Metabolic Regulation, Activity State, and Intracellular Binding of Glucokinase in Insulin-Secreting Cells

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Regulation of glucose-induced insulin secretion is crucially dependent on glucokinase function in pancreatic β-cells. Glucokinase mRNA expression was metabolically regulated allowing continuous translation into enzyme protein. Glucokinase enzyme activity in the β-cell was exclusively regulated by glucose. Using a selective permeabilization technique, different intracellular activity states of the glucokinase enzyme in bioengineered glucokinase-overexpressing RINm5F tissue culture cells were observed. These results could be confirmed in analogous experiments with dispersed islet cells. A diffusible glucokinase fraction with high enzyme activity could be distinguished from an intracellularly bound fraction with low activity. Glucose induced a significant long-term increase of the active glucokinase fraction. This effect was accomplished through the release of glucokinase enzyme protein from an intracellular binding site of protein character. The inhibitory function of this protein factor was abolished through proteolytic digestion or heat inactivation. Northern blot analyses revealed that this binding protein was not identical to the well-known liver glucokinase regulatory protein. This hitherto unknown new protein factor may have the function of a glucokinase regulatory protein in the pancreatic β-cell, which may regulate glucokinase enzyme activity in a glucose-dependent manner. *Diabetes* 48:514–523, 1999

The low-affinity glucose phosphorylating enzyme glucokinase (hexokinase type IV) couples changes in the millimolar glucose concentration range to glucose metabolism in pancreatic β-cells and liver (1–4). In β-cells, glucokinase is the glucose sensor (3–5) and serves as the signal-recognition enzyme for the initiation of insulin secretion (6). The regulation of glucokinase in pancreatic β-cells is more complex than in liver (7). Different studies have provided controversial results, in particular on the regulation of the glucokinase mRNA (8–11). On the other hand, there is a general consensus that glucokinase enzyme activity in β-cells is regulated by glucose through an as-yet-unknown mechanism on both the translational and posttranslational levels (12–17).

The regulation of this key enzyme has a central impact on β-cell function as demonstrated in studies on glucokinase mutations identified in patients suffering from maturity-onset diabetes of the young (MODY) (18–20) and on glucokinase knockout animal models, which present with hyperglycemia finally leading to death due to an impairment of glucose-induced insulin secretion (21,22).

To distinguish between effects of the glucose molecule itself and β-cell metabolism in the regulation of glucokinase, we studied the effects of glucose, the mitochondrial fuel α-ketoisocaproic acid, and the competitive glucokinase inhibitor mannoheptulose in cultured rat pancreatic islets by Northern and Western blot analyses and enzyme activity measurements. As shown here, this approach allowed the identification of β-cell metabolism as the principal regulator of β-cell glucokinase on the mRNA level, whereas the posttranslational regulation of enzyme activity was achieved exclusively by glucose.

The short-term regulation of liver glucokinase enzyme activity through a regulatory protein (23,24) raised the question whether a comparable principle of glucokinase regulation might exist in pancreatic β-cells. The liver glucokinase regulatory protein functions as an anchor protein in the intracellular matrix, which modulates the glucokinase activity status through inhibition of enzyme activity (25) and mediates translocation of the enzyme from and to the nuclear compartment (26–28). To investigate the principle of glucokinase binding for activity regulation in insulin-secreting cells, we used a technique of selective permeabilization of glucokinase-overexpressing RINm5F insulinoma cells as well as of mouse islet cells, adapting an experimental approach developed by Agius et al. (25,29,30) that significantly contributed to the understanding of glucokinase activity regulation in liver. Our results provide evidence for a nutrient-dependent regulation of glucokinase enzyme activity in insulin-secreting cells, through a protein factor different from the glucokinase regulatory protein of the liver (23,24,31–34). Such a factor of protein character with regulatory function can be of crucial importance for regulation of glycolytic flux and glucose-induced insulin secretion in pancreatic β-cells and may have far-reaching implications for new therapeutic concepts of non-insulin-dependent diabetes in the future.

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ECL, enhanced chemiluminescence; PVDF, polyvinylidine difluoride.

RESEARCH DESIGN AND METHODS

Materials. Collagenase P, restriction enzymes, the SP6/T7 Transcription Kit, and the DIG Nucleic Acid Detection Kit were obtained from Boehringer (Mannheim, Germany). Hybond N nylon membranes were from Amersham (Braunschweig, Germany) and Immobilon-P PVDF membranes from Millipore (Bedford, MA). Digitonin, peroxidase-labeled anti-rabbit immunoglobulin G antibody, D-mannoheptulose, and α-ketoisocaproic acid were from Sigma (St. Louis, MO). The enhanced chemiluminescence (ECL) detection system and autoradiography films were from Amersham. Guanidine thiocyanate was from Fluka (Neu-Ulm, Germany). Immobilized proteinase K, Triton X-100, and all other reagents of analytical grade were from Merck (Darmstadt, Germany). The cDNA coding for rat liver glucokinase was provided by Dr. P. Iynedjian (Geneva, Switzerland) and the cDNA coding for rat liver glucokinase regulatory protein by Dr. E. Van Schaftingen (Brussels, Belgium). All tissue culture equipment was from GIBCO Life Technologies (Gaithersburg, MD).

