Time-dependent potentiation of the β-cell is a Ca^{2+}-independent phenomenon

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Abstract

When isolated rat pancreatic islets are treated with 16·7 mM glucose, a time-dependent potentiation (TDP) of insulin release occurs that can be detected by subsequent treatment with 50 mM KCl. It has been thought that TDP by glucose is a Ca^{2+}-dependent phenomenon and only occurs when exposure to glucose is carried out in the presence of Ca^{2+}. In contrast to this, we now demonstrate TDP under stringent Ca^{2+}-free conditions (Ca^{2+}-free buffer containing 1 mM EGTA). In fact, under these Ca^{2+}-free conditions glucose caused an even stronger TDP than in the presence of Ca^{2+}. TDP induced by glucose in the absence of extracellular Ca^{2+} was unaffected by inhibitors of protein kinase C (PKC). However, cerulenin or tunicamycin, two inhibitors of protein acylation, eradicated TDP without affecting glucose metabolism. The TDP by glucose was not associated with an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$) during subsequent treatment with high K^+. Exposure of islets to forskolin under Ca^{2+}-free conditions did not cause TDP despite a large increase in the cellular cAMP levels. In conclusion, glucose alone induces TDP under stringent Ca^{2+}-free conditions when $[Ca^{2+}]i$ was significantly lowered. Protein acylation is implicated in the underlying mechanism of TDP.

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Introduction

The pancreatic β-cell is an excitable endocrine cell that displays a variety of electrophysiological and ionic responses upon stimulation with nutrients (Mears & Atwater 2000). Since the pioneering work of Grodsky and Bennett (1966), it has been accepted that extracellular Ca^{2+} is absolutely required for glucose stimulation of the β-cell (Wollheim & Sharp 1981). Thus, Ca^{2+} influx through L-type voltage-dependent Ca^{2+} channels and consequent elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$) has been thought to play an indispensable role in β-cell stimulus-secretion coupling (Wollheim & Sharp 1981, Komatsu *et al.* 1989). Moreover, glucose requires extracellular Ca^{2+} to increase cAMP production in the pancreatic β-cell (Charles *et al.* 1975). Glucose-induced ionic events have now been proven to originate, for the most part, from the closure of ATP-sensitive K^+ (KATP) channels and the resultant membrane depolarization, which is due to elevation of ATP or the ATP/ADP ratio produced by glucose metabolism (Seino *et al.* 2000).

In 1995, it was shown that glucose augments insulin release triggered by pharmacological activation of protein kinases C (PKC) and A (PKA), by phorbol 12-myristate 13-acetate (PMA) and forskolin respectively, under stringent Ca^{2+}-free conditions in the presence of EGTA (Komatsu *et al.* 1995). Stimulation of insulin release by a combination of a nutrient and 1-oleoyl-2-acetyl-glycerol, another activator of PKC, under the stringent Ca^{2+}-free conditions was also reported by others (Malaisse *et al.* 1985). This glucose stimulation of the β-cell was potent and occurred under significantly lowered $[Ca^{2+}]i$ (Komatsu *et al.* 1995, Straub *et al.* 2001). Yet, pharmacological activation of kinases, especially PKC, was a prerequisite for the Ca^{2+}-independent glucose stimulation of the β-cell to be observed. Partly due to such requirements for pharmacological manoeuvres, the physiological relevance of the Ca^{2+}-independent glucose action has been questioned (Sato *et al.* 1998).

As an extension of our efforts to establish the glucose action 'beyond ionic events' in the β-cell (Komatsu *et al.* 1997, Aizawa *et al.* 1998), identification of a novel Ca^{2+}-independent glucose action was made in the present study. We report here that time-dependent potentiation (TDP) of the islet β-cell by glucose is a Ca^{2+}-independent phenomenon. It had been reported previously that glucose

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failed to exhibit TDP under Ca^{2+}-free conditions in the presence of EGTA (Zawalich *et al.* 1988, Chalmers & Sharp 1989, Taguchi *et al.* 1995). However, TDP occurred when the priming exposure to glucose was carried out in the presence of diazoxide (Taguchi *et al.* 1995), a KATP channel activator, or in the nominally Ca^{2+}-free buffer without EGTA (Grill *et al.* 1978, 1979, Malaisse & Sener 1987, Taguchi *et al.* 1995). Furthermore, simple elevation of $[Ca^{2+}]i$ by high K^+ or sulfonylurea does not cause TDP (Taguchi *et al.* 1995). Taken together, it has been considered that 1) a glucose-induced rise in $[Ca^{2+}]i$ is not required for TDP, and that 2) extracellular Ca^{2+} concentrations ($[Ca^{2+}]o$) in the micromolar range and thus normal, resting $[Ca^{2+}]i$ are critically required for glucose-induced TDP. Contrary to these assumptions, we found that glucose alone, without any other stimulating agents, time-dependently potentiated insulin release under stringent Ca^{2+}-free conditions. Accordingly, a detailed characterization of this novel aspect of stimulus-secretion coupling in the β-cell was performed.

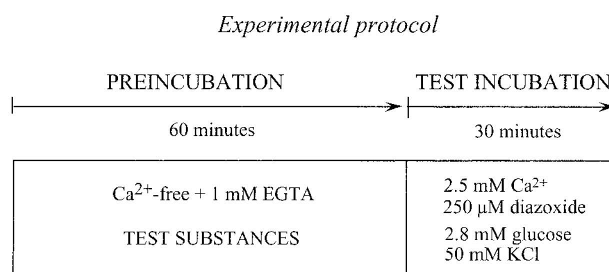

Figure 1 Schematic presentation of the protocol used for evaluation of Ca^{2+}-independent priming of insulin release. The islets were initially preincubated in Ca^{2+}-free KRB/EGTA buffer containing test substances (see text) at 37 °C for 60 min (Preincubation=priming period). Then they were stimulated with 50 mM KCl in regular KRB buffer containing 2·8 mM glucose and 2·5 mM $CaCl_2$ with 250 μM diazoxide (Test incubation). At the end of the Test incubation, the buffer was collected for radioimmunoassay of insulin.

