Insulin-stimulated Insulin Secretion in Single Pancreatic Beta Cells*

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Glucose is the principal regulator of insulin secretion from pancreatic beta cells in islets of Langerhans (1, 2); however, intra-islet communication through paracrine interactions may also exert an important level of control over insulin secretion and ultimately glucose homeostasis. For example, glucagon secreted from islet alpha cells potentiates insulin secretion (3), whereas somatostatin secreted from delta cells is a potent inhibitor of glucose-stimulated insulin secretion (4). Although these paracrine interactions are well established, the potential autocrine action of insulin upon insulin secretion remains unclear.

Several lines of evidence support the possibility of an autocrine action of insulin on beta cells. Insulin binds to the surface of beta cells (5, 6), and functional insulin receptors and receptor substrates identical to those found in peripheral tissues have been identified in both clonal and primary beta cells (6–9). Glucose stimulation of beta cell lines activates the beta cell insulin receptor in the same way as application of exogenous insulin, suggesting that insulin secreted from beta cells binds to the insulin receptor eliciting a physiological response (9, 10). The complete physiological consequences of insulin receptor activation of the beta cell have yet to be completely elucidated, but at least one effect is initiation of protein synthesis at both transcriptional and translational levels (10–12).

Although functional insulin receptors have been identified on beta cells, the possible effects on insulin secretion mediated by beta cell insulin receptors have not been firmly established. Several reports have shown that glucose-stimulated insulin or C-peptide secretion from islets or perfused pancreas is suppressed in the presence of exogenous insulin, leading many to believe that insulin inhibits secretion in beta cells (13–20). Under similar conditions however, some reports have shown no effect of insulin on glucose-stimulated insulin secretion (21–28). Furthermore, these data are difficult to interpret as direct autocrine action of insulin because: 1) intact organs or islets possess neuronal and hormonal regulatory mechanisms that could interact with exogenous insulin, 2) maintenance of normoglycemia during the time course of the experiments is often difficult because of the addition of exogenous insulin, and 3) high glucose levels used to evoke stimulation likely leads to substantial activation of beta cell insulin receptors by secreted endogenous insulin, masking the effect of exogenous insulin.

Experiments with purified beta cells and beta cell lines have also generated conflicting evidence for insulin feedback. Glucose-stimulated insulin secretion from purified rat beta cells was inhibited by 20% at exogenous insulin concentrations above 1 µM (28). In contrast, measurements of the effect of insulin on C-peptide secretion in βTC3 cells failed to show direct evidence of secretory regulation by insulin (9). Furthermore, transfected βTC6-F7 cells in which the insulin receptor was overexpressed showed enhanced basal and glucose-stimulated insulin secretion, but fractional secretory levels (percentage of total releasable cell insulin secreted) remained unchanged at all glucose concentrations whereas cells expressing kinase negative (inactive) insulin receptors showed decreased glucose-stimulated insulin secretion (11). Recent studies have confirmed that manipulation of IRS-1 levels of beta cell lines affect levels of insulin synthesis and secretion (29, 30). These results suggest an autocrine pathway regulating one or more of the following: insulin secretion, insulin synthesis, and glucose sensing/utilization.

Given the ambiguities of previous experiments, we have attempted to directly characterize the effect of exogenous insulin upon insulin secretion from single beta cells using amperometry (31, 32). In this technique, an amperometric electrode is positioned next to a single cell so that released secretory product can be detected with high sensitivity and temporal resolution. When secretion by vesicle fusion occurs, a current spike is recorded that corresponds to quantitative detection of packets of molecules released by exocytosis (31–33). Secreted insulin can be detected directly using a carbon fiber amperometric

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Insulin-stimulated Insulin Secretion

MATERIALS AND METHODS

Chemicals and Reagents—Bovine insulin, Type XI collagenase, HEPES, and tolbutamide were obtained from Sigma and used without further purification. Monoclonal anti-insulin, polyclonal anti-insulin receptor, and IgG were obtained from BioDesign International (Kennewick, ME) and were of rabbit origin. Unless otherwise stated, all chemicals for islet and cell culture were obtained from Life Technologies. All other chemicals were from Fisher unless noted and were of the highest purity available.

