Roles of Insulin Receptor Substrate-1, Phosphatidylinositol 3-Kinase, and Release of Intracellular Ca\(^{2+}\) Stores in Insulin-stimulated Insulin Secretion in \(\beta\)-Cells*

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The signaling pathway by which insulin stimulates insulin secretion and increases in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) in isolated mouse pancreatic \(\beta\)-cells and clonal \(\beta\)-cells was investigated. Application of insulin to single \(\beta\)-cells resulted in increases in [Ca\(^{2+}\)]\(i\) that were of lower magnitude, slower onset, and longer lifetime than that observed with stimulation with tolbutamide. Furthermore, the increases in [Ca\(^{2+}\)]\(i\), originated from interior regions of the cell rather than from the plasma membrane as with depolarizing stimuli. The insulin-induced [Ca\(^{2+}\)]\(i\) changes and insulin secretion at single \(\beta\)-cells were abolished by treatment with 100 nM wortmannin or 1 \(\mu\)M thapsigargin; however, they were unaffected by 10 \(\mu\)M U73122, 20 \(\mu\)M nifedipine, or removal of Ca\(^{2+}\) from the medium. Insulin-stimulated insulin secretion was also abolished by treatment with 2 \(\mu\)M bisindolylmaleimide I, but [Ca\(^{2+}\)]\(i\) changes were unaffected.

In an insulin receptor substrate-1 gene disrupted \(\beta\)-cell tumor line, insulin did not evoke either [Ca\(^{2+}\)]\(i\) changes or insulin secretion. The data suggest that autocrine-activated increases in [Ca\(^{2+}\)]\(i\), are due to release of intracellular Ca\(^{2+}\) stores, especially the endoplasmic reticulum, mediated by insulin receptor substrate-1 and phosphatidylinositol 3-kinase. Autocrine activation of insulin secretion is mediated by the increase in [Ca\(^{2+}\)]\(i\) and activation of protein kinase C.

Insulin secreted by pancreatic \(\beta\)-cells is the primary regulator of serum glucose concentrations in mammals. Although substantial progress has been made in elucidating the mechanisms responsible for normal regulation of insulin secretion from the \(\beta\)-cell, many aspects of this process remain unclear. In particular, chemical and physiological interactions between cells within the islet exert an important level of control in the physiological regulation of insulin secretion that is not entirely understood. Both hormonal and neuronal influences within islets may modulate \(\beta\)-cell activity and insulin secretion in vitro and in vivo (1–3). Although such influences have been demonstrated, the existence of significant autocrine effects of insulin on \(\beta\)-cells remained controversial for many years because a variety of studies yielded conflicting evidence on the modulation of insulin secretion by insulin in whole islets or in vivo. Recently, however, a variety of new methods have been utilized that demonstrate potent and possibly clinically important autocrine actions of insulin.

Several recent studies have indicated that \(\beta\)-cells express components of insulin signaling systems including insulin receptors (4–6), insulin receptor substrates (IRS-1 and IRS-2)\(^1\) (7–9), phosphatidylinositol 3-kinase (PI3-K) (10, 11), and protein kinase B (12). Evidence has also been obtained indicating that insulin released by glucose can activate these components in addition to other proteins in the cells. Insulin binds to receptors on the surface of \(\beta\)-cells (4, 13) and activates tyrosine phosphorylation of insulin receptors (6), insulin receptor substrate (8), and PHAS-I (an inhibitor of mRNA cap-binding protein) (14). Furthermore, maximal glucose-stimulated production of phosphatidylinositol 3,4,5-triphosphate (PIP\(_{3}\)), a major product of PI3-K activity, coincides with the early peak phase insulin secretion in islets and clonal \(\beta\)-cells (10). Thus, autocrine activation of the \(\beta\)-cell insulin receptors and several downstream proteins has been demonstrated.

Some of the physiological consequences of insulin receptor activation at \(\beta\)-cells have recently been revealed. Activation of the insulin signaling pathway in \(\beta\)-cells leads to initiation of insulin synthesis at both transcriptional and translational levels, increasing the cellular content of releasable hormone in primary and clonal \(\beta\)-cell cultures (14–16). In \(\beta\)TC6-F7 cells transfected to overexpress the insulin receptor, basal and glucose-stimulated insulin secretion was enhanced compared with kinase negative controls (15). In another report, clonal cells lacking the IRS-1 protein showed both decreased insulin content and glucose-stimulated secretion (17). These latter studies suggest that insulin can exert positive control over synthesis and/or secretion. Direct evidence for the effects of insulin on insulin secretion has been obtained by application of exogenous insulin to isolated \(\beta\)-cells and detecting secretion by amperometry (18). These data illustrate that insulin evokes insulin secretion mediated by the insulin receptor and that such positive feedback occurs during glucose stimulation. This report also showed that insulin could evoke an increase in intracellular [Ca\(^{2+}\)]\(i\) ([Ca\(^{2+}\)]\(i\)). A recent study with clonal \(\beta\)-cells demonstrated that overexpression of IRS-1 and insulin receptor elevated [Ca\(^{2+}\)]\(i\), levels and enhanced fractional insulin secretion (19), in good agree-

