Role of Cytosolic Ca^{2+} in Impaired Sensitivity to Glucose of Rat Pancreatic Islets Exposed to High Glucose In Vitro

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Sustained exposure to high concentrations of glucose selectively impairs the ability of pancreatic islets to secrete insulin in acute glucose stimulation. In order to evaluate the interrelationship between impaired insulin secretion and the dynamics of the cytosolic free Ca^{2+} level ($[Ca^{2+}]i$), we have investigated the effect of high glucose exposure on both $[Ca^{2+}]i$ dynamics in single rat β-cells and insulin release from rat pancreatic islets. Islets cultured at a high glucose concentration (16.7 mM) for 24 h showed significant reductions of the 16.7 mM GSIR compared with islets cultured at a normal glucose concentration (5.5 mM) (3.38 ± 0.24 vs. 4.26 ± 0.34%, respectively, $P < 0.05$). The capacity of glucose to raise the $[Ca^{2+}]i$ level also was significantly reduced in the β-cells maintained for 24 h at 16.7 mM glucose ($P < 0.001$). An additional culture in the medium with 5.5 mM glucose for 16 h restored both the GSIR and the $[Ca^{2+}]i$ response of islets cultured at high glucose. On the other hand, insulin release and $[Ca^{2+}]i$ rise in response to 20 mM L-Arg were well preserved. These observations confirm that exposure of pancreatic β-cells to high glucose concentrations induces a selective reduction of the GSIR and, further, shows that this impaired response is reversibly restored by an additional culture with normal glucose. We also suggest that the inability of glucose to provoke a $[Ca^{2+}]i$ rise, which is observed in the β-cells exposed to high glucose, may be responsible for the selective impairment of the GSIR. *Diabetes* 41:1555–61, 1992

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GSIR, glucose-stimulated insulin release; NIDDM, non-insulin-dependent diabetes mellitus; KRBB, Krebs-Ringer bicarbonate buffer; BSA, bovine serum albumin.

The decrease in GSIR together with the preservation of responsiveness to other secretagogues is a common characteristic of NIDDM (1,2). Selective insensitivity to glucose is reported to be improved by normalizing glycemic levels through diet, sulfonylureas, and insulin therapy (3). Experiments both in vivo (4–7) and in vitro (8–12) have shown that chronic hyperglycemia exerts a deleterious effect on pancreatic β-cell functions. This effect is referred to as glucose toxicity or desensitization (13,14) and is thought to induce more severe hyperglycemia and to influence the prognosis of patients with NIDDM (15). In vitro, rat pancreatic islets have been reported to become desensitized by continuous glucose stimulation with perifusion and batch incubation systems (11,12). However, the precise mechanisms responsible for this desensitization remain to be elucidated.

A rise in cytosolic free Ca concentration ($[Ca^{2+}]i$) is known to play a crucial role in the regulation of insulin secretion from pancreatic β-cells in response to various stimuli, including glucose (16–19). To clarify the role of $[Ca^{2+}]i$ in this phenomenon, we studied the chronic effects of high glucose exposure on insulin secretion using cultured rat pancreatic islets in vitro, and we monitored $[Ca^{2+}]i$ changes in single β-cells using a fluorescent probe fura 2. We show that both GSIR and $[Ca^{2+}]i$ rise are selectively impaired by sustained exposure of β-cells to high concentrations of glucose and that these impaired responses can be restored in a parallel fashion by normalization of ambient glucose levels.

RESEARCH DESIGN AND METHODS

Islets of Langerhans were isolated from male Wistar rats weighing 180–200 g by collagenase digestion (20), and groups of 200 islets were cultured under the following three experimental conditions. In experiment 1, islets

were cultured for 24 h at 5.5 (control) or 16.7 mM (high) glucose in 10 ml of RPMI-1640 medium containing 10% (vol/vol) heat-inactivated fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 37°C in humidified 5% CO_2. In experiment 2, islets were cultured for 24 h in the same RPMI-1640 medium containing 16.7 mM glucose in the presence and absence of 100 μM diazoxide. In experiment 3, the islets cultured for 24 h at 16.7 mM glucose were cultured for an additional 16 h in the same medium, but containing 5.5 or 16.7 mM glucose.

For the measurement of insulin secretion, cultured islets (10–15 in each tube) were washed and preincubated at 37°C for 30 min in 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 $NaHCO_3$, 10 mM HEPES, 3.3 mM glucose, and 0.2% BSA, pH 7.4 at 37°C. Islets then were incubated at 37°C for 30 min in KRBB with test materials. At the end of the incubation period, aliquots of the KRBB incubation medium were stored at −20°C until assayed, and total insulin contents were successively extracted by boiling the islets with 1 M acetic acid (7). In experiment 2, aliquots of the culture medium also were stored at −20°C for the measurement of insulin. The released insulin and the insulin content of the islets were measured by radioimmunoassay using rat insulin (Novo, Bagsvaerd, Denmark) as standard (7). Results are expressed either as $\mu U \cdot islet^{-1} \cdot 30\ min^{-1}$ or as the percentage of total islet insulin content, and the stimulated value of insulin release was normalized by taking basal insulin secretion (i.e., with a nonstimulatory glucose concentration of 3.3 mM) as 100%.

