A Defect Late in Stimulus-Secretion Coupling Impairs Insulin Secretion in Goto-Kakizaki Diabetic Rats

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A widely accepted genetically determined rodent model for human type 2 diabetes is the Goto-Kakizaki (GK) rat; however, the lesion(s) in the pancreatic islets of these rats has not been identified. Herein, intact islets from GK rats (aged 8–14 weeks) were studied, both immediately after isolation and after 18 h in tissue culture. Despite intact contents of insulin and protein, GK islets had markedly deficient insulin release in response to glucose, as well as to pure mitochondrial fuels or a non-nutrient membrane-depolarizing stimulus (40 mmol/l K^+). In contrast, mastoparan (which activates GTP-binding proteins [GBPs]) completely circumvented any secretory defect. Basal and stimulated levels of adenine and guanine nucleotides, the activation of phospholipase C by Ca^{2+} or glucose, the secretory response to pertussis toxin, and the activation of selected low–molecular weight GBPs were not impaired. Defects were found, however, in the autophosphorylation and catalytic activity of cytosolic nucleoside diphosphokinase (NDPK), which may provide compartmentalized GTP pools to activate G-proteins; a deficient content of phosphoinositides was also detected. These studies identify novel, heretofore unappreciated, defects late in signal transduction in the islets of our colony of GK rats, possibly occurring at the site of activation by NDPK of a mastoparan-sensitive G-protein–dependent step in exocytosis. *Diabetes* 48:1754–1762, 1999

The lesion intrinsic to the pancreatic β-cell in human type 2 diabetes is unknown. In the search to identify such a defect, animal models of diabetes have frequently been studied. The most widely accepted of these models is the Goto-Kakizaki (GK) rat, developed by repeated inbreeding of glucose-intolerant Wistar rats (1). Some investigators have considered the possibility (2–9) that the defect in the GK rat islet might lie in the glycerol phosphate shuttle (and possibly other hydrogen ion shuttles), which transports reducing equivalents from the cytosol to the mitochondria, where they donate electrons to the electron transport chain, promoting the synthesis of ATP. Furthermore, some studies have suggested that glucose as an agonist is selectively ineffective (3) in GK rats, compatible with a defect in the glycolytic cascade, which leads to the production of reducing equivalents in the cytosol. Therefore, it has been predicted (10) that glucose-stimulated ATP production might be deficient in the GK rat islet, leading to an inability of glucose to close plasma membrane ATP-sensitive K^+ channels, which in turn permits calcium to influx from the extracellular space. However, extant studies of ATP metabolism in GK rat islets are somewhat limited. In addition, some investigators (4,11) have failed to find that the defect in the GK islet is specific for glucose, but rather have suggested that it might lie in more distal steps in nutrient metabolism (i.e., a mitochondrial defect) or even in a step not specifically involving fuel metabolism. Furthermore, some studies have not found evidence of a causative role for the expression of mitochondrial glycerol-3-phosphate dehydrogenase in GK islets (6,12) or have suggested that any such defect might be secondary to the diabetic milieu in vivo (7).

Several other recent reports indicated that the insulin secretory response to (maximal) elevations in intracellular calcium concentration ($[Ca^{2+}_i]$) in permeabilized GK islets was normal, or even augmented (13,14); furthermore, the rise in $[Ca^{2+}_i]$ induced by β-cell agonists was largely intact, albeit delayed in one study (15). These reports might be interpreted to indicate that (one of) the primary defect(s) might lie distal to Ca^{2+} fluxes but proximal to the final calcium-mediated steps in exocytosis. Some experimental support for this has been presented by Abdel-Halim et al. (16). Because ATP is also necessary at late steps in stimulation-secretion coupling (17), deficient availability of adenine nucleotides might again be invoked to explain the GK defect. Recently, GTP has also been implicated as a distal cofactor in physiologic insulin secretion (18,19), but its availability in GK islets has not been formally assessed.

Therefore, the current study was designed to systematically assess several significant biochemical steps in signal transduction in the pancreatic islet, which might explain some of the secretory findings. Both freshly isolated and overnight-cultured islets were studied. In view of some reports of decreased intra-islet insulin content in the GK rat (15,20–24), we have assessed insulin content and excluded this as a relevant parameter.

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$[Ca^{2+}_i]$, intracellular calcium concentration; GBP, GTP-binding protein; Mas-17, mastoparan-17; NDPK, nucleoside diphosphokinase; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; Ptx, pertussis toxin.

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RESEARCH DESIGN AND METHODS

Materials. Sources of most materials used are itemized in previous publications from this laboratory (18,19,25–27). Pertussis toxin (Ptx) was obtained from List Biologicals (Campbell, CA). Mastoparan and mastoparan-17 (Mas-17) were purchased from Peninsula Labs (Belmont, CA). [^{3}H-methyl]methionine (70 Ci/mmol), [^{3}H(G)]hypoxanthine (17 Ci/mmol), S-adenosyl-[^{3}H-methyl]methionine (73 Ci/mmol), and [^{3}H]GDP (9.7 Ci/mmol) were purchased from Dupont-NEN (Boston, MA). Myo-[2-^{3}H]inositol (7.3 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Antisera directed against low–molecular mass GTPases were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Cdc42 (epitope corresponding to amino acid residues 166–182 near the COOH-terminus of Cdc42) is specific for Cdc42 and is non–cross-reactive with Rac1, Rac2, and other Ras-related GTPases. Anti-Rap1 (epitope corresponding to amino acid residues 121–136 of Rap1A) is specific for Rap1A and is nonreactive with Rap2. Antisera directed against various α-subunits (e.g., G_o, G_q, G_i) and the common β-subunit of G-proteins were obtained from Dupont-NEN. Specificity of these antisera has been described in our earlier publications (28).

