The Effects of Cerulenin, an Inhibitor of Protein Acylation, on the Two Phases of Glucose-Stimulated Insulin Secretion

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The potential role of protein acylation in the control of biphasic insulin secretion has been studied in isolated rat pancreatic islets. The protein acylation inhibitor cerulenin inhibited both phases of glucose-stimulated insulin secretion. However, it did not affect the secretory response to a depolarizing concentration of KCl in either the absence or presence of diazoxide. Therefore, cerulenin has no deleterious effect on the L-type Ca^{2+} channels or subsequent events in Ca^{2+} stimulus–secretion coupling. Advantage was taken of this to study the effect of cerulenin on the K_{ATP} channel–independent pathway of glucose signaling. In the presence of KCl and diazoxide, cerulenin powerfully inhibited the augmentation of insulin release by glucose and palmitate. Similar inhibition of the augmentation of release by glucose and palmitate was seen under Ca^{2+}-free conditions in the presence of 12-*O*-tetradecanoylphorbol-13-acetate and forskolin. As neither glucose oxidation nor the effect of glucose to inhibit fatty acid oxidation is affected by cerulenin, these data suggest that protein acylation is involved in the K_{ATP} channel–independent pathway of glucose signaling. *Diabetes* 51 (Suppl. 1): S91–S95, 2002

Glucose stimulus–secretion coupling in the pancreatic β-cell can be described in terms of two major signaling pathways. The K_{ATP} channel–dependent pathway acts via closure of K_{ATP} channels with subsequent depolarization of the cell, activation of voltage-dependent Ca^{2+} channels, increased influx of Ca^{2+}, elevated cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, and increased insulin release (1,2). The K_{ATP} channel–independent pathways discovered in 1992 (3–5) act in synergy with the K_{ATP} channel–dependent pathway to augment the release induced by elevated $[Ca^{2+}]_i$. They also operate in the absence of extracellular Ca^{2+} if protein kinase C (PKC) and protein kinase A are activated (6). The knowledge of the underlying mechanisms by which these K_{ATP} channel–independent pathways exert their effects is sparse. It is known that glucose metabolism is essential for their operation, but the link between glucose metabolism and increased exocytosis has not been established. Some potential mechanisms for glucose action include:

- A glucose-induced increase in malonyl-CoA, subsequent inhibition of carnitine palmitoyl transferase I and decreased fatty acid oxidation, and an increase in cytosolic long-chain acyl-CoAs. Increased cytosolic long-chain acyl-CoAs have the potential to act as second messengers *per se* or possibly activate second messengers like PKC isoforms. They may also act via protein acylation. This "malonyl-CoA hypothesis" has a great deal of supportive evidence (7–10) but is controversial because of recent studies (11,12).
- The glucose-induced increase in the ATP/ADP ratio (13).
- A putative glutamate signal (14), which is also controversial (15).
- Cytosolic NADPH produced by the mitochondrial pyruvate–malate shuttle (16).

The K_{ATP} channel–independent pathways are likely to consist of multiple signals that are coordinated with one another and with the K_{ATP} channel–dependent pathway. An increase in $[Ca^{2+}]_i$ alone will stimulate insulin release, as, for example, with sulfonylureas or depolarizing concentrations of KCl. However, the responses to these stimuli are different from the response to glucose. All cause a peak of first-phase release, but only glucose results in a prominent second phase, pointing to the involvement of the K_{ATP} channel–independent pathways in the generation of the second phase of release. Furthermore, as the first phase is thought to be due to a small pool of readily releasable granules, the second phase must be due to granules that have translocated from reserve pools to a releasable pool at the membrane. Therefore, in the presence of elevated $[Ca^{2+}]_i$, the K_{ATP} channel–independent pathways are responsible for the selection and translocation of insulin-containing granules from the reserve pools to the cell membrane, their assembly at the plasma membrane, priming to achieve fusion competence, and finally exocytosis.