For the experiments monitoring $[Ca^{2+}]i$, islet cells were cultured in a dissociated form. Single cells were prepared by shaking them in a Ca^{2+}-deficient medium, washing them twice, and subsequently culturing them on the 16 x 16 mm cover slips according to the same protocols as above. After a 24-h culture, the islet cells adhered to the cover slips either in a single-cell or small-cluster configuration. Islet cell viability, assessed by trypan blue exclusions, was >95% in each experimental group. Cultured islet cells then were loaded with 1.0 μM fura 2 acetoxymethylester (Molecular Probes, Eugene, OR) for 30 min in KRBB and placed in the square well (500 μl in bed volume) mounted on the stage of the microscope. Cells then were superfused with KRBB containing 3.3 mM glucose for 10 min and successively challenged with test materials at a flow rate of 2 ml/min for 20 min. Superfusion was performed at 34–37°C by heating the stage of the microscope and the medium. Dual-excitation wavelength measurement (340 and 360 nm) was permitted with an automatic device that allowed two wavelengths to be alternately selected using microcomputer-driven shutters. Islet cells were viewed with a x20 fluorescence objective (Nikon, Tokyo, Japan). Fluorescence emmision at 510 nm was monitored with a silicon-intensified target camera (C2400–08H, Hamamatsu Photonics, Hamamatsu, Japan), and the ratio calculation was digitized every 20 s by a computerized image processor (Argus-100/CA, Hamamatsu). The $[Ca^{2+}]i$ in 3–14 single β-cells could be monitored in individual experiments. All experiments were performed on individual cells >10 μm in diameter and not in contact with other cells, because non–insulin-producing cells reportedly are characterized by their smaller size (21). In vitro calibration was made in a range of pCa 9–5, and the 340/360 fluorescence ratio was converted into calibrated values of $[Ca^{2+}]i$ (22). After reconstructing the linear $[Ca^{2+}]i$ traces, the integrated $[Ca^{2+}]i$ values above the basal value were measured by cutting out and weighing 20-min sections of the traces on paper, expressed as mg of the paper.

All values are presented as means ± SE. The ratio of responsive cells that showed changes of $[Ca^{2+}]i$ were calculated as a percentage. Statistical analyses were performed using Student's unpaired *t* test for the significance of differences between groups and the χ^2 test for differential significance in the prevalence of responsive cells in $[Ca^{2+}]i$ monitoring.

RESULTS

In vitro effect of high glucose on insulin secretion (experiment 1). The basal insulin release in the presence of 3.3 mM glucose was significantly higher when cultured for 24 h at a high concentration of glucose (16.7 mM) than at the control value of 5.5 mM glucose in control islets (10.0 ± 0.7, $n = 22$ vs. 5.4 ± 0.4 $\mu U \cdot islet^{-1} \cdot 30\ min^{-1}$, $n = 21$, $P < 0.001$). By contrast, islets cultured at high glucose showed a significantly decreased insulin response to 16.7 mM glucose stimulation compared with control islets (15.2 ± 1.4, $n = 24$ vs. 28.1 ± 2.8 $\mu U \cdot islet^{-1} \cdot 30\ min^{-1}$, $n = 20$, $P < 0.001$).

The total islet insulin content was not significantly different between islets cultured for 24 h at high glucose and control islets (460.4 ± 39.9, $n = 76$ vs. 493.7 ± 31.8 μU/islet, $n = 73$). As shown in Fig. 1, when the data were expressed as the percentage of total islet insulin content, islets cultured at high glucose showed significantly higher insulin release in response to 3.3 mM glucose compared with controls (2.42 ± 0.15, $n = 22$ vs. 1.19 ± 0.10%, $n = 21$, $P < 0.001$), but significantly decreased insulin release was observed in response to 16.7 mM glucose (3.38 ± 0.24, $n = 24$ vs. 4.26 ± 0.34%, $n = 20$, $P < 0.05$).

In the presence of 3.3 mM glucose, 20 mM Arg elicited a significantly higher insulin response in islets cultured for 24 h at high glucose compared with controls (20.8 ± 1.7, $n = 18$ vs. 13.1 ± 1.2 $\mu U \cdot islet^{-1} \cdot 30\ min^{-1}$, $n = 20$, $P < 0.001$). When the data were expressed as the percentage of islet insulin content, the insulin response to Arg was 4.91 ± 0.42 and 2.66 ± 0.25%, respectively ($P < 0.001$, Fig. 1).

Decreased sensitivity to increasing glucose concentrations of islets cultured at 16.7 mM glucose became more obvious when the data were normalized by taking basal insulin secretion as 100% (% basal). In response to 8.3 and 16.7 mM glucose, islets cultured at 16.7 mM glucose secreted 122 ± 8 ($n = 12$) and 140 ± 10% ($n = 24$) of basal secretion, respectively, and control islets secreted 145 ± 15 ($n = 12$, $P < 0.05$) and 358 ± 29% ($n = 20$, $P < 0.001$) of basal insulin secretion, respectively.