Isolation and in Vitro Culture of Mouse Islets and Beta Cells—Islets were isolated from 20–30 g CD-1 mice following ductal injection with 30 mM KCl to dissolve the desired concentration of stimulant in KRB. 30 mM KCl was added to the buffer and allowed to incubate for 5 min. The same cell was then stimulated again with 100 mM insulin in the presence of the antibody. Following insulin stimulations, the cell was stimulated with 30 mM K+ in the presence of antibody to confirm cell viability.

Investigation of Autocrine Activation of Beta Cells—Direct autocrine activation of 5-HT-loaded beta cells was investigated by first establishing cells to be responsive to 100 mM insulin stimulation by detecting exocytosis of 5-HT by amperometry. The number of 5-HT spikes detected per stimulation was then compared at the various pH and Zn2+ concentrations.

Membrane Potential Measurements—Membrane potential measurements were made in the whole-cell perforated patch configuration at room temperature. Pipettes were pulled from borosilicate glass and had resistances between 4 and 6 megohm. Pipette solutions contained (in mM): 10 KCl, 76 K2SO4, 10 NaCl, 1 MgCl2, 10 HEPES, and 200 μM streptomycin and were used on days 2 to 4 after isolation.

Isolation and in Vitro Culture of Canine, Porcine, and Human Islets and Beta Cells—Pancreatic islets were isolated from canine, porcine, or human pancreas using controlled collagenase (Boehringer Mannheim) perfusion via the duct, automated dissociation, and discontinuous Euro-Ficoll purification using the COBE 2991 blood cell processor as described previously (35, 36). Islets were dispersed into single cells the next day using a previously described procedure (35). Cells were cultured 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 were used on days 2 to 4 after isolation.

Data Collection and Analysis—Microelectrodes were constructed that consisted of carbon fiber disks sealed in glass micropipettes and were polished to 30–45° angle immediately prior to use (32, 33). Amperometric measurements using a battery to apply potential to a sodium saturated calomel electrode (SSCE) as described previously (33). For measurements of 5-HT secretion, dispersed beta cells were incubated in tissue culture media containing 0.5 mM 5-hydroxytryptamine and 1 mM 5-hydroxytryptophan for 16 h at 37 °C, 5% CO2, pH 7.4 (33). Cells were used for secretion experiments immediately after loading. Measurements requiring direct detection of insulin were performed on beta cells that were not allowed to accumulate 5-HT prior to experimentation, and the microelectrode was chemically modified with a film of mixed valent cyanurathenate and ruthenium oxide as described elsewhere (32). For detection of 5-HT the potential at the working electrode was 0.65 V, whereas for detection of insulin the potential was 0.85 V. Data were low pass filtered at 100 Hz and collected at 500 Hz using a Digidata digitizer (Axon Instruments, Foster City, CA).

Extracellular Calcium Dependence of Insulin Stimulation—The extracellular calcium requirement for insulin stimulation was investigated using amperometry at 5-HT-loaded mouse beta cells. Cells were bathed in KRB containing 0 mM Ca2+ and stimulated with 100 mM insulin dissolved in KRB containing either 0 or 5 mM Ca2+. All data come from paired experiments of cells stimulated with both 0 and 5 mM Ca2+–containing stimulants.

Intracellular Calcium Measurements—Beta cells were incubated in 2 μM Fura-2/AM (Molecular Probes) dissolved in KRB at 37 °C, 5% CO2 for 30 min. Dye solution was then replaced with KRB, and coverslips with adherent cells were placed into a coverslip dish for immediate use. The resulting fluorescence from individual cells was collected at 1 Hz through a Plan/Ax 40 oil immersion objective (Zeiss), band pass filter (510 ± 10 nm), and 20 μm pinhole aperture onto a photocounter using a SPEX CMX cation measurement system and DM3000M data acquisition software (Instruments SA).