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1 The abbreviations used are: IRS, insulin receptor substrate; PI3-K, phosphatidylinositol 3-kinase; PIP\(_{3}\), phosphatidylinositol 3,4,5-triphosphate; DMEM, Dulbecco’s modified Eagle’s medium; KRB, Kreb’s Ringer buffer; 5-HT, 5-hydroxytryptamine; ROI, regions of interest; SERCA, sarco/endo-plasmic reticulum Ca\(^{2+}\)-ATPase; ER, endoplasmic reticulum; PLC, phospholipase C; PKC, protein kinase C.
ment with the studies on application of exogenous insulin.

The potential in vivo significance of positive autocrine feedback on insulin secretion and synthesis was revealed in experiments in which the gene for the β-cell insulin receptor was inactivated by use of the Cre-loxP system (20). Mice lacking the β-cell insulin receptor had lowered insulin response to glucose and impaired glucose tolerance, suggesting an important role for autocrine signaling in insulin secretion and glucose homeostasis in vivo. Further evidence for the importance of autocrine action was obtained when a polymorphism in IRS-1 in humans was associated with impaired insulin secretion and pathology of some forms of type 2 diabetes (21). The identical polymorphism expressed in clonal β-cells reduced glucose and sulfonylurea-stimulated insulin secretion.

The evidence so far has established that insulin activates the insulin receptor and that this effect results in enhanced insulin synthesis and insulin secretion. Derangement in this process leads to impaired insulin secretion similar to that seen in type 2 diabetes. Such results suggest a potential link between the symptoms of insulin resistance and impaired insulin secretion found in type 2 diabetes. Given the potential significance of autocrine activation of insulin secretion and [Ca^{2+}] changes, we have investigated some of the important elements that couple an insulin stimulus to insulin secretion and [Ca^{2+}] changes and further characterized the source and temporal characteristics of the [Ca^{2+}] changes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Type XI collagenase, HEPES, thapsigargin, Wortmannin, nifedipine, U73122, U73343, bisindolylmaleimide I, and bovine insulin were obtained from Sigma and used without further purification. Fluo-4 acetoxymethylester was from Molecular Probes. Unless otherwise stated, all chemicals for islet and cell culture were obtained from Life Technologies, Inc. All other chemicals were from Fisher unless noted and were of highest purity available.

**Isolation and In Vitro Culture of Mouse β-Cells**—Islets were isolated from 20–30 g of CD-1 mice. Briefly, islets were isolated by ductal injection with collagenase type XI and dispersed into single cells by shaking in dilute (0.025%) trypsin/EDTA for 8 min at 37 °C. Cells were cultured on 35-mm tissue culture dishes (Nunc) or 25-mm glass coverslips at 37 °C, 5% CO2, pH 7.4, in RPMI 1640 containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and used on days 2–4 following isolation.

**Culture of IRS-1 (+/−) and IRS-1(−/−) Cells**—Cell lines expressing the IRS-1 protein and IRS-1-specific knockouts were derived from breeding wild-type and IRS-1(+/−) mice with mice expressing the SV40 T antigen (RIP-Tag) on a β-cell-specific promoter similar to the procedure used by Efrat et al. (22) to derive βTC3 cells. Tumors from 12–14-week-old RIP-Tag/IRS-1(+/−) and RIP-Tag/IRS-1(−/−) mice were manually dissected and placed in DMEM supplemented with fetal calf serum, penicillin, and streptomycin. The tumor capsule was disrupted, and the cells were gently dispersed with forceps. Tumor cells were purified by gravity sedimentation and seeded in a 48-well plate containing DMEM. IRS-1(+/−) and IRS-1(−/−) clonal cells were grown to approximately 80% confluence and split 1:3 in DMEM supplemented with 20% fetal bovine serum. βTC3 cells were cultured in 200-ml tissue culture flasks in DMEM containing 25 mM d-glucose, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 5% CO2. Cells were passaged with 0.05% trypsin/EDTA at 70% confluence and plated onto 35-mm tissue culture plates (Nunc) for single cell experiments. Cells were used on days 2–4 following passage.

