

ORIGINAL

A new experimental model of ATP-sensitive K⁺ channel-independent insulinotropic action of glucose: a permissive role of cAMP for triggering of insulin release from rat pancreatic β-cells

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Abstract. In pancreatic β-cells, glucose metabolism leads to closure of ATP sensitive K⁺ channels (K_{ATP} channel) and Ca²⁺ influx, which is regarded as a required step for triggering of insulin release. Here, we demonstrate that glucose triggers rapid insulin release independent from its action on K_{ATP} channels given the cellular cAMP is elevated. We measured insulin release from rat pancreatic islets by static and perfusion experiments. Changes in cytosolic free Ca²⁺ concentration ([Ca²⁺]_i) were monitored using fura-2 loaded rat pancreatic β-cells. Glucose-induced insulin release was abolished when Ca²⁺ influx was inhibited by a combination of 250 μM diazoxide, an opener of K_{ATP} channel, and 10 μM nifedipine, a blocker of L-type voltage-dependent Ca²⁺ channels. However, with both nifedipine and diazoxide, glucose induced a 5-fold increase in insulin release in the presence of 10 μM forskolin, an activator of adenylyl cyclase. In the presence of diazoxide, nifedipine, and forskolin, 22 mM glucose sharply increased the rate of insulin release within 2 min which peaked at 6 min: this was followed by a further gradual increase in insulin release. In contrast, it lowered [Ca²⁺]_i with a nadir at 2-3 min followed by a gradual increase in [Ca²⁺]_i. The glucose effect was mimicked by 20 mM α-ketoisocaproic acid, a mitochondrial fuel, and it was nullified by 2 mM sodium azide, an inhibitor of mitochondrial electron transport. Cerulenin, an inhibitor of protein acylation, decreased the glucose effect. In conclusion, a rise in [Ca²⁺]_i through voltage-dependent Ca²⁺ channels is not mandatory for glucose-induced triggering of insulin release.

Key words: Glucose, Cyclic AMP, K_{ATP} channel, Insulin release

ELEVATION of cytosolic free calcium concentration ([Ca²⁺]_i) in endocrine and neuronal cells plays a pivotal role in triggering of hormones and neurotransmitters exocytosis [1]. In pancreatic β-cells, it is accepted that elevation of [Ca²⁺]_i is a required step for glucose triggering of insulin secretion [2, 3]. The process is envisaged as follows. Glucose enters the β-cell *via* glucose transporters, and it is phosphorylated by the glucokinase and subjected to subsequent metabolism. Resultant increases

in ATP and/or the ATP/ADP ratio closes ATP-sensitive K⁺ channels (K_{ATP} channels) causing membrane depolarization. Opening of voltage-dependent Ca²⁺ channels (VDCCs) and an elevation of [Ca²⁺]_i ensue, which rapidly enhances fusion of insulin granules and the plasma membrane [4] leading to acute rise in insulin exocytosis [5, 6]. At the same time, glucose produces signals that augment Ca²⁺-stimulated insulin release. This is referred to as the K_{ATP} channel-independent pathway because glucose robustly potentiates Ca²⁺-stimulated insulin release even when K_{ATP} channels are forced open by diazoxide [7, 8]. The K_{ATP} channel-independent glucose stimulation of insulin release can also be observed in the presence of sufficient concentrations of sulfonylurea with the channels being fully closed [9]. Later,

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we found that the K_{ATP} channel-independent glucose action can be experimentally demonstrated even under stringent Ca^{2+} -free conditions [10, 11]. Collectively, the K_{ATP} channel-dependent and -independent pathways have been called as the triggering and amplifying/augmentation pathways [5, 6], or fusion and replenishment pathways [4], respectively.

Incretin hormones bind to their specific receptors in the plasma membrane of pancreatic β -cells [12] and increase cAMP by activating adenylyl cyclase. Among them, glucagon-like peptide-1 (GLP-1), has attracted a great attention as a therapeutic tool in type 2 diabetes. Incretin-mimetics or dipeptidyl peptidase-4 inhibitors which raise the plasma active GLP-1 ameliorate hyperglycemia. With regard to the functional role of cAMP in pancreatic beta cells, we have previously demonstrated that cAMP selectively enhances K_{ATP} channel-independent glucose action using isolated rat pancreatic islets [13].