Animals and islet isolation. Pancreatic islets from fed male Wistar rats (300–400 g) or from *ob/ob* mice (50–60 g) were isolated by collagenase digestion. When other tissues were used, they were also taken from Wistar rats. Rat pancreatic islets were purified by Ficoll gradient centrifugation and transferred immediately to tissue culture.

Islet culture. Freshly isolated pancreatic islets from rats were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, penicillin, and streptomycin in a humidified atmosphere at 37°C and 5% CO_2. Batches of 200–300 pancreatic islets were distributed to 90-mm plastic dishes and incubated for 24 h with 2 mmol/l glucose, 10 mmol/l glucose, 2 mmol/l glucose plus 10 mmol/l mannoheptulose, 10 mmol/l glucose plus 10 mmol/l mannoheptulose, or 2 mmol/l glucose plus 10 mmol/l α-ketoisocaproic acid. Thereafter the islets were subjected to RNA and protein isolation or measurement of enzyme activities.

Northern blot analyses. Total RNA from rat tissues was isolated by combined water-saturated phenol-chloroform-isoamyl alcohol extraction according to Chomczynski and Sacchi (35) with an addition of ultrapure glycogen to achieve full precipitation of islet RNA. RNA was subjected to electrophoresis on denaturing formamide/formaldehyde 1% agarose gels and transferred to nylon membranes. Hybridization was performed at 68°C overnight in a solution containing 50% deionized formamide, 5× SSPE (180 mmol/l sodium chloride, 1 mmol/l EDTA, and 10 mmol/l NaH_2PO_4, pH 7.4), 10× Denhardt's solution, 0.5% SDS, 100 μg/ml sonicated nonhomologous DNA from herring sperm, and 11-DIG-UTP–labeled antisense cRNA probes coding for rat liver glucokinase (36), rat β-actin (37), or rat liver glucokinase regulatory protein (38). The DIG-labeled hybrids were detected by an enzyme-linked immunoassay using an anti–DIG-alkaline-phosphatase antibody conjugate. Hybrids were visualized by chemiluminescence detection on a light-sensitive film for quantification by densitometry with the National Institutes of Health Image 1.58 program (Bethesda, MD). Ribosomal bands were used as control markers for gel loading.

Western blot analyses. Cellular protein was purified from the phenol phase of RNA isolation according to Wu (39) with the modification that two acetone-precipitation steps were performed until the pellet was dissolved in 1× loading buffer (100 mmol/l Tris, pH 6.8, 2% SDS, 10% glycerol). Insoluble material was pelleted at 1,000*g* and 4°C for a period of 10 min. Protein content was determined by the BCA assay (Pierce, Rockville, MD). Thereafter, DTT and bromophenol blue were added from concentrated stocks to yield a final concentration of 100 mmol/l and 0.1%, respectively. Cellular protein (50 μg) was fractionated by reducing 10% SDS-PAGE and electroblotted to polyvinylidine difluoride (PVDF) membranes. The membranes were stained by Ponceau to verify the transfer of comparable amounts of cellular protein. Nonspecific binding sites of the membranes were blocked by nonfat dry milk for 1 h at 37°C. The blots were incubated with specific anti-glucokinase antibody, raised in our laboratory in rabbits against recombinant rat liver glucokinase (40), at a dilution of 1:10,000 overnight at 4°C, followed by a 2-h incubation period with peroxidase-labeled secondary antibody at a dilution of 1:15,000 at room temperature. The protein bands were visualized by chemiluminescence using the ECL detection system.

Glucose phosphorylation. Glucokinase and hexokinase enzyme activities were measured in sonicated islet homogenates and RINm5F-GK cellular fractions by a photometric assay containing glucose-6-P-dehydrogenase, 6-P-gluconate-dehydrogenase, ATP, and NADPH (41). Five islets per microliter were disrupted in glucokinase assay buffer without glucose, ATP, and enzymes by sonication (60 W, three bursts of 10 s each) and immediately used for spectrophotometric registration of glucose phosphorylation activity. Glucokinase activity was determined by subtracting the hexokinase activity measured at 1 mmol/l glucose from the activity measured at 100 mmol/l glucose. DNA was measured from the assay mixture of the cuvettes according to Rago et al. (42). Enzyme activities were expressed as units per gram DNA. One unit of enzyme activity was defined as 1 micromole of glucose-6-P formed from glucose and ATP per minute at 37°C.

Stable overexpression of glucokinase in RINm5F cells. Glucokinase-overexpressing permanent RINm5F insulinoma tissue culture cells (passages 55–60) (43,44) were generated through stable transfection of the human glucokinase cDNA (45) under control of the cytomegalovirus promoter in the pcDNA3 vector using lipofectamine as described (46). Selection was performed through resistance against G418. The RINm5F clone with the highest glucokinase activity level was used in this study. These RINm5F-GK cells showed glucokinase activities of 118 U/g DNA, 10-fold higher than in nontransfected control cells, and a glucokinase/hexokinase activity ratio of 2.2. Cells were kept in tissue culture in RPMI 1640 medium containing 200 μg/ml G418 and 10 mmol/l glucose.