**Amperometric Detection of Exocytosis**—Microelectrodes were constructed of carbon fibers sealed in glass micropipettes and were polished to a 45° angle and cleaned prior to use (23, 24). Amperometric measurements were made by positioning microelectrodes ~1 μm from a cell and applying stimulant from a micropipette ~30 μm from the cell as described elsewhere (23, 24). All experiments were performed on cells at 37 °C incubated in pH 7.4 Kreb’s Ringer buffer (KRB) containing 118 mM NaCl, 5.4 mM KCl, 2.4 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 3 mM NaHCO3, and 20 mM HEPES. Stimulant solutions (100 mM insulin, 17 mM glucose, or 200 μM tolbutamide) were prepared by diluting appropriate stock solutions into KRB.

Amperometry was performed using a battery to apply potential to a sodium-saturated calomel reference electrode as described previously (24). For measurements of 5-hydroxytryptamine (5-HT) secretion, dispersed β-cells were incubated in tissue culture medium containing 0.5

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**FIG. 1. [Ca^{2+}], changes in response to insulin stimulation.** Confocal images were collected at 1 Hz using the fluorescent Ca^{2+} indicator, fluo-4, and ratioed against the initial image in the series as described in text. Image series are control stimulation with KRB (A) and stimulation with 100 nM insulin for the same cell (B). Stimulant was applied from 22 to 52 s. The ratio value is indicated on the scale bar. The number in each image indicates the time in seconds that the image was collected. Note intracellular intensity increase at 38 s and the much greater intensity increase in the cell interior in B. C, time course of changes in [Ca^{2+}], in response to insulin stimulation (red line) and control (blue line) for the cell above. Relative fluorescence intensity ([F/F_o]) was obtained by drawing a ROI around the entire cell and applying the ROI to the entire series. The bar underneath the traces indicates the application of 100 nM insulin. D, comparison of [Ca^{2+}], increases in the interior region (red line) and the edge of the cell (blue line). The ROIs are indicated in the image, where the region inside the red circle is used as the interior region and the region between the concentric white circles is used as the edge region.
mm 5-hydroxytryptophan for 16 h at 37 °C, 5% CO₂, pH 7.4. Cells were used for secretion experiments immediately after loading. For detection of 5-HT, the potential at the working electrode was 0.65 V versus sodium-saturated calomel reference electrode. Data were low pass filtered at 100 Hz and collected at 500 Hz using a personal computer (Gateway 2000 P5-186) via a data acquisition board (Axon Instruments, DigiData 1200B). For amperometric data analysis, current spikes were used only if the signal-to-noise ratio was > 10.

Confocal Imaging of [Ca²⁺].—All imaging experiments were performed on a Nikon RCM 8000 laser scanning confocal microscope, consisting of a Nikon Diaphot 300 inverted microscope, an argon-ion laser (INNOVA Enterprise 622, Coherent), associated optics, and mechanical and computer control units. Prior to imaging experiments, 25-mm coverslips containing adherent cells were loaded with 1 μM fluo-4 acetoxymethylester in KRB for 30 min at 37 °C.

Dye solution was then replaced with KRB, and coverslips with adherent cells were placed into a 35-mm coverslip holder for immediate use. Temperature was maintained at 37 °C on the stage of the microscope through the use of a microincubator (Medical Systems, Corp., Greenvale, NY) and temperature controller (Warner Instruments, Hamden, CT). Experimental buffers were the same composition as those used for amperometric measurements. Images were collected at 1 Hz (average of 32 frames) through a Nikon 40×, (NA 1.15) water immersion objective and 520–60 nm band pass filter using the 488-nm excitation line of the laser. Images were stored on an optical disc back from the optical disc cartridge, captured, and analyzed using Simca image analysis software (C-IMAGING Systems, Cranberry Township, PA) in combination with an 8-bit frame grabber. Regions of interest (ROI) consisting of either the entire cell or localized intracellular regions were drawn by free hand and applied to a series of images.

The average intensity of the ROI was measured as a function of frame number. Because fluo-4 is a single wavelength dye, its emission is a function of both [Ca²⁺], and dye concentration. [Ca²⁺], changes were therefore expressed as F/F₀ ratios where F₀ was the fluorescence intensity of the initial image during the recording (25, 26). Ca²⁺ response was analyzed as a percentage of increase compared with basal, and the amount of insulin secretion was analyzed as spikes per stimulation. All means are reported as ± S.E. Statistical differences between means were evaluated using a two-tailed Student’s t test.

Thapsigargin, Wortmannin, Nifedipine, U73122, and Bisindolylmaleimide I Treatments—For amperometric experiments, mouse β-cells were stimulated with 100 nM insulin and exocytosis of 5-HT detected by amperometry to establish viability. Following successful repetitive stimulation with insulin, 100 nM wortmannin, 20 μM nifedipine, 10 μM U73122, or 2 μM bisindolylmaleimide I was added to the buffer and allowed to incubate for 5–10 min. The same cell was then stimulated again with insulin in the presence of the chemical added.