Materials 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 (Lacy & Kostianovsky 1967). Krebs–Ringer bicarbonate (KRB) buffer containing 129 mM NaCl, 5 mM $NaHCO_3$, 4·8 mM KCl, 1·2 mM KH_2PO_4, 1·2 mM $MgSO_4$, 2·5 mM $CaCl_2$, 5·6 mM glucose, 0·1% BSA, and 10 mM 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 and perifusion experiments. In static incubation, batches of 5 size-matched islets per tube were used. The typical experimental condition for static incubation is illustrated in Fig. 1. The islets were first incubated for 60 min at 37 °C in 1 ml Ca^{2+}-free KRB buffer containing 129 mM NaCl, 5 mM $NaHCO_3$, 4·8 mM KCl, 1·2 mM KH_2PO_4, 1·2 mM $MgSO_4$, 1 mM EGTA, and 0·2% BSA and 10 mM HEPES at pH 7·4 (Ca^{2+}-free KRB/EGTA buffer) including the indicated concentrations of glucose and/or test substances (Preincubation). At the end of the preincubation, all the tubes in a rack or racks were placed into an ice-cold water bath to stop glucose metabolism at once. Then the islets were washed with 1 ml ice-cold KRB buffer containing 2·5 mM Ca^{2+}, 2·8 mM glucose and 250 μM diazoxide. After the introduction of 1 ml fresh KRB buffer containing a depolarizing concentration (50 mM) of KCl, 2·5 mM Ca^{2+}, 2·8 mM glucose and 250 μM diazoxide, the rack(s) was placed into a 37 °C water bath again and additional incubation was carried out for 30 min (Test incubation). Thus, the medium used for the Test incubation was the same for all experiments, and did not contain test substances. The media change usually took 5 to 10 min. At the end of the Test incubation, the medium was collected and kept at − 20 °C until assayed for insulin.

In one series of experiments, buffer with various concentrations of Ca^{2+} was used during Preincubation to examine the dependency of the glucose effect on extracellular Ca^{2+} concentrations ($[Ca^{2+}]o$). In another series of experiments, 8·3 mM glucose was used in place of 50 mM K^+ as a secretagog during the Test incubation without diazoxide.

When the effects of cerulenin or tunicamycin, inhibitors of protein acylation (Schlesinger & Malfer 1982, Patterson & Skene 1994, Jochen *et al.* 1995, Hurley *et al.* 2000), were examined, islets were first incubated in regular KRB buffer (2·8 mM glucose and 2·5 mM $CaCl_2$) containing various concentrations of cerulenin or tunicamycin for 30 min at 37 °C. Then, after washing with 1 ml Ca^{2+}-free KRB/EGTA buffer containing 2·8 mM glucose, incubation experiments were carried out as described above. Cerulenin or tunicamycin was included throughout the experiment. Knowing that prolonged (>3 h) exposure of the cell to tunicamycin also causes inhibition of N-linked glycosylation of proteins (Kadowaki *et al.* 2000, Marmorstein *et al.* 2000), exposure to tunicamycin was limited to a maximum of 1 h. Although cerulenin inhibits fatty acid synthase (FAS) (Saitoh *et al.* 1996), little FAS activity was dected in the β-cell (Brun *et al.* 1996). Thus, cerulenin inhibition of FAS, if any, is not expected to significantly affect β-cell function. In the experiments with palmitate, the final concentration of palmitate was 0·6 mM in the presence of

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100 μM (0·68%) free fatty acid (FFA)-free BSA, which approximates to 10 μM of the unbound form of palmitate (Spector *et al.* 1971).

For perifusion experiments, 50 size-matched islets each were placed in 10 columns of identical size, and all columns were perifused in parallel at a flow rate of 1 ml/min at 37 °C (Sato *et al.* 1992). The islets were first perifused for 60 min with Ca^{2+}-free KRB/EGTA buffer containing 2·8 or 16·7 mM glucose. After that, the buffer containing 50 mM KCl, 2·5 mM $CaCl_2$, 2·8 mM glucose, and 250 μM diazoxide was used for perifusion for 30 min. Samples were collected at 1-, 2-, or 5-min intervals, and insulin in the perifusate was measured by radioimmunoassay.

In this paper, we define augmentation and TDP as follows. When high K^+-induced insulin release is enhanced by simultaneous application of a test substance, this is called augmentation, whereas when a prior application of a test substance causes an enhancement of insulin release subsequently triggered by high K^+ in the absence of the test substance, this is called TDP.

Measurement of cAMP

The cAMP content of the islet cells was measured as previously reported (Yajima *et al.* 1999). Briefly, 300 μl 0·2 M HCl were added to the glass tube containing 5 islets/100 μl KRB buffer. The tubes were placed in boiling water for 5 min with occasional vortexing for extraction of cAMP. Then, volatile constituents in the tube were evaporated using a SpeedVac system (Savant, Farmingdale, NY, USA) and the pellet was reconstituted with 50 μl distilled water. cAMP was determined using commercially available radioimmunoassay kits (Yamasa, Chiba, Japan).

Glucose oxidation

Glucose oxidation by the islet cells was measured as previously reported (Aizawa *et al.* 1994). In brief, 25 islets were first incubated in 0·5 ml KRB buffer with or without 30 μg/ml cerulenin or tunicamycin at 37 °C for 30 min. Then the buffer was aspirated and 100 μl KRB buffer containing 1 μCi D-[U-^{14}C]glucose with or without cerulenin or tunicamycin was introduced. The test incubation was carried out for 60 min at 37 °C. At the end of the incubation, $^{14}CO_2$ was trapped by methylbenzethonium hydroxide (Nacalai Tesque, Kyoto, Japan) after adding 200 μl 0·2 M HCl to the incubation mixture, and quantified by liquid scintillation spectrometry.

Preparation of single β-cells

Single β-cells were prepared as previously reported (Yada *et al.* 1997). Briefly, pancreatic islets were isolated from Wistar rats aged 10–12 weeks by collagenase digestion as described above. The isolated islets were dispersed into single cells by treatment with Ca^{2+}-free KRB buffer with 0·1 mM EGTA. The single cells were plated on coverslips and maintained in a short-term culture for up to 2 days in Eagle's minimum essential medium containing 5·6 mM glucose, 10% FBS, 100 μg/ml streptomycin and 100 units/ml penicillin at 37 °C in a 95% air and 5% CO_2 atmosphere. β-Cells were selected by morphological and physiological criteria as reported previously (Yada *et al.* 1997), or identified, post-experimentally, by immunostaining with antiserum against insulin.