In the present work, we have chosen to study the potential signaling role of protein acylation on the dynamic aspects of glucose stimulus–secretion coupling. We investigated the effects of cerulenin, an inhibitor of protein acylation, on the two phases of glucose-stimulated insulin release and on the K_{ATP} channel–independent pathway in

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BCH, 2-aminobicyclo(2.2.1)heptane; PKC, protein kinase C; SNAP-25, synaptosomal-associated protein-25; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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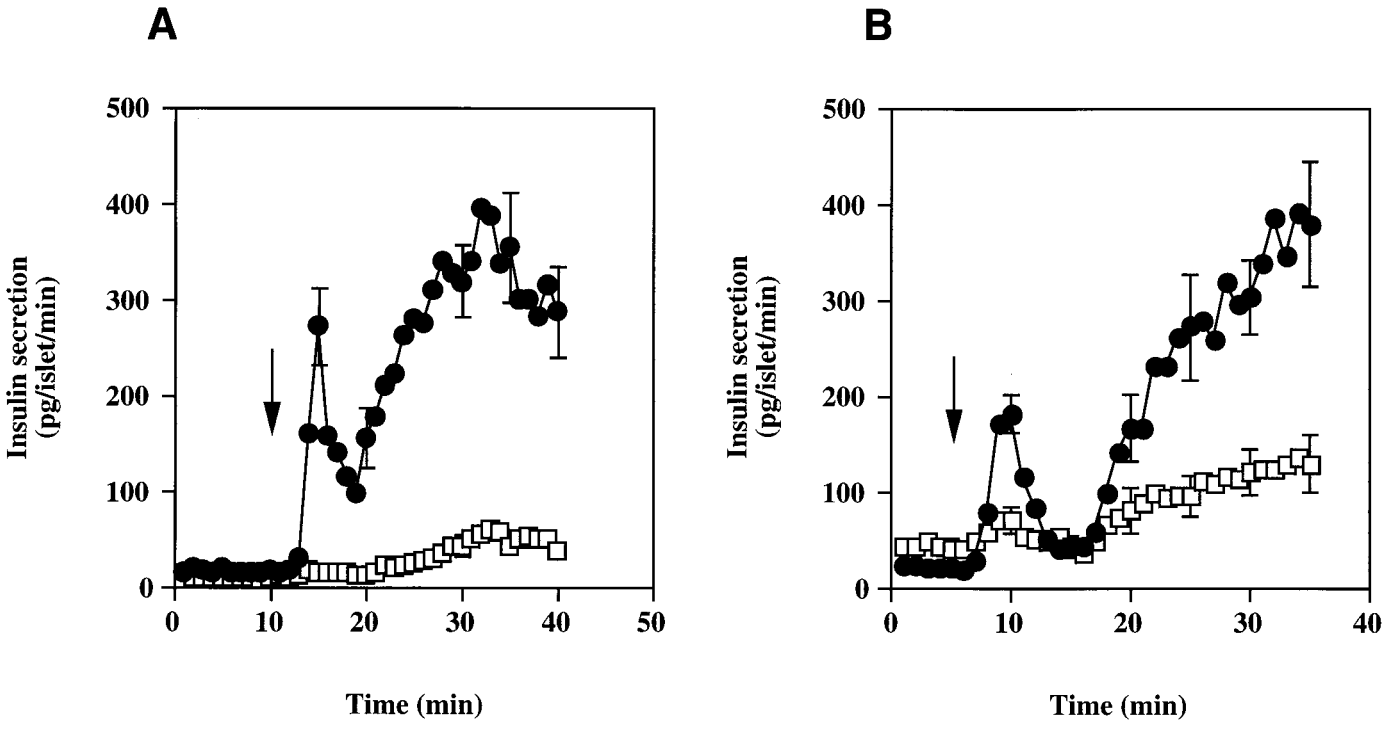

FIG. 1. The stimulation of insulin release by 16.7 mmol/l glucose (added at minute 10) (●) (*A*) and a combination of 10 mmol/l BCH and 10 mmol/l glutamine (added at minute 5) (●) (*B*), and the inhibitory effect of 100 μmol/l cerulenin (□). The basal glucose concentration was 2.8 mmol/l. Cerulenin was present only during the preincubation period. Mean ± SE, $n = 4$.

the presence and absence of extracellular Ca^{2+}. Cerulenin inhibits glucose-stimulated insulin release maximally at 100 μmol/l without affecting glucose or palmitate oxidation, or ATP content at low and high glucose concentrations (17). However, until now there has been no dynamic analysis of the effects on biphasic release.