Animals. GK rats were generously provided by Dr. Robert Farese (Tampa, FL). They were provided to our laboratory at the age of 8 weeks and were then housed in our animal care facility. Offspring were allowed to reach the ages of 8–14 weeks, when they were studied in parallel with age- and sex-matched Wistar rats (Harlan, Indianapolis, IN). The original colony of rats from which Dr. Farese's GK rodents were obtained was the colony studied by Drs. Suzuki and Toyota, Tohoku University (Sendai, Japan). Rats were housed in 12 × 14 × 12 inch cages, separated by sex; they were fed ad libitum (Purina 5008 lab chow; Purina, Richmond, IN). Blood samples were obtained by heart puncture after pentobarbital anesthesia and before the rats were killed.

Islet isolation. Islets were isolated from GK and control (Wistar) rats using previously published procedures (18,19,25–28). The following modifications were incorporated. Pancreases were inflated with collagenase solution (1 U/ml for Wistar rats, 2 U/ml for GK rats) in Hank's balanced salt solution supplemented with 1% fetal bovine serum. A first digestion period lasted 18 min; the pancreases were vigorously shaken for 20 s every 2 min. This was followed by a second digestion using half-strength collagenase, lasting for 16 min and involving vigorous shaking for 20 s every 2 min. Islets were washed twice, passed through mesh (92-µm pore size), and then purified on a Ficoll gradient. Islets were then hand-picked twice under stereomicroscopic control to exclude extra-islet debris. Some previous studies have indicated that a subpopulation of GK islets may appear misshapen because of increased fibrosis (1,23,29); we made certain to isolate islets with grossly normal size and appearance to minimize any extrinsic artifacts of the isolation procedure and the abnormal pancreatic morphology.

In some studies, control and GK islets were cultured overnight, as previously described (19,25,30), in RPMI 1640 medium (11.1 mmol/l glucose). The next day, these islets were harvested and studied.

Experimental and analytic procedures

Insulin secretion and content. Insulin content was assessed after extraction of the islets, as previously described (18,25). After a 45-min preincubation period in the absence of stimuli, insulin secretion into the medium was assessed in static 45-min incubations of 10 intact islets per tube, as previously described (18,19,25–27), in response to specific agonists, as described in RESULTS. To test the effects of Ptx, rats were pretreated with vehicle or Ptx (1.5 µg i.p. injected at 48 h, 24 h, and immediately before removal of the pancreas), followed by addition of 50 ng/ml Ptx to the overnight culture medium.

Purine nucleotides. Islets were cultured in groups of 150 islets as described (25). The next day, islets were transferred to siliconized tubes; culture medium was removed; and the purine nucleotide contents of islets were assessed basally (at 3.3 mmol/l glucose) and in response to acute glucose (16.7 mmol/l) stimulation. Stimulation was terminated by the rapid removal of medium, followed by a rapid wash in 1 ml of fresh ice-cold medium and then addition of ice-cold 10% trichloroacetic acid. Nucleotides were assessed after islet extraction, as previously described (25,26). Quantification was carried out in two ways: *1*) mass measurements of nucleotide content, using high-performance liquid chromatography coupled with ultraviolet spectroscopy, and *2*) via incorporation of the radiolabeled purine hypoxanthine (2 µCi/ml), provided overnight. This approach, to prelabel purine pools to assess new synthesis, has been previously described (26).

Phospholipase C and phosphoinositide substrates. The activation of phospholipase C (PLC) was assessed, as previously described in detail (27), during perifusions of intact rat islets (100 per tube) that had been prelabeled for 18 h with [^{3}H]inositol (25 µCi/ml in RPMI 1640 medium). We have previously documented (27) that such assessments represent a specific index of PLC activation, as verified by intracellular accumulation of inositol phosphates and degradation of phosphoinositides. Under such conditions, phosphatidylinositol (PI) 4-phosphate (PIP) and PI 4,5-bisphosphate (PIP_2) are labeled to full isotopic equilibrium, and PI is labeled to 75% of isotopic steady state; furthermore, in our previous studies, extending the labeling period to 50 h (such that even PI reached equilibrium labeling) did not materially change the assessments of PLC in intact rat islets. Measurements of phosphoinositides were carried out by similarly prelabeling control or experimental islets, followed by the extraction of their phospholipid content before any further experimental manipulation, as previously described, using thin-layer chromatography (27,31).

GTP-binding proteins. Our previous studies (28) suggested that specific low–molecular mass GTP-binding proteins (GBPs) have a requisite role in physiologic insulin release: specifically, Rho family proteins (especially Cdc42 and/or Rac), as well as Rap, have been implicated. To assess their function, such proteins were first assessed (for their presence and abundance) by use of immunoblots, as previously described (28). In addition, the activation of these GBPs was assessed via measurements of their carboxyl methylation. The latter is dependent on the availability of sufficient intracellular GTP and can be triggered by stimulation using a high glucose concentration (16.7 mmol/l). After such stimulation for 15–30 s, desired proteins were immunoprecipitated using specific antisera, and the degree of carboxyl methylation was assessed by vapor-phase equilibration assay (28). GTP-sensitive carboxyl methylation of GBPs was assessed in homogenates after incubation for 45 min in the absence or presence of 100 µmol/l GTPγS.

To examine the possibility that an inhibitory heterotrimeric GBP (such as G_i or G_o) was overactive in the GK rats, the responses to pretreatment with Ptx were assessed as described above. Finally, the islet responses to mastoparan, which activates many low– as well as high–molecular mass GBPs by stimulating GTP-for-GDP exchange (32), were assessed.

Nucleoside diphosphokinase. Nucleoside diphosphokinase (NDPK) activity is substantial in islet homogenates (33,34). It has been speculated (35) that NDPK might be responsible for converting GDP, via its transphosphorylation activity, to the active guanine nucleotide GTP on (or in the immediate vicinity of) specific GBPs. Thus, NDPK may activate a specific pool of relevant GBPs, even though the total cellular pool of GTP might not be changed detectably. Therefore, we assessed the activity of NDPK, as well as its autophosphorylation, on histidine residues, as previously described (33,34); this phosphorylation is an obligate step in the subsequent activation of NDPK enzymic activity. NDPK catalytic activity was assayed in homogenates and individual fractions (33,34) as the formation of [^{3}H]GTPγS from [^{3}H]GDP and ATPγS. The quantitation of NDPK phosphoenzyme formation was carried out in the presence of either [^{32}P]ATP or GTP as described in (33,34). Since the phosphohistidine of NDPK is heat labile, the phosphorylated samples were incubated in sample buffer at room temperature for 30 min before SDS-PAGE. To avoid removal of the acid-labile phosphohistidine, fixation times were minimized to 15 min, and gels were subsequently dried at room temperature. Labeled proteins were identified by autoradiography (33,34,36).