Measurements of [Ca^{2+}]i in single β-cells

[Ca^{2+}]i was measured by dual-wavelength fura-2 microfluorometry combined with digital imaging as previously reported (Yada *et al.* 1997). Briefly, cells on coverslips were loaded with fura-2 by incubation with 1 μM fura-2 acetoxymethylester in the KRB buffer containing 2·8 mM glucose for 60 min at 37 °C. The cells were then mounted in a chamber and superfused with KRB buffer at a rate of 1 ml/min at 37 °C. The cells were excited at 340 and 380 nm alternately every 2·5 s, emission signals at 510 nm were detected with an intensified charge-coupled device camera, and ratio images were produced by an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan). Ratio values were converted to [Ca^{2+}]i according to calibration curves. Nominal Ca^{2+}-free conditions were achieved in KRB buffer with no added Ca^{2+} (nominal Ca^{2+}-free KRB). Actual Ca^{2+} concentration of nominal Ca^{2+}-free buffer was 58 μM in our hands (Taguchi *et al.* 1995).

Materials

Forskolin, PMA, palmitate, α-ketoisocaproic acid (KIC), diazoxide, calphostin C, cerulenin, and tunicamycin were purchased from Sigma Chemical (St Louis, MO, USA). EGTA, fura-2 and fura-2 acetoxymethylester were obtained from Molecular Probes (Eugene, OR, USA). Bisindolylmaleimide was obtained from Calbiochem (La Jolla, CA, USA). Insulin was measured by radioimmunoassay using commercially available kits (Eiken Immunochemicals, Osaka, Japan) with rat insulin (Novo, Bagsvaerd, Denmark) as a standard (Komatsu *et al.* 1989, Sato *et al.* 1992).

Data analysis

Data are shown as means ± S.E. Statistical significance was evaluated by one-way ANOVA with a pairwise comparison by the Bonferroni method, or by Mann–Whitney U test. Differences were considered significant at $P<0.05$.

Results

TDP by glucose occurs independently of [Ca^{2+}]o

First, we systematically examined the effects of [Ca^{2+}]o on TDP. Insulin release during subsequent test incubations

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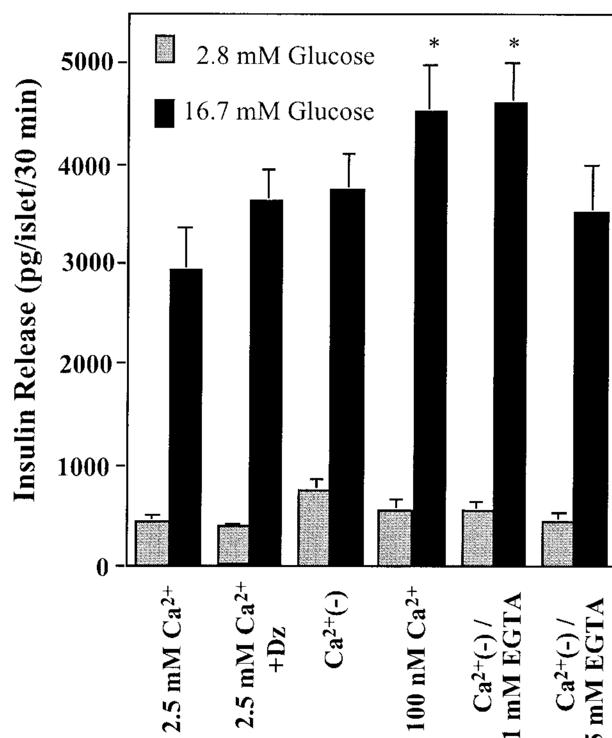

Figure 2 Effects of various $[Ca^{2+}]$o during the preincubation period on subsequent insulin release stimulated by 50 mM KCl. The islets were preincubated with low (2·8 mM) or high (16·7 mM) concentrations of glucose in the buffer with the indicated $[Ca^{2+}]$o for 60 min. They were then stimulated with 50 mM KCl in regular KRB buffer (containing 2·8 mM glucose and 2·5 mM $CaCl_2$) with 250 µM diazoxide (Dz) for 30 min. The amount of insulin released during the Test incubation is shown. Values are means ± S.E. of 8 determinations. Statistical significance was evaluated by one-way ANOVA with a pairwise comparison by the Bonferroni method. *$P<0·01$ compared with 16·7 mM glucose in 2·5 mM Ca^{2+}.

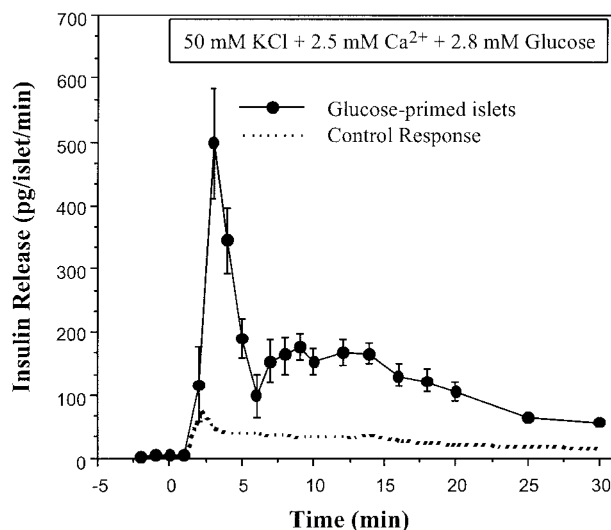

Figure 3 Temporal profile of Ca^{2+}-stimulated insulin release from the islets primed with 16·7 mM glucose in the Ca^{2+}-free KRB/EGTA buffer. Perifusion experiments were performed as described in Materials and Methods. The islets were first perifused with Ca^{2+}-free KRB/EGTA buffer containing 16·7 mM glucose for 60 min (− 60 to 0 min). At time 0, the solution was changed to the regular KRB buffer (containing 2·8 mM glucose and 2·5 mM $CaCl_2$) with 250 µM diazoxide and 50 mM KCl. Values are means ± S.E. of 10 determinations. The dashed line indicates the average 50 mM KCl-induced insulin release from the islets without any pretreatment.

with 50 mM K^+ is shown in Fig. 2. In the regular KRB buffer ($[Ca^{2+}]$o, 2·5 mM), 16·7 mM glucose strongly primed the β-cell, as established previously (Grill *et al.* 1978, 1979, Ashby & Shirling 1981, Grill 1981). The TDP by glucose was not at all attenuated when preincubation with high glucose was carried out in the buffer containing 250 µM diazoxide (a KATP channel opener). TDP by glucose was also apparent in KRB without added Ca^{2+}, in KRB with 0·6 mM $CaCl_2$ and 1 mM EGTA yielding a calculated $[Ca^{2+}]$o of 100 nM, and in KRB without added Ca^{2+} containing 1 or 5 mM EGTA (Fig. 2). Glucose-induced insulin release during the preincubation was totally inhibited by the five conditions: in the presence of diazoxide, in nominally Ca^{2+}-free KRB, in KRB with 100 nM Ca^{2+}, and in Ca^{2+}-free KRB (with 1 mM EGTA or 5 mM EGTA).

