Effects of Glucose, Exogenous Insulin, and Carbachol on C-peptide and Insulin Secretion from Isolated Perifused Rat Islets*

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Isolated perifused rat islets were stimulated with glucose, exogenous insulin, or carbachol. C-peptide and, where possible, insulin secretory rates were measured. Glucose (8–10 mM) induced dose-dependent and kinetically similar patterns of C-peptide and insulin secretion. The addition of 100 nM bovine insulin had no effect on C-peptide release in response to 8–10 mM glucose stimulation. The addition of 100 nM bovine insulin or 500 nM human insulin together with 3 mM glucose had no stimulatory effect on C-peptide secretion rates from perifused rat islets. Stimulation with carbachol plus 7 mM glucose enhanced both C-peptide and insulin secretion, and the further addition of 100 nM bovine insulin had no inhibitory effect on C-peptide secretory rates under this condition. Perfusion studies using pharmacologic inhibitors (genistein and wortmannin) of the kinases thought to be involved in insulin signaling potentiated 10 mM glucose-induced secretion. The results support the following conclusions: 1) C-peptide release rates accurately reflect insulin secretion rates from collagenase-isolated, perifused rat islets. 2) Exogenously added bovine insulin exerts no inhibitory effect on release to several agonists including glucose. 3) In the presence of 3 mM glucose, exogenously added bovine or human insulin do not stimulate endogenous insulin secretion.

Insulin secretion from the pancreatic β-cell is tightly regulated by stimulatory signals generated during the intracellular metabolism of glucose and by neurohumoral agonists operative at the cell membrane (1–5). Most recently an additional layer of complexity has been added by reports suggesting that insulin exerts an autocrine stimulatory effect on insulin secretion from the β-cell (6, 7). This concept was based primarily on amperometric measurements using β-cells preincubated in 5-hydroxytryptamine (5HT).1 Because 5HT exposure exerts inhibitory effects on insulin release (8–10), the precise physiologic significance of findings made with 5HT-preloaded β-cells is unclear. Moreover, previous studies exploring the potential role of insulin on insulin secretion showed no effect (11) or supported the concept that insulin exerts a negative, not positive, feedback on its own secretion (12–15).

There are at least three major issues that must be addressed in attempting to establish the impact of exogenously added insulin on endogenous insulin secretory rates. The first is technical; it is difficult to accurately measure endogenous insulin release rates in the presence of exogenous insulin. The second relates to concentration of insulin necessary to establish an effect of exogenously added hormone on the β-cell. Considering that the β-cell continually releases insulin into a small volume of interstitial fluid, the level of insulin bathing the β-cell may be quite high. For example, calculations based on islet cell volume, the insulin diffusion constant and insulin secretory rates, suggest that these levels may exceed 100 nM during glucose stimulation (16). Even at rest, levels of insulin far in excess of circulating plasma levels must exist at the interface of the β-cell membrane and the interstitium. Third, the contribution of constitutive insulin release to the secretory responses observed has to be considered. The necessary insulin signaling components identified in insulin-sensitive tissues including insulin receptors, insulin receptor substrate proteins, and phosphatidylinositol 3-kinase have been found in β-cells (17–19). Because there is constitutive secretion of insulin, a tonic level of insulin signaling in these cells may influence acute stimulatory responses and thus obscure any effect of exogenously added insulin. These three issues, in addition to the use of different species and disparate methodological approaches, may account in part for the discrepancies regarding the impact of insulin on its own secretion (11, 12, 14, 20, 21).

To circumvent the first problem, and to establish the effect of exogenously added insulin on its own secretion, we have measured connecting (C)-peptide secretion rates in response to a variety of agonists from isolated perifused rat islets. Finally, the impact of insulin signaling on 10 mM glucose-induced secretion was explored using several compounds known to antagonize the kinases activated by insulin.