RESULTS

Insulin-stimulated Insulin Secretion in Single Beta Cells—In view of the majority of prior results suggesting negative feedback of insulin on insulin secretion, it was surprising to find that application of bovine insulin to isolated mouse beta cells at nonstimulatory (3 mM) glucose concentrations stimulated exocytosis (Fig. 1A). This result was confined to beta cells as the cells that responded to insulin also exhibited exocytosis when stimulated with 17 mM glucose or 200 mM tolbutamide, stimulants known to act at beta cells (n = 0) (Fig. 1B). Furthermore, the effect did not result from a contaminant in the insulin-stimulatory solution as addition of anti-insulin to the stimulant solution abolished the tonic insulin response elicited by insulin stimulation in all cases (n = 7) (Fig. 1C). Finally, this effect is not an artifact of measuring accumulated 5-HT instead of insulin, as it was possible to observe secretion by direct measurement of insulin at single beta cells that had not been allowed to accumulate 5-HT (n = 5) (Fig. 1D). The observation of insulin-stimulated insulin secretion is not unique to mouse

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1 The abbreviations used were: 5-HT, 5-hydroxytryptamine; KRB, Kreb’s Ringer buffer.
cells as we observed similar stimulatory effects on insulin secretion following stimulation with exogenous insulin in human (n = 10), porcine (n = 7), and canine beta cells (n = 21) (Fig. 2).

Requirement of Beta Cell Insulin Receptor for Insulin-stimulated Exocytosis—To determine whether insulin-stimulated insulin secretion was mediated by the beta cell insulin receptor, we examined the antagonistic effect of anti-insulin receptor on insulin-stimulated insulin secretion (Fig. 3). For these experiments, cells were stimulated with 100 nM insulin to establish responsiveness, then incubated with 10 nM polyclonal anti-insulin receptor to block the insulin receptor, and stimulated again with 100 nM insulin. In no case was secretion detected following application of antibody (n = 4). Subsequent stimulation with 30 mM K⁺ in the presence of anti-insulin receptor evoked secretion in all cases establishing cell viability following antibody treatment. In control experiments, addition of immunoglobulins had no detectable effect on insulin-stimulated exocytosis (data not shown). We also found that the insulin-stimulated insulin secretion was dependent upon the concentration of applied insulin in the range of 1 to 100 nM as illustrated in Fig. 3.

Direct Autocrine Stimulation of Single Beta Cells—Next we investigated the possibility of direct autocrine action of insulin at single beta cells. Cells that had been established as insulin-responsive by detection of 5-HT secretion following insulin stimulation were stimulated with 30 mM K⁺ in the presence and absence of 25 nM anti-insulin receptor to prevent autocrine activation of the beta cell insulin receptor. Stimulation resulted in 20.2 ± 4.5 spikes per stimulation preceding addition of antibody and was reduced to 9.6 ± 2.4 spikes per stimulation (n = 13) upon addition of antibody (p < 0.05), indicating that antibody could block released insulin from further enhancing release.

As further confirmation that insulin could contribute to direct positive feedback, we investigated the effects of conditions that reduce the free insulin concentration at the cell surface. We have previously shown that increasing H⁺ and Zn²⁺ in the extracellular medium significantly decreases the extrusion rate of insulin from single secretory vesicles after vesicle fusion leading to a decrease in the concentration of free insulin at the cell surface in cells undergoing exocytosis (33, 34). Table I summarizes the amount of 5-HT secretion, measured as a number exocytosis events detected, evoked by 200 μM tolbutamide detected from cells incubated in control buffers and buffers containing varying H⁺ and Zn²⁺ concentrations. The number of spikes detected by stimulation is significantly reduced at higher H⁺ (p < 0.01) and Zn²⁺ (p < 0.005) concentrations. Thus, under conditions where the amount of free insulin is reduced at the cell surface after vesicle fusion, the number of exocytosis events is reduced. These results are consistent with the hypothesis that secreted free insulin activates further insulin secretion.