To study the temporal profile of Ca^{2+}-stimulated insulin release after TDP by glucose under stringent Ca^{2+}-free conditions, perifusion experiments were performed (Fig. 3). Here, the islets were first perifused with Ca^{2+}-free/1 mM EGTA buffer containing 16·7 mM glucose for 60 min, and the perifusion buffer was then changed to one with a depolarizing concentration of K^+ (50 mM), 2·5 mM Ca^{2+}, 2·8 mM glucose and 250 µM diazoxide. In the 16·7 mM glucose-potentiated islets, insulin release elicited by 50 mM K^+ was almost biphasic (Fig. 3). This was not the case with the rat islets without pre-exposure to high concentrations of glucose (shown by the dashed line in Fig. 3).

Characteristics of TDP by glucose under the stringent Ca^{2+}-free conditions

Glucose primed the β-cell under the stringent Ca^{2+}-free conditions in a concentration-dependent manner with an EC_{50} of 8·3 mM (Fig. 4, open circles). The minimum effective concentration of glucose was 5·6 mM and the maximum effect was observed at 22·2 mM (Fig. 4, open circles).

We next analyzed the kinetics of the glucose effect under the stringent Ca^{2+}-free conditions. The relationship between exposure time and the degree of TDP is shown in Fig. 5A. At least 5 min were needed for 16·7 mM glucose to prime the β-cell. When the exposure time to

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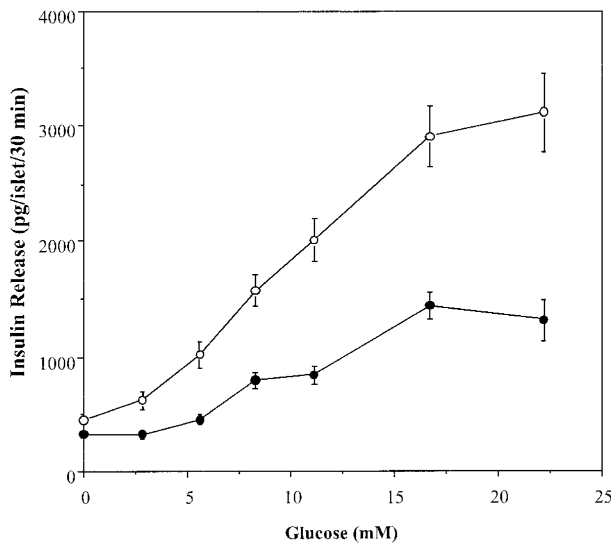

Figure 4 Concentration dependency of Ca^{2+}-independent, time-dependent potentiation (TDP) by glucose. Insulin release was measured in static incubation as described in Materials and Methods. The islets were preincubated with the respective concentrations of glucose in Ca^{2+}-free KRB/EGTA buffer for 60 min. After preincubation, they were exposed to 50 mM KCl for 30 min in the regular KRB buffer (containing 2·8 mM glucose and 2·5 mM $CaCl_2$) with 250 μM diazoxide (open circles) or regular KRB buffer (containing 2·5 mM $CaCl_2$ and 4·7 mM KCl) with 8·3 mM glucose (closed circles) for 30 min. The amount of insulin released during the Test incubation is shown. Values are means ± S.E. of 10 determinations.

high glucose was longer, the potentiating effect of high glucose increased time-dependently. The maximum effect occurred with a 60-min exposure (Fig. 5A). The time-dependent decay of TDP is shown in Fig. 5B. In this experiment, after 60 min of preincubation, the islets were incubated in regular KRB buffer containing 2·5 mM Ca^{2+}, 2·8 mM glucose and 250 μM diazoxide for 0–60 min, before exposure to 50 mM K^+. While there was no significant decay in TDP after 5 min, the TDP effect started to decrease thereafter and was absent after 60 min. The length of time for the TDP to become a half of the initial value ($T_{1/2}$) was approximately 10 min.

To examine whether the potentiated insulin release upon the second stimulation could take place not only with a depolarizing concentration of K^+, but also with a physiological concentration of glucose, 8·3 mM glucose was employed as the second stimulation in place of 50 mM K^+ (Fig. 4, closed circles). A concentration-dependent TDP was clearly detected in this experiment where 8·3 mM glucose was used as stimulation during the Test incubation.

Mediation of time-dependent potentiation by glucose under the stringent Ca^{2+}-free conditions

Effects of non-glucose nutrients and inhibitors of protein acylation As shown in Fig. 6, 20 mM α-KIC (a

