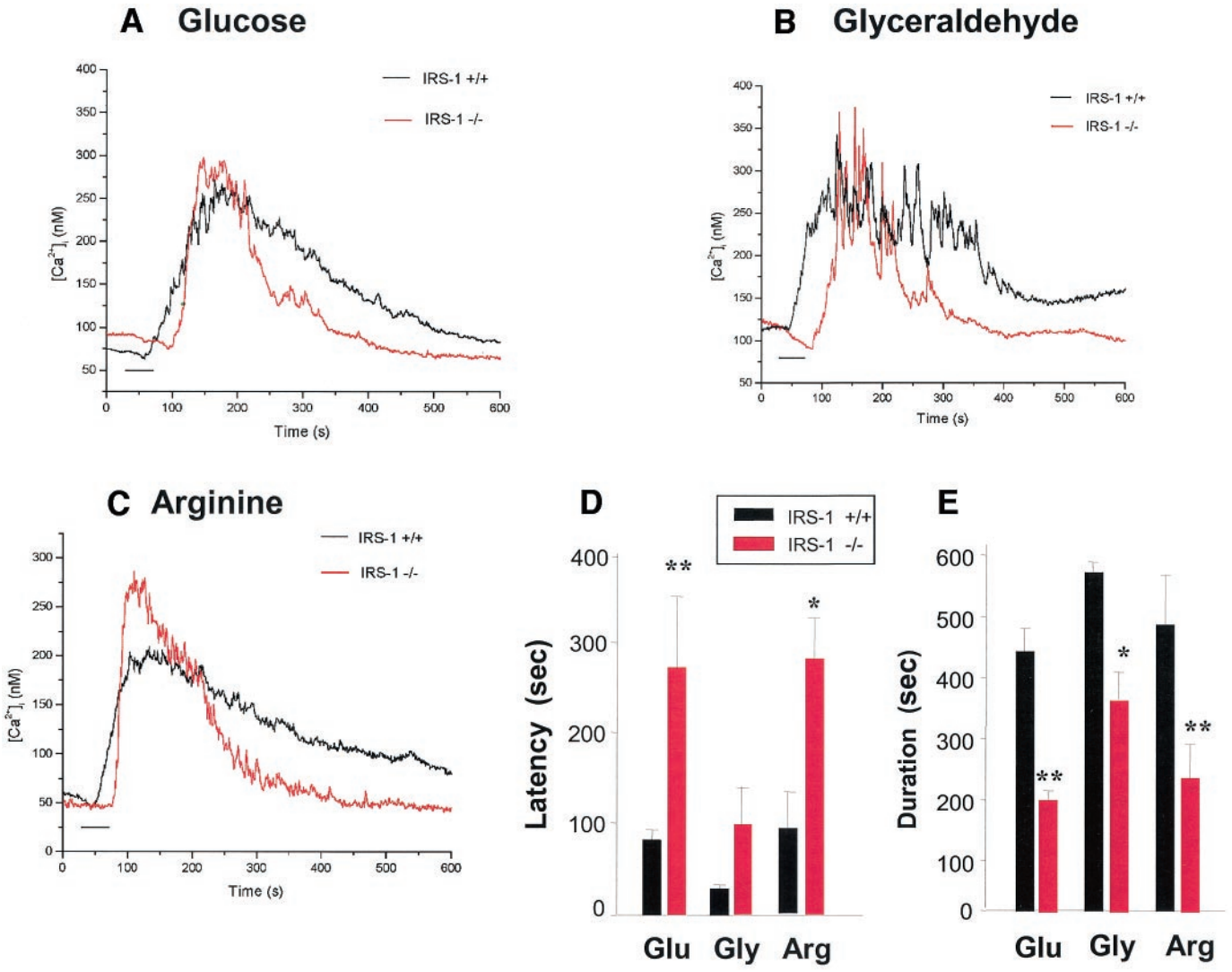

FIG. 1. IRS-1$^{-/-}$ primary β-cells show a longer latency of response and reduced total duration in the rise in $[Ca^{2+}]_i$. Changes in the $[Ca^{2+}]_i$ in IRS-1$^{+/+}$ and IRS-1$^{-/-}$ primary β-cells after stimulation with glucose (*A*), glyceraldehyde (*B*), and L-arginine (*C*) are shown. The latency and the total duration of increase in $[Ca^{2+}]_i$ are shown in *D* and *E*, respectively. The horizontal bar depicts the period of application of stimulus. **P < 0.001; *P < 0.05 IRS-1$^{+/+}$ vs IRS-1$^{-/-}$. All values are means ± SE (n = 4–8). Arg, L-arginine; Glu, glucose; Gly, glyceraldehyde.

wild-type β-cells, the number of exocytotic events in response to glucose (18 mmol/l) was quantitatively higher (14 ± 1 spikes per stimulation; n = 4, Fig. 3*A* and *D*) compared with the number observed with insulin alone (10 ± 1 spikes per stimulation; n = 7, $P < 0.05$ glucose vs. insulin stimulation). This can be interpreted as glucose inducing a greater secretory response than insulin or as the secretory response to glucose also including the autocrine effect of secreted insulin (46). Moreover, these