

# Ceruleinin, an Inhibitor of Protein Acylation, Selectively Attenuates Nutrient Stimulation of Insulin Release

## A Study in Rat Pancreatic Islets

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**Nutrients such as glucose stimulate insulin release from pancreatic  $\beta$ -cells through both ATP-sensitive  $K^+$  channel-independent and -dependent mechanisms, which are most likely interrelated. Although little is known of the molecular basis of ATP-sensitive  $K^+$  channel-independent insulinotropic nutrient actions, mediation by cytosolic long-chain acyl-CoA has been implicated. Because protein acylation might be a sequel of cytosolic long-chain acyl-CoA accumulation, we examined if this reaction is engaged in nutrient stimulation of insulin release, using cerulenin, an inhibitor of protein acylation. In isolated rat pancreatic islets, cerulenin inhibited the glucose augmentation of  $Ca^{2+}$ -stimulated insulin release evoked by a depolarizing concentration of  $K^+$  in the presence of diazoxide and  $Ca^{2+}$ -independent insulin release triggered by a combination of forskolin and phorbol ester under stringent  $Ca^{2+}$ -free conditions. Cerulenin inhibition of glucose effects was concentration dependent, with a 50% inhibitory concentration ( $IC_{50}$ ) of 5  $\mu$ g/ml and complete inhibition at 100  $\mu$ g/ml. Cerulenin also inhibited augmentation of insulin release by  $\alpha$ -ketoisocaproate, a mitochondrial fuel. Furthermore, cerulenin abolished augmentation of both  $Ca^{2+}$ -stimulated and  $Ca^{2+}$ -independent insulin release by 10  $\mu$ mol/l palmitate, which causes palmitoylation of cellular proteins. In contrast, cerulenin did not attenuate insulin release elicited by nonnutrient secretagogues, such as a depolarizing concentration of  $K^+$ , activators of protein kinases A and C, and mastoparan. Glucose oxidation, ATP content in islets, and palmitate oxidation were not affected by cerulenin. In conclusion, cerulenin inhibits nutrient augmentation of insulin release with a high selectivity. The finding is consistent with a prominent role of protein acylation in the process of  $\beta$ -cell nutrient sensing. *Diabetes* 49:712-717, 2000**

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BSA, bovine serum albumin;  $[Ca^{2+}]_i$ , cytosolic free  $Ca^{2+}$  concentration;  $K_{ATP}$  channel, ATP-sensitive  $K^+$  channel; KIC, ketoisocaproic acid; KRB, Krebs-Ringer bicarbonate; LC-CoA, long-chain acyl CoA; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

**A** new concept in glucose stimulus-secretion coupling in the pancreatic  $\beta$ -cell has emerged in recent years. Glucose metabolism, a mandatory initial step, activates three mutually interactive signaling pathways: the ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channel-dependent pathway, the  $K_{ATP}$  channel-independent and  $Ca^{2+}$ -dependent pathway, and the  $K_{ATP}$  channel-independent and  $Ca^{2+}$ -independent pathway (1,2). The first is the most extensively analyzed, and its molecular basis is well documented. Namely, elevation of ATP or the ATP/ADP ratio caused by glucose metabolism closes the  $K_{ATP}$  channels, leading to membrane depolarization, activation of voltage-dependent calcium channels, and  $Ca^{2+}$  influx. The resultant rise in cytosolic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) directly triggers insulin exocytosis. Once insulin exocytosis is triggered by  $Ca^{2+}$ , it is strongly augmented by glucose through the second signaling pathway. This can be demonstrated when the  $\beta$ -cell is stimulated with a combination of glucose and a depolarizing concentration of  $K^+$ , with full pharmacological activation of the  $K_{ATP}$  channels by diazoxide. Thus, the hexose augments insulin release triggered by depolarization, independently from its action on the  $K_{ATP}$  channels (3,4). A combination of forskolin and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), activators of adenylyl cyclase and protein kinase C, respectively, triggers insulin exocytosis, even under stringent  $Ca^{2+}$  deprivation (prolonged exposure to  $Ca^{2+}$ -omitted buffer with EGTA). This insulin release is potently augmented by glucose through either the second or possibly the third signaling pathway (5).