EXPERIMENTAL PROCEDURES

Islet Isolation—The detailed methodologies employed to assess insulin output from collagenase-isolated rat islets have been described previously (22, 23). Male Sprague-Dawley rats (350–475 g) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All animals were treated in a manner that complied with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 85–23, revised 1985). The animals were fed ad libitum. After intraperitoneal Nembutal (pentobarbital sodium, 50 mg/kg; Abbott Laboratories, Abbott Park, IL)-induced anesthesia, islets were isolated by collagenase digestion and handpicked, using a glass loop pipette, under a stereomicroscope. They were free of exocrine contamination.

Perfusion Studies—Groups of 14–18 freshly isolated islets were perifused with Krebs-Ringer bicarbonate at a flow rate of 1 ml/min for 30 or 45 min with 3 mM glucose to establish basal and stable insulin and C-peptide secretory rates. After this stabilization period they were then perifused with the appropriate agonist or agonist combinations as indicated in the figure legends and under “Results.” Perifusate solutions were gassed with 95% O2/5% CO2 and maintained at 37 °C. Insulin (24) and rat C-peptide released into the medium were measured by radio-

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1 The abbreviations used are: 5HT, 5-hydroxytryptamine; C-peptide, connecting-peptide; PMA, phorbol 12-myristate 13-acetate.
immunoassay; in the case of C-peptide measurements the protocol provided by the vendor was followed rigorously. C-peptide and, when possible, insulin release rates were measured in the same perfusate samples.

Studies with Cultured Islets—Groups of 14–18 islets were cultured as described previously (25, 26) for 18 h in CMRL-1066 containing 5.5 mM glucose and supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml) and glucose to achieve a final concentration of 2 mM. After this, the islets were perfused as described above.

Reagents—Hanks’ solution was used for the islet isolation. The Krebs-Ringer bicarbonate perfusion medium consisted of 115 mM NaCl, 5 mM KCl, 2.2 mM CaCl₂, 1 mM MgCl₂, 24 mM NaHCO₃, and 0.17 g/dl bovine serum albumin. The 125I-labeled insulin used for the insulin assay was purchased from PerkinElmer Life Sciences. Bovine serum albumin (RIA grade), glucose, carbachol, wortmannin, genistein, glutamic acid (50 μg/ml) and insulin to establish basal and stable rates of peptide secretion. They were then stimulated for 45 min with 10 mM glucose (G10) for 30 min, and perfusate samples were analyzed for C-peptide (closed circles) and insulin (open circles). Mean values ± S.E. are depicted in this and all subsequent figures. This and subsequent figures have not been corrected for the dead space in the perfusion apparatus, ~2.5 ml or 2.5 min with a flow rate of 1 ml/min.

RESULTS

Glucose-induced Insulin and C-peptide Secretion—In the initial series of experiments isolated perfused rat islets were stimulated with 10 mM glucose. Insulin and C-peptide secretory rates were measured in the same perfusate samples. As shown in Fig. 1, islet responses to 10 mM glucose (in terms of peptide secretory rates) were kinetically and quantitatively very similar. Both the C-peptide and insulin responses were biphasic in nature and, when compared with prestimulatory release rates measured in the presence of 3 mM glucose, the addition of 10 mM glucose resulted in 15-fold increases in the output of both peptides. Similar, although amplified, C-peptide and insulin responses were obtained when the perfusate glucose level was increased to 15 mM (results not shown).

Effects of Exogenous Insulin on Glucose-induced C-peptide Secretion—In the next experiment, islets were stimulated with 8 mM glucose, a level of the hexose that resulted in a modest 4–5-fold increase in insulin secretory rates (Fig. 2). For example, in the presence of 3 mM glucose islets released 30–35 pg of insulin/islet/min. After 40 min of stimulation with 8 mM glucose, the secretory rate increased to 143 ± 17 (n = 5) pg/islet/min. A similar response in terms of C-peptide secretion was also observed. Prestimulatory secretion rates (5–6 pg/islet/min) increased to 31 ± 5 pg/islet/min. To assess the potential impact of exogenous insulin on glucose-induced secretion, additional groups of islets were stimulated with 8 mM glucose plus 100 nM bovine insulin. The addition of exogenous insulin precluded the measurement of endogenous insulin secretion. However, using C-peptide secretion rates as an index of the endogenous insulin secretory response, no effect of added bovine insulin on C-peptide secretory rates was seen. 40 min after the onset of 8 mM glucose stimulation, C-peptide secretory rates averaged 32 ± 3 pg/islet/min in the presence of 100 nM bovine insulin.

