Effect of the Insulin Mimetic L-783,281 on Intracellular [Ca^{2+}] and Insulin Secretion From Pancreatic β-Cells

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L-783,281, an antidiabetic fungal metabolite that has previously been shown to activate insulin signaling in CHO cells, was tested for its effect on intracellular Ca^{2+} ($[Ca^{2+}]_i$) and insulin secretion in single mouse pancreatic β-cells. Application of 10 μmol/l L-783,281 for 40 s to isolated β-cells in the presence of 3 mmol/l glucose increased $[Ca^{2+}]_i$ to 178 ± 10% of basal levels ($n = 18$) as measured by fluo-4 fluorescence. L-767,827, an inactive structural analog of the insulin mimetic, had no effect on β-cell $[Ca^{2+}]_i$. The L-783,281–evoked $[Ca^{2+}]_i$ increase was reduced by 82 ± 4% ($n = 6$, $P < 0.001$) in cells incubated with 1 μmol/l of the SERCA (sarco/endoplasmic reticulum calcium ATPase) pump inhibitor thapsigargin and reduced by 33 ± 6% ($n = 6$, $P < 0.05$) in cells incubated with 20 μmol/l of the L-type Ca^{2+}-channel blocker nifedipine. L-783,281–stimulated $[Ca^{2+}]_i$ increases were reduced to 31 ± 3% ($n = 9$, $P < 0.05$) and 48 ± 10% ($n = 6$, $P < 0.05$) of control values by the phosphatidylinositol 3-kinase (PI3-K) inhibitors LY294002 (25 μmol/l) and wortmannin (100 nmol/l), respectively. In β-cells from IRS-$1^{-/-}$ mice, 10 μmol/l L-783,281 had no significant effect on $[Ca^{2+}]_i$ ($n = 5$). L-783,281 also resulted in insulin secretion at single β-cells. Application of 10 μmol/l L-783,281 for 40 s resulted in 12.2 ± 2.1 ($n = 14$) exocytotic events as measured by amperometry, whereas the inactive structural analog had no stimulatory effect on secretion. Virtually no secretion was evoked by L-783,281 in IRS-$1^{-/-}$ β-cells. LY294002 (25 μmol/l) significantly reduced the effect of the insulin mimetic on β-cell exocytosis. It is concluded that L-783,281 evokes $[Ca^{2+}]_i$ increases and exocytosis in β-cells via an IRS-1/PI3-K–dependent pathway and that the $[Ca^{2+}]_i$ increase involves release of Ca^{2+} from intracellular stores. *Diabetes* 51 (Suppl. 1):S43–S49, 2002

L-783,281 is a recently isolated fungal metabolite that has insulin mimetic properties (1,2). This compound stimulates insulin receptor tyrosine kinase (IRTK) activity in CHO cells that overexpress human insulin receptors by binding to the β-subunit of the insulin receptor (1). L-783,281–induced activation of IRTK stimulates downstream signaling proteins, including insulin receptor substrate 1 (IRS-1), Akt, and phosphatidylinositol 3-kinase (PI3-K). L-783,281 also induces glucose uptake in a dose-dependent manner from rat primary adipocytes and soleus muscle tissue from lean mice. In vivo administration of L-783,281 results in correction of hyperglycemia in *db/db* mice and improves glucose tolerance in *ob/ob* mice. Thus, L-783,281 is an insulin mimetic, in that it initiates insulin signaling and has antidiabetic effects in rodent models of insulin resistance.

The focus of prior studies with L-783,281 has been on its activation of IRTK and glucose uptake in nonendocrine cells; however, the presence of functional insulin receptors on pancreatic β-cells (3,4) suggests that L-783,281 could activate IRTK in β-cells. β-cell IRTK activity is initiated in vitro by insulin binding to insulin receptors after its secretion (5). This activity results in phosphorylation of insulin receptor substrates (6) and PHAS-I (an inhibitor of mRNA cap-binding protein, eukaryotic initiation factor–4E) (7). In addition, maximal insulin secretion corresponds with maximal production of phosphatidylinositol 3,4,5-trisphosphate, a major product of PI3-K activity (8). Insulin signaling in the β-cell has physiological responses that are mediated by different insulin receptor substrates. IRS-2 has been implicated in maintaining β-cell growth and development, evidenced by β-cell hypoplasia and a corresponding decrease in insulin secretion in IRS-2–null mice (9–11). Signaling through IRS-1 in β-cells is also important, as it appears to maintain intracellular insulin content and synthesis (12), regulate Ca^{2+} homeostasis (13), and promote insulin secretion (14). Activation of insulin receptor signaling in βTC6-F7 tumor β-cell lines by overexpression of IRS-1 or insulin receptors results in an increase in fractional insulin secretion and an increase in basal $[Ca^{2+}]_i$ (15). In RIN 1046–38 β-cells overexpressing a $Gly^{972} \rightarrow Arg$ mutant IRS-1, a decrease in binding of IRS-1 to the regulatory subunit of PI3-K is observed, and glucose- and sulfonylurea-stimulated insulin secretion is diminished (16). IRS-1–deficient islets and tumor cell lines have decreased insulin content and glucose- and arginine-stimulated insulin secretion, which can

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5-HT, 5-hydroxytryptamine; $[Ca^{2+}]_i$, intracellular Ca^{2+}; IRS-1, insulin receptor substrate-1; IRTK, insulin receptor tyrosine kinase; KRB, Krebs-Ringer buffer; PI3-K, phosphatidylinositol 3-kinase; SERCA, sarco/endoplasmic reticulum calcium ATPase.

