

Hyperresponse in calcium-induced insulin release from electrically permeabilized pancreatic islets of diabetic GK rats and its defective augmentation by glucose

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Summary In spontaneously diabetic GK rats, insulin secretion from pancreatic beta cells in response to glucose is selectively impaired, probably due to deficient intracellular metabolism of glucose and impaired closure of K_{ATP} channels during glucose stimulation. By using electrically permeabilized islets of GK rats, we explored the functional modulations in exocytotic steps distal to the rise in $[Ca^{2+}]_i$ in the diabetic condition. At 30 nmol/l Ca^{2+} (basal conditions) insulin release was similar between GK and non-diabetic control Wistar rats. In response to 3.0 μ mol/l Ca^{2+} (maximum stimulatory conditions), insulin release was significantly augmented in permeabilized GK islets ($p < 0.01$). Raising glucose concentrations from 2.8 to 16.7 mmol/l further augmented insulin release induced by 3.0 μ mol/l Ca^{2+} from permeabilized control islets ($p < 0.001$), but had no effect on that

from permeabilized GK islets. The stimulatory effect of glucose on insulin release from permeabilized control islets was partly inhibited by 2,4-dinitrophenol, an inhibitor of mitochondrial oxidative phosphorylation ($p < 0.01$). The hyperresponse to Ca^{2+} in GK islets may play a physiologically compensatory role on the putative functional impairment both in $[Ca^{2+}]_i$ rise and energy state in response to glucose in diabetic β cells, and may explain the relative preservation of insulin release induced by non-glucose depolarizing stimuli, such as arginine, from pancreatic islets in non-insulin-dependent diabetes mellitus. [Diabetologia (1995) 38: 772–778]

Key words Insulin release, intracellular calcium, exocytosis, GK rat, permeabilized islets.

The impairment of glucose-induced insulin secretion is known to be one of the major characteristics of non-insulin-dependent diabetes mellitus (NIDDM). Insulin release has been found to be selectively reduced in response to glucose, but not to other secreta-

gogues such as arginine, both in humans with NIDDM [1–3] and in animal models such as neonatally streptozotocin-induced diabetic rats (NSZ rats) [4,5].

Recently, the GK rat (Goto-Kakizaki rat) has been reported to be a novel spontaneous non-obese model of NIDDM [6–9]. The rats were developed by repetitive selective inbreeding of Wistar rats with abnormal glucose tolerance. After tens of generations, the diabetic state became stable. Thus, the diabetic aetiology of GK rats is thought to be exclusively genetic, and they show glucose intolerance at 2 weeks of age.

It is well established that a rise in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) is essential for insulin secretion from pancreatic beta-cells [10–12]. During glucose stimulation, metabolic fluxes such as production of ATP close ATP-sensitive K^+ channels (K_{ATP}

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Abbreviations: GK rat, Goto-Kakizaki rat; NIDDM, non-insulin-dependent diabetes mellitus; NSZ rats, neonatally streptozotocin-induced diabetic rats; KRBB, Krebs-Ringer bicarbonate buffer; BSA, bovine serum albumin; KA, potassium aspartate buffer; DNP, 2,4 dinitrophenol; CaM kinase, Ca^{2+} -calmodulin-dependent protein kinase.

channels). The resultant membrane depolarization brings about Ca^{2+} influx through voltage-dependent Ca^{2+} channels, leading to the rise in $[\text{Ca}^{2+}]_i$, which eventually activates exocytosis of insulin granules. We have recently reported that the inhibition of K_{ATP} channel activity in response to glucose is significantly reduced in diabetic beta-cells of NSZ [13] and GK rats [14], probably due to insufficient ATP production by a deficiency of intracellular glucose metabolism. These findings might suggest that the mechanism of impaired insulin secretion induced by glucose in NIDDM is based on the defective step(s) in insulin secretory machinery proximal to the $[\text{Ca}^{2+}]_i$ elevation. Accordingly, we have demonstrated that the rise in $[\text{Ca}^{2+}]_i$ in response to glucose is selectively impaired in beta-cells of diabetic NSZ rats [15], and also reported that pancreatic beta-cells maintained under prolonged hyperglycaemic conditions show deficient glucose-induced $[\text{Ca}^{2+}]_i$ rise together with the decreased insulin secretory capacity to glucose [16].

On the other hand, the exocytotic process distal to the rise in $[\text{Ca}^{2+}]_i$ may also be affected in diabetes, and can be explored independently of ionic events by permeabilizing the endocrine cells. The elevation of Ca^{2+} has been reported to be sufficient to activate exocytosis in the permeabilized insulin-secreting cells [17–20] as well as in other endocrine cells [21–24]. However, functional alterations in the insulin secretory machinery distal to $[\text{Ca}^{2+}]_i$ elevation in diabetic beta-cells are still to be elucidated. In this study, we have examined the modulation of $[\text{Ca}^{2+}]_i$ -activated exocytotic pathway in the diabetic state using electrically permeabilized pancreatic islets of GK rats.

Materials and methods

Animals. Male diabetic GK rats were donated by Pharmaceutical Research Laboratories-II (Takeda Chemical Industries Ltd., Osaka, Japan). Male non-diabetic age-matched Wistar rats were used as control animals. The animals were fed *ad libitum* with a standard pelleted chow and allowed free access to water in an air-conditioned room with a 12-h light/dark cycle until killing. All experiments were carried out with GK and control rats at 7- to 10-weeks of age.