Here, we report that glucose triggers rapid insulin release from the rat pancreatic β -cells when cAMP is elevated even when the K_{ATP} channel-dependent pathway is inhibited by simultaneous addition of diazoxide and nifedipine. On the basis of this finding, we propose a novel view on the stimulus-secretion coupling of pancreatic β -cells. Namely, glucose can stimulate insulin release not only independent from K_{ATP} channel closure, but also independent from Ca^{2+} influx through L-type VDCCs when intracellular cAMP is apparently elevated.

Materials and Methods

This study was approved by and the experiments were conducted in accordance with the guidelines of the Animal Research Committees of the respective institutions.

Isolation of pancreatic islets

Male Wistar rats weighing 250 – 450 g were killed by CO_2 asphyxiation. Immediately after death, the pancreas was surgically removed, and the islets were isolated by collagenase dispersion [14]. Krebs–Ringer bicarbonate 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 $CaCl_2$, 10 mM HEPES at pH 7.4 (KRBH) containing 5.6 mM glucose and 0.1% BSA was used for isolation and pooling of the islets.

Measurement of insulin release

We measured insulin release in both static incubation

and perfusion experiments, as described previously [7]. In static incubation, five size-matched batches of islets were incubated in 1 mL KRBH buffer containing 2.5 mM Ca^{2+} , 2.8 mM glucose and 0.2% BSA for 30 min at 37°C. This preincubation medium was aspirated off and fresh buffer with test material was added. To assess the effect of removal of extracellular Ca^{2+} , Ca^{2+} -free KRBH containing 1 mM EGTA was used, as described previously [10]. In all experiments except under the control conditions, insulin release was measured in the presence of 250 μ M diazoxide to open K_{ATP} channels and 10 μ M nifedipine to close L-type VDCCs. By doing so, membrane depolarization by the K_{ATP} channel closure and Ca^{2+} influx through the VDCC were inhibited and increase in $[Ca^{2+}]_i$ prevented. Forskolin (10 μ M), an activator of adenylyl cyclase, was used to increase intracellular concentrations of cAMP [13]. In perfusion experiments, 50-size matched islets were prepared and perfused with KRBH buffer containing 2.8 mM glucose and 0.2% BSA at 37°C. After the experiments, medium was kept at $-20^\circ C$ until measurement of insulin concentrations. Insulin was measured by radioimmunoassay. Rat insulin was used as a standard [15]. Forskolin, diazoxide, and nifedipine were included throughout the experiments, i.e., during the preincubation and baseline perfusion periods as well.

Measurement of $[Ca^{2+}]_i$ in single beta-cells

$[Ca^{2+}]_i$ was measured by dual-wavelength fura-2 microfluorometry combined with digital imaging, as reported previously [16]. Briefly, rat islets were dispersed by collagenase and the isolated cells on coverslips were loaded with fura-2 by incubation with 1 μ M fura-2 acetoxymethylester in KRBH buffer containing 2.8 mM glucose for 60 min at 37°C. The cells were then mounted in a chamber and superfused with KRBH 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. Data were taken only from the β -cells that were confirmed either immuno-cytochemically using anti-insulin antiserum or by morphological and physiological criteria as reported previously [17]. The emission signals at 510 nm were detected with an intensified charge-coupled device camera, and ratio images were produced with an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan).

Materials

Forskolin, diazoxide, nifedipine, fura-2 acetoxymethylester, α -ketoisocaproic acid (α -KIC), sodium

azide (NaN₃), cerulenin and collagenase were obtained from Sigma (St. Louis, MO).

Statistical analysis

Statistical analysis was performed by one-way ANOVA with pairwise comparison by Fisher's protected least significance difference test, unless otherwise indicated, and comparison of two mean values was performed by Mann-Whitney *U* test as required (StatView; SAS Institute, Inc., Cary, NC). *P* < 0.05 was considered significant. Data are expressed as the means ± SE.

Results

Effects of forskolin, diazoxide and nifedipine on glucose-induced insulin release in the presence of 2.5 mM Ca²⁺

In static incubation experiments (Fig. 1A), high concentration of glucose (22 mM) stimulated insulin release by 10-fold during 30 min. In the presence of forskolin (10 μM) that consistently increases intracellular cAMP concentration (13), glucose-induced insulin release was approximately doubled. Fig. 1B shows insulin release in the presence of diazoxide and nifedipine. Glucose-stimulated insulin release was completely abolished by the presence of 250 μM diazoxide and 10 μM nifedipine as expected. Nevertheless, 22 mM glucose induced a 5-fold increase in insulin release even in the presence of diazoxide and nifedipine if forskolin was present. All these data were obtained in the experiments with regular extracellular Ca²⁺ concentration (2.5 mM).