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be partially restored by reexpression of IRS-1 (17). Thus, insulin promotes release of intracellular Ca^{2+} and insulin secretion in a pathway involving insulin receptor, IRS-1, and PI3-K. Furthermore, defects in this pathway result in a reduction in insulin secretion (16–19). The in vivo significance of β-cell insulin signaling is demonstrated in β-cell insulin receptor knockout mice, which exhibit a reduction in glucose-stimulated insulin secretion and impaired glucose tolerance (19).

Although these recent studies have demonstrated positive feedback of insulin on secretion, several earlier studies concluded that insulin inhibits glucose-stimulated insulin secretion (20–23). The majority of these other studies were performed on islets or perfused pancreas with application of exogenous insulin, which makes interpretation as a direct effect on β-cell insulin receptors difficult due to neuronal or hormonal regulation that could interact with exogenous insulin and the presence of large amounts of endogenous insulin under the conditions used. Nevertheless, these discrepancies highlight the possibility that, while newer methods have demonstrated a positive effect of insulin on secretion in β-cells, in an intact system or in the presence of high insulin levels, other regulatory mechanisms may counteract this effect.

Due to the ability of L-783,281 to activate insulin signaling and the apparently important role of insulin in stimulating insulin secretion, we have examined the effect of L-783,281 on insulin secretion and $[Ca^{2+}]_i$ at the single-cell level. The results demonstrate that L-783,281 induces release of Ca^{2+} from thapsigargin-sensitive stores and initiates insulin secretion via a pathway similar to that of insulin at nonstimulatory glucose levels.

RESEARCH DESIGN AND METHODS

Materials. The insulin mimetic L-783,281 and its inactive analog L-767,827 were prepared at Merck Laboratories as described elsewhere (1). All chemicals for islet isolation and cell culture were from Life Technologies. All other chemicals were from Sigma Chemical (St. Louis, MO) unless otherwise stated and were of highest purity available. Experiments were performed with cells bathed in a modified Krebs-Ringer buffer (KRB) consisting of (in mmol/l): 118 NaCl, 5.4 KCl, 2.4 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 3.0 *d*-glucose, and 20 HEPES. Drugs were dissolved in DMSO and diluted in KRB to give the desired final concentration. A stock solution of LY294002 was dissolved in ethanol before diluting in KRB.

Cell culture and isolation. Single β-cells were prepared as previously described (24). Briefly, male CD1, C57Bl/6 IRS-$1^{+/+}$, or C57Bl/6 IRS-$1^{-/-}$ mice (25), weighing 20–30 g, were killed by cervical dislocation, and islets were isolated by collagenase digestion and hand-picked under a stereomicroscope. Islets were dispersed into single cells using a 0.025% trypsin/EDTA solution for 6 min at 37°C and plated on 35-mm tissue culture dishes for electrochemical experiments or 25-mm glass coverslips for Ca^{2+} imaging experiments. Cells were maintained in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, stored at 37°C in 5% CO_2, and used on days 2–4 following isolation.

Secretion measurements by amperometry. Cells were prepared for secretion measurements by incubating in RPMI medium supplemented with 1 mmol/l 5-hydroxytryptamine (5-HT) for 16 h before experiments. This treatment has previously been shown to cause 5-HT to accumulate in secretory vesicles of β-cells (26). During exocytosis, the accumulated 5-HT is coreleased with insulin (27), providing a marker of secretion that is reliably detected by amperometry using carbon-fiber microelectrodes. The validity of this method as a means to measure insulin exocytosis has been verified in several reports (27–29). To perform secretion measurements, RPMI medium containing 5-HT was replaced with KRB, and the culture plate was mounted on the stage of a Zeiss Axiovert 100 microscope equipped with a microincubator (Medical Systems, Greenvale, NY) to maintain temperature at 37°C. 5-HT released from the cells was detected with a 9-μm diameter carbon fiber disc electrode prepared as described elsewhere (30). For amperometric measurements, the electrode was poised at −0.65 V versus a sodium-saturated calomel reference electrode using a battery and voltage divider. Amperometric current was collected at 500 Hz and low-pass filtered at 100 Hz using a AI 403 current amplifier (Axon Instruments, Foster City, CA), a personal computer (Gateway 2000 P5–166), and a data acquisition board (Digidata 1200B; Axon Instruments). Amperometric traces were analyzed using software written in-house. For analysis, spikes with signal-to-noise ratio >10 were counted as significant.