For the [Ca²⁺] imaging experiments, cells were stimulated by 100 μM tolbutamide for 5 s to establish viability. Cells that responded to tolbutamide were then stimulated with 100 nM insulin, and temporal changes of [Ca²⁺], were monitored by fluo-4 fluorescence. Treatment with inhibitors was similar as in amperometric measurements. Before insulin stimulation, a control KRB stimulation was applied to the cell to ensure that Ca²⁺ changes and insulin secretion were not due to artifacts associated with the buffer application.

RESULTS

Insulin-stimulated Ca²⁺ Release from Intracellular Stores—We had previously reported that application of insulin to dispersed β-cells evoked a rise in [Ca²⁺], (18). Our initial experiments were to further characterize this change in [Ca²⁺], at
single cells. As shown in Fig. 1, application of 100 nM insulin, but not buffer solution, results in increased [Ca\textsuperscript{2+}], as monitored by fluo-4 fluorescence. Fig. 1C illustrates further analysis of the images shown in Fig. 1 (A and B) as a plot of relative fluorescence intensity (F_i/F_0) within the cell as a function of time. Insulin induced increases in [Ca\textsuperscript{2+}], were observed in 37 of 85 cells tested. All cells that were included in this sampling had first responded with a [Ca\textsuperscript{2+}] rise after tolbutamide stimulation. The cells that responded to insulin displayed a variety of temporal patterns of [Ca\textsuperscript{2+}] changes (Fig. 2). Of the 37 cells that responded to insulin, 9 (24%) exhibited biphasic or oscillatory response similar to that in Fig. 1C or 2D, 9 cells (24%) showed a transient peak response (Fig. 2A), 14 cells (38%) generated an elevated plateau of [Ca\textsuperscript{2+}], that lasted more than one minute after stimulation (Fig. 2B), and 5 cells (14%) had a slow increase of [Ca\textsuperscript{2+}], that did not peak or plateau after 2 min (Fig. 2C). The maximal [Ca\textsuperscript{2+}], increase induced by insulin was significantly smaller than that typically induced by a depolarizing stimuli, 200 \mu M tolbutamide (Fig. 2, D and E), and occurred with a much slower onset than that observed with tolbutamide. The latency of responses to insulin was 12 ± 10 s, whereas that for tolbutamide was 1.5 ± 1 s. The heterogeneity of temporal pattern is similar to that reported previously for single cell studies of [Ca\textsuperscript{2+}], changes induced by glucose in β-cells (27).

The relatively low success rate for insulin stimulation compared with tolbutamide stimulation is apparently due to cellular heterogeneity and the difficulty of detecting the insulin-induced signals. Cells that did not respond to insulin (n = 48) only gave a 72 ± 11% increase in [Ca\textsuperscript{2+}], with tolbutamide stimulation, whereas cells that did have a positive response to insulin (n = 37) averaged a 215 ± 38% increase in [Ca\textsuperscript{2+}], with tolbutamide stimulation. Thus, the nonresponding cells also had a statistically significant (p < 0.001) lower Ca\textsuperscript{2+} response to tolbutamide. Furthermore, the magnitude of the peak Ca\textsuperscript{2+} response for insulin stimulation is small, averaging <30% that of the tolbutamide (Fig. 2E). This small signal means that the insulin responses were more difficult to detect than tolbutamide responses. Thus, some of the cells that were counted as not responding may simply have had small responses that were not detectable. This conclusion is supported by the fact that the nonresponding cells consistently yielded lower [Ca\textsuperscript{2+}], responses to tolbutamide.

In 18 (~50%) of the cells that responded to insulin, the [Ca\textsuperscript{2+}], increase appeared initially in the interior of the cell rather than at the edge of the cell (Fig. 1B). In the remaining cells, the [Ca\textsuperscript{2+}], increase was observed simultaneously across the cell, suggesting that the temporal resolution of the measurement was too low to locate the initiation of [Ca\textsuperscript{2+}], because of rapid diffusion of the Ca\textsuperscript{2+}. A plot of the relative intensities (F_i/F_0) for the cell interior and the cytoplasm near the cell membrane is compared in Fig. 1D, which shows a higher [Ca\textsuperscript{2+}], increase in the interior region. The observation of larger [Ca\textsuperscript{2+}], increase in the cell interior is in contrast to Ca\textsuperscript{2+} imaging performed with depolarizing stimuli, such as glucose, that display higher [Ca\textsuperscript{2+}], increases at the edge of the cell because of Ca\textsuperscript{2+} entry through L-type Ca\textsuperscript{2+} channels (27). The apparent localization of the Ca\textsuperscript{2+} changes observed following application of insulin suggests that the increase in [Ca\textsuperscript{2+}], is a result of mobilization from intracellular Ca\textsuperscript{2+} stores. This conclusion was further supported by the observation that the magnitude of [Ca\textsuperscript{2+}], changes were unaffected by removing extracellular Ca\textsuperscript{2+} from the medium or by including 20 \mu M nifedipine, a blocker of L-type voltage-gated Ca\textsuperscript{2+} channels, in the medium (Fig. 4B). In addition, cells treated with 1 \mu M thapsigargin, a potent inhibitor of the SERCA pump that depletes endoplasmic reticulum (ER) stores of Ca\textsuperscript{2+}, had significantly lower insulin-induced [Ca\textsuperscript{2+}], changes (Figs. 3C and 4B) than control cells. These data suggest that the increase in [Ca\textsuperscript{2+}], evoked by insulin is a result of Ca\textsuperscript{2+} release from intracelluar stores, especially the ER.