Permeabilization of RINm5F cells and *ob/ob* mouse islet cells. For experiments on glucose dependence of intracellular glucokinase binding, RINm5F-GK cells were seeded at a density of 4×10^5 cells in 6-cm dishes for 24 h at 1 or 10 mmol/l glucose in RPMI 1640 medium. For experiments on Mg^{2+} dependence of intracellular glucokinase binding, cells were incubated for 30 min in HEPES-buffered (20 mmol/l) Krebs-Ringer bicarbonate medium (pH 7.4) (47) containing 0, 2, or 7.5 mmol/l $MgCl_2$. Permeabilization experiments on RINm5F-GK cells were conducted as described in detail by Agius et al. (29) with slight modifications. Cells were permeabilized with 3, 5, or 10 μg/ml digitonin in glucokinase assay buffer (20 mmol/l HEPES, 125 mmol/l KCl, pH 7.4) with the respective $MgCl_2$ concentrations for 5 min at room temperature. Thereafter the eluate was removed and the assay mixture was supplemented with 7.5 mmol/l $MgCl_2$, 5 mmol/l ATP, 0.5 mmol/l NAD, and 700 U/l glucose-6-P dehydrogenase from *Leuconostoc mesenteroides* for measurement of glucose phosphorylation activities. The permeabilized cell matrix was extracted with 0.5% Triton X-100 in glucokinase assay buffer. Enzyme activities in the eluate after permeabilization were expressed as percentage of the total activity in the eluate plus that in the extracted cell matrix. In experiments on the functional characterization of glucokinase binding proteins in digitonin eluates of RINm5F-GK cells, proteolytic digestion with immobilized proteinase K (5 U/ml) was performed for 5 min at 25°C. Thereafter catalytically active proteinase K was removed by spinning down the beads. Alternatively, protein components in the digitonin eluate were denatured by heating at 95°C for 5 min. For Western blot analyses of glucokinase, cellular proteins from the eluate and the extracted cell matrix were precipitated twice with a threefold volume of ice-cold acetone and pelleted by centrifugation at 10,000*g* for 10 min at 4°C. The pellet was rinsed twice with acetone and dissolved in gel loading buffer (100 mmol/l Tris, pH 6.8, 2% SDS, 10% glycerol). For permeabilization experiments on *ob/ob* mouse islet cells, isolated islets were dispersed into single cells as described (48). Thereafter islet cells in amounts equivalent to 250 *ob/ob* mouse islets were *1*) permeabilized with digitonin (10 μg/ml), *2*) permeabilized with digitonin plus addition of Triton X-100 (0.5% vol/vol) to the eluate after centrifugation of the cells, or *3*) lysed with Triton X-100 (0.5% vol/vol) for 5 min at room temperature. After short centrifugation, glucokinase and hexokinase activities were measured in the supernatant. In control experiments, it was confirmed with recombinant β-cell glucokinase (40) that 10 μg/ml digitonin and 0.5% Triton X-100 had no nonspecific effects on glucose phosphorylation activity measurements. The enzyme-coupled photometric assay was also not affected (data not shown). Thus all effects in the permeabilization and extraction experiments with glucokinase-overexpressing RINm5F-GK cells can be regarded as specific for intracellular binding or inhibition of the enzyme.

Statistical analyses. Data are expressed as means ± SE. Statistical analyses were performed using Student's *t* test.

RESULTS

Metabolic regulation of glucokinase expression in pancreatic islets

Gene expression. Glucokinase mRNA expression was increased in pancreatic islets incubated at 10 mmol/l glucose, reaching 281% of the control value at 2 mmol/l glucose (Fig. 1, Table 1). Co-incubation with 10 mmol/l mannoheptulose reduced this effect significantly (Fig. 1, Table 1). At 2 mmol/l glucose, mannoheptulose had no significant effect on glucokinase mRNA levels (Fig. 1, Table 1). α-Ketoisocaproic acid (10 mmol/l), an insulin secretagogue and β-cell substrate with an exclusively mitochondrial metabolic fate, also significantly increased glucokinase mRNA levels, by 92% (Fig. 1, Table 1), providing further evidence for the role of metabolism in the regulation of glucokinase mRNA. The effects of glucose and α-ketoisocaproic acid on glucokinase gene expression can be regarded as specific because the expression level of β-actin remained unchanged under these conditions (Fig. 1).