A picospritzer (General Valve, Fairfield, NJ) was used to deliver test solutions to the cells via a glass micropipette positioned ~30 μm from the cell, as described elsewhere (31). Independent experiments have confirmed that this method of stimulation delivers drug at the stated concentration to the cell for the duration of stimulation. After stimulation, the drug is quickly diluted to negligible levels by diffusion into the surrounding medium. For all secretion experiments, 10 μmol/l L-783,281 in KRB was applied to isolated cells for 40 s, while secretion was measured by amperometry. To compare the amount of exocytotic events induced by different secretagogues, 100 nmol/l insulin or 18 mmol/l glucose was also applied to single β-cells for 40 s. A control solution consisting of drug carrier was applied to the same cell under investigation to ensure amperometric spikes were not due to an artifact of stimulation. To determine LY294002 sensitivity, an individual β-cell was stimulated with 10 μmol/l L-783,281 twice, with 10 min between stimulations, to demonstrate cell activity. After successful stimulations, the cell was incubated with 25 μmol/l LY294002 for 10 min and the same cell stimulated with L-783,281 again to compare the amount of secretory events. Control experiments were performed in which cells were incubated with drug carrier using a similar protocol.

$[Ca^{2+}]_i$ measurements. β-cells attached to coverslips were incubated with 2 μmol/l fluo-4 acetoxymethyl ester or 2 μmol/l fura-2 acetoxymethyl ester in RPMI medium for 30 min at 37°C in 5% CO_2 before experiments to load dye into the cells (32). Cells were washed three times and bathed with KRB. Coverslips containing the cells formed the bottom of a microchamber kept at 37°C throughout all experiments via a microincubator (Medical Systems, Greenvale, NY).

Fluo-4 measurements were made using a Nikon RCM8000 confocal microscope that consisted of a Nikon Diaphot 100 inverted microscope and an Ar^+ laser (INNOVA Enterprise 622; Coherent, Santa Clara, Calif). The 488-nm line from the Ar^+ laser was focused onto cells by a 40×, 1.15 numerical aperture water immersion objective and scanned across the image plane via galvanometers. Emission was collected through the same objective, passed through a 520 ± 10 nm bandpass filter and onto a photomultiplier tube. Images were taken at 1 Hz and stored on an optical disk recorder (Panasonic TQ-FH332). For analysis, images were played back and the ratio (F_1/F_0) of each image (F_1) versus the first image (F_0) of the series was recorded (14).

Fura-2 fluorescence was measured using a SPEX CMX cation measurement system and DM3000M data acquisition software (Instruments SA Group, Edison, NJ) with a Zeiss Axiovert 100 microscope as previously described (18). Briefly, fura-2 loaded β-cells were excited at 1 Hz with 340 and 380 nm light focused onto cells by a 40×, 1.3 numerical aperture oil immersion objective. Emission was collected via the same objective, passed through a 400-nm long pass filter, 510 ± 10 nm bandpass filter, and 20 μm spatial filter and onto a photomultiplier tube. $[Ca^{2+}]_i$ was quantified as described elsewhere (33).

For $[Ca^{2+}]_i$ recordings, test solutions were applied to cells using the same protocol as described for secretion measurements. To test the sensitivity of L-783,281–induced Ca^{2+} to drugs, cells were incubated in KRB containing 100 nmol/l wortmannin, 25 μmol/l LY294002, 20 μmol/l nifedipine, or 1 μmol/l thapsigargin for 10 min before applying 10 μmol/l L-783,281 for 40 s. Only cells that responded to 200 μmol/l tolbutamide in the presence of wortmannin, LY294002, or thapsigargin were used in the study. The L-783,281–evoked increase in fluo-4 fluorescence recorded in the presence of drugs was compared with that obtained for identical stimulations in the absence of drugs.

All values are reported as means ± SE. Statistical significance of the difference between means was determined by a two-tailed Student's *t* test.

RESULTS

Intracellular Ca^{2+} release. Application of L-783,281 to isolated β-cells in the presence of 3 mmol/l glucose resulted in a concentration-dependent increase in $[Ca^{2+}]_i$, whereas no increase for the inactive structural analog, L-767,827, was observed (Fig. 1). At 10 μmol/l L-783,281, the average maximal increase in fluo-4 fluorescence was 178 ± 10% (n = 18 cells) with an average latency between stimulation and onset of the increase of 22 ± 17 s. The