Effect of lowering of extracellular Ca²⁺ on glucose stimulation of insulin release in the presence of forskolin, diazoxide, and nifedipine

Glucose-stimulated insulin release in the presence of forskolin, diazoxide, and nifedipine was progressively attenuated by lowering of extracellular Ca²⁺ (from 2.5 mM to zero) (Fig. 2). Stringent removal of extracellular Ca²⁺ with 1 mM EGTA nullified the insulinotropic effect of glucose. It should be noted that lowering extracellular Ca²⁺ concentration to 1.25 mM, which is equivalent to the physiological extracellular free Ca²⁺ concentration, produced only marginal reduction of the insulin release. This was in contrast to profound suppression of glucose-stimulated insulin release by reducing Ca²⁺ concentration from 2.5 to 1.25 mM under the regular condition, i.e., in the absence of forskolin, diazoxide and nifedipine (Komatsu *et al.* unpublished data).

Time course of glucose-induced insulin release and changes in [Ca²⁺]_i

Time course of insulin release stimulated by glucose in the presence of forskolin, diazoxide and nifedipine was studied in perfusion experiments (Fig. 3). Upon stimulation with 22 mM glucose, the rate of insulin release sharply increased within 2 min and peaked at 6 min. After that a transient marginal diminution of insulin release was followed by a gradual increase of it. Under regular conditions, high glucose increases insulin release within 2 min and the initial peak is observed at 4 min in our hands [15]. The time course of [Ca²⁺]_i

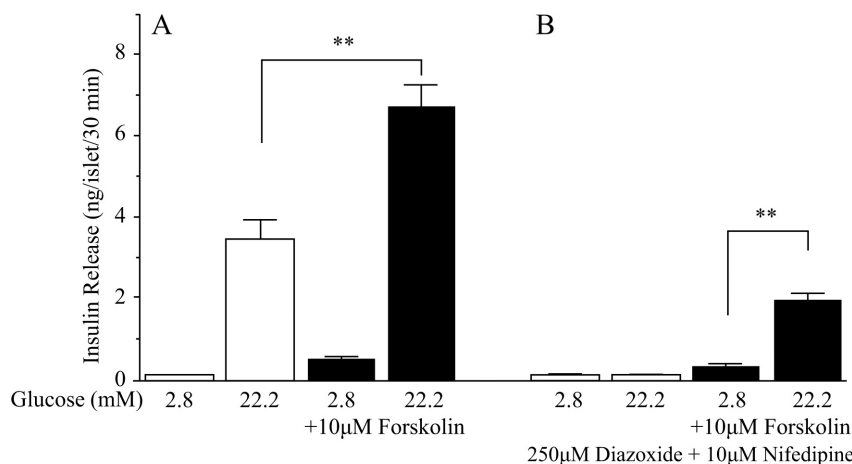


Fig. 1 Effects of forskolin, diazoxide and nifedipine on glucose-induced insulin release in the presence of 2.5 mM Ca²⁺ (A) Glucose-induced insulin release in rat pancreatic islets with (black) or without (white) forskolin in the absence of diazoxide and nifedipine (B) Glucose-induced insulin release in rat pancreatic islets with (black) or without (white) forskolin in the presence of diazoxide and nifedipine. Islets were pre-incubated with 2.8 mM glucose for 30 min. In the black columns, 10 μM forskolin was present. Insulin release during a 30 min-static incubation is shown. Values are mean ± se. n=10. **, *p*<0.01.