In 1989, it was proposed that action of glucose to increase malonyl-CoA in the  $\beta$ -cell would lead to decreased oxidation of fatty acids and an increase in long-chain acyl CoA (LC-CoA) in the cytosol and that the latter could be second messengers or activators of second messengers in stimulus-secretion coupling (6). Considerable evidence has accumulated to support this hypothesis (7-11), though with some evidence to the contrary (12). Recent direct supportive evidence includes the effect of fatty acids to augment insulin secretion (13,14). Furthermore, it has been suggested that malonyl-CoA and LC-CoA could be responsible for the  $K_{ATP}$  channel-independent pathways of glucose signaling (15,16). Following up on this hypothesis, we examined the effects of cerulenin, a fungal antibiotic that inhibits protein acylation

(17,18), on insulin release. We were interested in this drug because covalent protein acylation by LC-CoA is involved in vesicle translocation, membrane trafficking, and exocytosis in antigen-presenting cells, pituitary cells, and neuronal cells (19–22), and because acylation could be responsible for the augmentation of insulin release via the  $K_{ATP}$  channel-independent pathways. Accordingly, our expectation was that cerulenin, if it inhibits protein acylation in pancreatic  $\beta$ -cells as it does in other cells, would attenuate glucose augmentation of insulin release. In contrast, as stated above, the direct triggering of insulin release by  $Ca^{2+}$ , which is not related to LC-CoA, should not be affected by the drug. The data are in accord with the hypothesis that protein acylation is a key reaction involved in the  $K_{ATP}$  channel-independent augmentation of insulin release by glucose.

## RESEARCH DESIGN AND METHODS

**Isolation of pancreatic islets.** Male Wistar rats weighing 250–450 g were killed by  $CO_2$  asphyxiation. Immediately after death, the pancreases were surgically removed, and the islets were isolated by collagenase dispersion (23). Krebs-Ringer bicarbonate (KRB) buffer containing 129 mmol/l NaCl, 5 mmol/l  $NaHCO_3$ , 4.8 mmol/l KCl, 1.2 mmol/l  $KH_2PO_4$ , 1.2 mmol/l  $MgSO_4$ , 2.5 mmol/l  $CaCl_2$ , 5.6 mmol/l glucose, 0.1% bovine serum albumin (BSA), and 10 mmol/l HEPES at pH 7.4 was used for isolation and pooling of the islets.

**Measurements of insulin release.** Insulin release was measured in static incubation experiments at 37°C using KRB buffer containing 0.2% BSA. Batches of 5 size-matched islets per tube were used. The islets were first incubated in 0.5 ml KRB buffer containing 2.8 mmol/l glucose for 30 or 60 min (preincubation). After the preincubation, the buffer was removed by aspiration, 0.5 ml of fresh KRB buffer with test substance(s) was introduced, and the incubation was further continued for 30 or 60 min (test incubation). In some experiments, KRB buffer devoid of  $Ca^{2+}$  and containing 1 mmol/l EGTA was used. When palmitate was used, it was dissolved at a concentration of 600  $\mu$ mol/l in KRB buffer containing 0.68% (100  $\mu$ mol/l) fatty acid-free BSA (Sigma, St. Louis, MO). In the presence of 100  $\mu$ mol/l BSA, 600  $\mu$ mol/l palmitate gives an estimated free palmitate concentration of 10  $\mu$ mol/l (24). Cerulenin (Sigma) was present throughout the preincubation and incubation periods. Cerulenin stocks (2,000 $\times$ ) were made in DMSO. The same final concentrations of DMSO were present in paired control tubes. At the end of test incubations, the medium was aspirated and kept at  $-20^\circ C$  until radioimmunoassayed for insulin, where rat insulin was used as the standard (3,5,15,16).

**Glucose oxidation.** Glucose oxidation was measured as reported previously (25). In brief, 25 islets were preincubated in 0.5 ml KRB buffer with or without 30  $\mu$ g/ml cerulenin at 37°C for 30 min. At the end of the preincubation, the medium was removed, and 100  $\mu$ l KRB buffer with or without 30  $\mu$ g/ml cerulenin containing 1  $\mu$ Ci D-[ $U-^{14}C$ ]glucose and the required amount of glucose were added. The experimental incubation was carried out for 30 min. At the end of the incubation,  $^{14}CO_2$  was trapped by benzethonium hydroxide after adding 200  $\mu$ l 0.1 N HCl to the incubation mixture and quantitated by liquid scintillation spectrometry.

**Palmitate oxidation.** Palmitate oxidation was measured as previously reported (8). In brief, 25 islets were first incubated at 37°C for 30 min (preincubation) in 0.5 ml KRB buffer to which 0.8 mmol/l L-carnitine and BSA (to make a final concentration of 1%) with or without 30  $\mu$ g/ml cerulenin had been added. At the end of the preincubation, the medium was removed, 200  $\mu$ l KRB buffer (0.8 mmol/l L-carnitine, 1% BSA) with or without 30  $\mu$ g/ml cerulenin containing 0.2  $\mu$ Ci [ $1-^{14}C$ ]palmitate were added, and experimental incubation was carried out at 37°C for 120 min. At the end of the incubation,  $^{14}CO_2$  was trapped by benzethonium hydroxide after adding 200  $\mu$ l 0.2 N HCl to the incubation mixture and quantitated by liquid scintillation spectrometry.