RESEARCH DESIGN AND METHODS

Isolation of rat pancreatic islets and measurement of insulin secretion. Male Wistar rats weighing 300–400 g were killed by CO_2 asphyxiation, the pancreata were surgically removed, and islets were isolated by collagenase digestion in HEPES-buffered Krebs-Ringer bicarbonate solution (18,19). The islets were then subjected to a 45-min static incubation at 37°C in the presence or absence of 100 μmol/l cerulenin before they were transferred to perifusion chambers. A 40-min equilibration period was followed by perifusion under the indicated conditions, in which samples for insulin measurement were taken. Release rates were determined by radioimmunoassay using a charcoal separation technique (20). Since it has been shown that cerulenin covalently modifies sulfhydryl residues contained in the active sites of enzymes involved in fatty acid metabolism and therefore cannot be easily washed out (21), we added the compound only during the 45-min static incubations and not during the subsequent perifusion period. There was no evidence of reversibility of the effect of cerulenin over 90 min (21).

RESULTS

In initial control studies, 100 μmol/l cerulenin had no effect on the rate of basal insulin secretion in the presence or absence of extracellular Ca^{2+} (data not shown). This concentration of the inhibitor was then used throughout the study. When insulin secretion was stimulated by glucose or the combination of 10 mmol/l glutamine and 10 mmol/l 2-aminobicyclo(2.2.1)heptane (BCH; a nonmetabolizable analog of leucine, which activates glutamate dehydrogenase), cerulenin had a powerful inhibitory effect (Fig. 1*A* and *B*, respectively). Both the first and second phases of release were profoundly inhibited. The effect on the first phase suggested that the operation of either the K_{ATP} channels or voltage-dependent Ca^{2+} channels had been compromised. To determine if this was the case, the effect of cerulenin on the response to a depolarizing concentration of KCl was studied. As can be seen from the results in Fig. 2, cerulenin had no effect on the response to KCl. Thus, the Ca^{2+} channels are operating normally in response to depolarization, and the subsequent secretory response to elevated $[Ca^{2+}]_i$ is also unaffected. It is concluded that cerulenin either prevents closure of the K_{ATP} channels in response to nutrient stimulation or is actually activating the channels. Because of this effect, and because the K_{ATP} channel–independent pathway acts in synergy with increased $[Ca^{2+}]_i$, it was necessary to study the effect of cerulenin on the K_{ATP} channel–independent pathway in isolation. This was achieved by activating the K_{ATP} channels with diazoxide, depolarizing the cell with KCl, and then measuring the effect of 16.7 mmol/l glucose on insulin release independently of the K_{ATP} channels. The results are shown in Fig. 3. The control response to 40 mmol/l KCl in the presence of 250 μmol/l diazoxide was monophasic, with the peak secretion rate after 1 min and a return to basal levels after 10–15 min. The K_{ATP} channel–independent response to glucose under these conditions was a large increase in release over and above that of KCl alone. Peak rates were achieved after 10 min, and insulin secretion remained at high levels throughout the course of the experiment. Cerulenin strongly inhibited this response. The initial rise in insulin secretion to the KCl peak at 1 min was unaffected. However, from minute 2, the secretion rate began to decline as the glucose response was inhibited. Thus, cerulenin inhibits the K_{ATP} channel–independent pathway of glucose signaling. Because the main

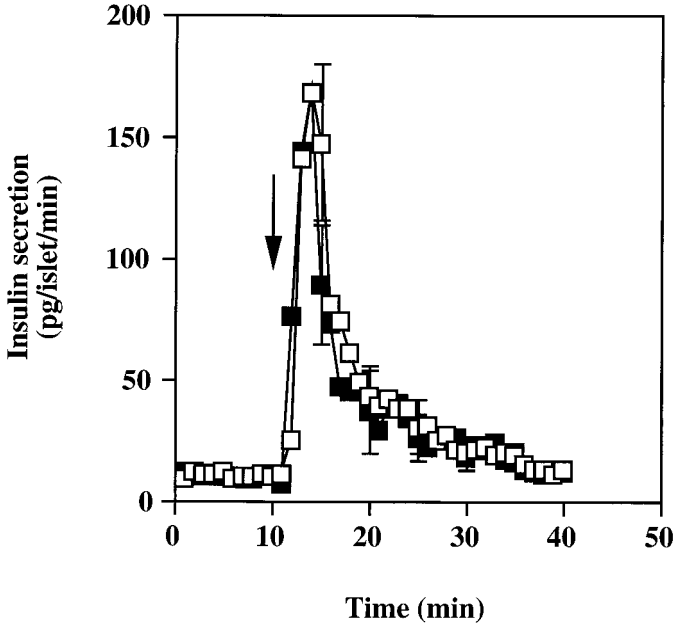

FIG. 2. The effects of 40 mmol/l KCl (■) (added at minute 10) to stimulate insulin release, and the lack of inhibition of this response by cerulenin (100 μmol/l) (□). The basal glucose concentrations was 2.8 mmol/l. Cerulenin was present only during the preincubation period. Mean ± SE, $n = 5$.

purpose of these studies is to determine whether acylation is involved in stimulus-secretion coupling, the K_{ATP} channel–independent effect of palmitate was studied under the same conditions with KCl and diazoxide (22). The results of these experiments are shown in Fig. 4. It can be seen that the response to palmitate was also completely blocked by cerulenin.

