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Necessity of Endogenous GTP Derived from Glucose-6-phosphate for Insulin Secretion Augmented by Glucose under Protein Kinase A Activation

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To investigate the possible involvement of some intracellular metabolic signaling other than the ATP derived from glucose metabolism under protein kinase A (PKA) activation, we measured the insulin secretory capacity stimulated by glucose and other fuel secretagogues using diazoxide-treated pancreatic islets. Under these conditions, we found a signal from a site proximal to glyceraldehyde-3-phosphate (GA-3-P) in the glycolysis to be necessary for glucose-induced insulin secretion. By using several different glycolytic enzyme inhibitors, we found that this proximal signal is derived from glucose-6-phosphate (G-6-P), and that metabolic signaling distal to GA-3-P also is necessary. Mycophenolic acid completely inhibited the augmented glucose-induced insulin secretion, which guanosine could reverse, indicating that the proximal signaling is coupling with endogenous GTP production. In this novel system of metabolic signaling, endogenous GTP derived from G-6-P in the glycolysis elicits the augmentation of glucose-induced insulin secretion under PKA activation in diazoxide-treated pancreatic islets.

Intracellular metabolic signals derived from glucose metabolism in addition to ATP are necessary to elicit the glucose-induced insulin secretion in β cells. The ATP generated through intracellular glucose metabolism closes the ATP-sensitive K^+ (K_{ATP}) channels, depolarizing the plasma membrane and elevating $[Ca^{2+}]_i$ through voltage-dependent Ca^{2+} channels and triggering insulin secretion. Another mechanism, which does not require K_{ATP} channel closure, has been identified recently (1–4) in which guanine and adenine nucleotides may participate in the regulation of glucose-induced insulin secretion (5), but it is similarly dependent on $[Ca^{2+}]_i$ elevation through the voltage-dependent Ca^{2+} channels. Another mechanism has been very recently reported, a glucose-stimulated exocytotic mechanism not necessarily dependent on $[Ca^{2+}]_i$ elevation, in a study using islets perifused by calcium-depleted medium under activated conditions of protein kinases (6).

It is well known that glucose-induced insulin secretion from pancreatic β cells is augmented by the action of protein kinase A (PKA). This augmentation has a significant physiological role on the entero-insular axis for glucose-induced insulin release mediated by gastric inhibitory polypeptide (GIP) and glucagon-like peptide (GLP-1) (7). The intracellular mechanism of the secretory augmentation by PKA activation, i.e., an accelerating effect on the exocytotic process after elevation of the intracellular calcium level ($[Ca^{2+}]_i$), has been clarified using permeabilized pancreatic islets (8–10). However, the involvement of other mechanisms in this phenomenon is unknown.

In the present study we used pancreatic islets treated with diazoxide, a known K_{ATP} channel opener, to investigate intracellular signalings besides the detectable $[Ca^{2+}]_i$ elevation for the augmentation of glucose-induced insulin secretion under PKA activation. We focus on the involvement of an endogenous substrate of metabolic signaling distinct from the ATP derived from glucose metabolism, and on the site in the glycolytic pathway in pancreatic β cells enabling the signal to generate this augmentation of glucose-induced insulin secretion.

MATERIALS AND METHODS

Materials. GIP (human fragment 1~42) was purchased from Peptide Institute (Osaka, Japan), and dibutyryl cyclic AMP (db-cAMP), mannoheptulose, mycophenolic acid (MPA), and diazoxide were purchased from Sigma Chemical Co. (St. Louis, MO). Other agents were obtained through Nacalai Tesque (Kyoto, Japan) unless stated otherwise.