Subcellular fractionation of pancreatic islets. In some experiments, homogenates of Wistar and GK rat islets were spun at 105,000*g* for 90 min (Beckman Ultima TL-100; Beckman-Coulter, Fullerton, CA) to obtain total particulate (pellet) and soluble (supernatant) fractions.

Protein content. Protein concentrations in samples were determined by Bio-Rad (Hercules, CA) assay using bovine serum albumin as standard (33).

Statistical methods. Data are expressed as means ± SE for n experiments or determinations, as indicated. For single comparisons, t or Mann-Whitney testing was used as appropriate. For multiple comparisons, analysis of variance was used, followed by post hoc analysis as appropriate. Significance was taken at $P < 0.05$.

RESULTS

The animal model: insulin secretion. GK rats had consistently higher plasma glucose concentrations in the fed state compared with age- and sex-matched Wistar control rats, and these glycemic indices were stable throughout the study (Table 1). However, plasma insulin values were identical in control and GK rats, indicating inadequate insulin secretion by GK rats in the face of marked hyperglycemia. The weights of 11- to 14-week-old GK rats were 20% less than those of age-matched Wistar rats.

When islets were freshly isolated from such animals and studied during static incubations, the insulin response to glucose was, as expected, markedly impaired, especially at higher glucose concentrations (Fig. 1), with an apparent shift to the right in glucose dose/insulin–response curve. Interestingly, however, both basal release rates and the secretory responses to a pure mitochondrial fuel, succinic acid methyl ester (20 mmol/l), were also significantly depressed, as was the response to a depolarizing concentration (40 mmol/l) of

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TABLE 1
Characteristics of control (Wistar) and GK rats

	Control	GK
Weight (g)	320 ± 87 (24)	256 ± 49 (29)*
Plasma glucose (mg/dl)		
Initial	226 ± 33 (28)	429 ± 59 (19)
After 12 months' propagation	248 ± 16 (4)	458 ± 43 (39)
After 17 months' propagation	228 ± 58 (16)	513 ± 92 (24)
Plasma insulin (μU/ml)	51 ± 31 (17)	51 ± 33 (17)

Data are means + SD (n). Rats were 11–14 weeks old. Plasma glucose and insulin values are from nonfasting rats (fed ad libitum). Blood was obtained by heart puncture. *P = 0.031 compared with control rats.

potassium, both being studied at 3.3 mmol/l glucose (Fig. 2). In preliminary studies, the insulin response to ketoisocaproate (15 mmol/l) was similarly impaired in each of three experiments (to 60 ± 5% of control). The insulin content of freshly isolated GK islets was unaltered compared with control (Table 2). After overnight culture, insulin secretory responses remained markedly impaired in GK islets (Fig. 3). Insulin content was actually greater in GK islets than in control islets after culture (Table 2), indicating that an even more dramatic impairment of fractional insulin secretion existed.

In stark contrast, the insulin secretory responses to mastoparan (30 μmol/l), an activator of GBPs by virtue of promoting GTP-for-GDP exchange (32,34), were not only intact, but in fact were exaggerated in GK islets (Fig. 4), both at very low glucose concentrations (1.7 mmol/l; Fig. 4) and at a slightly higher level (3.3 mmol/l), at which the response to mastoparan in GK rat islets was approximately twice that of control islets (data not shown). Mas-17, a structural analog of mastoparan that does not activate GBPs (34), had no effect in control or GK islets (Fig. 4). Pretreatment with Ptx did not significantly reduce mastoparan-induced insulin secretion in Wistar control islets or in GK islets (not shown).

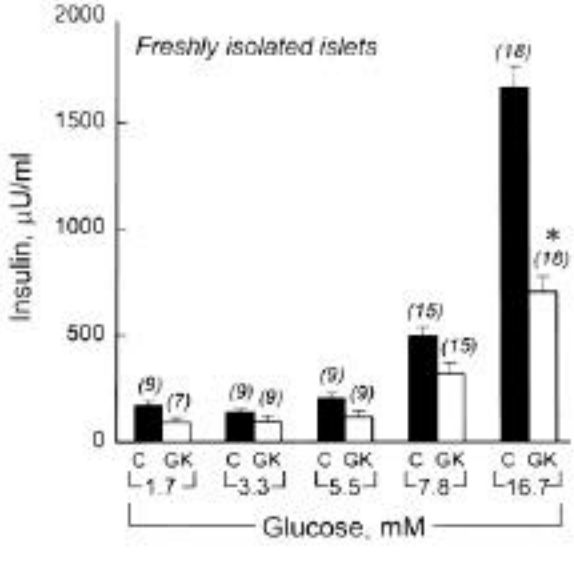

FIG. 1. Insulin secretory responses to increasing concentrations of glucose in freshly isolated control Wistar (C) compared with GK rat islets. Numbers in parentheses represent the number of determinations. *P < 0.05.

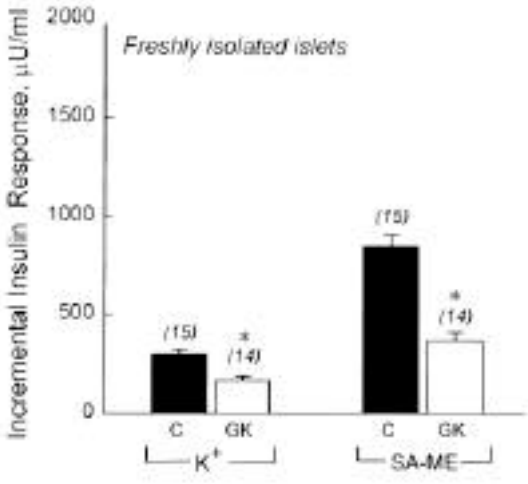

FIG. 2. Incremental insulin responses to 40 mmol/l K^+ or 20 mmol/l succinic acid methyl ester (SA-ME) in freshly isolated Wistar (C) compared with GK rat islets. Data are pooled from three independent experiments. Numbers in parentheses represent number of determinations. *P < 0.05.