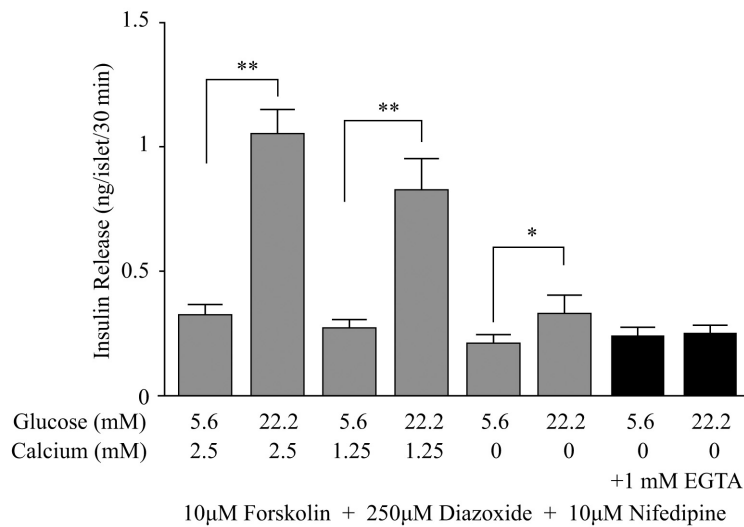


Fig. 2 Effect of lowering of extracellular Ca^{2+} on glucose stimulation of insulin release in the presence of forskolin, diazoxide and nifedipine. Basal (5.6 mM) and high (22.2 mM) glucose-induced insulin release were assessed in the presence of 10 μM forskolin, 250 μM diazoxide and 10 μM nifedipine, at the indicated concentration of extracellular Ca^{2+} . In the black columns, 1 mM EGTA was added to completely remove extracellular Ca^{2+} . Insulin release during a 30 min-static incubation is shown. Values are mean \pm se. $n=8$. **, $p<0.01$; *, $p<0.05$.

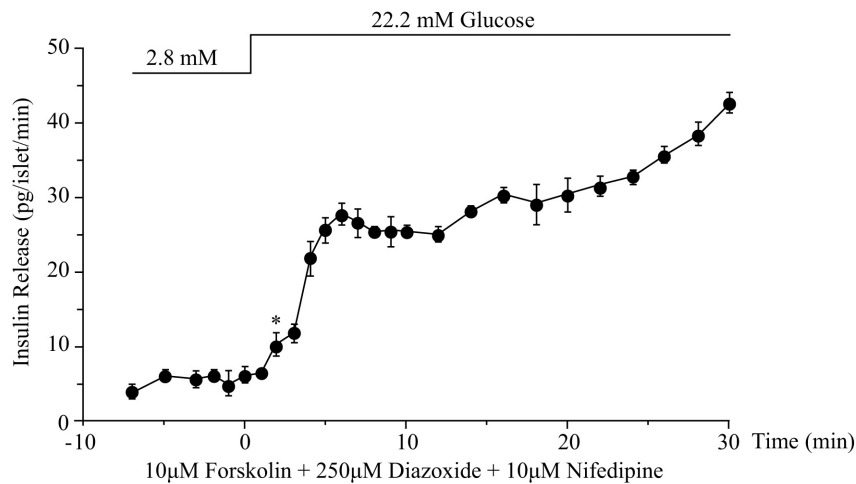


Fig. 3 Time course of glucose-induced insulin release in the presence of forskolin, diazoxide and nifedipine. High (22.2 mM) glucose-induced insulin release were measured by perfusion experiments in the presence of 10 μM forskolin, 250 μM diazoxide and 10 μM nifedipine. Values are mean \pm se. $n=10$. *, the first time point when the rate of insulin release became significantly ($p<0.05$) higher than the basal value.

change in isolated single pancreatic β cells under the same condition was shown in Fig. 4. Upon stimulation with 22.2 mM glucose, $[\text{Ca}^{2+}]_i$ decreased sharply within 3 min and remained low for the next 2 to 3 min; it gradually increased thereafter. The $[\text{Ca}^{2+}]_i$ level remained higher than the basal level after 10 min of stimulation. Nevertheless, typical oscillation of $[\text{Ca}^{2+}]_i$ seen after glucose stimulation under regular condition [17] was absent in all of the isolated β -cells. Thus,

under the condition with forskolin, diazoxide, and nifedipine, high concentration of glucose could induce 1st phase-like insulin release without rapid rise in $[\text{Ca}^{2+}]_i$ that is normally evoked by glucose stimulation under the control condition. By contrast, a large 2nd phase-like increase in insulin release may be produced by apparent and gradual elevation of $[\text{Ca}^{2+}]_i$, stimulatory signals generated by glucose metabolism, and permissive action of increased cAMP.