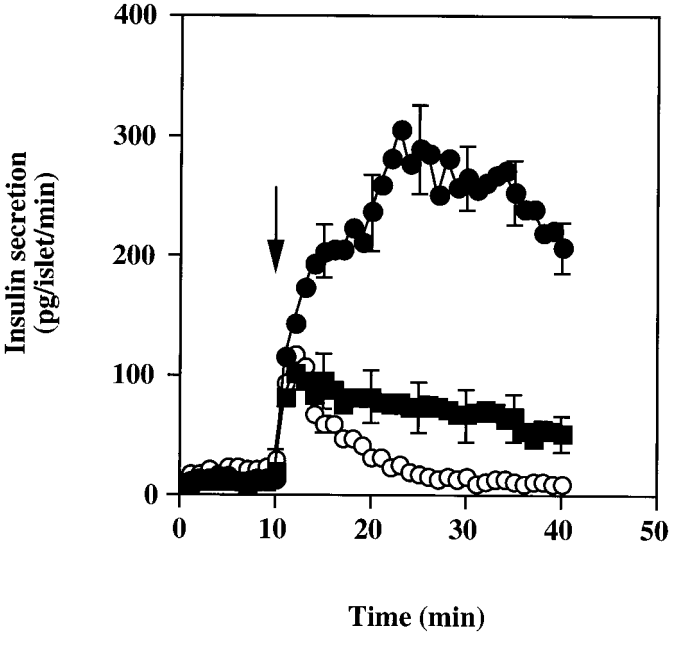

FIG. 3. The effects of 40 mmol/l KCl and 250 μmol/l diazoxide (○) on insulin release, the augmentation in the presence of 16.7 mmol/l glucose (●) (both added at minute 10), and the inhibition of the glucose response after treatment with cerulenin (100 μmol/l) (■). The basal glucose concentration was 2.8 mmol/l. Cerulenin was present only during the preincubation period. Mean ± SE, $n = 5$.

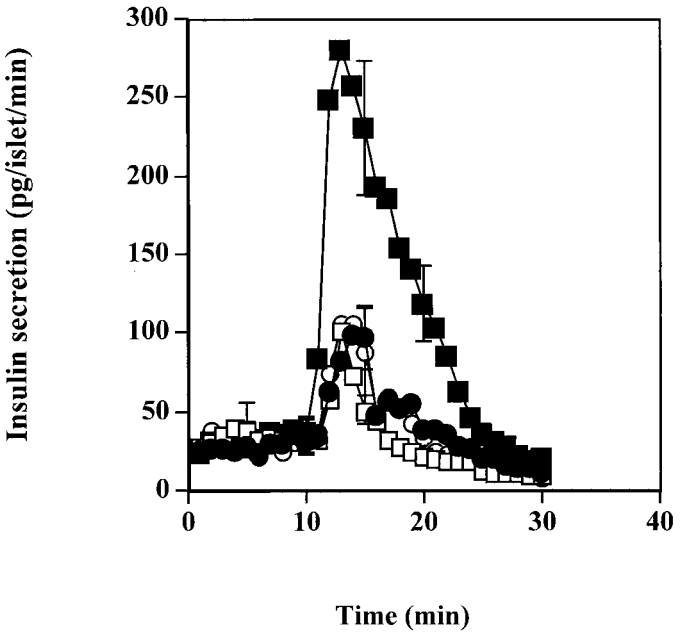

FIG. 4. The effects of 40 mmol/l KCl (●) on insulin release, and the lack of effect of cerulenin (100 μmol/l) (○) on KCl-induced secretion. Also shown is the enhancement of the response to KCl by 10 μmol/l free palmitate (■) and its inhibition by cerulenin (□). Palmitate was present during the preperifusion and perifusion period. The basal glucose concentration was 2.8 mmol/l and KCl was added at minute 10. Cerulenin was present only during the preincubation period. Mean ± SE, $n = 5$.