changes were in parallel to the observations in $[Ca^{2+}]_i$ responses described above. By contrast, in the IRS-1 KO cells, although a response to glucose was detectable in the IRS-1 KO cells, the average number of current spikes detected were significantly fewer when compared with the response to insulin, indicating suppressed insulin release in the knockouts (glucose 7 ± 1 vs. insulin 0.1 ± 0.1 spikes per stimulation; n = 6, $P < 0.001$; Fig. 2*B*; Fig. 3*A* and *D*). These data indicate that glucose-stimulated insulin secretion is detectable but significantly reduced in single β-cells lacking IRS-1 compared with wild-type β-cells. These results agree with previous results of reduced glucose-

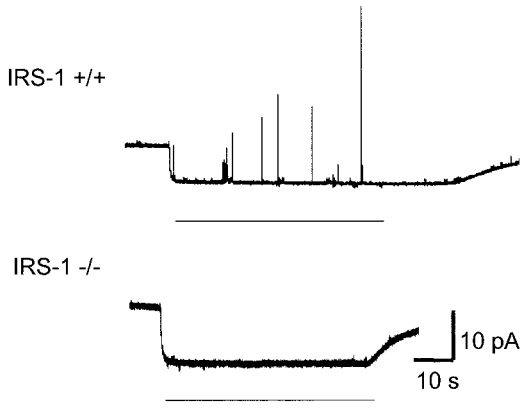

FIG. 2. Absence of insulin-stimulated insulin secretion in IRS-1$^{-/-}$ primary β-cells. Addition of exogenous insulin (100 nmol/l) to IRS-1$^{+/+}$ primary β-cells (*A*) and IRS-1$^{-/-}$ β-cells (*B*) is shown. A representative current spike pattern is shown recorded on the amperometric trace as described in RESEARCH DESIGN AND METHODS (n = 6). The horizontal bar under the trace indicates the period of stimulation.