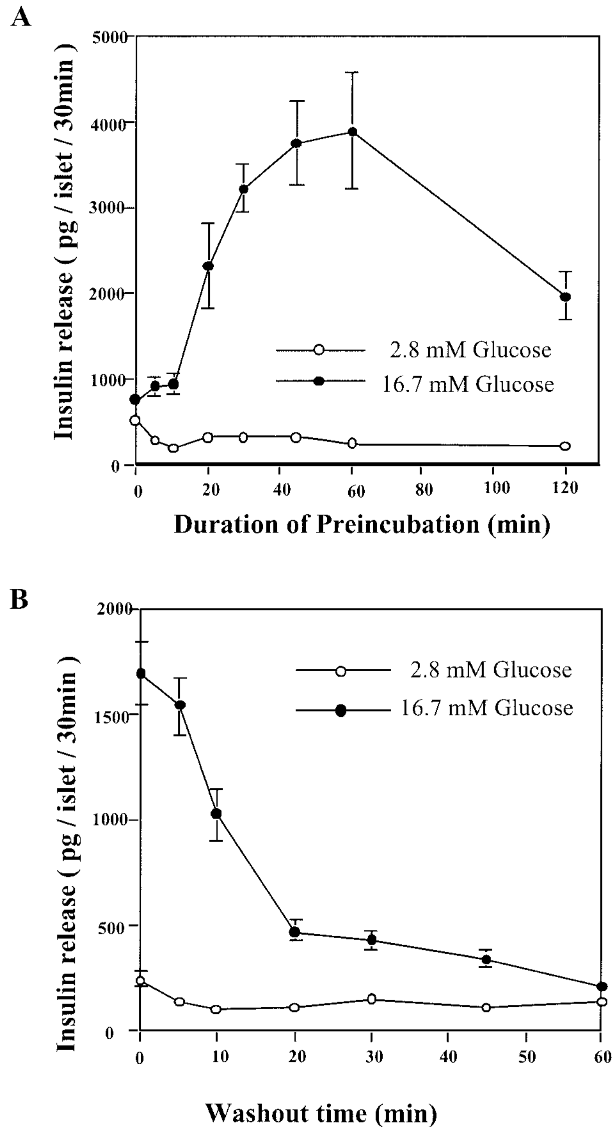

Figure 5 (A) Effects of the length of preincubation under Ca^{2+}-deprived conditions on subsequent insulin release stimulated by 50 mM KCl. The islets were preincubated for 0–120 min in Ca^{2+}-free KRB/EGTA buffer containing 2·8 (open circles) or 16·7 (closed circles) mM glucose and then stimulated with 50 mM KCl for 30 min in the regular KRB buffer (containing 2·8 mM glucose and 2·5 mM $CaCl_2$) with 250 μM diazoxide. The amount of insulin released during the Test incubation is shown. (B) Effects of the length of washout on glucose-induced, Ca^{2+}-independent TDP. After the TDP by glucose under stringent Ca^{2+}-free conditions for 60 min, the islets were incubated in 1 ml regular KRB buffer (containing 2·8 mM glucose and 2·5 mM $CaCl_2$) with 250 μM diazoxide for the indicated periods (0–60 min) at 37 °C. They were then exposed to 50 mM KCl for 30 min (Test incubation) in the regular KRB buffer with 250 μM diazoxide. The amount of insulin released during the Test incubation is shown. Values are means ± S.E. of 10 determinations.

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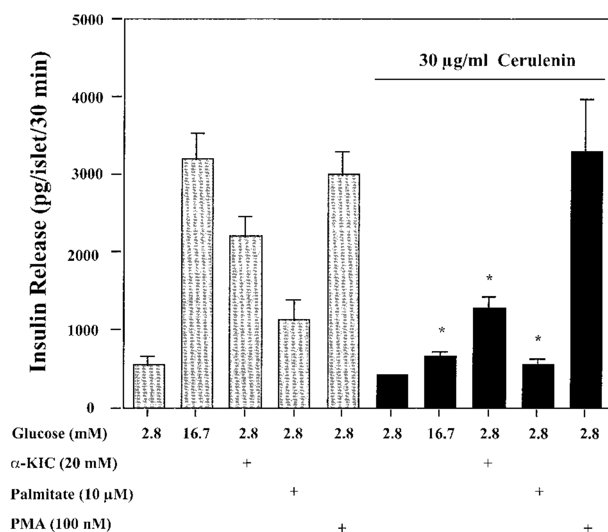

Figure 6 Effects of cerulenin, an inhibitor of protein acylation, on TDP by glucose under stringent Ca^{2+}-free conditions. The islets were first incubated in regular KRB buffer with or without 30 μg/ml (134 μM) cerulenin for 30 min. Then the islets were washed once with Ca^{2+}-free KRB/EGTA buffer containing 2·8 mM glucose and incubated in the Ca^{2+}-free KRB/EGTA buffer containing 16·7 mM glucose, 20 mM α-ketoisocaproic acid (α-KIC), 10 μM palmitate or 100 nM phorbol 12-myristate 13-acetate (PMA) in the presence or absence of 30 μg/ml cerulenin. Insulin release was finally stimulated with 50 mM KCl for 30 min in the regular KRB buffer (containing 2·8 mM glucose and 2·5 mM $CaCl_2$) with 250 μM diazoxide. The amount of insulin released during the Test incubation is shown. Values are means ± S.E. of 10 determinations. Statistical significance was evaluated by Mann–Whitney U test. *$P<0·01$ compared with corresponding values in the absence of cerulenin.

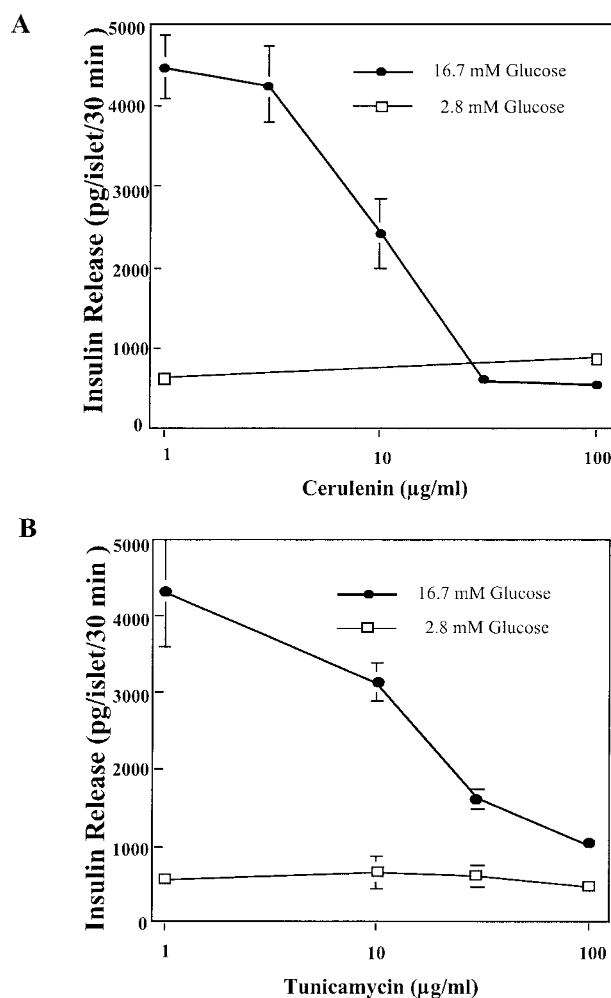

Figure 7 Concentration-dependent inhibition of glucose-induced TDP under stringent Ca^{2+}-free conditions by inhibitors of protein acylation. (A) Effects of cerulenin. Cerulenin was present in pretreatment (30 min) and Preincubation (60 min). (B) Effects of tunicamycin. Tunicamycin was present in Preincubation (60 min). Preincubation was performed in Ca^{2+}-free KRB/EGTA buffer containing 2·8 or 16·7 mM glucose. Insulin release was subsequently stimulated with 50 mM KCl in the regular KRB buffer (containing 2·8 mM glucose and 2·5 mM $CaCl_2$) with 250 μM diazoxide for 30 min in the absence of the inhibitors. The amount of insulin released during the Test incubation is shown. Values are means ± S.E. of 7–9 determinations.