Measurement of serum glucose and insulin. Blood was drawn from the left renal vein immediately before isolation of the pancreatic islets of Langerhans under pentobarbital anaesthesia (40 mg/kg body weight). Sera obtained by centrifugation were used for the measurements of glucose and immunoreactive insulin. Serum glucose was measured by the glucose-oxidase method [25]. Immunoreactive insulin was determined by radioimmunoassay using the polyethylene glycol method with rat insulin (Novo Nordisk, Copenhagen, Denmark) as the standard [4].

Measurement of insulin release from intact islets. Islets of Langerhans were isolated from GK and control rats by collagenase digestion as described previously [26]. The islets then

were preincubated at 37°C for 30 min in Krebs-Ringer bicarbonate buffer (KRBB)[16] supplemented with 2.8 mmol/l glucose and 0.2% bovine serum albumin (BSA). Groups of five islets were then batch-incubated for 60 min at 37°C in 0.4 ml KRBB with test materials. At the end of the incubation period, islets were pelleted by centrifugation ($10,000 \times g$, 60 s) and aliquots of the buffer were removed and diluted to one-twentieth for insulin assay.

Measurement of insulin release from permeabilized islets. After the preincubation as described above, the islets were washed twice in cold potassium aspartate buffer (KA buffer) containing 140 mmol/l potassium aspartate, 7 mmol/l MgSO_4 , 5 mmol/l ATP, 2.8 mmol/l glucose, 2.5 mmol/l EGTA, 30 mmol/l HEPES, 0.5% BSA (pH 7.0), with CaCl_2 added to give a Ca^{2+} concentration of 30 nmol/l. The islets were then permeabilized by high voltage discharge (four exposures each of 450- μs duration to an electrical field of 4.0 kV/cm) in KA buffer and washed once with the same buffer. Groups of five electrically permeabilized islets were then batch-incubated for 60 min at 37°C in 0.4 ml KA buffer with various concentrations of Ca^{2+} (from 30 nmol/l to 10.0 $\mu\text{mol/l}$) and test materials. Aliquots of the buffer were assayed for insulin using the same protocol described above. Ca^{2+} concentrations in the KA buffer were calculated by using the dissociation constants of Martell and Smith [27]. In some series of experiments, insulin was extracted from groups of five islets with 0.4 ml 1 mol/l acetic acid both before and after the permeabilization procedure and the content was determined by radioimmunoassay [4].

Materials. Potassium aspartate was obtained from Sigma Chemicals Co. (St. Louis, Mo., USA); ATP was obtained from Kohjin Co., Ltd. (Tokyo, Japan); and all other reagents were of analytical grade and were obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Statistical analysis. All results are presented as mean \pm SEM for the indicated number of observations. Data were analysed by Student's *t*-test for unpaired data. Differences were considered significant at $p < 0.05$.

Results

Characteristics of GK and control rats. The *in vivo* characteristics of diabetic GK and non-diabetic Wistar rats are summarized in Table 1. The body weight and the serum insulin level were not significantly different between the two groups. The serum glucose concentration in the GK rats was significantly higher ($p < 0.001$) than that of the controls. In each experiment, GK rats stably exhibited higher glucose levels than controls.

Insulin release from intact islets. Figure 1 shows the insulin secretory capacity in response to glucose and arginine. Insulin release in response to 2.8 or 8.3 mmol/l glucose was not significantly different between GK and control rats. Basal insulin secretion at 2.8 mmol/l glucose was $0.62 \pm 0.03 \text{ ng} \cdot \text{islet}^{-1} \cdot 60 \text{ min}^{-1}$ ($n = 25$) in control and $0.53 \pm 0.04 \text{ ng} \cdot \text{islet}^{-1} \cdot 60 \text{ min}^{-1}$ ($n = 24$) in GK rats. Insulin response to 16.7 mmol/l glucose was significantly decreased in GK rats

Table 1. Body weight, serum glucose, and serum insulin of control and GK rats

	Control	GK
Body weight (g)	274.7 ± 6.4	256.6 ± 8.3
Serum glucose (mmol/l)	10.9 ± 0.3	21.1 ± 0.5 ^a
Serum insulin (ng/ml)	2.28 ± 0.24	2.49 ± 0.30

Values are means ± SEM for 39 control and 43 GK rats. All animals were in the fed state. ^a $p < 0.001$ vs control

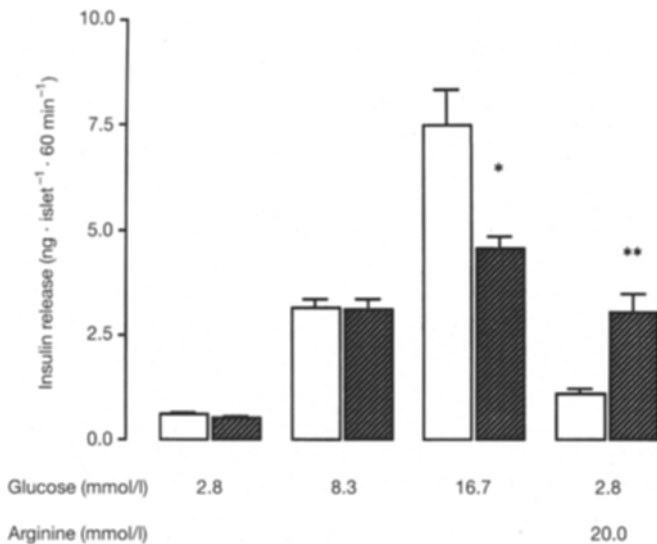


Fig. 1 Effects of glucose and arginine on insulin release from isolated pancreatic islets of control (□) and GK rats (▨). The response was tested for 60 min at 37°C in KRBB under the indicated conditions. Bars represent mean ± SEM for 15–25 batches of islets from three separate experiments. * $p < 0.01$, ** $p < 0.001$ vs control