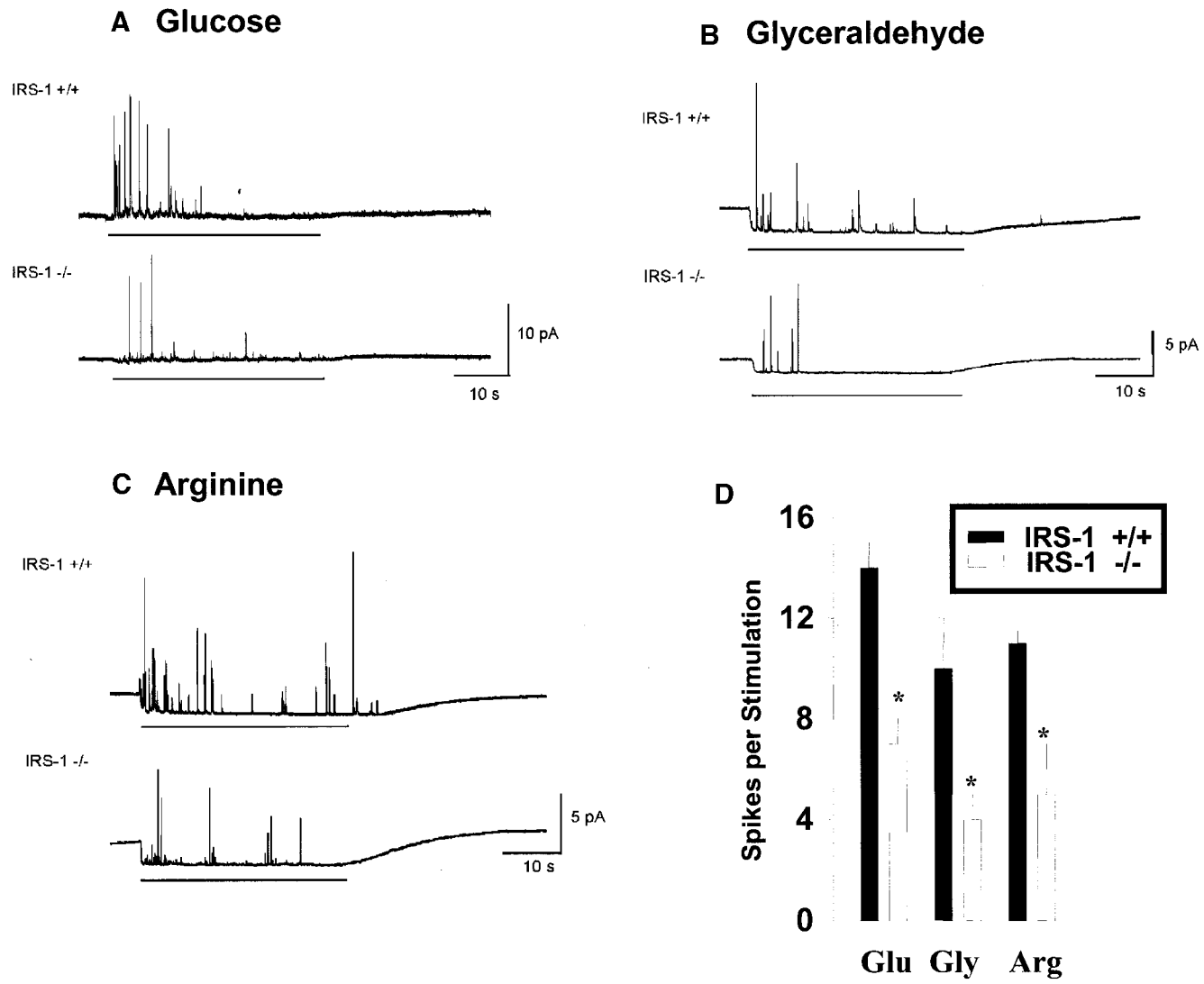

FIG. 3. IRS-$1^{-/-}$ primary β-cells exhibit reduced exocytosis in response to common insulin secretagogues. Representative traces of a current spike pattern in IRS-$1^{+/+}$ and IRS-$1^{-/-}$ primary β-cells after stimulation with glucose (*A*), glyceraldehyde (*B*), and L-arginine (*C*) are shown. The horizontal bar under each trace shows the period of application of stimulus. No responses were observed when 2-deoxyglucose was applied to the cells indicating evident trace in *D*. The bar chart shows the mean number of spikes after stimulation with glucose, glyceraldehydes, and L-arginine in IRS-$1^{+/+}$ and IRS-$1^{-/-}$ primary β-cells. *$P < 0.05$, IRS-$1^{+/+}$ vs. IRS-$1^{-/-}$. All values are means ± SE ($n = 4$–9). Arg, L-arginine; Glu, glucose; Gly, glyceraldehyde.

stimulated secretion in vivo (10) and from islets (10,11) lacking IRS-1 and extend them to single β-cells, illustrating that the effect is unlikely due to paracrine factors within islets.