mitochondrial fuel) and 10 μM palmitate (a putative enhancer of intracellular lipid messengers) also primed the β-cell under the stringent Ca^{2+}-free conditions, indicating that a product or products of mitochondrial metabolism and/or accumulation of lipid messengers may be mediating the TDP by glucose. Therefore, cerulenin (30 μg/ml = 134 μM), an inhibitor of protein acylation (Schlesinger & Malfer 1982, Jochen *et al.* 1995), was tested. We have recently identified that cerulenin specifically attenuates nutrient-induced insulin release (Yajima *et al.* 2000). Treatment with cerulenin eradicated 16·7 mM glucose- or 10 μM palmitate-induced TDP (Fig. 6). It also suppressed 20 mM α-KIC-induced TDP. Note that 100 nM PMA-induced TDP was unaffected by the treatment with cerulenin. The effect of cerulenin was concentration-dependent with an IC_{50} of 10 μg/ml. Complete inhibition of glucose-induced TDP was seen at 30 μg/ml or higher. A high K^+-induced insulin release in non-primed islets was not inhibited by 100 μg/ml cerulenin (Fig. 7A).

When cerulenin was applied after glucose exposure, cerulenin did not inhibit TDP by glucose. The islets were preincubated in Ca^{2+}-free/1 mM EGTA buffer in the presence of 2·8 mM or 16·7 mM glucose for 60 min (TDP periods). Then the islets were incubated with basal KRB containing 2·8 mM glucose and 2·5 mM Ca^{2+} in the presence or absence of 30 μg/ml cerulenin for 15 min, and the Ca^{2+}-stimulated insulin release was measured. Exposure to 16·7 mM glucose potentiated the Ca^{2+}-stimulated insulin release irrespective of the presence or absence of subsequent cerulenin exposure (2·8 mM glucose 222 ± 29 pg/islet/30 min vs 16·7 mM glucose

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Table 1 Effects of cerulenin and tunicamycin in the presence of 2·8 mM or 16·7 mM glucose on glucose oxidation in rat pancreatic islets. Values are means ± S.E. Number of determinations are indicated in parentheses

	Control		**Cerulenin** (30 μg/ml)		**Tunicamycin** (30 μ/ml)	
	2·8 mM glucose	16·7 mM glucose	2·8 mM glucose	16·7 mM glucose	2·8 mM glucose	16·7 mM glucose
Glucose oxidation (pmol/islet*60 min)	34·6 ± 5·2 (13)	114·1 ± 17·0 (13)	22·6 ± 2·3 (10)	151·1 ± 33·9 (8)	54·8 ± 4·9 (6)	177·3 ± 30·4 (7)*

*$P<0·05$ compared with the corresponding value in the control group (one-way ANOVA with a pairwise comparison by the Bonferroni method).

1007 ± 31 pg/islet/30 min, without cerulenin exposure; 2·8 mM glucose128 ± 31 pg/islet/30 min vs 16·7 mM glucose 882 ± 143 pg/islet/30 min, with exposure to 30 μg/ml cerulenin). This suggests that cerulenin needs to be present with high glucose to inhibit TDP. Examination of the effects of longer exposure to cerulenin *after* removal of high glucose was not possible because glucose-induced TDP *per se* decays with a $T_{1/2}$ of 10 min (see above). Another inhibitor of protein acylation, tunicamycin (Patterson & Skene 1994, Hurley *et al.* 2000), also attenuated 16·7 mM glucose-induced TDP in a concentration-dependent manner (Fig. 7B).

Neither cerulenin nor tunicamycin suppressed glucose oxidation by the islet cells (Table 1), implying that the inhibitory effects of the drugs are not due to metabolic inhibition in the β-cell. In fact, treatment with tunicamycin modestly augmented glucose oxidation in the presence of 16·7 mM glucose.

No evidence for involvement of PKC and PKA

Activation of PKC by 100 nM PMA primed the β-cell as shown by the shaded bars in Fig. 8A and B. However, PKC was ruled out as a mediator of glucose-induced TDP of the β-cell for the following reasons. Bisindolylmaleimide and calphostin C, inhibitors of PKC, which attenuated PMA-induced TDP, did not attenuate glucose-induced TDP at all (the solid bars in Fig. 8A and B). Also, addition of PMA, with a near maximum concentration of glucose, further primed the β-cell, suggesting that mediation of TDP by glucose is distinct from that by PMA.

As shown in Fig. 9, treatment of islets with forskolin did not prime the β-cell, despite a large increase in cAMP content. As expected, glucose- and PMA-induced TDP of the β-cell occurred without any measurable increase of cAMP in the islet cells. Direct evidence that PMA activates PKC under the stringent Ca^{2+}-free conditions is lacking. However, the fact that bisindolylmaleimide and calphostin C suppressed the PMA effect under the stringent Ca^{2+}-free conditions indirectly suggests that PKCs are involved in the PMA-induced TDP.