**Measurement of ATP content.** ATP content in islets was measured as previously reported with minor modifications (26). In brief, batches of 10 size-matched islets were first incubated in 0.5 ml KRB buffer containing 2.8 mmol/l glucose with or without 30  $\mu$ g/ml cerulenin at 37°C for 30 min (preincubation). After the preincubation, the buffer was removed by aspiration, 0.4 ml of fresh KRB buffer containing 2.8 or 16.7 mmol/l glucose with or without 30  $\mu$ g/ml cerulenin was introduced, and the incubation was continued for another 30 min. The incubations were stopped by addition of 0.6 ml of ice-cold trichloroacetic acid to a final concentration of 5%. The tubes were then vortexed, left on ice for 15 min, and centrifuged for 5 min at 2,000 rpm. A fraction (400  $\mu$ l) of the supernatant was then thoroughly mixed with 1.5 ml diethyl ether, and the ether phase containing trichloroacetic acid was discarded. This step was repeated twice. Then, 1.5 ml diethyl ether was added for the third time and vortexed. A fraction (200  $\mu$ l) of the water phase was collected; diluted with 200  $\mu$ l of a buffer containing 20 mmol/l HEPES, 3 mmol/l

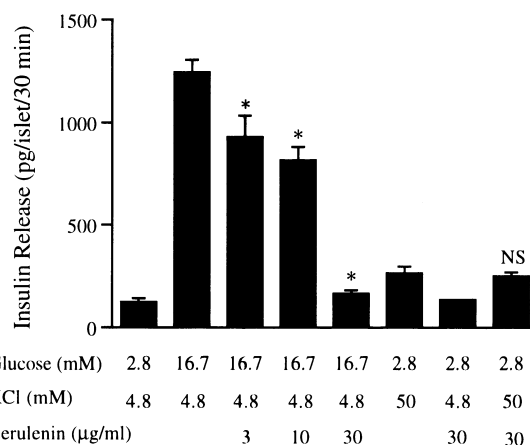
$MgCl_2$ , and KOH to adjust pH at 7.75; and stored at  $-20^\circ C$  until assayed. ATP was assayed by a luminometric method by using commercially available kits (ATP Assay System; LL-100-1, Toyo Ink, Tokyo).

**Data analysis.** All data are shown as mean  $\pm$  SE. The differences were tested by the Mann-Whitney *U* test. A *P* value  $<0.05$  was considered significant.

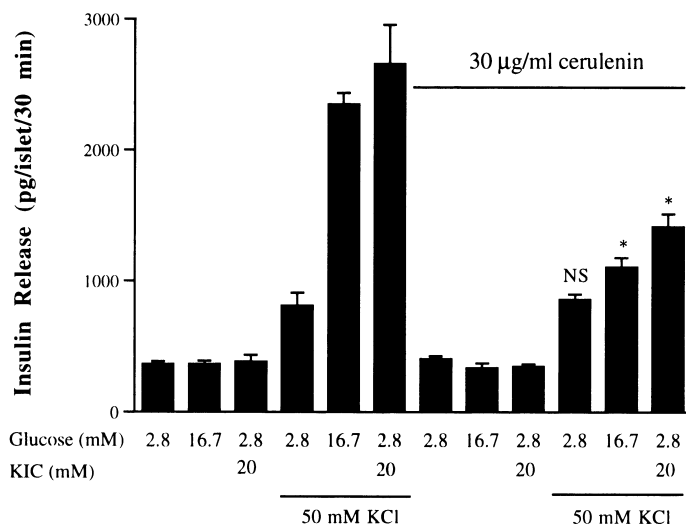
## RESULTS

**Effects of cerulenin on glucose- and KCl-induced insulin release under normal conditions.** As shown in Fig. 1, 16.7 mmol/l glucose caused a 10-fold increase in insulin release during 30 min, which was inhibited by cerulenin in a concentration-dependent manner. Cerulenin at 30  $\mu$ g/ml inhibited the glucose-induced insulin release by 87%. Basal insulin release in the presence of 2.8 mmol/l glucose was not affected by 30  $\mu$ g/ml cerulenin (7th column from the left in Fig. 1). To examine the effect of cerulenin on  $Ca^{2+}$ -stimulated exocytosis per se, we measured insulin release in response to 50 mmol/l KCl in the presence of 30  $\mu$ g/ml cerulenin. Application of a depolarizing concentration of  $K^+$  triggers insulin release due simply to elevation of  $[Ca^{2+}]_i$ . The depolarizing concentration of KCl doubled the release (1st column vs. 6th column from the left in Fig. 1), and the treatment with 30  $\mu$ g/ml cerulenin had no effect on KCl-induced ( $Ca^{2+}$ -stimulated) insulin release. Thus, cerulenin inhibited glucose stimulation of insulin release without perturbation of the  $Ca^{2+}$ -responsive exocytotic machinery per se.