Protein expression. In contrast with glucokinase mRNA expression, the levels of glucokinase protein were not affected by glucose, α-ketoisocaproic acid, or mannoheptulose

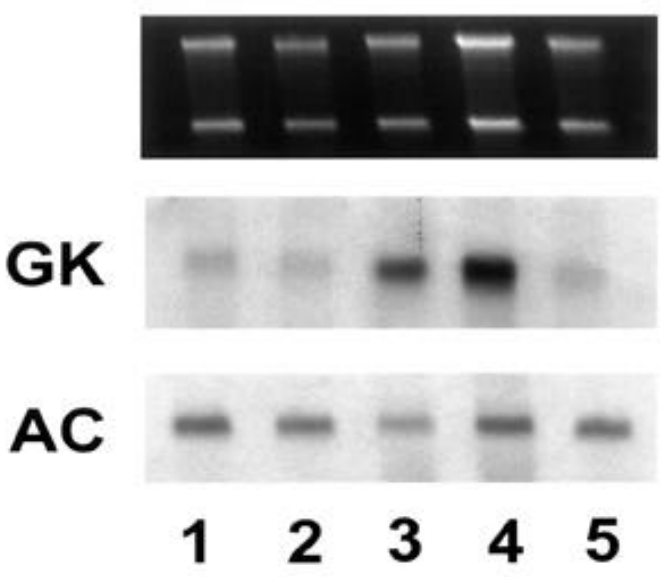

FIG. 1. Effects of glucose, mannoheptulose, and α-ketoisocaproic acid on glucokinase and β-actin gene expression in cultured rat pancreatic islets. *Lane 1*, 2 mmol/l glucose; *lane 2*, 2 mmol/l glucose + 10 mmol/l mannoheptulose; *lane 3*, 2 mmol/l glucose + 10 mmol/l α-ketoisocaproic acid; *lane 4*, 10 mmol/l glucose; *lane 5*, 10 mmol/l glucose + 10 mmol/l mannoheptulose. Isolated rat pancreatic islets were incubated with the various test compounds for 24 h. Total RNA (5 µg) was analyzed by Northern blot hybridization using DIG-UTP–labeled cRNA probes coding for rat liver glucokinase (GK) or rat β-actin (AC). Shown are representative blots of at least four independent experiments.

(Fig. 2, Table 1). This difference is particularly evident, since RNA and protein were isolated in a single-step method from the same batch of islets. Thus the increased glucokinase mRNA levels after stimulation by glucose or α-ketoisocaproic acid were apparently not translated into protein.

Enzyme activity. Glucokinase enzyme activity increased after incubation at 10 mmol/l glucose by 116% compared with 2 mmol/l glucose (Table 2). Co-incubation with mannoheptulose decreased glucokinase activity below the basal level at 2 mmol/l glucose (Table 2). Interestingly, the mitochondrial β-cell substrate α-ketoisocaproic acid (10 mmol/l) did not increase glucokinase enzyme activity (Table 2). Hexokinase enzyme activity remained unaffected by glucose, α-ketoisocaproic acid, and mannoheptulose (Table 2).

Regulation of glucokinase activity in glucokinase-overexpressing RINm5F-GK insulinoma cells

Intracellular compartmentalization of glucose phosphorylation activities. Digitonin permeabilization of RINm5F-GK cells released glucokinase enzyme activity in a concentration-dependent manner, amounting to ≤90% of total cellular glucokinase activity (Fig. 3). In contrast with glucokinase, only 30% of cellular hexokinase activity was detected maximally in the eluate at 10 µg/ml digitonin (Fig. 3). In the next step, glucokinase enzyme activity in the digitonin eluates was compared with the amounts of released glucokinase protein. Glucokinase protein, measured by Western blot analysis, was also released in a concentration-dependent manner after digitonin permeabilization in the range between 1 and 20 µg/ml digitonin (Fig. 4). However, there was an apparent discrepancy between the amounts of glucokinase protein and the respective enzyme activity detected in the digitonin eluates and the Triton X-100 extracted matrix. This discrepancy reflects different specific glucokinase enzyme activities.

At 10 µg/ml digitonin, 90% of total cellular glucokinase activity was found in the eluate, but this corresponded to only 45% of the total cellular glucokinase protein (Figs. 3 and 4). Calculation of the glucokinase activity/protein ratio showed that the specific activity of the Triton X-100 matrix-bound glucokinase decreased depending on the digitonin concentration (Fig. 5). On the other hand, the specific activity of the soluble glucokinase fraction released by digitonin permeabilization remained nearly constant irrespective of the concentration of digitonin (Fig. 5). Thus, the specific activity of glucokinase was apparently modulated in the matrix-bound fraction that was extracted by Triton X-100.

Evidence for a glucokinase regulatory protein factor. Although 0.5% Triton X-100 did not interfere with the glucokinase enzyme assay using purified recombinant glucokinase, the results obtained on matrix-bound glucokinase prompted us to study the effects of Triton X-100 on glucokinase activities in the cellular environment. Therefore, we compared the effects of 0.5% Triton X-100 on glucokinase activities in sonicated homogenates and digitonin eluates from RINm5F-GK cells (Fig. 6). The addition of 0.5% Triton X-100 to the 10,000*g* supernatant of sonicated homogenates from RINm5F-GK cells resulted in a decrease of the glucokinase activity by 52%, whereas hexokinase activity increased by 33%, apparently through mobilization of mitochondrially bound enzyme (Fig. 6). Similar results were obtained after direct lysis

TABLE 1
Effects of glucose, mannoheptulose, and α-ketoisocaproic acid on glucokinase and β-actin expression in cultured pancreatic islets