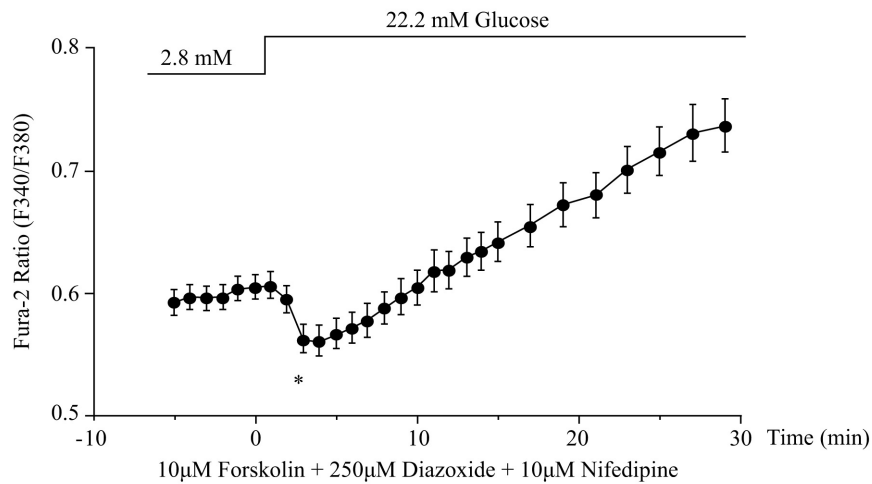


Fig. 4 Time course of glucose-induced changes in $[Ca^{2+}]_i$ in the presence of forskolin, diazoxide and nifedipine. Isolated rat pancreatic beta cells were used. Intracellular Ca^{2+} were measured by dual-wave length fura-2 microfluorometry combined with digital imaging. Values are mean \pm se. $n=10$. *, $p<0.01$ vs the values at 0 min.

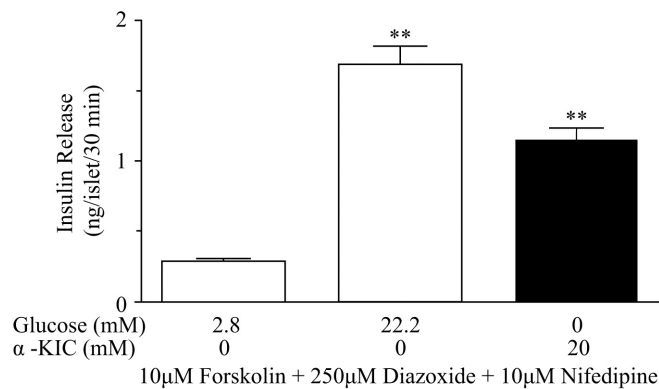


Fig. 5 Effect of α -KIC on insulin release in the presence of forskolin, diazoxide and nifedipine. Basal (2.8 mM) and high (22.2 mM) glucose-induced and α -ketoisocaproic acid (KIC, 20 mM)-induced insulin release in rat pancreatic islets in the presence of 10 μ M forskolin, 250 μ M diazoxide and 10 μ M nifedipine are shown. Islets were pre-incubated with 2.8 mM glucose for 30 min. In the black column, α -KIC was added instead of glucose. Insulin release during a 30 min-static incubation is shown. Values are mean \pm se. $n=8$. **, $p<0.01$ vs 2.8 mM glucose.

Effect of mitochondrial fuel, metabolic inhibition and inhibition of protein acylation on glucose-induced insulin release in the presence of forskolin, diazoxide and nifedipine

α -KIC, a mitochondrial fuel, robustly stimulated insulin release as a high concentration of glucose in the presence of diazoxide and nifedipine if forskolin was included in the incubation medium (Fig. 5). At a concentration of 20 mM, the amino-acid derivative induced a 4-fold increase in insulin release, which is approximately 70% of 22 mM glucose-induced insulin release. Effects of metabolic inhibition on insulin release under the same conditions were also examined (Fig. 6). NaN_3 (2 mM) which selectively and reversibly inhibits mito-

chondrial electron transport [18] strongly inhibited glucose-stimulated insulin release in the presence of forskolin, diazoxide and nifedipine. On the other hand, NaN_3 did not affect basal insulin release in the presence of 2.8 mM glucose.

Cerulein, an inhibitor of protein acylation [19-22], suppressed glucose-induced insulin release in the presence of forskolin, diazoxide and nifedipine in a concentration-dependent manner (Fig. 7). Because cerulein inhibits protein acylation in pancreatic β -cells [20, 23] and does not perturb glucose metabolism in rat pancreatic islets [19], the insulinotropic action of glucose shown in this study may be mediated by protein acylation.