Although not shown, we could detect no inhibitory effect of 100 nM bovine insulin on C-peptide responses to 7 or 10 mM glucose, levels of the hexose that increase endogenous insulin secretion about 2- or 10–15-fold, respectively, above those observed with 3 mM glucose.

Effects of Exogenous Insulin on Insulin Secretion—It has been reported that 100 nM exogenous bovine insulin in the presence of 3 mM glucose increases insulin secretion from β-cells, a response monitored not by the release of insulin but by 5HT release from 5HT-prelabeled β-cells (6). We directly tested this concept in perfused islets, which retain a level of physiologic sensitivity to glucose stimulation comparable with that observed with the perfused rat pancreas preparation (27–29). After a 45-min stabilization period in the presence of 3 mM glucose, islets were perfused with 100 nM bovine insulin or 500 nM human insulin in the continued presence of 3 mM glucose. There was no stimulatory effect of either insulin preparation on C-peptide secretion rates (Fig. 3). Throughout the perfusion, basal and stable rates of C-peptide secretion were noted.

Carbachol-induced C-peptide and Insulin Secretion—We considered that the process of collagenase isolating islets may disrupt β-cell membrane integrity and that the lack of any effect of insulin on C-peptide secretion may be a consequence of this potential adverse effect of the isolation procedure. To address this concern, two sets of additional experiments were conducted. In the first set of experiments (Fig. 4) islets were stimulated with 7 mM glucose plus 5 μM carbachol, a cholinergic agonist that activates phospholipase C via a membrane muscarinic receptor (30–32). Islets stimulated with 7 mM glucose...
The further addition of 100 nM insulin had no effect on the addition of human insulin was without any stimulatory effect on C-peptide secretion rates. Because culturing impairs islet sensitivity to glucose stimulation alone (25, 33, 34), these islets were then stimulated with the combination of 20 mM glucose plus 500 nM PMA (Fig. 5). This agonist combination resulted in an ~25-fold increase in C-peptide secretion rates from both control and prior insulin-stimulated islets.

In the next set of experiments, islets were first cultured for 18 h to allow more complete recovery of any potential adverse impact of the collagenase isolation procedure. Islets were then perfused with 3 mM glucose to establish basal and stable rates of peptide secretion. They were then stimulated with 7 mM glucose plus 5 μM carbachol plus (n = 3) or minus (n = 5) the further addition of human bovine insulin. Insulin release rates (open circles), and C-peptide secretion rates (closed circles, solid line, no added bovine insulin; closed circles, dashed line, 100 nM bovine insulin added together with 7 mM glucose plus 5 μM carbachol). Plus 5 μM carbachol responded with an approximate 4-fold increase in both insulin and C-peptide secretion rates. The response to this agonist combination was ~2.5-fold greater than the response to 7 mM glucose alone (results not shown). For example, 20, 30 or 40 min after the onset of stimulation with 7 mM glucose alone insulin release rates averaged 53 ± 9, 57 ± 10, or 58 ± 12 pg/islet/min (n = 8), respectively. The addition of carbachol increased the values at these times to 109 ± 29, 125 ± 18, or 126 ± 19 pg/islet/min (n = 5), respectively. The further addition of 100 nM insulin had no effect on C-peptide secretion to glucose plus carbachol stimulation (Fig. 4).