	Glucokinase mRNA (%)	β-actin mRNA (%)	Glucokinase protein (%)
2 mmol/l glucose (reference value)	100 ± 0 (6)	100 ± 0 (6)	100 ± 0 (6)
2 mmol/l glucose + 10 mmol/l mannoheptulose	94 ± 13 (6)	110 ± 9 (6)	87 ± 6 (4)
2 mmol/l glucose + 10 mmol/l α-ketoisocaproic acid	192 ± 29 (5)*	102 ± 24 (6)	100 ± 9 (5)
10 mmol/l glucose	281 ± 40 (5)*	85 ± 24 (5)	107 ± 11 (4)
10 mmol/l glucose + 10 mmol/l mannoheptulose	151 ± 20 (6)†	161 ± 27 (6)	120 ± 12 (4)

Data are means ± SE (number of experiments). Isolated pancreatic islets were incubated for 24 h with glucose, mannoheptulose, or α-ketoisocaproic acid. RNA (10 µg) from pancreatic islets was hybridized with antisense cRNA probes coding for rat cytoplasmic glucokinase or rat β-actin. Total cellular protein (50–100 µg) from pancreatic islets was subjected to Western blot analysis using a specific antibody against rat liver glucokinase. *$P < 0.05$ compared with 2 mmol/l glucose; †$P < 0.05$ compared with 10 mmol/l glucose.

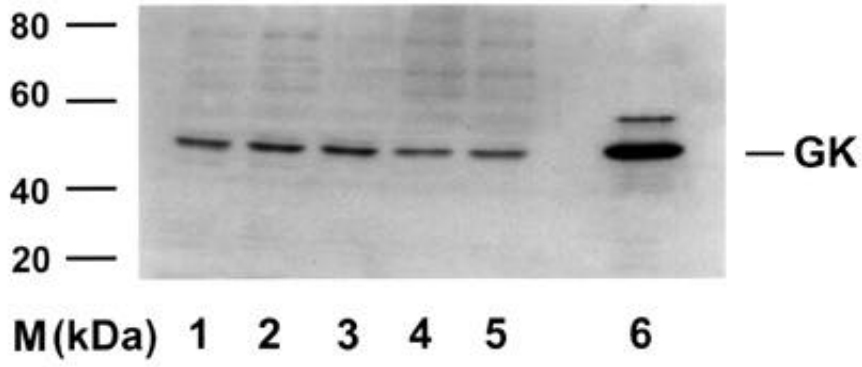

FIG. 2. Effects of glucose, mannoheptulose, and α-ketoisocaproic acid on glucokinase protein expression in cultured rat pancreatic islets. *Lane 1*, 2 mmol/l glucose; *lane 2*, 2 mmol/l glucose + 10 mmol/l mannoheptulose; *lane 3*, 2 mmol/l glucose + 10 mmol/l α-ketoisocaproic acid; *lane 4*, 10 mmol/l glucose; *lane 5*, 10 mmol/l glucose + 10 mmol/l mannoheptulose; *lane 6*, 5 µg cytoplasmic protein from fed rat liver. Isolated rat pancreatic islets were incubated with the various test compounds for 24 h. Cellular protein (10 µg) was analyzed by immunoblotting using a specific antibody against rat liver glucokinase. Shown is a representative blot of at least four independent experiments.

of RINm5F-GK cells by 0.5% Triton X-100 (Fig. 6). Permeabilization by digitonin induced a similar release of glucokinase, as did sonication (Fig. 6). When 0.5% Triton X-100 was added to the digitonin eluate, glucokinase activity decreased significantly by 55%, whereas hexokinase activity remained unaffected (Fig. 6). The data suggested that either an inhibitory cellular factor might have been activated or a stimulatory factor might have been inactivated by Triton X-100 treatment of cellular extracts. To test these hypotheses, purified recombinant β-cell glucokinase was mixed with digitonin eluates from RINm5F-GK cells that had been pretreated with proteinase K or had been heated to achieve an inactivation of the proteins (Fig. 7). The addition of 0.5% Triton X-100 to the native digitonin eluate significantly decreased the activity level of spiked recombinant glucokinase, by 38% (Fig. 7). Inactivation of cellular proteins in the digitonin eluate by either proteinase K digestion or heat treatment prevented the activity decrease of recombinant spiked glucokinase (Fig. 7), indicating that a factor of proteinaceous character might be involved in glucokinase activity regulation.

Expression of the liver glucokinase regulatory protein. Northern blot analyses showed no significant hybridization signals with a liver glucokinase regulatory protein probe (38) with rat islet and RINm5F cell RNA (Fig. 8). In addition to liver, unexpectedly, hybridization signals of 2.4 kb were detected also in brain, muscle, heart, and lung tissue (Fig. 8). The intensities of the bands of these latter organs were virtually abolished after increasing the stringency of the washing conditions to 75°C (data not shown). Thus these bands cannot be regarded as specific for the mRNA of the liver glucokinase regulatory protein but may share some degree of homology with a protein of different cellular function.