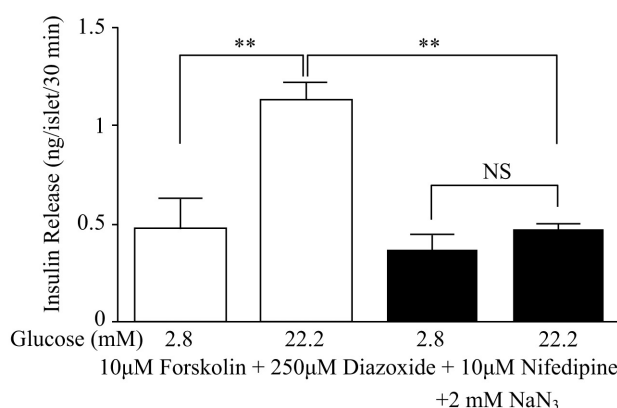


Fig. 6 Effect of NaN₃ on glucose stimulation of insulin release in the presence of forskolin, diazoxide, and nifedipine. Basal (2.8 mM) and high (22.2 mM) glucose-induced insulin release in rat pancreatic islets with or without sodium azide (NaN₃, 2 mM) in the presence of 10 μM forskolin, 250 μM diazoxide, and 10 μM nifedipine are shown. Islets were pre-incubated with 2.8 mM glucose for 30 min. In the black columns, NaN₃ was added. Insulin release during a 30 min-static incubation is shown. Values are mean ± se. n=8. **, *p*<0.01.

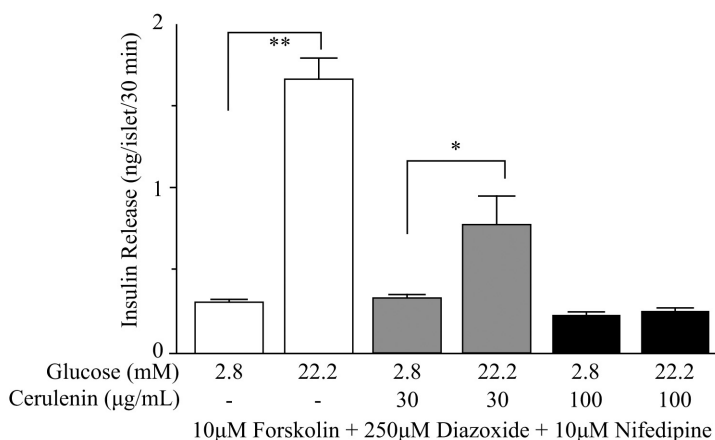


Fig. 7 Effect of cerulenin on glucose stimulation of insulin release in the presence of forskolin, diazoxide and nifedipine. Basal (2.8 mM) and high (22.2 mM) glucose-induced insulin release in rat pancreatic islets with 0, 30, 100 μg/mL of cerulenin in the presence of 10 μM forskolin, 250 μM diazoxide and 10 μM nifedipine are shown. In the gray and the black columns, 30 μg/mL and 100 μg/mL of cerulenin were added, respectively. Insulin release during a 30 min static incubation is shown. Values are mean ± se. n=10. **, *p*<0.01; *, *p*<0.05.

Discussion

In this study, we demonstrated that high concentration of glucose stimulates insulin release under the conditions where Ca²⁺ influx is inhibited by diazoxide and nifedipine, an opener of K_{ATP} channels and a blocker of L-type VDCCs, respectively, provided cellular cAMP is increased by forskolin. Strikingly, immediately after stimulation with high glucose under the same condition, glucose raises the rate of insulin release but unequivocally decreases [Ca²⁺]_i. The lowering of [Ca²⁺]_i is most likely due to sequestration of Ca²⁺ into the intracellular store sites occurring during the initial phase of glucose stimulation [24, 25]. It should be noted

that Ca²⁺ influx *via* voltage-independent Ca²⁺ channels must be taking place because of the presence of diazoxide, which hyperpolarized the β-cells, namely this Ca²⁺ influx is initially masked by profound Ca²⁺ sequestration into the intracellular stores [26]. Thus it is possible that the voltage-independent Ca²⁺ influx triggers insulin release. Therefore, increase in Ca²⁺ influx *via* L-type VDCCs is not a mandatory step for glucose-induced insulin release provided cellular cAMP is increased. However, glucose-induced insulin release under such condition was mitigated by stringent removal of extracellular Ca²⁺ with EGTA, which may bring about excessively low, unphysiological Ca²⁺ concentration immediately beneath the plasma mem-