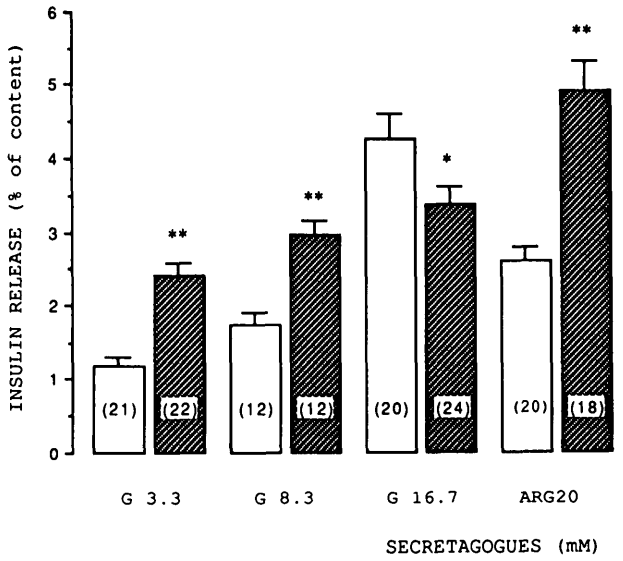

FIG. 1. Effects of glucose and Arg on insulin release from islets cultured for 24 h in RPMI-1640 medium containing 5.5 (□) or 16.7 mM glucose (▨). The response was tested for 30 min at 37°C in KRBB containing 3.3, 8.3, or 16.7 mM glucose *(G)*, and 20 mM Arg in the presence of 3.3 mM glucose *(ARG20)*. Data are expressed as the percentage of total islet insulin content; each bar represents means ± SE. *(n)*, number of observations; *$P < 0.05$, **$P < 0.001$ vs. islets cultured for 24 h with 5.5 mM glucose.

In vitro effect of diazoxide on GSIR (experiment 2). Insulin release into the RPMI-1640 medium after 24-h culture at 16.7 mM glucose was significantly higher than at 5.5 mM glucose (5.15 ± 0.88, $n = 5$, vs. 1.20 ± 0.35 ng/ml, $n = 5$, $P < 0.01$). The addition of 100 μM diazoxide to the RPMI-1640 medium containing 16.7 mM glucose markedly suppressed the insulin concentration (1.20 ± 0.20, $n = 5$, $P < 0.01$ vs. 16.7 mM glucose alone). The insulin response to 16.7 mM glucose in islets cultured at high glucose plus diazoxide was significantly higher than in islets cultured at high glucose alone and was comparable with control islets (19.3 ± 2.5, $n = 13$ vs. 12.5 ± 1.4, $n = 8$, $P < 0.05$, vs. 20.4 ± 3.4 μU · islet^{-1} · 30 min^{-1}, $n = 7$). The total islet insulin content was not significantly affected in islets cultured with 5.5 mM glucose, 16.7 mM glucose, and 16.7 mM glucose plus 100 μM diazoxide (479.8 ± 38.1, $n = 14$ vs. 477.4 ± 51.7, $n = 16$ vs. 458.7 ± 23.0 μU/islet, $n = 25$). As shown in Table 1, the decreased glucose sensitivity observed in islets cultured at high glucose was restored by the simultaneous culture with 100 μM diazoxide, and the increased basal insulin secretion was not statistically reversed.

Reversibility of the in vitro effect of high glucose on insulin secretion (experiment 3). In this experiment, the insensitivity to glucose of the islets in the 24-h culture with 16.7 mM glucose was restored by an additional culture for 16 h at 5.5 mM glucose. The insulin response to 16.7 mM glucose in these experimental islets was significantly higher than in islets cultured for 40 h at high glucose throughout (20.3 ± 2.3, $n = 7$ vs. 14.0 ± 1.7 μU · islet^{-1} · 30 min^{-1}, $n = 6$, $P < 0.05$). The total islet insulin content was not statistically different between these groups (388.2 ± 36.1, $n = 15$ vs. 413.6 ± 35.8 μU/islet, $n = 13$, respectively). As shown in Fig. 2, 16.7 mM glucose elicited an insulin response in experimental islets significantly higher than in islets cultured for 40 h at high glucose throughout (5.22 ± 0.58, $n = 7$ vs. 3.38 ± 0.41%, $n = 6$, $P < 0.05$). In contrast, the basal insulin secretion of experimental islets was significantly lower than that of the islets cultured for 40 h at high glucose (0.91 ± 0.07, $n = 8$ vs. 2.16 ± 0.31%, $n = 7$, $P < 0.001$).

Changes in cytosolic free Ca concentrations. As has been reported previously (18), the changes in $[Ca^{2+}]i$ in response to glucose were heterogeneous among the β-cells examined. Response patterns of a gradual and sustained rise, a biphasic rise with an initial phase followed by a nadir and second plateau phase, and a gradual rise followed by a spontaneous decline were similarly observed in β-cells cultured for 24 h at both high (16.7 mM) and control (5.5 mM) glucose. Figure 3 shows representative traces of the $[Ca^{2+}]i$ rise in response to 16.7 mM glucose in both groups of β-cells of the last pattern, which was most common. Although glucose did not elicit a $[Ca^{2+}]i$ rise in all β-cells examined, the population of responsive β-cells to 16.7 mM glucose was almost identical between β-cells cultured at high glucose and controls (57% [52 of 92 observations] vs. 56% [45 of 81 observations], in 10 separate experiments).