Regulation of intracellular glucokinase binding by glucose. To address the functional significance of intracellular glucokinase binding in insulin-secreting cells, RINm5F-GK cells were incubated for 24 h at 1 or 10 mmol/l glucose. Incubation at 10 mmol/l glucose significantly increased the diffusible portion of total glucokinase activity when the cells were permeabilized with 3 or 5 µg/ml digitonin (Table 3). At 10 µg/ml digitonin, the differences between 1 and 10 mmol/l glucose were much smaller, indicating a dependence on the extent of plasma membrane disintegration by digitonin (Table 3). In contrast with glucokinase, the diffusible intracellular portion of hexokinase was not significantly affected by glucose (Table 3). Thus, the glucose regulation of glucokinase binding in RINm5F-GK cells can be interpreted as a specific nutrient effect that modulates the glucokinase/hexokinase phosphorylation ratio.

In short-term incubation protocols (5 or 30 min), glucose did not significantly affect glucokinase or hexokinase binding in RINm5F-GK cells (data not shown). However, a selective short-term effect on glucokinase regulation in RINm5F-GK insulin-secreting cells was observed depending on the Mg^{2+} concentration in the permeabilization medium (Table 4). Raising the Mg^{2+} concentration from 0 to 2 or 7.5 mmol/l decreased the portion of diffusible glucokinase activity (Table 4). In contrast, digitonin-releasable hexokinase activity was not affected by Mg^{2+} (Table 4).

Regulation of glucokinase activity in* ob/ob *mouse pancreatic islet cells. Normal pancreatic β-cells differ from RINm5F-GK insulin-secreting cells not in their glucokinase/hexokinase activity ratio but in the specific activities of both enzymes, which are lower by a factor of 10 in β-cells than in RINm5F-GK insulin-secreting cells. We therefore also

TABLE 2
Effects of glucose, mannoheptulose, and α-ketoisocaproic acid on glucokinase and hexokinase enzyme activities in cultured pancreatic islets

	Glucokinase (U/g DNA)	Hexokinase (U/g DNA)
2 mmol/l glucose	12.7 ± 1.5 (9)	7.7 ± 1.4 (9)
2 mmol/l glucose + 10 mmol/l mannoheptulose	6.5 ± 1.3 (4)*	10.1 ± 1.7 (4)
2 mmol/l glucose + 10 mmol/l α-ketoisocaproic acid	12.6 ± 1.1 (6)	5.6 ± 0.5 (6)
10 mmol/l glucose	27.5 ± 4.1 (8)*	9.6 ± 1.3 (8)
10 mmol/l glucose + 10 mmol/l mannoheptulose	9.0 ± 2.9 (6)†	10.6 ± 1.3 (6)

Data are means ± SE (number of experiments). Isolated pancreatic islets were incubated for 24 h with glucose, mannoheptulose, or α-ketoisocaproic acid. Glucose phosphorylation activities were measured in sonicated cellular homogenates by an enzyme-coupled spectrophotometric assay. *$P < 0.05$ compared with 2 mmol/l glucose; †$P < 0.05$ compared with 10 mmol/l glucose.

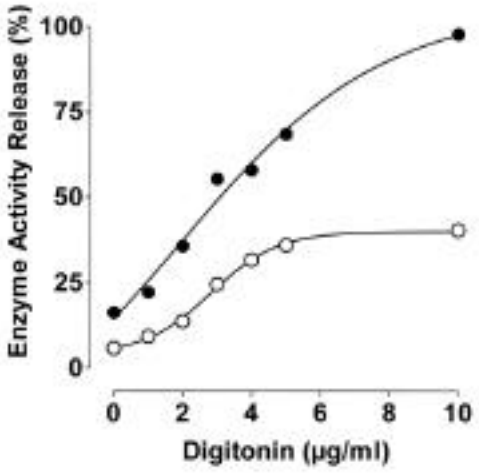

FIG. 3. Concentration-dependent release of glucokinase (●) and hexokinase (○) enzyme activities with digitonin from RINm5F-GK cells. Cells (4×10^5) were grown for 24 h in RPMI 1640 medium at 1 mmol/l glucose. Cells were permeabilized with 0, 1, 2, 3, 4, 5, or 10 μg/ml digitonin in glucokinase assay buffer for 5 min at room temperature. Thereafter, the cell matrix was extracted by 0.5% Triton X-100 in glucokinase assay buffer for 5 min at room temperature. Glucokinase and hexokinase enzyme activities were measured by a spectrophotometric assay in the eluate and the matrix extracts. The activities in the eluate are expressed as % of total activity in eluate plus matrix extract. Shown are means ± SE from four experiments.