**Effects of cerulenin on nutrient augmentation of  $Ca^{2+}$ -stimulated insulin release.** Glucose and other metabolizable nutrients augment insulin release, which is mediated by non- $Ca^{2+}$  signal(s). Therefore, we next examined effects of cerulenin on the nutrient-induced augmentation of insulin release. Here, we stimulated the islets with a combination of depolarizing concentration (50 mmol/l) of  $K^+$  and a nutrient in the presence of 250  $\mu$ mol/l diazoxide, a  $K_{ATP}$  channel activator, to prevent the nutrient action on the  $K_{ATP}$  channels and thereby on membrane potential. The results of such experiments are shown in Fig. 2. The KCl-induced insulin release was three times greater in the presence of 16.7 mmol/l glucose than in the presence of



**FIG. 1.** Effects of cerulenin on glucose- and KCl-induced insulin release under normal conditions. Cerulenin was present throughout the preincubation and test incubation periods. Values are mean  $\pm$  SE from 8–10 determinations. Conversion factor of picograms per islet per minute to nanomoles per islet per minute is 0.1739. \**P*  $<0.01$  vs. a corresponding value in the absence of cerulenin. NS, no significant difference vs. a corresponding value in the absence of cerulenin.

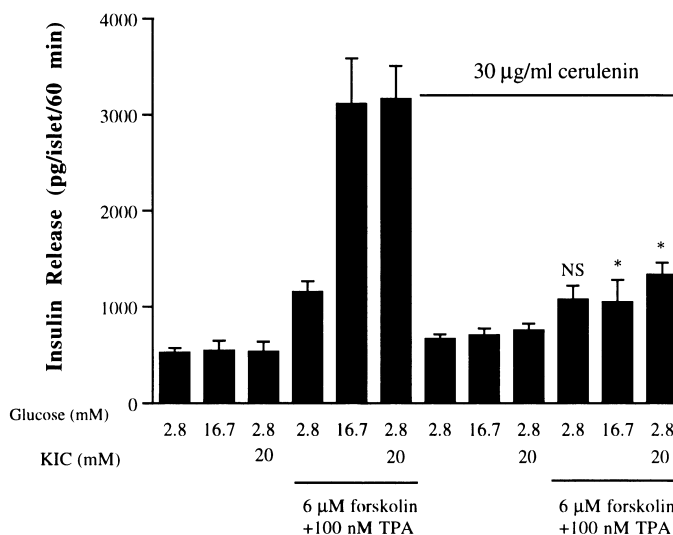


**FIG. 2.** Effects of cerulenin on nutrient augmentation of  $\text{Ca}^{2+}$ -stimulated insulin release. Diazoxide (250  $\mu\text{mol/l}$ ) was present in both preincubation and test incubation periods. Cerulenin was present throughout the preincubation and test incubation periods in the 6 conditions presented on the right. Values are mean  $\pm$  SE from 10 determinations. Conversion factor of picograms per islet per minute to nanomoles per islet per minute is 0.1739. \* $P < 0.01$  vs. a corresponding value in the absence of cerulenin. NS, no significant difference vs. a corresponding value in the absence of cerulenin.

2.8 mmol/l glucose. Such glucose augmentation was profoundly attenuated (by 85%) by 30  $\mu\text{g/ml}$  cerulenin. Similarly, augmentation of insulin release by 20 mmol/l ketoisocaproic acid (KIC), a mitochondrial fuel, was significantly suppressed (by 60%) by 30  $\mu\text{g/ml}$  cerulenin. As in Fig. 1, KCl-induced insulin release itself was not affected by cerulenin, implying that  $\text{Ca}^{2+}$ -stimulated insulin exocytosis per se is not suppressed by the drug.