The $[Ca^{2+}]i$ response to glucose stimulation in β-cells

TABLE 1
In vitro effect of diazoxide on GSIR

Culture condition	Insulin secretion: In 3.3 mM glucose — Percentage of total islet insulin content (%)	n	In 16.7 mM glucose — Percentage of total islet insulin content (%)	n
5.5 mM glucose	1.60 ± 0.17	7	4.26 ± 0.70	7
16.7 mM glucose	2.33 ± 0.23*	8	2.61 ± 0.29*	8
16.7 mM glucose plus 100 μM diazoxide	1.96 ± 0.32	12	4.20 ± 0.54†	13

Values are means ± SE. *n*, number of observations. Pancreatic islets were cultured for 24 h in RPMI-1640 medium containing glucose and/or diazoxide at indicated concentrations. Insulin secretion was measured after incubating islets for 30 min in KRBB containing 3.3 or 16.7 mM glucose.
*$P < 0.05$ vs. 5.5 mM glucose.
†$P < 0.05$ vs. 16.7 mM glucose.

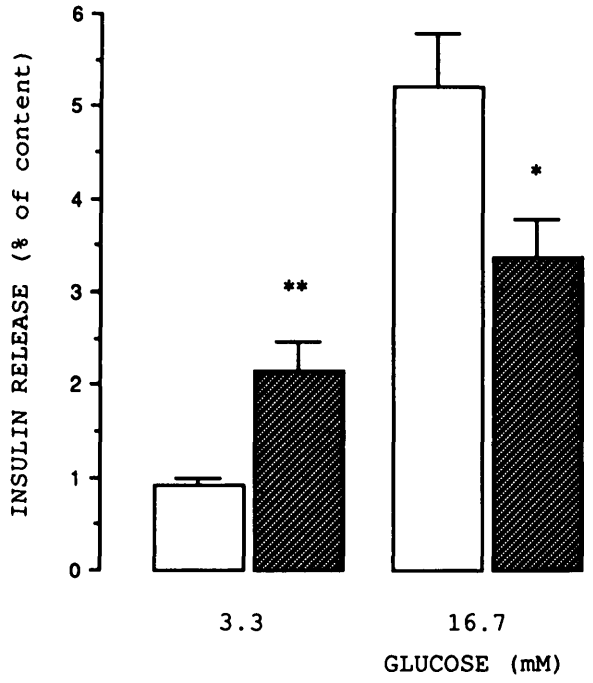

FIG. 2. Reversibility of insulin release from islets cultured for 24 h at 16.7 mM glucose. Islets cultured for 24 h at 16.7 mM glucose were cultured for an additional 16 h in RPMI-1640 medium containing 5.5 (□) or 16.7 mM glucose (▨). The response was tested for 30 min at 37°C in KRBB containing 3.3 or 16.7 mM glucose. Data are expressed as the percentage of total islet insulin content; each bar represents means ± SE for 6–8 experiments. *$P < 0.05$, **$P < 0.001$.

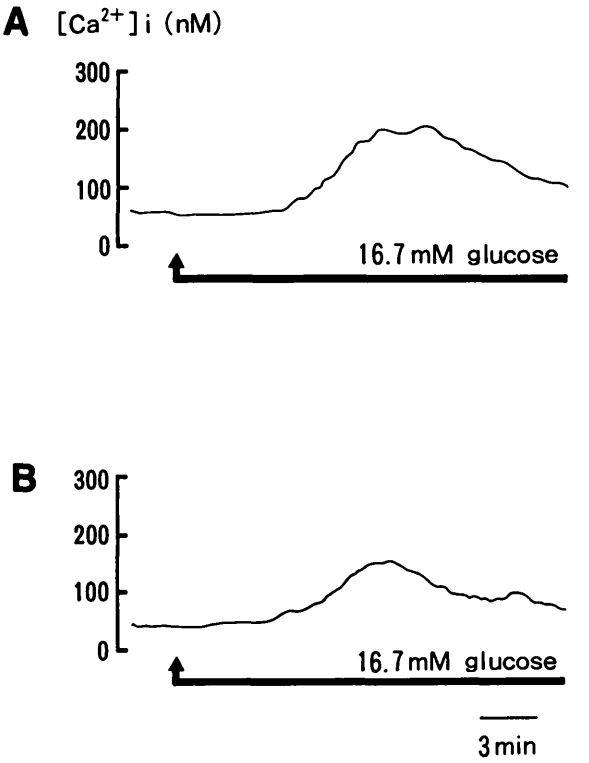

FIG. 3. Changes in $[Ca^{2+}]i$ stimulated by 16.7 mM glucose in single fura 2–loaded β-cells cultured for 24 h either at 5.5 *(A)* or 16.7 mM *(B)* glucose. The traces are representative of 45 and 52 observations, respectively.

cultured at 16.7 mM glucose was characterized by a relative diminution of the maximal amplitude. As indicated in Table 2, although no difference was found in basal $[Ca^{2+}]i$ level at 3.3 mM glucose between the two groups, both the maximal level of $[Ca^{2+}]i$ observed within 20-min stimulation and the incremental $[Ca^{2+}]i$ rise, which is calculated by subtracting the basal $[Ca^{2+}]i$ value from the maximal value, were significantly decreased in β-cells cultured at 16.7 mM glucose. Because of the large degree of heterogeneity in the response patterns of glucose stimulation, the integrated $[Ca^{2+}]i$ levels above the basal also were measured by cutting out and weighing 20-min sections of the $[Ca^{2+}]i$ traces on paper. The integrated $[Ca^{2+}]i$ expressed as mg of the paper also was significantly decreased in β-cells cultured at high glucose compared with control β-cells (10.11 ± 0.85, $n = 52$ vs. 16.99 ± 1.15 mg, $n = 45$, $P < 0.001$).