(4.52 ± 0.30 ng · islet⁻¹ · 60 min⁻¹, $n = 23$, $p < 0.01$) compared with controls (7.48 ± 0.85 , $n = 23$). The rise in glucose concentration from 8.3 to 16.7 mmol/l in the incubation medium did not elicit further insulin secretion from islets of GK rats. In contrast, 20 mmol/l arginine in the presence of 2.8 mmol/l glucose markedly enhanced insulin release from islets of GK rats (3.02 ± 0.43 ng · islet⁻¹ · 60 min⁻¹, $n = 15$, $p < 0.001$) compared with that of controls (1.09 ± 0.10 , $n = 14$). In another series of experiments, the effect of depolarizing concentrations of potassium was tested. In the presence of 2.8 mmol/l glucose, insulin secretion induced by 25 mmol/l potassium also was significantly higher in GK rats (by 3.10 ± 0.28 -fold, $n = 8$, $p < 0.05$) when compared with controls (by 1.67 ± 0.28 -fold, $n = 8$).

Characteristics of the Ca²⁺-induced insulin release from electrically permeabilized normal pancreatic islets. In permeabilized insulin-secreting cells, the elevation of Ca²⁺ levels directly stimulates exocytosis of insulin, as has been previously reported [17–20]. When permeabilized islets were incubated in solu-

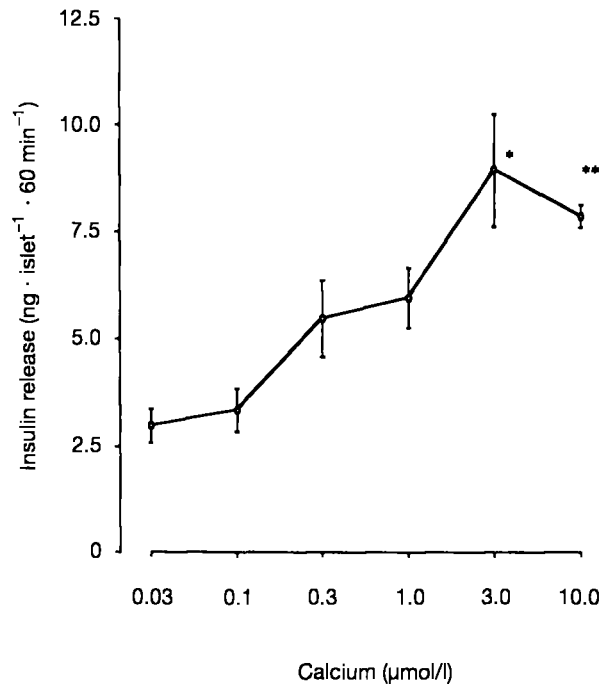


Fig. 2 Ca²⁺-induced insulin release from electrically permeabilized islets. Electrically permeabilized islets obtained from control Wistar rats were incubated for 60 min in solutions containing Ca²⁺ in increasing amounts from 0.03 to 10.0 µmol/l. Results are means ± SEM for four batches of islets. * $p < 0.05$, ** $p < 0.01$ vs the value at 0.03 µmol/l Ca²⁺

tions containing increasing Ca²⁺ concentrations from 30 nmol/l to 10.0 µmol/l, insulin release was dose-dependently stimulated by about three-fold (Fig. 2). Secretion was found to be augmented half-maximally by about 0.5 µmol/l Ca²⁺ and maximally by 3.0 µmol/l Ca²⁺. The lactate dehydrogenase content of the electrically permeabilized islet was measured as a marker of high molecular weight cytosolic proteins. Compared with the enzyme content in intact islets, that of permeabilized islets was $65.4 \pm 2.6\%$ ($n = 4$) immediately after electric permeabilization, and $64.5 \pm 2.6\%$ ($n = 4$) after a 60-min incubation at 30 nmol/l Ca²⁺, indicating that no significant leakage occurred during the incubation period. The staining of islets with trypan blue after the incubation period showed that essentially all of the islet cells were still positive (data not shown), indicating that islets can be kept permeabilized during the incubation period, a finding consistent with results in a previous report [28].