In the next set of experiments, islets were first cultured for 18 h. One group (open circles, n = 4) was perfused for 90 min with 3 mM prior to stimulation with 20 mM glucose plus 500 nM PMA for 20 min. Only the final 25 min of this perifusion is shown. The second group (closed circles, n = 3) was perfused for 30 min with 3 mM glucose, 30 min with 3 mM glucose plus 500 nM human insulin, 30 min with 3 mM glucose, and 20 min with 20 mM glucose plus 500 nM PMA.

Effects of Genistein and Wortmannin on Glucose-induced Release—In an attempt to disrupt the contribution of constitutive insulin signaling on glucose-stimulated β-cell responses of our perifused islet preparation, additional studies were conducted with the tyrosine kinase inhibitor genistein (10 μM) and the phosphatidylinositol 3-kinase inhibitor wortmannin (50 nM). Both types of kinases are established participants in insulin signaling (35, 36). As shown in Fig. 6, both inhibitors significantly potentiated 10 mM glucose-induced secretion.

DISCUSSION

Several important considerations have emerged in our attempt to establish the precise impact of exogenously added insulin on endogenous insulin secretory rates. First is the realization that the levels of insulin bathing the β-cell, even under basal nonstimulatory conditions, must far exceed those normally bathing other tissues. This has to do with islet cell volume, limited interstitial space distribution, and constitutive rates of hormone output (16). Any attempt to establish either an inhibitory or excitatory effect of exogenous insulin on endogenous secretion must contend with this. For example, if insulin does indeed inhibit its own release as suggested in other reports (12, 14, 20), an inhibitory effect of added exogenous insulin on the secretory response to glucose might not be observed if saturating (with regards to insulin signaling) endogenous hormone concentrations already exist in the interstitial space and tonically influence secretion. In addition, it is technically difficult to measure endogenous insulin release rates in the presence of high levels of exogenously added hormone. In an attempt to circumvent these issues two approaches were utilized. First, we used C-peptide secretion rates as a surrogate marker for endogenous insulin secretion (37). Second, constitutive insulin signaling in the β-cell was disrupted using inhibitors known to interfere with the kinases involved in the insulin signaling cascade.

Several salient points emerge from the present studies. First, C-peptide secretion rates accurately reflect both quantitatively and qualitatively the kinetics and amplitude of insulin secretion. Like insulin output in response to 10 mM glucose, it is biphasic in nature. Most importantly in terms of sensitivity, small increments in glucose-induced insulin release evoked by 7–8 mM glucose evoke small, easily measurable increments in C-peptide release. Third, in terms of inhibition of insulin se-
cretion, we could not detect any inhibitory effect of exogenously added bovine insulin on glucose-induced insulin secretion. Fourth, neither bovine nor human insulin at levels of 100–500 nM had any discernible stimulatory effect on C-peptide secretion rates and, based on the tight coupling between C-peptide and insulin secretion demonstrated in these studies, exerted no stimulatory effect on insulin release as well. Fifth, addition of a membrane-active agonist, carbachol, evoked substantial insulin and C-peptide responses indicating that at least for this agonist the functional integrity of its membrane receptor has been maintained during the isolation procedures. However, because the insulin receptor may be more vulnerable to collagenase than the muscarinic cholinergic receptor, islets were allowed to recover from the isolation procedure during an 18-h culturing period. These islets still failed to respond to exogenously added insulin by increasing C-peptide secretion rates. We were unable to document any inhibitory effect of exogenously added insulin on its own secretion using C-peptide as the surrogate marker for insulin release. Does this failure exclude an inhibitory effect of endogenously released insulin on the insulin release process? Does constitutive insulin secretion tonically influence stimulated secretion? We attempted to address this issue by using two different inhibitors known to disrupt the kinases involved in insulin signaling. For example, islets deficient in the insulin receptor substrate-2 protein, or genistein-treated islets at this time point.