To determine whether overactivity of a Ptx-sensitive inhibitory trimeric GBP could explain the reduction of insulin secretion in GK rats, the effects of Ptx were explored further in overnight-cultured islets (Table 3), with results expressed as fractional secretion because Ptx reduced insulin content throughout the study (not shown). Glucose-induced secretion (in the absence of Ptx) was reduced by 68% in GK islets. Ptx augmented glucose-induced (16.7 mmol/l) secretion in both control and GK islets by nearly identical increments (Table 3); however, glucose-induced insulin secretion remained blunted in the GK islets. Thus, Ptx improved insulin secretion in GK islets but did not reverse the secretory defect. At 1.7 mmol/l glucose, mastoparan again tended (albeit nonsignificantly in this particular experiment) to elicit greater secretion in GK islets, and this increment was

TABLE 2
Insulin and protein content of Wistar (control) compared with GK islets

	Control	GK
Insulin content		
Freshly isolated islets (μU/islet)		
1.7 mmol/l glucose	1,806 ± 96 (24)	1,750 ± 119 (23)
Cultured islets		
1.7 mmol/l glucose	1,282 ± 51 (6)	1,739 ± 103 (6)*
3.3 mmol/l glucose	1,372 ± 135 (10)	2,055 ± 39 (10)*
16.7 mmol/l glucose	1,218 ± 47 (23)	1,830 ± 41 (23)*
1.7 mmol/l glucose + 40 mmol/l K^+	1,274 ± 66 (8)	1,729 ± 63 (8)*
3.3 mmol/l glucose + 40 mmol/l K^+	993 ± 46 (8)	1,802 ± 62 (8)*
Protein content (μg protein/islet)	0.44 ± 0.02 (58)	0.42 ± 0.02 (22)

Data are means ± SE (n determinations), each representing the content of one islet. Insulin content was measured after a 45-min incubation period in the presence of the additions listed. For protein content, islets had been cultured overnight. *Difference is statistically significant at the level of $P \leq 0.003$.

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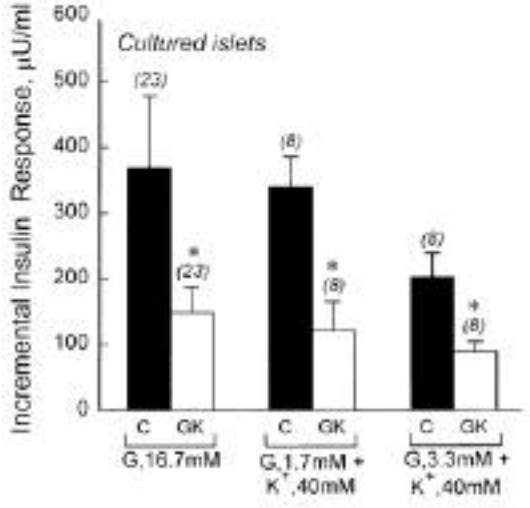

FIG. 3. Incremental insulin responses to 16.7 mmol/l glucose or 40 mmol/l K^+ (at 1.7 or 3.3 mmol/l glucose) in overnight-cultured control Wistar (C) compared with GK rat islets. Numbers in parentheses represent number of determinations. *P < 0.05.

actually exaggerated by Ptx. Thus, when the influence of inhibitory trimeric GBPs (which are also activated by mastoparan) were abrogated by Ptx, an even greater accentuation of mastoparan-induced secretion was seen in GK islets compared with controls (Table 3). Note that in contrast to Sprague-Dawley rats (37), Ptx did not reduce mastoparan-induced secretion from Wistar control rats.

Purine nucleotide metabolism. The time course of the purine nucleotide responses to stimulation by high glucose was first ascertained using Wistar (control) rats (Fig. 5). ATP/ADP and GTP/GDP ratios were significantly elevated by glucose by 15–30 s, with the elevations being maintained from 1 through 30 or 60 min. Absolute values for ATP rose in response to high glucose by 1 min and all points thereafter,

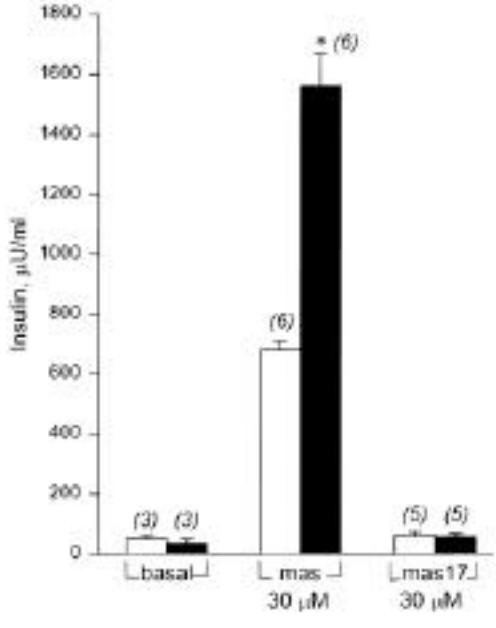

FIG. 4. Insulin secretory responses to 30 µmol/l mastoparan or 30 µmol/l Mas-17 in islets from control Wistar (□) compared with GK (■) rat islets during 45-min incubations at 1.7 mmol/l glucose. *P < 0.05.