In both groups of β-cells, 20 mM Arg elicited a rapid (within 20 s) and sustained $[Ca^{2+}]i$ rise (Fig. 4). The $[Ca^{2+}]i$ response was observed in all β-cells examined (26 control cells and 20 cells cultured at 16.7 mM glucose in 7 and 5 separate experiments, respectively). The basal level of $[Ca^{2+}]i$, the maximal level of $[Ca^{2+}]i$, and the incremental $[Ca^{2+}]i$ were not statistically different between the two groups (Table 2). Furthermore, the integrated $[Ca^{2+}]i$ levels also were similar in both groups (16.74 ± 1.53, $n = 26$ vs. 16.65 ± 2.23 mg, $n = 20$, respectively).

TABLE 2
In vitro effect of glucose on $[Ca^{2+}]i$ changes in pancreatic β-cells

Stimuli	Culture condition	n	Calcium concentration (nM)		
			Basal	Maximal	Increment
16.7 mM glucose	5.5 mM glucose	45	42.4 ± 2.7	164.3 ± 8.5	121.8 ± 7.7
	16.7 mM glucose	52	42.5 ± 2.2	115.2 ± 7.1*	72.8 ± 6.2*
20 mM Arg	5.5 mM glucose	26	65.9 ± 4.3	229.7 ± 15.8	163.8 ± 13.5
	16.7 mM glucose	20	72.7 ± 5.4	216.1 ± 17.5	143.7 ± 14.3

Values are means ± SE. n, number of observations. Pancreatic β-cells were cultured for 24 h in RPMI-1640 medium containing 5.5 or 16.7 mM glucose. After equilibration with KRBB containing 3.3 mM glucose, the β-cells were challenged with 16.7 mM glucose or 20 mM Arg in the presence of 3.3 mM glucose. The peak $[Ca^{2+}]i$ value observed during the 20 min stimulation is expressed as maximal. The incremental rise in $[Ca^{2+}]i$ is calculated by subtracting the basal value from the peak value in individual observations.
*$P < 0.001$.

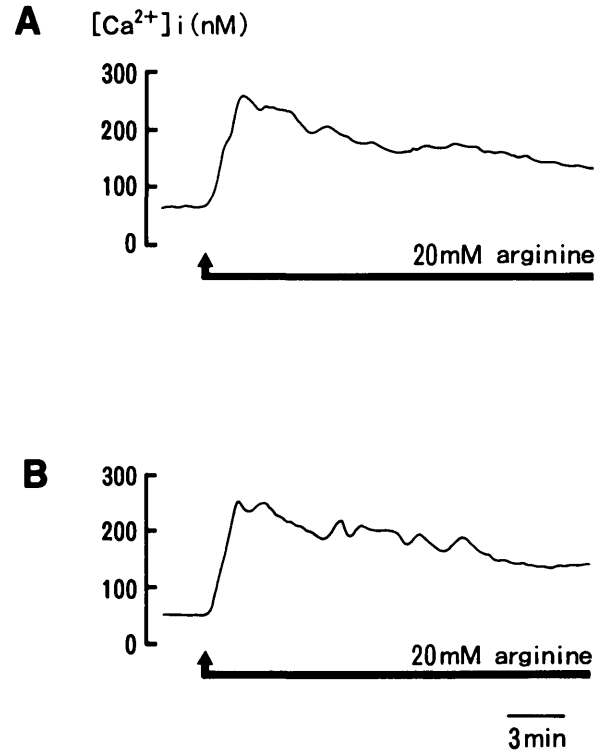

FIG. 4. Effects of 20 mM Arg on [Ca^{2+}]i in single fura 2–loaded β-cells cultured for 24 h either at 5.5 *(A)* or 16.7 mM *(B)* glucose. The traces are representative of 26 and 20 observations, respectively.

In experiment 2, the impairment of the glucose-induced [Ca^{2+}]i rise observed in β-cells cultured at 16.7 mM glucose was restored by the concomitant culture for 24 h with 100 μM diazoxide (Table 3). The integrated [Ca^{2+}]i response to 16.7 mM glucose in β-cells cultured with high glucose plus diazoxide was significantly higher than in β-cells cultured at high glucose alone and was comparable with that in β-cells cultured at 5.5 mM glucose (18.18 ± 0.83, n = 35 vs. 11.53 ± 0.83, n = 25, $P < 0.001$ vs. 19.79 ± 1.87 mg, n = 21, respectively). The population of responsive β-cells to 16.7 mM glucose was not significantly different among β-cells cultured at 5.5 mM glucose or 16.7 mM glucose with or without diazoxide (54% [21 of 39 in 5 experiments] vs. 59% [35 of 59 in 7 experiments] vs. 58% [25 of 43 in 5 experiments]).

In experiment 3, the decreased [Ca^{2+}]i response to glucose observed in β-cells cultured for 24 h at 16.7 mM glucose was reversed by an additional 16-h culture at 5.5 mM glucose (Table 4). The integrated [Ca^{2+}]i also was reversed to a value significantly higher than that in β-cells cultured for 40 h at 16.7 mM glucose throughout (15.70 ± 2.12, n = 16 vs. 8.63 ± 1.48 mg, n = 14, $P < 0.01$). The population of β-cells responsive to 16.7 mM glucose was not significantly different between these islets and β-cells cultured for 40 h at 16.7 mM glucose throughout (50% [16 of 32] vs. 52% [14 of 27] in 3 separate experiments).