brane. In our experimental condition, Ca²⁺ influx *via* voltage-independent Ca²⁺ channels might have a triggering role for insulin exocytosis [26]. Glucose also provides an insulintropic signal(s) leading to insulin exocytosis in addition to Ca²⁺ influx. Thus, we conclude that insulin exocytosis *per se* is Ca²⁺-dependent but glucose enhancement of it is not necessarily dependent on Ca²⁺ influx *via* the K_{ATP} channels/L-type VDCCs pathway. This novel idea does not contradict with, nor deny, the finding that elevation of [Ca²⁺]_i *via* K_{ATP} channel-dependent pathway rapidly increases the rate of insulin release [2, 3].

Under physiological conditions, pancreatic β-cells are under multifactorial control [27]. The β-cells are stimulated by not only glucose, but also amino acids, fatty acids, and neural and hormonal inputs including incretins which increase cellular cAMP [12]. Therefore, it is most likely that elevation of extracellular glucose triggers insulin release through multiple interrelated mechanisms, some of which may well be independent of Ca²⁺ influx as shown here. Thus, the simplistic view that elevation of [Ca²⁺]_i *via* K_{ATP} channel-dependent pathway is always required for rapid rise of insulin secretion upon glucose stimulation needs a critical reconsideration.

We use 10 μM forskolin to increase cellular cAMP, because the purpose of this study is to provide a new experimental model of K_{ATP} channel-independent insulintropic action of glucose, and this concentration of forskolin is strong and constant enough to exhibit pharmacological effect of cAMP [13]. In future, significance of this model under physiological conditions must be examined by using various concentrations of glucose and incretin hormones such as glucagon like peptide-1.

Regarding the conveyer of the novel glucose action described here, metabolic signal(s) linked to protein acylation is likely [22, 23]. This is because mitochondrial metabolism was necessary for the glucose action and a mitochondrial fuel mimicked it. Furthermore, an inhibitor of protein acylation suppressed the glucose action in a concentration-dependent manner. Thus, the glucose action demonstrated in this study shares the similar mechanisms as K_{ATP} channel-independent glucose action that we demonstrated previously [28, 29]. Alternatively, Epac2 [30] might be a mediator of the novel glucose action that is critically dependent on cAMP.

A robust insulin secretion in response to the intravenous infusion of glucose has been reported in a patient with a loss of function sulfonylurea receptor mutations

[31]. Furthermore, it has been reported that rapid insulin secretion in response to glucose stimulation occurs from the islets of mice lacking K_{ATP} channels [32]. Ravier *et al.* speculated that glucose caused membrane depolarization *via* a route other than the K_{ATP} channel closure, such as closure of barium-sensitive K⁺ channels. However, a sufficient concentration of nifedipine was present during experiments in our study so that opening of VDCC and subsequent Ca²⁺ influx and [Ca²⁺]_i elevation must not have occurred. Indeed, glucose did not increase [Ca²⁺]_i during acute triggering of insulin release in our study. Accordingly, we consider that the novel action of glucose described here was occasionally and fragmentally demonstrated previously, but the idea proposed here was not clearly formulated.

In conclusion, we established that high glucose elicits quasi-biphasic insulin release with Ca²⁺ influx through VDCCs being fully blocked by the presence of diazoxide (K_{ATP} channel opener) and nifedipine (calcium channel blocker), if cellular cAMP was elevated by forskolin. Interestingly, a sharp rise in the rate of insulin release peaking at 6 min after the glucose stimulation was temporally associated with a significant dropping of [Ca²⁺]_i. It should be noted, however, that a tiny Ca²⁺ influx which is masked by apparent Ca²⁺ uptake into endoplasmic reticulum, could contribute to 1st phase-like insulin release. Indeed, the role of voltage-independent Ca²⁺ influx through TRPM2 channel is reported in insulin secretion [26]. The fact that oscillation of [Ca²⁺]_i was absent throughout suggests that depolarization-induced Ca²⁺ influx are required for generation of glucose-induced [Ca²⁺]_i oscillations. Existence of novel non-ionic molecular mechanism(s) for glucose stimulation, *i.e.*, both triggering and augmentation/amplification, of insulin release was strongly suggested. To fully understand the physiological regulation of insulin secretion from pancreatic β-cells, further studies are required.

Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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