**Effects of cerulenin on nutrient augmentation of  $\text{Ca}^{2+}$ -independent insulin release.** The metabolizable nutrients have another mode of augmentation of insulin release that is  $\text{Ca}^{2+}$ -independent. In the complete absence of extracellular  $\text{Ca}^{2+}$ , the nutrients augment insulin release induced by a simultaneous activation of protein kinases A and C by forskolin and TPA, respectively. In Fig. 3, the effects of 30  $\mu\text{g/ml}$  cerulenin on this nutrient action are shown. Under stringent  $\text{Ca}^{2+}$ -free conditions (no addition of  $\text{Ca}^{2+}$  plus 1 mmol/l EGTA),  $\text{Ca}^{2+}$ -independent insulin release was evoked by the simultaneous addition of 6  $\mu\text{mol/l}$  forskolin and 100 nmol/l TPA. The combination of forskolin and TPA doubled the rate of insulin release in the presence of 2.8 mmol/l glucose, and such release was augmented ~2-fold by 16.7 mmol/l glucose or 20 mmol/l KIC. Here again, treatment with 30  $\mu\text{g/ml}$  cerulenin abolished the glucose augmentation of the  $\text{Ca}^{2+}$ -independent insulin release and greatly attenuated (~90%) the KIC augmentation.  $\text{Ca}^{2+}$ -independent insulin release induced by the combination of forskolin and TPA in the presence of low glucose (2.8 mmol/l) was not affected by the treatment with 30  $\mu\text{g/ml}$  cerulenin (4th column vs. 10th column from the left in Fig. 3).

**Concentration dependency of cerulenin effects on the two augmentation pathways.** In Fig. 4A, the effects of 3–100  $\mu\text{g/ml}$  cerulenin on glucose augmentation of  $\text{Ca}^{2+}$ -stimulated insulin release are shown.  $\text{Ca}^{2+}$ -stimulated

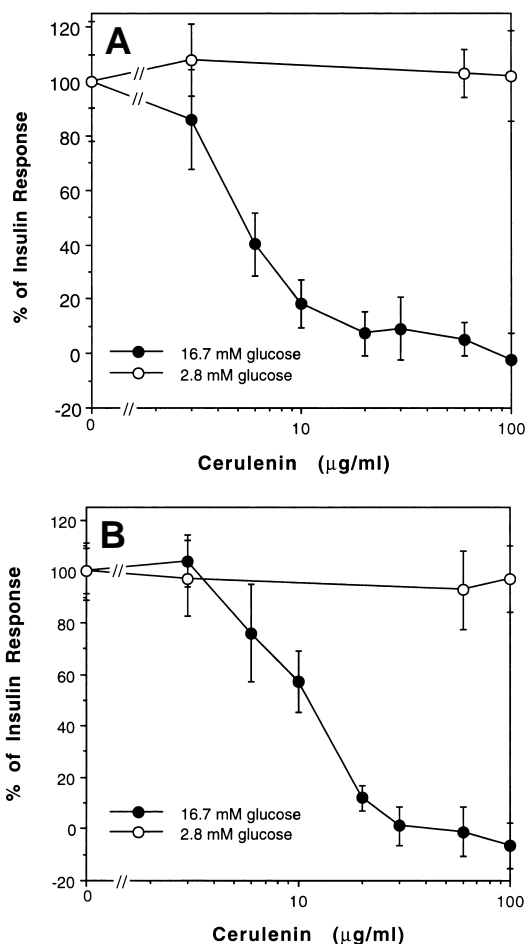


**FIG. 3.** Effects of cerulenin on nutrient augmentation of  $\text{Ca}^{2+}$ -independent insulin release. The experiment was performed under stringent  $\text{Ca}^{2+}$ -free conditions in KRB buffer devoid of  $\text{Ca}^{2+}$  and containing 1 mmol/l EGTA. Cerulenin was present throughout the preincubation and test incubation periods in the 6 conditions shown on the right.  $\text{Ca}^{2+}$ -independent insulin release was evoked by a combination of 6  $\mu\text{mol/l}$  forskolin and 100 nmol/l TPA. Values are mean  $\pm$  SE from 10 determinations. Conversion factor of picograms per islet per minute to nanomoles per islet per minute is 0.1739. \* $P < 0.01$  vs. a corresponding value in the absence of cerulenin. NS, no significant difference vs. a corresponding value in the absence of cerulenin.

insulin release was evoked by 50 mmol/l KCl, and the glucose augmentation of it was inhibited by cerulenin in a concentration-dependent manner. The 50% inhibitory concentration ( $\text{IC}_{50}$ ) of cerulenin inhibition was 5  $\mu\text{g/ml}$ , and complete inhibition was observed at 100  $\mu\text{g/ml}$ . As can be seen in Fig. 4B, the concentration dependency of cerulenin inhibition was similar when its effect on the glucose augmentation of  $\text{Ca}^{2+}$ -independent insulin release was examined.