Ca²⁺ dose-response of insulin release was augmented in electrically permeabilized pancreatic islets of GK rats. Insulin secretory capacity in response to increasing Ca²⁺ concentrations was compared between electrically permeabilized pancreatic islets of GK and control rats. Although the insulin response to non-stimulatory concentrations of Ca²⁺ was similar in both groups of islets, the response to stimulatory Ca²⁺ concentrations was significantly augmented in the

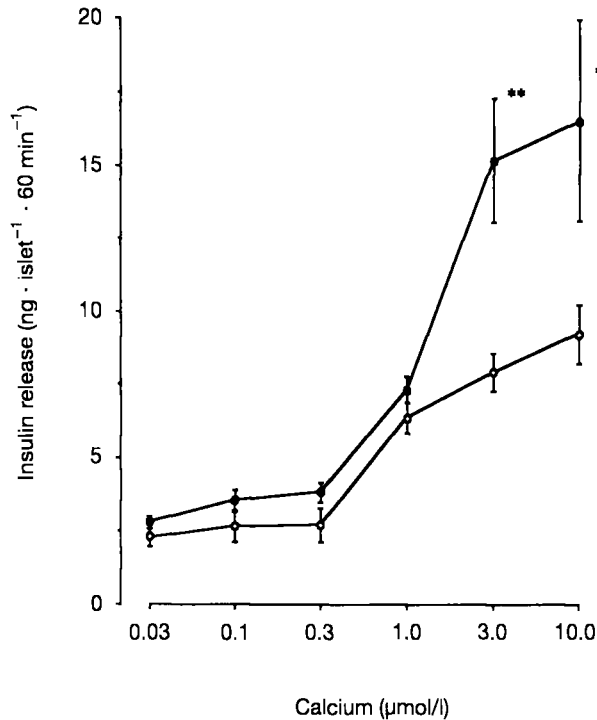


Fig. 3 Augmented Ca^{2+} dose-response of insulin release in electrically permeabilized pancreatic islets of GK rats. Insulin release from electrically permeabilized islets of control (○) and GK rats (●) were tested for 60 min in solutions containing increasing amounts of Ca^{2+} from 0.03 to 10.0 $\mu\text{mol/l}$ in the presence of 2.8 mmol/l glucose. Results are means \pm SEM for 11–12 batches of islets from two separate experiments. * $p < 0.05$, ** $p < 0.01$ vs control

Table 2. Effect of glucose on Ca^{2+} -induced insulin secretion from electrically permeabilized control islets

Agents	Ca^{2+} ($\mu\text{mol/l}$)	
	0.03	3.0
2.8 mmol/l Glucose	100 (22)	100 (22)
16.7 mmol/l Glucose	102 \pm 10 (21)	281 \pm 23 (22) ^a
16.7 mmol/l Glucose + 100 $\mu\text{mol/l}$ 2,4-dinitrophenol	95 \pm 9 (15)	184 \pm 16 (26) ^{a, b}

Electrically permeabilized control pancreatic islets were incubated for 60 min in KA buffer containing the indicated agents. Data are normalized by taking the secretion at 2.8 mmol/l glucose as 100%, and expressed as mean \pm SEM. Number of batches from three separate experiments are shown in parentheses. Secretion in the presence of 2.8 mmol/l glucose was 1.74 ± 0.26 ng \cdot islet $^{-1} \cdot$ 60 min $^{-1}$ at 0.03 $\mu\text{mol/l}$ Ca^{2+} and 4.99 ± 0.50 ng \cdot islet $^{-1} \cdot$ 60 min $^{-1}$ at 3.0 $\mu\text{mol/l}$ Ca^{2+} ($p < 0.001$).

^a $p < 0.001$ vs 2.8 mmol/l glucose; ^b $p < 0.01$ vs 16.7 mmol/l glucose

GK rat islets (Fig. 3). In the presence of 2.8 mmol/l glucose, insulin response to 30 nmol/l Ca^{2+} was 2.34 ± 0.36 ng \cdot islet $^{-1} \cdot$ 60 min $^{-1}$ ($n = 12$) in control and 2.81 ± 0.21 ng \cdot islet $^{-1} \cdot$ 60 min $^{-1}$ ($n = 11$) in GK rats. At the maximally stimulatory concentration of 3.0 $\mu\text{mol/l}$ Ca^{2+} , however, insulin release was 15.12 ± 2.12 ng \cdot islet $^{-1} \cdot$ 60 min $^{-1}$ ($n = 12$) in GK rats,

which was significantly higher than the value of 7.89 ± 0.66 ($n = 12$, $p < 0.01$) in control rats. Thus, the increase in Ca^{2+} concentrations from 30 nmol/l to 3.0 $\mu\text{mol/l}$ stimulated insulin release by 5.4-fold in GK rats and by 3.4-fold in control rats.

Insulin content of intact islets was not statistically different between control (49.0 ± 4.3 ng/islet, $n = 15$) and GK islets (52.1 ± 3.3 ng/islet, $n = 15$). In the case of electrically permeabilized islets, insulin content was also not statistically different between the two groups (35.6 ± 2.8 ng/islets, $n = 15$ vs 37.3 ± 3.2 , $n = 15$, in control and GK rats, respectively). This indicates that insulin content was as well preserved as with the permeabilization procedure in GK (71.5%) and control rats (72.6%).