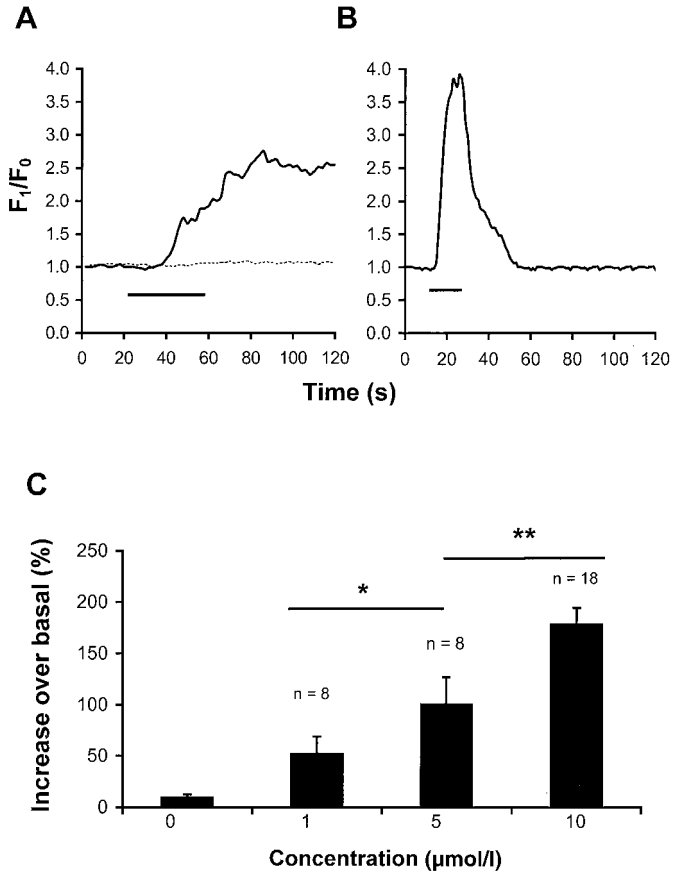

FIG. 1. L-783,281 increases $[Ca^{2+}]_i$ in pancreatic β-cells at 3 mmol/l glucose. *A*: Representative trace of relative fluo-4 fluorescence intensity versus time for stimulation with 10 μmol/l L-783,281 (solid line) and 10 μmol/l of the structural analog, L-767,827 (dashed line), in a single β-cell. Duration of stimulation is indicated by the horizontal bar under trace. Fluo-4 fluorescence was measured by drawing a region of interest around the entire cell and plotted as relative fluorescence intensity (F_1/F_0) as described in the text. *B*: Fluo-4 fluorescence from a β-cell stimulated with 200 μmol/l tolbutamide. Note the decrease in fluo-4 fluorescence after stimulation with tolbutamide ended versus that with L-783,281 in *A*. *C*: Average stimulated fluo-4 fluorescence versus concentration of L-783,281 for the number of cells indicated. Data are shown as means ± SE *$P < 0.01$, **$P < 0.001$.

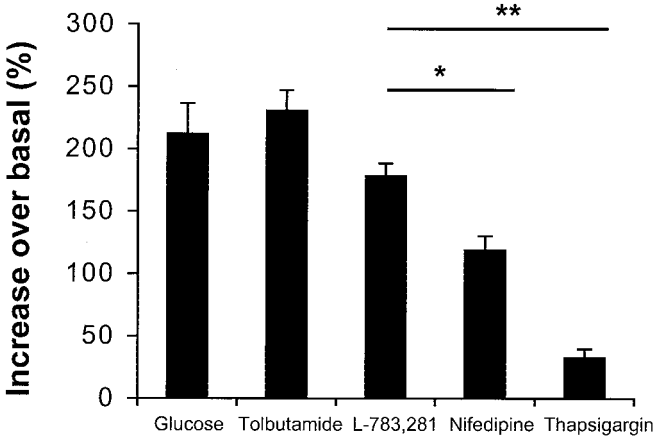

FIG. 2. Increases in $[Ca^{2+}]_i$ induced by L-783,281. Single pancreatic β-cells were incubated in 3 mmol/l glucose and stimulated for 40 s with 18 mmol/l glucose ($n = 7$ cells), for 10 s with 200 μmol/l tolbutamide ($n = 6$ cells), or for 40 s with 10 μmol/l L-783,281 ($n = 18$ cells), and the peak fluo-4 fluorescence increase over baseline was recorded. To determine the role of intracellular Ca^{2+} stores in the L-783,281–induced $[Ca^{2+}]_i$ change, cells were incubated for 10 min in 20 μmol/l nifedipine ($n = 6$) or 1 μmol/l thapsigargin ($n = 6$) and stimulated with 10 μmol/l L-783,281. All values are reported as mean ± SE. Testing of statistical significance for differences in maximal increase over baseline fluorescence was performed versus L-783,281 stimulation in the absence of any drugs. *$P < 0.05$, **$P < 0.001$.

maximal increase was comparable to that for glucose and tolbutamide stimulations (Fig. 2). The increase in $[Ca^{2+}]_i$ was long-lived and generally lasted for the entire recording time of 200 s. Further measurements revealed that the $[Ca^{2+}]_i$ stayed within 10% of the maximum stimulated level more than 5 min after the end of stimulation. For comparison, a typical trace obtained with application of 200 μmol/l tolbutamide to a single cell is shown in Fig. 1. This trace is typical of traces obtained from six different cells.