The secretory defect in the KO cells was also observed with other secretagogues. Thus, the total number of exocytotic events over the duration of stimulation with glyceraldehyde was significantly lower in the KO cells ($n = 6$) than in wild-type cells ($n = 5$) (Fig. 3*B* and *D*). Although we did not observe a robust potentiation in exocytosis when L-arginine was used in combination with glucose to stimulate wild-type β-cells, we did observe fewer exocytotic events in response in the KO cells ($n = 8$) compared with wild-type controls ($n = 9$) (Fig. 3*C* and *D*). The mechanism(s) by which L-arginine potentiates glucose-stimulated insulin release is not fully understood, and further work is clearly necessary to address this question in single β-cells. However, the lack of amperometric current spikes in response to application of nonmetabolizable sugars, such as 2-deoxy-D-glucose, in wild-type β-cells (Fig. 4*B*) confirms the specificity of responses to metabolizable fuels (Fig. 4*A* and Fig. 3*A*–*C*).

These data complement the observations on the changes in $[Ca^{2+}]_i$ described above and indicate that the reduced insulin exocytosis is observed with multiple stimuli. Given the prominent role of Ca^{2+} signaling in stimulating exocytosis, it is reasonable to conclude that the reduced Ca^{2+} signaling is at least partially responsible for the reduced exocytosis.

Abnormal glucose-stimulated oscillatory $[Ca^{2+}]_i$ and O_2 consumption in IRS-1 KO islets. The characteristic pulsatile pattern of insulin secretion that has been observed in vivo (47,48) and in vitro (49–52) has been suggested to be driven by oscillations in $[Ca^{2+}]_i$ (29). If IRS-1 does influence Ca^{2+} handling, we hypothesized that deletion of IRS-1 would disrupt normal oscillations. To test this hypothesis, we measured $[Ca^{2+}]_i$ in freshly isolated islets from wild-type and IRS-1 KO mice. Stimulation of the islets with 3 mmol/l glucose did not show significant differences between the wild-type and IRS-1 KO groups

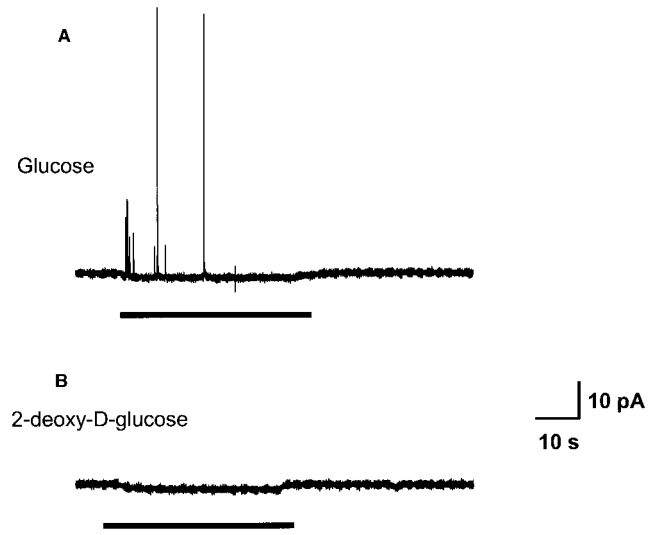

FIG. 4. Amperometric current spikes only occur in the presence of metabolizable fuels. Representative traces of a current spike pattern in IRS-1$^{+/+}$ primary β-cells (n = 9) after stimulation with 10 mmol/l glucose (*A*) and 10 mmol/l 2-deoxy-D-glucose (*B*) are shown. The horizontal bar under each trace shows the period of application of stimulus. Cells stimulated with glucose had an average of 12.4 ± 2.3 current spikes, whereas stimulation with 2-deoxy-D-glucose evoked no current spikes. All values are means ± SE.