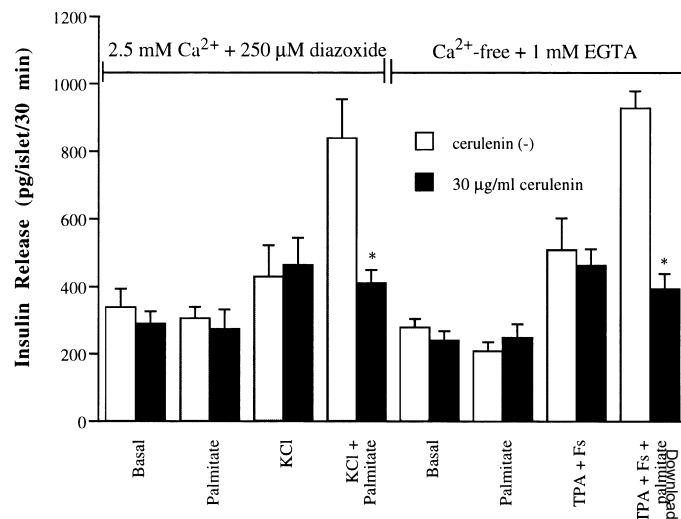
Even at a very high concentration (100  $\mu\text{g/ml}$ ), cerulenin did not suppress insulin release evoked by 50 mmol/l KCl or by the combination of forskolin and TPA in the presence of a non-stimulatory concentration of glucose (2.8 mmol/l) (Fig. 4A and B). Therefore, selectivity of cerulenin on nutrient action is clearly confirmed.

**Effects of cerulenin on palmitate augmentation of  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent insulin release.** In previous studies, we demonstrated that palmitate had an augmentation effect similar to that of glucose on both  $\text{Ca}^{2+}$ -induced and  $\text{Ca}^{2+}$ -independent (forskolin and TPA-induced) insulin release (15,16). Knowing that cerulenin is an inhibitor of palmitoylation, we examined the effects of cerulenin on the augmentation of insulin release by palmitate (Fig. 5). As shown in the left panel of Fig. 5, inclusion of 10  $\mu\text{mol/l}$  palmitate caused an augmentation of the  $\text{Ca}^{2+}$ -induced insulin release as previously reported, and such augmentation was totally eliminated by 30  $\mu\text{g/ml}$  cerulenin. Similar results were obtained in the case of augmentation of  $\text{Ca}^{2+}$ -independent insulin release, as shown in the right panel of Fig. 5. Namely, 10  $\mu\text{mol/l}$  palmitate augmented  $\text{Ca}^{2+}$ -independent (forskolin and TPA-induced) insulin release, and inclusion of 30  $\mu\text{g/ml}$  cerulenin completely obliterated the augmentation effect of palmitate.



**FIG. 4.** Concentration dependency of cerulein inhibition on glucose augmentation of Ca<sup>2+</sup>-stimulated and Ca<sup>2+</sup>-independent insulin release. Cerulein was present throughout the preincubation and test incubation periods. Insulin release was expressed as the percentage of control in which no cerulein was included. *A*: Effects of various concentrations of cerulein on Ca<sup>2+</sup>-stimulated insulin release in the presence of 2.8 or 16.7 mmol/l glucose. The experiments were performed in the presence of 250 μmol/l diazoxide. Ca<sup>2+</sup>-stimulated insulin release was evoked by 50 mmol/l KCl. *B*: Effects of various concentrations of cerulein on Ca<sup>2+</sup>-independent insulin release in the presence of 2.8 or 16.7 mmol/l glucose. The experiments were performed under stringent Ca<sup>2+</sup>-free conditions in KRB buffer devoid of Ca<sup>2+</sup> and containing 1 mmol/l EGTA. Ca<sup>2+</sup>-independent insulin release was evoked by the simultaneous addition of 6 μmol/l forskolin and 100 nmol/l TPA. Values are mean ± SE from 15–20 determinations. Conversion factor of picograms per islet per minute to nanomoles per islet per minute is 0.1739.

**Lack of effect of cerulein on insulin release elicited by strong nonnutrient stimuli.** In the experiments presented so far, cerulein did not suppress insulin release elicited by nonnutrient secretagogues. However, in those experiments, the insulin release evoked by nonnutrient secretagogues was not comparable to that elicited by nutrient stimulation. To definitively prove the selectivity of cerulein effect on nutrients, potent nonnutrient stimuli were applied in the absence of glucose, and the effects of cerulein were examined. As shown in Fig. 6, a combination of 50 mmol/l KCl, 6 μmol/l forskolin, and 100 nmol/l TPA produced a 4-fold increase in insulin release in the absence of glucose. It was not inhibited by 30 μg/ml cerulein. As the other mode of potent non-



**FIG. 5.** Effects of cerulein on palmitate augmentation of Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent insulin release. Test incubation was performed in the presence of 2.8 mmol/l glucose in this experiment. The experiment was performed in the presence of 0.68% BSA (100 μmol/l). The total concentration of palmitate was 600 μmol/l, resulting in the estimated free concentration of 10 μmol/l. Cerulein was present throughout the preincubation and test incubation periods. The left half of the figure shows the insulin release in the presence of 2.5 mmol/l Ca<sup>2+</sup> and 250 μmol/l diazoxide. The right half shows the release in the absence of Ca<sup>2+</sup> and the presence of 1 mmol/l EGTA. Values are mean ± SE from 10 determinations. Conversion factor of picograms per islet per minute to nanomoles per islet per minute is 0.1739. Fs, forskolin. \**P* < 0.01 vs. a corresponding value in the absence of cerulein.