DISCUSSION

In this study, we have shown that insulin release from islets exposed for 24 h at 16.7 mM glucose becomes refractive to subsequent acute glucose stimulation, compared with islets maintained at 5.5 mM glucose concentration, and that this impaired insulin response is restored by subsequent 16-h culture at 5.5 mM glucose concentration. This finding confirms that high concentrations of glucose has a deleterious effect on pancreatic β-cell function, referred to as glucose toxicity or desensitization (13,14). In contrast, Arg stimulation elicited similar insulin responses in both groups of islets, and, in absolute values, the insulin release was rather potentiated in islets cultured for 24 h at 16.7 mM glucose. These observations clearly indicate that the impairment of insulin secretion from pancreatic β-cells occurs selectively in response to the glucose, but not to the Arg stimulation. Taken together, these in vitro culture models would provide a tool for better understanding of the details of the intracellular mechanism responsible for the selectively impaired insulin response to glucose in desensitized β-cells.

The rise in cytosolic free Ca concentration ([Ca^{2+}]i) has been shown to play a crucial role in the regulation of insulin secretion from the pancreatic β-cells (16). It would seem, therefore, to be of interest to examine the role of [Ca^{2+}]i in the glucose-induced desensitization. In our study, [Ca^{2+}]i was monitored at a single β-cell level because desensitization has been shown to occur in a pure β-cell preparation (8) and is not related to the paracrine effect in vitro (23). In β-cells cultured for 24 h at

TABLE 3
In vitro effect of diazoxide on glucose-induced [Ca^{2+}]i changes in pancreatic β-cells

Culture condition	n	Calcium concentration (nM)		
		Basal	Maximal	Increment
5.5 mM glucose	21	39.9 ± 3.1	173.5 ± 13.7	133.6 ± 13.3
16.7 mM glucose	25	44.8 ± 2.8	115.6 ± 5.6*	73.8 ± 4.7*
16.7 mM glucose plus 100 μM diazoxide	35	49.6 ± 3.8	187.1 ± 11.7†	137.5 ± 10.9†

Values are means ± SE. n, number of observations. Pancreatic β-cells were cultured for 24 h in RPMI-1640 medium containing glucose and/or diazoxide at indicated concentrations. After equilibration with KRBB containing 3.3 mM glucose, the β-cells were challenged with 16.7 mM glucose. The peak [Ca^{2+}]i value observed during the 20-min stimulation is expressed as maximal. The incremental rise in [Ca^{2+}]i is calculated by subtracting the basal value from the peak value in individual observations.
*$P < 0.001$ vs. 5.5 mM glucose.
†$P < 0.001$ vs. 16.7 mM glucose.

TABLE 4
Reversibility of glucose-induced $[Ca^{2+}]i$ changes in pancreatic β-cells

Culture condition	*n*	Calcium concentration (nM)		
		Basal	Maximal	Increment
16.7 mM glucose for 24 h plus 5.5 mM glucose for 16 h	16	50.7 ± 4.5	160.3 ± 16.8	109.6 ± 15.0
16.7 mM glucose for 40 h	14	50.1 ± 2.4	114.5 ± 9.6*	64.4 ± 9.5*

Values are means ± SE. *n*, number of observations. Pancreatic β-cells cultured for 24 h at 16.7 mM glucose were cultured for an additional 16 h at 5.5 or 16.7 mM glucose. After equilibration with KRBB containing 3.3 mM glucose, the β-cells were challenged with 16.7 mM glucose. The peak $[Ca^{2+}]i$ value observed during the 20-min stimulation is expressed as maximal. The incremental rise in $[Ca^{2+}]i$ is calculated by subtracting the basal value from the peak value in individual observations.
*$P < 0.05$.

16.7 mM glucose, the peak increase and incremental rise in $[Ca^{2+}]i$ evoked by glucose was significantly decreased compared with control cells, although the population of responsive β-cells was the same in both groups of islets. Furthermore, this impaired response was fully reversible by further culture of these islets in normoglycemic medium. Because of the large degree of heterogeneity in the response patterns of glucose stimulation, we examined the integrated $[Ca^{2+}]i$ levels above the basal. This parameter also was impaired in β-cells cultured at a high concentration of glucose and was restored by subsequent exposure of the cells to low glucose. Because a significant correlation exists between changes in $[Ca^{2+}]i$ increase and insulin secretion in desensitized islets, we suggest that the relative diminution in $[Ca^{2+}]i$ increase in response to subsequent 16.7 mM glucose in individual pancreatic β-cells may be, at least in part, responsible for the impairment of GSIR in the desensitized islets of this in vitro model. The measured $[Ca^{2+}]i$ values in this study seem to be lower than those obtained by in vivo calibration (18,19), but the difference in the methodology of calibration might well explain this phenomenon, as has been suggested by Williams et al. (22).