(data not shown). However, stimulation with 10 mmol/l glucose resulted in a reproducible oscillatory pattern of $[Ca^{2+}]_i$ similar to that reported by other investigators (Fig. 5*A*) (25,53). The IRS-1 KO islets showed a longer latency in response that was similar to that observed in primary IRS-1 KO β-cells and showed a slower rise to peak compared with the rise in wild-type islets (Fig. 5*A*).

Most wild-type islets (12 of 16) exhibited fast spikes superimposed on slow oscillations that showed a periodicity of 136 ± 8 s and an amplitude change of 68 ± 9 nmol/l (n = 6). (Of the four other islets, one exhibited only slow oscillations and three had only fast oscillations.) In contrast, most of the IRS-1 KO islets (10 of 13) showed only the fast spikes with a period of 13 ± 3 s and an amplitude change of 13 ± 2 nmol/l, and only 1 of the 11 IRS-1 KO islets tested showed a slow oscillatory pattern that appeared to resemble the wild-type islets (period, 88.5 s; amplitude change, 30.2 nmol/l). (The other two IRS-1 KO islets displayed no oscillations.) These data suggest that the normal feedback effects that regulate $[Ca^{2+}]_i$ and the glucose-evoked slow oscillations are disrupted when IRS-1 is absent.

It has been reported that the increase in oxygen (O_2) consumption in islets after glucose stimulation is pulsatile (25,31). These changes in oxygen consumption parallel the oscillatory changes in $[Ca^{2+}]_i$ and insulin release, consistent with a stimulus-secretion coupling process (31). In agreement with these studies, we observed that oscillations in O_2 consumption paralleled the oscillations in $[Ca^{2+}]_i$ in both wild-type and KO islets (Fig. 5*B*). A total of 74% of the wild-type islets tested (n = 14 of 19) showed