To determine if the increase in $[Ca^{2+}]_i$ was associated with Ca^{2+} entry through voltage-dependent Ca^{2+} channels, the effect of nifedipine was investigated on the L-783,281–evoked $[Ca^{2+}]_i$ response. As shown in Fig. 2, pretreatment with 20 μmol/l nifedipine reduced the L-783,281–induced Ca^{2+} increase 33 ± 6% ($n = 6$ cells, $P < 0.05$). At this concentration, nifedipine completely abolishes the $[Ca^{2+}]_i$ increases because of stimulation by K^+ or tolbutamide (data not shown), compounds that evoke an influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels. To determine if the rise in $[Ca^{2+}]_i$ was associated with release from intracellular Ca^{2+} stores, the effect of thapsigargin was examined. Thapsigargin has been shown to deplete intracellular Ca^{2+} stores by inhibiting sarco/endoplasmic reticulum Ca^{2+}-ATPases (34). As shown in Fig. 2, pretreatment with 1 μmol/l thapsigargin for 10 min reduced the L-783,281–induced $[Ca^{2+}]_i$ change by 82 ± 4% ($n = 6$ cells, $P < 0.001$). (Although thapsigargin increases intracellular Ca^{2+} as it depletes the stores, this effect is transient, and measurements were made after $[Ca^{2+}]_i$ had returned to baseline.) Combined, these results suggest that most of the increase in $[Ca^{2+}]_i$ was due to intracellular release from thapsigargin-sensitive stores.

L-783,281–induced insulin secretion. Application of L-783,281 to β-cells in the presence of 3 mmol/l glucose resulted in a series of current spikes in the amperometric trace indicative of exocytosis (Fig. 3*A*). An average of 12.2 ± 2.1 exocytotic events per stimulation ($n = 14$ cells) were detected following application of L-783,281, whereas only 1.2 ± 0.3 exocytotic events per stimulation ($n = 8$ cells) were detected with 10 μmol/l L-767,827, the inactive structural analog. For comparison, stimulation with insulin or glucose for an identical period of time resulted in a similar number of spikes detected by amperometry as with L-783,281 (Fig. 3*B*). To confirm that this response was due to effects at the insulin receptor, cells were preincubated with 100 nmol/l insulin for 10 min before application of 10 μmol/l L-783,281. Application of 10 μmol/l L-783,281 to these cells caused 0 ± 0 exocytotic events per stimulation, even though the cells were still viable as application of 40 mmol/l K^+ to the same cells resulted in 14.8 ± 0.5 spikes ($n = 4$ cells, $P < 0.05$).

Effect of L-783,281 on $[Ca^{2+}]_i$ and insulin secretion in β-cells with IRS-1 knockout. The potential role of IRS-1 in mediating the insulin secretion and increase in $[Ca^{2+}]_i$ evoked by L-783,281 was investigated by comparing the effect of the drug on β-cells from IRS-$1^{-/-}$ and IRS-$1^{+/+}$ C57Bl/6 mice. As seen in Fig. 4, stimulation of IRS-$1^{-/-}$ cells with L-783,281 resulted in no significant increases in

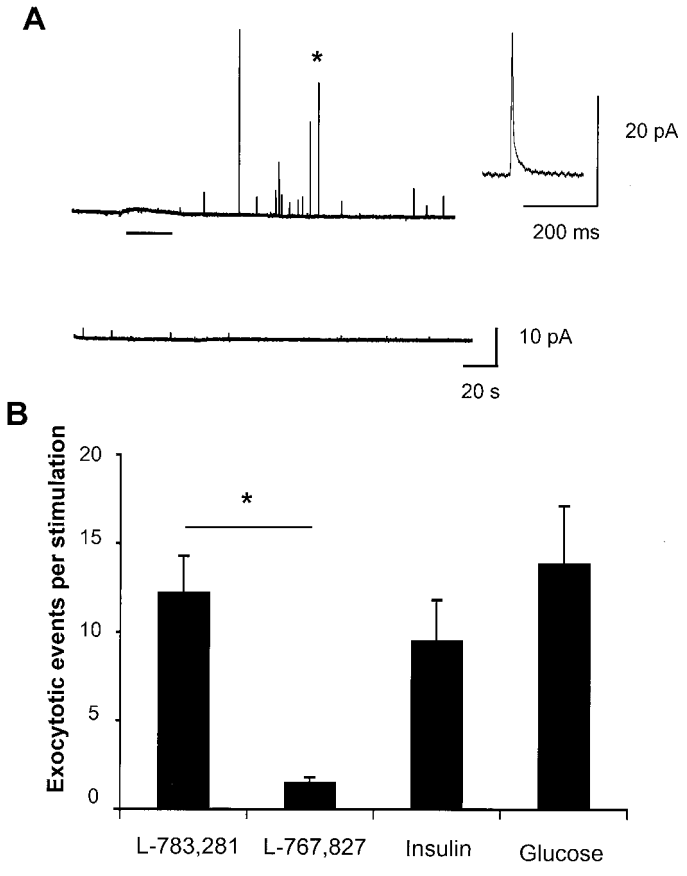

FIG. 3. L-783,281 induces exocytosis from β-cells in cells incubated with 3 mmol/l glucose. *A*: Application of L-783,281 (top) to a single β-cell results in current spikes on amperometric trace indicative of exocytosis. β-Cells were loaded with 5-HT as described in the text, and release events were measured by amperometry using carbon fiber microelectrodes. The inset shows an expanded view of a current spike (indicated by *). Stimulation with 10 μmol/l of the inactive analog, L-767,827 (bottom) lasted for the duration indicated by the horizontal bar under the traces. *B*: Number of exocytotic events induced by a 40-s stimulation with 10 μmol/l L-783,281, 10 μmol/l L-767,827, 100 nmol/l insulin, or 18 mmol/l *d*-glucose. Results are given as means ± SE