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Isolation of rat pancreatic islets and assessment of insulin secretory capacity. Islets of Langerhans were isolated from male Wistar rats weighing 180∼200g using the collagenase digestion method, as previously described (11,12). The insulin secretion from the isolated rat pancreatic islets was measured by batch incubation. Groups of 8∼12 islets were preincubated for 30 min at 37 °C in Krebs-Ringer-bicarbonate buffer (KRBB) containing 129.4 mM NaCl, 5.2 mM KCl, 2.7 mM $CaCl_2$, 1.3 mM KH_2PO_4, 1.3 mM $MgSO_4$, 24.8 mM $NaHCO_3$, and 10 mM HEPES-NaOH (pH 7.4 at 37 °C) supplemented with 3.3 mM glucose and 0.2 % bovine serum albumin (BSA) and gassed with 95 % O_2 and 5 % CO_2. Islets were then incubated at 37 °C for 30 min in KRBB with the test materials described below. At the end of the incubation period, a 0.1 ml aliquot was withdrawn and diluted to 10 % for insulin measurement. The released insulin was measured by radioimmunoassay using the polyethylene glycol method with rat insulin (Novo, Bagsvaerd, Denmark) as standard (12). Diazoxide was used to prevent the K_{ATP} channels from closing in spite of the stimulation by glucose, in order to examine the pathway in which glucose controls insulin secretion from pancreatic β cells independent of K_{ATP} channel closure. In addition, we evaluated the insulin secretory capacity induced by glyceraldehyde (GA) or ketoisocaproate (KIC) in place of glucose, fuel substrates metabolized through glycolytic intermediates, under the same conditions described above. Mannoheptulose (MH) and 2-deoxyglucose (2-DG) were used to block the activity of glucokinase and glucose-phosphate isomerase, which inhibit the glycolytic pathway at the steps of conversion of glucose into glucose-6-phosphate (G-6-P) and of G-6-P into fructose-6-phosphate (F-6-P), respectively (1,13). In addition, we treated isolated rat islets with mycophenolic acid (MPA), an inhibitor of *de novo* GTP synthesis (14–16), or with MPA and guanosine simultaneously (17), to examine the involvement of endogenous GTP. For the treatment with MPA alone or MPA and guanosine, islets were cultured with these agents overnight (18 h) at 37 °C in RPMI 1640 medium containing 10 % fetal calf serum, 11.1 mM glucose, 100 units/ml penicillin, and 100 μg/ml of streptomycin in humidified air with 5 % CO_2. The cultured islets were then washed five times with ice-cold KRBB supplemented with 3.3 mM glucose and 0.2 % BSA. Groups of 8∼12 islets were preincubated for 30 min at 37 °C and then incubated for 30 min at 37 °C in KRBB with test materials for the insulin secretory study, as mentioned above.

Measurement of intracellular calcium concentrations in pancreatic β cells. Isolated pancreatic islets were dispersed according to the method previously described (18) and single islet cells subsequently adhered to the 16 × 16 mm cover slips using Cell Tak (Collaborative Research, Redford, MA). The islet cells were then loaded with 1.0 μM fura-2 acetoxymethylester (Molecular Probes, Eugene, OR) for 30 min in KRBB at 37 °C as reported previously (19), and then the cells were superfused at 37 °C with KRBB containing 3.3 mM glucose plus 100 μM diazoxide. They were successively challenged with either 2 mM db-cAMP or 25 mM potassium chloride for 5 min, and then the glucose concentration was raised to 16.7 mM in the presence of the agent for 12 min at a flow rate of 2 ml/min. The effects of 10 μM nitrendipine on intracellular calcium were also observed. Dual-excitation wavelength measurement (340 and 360 nm) was done and fluorescence emission at 510 nm was monitored with a silicon-intensified target camera (C2400-08H, Hamamatsu Photonics, Hamamatsu, Japan). The ratio calculation was digitized every 20 seconds by a computerized image processor (Argus-100/CA, Hamamatsu Photonics). All experiments were performed on individual cells of more than 10 μm in diameter which were not in contact with other cells, since it has been reported that non-insulin producing cells are characterized by smaller size (20). *In vitro* calibration was made in a range of pCa 9∼5 and the 340/360 fluorescence ratio was converted into calibrated values of intracellular calcium concentration ($[Ca^{2+}]_i$), as described previously (19,21).

Data presentation and statistical analysis. Insulin release is expressed as microunits/islet/30 min. All values are presented as mean ± S.E., with (n) representing the number of observations. Statistical significance was evaluated by unpaired Student's *t* test and *p* less than 0.05 was considered significant.