TABLE 3
Effects of Ptx on mastoparan-induced fractional insulin release

	Fractional insulin release (% per 45 min)		
	Wistar	GK	P
Mastoparan			
30 µmol/l (1.7 mmol/l glucose)	9.62 ± 0.48	11.59 ± 1.05	
30 µmol/l + Ptx (1.7 mmol/l glucose)	8.85 ± 1.03	13.78 ± 0.54	0.005
Glucose			
16.7 mmol/l	3.75 ± 0.16	1.19 ± 0.14	<0.001
16.7 mmol/l + Ptx	7.54 ± 0.17	5.80 ± 0.32	0.003

Data are means ± SE. n = 4 determinations each. Ptx-induced increments at 16.7 mmol/l glucose were 3.79 ± 0.16 in control islets and 4.61 ± 0.32 in Wistar islets (% per 45 min, NS).

whereas a significant response in GTP was delayed compared with ATP (not shown). Based on these studies, the 1- and 15-min time points were chosen for comparison of Wistar and GK islets to sample both early and more sustained time points. Since β-cell secretory granules contain an inert, but releasable, pool of purine nucleotides (38), it is conceivable that abnormalities in insulin secretory dynamics in GK islets might perturb, or even mask, abnormalities in these GTP or ATP measurements. However, epinephrine (1 µmol/l), which totally obliterated glucose-induced insulin

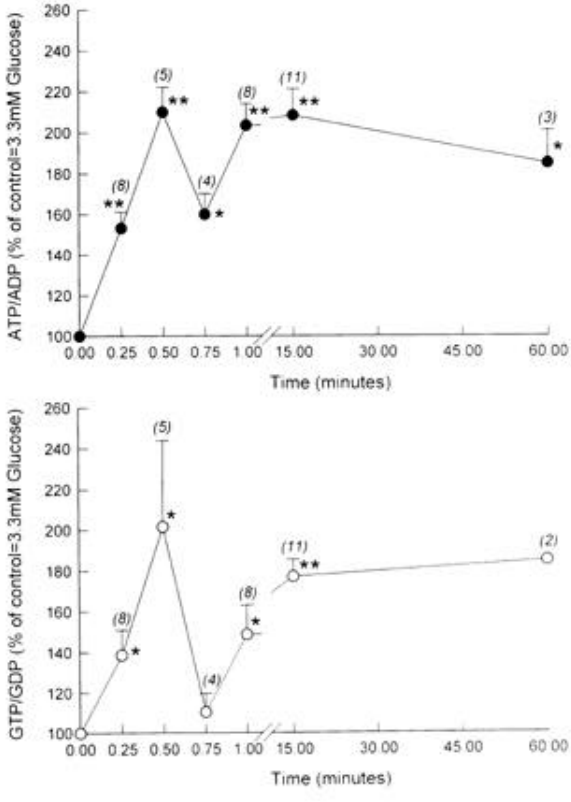

FIG. 5. Time course of ATP/ADP and GTP/GDP ratios in Wistar rat islets exposed to 16.7 mmol/l glucose and expressed as percent of control values (at identical time points, but in the presence of 3.3 mmol/l glucose). Data are taken from nine experiments. *P < 0.05 vs. control; **P < 0.01 vs. control.

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TABLE 4
Glucose stimulation of adenine and guanine nucleotides in GK and Wistar rats: mass measurements

	ATP (pmol/islet)	ATP/ADP ratio	ATP/ADP ratio % of control	GTP (pmol/islet)	GTP/GDP ratio	GTP/GDP ratio % of control
Wistar						
1 min						
3.3 mmol/l glucose	11.48 ± 0.81 (9)	14.10 ± 1.5 (9)	100 ± 2 (9)	2.51 ± 0.28 (9)	12.8 ± 3.2 (7)	100 ± 2 (7)
16.7 mmol/l glucose	12.85 ± 0.97 (9)	23.00 ± 1.17 (9)*	180 ± 20 (9)*	2.80 ± 0.32 (9)	15.3 ± 2.26 (8)	144 ± 15 (8)*
15 min						
3.3 mmol/l glucose	16.74 ± 1.18 (8)	7.04 ± 0.94 (8)	100 ± 2 (8)	4.26 ± 0.32 (8)	5.46 ± 1.04 (8)	100 ± 4 (8)
16.7 mmol/l glucose	18.60 ± 0.66 (8)	12.95 ± 1.75 (8)*	185 ± 7 (8)*	4.64 ± 0.18 (8)	8.01 ± 2.03 (8)	137 ± 9 (8)*†
GK						
1 min						
3.3 mmol/l glucose	10.51 ± 0.81 (9)	15.00 ± 1.3 (9)	100 ± 4 (9)	2.48 ± 0.27 (9)	12.4 ± 2.29 (9)	100 ± 5 (9)
16.7 mmol/l glucose	11.56 ± 1.01 (9)	21.30 ± 0.75 (9)*	150 ± 15 (9)*	2.65 ± 0.29 (9)	14.4 ± 2.47 (8)	117 ± 12 (8)
15 min						
3.3 mmol/l glucose	15.36 ± 0.44 (9)	5.40 ± 0.6 (9)	100 ± 3.8 (9)	3.98 ± 0.14 (9)	5.05 ± 0.82 (9)	100 ± 3 (9)
16.7 mmol/l glucose	17.33 ± 0.64 (8)	10.72 ± 1.5 (8)*	207 ± 12 (8)*	4.33 ± 0.17 (8)	9.28 ± 1.86 (8)	176 ± 11 (8)*

Data are means ± SE (n). Islets were preincubated for 15 min at 3.3 mmol/l glucose and then incubated for 1 or 15 min at 3.3 or 16.7 mmol/l glucose. n represents the number of determinations from three separate experiments. *$P < 0.05$ for 3.3 compared with 16.7 mmol/l glucose; †$P < 0.05$ for GK compared with Wistar islets.

release at 15 min (data not shown), failed to significantly alter ATP or GTP contents or ATP/ADP or GTP/GDP ratios at either low or high glucose concentrations. For example, ATP/ADP and GTP/GDP ratios rose, respectively, from 4.37 ± 0.04 and 4.63 ± 0.34 at 3.3 mmol/l glucose to 9.41 ± 1.11 and 13.75 ± 0.97 at 16.7 mmol/l glucose in control islets; in the concurrent presence of epinephrine, these values were 4.32 ± 0.08, 4.97 ± 0.55, 10.03 ± 2.01, and 13.17 ± 1.98 (all changes NS). Thus, our measurements of the purine nucleotide seem physiologically relevant.