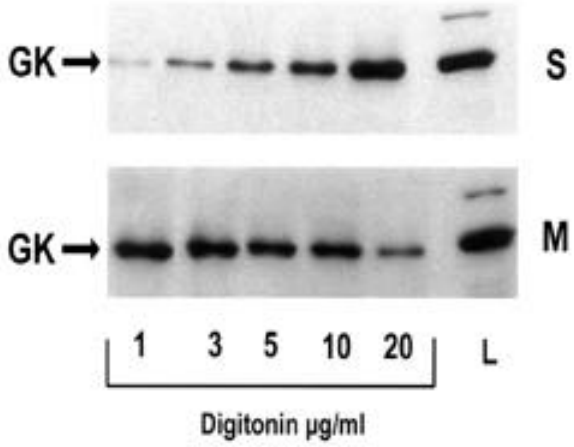

FIG. 4. Concentration-dependent release of glucokinase protein with digitonin from RINm5F-GK cells. Cells (4×10^5) were grown for 24 h in RPMI 1640 medium at 1 mmol/l glucose. Cells were permeabilized as described in Fig. 3 with the modification that digitonin was dissolved in Krebs-Ringer bicarbonate medium. Cellular proteins in the eluates and matrix extracts were precipitated twice by acetone. The protein pellet was subjected to Western blot analyses of glucokinase. S, soluble fraction in the eluate; M, matrix-bound fraction; L, 10 μg liver cytoplasmic protein. Shown is a representative blot from four experiments.

studied the effects of digitonin and Triton X-100 in dispersed cells from *ob/ob* mouse islets, which contain more than 90% β-cells (49). The addition of Triton X-100 (0.5%) to the eluate after permeabilization with digitonin (10 μg/ml) significantly reduced the glucokinase activity level by 35% (Fig. 9), comparable to results obtained on RINm5F-GK insulin-secreting cells (Fig. 6). The direct lysis of *ob/ob* mouse islet cells by Triton X-100 decreased glucokinase activity by 75% compared with the activity in the digitonin eluates (Fig. 9). In contrast, hexokinase enzyme activity was significantly increased in the Triton X-100 lysate by more than 300%, apparently because of dissociation of the enzyme from mitochondria (Fig. 9). The inhibitory effect of Triton X-100 on glucokinase activity was only observed in a cellular environment but not in an assay system with purified recombinant glucokinase (data not shown).

DISCUSSION

Our results show that the principles of β-cell glucokinase regulation are different from those in the liver on the level of mRNA, enzyme protein, and intrinsic activity. Glucose increased glucokinase mRNA expression in isolated rat pancreatic islets, thus confirming earlier studies conducted under physiologic conditions of fasting and refeeding (10,11,50). Interestingly, we could observe in the present study that the mitochondrial substrate α-ketoisocaproic acid mimicked the stimulatory effect of glucose. Thus metabolism apparently regulates pancreatic β-cell glucokinase mRNA expression. Substrates other than glucose may secure high glucokinase mRNA levels sufficient for continuous translation of glucokinase protein to maintain a pool of glucokinase protein that guarantees glucose recognition by β-cells also after periods of food deprivation. Impaired glucose recognition in β-cells from glucokinase knockout mice indicates that a basal level of glucokinase mRNA expression is necessary to maintain a sufficient level of enzyme activity for normal β-cell function (21,22). However, we did not find a concordance between changes in glucokinase mRNA and glucokinase protein levels in response to glucose and α-ketoisocaproic acid. This dichotomy of increased glucokinase mRNA with no change in glucokinase protein may be explained either by a delayed translational activation during the 24-h incubation period or by an increased turnover rate of glucokinase, both of which can result in a constant steady-state level of enzyme protein. Pulse-chase labeling experiments followed by glucokinase immunoprecipitation were not sufficiently sensitive to clarify

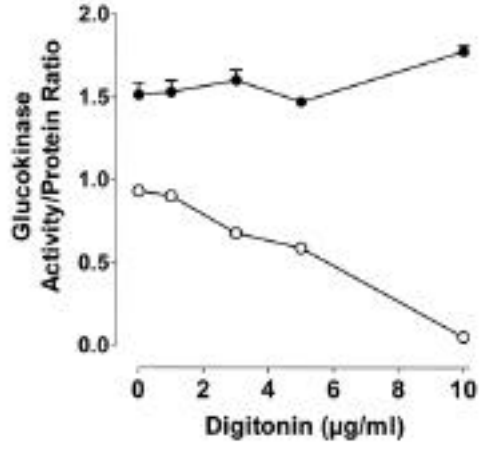

FIG. 5. Effects of digitonin on glucokinase activity/protein ratios in the soluble (●) and matrix-bound (○) fractions of RINm5F-GK cells. Ratios were calculated from the % values of eluate and matrix-bound glucokinase activities in Fig. 3 and densitometrically quantified glucokinase protein in Fig. 4. The ratios reflect the intrinsic activities of the released glucokinase protein. Shown are means ± SE from four experiments.