RESULTS

Insulin Secretion under PKA Activation from Diazoxide-Treated Pancreatic Islets Failed to be Augmented by Fuel Secretagogues other Than Glucose

Insulin response in the presence of 2 mM db-cAMP was significantly augmented by 16.7 mM glucose even under 100 μM diazoxide (9.89 ± 0.87 μU/islet/30min and 36.40 ± 1.97, in the absence and presence of db-cAMP, respectively, $p<0.001$ (n = 7). To find if we could get the same enhancement effect as in the case of glucose, we instead used the metabolic intermediates of glycolysis, glyceraldehyde (GA) or ketoisocaproate (KIC). However, we failed to get any secretory augmentation with 10 mM GA (7.30 ± 0.56 μU/islet/30min and 8.02 ± 0.34, in the absence and presence of 2 mM db-cAMP, respectively, not significant (n = 7)) or 10 mM KIC (6.32 ± 0.13 μU/islet/30min and 5.92 ± 0.46, in the absence and presence of 2 mM db-cAMP, respectively, not significant (n = 7)).

Requirement of Proximal Signaling to GA-3-P in the Glycolytic Pathway in Addition to Distal Signaling for Secretory Augmentation with Glucose under PKA Activation

Dose dependency of GA or KIC, which provides distal signaling to GA-3-P in insulin secretory mechanism, on insulin release induced by 2 mM db-cAMP was evaluated in diazoxide-treated islets. No augmentation of db-cAMP-induced insulin secretion under 100 μM diazoxide was, however, seen at any concentration of GA examined (6.58 ± 0.47 μU/islet/30min, 6.34 ± 0.23, 6.79 ± 0.56, 7.34 ± 0.42, 7.08 ± 0.71, and 6.31 ± 0.41 at 0 mM, 1 mM, 5 mM, 10 mM, 20 mM, and 100 mM GA, respectively (n = 7)) or of KIC examined (4.74 ± 0.87 μU/islet/30min, 4.31 ± 0.34, 4.85 ± 0.49, 4.78 ± 0.51, 4.91 ± 0.74, 4.81 ± 0.66 at 0 mM, 1 mM, 5 mM, 10 mM, 20 mM, 100 mM KIC, respectively (n = 7)).

Confirmation of the Evidence of the Proximal Signal from G-6-P for Secretory Augmentation

The effects of 2-DG, MH, and GA or KIC on insulin release were examined in the presence of 2 mM db-cAMP, as shown in Figs. 1A and 1B. Firstly, the insulin secretion augmented by 16.7 mM glucose under PKA activation was thoroughly inhibited to the basal level (in the presence of 3.3 mM) by 20 mM 2-DG (4.57 ± 0.21 μU/islet/30 min at 3.3 mM glucose, 41.65 ± 2.48 at 16.7 mM glucose: $p<0.001$ vs. 3.3 mM glucose alone, 5.42 ± 0.38 at 16.7 mM glucose + 20 mM 2-DG: $p<0.001$ vs. 16.7 mM glucose alone). Secondly, the addition of 10

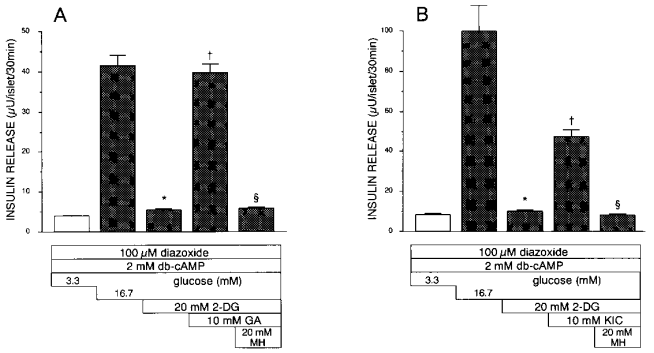

FIG. 1. Effects of 2-DG, MH and (A) GA or (B) KIC on db-cAMP-induced insulin secretion independent of K_{ATP} channel closure. The response was tested for 30 min. at 37°C in KRBB containing 3.3 mM glucose (□) or 16.7 mM glucose (▨). Two-DG significantly inhibited glucose-induced insulin secretion under PKA activation to the basal level in diazoxide-treated islets. Then, (A) GA or (B) KIC significantly enhanced insulin secretion. The addition of MH completely inhibited the secretion. Each bar represents mean ± S.E. Number of observations is 7. *$p<0.001$ vs. 16.7 mM glucose, †$p<0.001$ vs. 16.7 mM glucose + 20 mM 2-DG, § $p<0.001$ vs. 16.7 mM glucose + 20 mM 2-DG + 10 mM GA, respectively.