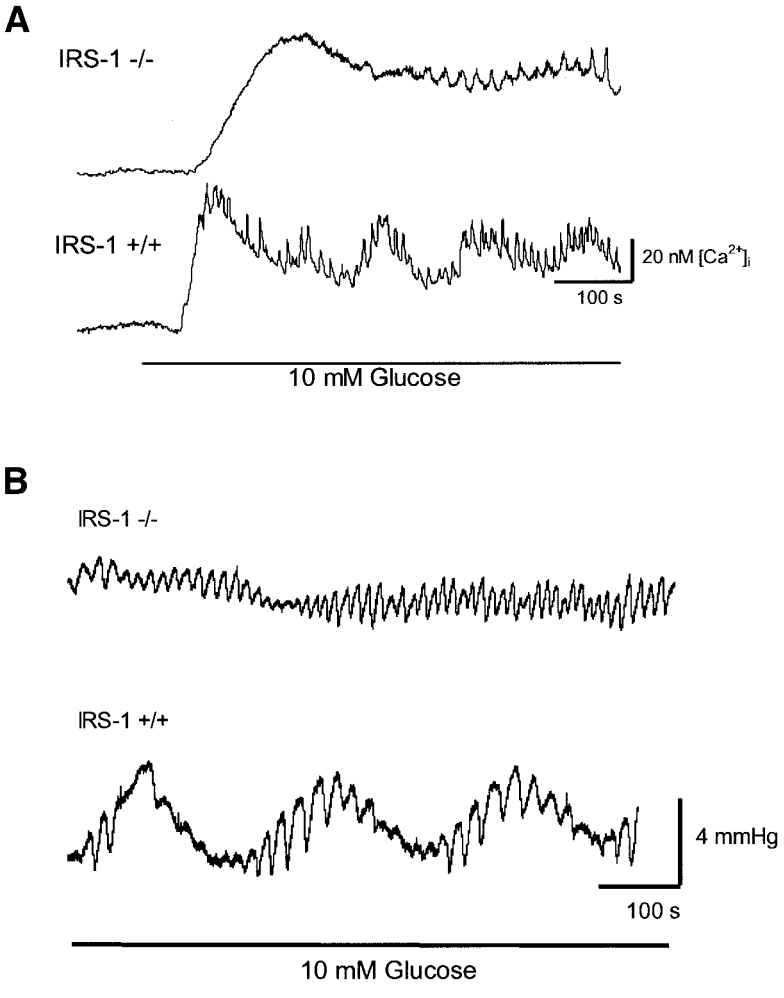

FIG. 5. Glucose stimulation of IRS-1$^{-/-}$ islets leads to dampened oscillations in $[Ca^{2+}]_i$ and O_2 consumption. Representative trace of oscillations in $[Ca^{2+}]_i$ (*A*) and in O_2 consumption (*B*) after stimulation of freshly isolated islets from IRS-1$^{-/-}$ mice (*upper panel*) and IRS-1$^{+/+}$ mice (*lower panel*). The horizontal bar under the trace indicates the period of stimulation with 10 mmol/l glucose.

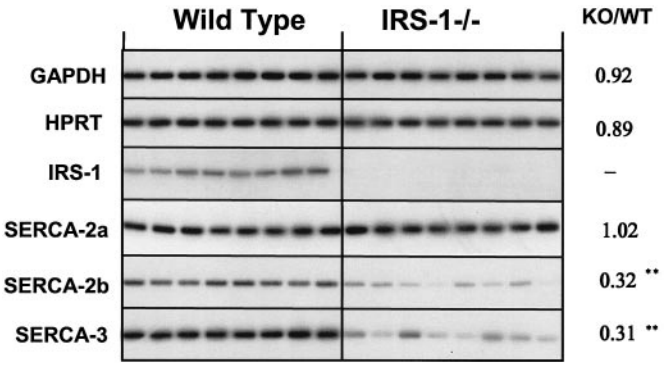

FIG. 6. Dramatic reduction in steady-state mRNA levels of SERCA proteins in IRS-1$^{-/-}$ islets. RT-PCR analysis of Ca-ATPases SERCA-2a, -2b, and -3 in islets isolated from eight individual IRS-1$^{+/+}$ and IRS-1$^{-/-}$ mice. Samples were normalized using the mitochondrial marker hypoxanthine-guanine phosphoribosyltransferase (HPRT) as described in RESEARCH DESIGN AND METHODS. Quantitative measurements were obtained by densitometry and expressed as a ratio of the mean values of IRS-1$^{-/-}$ KO to wild-type (WT) mice. **P < 0.0001 WT vs. IRS-1 KO.

slow oscillations with a periodicity of 210 ± 37 s compared with only 10% of the IRS-1 KO islets (1 of 11 tested; periodicity 203.0 s, amplitude 1.8 mmHg). All the wild-type islets exhibited fast oscillations superimposed on the slow waves. Similar to the observations in $[Ca^{2+}]_i$, most of the IRS-1 KO islets (10 of 11) showed only fast oscillations with a period of 22.5 ± 5.1 and an amplitude change of 0.8 ± 0.5 mmHg. Thus, the dampened slow waves in the Ca^{2+} oscillations observed in the KO islets were also observed in the O_2 consumption pattern, indicating a role for IRS-1 in the maintenance of normal intracellular Ca^{2+} levels and in the metabolic effects associated with glucose metabolism.

Reduced gene expression of islet SERCA proteins in IRS-1 KO mice. Alterations in $[Ca^{2+}]_i$ levels in the β-cells after glucose stimulation are a result of changes in endoplasmic reticulum Ca^{2+} stores and an influx from extracellular Ca^{2+}. To begin to localize the defect in the KO islets created by the loss of IRS-1, we examined the levels of expression of SERCA proteins. We performed semiquantitative RT-PCR using RNA prepared from freshly isolated islets from individual wild-type and IRS-1 KO mice. A relatively similar expression was observed among individual wild-type mice of all three SERCA proteins (Fig. 6). By contrast, in the IRS-1 KO islets, there was a marked decrease (>70%) in the expression of SERCA-2b and -3 (Fig. 6). No differences were evident in the expression of SERCA-2a between the groups.