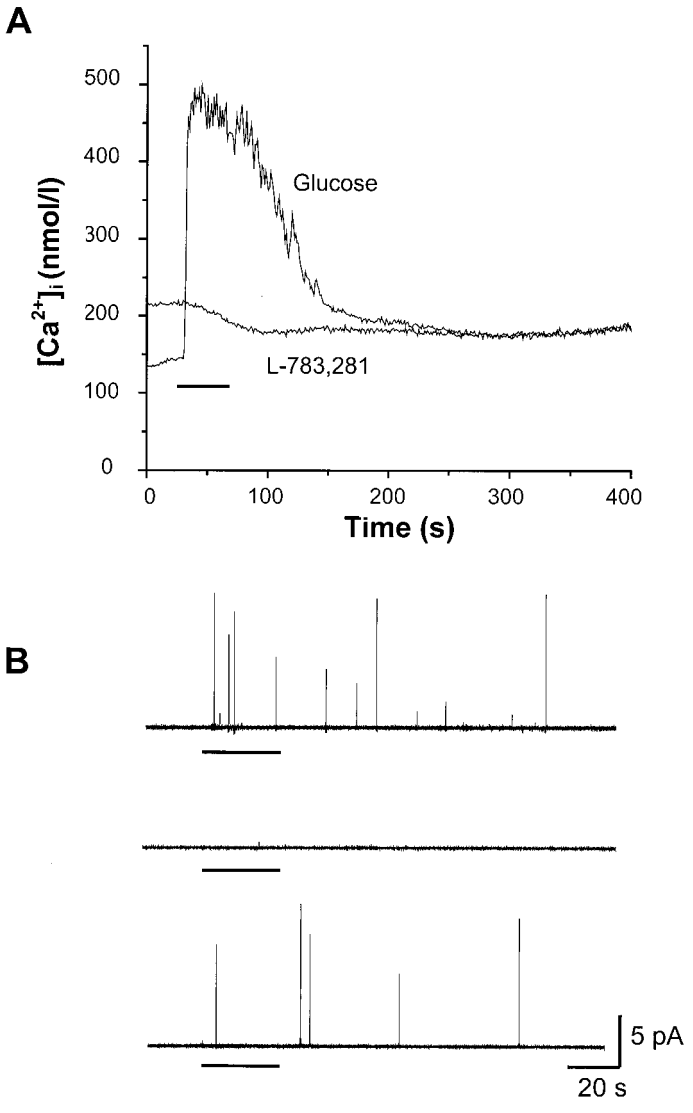

FIG. 4. Involvement of IRS-1 in L-783,281–stimulated increases in [Ca^{2+}]$_i$ and exocytosis. *A*: Representative Ca^{2+} trace of a fura-2 loaded IRS-1$^{-/-}$ β-cell stimulated with 18 mmol/l glucose and the same cell stimulated with 10 μmol/l L-783,281. The horizontal bar under the trace indicates time of stimulation. *B*: Amperometric trace of a single IRS-1$^{+/+}$ β-cell stimulated with 10 μmol/l L-783,281 (top), a IRS-1$^{-/-}$ β-cell stimulated with 10 μmol/l L-783,281 (middle), and the same IRS-1$^{-/-}$ β-cell stimulated with 18 mmol/l glucose (bottom).

[5Ca^{2+}]$_i$, even though 18 mmol/l glucose was able to evoke substantial increases in [Ca^{2+}]$_i$ in these cells. The average increase induced by L-783,281 was 8 ± 3% (n = 5 cells) over basal values, which was not significantly different from the effect of drug carrier alone (data not shown) and significantly less ($P < 0.05$) than the increase of 119 ± 25% (n = 5 cells) observed in the IRS-1$^{+/+}$ β-cells from the same type of mouse. Stimulation of β-cells from IRS-1$^{-/-}$ mice with L-783,281 resulted in 1.3 ± 0.1 (n = 5 cells) exocytotic events, which was significantly less ($P < 0.05$) than the 11.0 ± 2.0 (n = 5 cells) observed in control cells. The IRS-1$^{-/-}$ cells still responded to stimulation with 18 mmol/l glucose, with an average of 6.8 ± 0.1 (n = 8 cells) exocytotic events detected per cell (see Fig. 4*B*). These results indicate that the presence of IRS-1 is required for L-783,281 to evoke increases in [Ca^{2+}]$_i$ and insulin secretion in β-cells.

Role of PI3-K in Ca^{2+} release and insulin secretion. Pancreatic β-cells were tested to determine if L-783,281–induced [Ca^{2+}]$_i$ changes were sensitive to the PI3-K inhibitors LY294002 and wortmannin. When cells were pretreated with 25 μmol/l LY294002, application of L-783,281 still increased fluo-4 fluorescence; however, the increase was just 31 ± 3% (n = 9 cells) of that evoked without LY294002. Similarly, application of L-783,281 in the presence of 100 nmol/l wortmannin caused an increase that was 48 ± 10% (n = 6 cells) of that observed without wortmannin. Both of these increases were significantly less ($P < 0.05$) than that observed without PI3-K inhibition.