**Requirement of Intracellular Ca\textsuperscript{2+} Mobilization for Insulin-stimulated Exocytosis**—After observing intracellular Ca\textsuperscript{2+} mobilization following insulin stimulation, we investigated the possible requirement of Ca\textsuperscript{2+} mobilization on insulin-stimulated exocytosis. Exocytosis was monitored by amperometrically detecting release of 5-HT that had been allowed to accumulate in the periphery granules of the β-cells. The 5-HT method was used instead of direct detection of insulin at a modified electrode (23) because of the relative simplicity of this approach, especially for autocrine studies. The validity of the 5-HT method has been established by previous studies that demonstrated: 1) detection of exocytosis with a variety of insulin secretagogues (24, 28, 29); 2) that insulin and 5-HT are released exclusively from the same vesicles (30); and 3) that direct detection of insulin and detection of 5-HT give comparable results for insulin stimulation (18). As seen in Fig. 3A, application of 100 nM insulin to a single, isolated β-cell results in detection of a series of current spikes at the microelectrode indicative of exocytosis and subsequent detection of packets of 5-HT diffusing to the electrode (24, 28, 29). Incubation of cells
with 1 μM thapsigargin completely abolished insulin-induced exocytosis in all cells tested (n = 4; Figs. 3B and 4A). In addition, insulin-stimulated insulin secretion, measured as number of exocytosis events detected per stimulation per cell, was not significantly affected by removal of extracellular Ca^{2+} or by treatment with 20 μM nifedipine (Fig. 4A). The results from the thapsigargin treatment suggest an important role for Ca^{2+} released from the ER in evoking secretion. In addition, the Ca^{2+}-free and nifedipine results indicate a minor, if any, role for extracellular Ca^{2+} entering the cell in insulin activation of exocytosis.

Involvement of IRS-1 in Insulin-stimulated Exocytosis and Increases in [Ca^{2+}].—We have previously demonstrated that insulin-stimulated insulin secretion in β-cells is mediated by β-cell insulin receptors (18). Insulin receptor substrates are intimately coupled to the insulin receptor and hence the insulin signaling pathway (31). To investigate the potential involvement of IRS-1 in autocrine activation of β-cell secretion, we measured insulin-stimulated insulin secretion and [Ca^{2+}] changes in wild-type βTC3 cells (IRS-1+/−) and IRS-1 knockout cells (IRS-1−/−) as illustrated in Fig. 5. We observed that much like primary β-cells, wild-type cells exhibited insulin-induced exocytosis and [Ca^{2+}] changes. Wild-type cells stimulated with 100 nM insulin evoked detectable exocytosis in 16 of 33 cells attempted. In the IRS-1 knockout cells, no secretory activity was detected upon application of 100 nM insulin (25 of 25 cells) (Fig. 5C), even though these same cells exhibited secretion from tolbutamide stimulation (Fig. 5C). Insulin-induced increases in [Ca^{2+}] were observed in 7 of 16 wild-type cells. The various patterns of [Ca^{2+}], increase seen with primary β-cells were also seen with the wild-type cells. No increase in [Ca^{2+}], was observed for IRS-1 knockout cells (13 of 13 cells) following insulin stimulation, but all the cells used for insulin stimulation showed a [Ca^{2+}] increase following 200 μM tolbutamide stimulation (Fig. 5A). To summarize, in wild-type βTC3 cells, insulin evoked exocytosis and [Ca^{2+}] changes with a similar frequency and magnitude to that observed in primary cells; however, in the IRS-1 knockout βTC3 cells, insulin did not evoke either [Ca^{2+}] changes or exocytosis. These results illustrate a critical role for IRS-1 in mediating the autocrine [Ca^{2+}], changes and insulin secretion.