DISCUSSION

Insulin secretion and biosynthesis from pancreatic islets depends primarily on glucose stimulation and the effect of some gastrointestinal hormones such as glucagon-like peptide 1 (54) and gastric inhibitory peptide (55). Recently, it has become evident that insulin itself can stimulate its receptor on the β-cell and can activate pathways that also lead to insulin secretion and synthesis (1). The insulin/IGF-I receptor substrates IRS-1 and IRS-2 and PI 3-kinase have been shown to play roles in insulin secretion and/or islet development (10,11,56), potentially serving as mediators of insulin, IGF-I, growth hormone, and other cytokines. In the present study, we provide evidence that lack of IRS-1 is associated with altered β-cell $[Ca^{2+}]_i$ and expression of SERCA-2b and -3 in the islets. Furthermore, we extend our previous observations to demonstrate reduced fuel-stimulated exocytosis in primary β-cells isolated from IRS-1 null mice.

Loss of IRS-1 leads to a complete absence of insulin-stimulated insulin release in primary β-cells, indicating the central role of IRS-1 in this secretory response (8 and present study). Furthermore, the longer latency of onset and the reduced increment of cytosolic Ca^{2+} are associated with a decrease in insulin exocytosis in IRS-1 KO cells, indicating that this protein is required for a normal glucose-stimulated insulin secretory response. This effect is also observed with glyceraldehyde and L-arginine. All of these stimuli increase $[Ca^{2+}]_i$ by activating the voltage-dependent Ca^{2+} channels before insulin exocytosis (15). It is possible that part of the rise in cytosolic Ca^{2+} activated by glucose stimulation is mediated by the ability of IRS-1 to influence endoplasmic reticulum uptake of Ca^{2+} (8). Thus, the prolonged latency, slow rise, and rapid decline in $[Ca^{2+}]_i$ levels after stimulation of IRS-1 KO β-cells may reflect the lack of insulin-stimulated increase in $[Ca^{2+}]_i$ normally mediated by IRS-1 (8,57).

We find a substantial reduction, but not total ablation, in the expression of SERCA-3 and -2b but not SERCA2a in IRS-1 KO islets, suggesting that some activity of SERCA proteins is still present in the mutant islet cells. The precise roles of each of these isoforms in islet function are not fully understood. For example, loss of SERCA-3 is associated with abnormalities in Ca^{2+} signaling in several rodent models of diabetes including the *db/db* and the Goto Kakizaki (GK) rat (20,58). In fact, a selective reduction in the islet expression of SERCA-3 but not in SERCA-2 has been reported in the GK rat model of type 2 diabetes (19). One potential explanation for the selective reduction in SERCA3 but not other isoforms in the islets is the presence of independent regulatory mechanisms for each isoform that has been reported to occur in other cell types (19,21). Recently, ablation of SERCA3 in mice has been reported not to impair insulin secretion (59), whereas studies by Borge and Wolf (33) indicate that IRS-1 and SERCA-3b are colocalized in the endoplasmic reticulum and play a role in the insulin secretory process. Furthermore, IRS-1 overexpressing β-cells show an increase in insulin secretion that is associated with altered levels of SERCA-3b expression but without affecting the levels of SERCA-2b mRNA levels (57). Although the findings in the overexpression study appear to be at deference to our findings using IRS-1 KOs in the present article, it is possible that overexpressing and/or "knocking out" IRS-1 alters several other signaling pathways known to use IRS-1 in the modulation of β-cell function, including those used by IGF-I, prolactin, placental lactogen, and growth hormone (60,61). A detailed examination of the effects of each of these ligands on β-cell function, in both the IRS-1 overexpressing and the IRS-1 KO β-cell, is necessary to address this issue.

The precise factors that regulate the different isoforms of SERCA that serve specific cellular functions are not fully elucidated. IRS-1 has been shown to influence SERCA-1 and -2 in skeletal and cardiac muscle cells in a phosphorylation-dependent manner (32) and has been

suggested to colocalize with SERCA-3 in the β-cell endoplasmic reticulum (33,57). The presence of a secretory defect in the KO islets and primary β-cells derived from them, in the present study, is suggestive that the Ca-ATPase isoforms play a role in IRS-1–mediated effects on intracellular Ca^{2+} flux in response to insulin and glucose stimulation. Thus, these data provide evidence for a direct connection between the insulin-signaling pathway and Ca^{2+} signaling in β-cells.