Glucose Dependence of Insulin-stimulated Insulin Secretion—The primary physiological action of insulin is to stimu-
late glucose uptake and utilization, therefore we examined the interaction between exogenous insulin stimulation and extracellular glucose concentration by measuring the effect of insulin stimulation upon insulin secretory activation in different glucose conditions. At 0 and 3 mM glucose, application of 100 nM insulin evoked a similar number of exocytosis events per stimulation (n = 7 and 8 for 1 and 100 nM insulin, respectively) in mouse beta cells. Statistical significance for \( p < 0.025 \). F, dependence of secretory activity, measured as number of exocytosis events detected, upon concentration of insulin in stimulant solution (n = 4, 14, and 12 for 1, 10, and 100 nM insulin, respectively) in canine beta cells. Statistical significance for \( p < 0.05 \). Higher concentrations of insulin (1 \( \mu \)M) did not evoke a significantly different number of exocytosis events.

Effects of Insulin on Membrane Potential and Intracellular \( \text{Ca}^{2+} \) The novelty of insulin as an insulin secretagogue prompted us to explore other effects of insulin on stimulus-secretion pathways. Many insulin secretagogues such as glucose, sulfonylureas, and \( \text{K}^+ \) depolarize the plasma membrane leading to opening of L-type voltage-gated \( \text{Ca}^{2+} \) channels and thereby allowing \( \text{Ca}^{2+} \) entry into the cell and initiation of exocytosis. Insulin however, did not depolarize the membrane in beta cells that could be depolarized by 200 \( \mu \)M tolbutamide (Fig. 4A). The average change in membrane potential from baseline to plateau (not including action potential) following tolbutamide stimulation was 32.5 ± 1.58 mV with the occurrence of action potentials although the average change following insulin stimulation was 1.47 ± 1.52 mV (n = 13). (Although some cells showed a small depolarization, such as that shown in Fig. 4A, others had no effect or slight hyperpolarization.) Insulin-stimulated secretion was not dependent upon extracellular \( \text{Ca}^{2+} \) (Fig. 4B) as the numbers of exocytosis events were not significantly different in cells bathed in \( \text{Ca}^{2+} \)-free KRB and stimulated with 100 nM insulin containing 5 mM or 0 mM \( \text{Ca}^{2+} \) (n = 10). This result is not an artifact of low levels of \( \text{Ca}^{2+} \) in the media as we have used similar protocols to demonstrate the \( \text{Ca}^{2+} \) dependence of glucose, tolbutamide, and K+ stimulation (32). Although extracellular \( \text{Ca}^{2+} \) was not required to initiate secretion, stimulation with insulin did cause increases in intracellular calcium ([\( \text{Ca}^{2+} \)]) (n = 5) as seen in Fig. 4C. Also seen in Fig. 4C, the insulin-evoked [\( \text{Ca}^{2+} \)] changes were smaller than those caused by K+ but were of longer duration.

**DISCUSSION**

We have demonstrated for the first time that insulin can stimulate insulin secretion in pancreatic beta cells (Fig. 1D). This effect is mediated by the beta cell insulin receptor as evidenced by the antagonistic effect of anti-insulin receptor (Fig. 3). Furthermore, the insulin concentrations necessary for this effect are in the nanomolar range, which is reasonable because the EC_{50} of the beta cell insulin receptor is \( \sim 4 \) nM (9). Insulin released from a single cell is present at a sufficient level to activate the receptor and enhance secretion as demonstrated by the following results: 1) decreased secretion stimulated by K+ when the insulin receptor is blocked by anti-insulin; 2) decreased tolbutamide-evoked secretion when the cell surface concentration of free insulin is reduced by increases in H+ and \( \text{Zn}^{2+} \) extracellular concentration (Table 1); and 3) the relatively minor enhancement of secretion by exogenous insulin during stimulation with 20 mM glucose, a condition in which released endogenous insulin may be expected to activate receptors to near maximal levels. Our observation that released insulin can activate insulin autoreceptors is in agreement with previous results which demonstrated that beta cell insulin receptors are activated by glucose (9). Taken together, these results suggest that a portion of secretion that is normally observed from single beta cells is because of positive autocrine feedback upon beta cell exocytosis acting through the beta cell insulin receptor.