Wortmannin Sensitivity of Insulin-stimulated Exocytosis and Ca^{2+} Response.—After observing IRS-1 involvement in insulin stimulation of exocytosis in the β-cell, we investigated the role of PI3-K in insulin-stimulated secretion and [Ca^{2+}] changes in primary β-cells. Following repetitive stimulation with 100 nM insulin to ensure that a cell responded to insulin, β-cells were incubated with 100 nM wortmannin, a potent inhibitor of
P3-K, for 5 min. As shown in Fig. 4A, wortmannin completely abolished exocytosis from the β-cells, suggesting the requirement of P3-K activation in the insulin-stimulated insulin secretion pathway. The requirement of P3-K activation was also investigated for insulin-stimulated [Ca²⁺]ᵢ changes. In the presence of 100 nM wortmannin, the insulin-induced Ca²⁺ response was significantly reduced (Fig. 4B).

Roles of Phospholipase C (PLC) and PKC in Insulin-stimulated Insulin Secretion—The activation of P3-K leads to production of PIP₃, which could activate PLC-γ (32, 33) and lead to release of Ca²⁺ from inositol 1,4,5-trisphosphate-sensitive Ca²⁺ stores. The effect of PLC activation on insulin-stimulated insulin secretion was investigated by using the PLC inhibitor U73122. As shown in Fig. 4A, in the presence of the inhibitor, secretion was reduced to 82% of control; however, the difference was not statistically significant. In the calcium measurement, we used the structural analog of U73122, U73343, which does not inhibit PLC; as a control. No difference between the effect of U73122 and U73343 control was observed (Fig. 4B). In a positive control, treatment with U73122 completely abolished the secretory and Ca²⁺ response evoked by carbachol stimulation, which is known to release Ca²⁺ through the PLC-inositol 1,4,5-trisphosphate pathway. These data suggest that PLC is not involved in the insulin-stimulated exocytosis signaling pathways.

Next, we investigated the effect of PKC inhibition on insulin-stimulated insulin secretion and [Ca²⁺]ᵢ changes. As shown in Fig. 4, treatment of cells with the PKC inhibitor bisindolylmaleimide I completely abolished the insulin-stimulated insulin secretion but had an insignificant effect on [Ca²⁺]ᵢ changes evoked by insulin. These data indicate that activation of PKC is not required for Ca²⁺ mobilization but is strongly involved in the secretory effect induced by insulin.

DISCUSSION

The discovery that β-cell insulin receptors play a role in normal regulation of insulin secretion provides a potential direct link between impaired insulin secretion and insulin resistance in type 2 diabetes (18, 20, 34, 35). Investigation of the signal transduction mechanisms by which insulin exerts the stimulatory effect on insulin secretion from the β-cell is therefore essential. Our data have shown that insulin-stimulated insulin secretion is mediated by functional insulin receptors (18), IRS-1, and P3-K activation. Activation of these substrates by insulin in β-cells has previously been reported (16, 19), and our results illustrate that these effects are directly linked to insulin secretion and increases in [Ca²⁺]ᵢ.

The increase in [Ca²⁺]ᵢ evoked by insulin appears to be mediated by release of Ca²⁺ from intracellular Ca²⁺ stores based on the localization of the increase, the effects of thapsigargin, and the occurrence of [Ca²⁺]ᵢ changes in the absence of extracellular Ca²⁺. The release of intracellular Ca²⁺ requires activation of IRS-1/P3-K; however, the complete biochemical mechanism is not clear. The PLC inhibitor study indicates that Ca²⁺ release does not result from PIP₃ activation of PLC via P3-K; however, because multiple isomers of PLC exist and the inhibitor used may not cross-react with all isoforms (36), it is not possible to completely rule out a role for any isoform of PLC.
in the insulin signaling pathway. One possible explanation of the increases in [Ca\(^{2+}\)]\(_i\), is due to the inhibition of SERCA pumps on the ER. IRS-1 has been shown to interact with SERCA proteins (37), and it has recently been demonstrated that overexpression of IRS-1 in the clonal SERCA proteins (37), and it has recently been demonstrated that PKC activation can elicit exocytosis in β-cells independent of entry of Ca\(^{2+}\) from the extracellular environment (39). Our data would support the hypothesis that IRS-1 mediates increases in [Ca\(^{2+}\)]\(_i\), necessary for insulin-evoked exocytosis and that the phosphorylated IRS-1 pathway is sufficient for activating secretion; however, we cannot rule out that IRS-3 has other roles in activating exocytosis. Several lines of evidence suggest that phosphorylated products of PI3-K play critical functions in the regulation of membrane trafficking along the secretory pathway (40, 41). PI3-K also forms an essential link between the insulin receptor and glucose transporter translocation and intracellular vesicular trafficking (42). In addition, PI3-K has been shown to be involved in regulated exocytosis in adenyl chromaffin cells by an interaction with the actin cytoskeleton independent of any effects on Ca\(^{2+}\)\(_i\). A direct link between PI3-K and the late granular docking step of regulated exocytosis was also suggested from a recent report that synaptotagmin interacts with PIP\(_2\) and PIP\(_2\) in a Ca\(^{2+}\)\(_i\)-dependent manner (44). Thus, the involvement of PI3-K in autocrine activation of insulin secretion opens up a number of possible routes for secretory regulation in β-cells.