Secretion was also sensitive to LY294002, as the number of detected exocytotic events was reduced to 21 ± 6% (n = 4 cells, $P < 0.05$) of the L-783,281–induced secretion in the presence of no LY294002 at a given cell (see Fig. 5). Without application of LY294002, successive applications of L-783,281 at a 10-min interval resulted in no reduction of spike number as the second stimulation yielded 97 ± 5% of the spikes from the first stimulation (n = 8 cells).

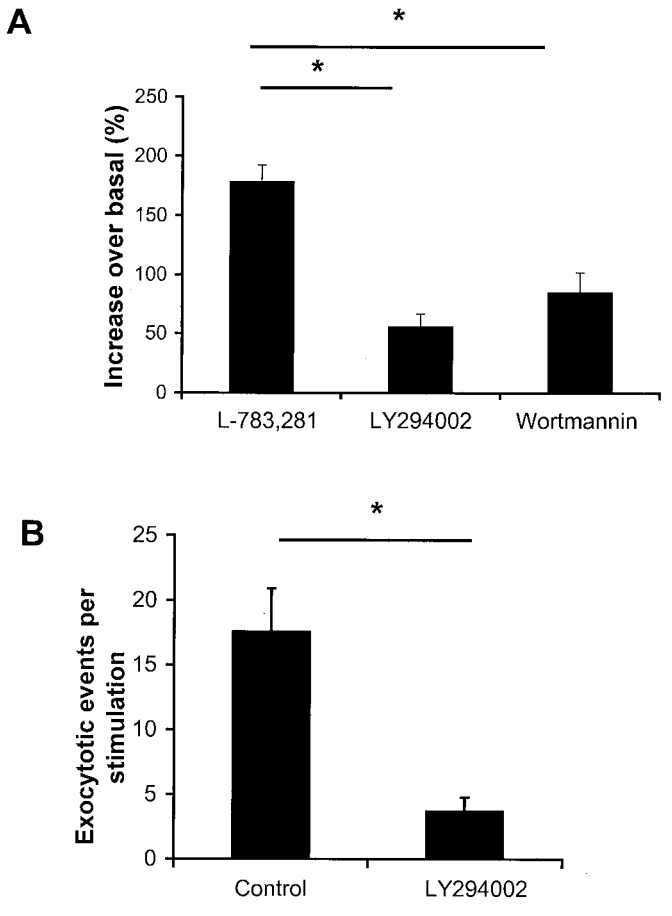

FIG. 5. Effect of PI3-K on L-783,281–induced $[Ca^{2+}]_i$ changes and exocytosis. *A*: β-Cells were incubated with 25 μmol/l LY294002 or 100 nmol/l wortmannin for 10 min and stimulated with 10 μmol/l L-783,281, and $[Ca^{2+}]_i$ changes were monitored by fluo-4 fluorescence as described in the text and in Fig. 1. Control indicates the average increase over basal induced by L-783,281 in the absence of drugs. Statistical significance was determined by comparing the mean L-783,281–induced increase in the absence and presence of the indicated drug. *B*: The number of exocytotic events induced by 10 μmol/l L-783,281 was recorded and compared with the number of exocytotic events induced by 10 μmol/l L-783,281 in the presence of 25 μmol/l LY294002. Stimulations were performed with cells in 3 mmol/l glucose. *$P < 0.05$.

DISCUSSION

Our data support the conclusions that L-783,281 evokes increases in $[Ca^{2+}]_i$ and insulin exocytosis by an IRS-1/PI3-K sensitive mechanism at a nonstimulatory glucose concentration. Given the known ability of L-783,281 to activate IRTK and its downstream cascade, including IRS-1 and PI3-K in CHO cells, and the similarity of L-783,281 and insulin effects on β-cell $[Ca^{2+}]_i$ and secretion, it is reasonable to conclude that the effects of L-783,281 on β-cells are mediated by insulin receptor activation.

Comparison of the effects of insulin and L-783,281 on β-cells. The effects of insulin signaling on β-cell $[Ca^{2+}]_i$ and exocytosis have been reported previously (13–15,18). These data support the idea that insulin binding to the insulin receptor promotes insulin secretion and results in increases in $[Ca^{2+}]_i$ via release of Ca^{2+} from the endoplasmic reticulum by a pathway that involves IRS-1 and PI3-K. Most relevant to the present study is the report of direct application of 100 nmol/l insulin to β-cells, which resulted in increases in $[Ca^{2+}]_i$ and exocytosis at β-cells (14,18).