Next, we studied the K_{ATP} channel–independent effect of glucose under Ca^{2+}-free conditions (in the presence of 1 mmol/l EGTA) and also took advantage of the marked responses to both glucose and palmitate that occur in the absence of extracellular Ca^{2+} (23). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA; 100 nmol/l) and forskolin (6 μmol/l) applied at 10 min caused a small increase in insulin release as anticipated. After 20 min, the addition of 11.1 mmol/l glucose (the maximally effective concentration under these conditions) caused a threefold increase in insulin secretion, which was totally blocked by cerulenin (Fig. 5*A*). Similar results were obtained when the inhibitor was tested against the response to 10 μmol/l free palmitate (Fig. 5*B*). Palmitate (in the presence of TPA and forskolin) induced a rise in the rate of insulin secretion that rose to three times the basal rate. As was the case with glucose, cerulenin completely abolished the response.

DISCUSSION

In this study, we provide further evidence that nutrient-stimulated insulin release and specifically the nutrient effects on the K_{ATP} channel–independent pathways of secretion can be almost completely eliminated by the irreversible protein acylation inhibitor cerulenin. Strikingly, this antibiotic does not affect secretory responses elicited by nonnutrients like KCl, which increases intracellular Ca^{2+}, or the stimulation caused by the combination of TPA and forskolin. Caution has to be paid to possible other specific (by inhibition of acylation) and/or nonspecific effects that cerulenin might exert in the β-cell, where it clearly inhibits the glucose effect on the first phase of secretion. The inhibition of the first phase of secretion by cerulenin must be due to a failure of glucose to increase