FIG. 6. Effects of genistein and wortmannin on glucose-induced insulin secretion. Groups of 14–18 islets were perfused with 3 mM glucose (G3) for 30 min. Islets were then stimulated (indicated by the vertical line) with 10 mM glucose alone (open circles), 10 mM glucose plus 10 μM genistein (closed circles, dashed line), or 10 mM glucose plus 50 nM wortmannin (closed triangles). The asterisk indicates a significant difference between the G10 controls and the G10 plus wortmannin- or genistein-treated islets at this time point.

Our working hypothesis is that constitutive insulin release from islets, even under nonstimulatory conditions, exerts a tonic inhibitory effect on stimulated secretion. The addition of exogenous insulin to a system already tonically inhibited has little further inhibitory effect as demonstrated here, but its disruption using several inhibitors markedly improves the secretory performance of the β-cell. This hypothesis is consistent with findings made using knockout mice where insulin signaling has been disrupted and by pharmacologic inhibition of kinases thought to be involved in insulin signaling. For example, islets deficient in the insulin receptor substrate-2 protein or the p85α regulatory subunit of phosphatidylinositol 3-kinase hyper-respons to glucose stimulation (41, 44). Of particular physiologic significance, perhaps, is the observation that basal secretion is not augmented from these β-cells. The impact of these genetic manipulations becomes manifest only in studies in which glucose stimulates release. This finding suggests that only under conditions in which insulin release is stimulated by glucose does insulin signaling negatively impact release. This situation is analogous to the α2 adrenergic effects in the central nervous system where presynaptically released catecholamines feed back in a negative fashion on the cell that released it in order to restrain the secretion of additional neurotransmitter (45).

Our studies, as well as those of others, using pharmacologic inhibition of the kinase involved with insulin signaling are also consistent with the hypothesis that constitutive insulin signaling acts to restrain stimulated insulin secretion. For example, neither wortmannin nor genistein potentiates release from islets in the presence of low nonstimulatory glucose (23, 38). Their positive effect on secretion only becomes manifest when stimulatory glucose is employed. Although the specificity of these inhibitors on islet kinases remains to be determined, it has to be emphasized that these inhibitors reversibly potentiates glucose-induced insulin secretion, thus ruling out any untoward nonspecific toxic action. For wortmannin at least, and from a quantitative perspective, its potentiating effect is comparable with clinically utilized insulin secretagogues.

Recent interest in the potential role of insulin signaling on β-cell response patterns has been generated largely as a result of studies in insulin signaling knockout animals (46–48). Amperometric measurements of 5HT release from normal or abnormal β-cells have been used to support the concept that insulin stimulates its own secretion (6, 7). Our C-peptide measurements failed to reveal any stimulatory effect of exogenous insulin on C-peptide release, a surrogate marker of insulin secretion that accurately reflects small changes in the kinetics and amplitude of insulin release. The assay used to measure C-peptide secretion appears sensitive enough to measure small increments in insulin secretion. For example, the modest 4–5-fold increase in 8 mM glucose-induced release was paralleled by a modest 4–5-fold increase in C-peptide secretion. Even with 7 mM glucose alone, small but parallel 2-fold increments in both insulin and C-peptide release were recorded.

In conclusion, C-peptide release rates from perfused rat islets reflect accurately the kinetics and magnitude of glucose- and carbachol-induced insulin release. In perfused rat islets, exogenously added insulin has no inhibitory effect on endogenous insulin secretion monitored by C-peptide secretion rates. Exogenously added bovine or human insulin do not stimulate C-peptide release from islets and, by inference, also fail to exert any autocrine insulin stimulatory effect. Human insulin failed to affect C-peptide secretion from cultured islets as well. Known inhibitors of the kinases that participate in insulin signaling in other tissues significantly amplify glucose-induced secretion. Although the tonic impact of endogenously released insulin makes it technically difficult to establish an inhibitory
effect of exogenously added hormone on the release process, any small autocrine stimulatory effect of added insulin, if it occurred, should have been readily detected considering the secretory capacity of the β-cell and the sensitivity of the C-peptide assay. This was, however, not the case.

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REFERENCES

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