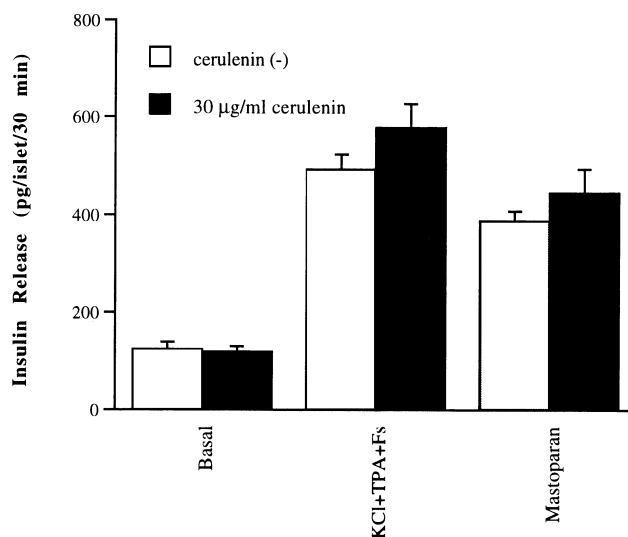
nutrient secretagogue of insulin release, mastoparan, a wasp venom that directly activates distal exocytotic machinery in the β-cell (27), was used. A dose of 10 μmol/l mastoparan caused a 3.5-fold increase in insulin release, and 30 μg/ml cerulein did not suppress the mastoparan effect.

**Effect of cerulein on glucose oxidation and ATP content in rat pancreatic islets at low and high concentrations of glucose.** As shown in Table 1, in the islets exposed to 16.7 mmol/l glucose for 30 min, glucose oxidation was 35 times more than that in the islets exposed to 2.8 mmol/l glucose. The presence of 30 μg/ml cerulein did not alter the glucose oxidation at either low or high glucose concentrations (Table 1). ATP content was increased ~20% after exposure to 16.7 mmol/l glucose for 30 min. Cerulein had no effect on the islet ATP content at low or high glucose concentrations.

**Effect of cerulein on palmitate oxidation in rat pancreatic islets at low and high glucose.** Finally, we examined the effect of cerulein on fatty acid metabolism in rat pancreatic islets. As shown in Table 1, oxidation of palmitate in the presence of 16.7 mmol/l glucose was significantly lower than that in the presence of 2.8 mmol/l glucose. The suppression of palmitate oxidation by 16.7 mmol/l glucose was unaffected by the presence of 30 μg/ml cerulein, although the oxidation tended to be less in the presence of cerulein at both the low and high concentrations of glucose.

## DISCUSSION

In the present study, we found that cerulein, an inhibitor of protein acylation (17,18), inhibits stimulation of insulin release by glucose, KIC, and palmitate. In contrast, cerulein



**FIG. 6. Lack of effect of cerulenin on insulin release induced by non-nutrient stimuli.** Both preincubation and test incubation were performed in the absence of glucose in this experiment. Insulin release was evoked by a combination of 50 mmol/l KCl, 6 µmol/l forskolin, and 100 nmol/l TPA or by 10 µmol/l mastoparan. Cerulenin was present throughout the preincubation and test incubation periods. Values are mean ± SE from 10 determinations. Conversion factor of picograms per islet per minute to nanomoles per islet per minute is 0.1739. Fs, forskolin.

did not inhibit insulin exocytosis triggered by nonnutrient secretagogues, such as a depolarizing concentration of K<sup>+</sup>, forskolin plus TPA, K<sup>+</sup> plus forskolin plus TPA, and mastoparan. Therefore, cerulenin is acting on the step commonly shared by the three nutrient secretagogues but not by the nonnutrient secretagogues. The concentration dependency of cerulenin inhibition of nutrient stimulation of insulin release is similar to the one reported for the drug-induced inhibition of protein acylation (17,18), supporting the idea that cerulenin is acting as an inhibitor of protein acylation under the experimental conditions used here. In fact, protein acylation may well be the key step common to glucose, KIC, and palmitate stimulation of the β-cell because anaplerotic metabolism of glucose and KIC raises cytosolic malonyl-CoA concentration, which leads to suppression of carnitine palmitoyl-transferase I activity and, eventually, accumulation of LC-CoA (6,7,9). Accumulation of cytosolic LC-CoA, in turn, will result in increased acylation of the mol-

ecule linked to insulin exocytosis (28). Regarding β-cell stimulation by palmitate, exogenous application is thought to cause protein palmitoylation, a well-documented example of acylation, which is expected to be inhibited by cerulenin.