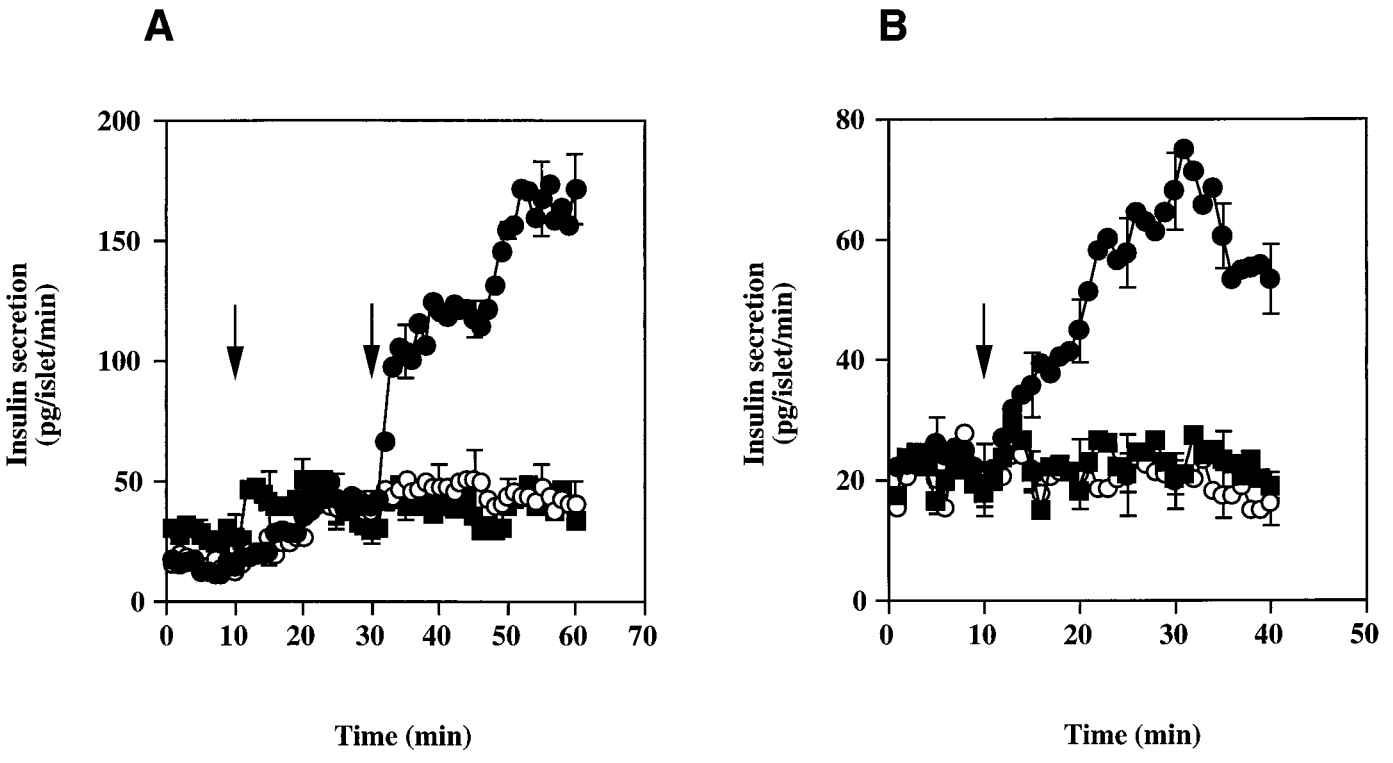

FIG. 5. *A*: **The stimulation of secretion by TPA (100 nmol/l) and forskolin (6 μmol/l) (○) (added at minute 10) in the absence of extracellular Ca^{2+}; the augmentation of secretion by 11.1 mmol/l glucose (added at minute 30) (●); and the inhibition of secretion by cerulenin (■).** *B*: **The effect of 10 μmol/l free palmitate alone (○) (present throughout the preperifusion and perifusion period), in the presence of TPA and forskolin (●), and when inhibited by cerulenin (■). Basal glucose concentrations were 2.8 mmol/l. Cerulenin was present only during the preincubation period. Mean ± SE, $n = 5$.**

$[Ca^{2+}]_i$ because the response to increased $[Ca^{2+}]_i$ by KCl is not affected by cerulenin. Such an effect might be exerted directly on the K_{ATP} channel or indirectly by virtue of an effect on glucose metabolism. In this respect, however, it is important to note that cerulenin treatment does not affect ATP production, glucose oxidation, or the effect of glucose to suppress palmitate oxidation (17). It is possible, therefore, that cerulenin has an effect on the K_{ATP} channel itself. The fact that cerulenin has no effect on the secretory response to a depolarizing concentration of KCl and raised $[Ca^{2+}]_i$ demonstrates that cerulenin has no effect on Ca^{2+} stimulus–secretion coupling. Its inhibitory effects are therefore exerted on the nutrient augmentation pathways.

Additional potential targets for inhibition by cerulenin are enzymatic activities like fatty acid synthase (23), although its activity is very low in the β-cell, and acetyl-CoA carboxylase (24). Whereas an inhibition of acetyl-CoA carboxylase cannot be excluded as a cause of the inhibition of the response to glucose (although the glucose suppression of fatty acid oxidation was not affected by cerulenin), we were able to bypass this enzyme in the experiments in which palmitate was used as the stimulus in both the presence and the absence of Ca^{2+} and in which cerulenin still exerted its full inhibitory capacity. Other described effects of the compound, e.g., reduced apoptosis (25) and inhibition of RNA (26) and protein synthesis (23), are not likely to explain our results because of the short exposure time to cerulenin and the lack of effect on the KCl response.

Protein acylation is a post-translational event, which usually links palmitate in the form of a fatty acyl-CoA, as the preferred substrate, to a cysteine residue through a thioester linkage in a variety of proteins (e.g., G_α subunits, *ras* [27], Ca^{2+} channels [28], etc.), and it has only recently been established that an enzymatic activity (protein *S*-acyltransferase) is likely to represent the predominant mechanism for thioacylation (29). Because it is a reversible modification with dynamic cycles of acylation and deacylation, it is capable of playing a role in signal transduction. One possible target for acylation has been recently identified as a PKC of 80 kDa whose translocation to membrane bilayers was facilitated upon palmitoylation (30). Moreover, acylation has also been shown to occur on proteins directly linked to exocytosis like synaptotagmin (31), which is a putative Ca^{2+} sensor, and SNAP-25 (synaptosomal-associated protein-25) (32), a component of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex, and it therefore might reflect a distal event in secretion. This assumption is supported by a study in HIT-T15 cells, where an enhancement of Ca^{2+}-induced insulin secretion by long chain-acyl CoAs was reported (33). However, nonspecific interactions of acyl-CoAs at high concentrations with cell membranes and subsequent facilitation of fusion processes cannot yet be excluded. In preliminary studies where rat pancreatic islets were labeled with [^{3}H]palmitate and subjected to SDS-PAGE and fluorography, we were able to distinguish several discrete bands, of which one had a molecular weight of 25,000, the same as SNAP-25. Obviously, more detailed studies are needed to determine an involvement of protein acylation in the course of the K_{ATP} channel–independent pathways.

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