As in normal Sprague-Dawley rats (26), [^{3}H]hypoxanthine was briskly incorporated into adenine and guanine nucleotides in both Wistar and GK islets, indicating an intact purine nucleotide salvage pathway in both groups. The basal ATP content, ATP/ADP ratio, GTP content, and GTP/GDP ratio (expressed in terms of mass and/or labeling and specific activity) were generally similar in GK rats compared with Wistar controls after 1- or 15-min incubations (Tables 4 and 5). Absolute peak values achieved after stimulation using 16.7 mmol/l glucose were also comparable, as were those expressed as a percentage of values at low glucose, although they appeared to be somewhat delayed in GK rats, reaching slightly higher levels at 15 min rather than at 1 min. Islet contents of the pyrimidine nucleotide UTP were also similar after 15 min of incubation at both low and high glucose concentrations (control at low glucose = 2.44 ± 0.21 pmol/islet, control at high glucose = 3.02 ± 0.17 pmol/islet, $P < 0.05$; GK at low glucose = 2.06 ± 0.12 pmol/islet, GK at high glucose = 2.78 ± 0.13 pmol/islet, $P < 0.05$; n = 8–9 determinations each), although both values were slightly reduced (–25%; $P < 0.05$) in GK rats at 1 min.

PLC. In the pancreatic islet, PLC is glucose-stimulated and Ca^{2+}-influx dependent (27,30). The stimulation of PLC in GK islets was entirely normal (both temporally and quantita-

TABLE 5
Glucose stimulation of adenine and guanine nucleotides in GK and Wistar rats: radiolabeling experiments

	GK		Wistar	
	dpm	Specific activity	dpm	Specific activity
ATP				
3.3 mmol/l glucose	1,167 ± 116 (9)	76 ± 7 (9)	1,195 ± 130 (8)	75 ± 10 (8)
16.7 mmol/l glucose	1,414 ± 186 (8)	81 ± 10 (8)	1,342 ± 160 (8)	73 ± 9 (8)
ADP				
3.3 mmol/l glucose	297 ± 9 (6)†	82 ± 6 (6)	219 ± 28 (5)	76 ± 10 (9)
16.7 mmol/l glucose	151 ± 9 (6)*	74 ± 7 (6)	158 ± 10 (4)	82 ± 0.9 (4)
GTP				
3.3 mmol/l glucose	213 ± 11 (9)	54 ± 3 (9)	199 ± 17 (8)	48 ± 4 (8)
16.7 mmol/l glucose	260 ± 17 (8)*	58 ± 3 (8)†	221 ± 17 (8)	48 ± 3 (8)
GDP				
3.3 mmol/l glucose	75 ± 8 (6)	111 ± 26 (6)	55 ± 5 (5)	100 ± 34 (5)
16.7 mmol/l glucose	46 ± 1.2 (5)*	108 ± 37 (5)	43 ± 3 (4)	104 ± 36 (4)

Data are means ± SE (n). dpm, disintegrations per minute. Islets were labeled for 18 h with 2 μCi/ml [^{3}H]hypoxanthine in RPMI 1640 media and then incubated for 15 min at 3.3 or 16.7 mmol/l glucose. n represents the number of determinations from three separate experiments. *$P < 0.05$ for 3.3 compared with 16.7 mmol/l glucose; †$P < 0.05$ for GK compared with Wistar rats.

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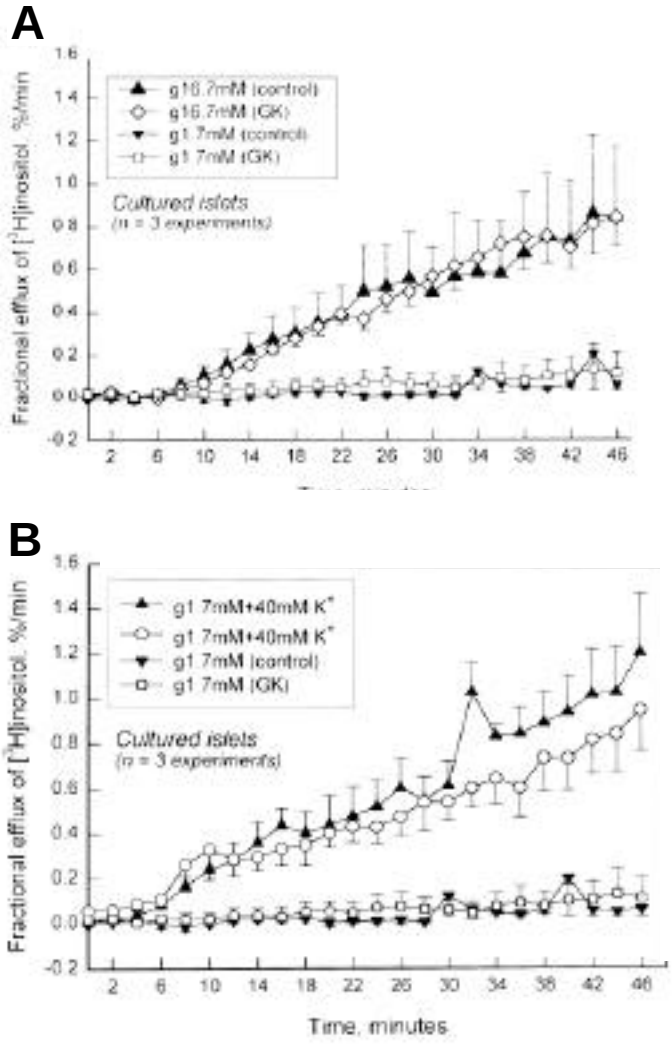

FIG. 6. PLC activation (expressed as fractional efflux of inositol) in response to 16.7 mmol/l glucose (*A*) or 40 mmol/l K^+ at 1.7 mmol/l glucose (*B*) in overnight-cultured control Wistar compared with GK rat islets. In *B*, ▲ indicates control data from control Wistar rat islets in the presence of 40 mmol/l K^+ and ○ represents corresponding data from GK islets.

tively) in response to either glucose (Fig. 6*A*) or 40 mmol/l potassium (Fig. 6*B*). These findings suggest that the influx of Ca^{2+} in response to glucose or K^+ and the sensitivity of PLC to Ca^{2+} are all intrinsically intact in GK rats, at least ex vivo. During these perifusion studies, insulin secretion induced by 40 mmol/l K^+ (at 3.3 mmol/l glucose) or by 16.7 mmol/l glucose was markedly diminished in GK rat islets.