The exact mechanism underlying the defect in the glucose-induced $[Ca^{2+}]i$ rise in β-cells exposed to high glucose remains unclear. It is generally accepted that glucose promotes closure of ATP-sensitive K^+ channels, resulting in depolarization and opening of voltage-dependent Ca^{2+} channels (24). Note that, in this study, both GSIR and the $[Ca^{2+}]i$ response were restored when pancreatic islets were cultured at 16.7 mM glucose with diazoxide, an opener of ATP-sensitive K^+ channels. This agent has been known to hyperpolarize β-cell membranes and to prevent GSIR (25). We speculate, therefore, that β-cell desensitization is the consequence either of excessive stimulation with high concentrations of glucose or of ambient high concentrations of insulin, as has been suggested by Sako et al. (26).

Because the $[Ca^{2+}]i$ response to Arg was not different from control β-cells and because this positively charged amino acid has been suggested to exert its insulinotropic effect mainly by causing depolarization of the plasma membrane with subsequent gating of voltage-dependent Ca^{2+} channels (27), the voltage dependency of this Ca^{2+} channel might not be affected in desensitized β-cells. This notion might be supported further by a previous report that in glucose-responsive rat insulinoma cells maintained in culture, the apparent number of channels and their affinity for the Ca^{2+} channel-blocker nitrendipine was shown not to be affected by the glucose concentration of the culture medium (5 and 30 mM), even though the binding capacity for nitrendipine is significantly decreased under these culture conditions, compared with those in freshly isolated cells (28). However, the voltage-dependent Ca^{2+} currents that are resistant to the Ca^{2+} channel-blocker nimodipine have been reported to be present in insulin secreting cells (29,30). Thus, further studies are required to ascertain whether alterations of the Ca^{2+} channel activity are responsible for the mechanisms of β-cell desensitization. Failure of closure of the ATP-sensitive K^+ channel might explain the decrease in $[Ca^{2+}]i$ rise in the desensitized islets because the decline of ^{86}Rb efflux has been shown to be significantly impaired in islets maintained at high glucose concentrations (9), which could result in the reduction of both membrane depolarization and a subsequent activation of voltage-dependent Ca^{2+} channels. In contrast to the glucose stimulation, the insulin response to Arg was rather potentiated in desensitized islets, whereas the $[Ca^{2+}]i$ rise was evoked equally when compared with control islets. Possibly, therefore, the calcium-activated intracellular mechanisms for the transfer of insulin secretory granules and their exocytosis may be somewhat enhanced to compensate for the impaired $[Ca^{2+}]i$ rise induced by glucose.

Glucose also is known to accelerate membrane phosphoinositide hydrolysis, resulting in the generation of diacylglycerol and inositol trisphosphate (31). GSIR and phosphoinositide hydrolysis reportedly are impaired in a parallel fashion by chronic hyperglycemia both in vivo (32) and in vitro (33). These observations might suggest that Ca release from the endoplasmic reticulum also is affected by the decreased generation of Ca-mobilizing inositol trisphosphate in these desensitized β-cells.

In summary, this study shows clearly that the insulin secretion from islets exposed to high glucose concentrations is affected selectively in response to further glucose stimulation and is reversible by subsequent culture in a normoglycemic medium. We also have shown that this phenomenon correlates well with changes in the elevation of $[Ca^{2+}]i$ in individual β-cells. Whether the decreased insulin response seen in patients with NIDDM is a reflection of diminished $[Ca^{2+}]i$ response in pancreatic

β-cells as observed in this experiment requires further examination.