Fig. 6 presents a summary of the possible pathways for the effects of insulin on Ca\(^{2+}\)\(_i\) and insulin secretion within the β-cell based on the data presented here. Autocrine activation of insulin secretion in the β-cell is mediated by activation of IRS-1 and PI3-K. PI3-K or its phosphatidylinositol products may be involved, with Ca\(^{2+}\), in direct activation of the exocytosis machinery of the cell. IRS-1/PI3-K also evokes release of Ca\(^{2+}\) from the ER by an as yet unknown mechanism. The Ca\(^{2+}\) may be directly involved in activating exocytosis; however, our data favor a requirement for PKC activation. Although our results have emphasized autocrine activation of an insulin receptor/IRS-1 pathway, previous investigations have demonstrated a significant role for IRS-2 activation as well. Increased insulin biosynthesis may be mediated by autocrine activation of IRS-2 (16). In addition, mice with IRS-2 gene knockouts show defects in islet development (45) and IRS-2 in β-cells may mediate IGF-1 receptor effects on β-cell development and peripheral insulin signaling (46).

Our data have identified some important contributors to the observed activation of insulin secretion and increased [Ca\(^{2+}\)]\(_i\), evoked by insulin at the β-cell. These mechanisms are presumably activated by insulin released during normal glucose stimulation in vivo. The importance of these effects for normal glucose homeostasis has been demonstrated by the glucose intolerance and reduction of first phase glucose-stimulated insulin secretion in mice lacking the β-cell insulin receptor (20) and impaired insulin secretion associated with IRS-1 polymorphisms (21). Further studies are needed to understand the linkage between factors regulated by glucose versus insulin and possible interactions of insulin with metabolism in the β-cell. Defects in any of the components of the insulin signaling pathway could be involved in impaired insulin secretion and insulin resistance seen in diabetes; however, the actual role of autocrine regulation in diabetes remains to be determined.

REFERENCES
1. Schuit, F. C., Derde, M. P., and Pipeleers, D. G. (1989) Diabetologia 32, 207–212
2. Norman, A. W., and Litwack, G. (1997) Hormones, 2nd Ed., pp. 193–227, Academic Press, San Diego, CA
3. Moens, K., Himberg, H., Flaméz, D., Huybens, P., Quartier, E., Ling, Z., Pipeleers, D., Gremlich, S., Thorens, B., and Schuit, F. (1996) Diabetes 45, 257–261
4. Verheij, K. J., and Ammon, H. P. T. (1980) J. Clin. Invest. 65, 1230–1237
5. Gazzano, H., Halban, P., Pretti, M., Ballotti, R., Brandenburg, D., Fehmll, M., and Van Obberghen, E. (1985) Biochem. J. 226, 867–872
6. Rothenberg, P. L., Willem, L. D., Simon, J., and Wolf, B. A. (1995) Diabetes 44, 820–829
7. Harbeck, M. C., Louie, D. C., Howland, J. W., Wolf, B. A., and Rothenberg, P. L. (1996) Diabetes 45, 717–721
8. Vellais, L., Carneiro, M. E., Crepaldi, S. C., Beschorner, A. C., and Saad, M. J. A. (1995) FERS LETT. 353–357
9. Sun, X. J., Pons, S., Wang, L. M., Zhang, Y. T., Yenush, L., Burke, D., Myers, M. G., Glasheen, E., Copeland, N. G., Jenkins, N. A., Pierce, J. H., and White, M. F. (1997) Mol. Endocrinol. 11, 251–262
10. Alter, C. A., and Wolf, B. A. (1995) Biochem. Biophys. Res. Commun. 208, 190–197
11. Gao, Z-Y., Konrad, R. J., Collins, H., Matsumaki, F. M., Rothenberg, P. L., and Wolf, B. A. (1996) Diabetes 45, 854–862
12. Holst, L. S., Muder, H., Manganelli, V., Bundler, F., Ahren, B., Holm, C., and Degerman, E. (1998) Biochem. Biophys. Res. Commun. 256, 181–186
13. Patel, Y. C., Amherdt, M., and Orci, L. (1982) Science 217, 1155–1156
14. Xu, G., Marshall, C. A., Lin, T.-A., Kwon, G., Munkvendatrapa, R. B., Hill, J. R., Lawrence, J. C., Jr., and McDaniel, M. L. (1998) J. Biol. Chem. 273, 4485–4491
15. Xu, G., and Rothenberg, P. L. (1998) Diabetes 47, 1243–1252
16. Leibiger, I. B., Leibiger, B., Jone, D., and Berggreen, P. O. (1998) Mol. Cell 1, 273–288
17. Kulkarni, R. N., Winny, J. N., Brüning, J. C., and Hanahan, D. (1998) Diabetes 47, A57
18. Aspinwall, C. A., Lakey, J. R. T., and Kennedy, R. T. (1999) J. Biol. Chem. 274, 6360–6365
19. Xu, G. O., Gao Z. Y., Borge, P. D., and Wolf B. A. (1999) J. Biol. Chem. 274, 10987–10974
20. Kulkarni, R. N., Brüning, J. N., Postic, C., Magnuson, M. A., and Kahn, C. R. (1999) J. Cell 96, 329–339
21. Porzio, O., Federici, M., Hribi, M. L., Dauro, D., Aceili, D., Dauro, R., Borboni, G., and Seis, G. (1999) J. Clin. Invest. 104, 357–364
22. Erfat, S., Linde, S., Kofod, H., Spector, D., Delanney, M., Grant, S., Hanahan, D., and Baeckkeskov, S. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9037–9041
23. Huang, L., Shen, H., Atkinson, M. A., and Kennedy, R. T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9608–9612
24. Aspinwall, C. A., Brooks, S. A., Kennedy, R. T., and Lakey, J. R. T. (1997) J. Biol. Chem. 272, 31308–31314
25. Guerineau, N. C., Bonnefoix, X., Stoeckler, L., and Mollard, P. (1998) J. Biol. Chem. 273, 10389–10395
26. Chen, M., Mollard, P., Madia, P., Suter, S., and Jonesma, H. J. (1999) J. Biol. Chem. 274, 2925–2937
27. Thaler, J. M., Mollard, P., Guerineau, N., Vacher, J. P., Schlegel, W., and Wellheim, W. B. (1992) J. Biol. Chem. 267, 18110–18117
28. Smith, P. A., Duchen, M. R., and Ascheroff, F. M. (1995) Pfluegers Arch. Eur. J. Physiol. 430, 808–818
29. Zhou, Z., and Misler, S. (1999) J. Biol. Chem. 274, 3498–3505
30. Aspinwall, C. A., Huang, L., Lakey, J. R. T., and Kennedy, R. T. (1999) Annu. Rev. Physiol. 45, 883–905
31. White, M. F. (1997) Diabetologia 40, S2–S17
32. Bae, Y. S., Cantley, L. C., Chen, C.-S., Kim, S.-R., Kwon, K.-S., and Rhee, S. G. (1998) J. Biol. Chem. 273, 4485–4490