In previous reports, it was shown that cerulenin has actions other than inhibition of protein acylation in various cells. These include apoptosis in cancer cells (29,30) and inhibition of fatty acid, cholesterol, RNA, and protein synthesis (17,18). In addition, increase in basal glucose oxidation, reduction in insulin-stimulated glucose oxidation, and inhibition of acetyl-CoA carboxylase (18) by cerulenin were documented. Among these other actions of cerulenin, apoptosis, inhibition of RNA, and protein synthesis can be excluded as mechanisms of cerulenin inhibition of insulin release in our acute experimental conditions. Also, inhibition of fatty acid synthase by cerulenin is considered not responsible for the effect shown in this study because 1) the activity of the enzyme is very low in the pancreatic β-cell (31); 2) activation of fatty acid synthase by glucose, KIC, or palmitate has not been demonstrated in the β-cell; and 3) exogenous palmitate bypasses this step. Furthermore, we found that cerulenin had no effect on increased glucose oxidation and ATP content after exposure to high glucose. Thus, inhibition of glucose metabolism by cerulenin is most unlikely.

If cerulenin inhibits acetyl-CoA carboxylase in rat pancreatic islets, it is expected that cerulenin increases β-oxidation of fatty acid on one hand and blunts the suppressive effect of glucose on the fatty acid oxidation on the other. This is because acetyl-CoA carboxylase is a rate-limiting enzyme for malonyl-CoA production, and malonyl-CoA inhibits carnitine palmitoyltransferase I, an enzyme required for fatty acid incorporation into the mitochondrion for oxidation. However, we found that treatment of the islets by cerulenin did not increase basal palmitate oxidation (in the presence of low concentration of glucose) and did not alter glucose-induced suppression of palmitate oxidation. This finding suggests that the inhibitory effect of cerulenin on insulin release in this study is not caused by inhibition of acetyl-CoA carboxylase.

To definitively establish that protein acylation is involved in nutrient-induced insulin release, a direct demonstration of protein acylation is required. Jochen et al. (18) could detect protein acylation in adipocytes by incubation of the cells with radioactive palmitate (18). Accordingly, we incubated the islets with [<sup>3</sup>H]- or [<sup>14</sup>C]palmitate under various conditions and tried to identify palmitoylation of protein(s) by the autoradiography after electrophoresis. Although we could detect sev-

**TABLE 1**  
Effects of cerulenin on glucose oxidation, ATP content, and palmitate oxidation in rat pancreatic islets

Experiments	Cerulenin (-)		30 µg/ml Cerulenin	
	2.8 mmol/l glucose	16.7 mmol/l glucose	2.8 mmol/l glucose	16.7 mmol/l glucose
Glucose oxidation (pmol · islet <sup>-1</sup> · 30 min <sup>-1</sup> )	1.1 ± 0.1 (8)	3.5 ± 0.3 (8)†	1.0 ± 0.1 (7)	3.5 ± 0.4 (8)†
ATP (pmol/islet)	18.0 ± 1.3 (13)	21.5 ± 1.1 (12)*	16.2 ± 1.0 (12)	22.7 ± 1.6 (13)*
[ <sup>14</sup> C]palmitate converted to CO <sub>2</sub> (fmol · islet <sup>-1</sup> · 120 min <sup>-1</sup> )	98.9 ± 5.0 (8)	83.8 ± 3.1 (7)*	86.5 ± 6.4 (7)	66.4 ± 8.1 (7)*

Data are means ± SE (number of determinations). Glucose oxidation, ATP content, and palmitate oxidation were measured as described in RESEARCH DESIGN AND METHODS. \*†Significantly different from the value in the presence of 2.8 mmol/l glucose: \*P < 0.05; †P < 0.01. None of the comparisons between the corresponding values in the absence and presence of cerulenin in each experiment was significant. The same batch of islets were used for 4 conditions in each experiment.

eral protein bands in the extract of adipocytes, as reported by Jochen et al. (data not shown), we could not detect significant labeling of protein(s) in the extract of the islets incubated with the labeled palmitate. This might be because the amount of protein palmitoylation is too small to detect by the method used. Further experiments are clearly needed.

In conclusion, cerulenin unequivocally inhibits stimulation of insulin release by glucose, KIC, and palmitate but not by nonnutrients. The finding strongly suggests a prominent role of protein acylation in the  $\beta$ -cell nutrient sensing apparatus and adds further support to the hypothesis that increased malonyl-CoA leading to increased cytosolic LC-CoA is an obligatory step in nutrient-stimulated insulin secretion.

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