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REFERENCES

1. Pfeifer MA, Halter JB, Porte D Jr: Insulin secretion in diabetes mellitus. *Am J Med* 70:579–88, 1981
2. Robertson RP, Porte D Jr: The glucose receptor: a defective mechanism in diabetes mellitus distinct from the beta adrenergic receptor. *J Clin Invest* 52:870–76, 1973
3. Kosaka K, Kazuya T, Akanuma Y, Hagura R: Increase in insulin response after treatment of overt maturity-onset diabetes is independent of the mode of treatment. *Diabetologia* 18:23–28, 1980
4. Rossetti L, Gerald IS, Zawalich W, DeFronzo RA: Effect of chronic hyperglycemia on in vivo insulin secretion in partially pancreatectomized rats. *J Clin Invest* 80:1037–44, 1987
5. Leahy JL, Cooper HE, Weir GC: Impaired insulin secretion associated with near normoglycemia: study in normal rats with 96-h in vivo glucose infusions. *Diabetes* 36:459–64, 1987
6. Giriox MH, Portha B, Kergoat M, Bailbe D, Picon L: Glucose insensitivity and amino-acid hypersensitivity of insulin release in rats with non-insulin-dependent diabetes: a study with the perfused pancreas. *Diabetes* 32:445–51, 1983
7. Tsuji K, Taminato T, Usami M, Ishida H, Kitano N, Fukumoto H, Koh G, Kurose T, Yamada Y, Yano H, Seino Y, Imura H: Characteristic features of insulin secretion in the streptozotocin-induced NIDDM rat model. *Metabolism* 37:1040–44, 1988
8. Purrello F, Vetri M, Gatta C, Gullo D, Vigneri R: Effects of high glucose on insulin secretion by isolated rat islets and purified β-cells and possible role of glycosylation. *Diabetes* 38:1417–22, 1989
9. Purrello F, Vetri M, Vinci C, Gatta C, Buscema M, Vigneri R: Chronic exposure to high glucose and impairment of K^+-channel function in perifused rat pancreatic islets. *Diabetes* 39:397–99, 1990
10. Davalli AM, Ricordi C, Socci C, Braghi S, Bertuzzi F, Fattor B, Carlo VD, Pontiroli AE, Pozza G: Abnormal sensitivity to glucose of human islets cultured in a high glucose medium: partial reversibility after an additional culture in a normal glucose medium. *J Clin Endocrinol Metab* 72:202–208, 1991
11. Bolaffi JL, Heldt A, Lewis LD, Grodsky GM: The third phase of in vitro insulin secretion: evidence for glucose insensitivity. *Diabetes* 35: 370–73, 1986
12. Grodsky GM: A new phase of insulin secretion: how will it contribute to our understanding of β-cell function? *Diabetes* 38:673–78, 1989
13. Rossetti L, Giaccari A, DeFronzo RA: Glucose toxicity. *Diabetes Care* 13:610–30, 1990
14. Robertson RP: Type II diabetes, glucose "Non-Sense," and islet desensitization. *Diabetes* 38:1501–505, 1989
15. Unger RH, Grundy S: Hyperglycaemia as an inducer as well as a consequence of impaired islet cell function and insulin resistance: implications for the management of diabetes. *Diabetologia* 28:119–21, 1985
16. Prentki M, Matschinsky FM: Ca^{2+}, cAMP and phospholipid-derived messengers in coupling mechanisms of insulin secretion. *Physiol Rev* 67:1185–1248, 1987
17. Sussman KE, Leitner JW, Draznin B: Cytosolic free-calcium concentrations in normal pancretic islet cells: effect of secretagogues and somatostatin. *Diabetes* 36:571–77, 1987
18. Pralong WF, Bartley C, Wollheim CB: Single islet β-cell stimulation by nutrients: relationship between pyridine nucleotides, cytosolic Ca^{2+} and secretion. *EMBO J* 9:53–60, 1990
19. Longo EA, Tornheim K, Deeney JT, Varnum BA, Tillotson D, Prentki M, Corkey BE: Oscillations in cytosolic free Ca^{2+}, oxygen consumption, and insulin secretion in glucose-stimulated rat pancreatic islets. *J Biol Chem* 266:9314–19, 1991
20. Gotoh M, Maki T, Kiyoizumi T, Satomi S, Monaco AP: An improved method for isolation of mouse pancreatic islets. *Transplantation* 40:437–41, 1985
21. Pipeleers DG, In't Veld PA, Van de Winkel M, Maes E, Schuit FC, Gepts W: A new in vitro model for the study of pancreatic A and B cells. *Endocrinology* 117:806–17, 1985
22. Williams DA, Fogarty KV, Tsien RY, Fay FS: Calcium gradients in single smooth muscle cells revealed by the digital imaging microscope using Fura-2. *Nature (Lond)* 318:558–61, 1985
23. Bolaffi JL, Rodd G, Ma Y, Grodsky GM: Effect of glucagon or somatostatin on desensitized insulin secretion. *Endocrinology* 126: 1750–55, 1990
24. Arkhammar P, Nilsson T, Rorsman P, Berggren PO: Inhibition of ATP-regulated K^+ channels precedes depolarization-induced increase in cytoplasmic free Ca^{2+} concentration in pancreatic β-cells. *J Biol Chem* 262:5448–54, 1987
25. Trube G, Rorsman P, Shosaku O: Opposite effect of tolbutamide and diazoxide on the ATP dependent K^+ channel in mouse pancreatic β-cells. *Pflüegers Arch Eur J Physiol* 407:493–99, 1986
26. Sako Y, Grill VE: Coupling of β-cell desensitization by hyperglycemia to excessive stimulation and circulating insulin in glucose-infused rats. *Diabetes* 39:1580–83, 1990
27. Blachier F, Mourtada A, Sener A, Malaisse WJ: Stimulus-secretion coupling of arginine-induced insulin release: uptake of metabolized and nonmetabolized cationic amino acids by pancreatic islets. *Endocrinology* 124:134–41, 1989
28. Hoenig M, Russo LL, Ferguson DC: Characterization of calcium channels of a glucose-responsive rat insulinoma. *Am J Physiol* 256:E488–93, 1989
29. Hopkins WM, Satin LS, Cook DL: Inactivation kinetics and pharmacology distinguish two calcium currents in mouse pancreatic β-cells. *J Membr Biol* 119:229–39, 1991
30. Satin LS, Cook DL: Calcium current inactivation in insulin-secreting cells is mediated by calcium influx and membrane depolarization. *Pflügers Arch Eur J Physiol* 414:1–10, 1989
31. Best L, Malaisse WJ: Nutrient and hormone-neurotransmitter stimuli induce hydrolysis of polyphosphoinositides in rat pancreatic islets. *Endocrinology* 115:1814–20, 1984
32. Zawalich WS, Zawalich KC, Shulman GI, Rossetti L: Chronic in vivo hyperglycemia impairs phosphoinositide hydrolysis and insulin release in isolated perifused rat islets. *Endocrinology* 126:253–60, 1990
33. Zawalich WS, Zawalich KC, Rasmussen H: The conditions under which rat islets are labeled with [^{3}H]inositol alter the subsequent responses of these islets to a high glucose concentration. *Biochem J* 259:743–49, 1989