Interestingly, however, the content of phosphoinositide substrates was reduced, with PI, PIP, and PIP_2 contents being inhibited by 29–31% (Table 6). This effect did not appear to be due to an impairment of [^{3}H]inositol uptake into the cells, since the inositol content of the aqueous fraction of the cells after extraction was not clearly or commensurately reduced (–2.5%; mean of two independent experiments).

GBP. In initial screening studies (not shown), Western blots for the following GBPs revealed no discernible abnormalities between control and experimental islets: α-subunits of G_o, G_q, and G_{i3}; and low–molecular weight GBPs: Cdc42 and Rap 1. Furthermore, no significant difference was demonstrable in

TABLE 6
Phosphoinositide content of Wistar control compared with GK islets

	PI	PIP	PIP_2
Control	80,674 ± 2,249	1,155 ± 80	2,255 ± 80
GK	60,991 ± 2,116	793 ± 42	1,564 ± 58
Percentage (%)	–24	–31	–31
P	<0.001	0.007	<0.001

Data are means ± SE, unless otherwise indicated, for four replicate determinations (tubes of islets) each. Results were confirmed in a second experiment with nearly identical results. 100 islets per condition were labeled for 18 h in 25 µCi/ml [^{3}H]inositol as described in METHODS. The next morning (before any incubations), islets were harvested and extracted, as described. Organic phases were analyzed using thin-layer chromatography, using silica plates pretreated with oxalate; 100 µl of each organic phase was applied to the plates.

the ability of GTPγS to stimulate the carboxyl methylation of the protein band at 23 kDa in islet homogenates from Wistar control islets (1.64 ± 0.2–fold; mean of two experiments) and GK rat islets (1.66 ± 0.3–fold; mean of two experiments). The ability of glucose to stimulate the carboxyl methylation of low–molecular weight proteins (28) such as Cdc42 was also unaltered in diabetic rat islets. After prelabeling of islets for 60 min with [^{3}H]methionine, glucose (16.7 mmol/l) stimulated the carboxyl methylation of Cdc42 by 2.4 ± 0.8–fold in control islets (compared with 3.3 mmol/l glucose) and 1.83 ± 0.33–fold in freshly isolated GK rat islets.

NDPK. The histidine autophosphorylation of NDPK, as assessed in Wistar and GK rat islets using either [^{32}P]ATP or GTP as phosphoryl donor, was markedly reduced in GK islets compared with Wistar islets (not shown). Furthermore, the catalytic activity of NDPK was also decreased in homogenates of either freshly isolated or cultured islets (Fig. 7). This reduction in NDPK activity was demonstrable principally in the cytosolic fraction (–39 ± 11% of control; mean of three individual fractionations). In contrast, in preliminary studies, the histidine phosphorylation of the β-subunit of trimeric GBPs in the islet membrane fraction (36) appeared to be intact (not shown). Furthermore, the reduced activity of NDPK also did not seem to be due to defects in its expression, since immunoblots for NDPK did not reveal any apparent reduction in GK islets (not shown).

DISCUSSION

In these studies, pancreatic islets were isolated from overtly diabetic but nonobese GK rats (a genetic model for human type 2 diabetes) and studied ex vivo in comparison to age- and sex-matched control (Wistar) rats. Despite studying both freshly isolated and overnight-cultured islets, we cannot totally exclude some contribution to the islet dysfunction by the antecedent ambient diabetic milieu in vivo, since we have not attempted to restore normoglycemia in vivo or to culture the islets in vitro for prolonged periods at a normal glucose concentration. Therefore, some degree of glucotoxicity (39) or lipotoxicity (40) might contribute to our findings. Indeed, some authors have observed at least partial reversal of GK islet defects by in vivo normalization of hypoglycemia (7) or in vitro by exposure of perfused pancreases to glucose-free buffer (21). However, certain characteristics typical of glucose

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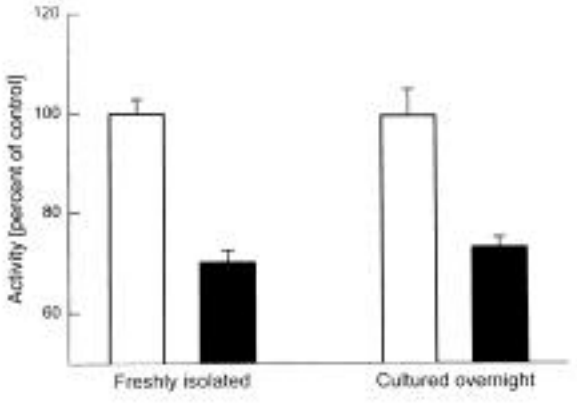

FIG. 7. NDPK activity in control Wistar (□) and GK (■) rat islet homogenates. NDPK activity was assayed in homogenates from Wistar and GK rat islets (freshly isolated or cultured overnight) as described in METHODS. Data are means ± SE from two to three islet preparations in each case, performed using triplicate measurements with 8 µg of homogenate protein per assay.

toxicity were absent from the GK islets, including relative preservation of responses to mitochondrial fuels (41), inhibition of PLC (42), and reduction in insulin stores (39). Similarly, plasma levels of free fatty acids and intra-islet content of triglycerides, the hallmarks of lipotoxicity (43), are not elevated in plasma of GK rats (44). Additionally, the defect induced by fatty acids (at least in vitro) rapidly reverts after their removal (43); fatty acid exposure usually elevates basal insulin release (45), tends to spare responsivity to mitochondrial fuels (43), and reduces insulin content (45), all in contradistinction to the defect(s) in the islets from our GK colony. Thus, it seems likely that the majority of the defects observed are intrinsic to the GK islet.