Effect of glucose on Ca^{2+} -induced insulin secretion from electrically permeabilized normal pancreatic islets. The effect of glucose on Ca^{2+} -induced insulin secretion was assessed in electrically permeabilized normal pancreatic islets by raising the glucose concentration in the KA buffer from 2.8 to 16.7 mmol/l. In addition, we examined the effect of 2,4-dinitrophenol (DNP), an inhibitor of mitochondrial oxidative phosphorylation, to explore the underlying mechanism of the glucose effect. In intact control islets incubated in KRBB, insulin release of 8.25 ± 1.03 ng \cdot islet $^{-1} \cdot$ 60 min $^{-1}$ ($n = 5$) induced by 16.7 mmol/l glucose was found to be markedly inhibited by 100 $\mu\text{mol/l}$ DNP to 0.55 ± 0.14 ng \cdot islet $^{-1} \cdot$ 60 min $^{-1}$ ($n = 5$), corresponding to the basal insulin secretion at 2.8 mmol/l glucose (0.65 ± 0.09 ng \cdot islet $^{-1} \cdot$ 60 min $^{-1}$, $n = 5$). As shown in Table 2, 16.7 mmol/l glucose did not enhance the insulin release from electrically permeabilized control islets in the presence of non-stimulatory 30 nmol/l calcium. In contrast, 16.7 mmol/l glucose significantly augmented insulin release in the presence of 3.0 $\mu\text{mol/l}$ calcium ($p < 0.001$ vs secretion at 2.8 mmol/l glucose). DNP at 100 $\mu\text{mol/l}$ significantly suppressed the augmentative effect of 16.7 mmol/l glucose ($p < 0.01$), but this inhibitory effect on glucose-augmented insulin secretion was only partial; the agent did not completely inhibit the augmented insulin secretion to the secretory level at 2.8 mmol/l glucose.

It should be noted that the rise in glucose concentration from 2.8 to 16.7 mmol/l failed to increase the insulin release from intact control pancreatic islets in KA buffer containing 3.0 $\mu\text{mol/l}$ Ca^{2+} (0.23 ± 0.04 ng \cdot islet $^{-1} \cdot$ 60 min $^{-1}$, $n = 5$, vs 0.29 ± 0.05 ng \cdot islet $^{-1} \cdot$ 60 min $^{-1}$, $n = 5$). This finding excludes the possibility that residual intact beta-cells in electrically permeabilized islets might perturb the secretory data in this experiment.

Impaired glucose augmentation of Ca^{2+} -induced insulin release in electrically permeabilized pancreatic islets of GK rats. The augmentative glucose effect on

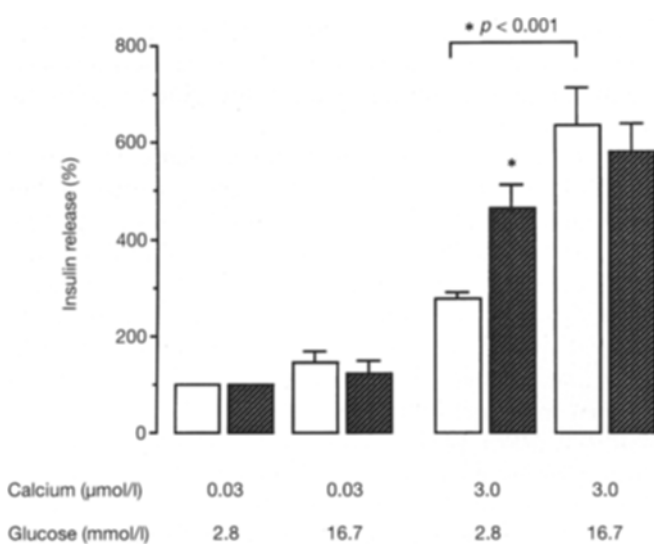


Fig. 4 Impairment of glucose effect on Ca^{2+} -induced insulin secretion from electrically permeabilized GK islets. The stimulatory effect of glucose on insulin release from electrically permeabilized islets of control (\square) and GK rats (▨) was tested for 60 min in KA buffer containing either 0.03 or 3.0 $\mu\text{mol/l}$ Ca^{2+} . Data are normalized by taking the basal insulin secretion at 0.03 $\mu\text{mol/l}$ Ca^{2+} in the presence of 2.8 mmol/l glucose by 100 %, and expressed as mean \pm SEM for 15–16 batches from two separate experiments. * $p < 0.001$ vs control

Ca^{2+} -induced insulin release was compared between electrically permeabilized pancreatic islets of control and GK rats (Fig. 4). Basal secretion at 30 nmol/l Ca^{2+} in the presence of 2.8 mmol/l glucose was not statistically different between control (1.97 ± 0.15 ng \cdot islet $^{-1} \cdot 60$ min $^{-1}$) and GK rats (1.58 ± 0.16 ng \cdot islet $^{-1} \cdot 60$ min $^{-1}$). When the data were normalized by taking the basal secretion in each group as 100 %, insulin release induced by 3.0 $\mu\text{mol/l}$ Ca^{2+} in the presence of 2.8 mmol/l glucose was significantly higher in GK islets, which again confirms the hyperresponse to Ca^{2+} in GK rats. In electrically permeabilized control pancreatic islets, the rise in glucose concentration from 2.8 to 16.7 mmol/l further augmented insulin release induced by 3.0 $\mu\text{mol/l}$ Ca^{2+} ($p < 0.001$). In contrast, the rise in glucose concentration had no effect on Ca^{2+} -induced insulin release from electrically permeabilized GK islets.

Discussion

The GK rat has been shown to be a novel genetic animal model of NIDDM [6–9]. In the present study, the serum glucose level of GK rats was found to be consistently higher than that of control rats. Furthermore, the insulin release induced by glucose *in vitro* from isolated pancreatic islets of GK rats was significantly decreased compared with control Wistar rats, whereas the insulin release induced by other secretagogues such as arginine was rather augmented.