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33. Falasca, M., Logan, S. K., Lehto, V. P., Baccante, G., Lemmon, M. A., and Schlessinger, J. (1998) *EMBO J.* 17, 414–422
34. Rutter, G. A. (1999) *Curr. Biol.* 9, 443–445
35. Taylor, S. I. (1999) *Cell* 97, 9–12
36. Zawalich, W. S., Zawalich, K. C., and Kelley, G. G. (1995) *Endocrinology* 136, 4903–4909
37. Algenstaedt, P., Antonetti, D. A., Yaffe, M. B., and Kahn, C. R. (1997) *J. Biol. Chem.* 272, 23696–23702
38. Ashcroft, S. J. (1994) *Diabetologia* 37, (Suppl. 2) S21–S29
39. Eliasson, L., Renstrom, E., Ammala, C., Berggren, P. O., Bertorello, A. M., Bokvist, K., Chibalin, A., Deeney, J. T., Flatt, P. R., Gabel, J., Gromada, J., Larsson, O., Lindstrom, P., Rhodes, C. J., and Rorsman, P. (1996) *Science* 271, 813–5
40. De Camilli, P., Scott, D. E., McPherson, P. S., and Novick, P. (1996) *Science* 271, 1533–1539
41. Shepherd, P. R., Reaves, B. J., and Davidson, H. W. (1996) *Trends Cell Biol.* 6, 92–97
42. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) *Cell. Biol.* 14, 4902–4911
43. Chasserot-Golaz, S., Hubert, P., Thierse, D., Dirrig, S., Vlahos, C. J., Aunis, D., and Bader, M. F. (1998) *J. Neurochem.* 70, 2347–2356
44. Schiavo, G., Gu, Q. M., Pretwich, G. D., Sollner, T. H., and Rothman, J. E. (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93, 13327–13332
45. Withers, D. J., Gutierrez, J. S., Towery, H., Burks, D. J., Ren, J. M., Previs, S., Zhang, Y., Iberal, D., Pons, S., Shulman, G. I., Bonner-Weir, S., and White, M. F. (1998) *Nature* 391, 900–904
46. Withers, D. J., Burks, D. J., Towery, H., Almamuro, S. L., Flint, C. L., and White, M. F. (1999) *Nat. Genet.* 23, 32–40