Time-dependent potentiation by glucose is not caused by an increase in $[Ca^{2+}]i$

Single β-cells were preincubated for 80 min at 37 °C in the nominal Ca^{2+}-free KRB buffer containing 2·8 mM (expt. 1) or 16·7 mM glucose (expt. 2), during which periods fura-2 loading was carried out simultaneously. In each experimental group, the cells were washed three times with the same solution without fura-2 acetoxymethylester, and superfusion with the same solution was started. $[Ca^{2+}]i$ measurements were carried out at 37 °C. When stable $[Ca^{2+}]i$ levels were achieved, the superfusion solutions were changed from nominally Ca^{2+}-free-2·8 mM glucose (expt. 1) or nominally Ca^{2+}-free-16·7 mM glucose (expt. 2) to that containing 2·5 mM Ca^{2+}, 2·8 mM glucose, 50 mM KCl and 250 μM diazoxide in both groups. The stimulation with 50 mM KCl increased $[Ca^{2+}]i$ in a biphasic manner: a rapid initial rise, a nadir, followed by a sustained plateau (Fig. 10). The peak level of the initial rise observed at 0·5 min after the stimulation with 50 mM KCl was 841 ± 64 nM (n=20) in expt. 1 and 814 ± 57 nM (n=20) in expt. 2 ($P>0·05$). The peak of the sustained $[Ca^{2+}]i$ rise at 20 min after the stimulation was 730 ± 43 nM (n=20) in expt. 1 and 687 ± 69 nM (n=20) in expt. 2 ($P>0·05$). In both the initial and sustained phases, the elevation of $[Ca^{2+}]i$ in β-cells preincubated with 16·7 mM glucose was slightly lower but not significantly different from that in β-cells preincubated with 2·8 mM glucose. In other words, the temporal profile and the magnitude of the $[Ca^{2+}]i$ increase were not affected by the pretreatment with glucose. In sharp contrast, glucose-induced TDP was apparently seen even when the single β-cells, not islets, were used for secretion experiments: pretreatment with 2·8 mM glucose, 0·39 ± 0·04% of insulin content n=8; pretreatment with 16·7 mM glucose, 1·04 ± 0·07% of insulin content n=10, $P<0·01$.

Discussion

We have re-examined the question as to whether TDP in the β-cell is a Ca^{2+}-dependent process. Contrary to currently held views, we found that glucose induced a strong TDP in the β-cell under stringent Ca^{2+}-free conditions, with significantly lowered $[Ca^{2+}]i$ and without any elevation of $[Ca^{2+}]i$. Activation of PKC or PKA was not required for the glucose effect. Instead, protein acylation appeared to be an obligatory step because glucose-induced TDP was attenuated by two different types of acylation inhibitors. In the physiological setting in

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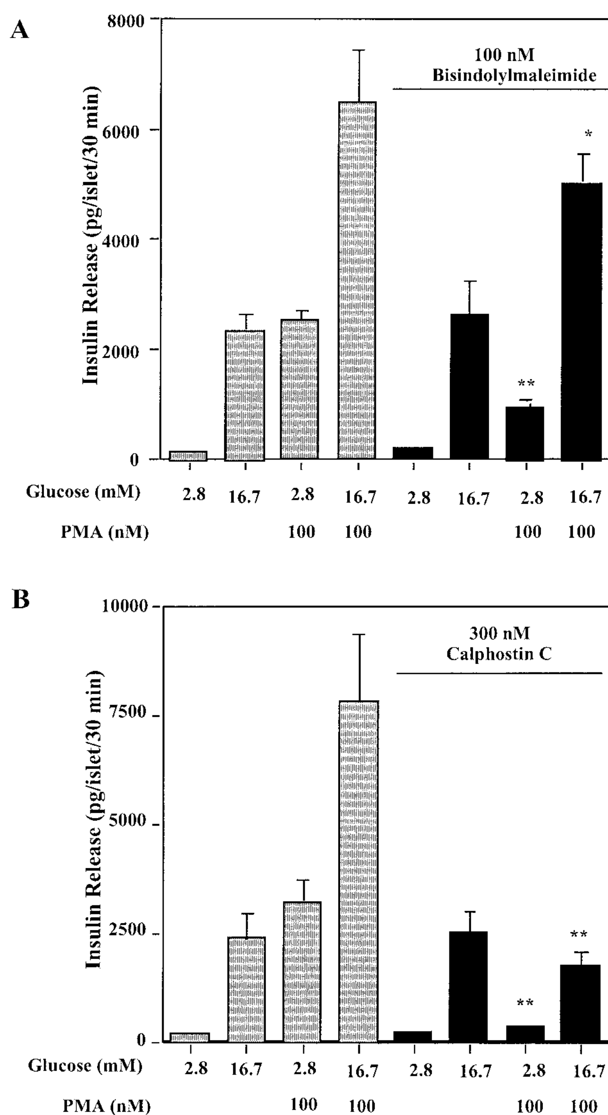

Figure 8 Effects of inhibitors of protein kinase C on glucose and PMA-induced TDP under stringent Ca^{2+}-free conditions. Effects of 100 nM bisindolylmaleimide (A) or 300 nM calphostin C (B). The islets were preincubated in Ca^{2+}-free KRB/EGTA buffer containing the indicated concentrations of glucose and/or 100 nM PMA in the presence or absence of the inhibitors. Then, insulin release was stimulated with 50 mM KCl in the regular KRB buffer (containing 2·8 mM glucose and 2·5 mM $CaCl_2$) with 250 μM diazoxide for 30 min. Values are means ± S.E. of 7 (A) and 8 (B) determinations. Statistical significance was evaluated by Mann–Whitney U test. *P<0·05 and **P<0·01 compared with corresponding values in the absence of inhibitors.

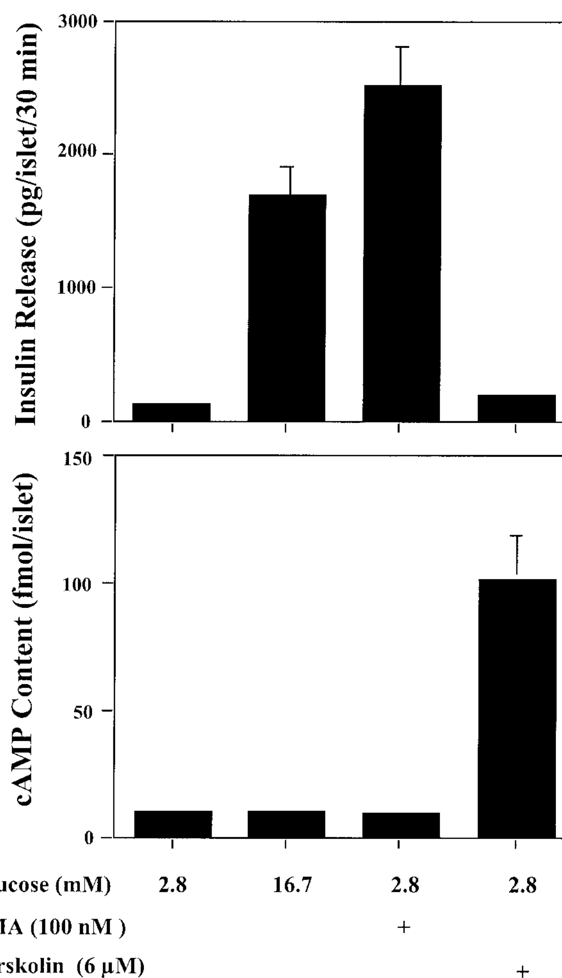

Figure 9 Comparison of TDP effect and cAMP content. The islets were preincubated with 16·7 mM glucose, 100 nM PMA or 6 μM forskolin as indicated at the bottom of the figure in Ca^{2+}-free KRB/EGTA buffer. Then, insulin release was stimulated with 50 mM KCl in the regular KRB buffer (containing 2·8 mM glucose and 2·5 mM $CaCl_2$) with 250 μM diazoxide for 30 min. The upper panel shows insulin release during the Test incubation with 50 mM KCl. The lower panel shows cAMP content of the islets at the end of the Test incubation. Values are means ± S.E. of 10 determinations.