These data confirm the previous findings reported both *in vivo* [7,9] and *in vitro* [8,9,29,30], indicating a selective reduction of glucose-induced insulin secretion of GK rats. The GK rat thus provides a very suitable model for investigating the pathophysiological aspects of alterations in the insulin secretory machinery of NIDDM beta cells.

A rise in $[\text{Ca}^{2+}]_i$ is believed to play a crucial role in the regulation of insulin secretion from pancreatic beta-cells in response to various stimuli, including glucose [10–12]. Investigations of permeabilized insulin-secretory cells have shown that the elevation in $[\text{Ca}^{2+}]_i$ is sufficient to activate exocytosis of insulin granules [17–20]. We have previously reported that the selective impairment of glucose-induced insulin secretion observed in NIDDM beta-cells is associated with the defective $[\text{Ca}^{2+}]_i$ elevation by glucose, but not by arginine [15], and this phenomenon may be explained in part by defects in the steps proximal to the $[\text{Ca}^{2+}]_i$ elevation in glucose stimulation, including deficient intracellular glucose metabolism [13–15]. However, it is also of great interest to explore the functional modulations in steps distal to the rise in $[\text{Ca}^{2+}]_i$ in beta cells in the diabetic condition.

It is noteworthy that in this study insulin release in response to stimulatory concentrations of Ca^{2+} was markedly augmented in electrically permeabilized islets of GK rats. This finding may well explain the hyperresponse of GK rats to depolarizing stimuli, such as arginine, found in this study and previous reports [9], because this positively charged amino acid has been suggested to exert its insulinotropic effect mainly by causing direct depolarization of the plasma membrane, resulting in an increase in $[\text{Ca}^{2+}]_i$ via Ca^{2+} influx through the voltage-dependent Ca^{2+} channels [31].

Portha et al. [9] have reported that the hyperresponse to arginine, together with the decreased insulin release in response to glucose, were partially but not completely improved by perfusion of GK pancreas with buffer containing no glucose. Thus, it can be speculated that the increased insulin response to Ca^{2+} in electrically permeabilized islets of GK rats may partly be explained by so-called glucose toxicity [32,33], although the involvement of genetic background cannot be excluded. The hyperresponse to arginine at least in the presence of a non-stimulatory concentration of glucose has also been demonstrated in various hyperglycaemic models including NSZ rats [4,5,34], partially pancreatectomized rats [35], *in vivo* glucose-infused rats [36], and another genetically-diabetic rat model [37]. In the report by Leahy et al. [34], the hyperresponse to arginine in NSZ rats was recovered by 24-h insulin treatment, which again suggests that the increased Ca^{2+} -induced insulin release from permeabilized diabetic islets may be ascribed to prevailing hyperglycaemia. In this re-

spect, we have preliminarily observed that Ca^{2+} -induced insulin release is similarly augmented in an electrically permeabilized beta cell line, HIT-T 15 cells cultured for 24 h at high concentrations of glucose (Y. Okamoto and H. Ishida, unpublished observations), whereas the glucose-induced insulin release from the intact cells is found to be blunted as previously demonstrated [38].

The presence of intracellular defects of glucose metabolism has been suggested in beta cells of GK rats [14,29,30,39]. Thus, we speculate that the significant enhancement of Ca^{2+} -activated insulin secretion, which was observed in electrically permeabilized islets of GK rats, might play a physiological compensatory role in the putative impairment both in $[\text{Ca}^{2+}]_i$ rise and energy state after stimulation with glucose in diabetic beta cells. In electrically permeabilized control pancreatic islets, stimulatory concentrations of glucose were found to augment Ca^{2+} -induced insulin release in a dose-dependent manner. This stimulatory effect of glucose on Ca^{2+} -induced insulin release was significantly inhibited by DNP, a metabolic uncoupler which inhibits oxidative phosphorylation. This finding strongly suggests that glucose can augment insulin secretion from electrically permeabilized islets via its intracellular metabolism, even though it is possible that glycolytic enzymes might be lost to some extent during the permeabilization procedure. DNP at 100 $\mu\text{mol/l}$ completely inhibits glucose-induced insulin release from intact pancreatic islets, but its suppression of the stimulatory glucose effect on Ca^{2+} -induced insulin release from electrically permeabilized islets was incomplete. It is, therefore, also suggested that glucose can exert its stimulatory effect through mechanisms other than the generation of ATP. Although the precise mechanisms underlying augmentation by glucose are still to be elucidated, the putative metabolic signals derived from glycolytic intermediates might participate in the stimulatory glucose effect on Ca^{2+} -induced insulin release from electrically permeabilized pancreatic islets. In addition, activation of protein kinases, which facilitate the effect of Ca^{2+} on the exocytotic pathway, may also play a role.

Using intact pancreatic islets treated with diazoxide and depolarized by potassium, it has been demonstrated that glucose can control the Ca^{2+} -activated exocytotic pathway independently from the ionic events including K_{ATP} channels, presumably by activating protein kinases [40,41] or changing energy state [42]. The present finding seems to be consistent with these reports. In permeabilized GK islets, however, glucose failed to enhance the calcium-induced insulin release. This defective augmentation by glucose on the Ca^{2+} -activated effector system could cause the selective impairment of glucose-induced insulin release from intact GK rats in conjunction with

the putative impaired elevation and function of intracellular Ca^{2+} probably due to the disturbed intracellular glucose metabolism after the glucose stimulation [14].