Several similarities are observed in the acute effects of direct application of exogenous insulin and L-783,281 on β-cell $[Ca^{2+}]_i$ and secretion. In both cases, the $[Ca^{2+}]_i$ increase is characterized by a latency after application of stimulus, 20 ± 17 s and 12 ± 10 s for L-783,281 and insulin, respectively, followed by a slow increase to a maximum value (~30 s) that plateaus and persists for over 120 s. This time course is in contrast to stimulation with depolarizing agents, such as tolbutamide, which have been shown to have a latency of 1 to 2 s, a peak in $[Ca^{2+}]_i$ at ~5 s, and a return to near baseline within a minute after stimulation (Fig. 1) (14). The pharmacology of the L-783,281–induced $[Ca^{2+}]_i$ increase also resembles that initiated by insulin. Nifedipene has no effect on insulin-induced increases (14) and decreases the L-783,281–evoked increase by just 33%. In contrast, thapsigargin has strong effects, resulting in a decrease of 75 and 82% for insulin (14) and L-783,281–initiated $[Ca^{2+}]_i$ increases, respectively. PI3-K inhibitors such as wortmannin also significantly inhibit the $[Ca^{2+}]_i$ increases evoked by both insulin and L-783,281. Insulin and L-783,281 had essentially no effect on β-cell $[Ca^{2+}]_i$ in the IRS-1 knockout model. Likewise, exocytosis evoked by insulin and L-783,281 was strongly inhibited by PI3-K inhibitors and was nonexistent in β-cells from IRS-1–null mice. The similarities between the effects of L-783,281 and insulin on β-cell secretion and $[Ca^{2+}]_i$ support the idea that these compounds exert their effects through a similar mechanism that involves activation of IRS-1/PI3-K.

Another similarity involved in insulin signaling by insulin and L-783,281 is in the effect of regulating insulin gene expression in β-cells. Insulin upregulates its transcription in an autocrine manner (12). Similarly, L-783,281 acts to enhance insulin transcription by acting through the insulin receptor/IRS-1 pathway (13). Thus, insulin signaling helps to regulate its bioavailability and secretion.

Mechanism of the effects of L-783,281. Our data indicate that the majority of the $[Ca^{2+}]_i$ increase that occurs during application of L-783,281 comes from thapsigargin-sensitive stores. Generally, these intracellular stores are considered to be the endoplasmic reticulum and are regulated by IP_3 or ryanodine receptors; however, $[Ca^{2+}]_i$ can also be increased by inhibition of SERCA (sarco/endoplasmic reticulum calcium ATPase) pumps, resulting in passive diffusion of Ca^{2+} out of the endoplasmic reticulum. Several lines of evidence point to SERCA inhibition resulting from activation of IRTK or its downstream effectors, including the ability of IRS-1 to interact with SERCA pumps (35), increased basal $[Ca^{2+}]_i$ by IRS-1 inhibition of SERCA pumps, thereby inhibiting Ca^{2+} uptake into ER (15), and regulation of SERCA gene expression by activation of insulin signaling (13). The present study agrees with these previous reports in that deletion of IRS-1 inhibits the L-783,281–stimulated $[Ca^{2+}]_i$ increase. The observations of the effects of insulin signaling on $[Ca^{2+}]_i$ are consistent with the hypothesis that activation of the insulin receptor, whether by insulin or L-783,281, releases Ca^{2+} from the endoplasmic reticulum via an interaction involving IRS-1 and SERCA. Whether this Ca^{2+} is responsible for activating exocytosis has yet to be proven.

Implications for in vivo effects of L-783,281. Initial studies of L-783,281 demonstrated that this compound enhanced glucose uptake in vitro and was antidiabetic

inasmuch as it restored normoglycemia in *db/db* and *ob/ob* mice (1). It is tempting to speculate that part of the reason for an antidiabetic effect of L-783,281 was an increase in insulin secretion due to activation of the pathway observed here; however, it would be premature to reach this conclusion, since the experiments in this report involved only transient application of L-783,281 in the presence of 3 mmol/l glucose. Further experiments are necessary to determine if L-783,281 affects insulin secretion at stimulatory glucose levels, with longer-term stimulation, in whole islets, and in vivo. Demonstration of enhanced insulin secretion in vivo may be difficult to demonstrate because of multiple effects of the drug, and in vivo glucose homeostasis mechanisms may mask effects at the level of β-cells. For example, L-783,281 lowers glucose levels by activating glucose transport and potently increases insulin sensitivity in peripheral tissues (1); therefore, insulin demand during a glucose challenge is likely less, which could result in a net decrease in insulin secretion even if the drug has a stimulatory effect on insulin secretion by direct action on β-cells.

CONCLUSIONS

Type 2 diabetes is characterized by both insulin resistance and impaired insulin secretion, with the possible link between these two symptoms remaining obscure; however, the recent discovery of active insulin receptors on β-cells that promote insulin secretion, insulin synthesis, and β-cell growth has led to the hypothesis that impaired insulin signaling could account for both symptoms (36–39). We have demonstrated that the insulin mimetic L-783,281, a fungal metabolite previously shown to increase glucose uptake in peripheral tissues, also induces an increase in $[Ca^{2+}]_i$ and insulin secretion from pancreatic β-cells at nonstimulatory glucose concentrations. The effects appear to involve insulin signaling, since they are blocked by knockout of IRS-1 and are sensitive to inhibitors of PI3-K. Other groups have demonstrated that L-783,281 promotes insulin synthesis (13). These results suggest that the actions of insulin mimetics in β-cells should be further investigated as a possible tool in studying insulin signaling in β-cells and for a potential role in diabetes treatments.

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