Although insulin evokes secretion, it does not evoke membrane depolarization and subsequent \( \text{Ca}^{2+} \) entry to cause se-
cretion as evidenced by the minimal effect on membrane potential (Fig. 4A) and independence of the insulin-stimulatory effect on extracellular Ca\(^{2+}\) (Fig. 4B). Insulin-stimulated insulin secretion is not mediated by glucose or increases in glucose utilization as the effect occurs even at 0 mM glucose. Insulin does, however, evoke a rise in \([Ca^{2+}]_i\). These results are consistent with a mechanism of stimulation in which insulin evokes release of intracellular calcium stores to initiate exocytosis reminiscent of ATP binding to P2y receptors in beta cells (40, 41).

Autoreceptor effects on hormone or neurotransmitter secretion are well known; however, most autoreceptors mediate negative feedback on secretion. The beta cell-insulin system appears to be a rare example of positive feedback on secretion. The interplay of this positive feedback effect with other regulatory mechanisms to control insulin secretion and glucose homeostasis in vivo is likely complex. It is reasonable to speculate that positive feedback would cause augmented secretion during the initial stages of elevated glucose levels giving rise to a greater bolus of insulin release; however, other mechanisms must eventually take over to suppress release. Such a sequence could contribute to the rapid increase observed in first phase insulin secretion and the sustained, lower secretion during second phase. The possibility that insulin has a local effect on secretion also raises the possibility of novel regulatory mechanisms that might occur within islets. For example, because Zn\(^{2+}\) and H\(^+\) can control the cell-surface concentration of insulin after vesicular fusion (33), these ions could play a role in regulating insulin secretion by affecting positive feedback. Serum concentrations of Zn\(^{2+}\) are in the range of 15–25 μM (42), which would be sufficient to have a large effect on free insulin level around the beta cell.

The existence of positive feedback may allow explanation of several phenomena in beta cells. For example, cultured beta cells in contact with other beta cells have been shown to secrete more insulin than isolated cells (43, 44). This result has not been explained but could be mediated by insulin from one cell stimulating further release in neighboring cells. In addition, insulin secretion from islets has been demonstrated to be oscillatory in nature and many models for oscillation have assumed some form of positive feedback by a diffusible factor released from beta cells (45, 46). No compound has been satisfactorily identified that could serve this role; however, these results indicate insulin as a possible candidate. Oscillations in insulin release are of significant interest because loss of oscillatory release is an early symptom of type-II diabetes (47).

Finally, it has been demonstrated that many type II diabetics have a marked reduction in first phase insulin secretion (48), which to date has remained unexplained but could be envisioned as involving lack of positive feedback from the beta cell insulin receptor. Supporting the possibility that autocrine activation of the beta cell insulin receptor is involved in early secretory responses is recent work in which mice with knockouts of the beta cell insulin receptor have impaired early insulin secretion and concomitant glucose intolerance (49).

Perhaps most importantly, these results suggest a possible
link between impaired insulin secretion and insulin resistance, both of which can lead to hyperglycemia and are considered hallmarks of type-II diabetes (50). Considerable controversy surrounds the issue of which of these deficiencies is the primary cause of diabetes. In some studies, the earliest observed defect is dysfunctional secretion (45, 47) and in others insulin resistance appears to be the first detectable problem (51). The observation that insulin receptors on beta cells mediate insulin secretion and synthesis (10, 11, 12), in addition to the well known role in activating glucose utilization, leads to the possibility of a direct link between dysfunctional insulin secretion and insulin resistance. Such a link is supported by evidence that disruption of the beta cell insulin receptor (49) or beta cell receptor substrates (11, 29, 30) induces defects in secretion whereas disruption in insulin receptor substrates induces insulin resistance (52, 53).

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