We did not formally assess DNA content of the islets or their β-cell mass; the latter has been reported to be reduced in some (22,23), but not in other (29,46), studies of GK islets. In our studies, however, insulin and protein contents and stereomicroscopic morphology were totally intact in islets from GK rats; and insulin secretion, even when expressed as fractional release, was still impaired, arguing against a selective drop-out of β-cells as the (sole) islet lesion. Furthermore, in all reports of which we are aware in which relative β-cell volume or DNA content was compared with insulin content in GK islets, the latter was invariably reduced as much as, if not more than, was β-cell mass and was at least as sensitive an index of decreased β-cell mass as DNA (9,11,22,23,46). For all of these reasons, we feel that a decrease in β-cell number cannot explain any of our major findings in the GK islets. However, we emphasize that our studies focus on functional, not anatomic, defects.

Clear secretory defects were observed in response to glucose, pure mitochondrial agonists, or 40 mmol/l K^+, findings that are in accord with studies by some other investigators (11,13), but not all (14,47). While the reason(s) for these discrepancies is unclear, it is becoming increasingly clear that there is significant variability between different GK colonies, a fact that demands caution in generalizing our findings, as might variations in age, diet, and experimental protocols used (e.g., pancreas perfusion compared with static incubations or perifusions of isolated islets). A lesion in early steps in the glycolytic metabolism of glucose would not be sufficient to explain all of the secretory abnormalities, since several of our experiments were carried out at very low glucose concentrations (1.7–3.3 mmol/l). Likewise, an intact activation by glucose of PLC fails to provide support for the concept of a defect in glucose metabolism. Indeed, others have observed that the intrinsic capacity for glucose metabolism is intact in GK islets (20,48). With regard to purine nucleo-tides, the nondiffusible pools of purine nucleotides within secretory granules do not change upon acute stimulation by glucose (49); therefore, it is clear that the normal increments in ATP/ADP and GTP/GDP ratios (which we assessed by both radiolabeling and mass measurements) seen in GK islets must derive from the physiologically meaningful cytosolic (diffusible) compartment (49) and were not masked by chronic abnormalities related to granulation of the cells (and their corresponding content of nucleotides).

Some data (3,10,50) have implied that ATP, ATP/ADP, and the subsequent closure of the ATP-sensitive K^+ channel might be deficient in diabetic islets in response to glucose. In one study, however, old GK rats (nearly 18 weeks) were studied, and measurements were made only after 60 min of incubation. ATP/ADP ratios (less than 3) were inexplicably low even in control rats (10). Hughes et al. (51) reported that ATP rose somewhat less after exposure of GK islets to 10 mmol/l glucose than what occurred in control islets; however, ATP/ADP ratios were not reported. In contrast, Zong-Chao et al. (48) reported that isolated mitochondria from GK islets synthesize ATP essentially normally. Finally, it should be noted that a defect in glucose-potentiated secretion occurs in GK islets even when the role of K^+ (ATP) channels in insulin release is vitiated (16,51).

Furthermore, the normal activation of PLC (in response to either glucose or 40 mmol/l K^+) suggests that steps that are critical to glucose-induced PLC activation (e.g., glucose metabolism, calcium influx [27], GTP content [27,30,31], and Ca^{2+}-activated PLC activity) are all relatively preserved in GK islets. GTP and GTP/GDP ratios were measured and found to be normal. Activation of low–molecular weight GBPs coupled with the activation of calcium-stimulated PLC in pancreatic islets (28) is apparently also normal in GK islets, since the GBPs most likely to be implicated in this event (Cdc42 and/or Rap 1) had normal abundance and were normally carboxyl methylated in response to GTP or glucose. Our study did not directly address cytosolic or mitochondrial calcium levels in GK islets, and there are few extant studies of intracellular calcium levels in such animals. However, some investigators have suggested that calcium currents and intracellular concentrations are normal or even increased, or display only minor delays (15,47,51), although at least one study did find significant impairment in the increments in cytosolic calcium concentrations induced by glucose (47). However, any postulate that Ca^{2+} fluxes are abnormal in GK islets would not be readily reconciled with the finding that glucose-activated PLC is normal (both temporally or quantitatively), since the latter is strictly dependent on Ca^{2+} influx (27).

Since exocytosis is normal, or even hyperresponsive to, Ca^{2+} in permeabilized GK islet cells (13,14) but its potentiation by glucose is impaired (16,51), some glucose-activated signal(s) that potentiates exocytosis may be defective in the GK β-cell. This process appears to be GTP-dependent (30). Mastoparan stimulates one such pathway in β-cells (52,53). The apparently selective circumvention of the defect(s) in GK islets by

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mastoparan (but not by the inactive analog Mas-17) suggests that activation of a GBP is involved. This finding may be in accord with findings by others (54) of an intact response in GK islets to galparan (a hybrid molecule of mastoparan and galanin, which shares several properties with mastoparan alone). This putative defect would presumably involve GTP loading onto a Ptx-insensitive GBP, since mastoparan promotes GTP-for-GDP exchange (32,55). Interestingly, NDPK, the activity of which was defective in GK islets, has been speculated to channel the GTP it produces to specific GBPs (56,57), and NDPK may interact with mastoparan to activate GBPs (56). Thus, we speculate that mastoparan might be acting to bypass the defective activation of a critical GBP due to a reduced activity of NDPK in GK islets.

A second abnormality in GK islets was a reduced content of all three major phosphoinositides (PI, PIP, and PIP_2). Aside from their effects as substrates for PLC, phosphoinositides have direct effects to promote several cell functions, including GTP-for-GDP exchange onto GBPs (58) and secretion at steps just before the exocytotic process itself (17,59,60). Synthesis of phosphoinositides, in turn, may be regulated by GBPs (61,62) in a mastoparan-sensitive fashion (63). Therefore, mastoparan might be able to bypass the GK defect either by *1*) first activating a critical but unidentified GBP (and perhaps thereby increasing phosphoinositide synthesis and secretion), or, alternatively, by *2*) first increasing phosphoinositide content, thereby activating a critical GBP and, with it, exocytosis. Additional studies will be required to examine these (and other) formulations.

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