The detailed molecular mechanisms by which Ca^{2+} acts on exocytosis are still unclear, but studies particularly using chromaffin granules have reported that some cytosolic proteins such as calmodulin and members of the annexin family can bind to secretory granules in a Ca^{2+} -dependent manner [24,43]. In secretory granules of pancreatic islets, the presence of Ca^{2+} -calmodulin-dependent protein kinases (CaM kinases) and endogenous substrates have also been demonstrated [44]. Recently, it has been reported that glucose activates CaM kinase II in pancreatic islets over a similar concentration range to glucose-induced insulin secretion [45]. The precise mechanism of the increased Ca^{2+} sensitivity at non-stimulatory concentrations of glucose and of the absence of glucose effect to enhance the secretory response to $[\text{Ca}^{2+}]_i$ elevation in diabetic beta cells is still under investigation, but it is possible that the phosphorylation state of exocytosis-related Ca^{2+} -dependent proteins might be altered through putative metabolic abnormalities under prolonged hyperglycaemic conditions. The further clarification of abnormal regulatory systems in intracellular signalling for insulin secretion is necessary to elucidate the underlying mechanism of the selective impairment in glucose-induced insulin secretory capacity characteristic of beta cells in NIDDM.

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References

1. Pfeiffer MA, Halter JB, Porte D Jr (1981) Insulin secretion in diabetes mellitus. *Am J Med* 70: 579–588
2. Ward WK, Beard JC, Halter JB, Pfeiffer MA, Porte D (1984) Pathophysiology of insulin secretion in non-insulin-dependent diabetes mellitus. *Diabetes Care* 7: 491–502
3. Leahy JL (1990) Natural history of β -cell dysfunction in NIDDM. *Diabetes Care* 13: 992–1010
4. Tsuji K, Taminato T, Usami M et al. (1988) Characteristic features of insulin secretion in the streptozotocin-induced NIDDM rat model. *Metabolism* 37: 1040–1044
5. Giroix MH, Portha B, Kergoat M, Bailbe D, Picon L (1983) Glucose insensitivity and amino-acid hypersensitivity of insulin release in rats with non-insulin-dependent diabetes. *Diabetes* 32: 445–451