The dynamics of the Ca^{2+} response support the idea that the link between IRS-1 and Ca^{2+} signaling involves modulation of SERCA by an IRS-1–dependent pathway as previously suggested (8,33). However, IRS-1–mediated modulation of Ca^{2+} sequestering activity of SERCA, stimulated by release of insulin, could contribute to the rapid rise and long decay of intracellular Ca^{2+} signals during transient secretagogue application. In the IRS-1 KO cells, SERCA does not become inhibited and, by remaining active, slows the rise of Ca^{2+} and rapidly brings it to basal levels after secretagogue is removed by sequestering Ca^{2+} that enters the cells. This effect occurs despite the reduced expression of SERCA-2b and -3 in the IRS-1 KO cells, suggesting the presence of some remnant SERCA activity in the mutant cells. Furthermore, the slopes representing the initial lowering of Ca^{2+} preceding the glucose-induced rise, which has been suggested to be an indicator of SERCA-2b activity (59), were not significantly different between IRS-1 KO and wild-type groups, suggesting that SERCA-2b activity is not totally absent in IRS-1 KO islets.

Another feature we observed in the IRS-1 KO islets is the loss of normal oscillations in Ca^{2+} and O_2 consumption in response to glucose stimulation. Oscillations in both of these parameters are closely associated with the pulsatility in insulin release. The importance of oscillations in insulin secretion is highlighted by the observation of a frequently altered or impaired oscillatory behavior in insulin secretion in relatives of patients with type 2 diabetes (24). Oscillatory insulin secretion in isolated islets has been suggested to be driven by oscillatory $[Ca^{2+}]_i$ that is accompanied by oscillations in glucose metabolism (26,29). Interestingly, a loss of pulsatile insulin secretion in the Zucker diabetic fatty rat is associated with a reduction in the mRNA encoding the C- and D-isoforms of α1-subunits of β-cell L-type Ca^{2+} channels (62) and thereby linking Ca^{2+} signaling with oscillations. Other studies suggest a primary role for glucose metabolism leading to pulsatility in the ATP/ADP ratio that ultimately leads to oscillations in voltage-gated Ca^{2+} channel activity (25). It is possible that in mouse islets, IRS-1 contributes to a normal feedback mechanism that modulates cytosolic Ca^{2+} levels by regulating the expression and activity of SERCA-2b and/or -3 and that the disruption of this feedback may contribute to the observed loss of oscillations in Ca^{2+} in the KO islets and in turn to reduced insulin secretion. Thus, the low levels of expression of SERCA-2b and/or -3 isoforms in the islets likely lead to aberrant Ca^{2+} sequestration within the β-cells, and consequently the normal insulin secretory response would be disrupted.

Taken together, these data provide direct evidence for a relationship between IRS-1 and the regulation of Ca^{2+} homeostasis in islets that can potentially link insulin resistance with a secretory defect in the endocrine pancreas. Interestingly, the SERCA-3 locus has been associated with genetic susceptibility to type 2 diabetes in a Caucasian population (63). However, SERCA-3 KO mice do not show defects in insulin secretion (59), and it is unclear whether the genetic susceptibility in the patients who develop type 2 diabetes is primarily due to a defect in SERCA3 itself or due to the ambient hyperglycemia (64,65). On the other hand, polymorphisms in the IRS-1 gene in humans occur at a twofold higher frequency in patients with type 2 diabetes than in the healthy population (66,67), and these patients exhibit significantly lower basal circulating insulin levels (66). Further experiments including measurement of ATP levels, the ATP/ADP ratio, and activity of ATP-dependent K^+ channels (68,69) in IRS-1 KO islets or potential rescue of secretory defects by overexpression of one or more SERCA proteins may provide insight into associated mechanisms that likely influence the oscillatory process and/or the modulation of β-cell membrane potential by the endoplasmic reticulum.

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