mM GA significantly augmented the secretion (39.74 ± 2.15: $p<0.001$ vs. 16.7 mM glucose + 2-DG). Thirdly, when we added 20 mM MH, the secretion was completely inhibited (5.83 ± 0.37: $p<0.001$ vs. 16.7 mM glucose + 2-DG + GA, not significant vs. 3.3 mM glucose alone). Similar results were obtained when 10 mM KIC was used instead of 10 mM GA (Fig. 1B). On the other hand, 20 mM MH alone had no effect on GA- or KIC-induced insulin secretion in any concentration examined in intact islets (25.25 ± 0.49 μU/islet/30min and 25.99 ± 0.64 at 10 mM GA, 28.54 ± 0.56 and 28.68 ± 0.66 at 50 mM GA, and 27.11 ± 0.40 and 27.31 ± 0.29 at 100 mM GA in the absence and presence of 20 mM MH, respectively, not significant (n = 7); and 25.97 ± 0.50 and 26.04 ± 0.81 at 10 mM KIC, 38.94 ± 1.76 and 39.17 ± 1.98 at 50 mM KIC, and 33.37 ± 1.48 and 33.59 ± 1.83 at 100 mM KIC in the absence and presence of 20 mM MH, respectively, not significant (n = 7)). Moreover, 10 nM GIP or 10 μM forskolin in place of 2 mM db-cAMP gave a similar result (in the case of GIP and forskolin; 4.40 ± 0.54 μU/islet/30 min and 6.01 ± 0.67 at 3.3 mM glucose, 29.50 ± 2.15 and 181.77 ± 8.60 at 16.7 mM glucose, 4.13 ± 0.26 and 35.32 ± 4.72 at 16.7 mM glucose + 20 mM 2-DG, 31.05 ± 2.19 and 157.29 ± 15.74 at 10 mM GA in addition to the former condition, and 5.99 ± 0.68 and 31.99 ± 2.29 at 20 mM MH in further addition to the former condition (n = 7)).

Participation of Endogenously Produced GTP in the Proximal Signal

As shown in Fig.2, the treatment with MPA (25 μg/ml) markedly inhibited the augmented insulin release induced by 16.7 mM glucose + 20 mM 2-DG + 10 mM GA under PKA activation in the presence of diazoxide (25.05 ± 2.25 μU/islet/30min and 4.66 ± 0.41, with and without MPA treatment, respectively, $p<0.001$). Furthermore, this inhibition was completely reversed by the simultaneous incubation with 500 μM guanosine (29.13 ± 2.56: $p<0.001$ vs. 16.7 mM glucose + 20 mM 2-DG + 10 mM GA with MPA treatment).

Effects of Nitrendipine on $[Ca^{2+}]_i$ Dynamics and Insulin Release under PKA Activation in the Presence of Diazoxide

Figs.3A and 3B show representative traces of the $[Ca^{2+}]_i$ dynamics in response to high potassium (25 mM) or 2 mM db-cAMP in the presence of 100 μM diazoxide. In contrast to the case of high potassium, 2 mM db-cAMP elicited no detectable $[Ca^{2+}]_i$ changes. As shown in Fig. 3A, 10 μM nitrendipine markedly reduced the elevated level of $[Ca^{2+}]_i$ induced by high po-