6. Goto Y, Kakizaki M, Masaki N (1975) Spontaneous diabetes produced by selective breeding of normal Wistar rats. *Proc Jpn Acad* 51: 80–85
7. Goto Y, Kakizaki M (1981) The spontaneous-diabetes rat: a model of noninsulin dependent diabetes mellitus. *Proc Jpn Acad* 57: 381–384
8. Kimura K, Toyota T, Kakizaki M, Kudo M, Takebe K, Goto Y (1982) Impaired insulin secretion in the spontaneous diabetic rat. *Tohoku J Exp Med* 137: 453–459
9. Portha B, Serradas P, Bailbe D, Suzuki K, Goto Y, Giroix MH (1991) β -cell insensitivity to glucose in the GK rat, a spontaneous nonobese model for type 2 diabetes. *Diabetes* 40: 486–491
10. Wollheim CB, Biden TJ (1986) Signal transduction in insulin secretion: comparison between fuel stimuli and receptor agonists. *Ann NY Acad Sci* 488: 1043–1052
11. Prentki M, Matschinsky FM (1987) Ca^{2+} , cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion. *Physiol Rev* 67: 1185–1248
12. Rajan AS, Aguilar-Bryan L, Nelson DA et al. (1990) Ion channels and insulin secretion. *Diabetes Care* 13: 340–363
13. Tsuura Y, Ishida H, Okamoto Y et al. (1992) Impaired glucose sensitivity of ATP-sensitive K^+ channels in pancreatic β cells in streptozotocin-induced NIDDM rats. *Diabetes* 41: 861–865
14. Tsuura Y, Ishida H, Okamoto Y et al. (1993) Glucose sensitivity of ATP-sensitive K^+ channels is impaired in β cells of the GK rat, a new genetic model of NIDDM. *Diabetes* 42: 1446–1453
15. Tsuji K, Taminato T, Ishida H, Okamoto Y et al. (1993) Selective impairment of the cytoplasmic Ca^{2+} response to glucose in rat pancreatic β -cells of streptozotocin-induced noninsulin-dependent diabetic rats. *Metabolism* 42: 1424–1428
16. Okamoto Y, Ishida H, Taminato T et al. (1992) Role of cytosolic Ca^{2+} in impaired sensitivity to glucose of rat pancreatic islets exposed to high glucose in vitro. *Diabetes* 41: 1555–1561
17. Pace CS, Tarvin JH, Neighbors AS, Pirkle JA, Greider MH (1980) Use of a high voltage technique to determine the molecular requirements for exocytosis in islet cells. *Diabetes* 29: 911–918
18. Yaseen MA, Pedley KC, Howell SL (1982) Regulation of insulin secretion from islets of Langerhans rendered permeable by electric discharge. *Biochem J* 206: 81–87
19. Jones PM, Persaud SJ, Howell SL (1989) Time-course of Ca^{2+} -induced insulin secretion from perfused, electrically permeabilized islets of Langerhans: effects of cAMP and a phorbol ester. *Biochem Biophys Res Commun* 162: 998–1003
20. Ullrich S, Prentki M, Wollheim CB (1990) Somatostatin inhibition of Ca^{2+} -induced insulin secretion in permeabilized HIT-T15 cells. *Biochem J* 270: 273–276
21. Ronning SA, Martin TFJ (1986) Characterization of Ca^{2+} -stimulated secretion in permeable GH3 pituitary cells. *J Biol Chem* 261: 7834–7839
22. Oetting M, LeBoff MS, Levy S et al. (1987) Permeabilization reveals classical stimulus-secretion coupling in bovine parathyroid cells. *Endocrinology* 121: 1571–1576
23. Guild S, Frey EA, Pocotte SL, Kebabian JW (1988) Adenosine 3', 5'-cyclic monophosphate-mediated enhancement of calcium-evoked prolactin release from electrically permeabilized 7315c tumour cells. *Br J Pharmacol* 94: 737–744
24. Burgoyne RD (1991) Control of exocytosis in adrenal chromaffin cells. *Biochim Biophys Acta* 1071: 174–202
25. Marks V, Lloyd K (1963) Preservation of blood sample for glucose analysis by glucose oxidase. *Clin Chem Acta* 8: 326
26. Sutton R, Peters M, Mcshane P, Gray DWR, Morris P (1986) Isolation of rat pancreatic islets by ductal injection of collagenase. *Transplantation (Baltimore)* 42: 689–691
27. Martell AE, Smith RM (1974) Critical stability constants: amino acids (vol 1). Plenum Press, New York, p 269
28. Jones PM, Persaud SJ, Howell SL (1992) Ca^{2+} -induced insulin secretion from electrically permeabilized islets. *Biochem J* 285: 973–978
29. Giroix M-H, Vesco L, Portha B (1993) Functional and metabolic perturbations in isolated pancreatic islets from the GK rat, a genetic model of noninsulin-dependent diabetes. *Endocrinology* 132: 815–822
30. Östenson C-G, Khan A, Abdel-Halim SM, Guenifi A, Suzuki K, Goto Y, Efendic S (1993) Abnormal insulin secretion and glucose metabolism in pancreatic islets from the spontaneously diabetic GK rat. *Diabetologia* 36: 3–8
31. Blachier F, Mourtada A, Sener A, Malaisse WJ (1989) Stimulus-secretion coupling of arginine-induced insulin release: uptake of metabolized and nonmetabolized cationic amino acids by pancreatic islets. *Endocrinology* 124: 134–141
32. Rossetti L, Giaccari A, DeFronzo RA (1990) Glucose toxicity. *Diabetes Care* 13: 610–630
33. Leahy JL, Bonner-Weir S, Weir GC (1992) β -cell dysfunction induced by chronic hyperglycaemia. *Diabetes Care* 15: 442–455
34. Leahy JL, Bonner-Weir S, Weir GC (1985) Abnormal insulin secretion in a streptozotocin model of diabetes. Effects of insulin treatment. *Diabetes* 34: 660–666
35. Rossetti L, Gerald IS, Zawulich W, DeFronzo RA (1987) Effect of chronic hyperglycaemia on in vivo insulin secretion in partially pancreatectomized rats. *J Clin Invest* 80: 1037–1044
36. Leahy JL, Cooper HE, Weir GC (1987) Impaired insulin secretion associated with near normoglycemia. Study in normal rats with 96-h in vivo glucose infusions. *Diabetes* 36: 459–464
37. Voyles N R, Powell AM, Timmers KI et al. (1988) Reversible impairment of glucose-induced insulin secretion in SHR/N-*cp* rats. *Diabetes* 37: 398–404
38. Inagaki N, Yasuda K, Inoue G, Okamoto Y et al. (1992) Glucose as regulator of glucose transport activity and glucose-transporter mRNA in hamster β -cell line. *Diabetes* 41: 592–597
39. Östenson C-G, Abdel-Halim SM, Rasschaert J et al. (1993) Deficient activity of FAD-linked glycerophosphate dehydrogenase in islets of GK rats. *Diabetologia* 36: 722–726
40. Gembal M, Gilon P, Henquin JC (1992) Evidence that glucose can control insulin release independently from its action on ATP-sensitive K^+ channels in mouse B cells. *J Clin Invest* 89: 1288–1295
41. Sato Y, Aizawa T, Komatsu M, Okada N, Yamada T (1992) Dual functional role of membrane depolarization/ Ca^{2+} influx in rat pancreatic B-cell. *Diabetes* 41: 438–443
42. Gembal M, Detimary P, Gilon P, Gao ZY, Henquin JC (1993) Mechanisms by which glucose can control insulin release independently from its action on adenosine triphosphate-sensitive K^+ channels in mouse B cells. *J Clin Invest* 91: 871–880
43. Burgoyne RD (1990) Secretory vesicle-associated proteins and their role in exocytosis. *Annu Rev Physiol* 52: 647–659
44. Watkins DT (1991) Ca^{2+} -calmodulin dependent phosphorylation of islet secretory granule proteins. *Diabetes* 40: 1063–1068
45. Wenham RM, Landt M, Easom RA (1994) Glucose activates the multifunctional Ca^{2+} /calmodulin-dependent protein kinase II in isolated rat pancreatic islets. *J Biol Chem* 269: 4947–4952