humans, plasma glucose concentration gradually changes within the range of 4 to 8 mM, at which level TDP by glucose takes place. This means that β-cells are almost always 'primed' with glucose *in vivo*, so TDP must be a mechanism involved in the physiological regulation of insulin secretion. Of course, $[Ca^{2+}]$o is in the millimolar range in the body and $[Ca^{2+}]$i in the β-cell is estimated to be in the nanomolar to micromolar range (Martin *et al.* 1997), and glucose stimulation of the β-cell is occurring *in vivo* in this setting. Obviously the stringent Ca^{2+}-free conditions imposed here are not physiologically relevant but are used only to understand the mechanisms underlying TDP.

In view of the possible involvement of protein acylation in TDP, we emphasize the relevancy of the malonyl-CoA pathway (Corkey *et al.* 1989, Prentki *et al.* 1992, Chen *et al.* 1994, Brun *et al.* 1996). The anaplerotic metabolism of glucose is thought to raise the cytosolic malonyl-CoA

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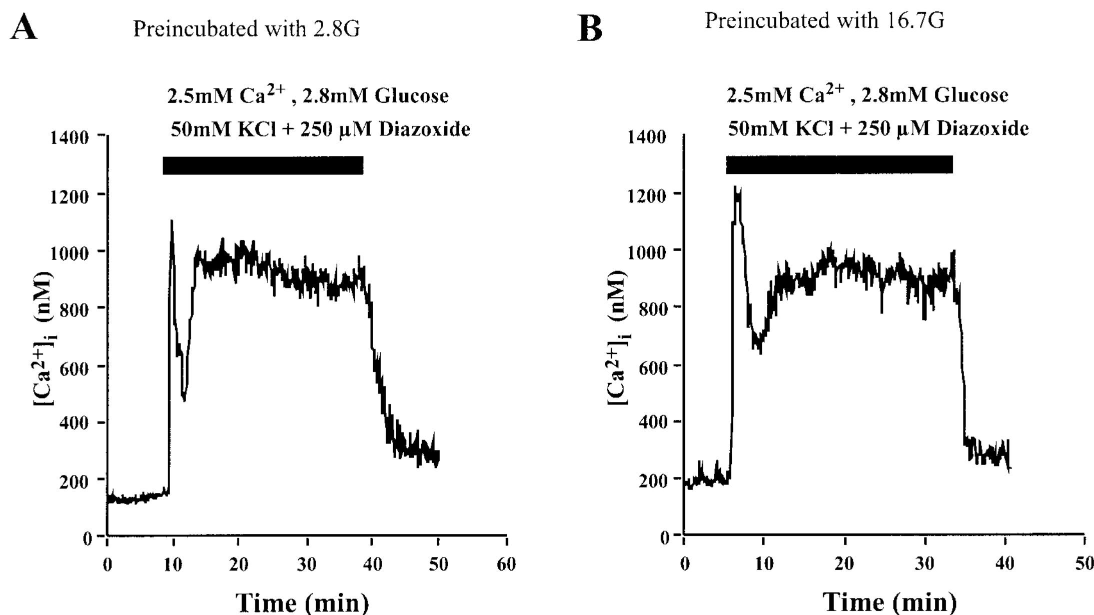

Figure 10 $[Ca^{2+}]$i responses to 50 mM KCl in the presence of 250 µM diazoxide, 2·5 mM Ca^{2+} and 2·8 mM glucose under superfusion conditions at 37 °C. Single β-cells were preincubated for 80 min at 37 °C in nominal Ca^{2+}-free conditions with (A) 2·8 mM glucose (G; expt. 1) or (B) 16·7 mM glucose (expt. 2). The results shown are representative of 20 cells each in A and B.

concentration (Corkey *et al.* 1989), which leads to suppression of carnitine palmitoyl-transferase 1 activity and, eventually, accumulation of long chain acyl CoA (LC-CoA) (Liang & Matschinsky 1991). Accumulation of cytosolic LC-CoA could facilitate acylation of molecule(s) involved in exocytosis. Thus, this hypothesis provides, at least in part, the mechanistic background for our findings. We have previously reported that FFA by itself strongly potentiates glucose-induced insulin release (Komatsu & Sharp 1998, Komatsu *et al.* 1999). Because palmitate-induced TDP was also abolished by cerulenin, it can be speculated that increased cytosolic LC-CoA converted from FFA might be a source of protein acylation.

Previously, it was considered that $[Ca^{2+}]$o in the micromolar range and thus normal, resting $[Ca^{2+}]$i is required for glucose-induced TDP (Ashby & Shirling 1981, Malaisse & Sener 1987, Zawalich *et al.* 1988, Chalmers & Sharp 1989, Taguchi *et al.* 1995). There are several differences in the experimental conditions between the current study and the previous one (Taguchi *et al.* 1995), which may be responsible for the discrepancy. These include the duration of TDP (60 min vs 45 min), the time elapsed before the test incubation (0 vs 20 min), the concentration of K^+ during the test incubation (50 mM vs 25 mM K^+), and the $[Ca^{2+}]$o during the test incubation (2·5 mM vs 1·9 mM). However, the exact reasons why glucose-induced TDP under the stringent Ca^{2+}-free conditions was not seen in the previous studies remain to be established.

In summary, we have revealed a novel aspect of stimulus-secretion coupling in pancreatic β-cells. Contrary to the current consensus, TDP by glucose in the β-cell is a Ca^{2+}-independent phenomenon. Protein acylation might be a required step for this glucose action in the β-cell.

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