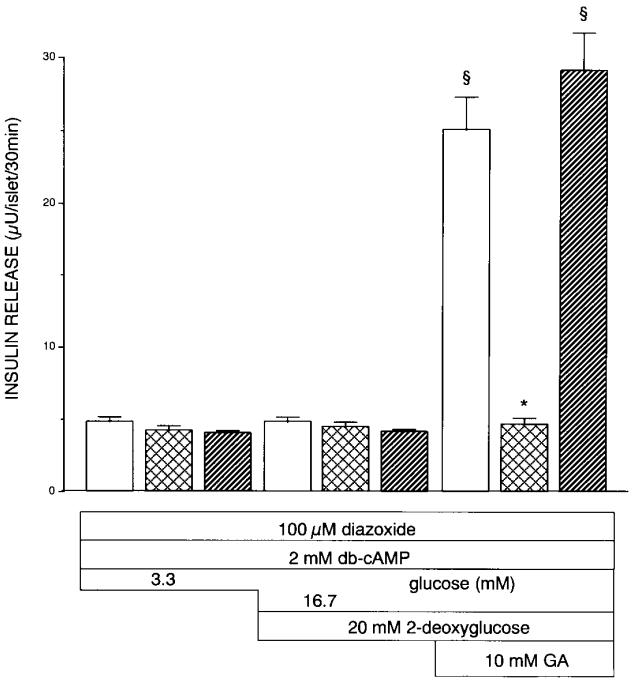

FIG. 2. Influences of MPA and guanosine on db-cAMP-induced insulin secretion independent of K_{ATP} channel closure. The response was tested overnight at 37 °C in RPMI 1640 medium containing 11.1 mM glucose in the absence (□) or in the presence of 25 μg/ml MPA alone (▨), or in the presence of 500 μM guanosine under 25 μg/ml MPA (▨). After the overnight culture (18 h), the islets were preincubated and then incubated as described in Materials and Methods. MPA completely inhibited the augmented glucose-induced insulin release under PKA activation in the presence of diazoxide. However, the addition of guanosine completely reversed the MPA-induced inhibition. Each bar represents mean ± S.E. Number of observations is 7. § $p<0.001$ vs. 16.7 mM glucose + 20 mM 2-DG without MPA. *no significance vs. 16.7 mM glucose + 20 mM 2-DG with MPA alone. † $p<0.001$ vs. 16.7 mM glucose + 20 mM 2-DG with guanosine under MPA. # no significance vs. 16.7 mM glucose + 20 mM 2-DG + 10 mM GA in the absence of MPA.

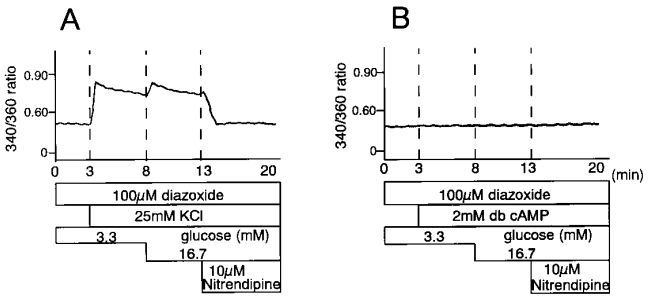

FIG. 3. Changes in $[Ca^{2+}]_i$ stimulated by high potassium chloride or db-cAMP and 10 μM nitrendipine in single Fura-2-loaded β cells under 100 μM diazoxide. Fig.3A shows that nitrendipine markedly reduced the augmented $[Ca^{2+}]_i$ induced by high potassium to basal concentration. Fig.3B shows that 2 mM db-cAMP elicited no detectable change in $[Ca^{2+}]_i$ and that nitrendipine had apparently no effects on the $[Ca^{2+}]_i$ dynamics with db-cAMP. The traces are representative of 50 observations.

tassium under 100 μM diazoxide to the basal concentration (235 $\pm$ 14 nM and 43 $\pm$ 4 in the absence and presence of nitrendipine, respectively: $p<0.001$, ($n = 50$)). On the other hand, no detectable effects of nitrendipine were found on the $[Ca^{2+}]_i$ with db-cAMP (45 $\pm$ 3 nM and 44 $\pm$ 3 in the absence and presence of nitrendipine, respectively: not significant ($n = 50$); Fig.3B).

The augmented insulin secretion induced by 16.7 mM glucose in the presence of high potassium under diazoxide was inhibited dramatically by 10 μM nitrendipine to the basal level (120.83 $\pm$ 7.89 μU/islet/30min and 10.97 $\pm$ 0.94, in the absence and presence of nitrendipine, respectively, $p<0.001$ ($n = 7$)). In addition, the 16.7 mM glucose-induced insulin release in the presence of 2 mM db-cAMP was also inhibited markedly to the basal level by 10 μM nitrendipine under diazoxide (101.76 $\pm$ 11.78 μU/islet/30min and 9.48 $\pm$ 0.64, in the absence and presence of nitrendipine, respectively, $p<0.001$ ($n = 7$)).

DISCUSSION

In this study, we have determined the presence of a metabolic signal for insulin secretion distinct from ATP derived from glycolysis and intramitochondrial oxidative phosphorylation, and have identified the site responsible in the glucose metabolic pathway in a pancreatic β cell which generates the signal affecting glucose-induced insulin secretion in diazoxide-treated islets under PKA activation.

The glucose augmentation of the insulin secretory mechanism under PKA activation in the presence of diazoxide was not found to occur without the signal derived from intermediate metabolic steps prior to GA-3-P in the glycolytic pathway. No augmentation of insulin secretion under PKA activation was observed when GA or KIC alone was used in diazoxide-treated islets instead of glucose. To ascertain which metabolite is responsible, we inhibited glucose-phosphate isomerase by 2-DG, as reported previously (13). The glucose-augmented insulin release was totally inhibited to the basal level by 20 mM 2-DG (Fig.1). Afterwards, the release was again significantly augmented by adding 10 mM GA or 10 mM KIC, showing that the proximal signal is derived from steps proximal to G-6-P, because 2-DG blocks the conversion of G-6-P to F-6-P, and also that the signaling distal to GA-3-P is additionally necessary for the secretory augmentation. Since the secretion augmented by the addition of GA or KIC was completely inhibited by the addition of 20 mM MH, a glucokinase inhibitor, the proximal signal must be derived from G-6-P, but not from glucose itself. The existence of this proximal signaling in diazoxide-treated islets can be again confirmed from the fact that 20 mM MH exhibited no effect on the distal signaling system in insulin secretion induced by GA or KIC alone in intact islets.

We then traced the metabolism from G-6-P as the site responsible for the proximal signaling. The pentose pathway has been known to produce endogenous GTP via phosphoribosylpyrophosphate (14). It has been recently reported that this endogenous GTP plays an important role in the mechanism of glucose-induced insulin secretion (5,14–16). Accordingly, we investigated to find if the metabolic pathway derived from G-6-P could be coupling with endogenous GTP production, by using MPA, a selective inhibitor of *de novo* GTP synthesis. As shown in Fig.2, the treatment with MPA completely inhibited the augmented glucose-induced insulin release under PKA activation, and this inhibition was found to be completely reversed by the simultaneous incubation with guanosine. Although it has been reported that MPA can additionally reduce ATP production (14,22), the reversibility by guanosine, a substrate for exogenous GTP, indicates that the defect of endogenous GTP production induced by MPA is probably, at least in part, responsible for the inhibition of the augmented insulin release.

Since we have a similar result when using GIP in place of db-cAMP, we suggest that this proximal signaling system might play a physiologically important role on the glucose-induced augmentation of insulin secretion in the entero-insular axis (7).

Furthermore, the glucose-augmented insulin secretion under PKA activation in diazoxide-treated islets was markedly inhibited by nitrendipine, a blocker of voltage-dependent calcium channels, whereas no detectable changes in the $[Ca^{2+}]_i$ in β cells were found by the agent. From this apparently discrepant effect of nitrendipine on the $[Ca^{2+}]_i$ dynamics and the insulin secretion, the mechanism of the augmentation of insulin release would seem to be coupling with $[Ca^{2+}]_i$ rises which may have been too small for detection by the conventional fura-2 method. Under PKA activation it

is possible that the exocytotic process after the Ca^{2+} influx becomes hyperreactive to $[Ca^{2+}]_i$ changes, since it has been reported that cAMP initiates exocytosis at a $[Ca^{2+}]_i$ concentration which, by itself, does not elicit secretion (23). In addition, it has been demonstrated that voltage-dependent calcium channels are directly sensitized to depolarization through glucose metabolism, and that cAMP increases the number of β cells responding to glucose with K_{ATP} channel closure (24). A localized $[Ca^{2+}]_i$ elevation beneath the plasma membrane is, therefore, possible following partial closure of the K_{ATP} channels. Subcellular imaging techniques will be necessary to resolve this, since it has been shown that the single action potential evoked by glucose causes $[Ca^{2+}]_i$ rises that are restricted to the cell boundary (25).

In conclusion, there is a novel system of metabolic signaling by endogenous GTP derived from G-6-P in a proximal site of the glycolytic pathway in pancreatic β cells which has a role in the augmentation of glucose-induced insulin secretion under PKA activation in diazoxide-treated islets. It seems probable that insulin exocytosis can occur through the coupling of endogenous GTP with minute $[Ca^{2+}]_i$ elevations under PKA activation. It should be helpful clinically to clarify through further examination details of the mechanisms of pathophysiological significance of these signals in the altered insulin secretory capacity found in the diabetic state.

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