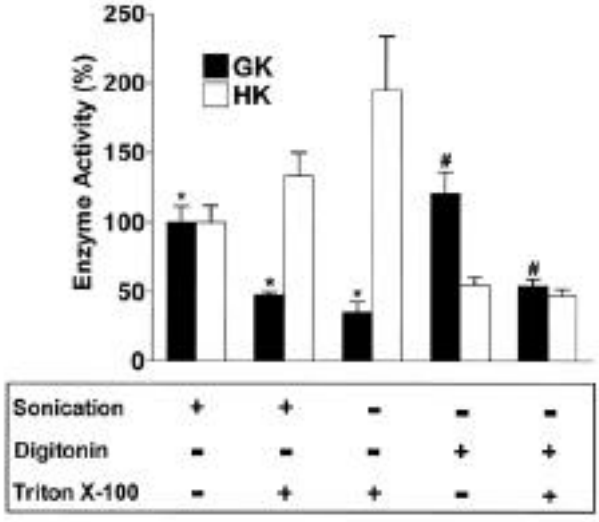

FIG. 6. Effects of sonication, digitonin, and Triton X-100 on glucokinase (GK) and hexokinase (HK) activity release from RINm5F-GK cells. RINm5F-GK cells (4×10^5) were grown in a 6-cm dish overnight at 1 mmol/l glucose. Thereafter, cells were either homogenized by sonication followed by centrifugation at 10,000*g* to remove cellular debris or exposed to 10 µg/ml digitonin or 0.5% Triton X-100 in 1 ml glucokinase assay buffer. In experiments with combined sonication and Triton X-100 extraction, the detergent was added to the 10,000*g* supernatant after sonication. In experiments with combined digitonin and Triton X-100, cells were first permeabilized with digitonin followed by the addition of Triton X-100 to the eluate. Enzyme activities were measured by a spectrophotometric assay. Data are expressed in % of the enzyme activities measured in sonicated homogenates. Shown are means ± SE from 8 to 14 experiments. *$P < 0.05$ compared with glucokinase activity in sonicated homogenates. #$P < 0.05$ compared with glucokinase activity in digitonin eluates.

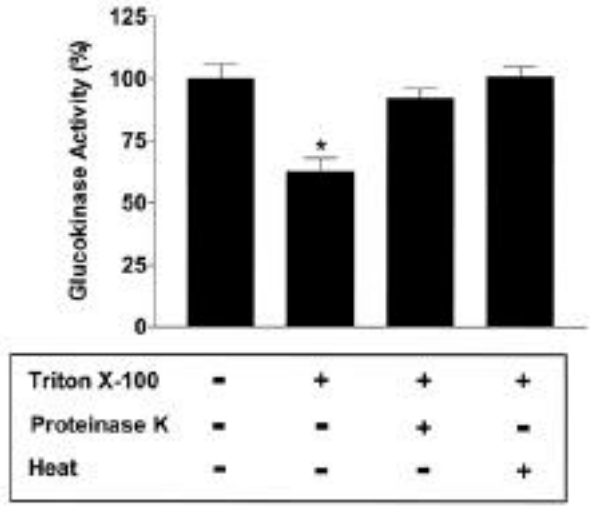

FIG. 7. Effects of proteinase K or heat inactivation on Triton X-100–induced inhibition of recombinant glucokinase in digitonin eluates from RINm5F-GK cells. RINm5F-GK cells (4×10^5) were grown in a 6-cm dish overnight at 1 mmol/l glucose. Cells were permeabilized by 10 µg/ml digitonin in 1 ml glucokinase assay buffer. The eluates were treated with proteinase K (5 min, 25°C) or heat-inactivated (5 min, 95°C). Thereafter, Triton X-100 was added to a final concentration of 0.5% followed by the addition of 20 mU recombinant human β-cell glucokinase. Glucokinase activities were measured by a spectrophotometric assay. Data are expressed in % of the recombinant glucokinase enzyme activities in digitonin eluates without Triton X-100. Shown are means ± SE from 6 to 12 experiments. *$P < 0.05$ compared with recombinant glucokinase enzyme activities in digitonin eluates without triton X-100.

this question because of the low levels of glucokinase protein in rat islets (M.T., unpublished observations). Irrespective of the unknown balance between translational activation and protein degradation, the apparent dichotomy of glucokinase mRNA and protein levels implies that transcriptional activation of glucokinase by glucose or α-ketoisocaproic acid does not play a significant role in functional regulation of glucokinase enzyme activity in the first 24 h of nutrient stimulation.

Previous studies demonstrated that islet glucokinase is regulated by glucose on the levels of both protein content and intrinsic enzyme activity (13–16). Within this dual regulatory system (15), increases of islet glucokinase activity without concomitant increases of glucokinase protein were observed, in the early phase, 6 h after of glucose stimulation (15). Twenty-four hours after stimulation with 16.7 mmol/l glucose, the increase of glucokinase activity could be observed even when the 40% increase of glucokinase protein content was suppressed by cycloheximide (15). Slight variations of islet protein synthesis are probably already sufficient to explain the constant glucokinase protein content at 10 mmol/l glucose, along with a 117% increase of glucose enzyme activity in our study. Conclusively, all studies support the contention that a glucose-induced posttranslational increase of glucokinase enzyme activity can take place without a concomitant change in glucokinase protein content of comparable magnitude. The stimulatory effect of glucose is a very reproducible phenomenon in glucokinase regulation. It has been documented both in vivo in dependence on the nutrient status (12,51–54) and in vitro in cultured islets (13–15). In contrast with its effect on glucokinase mRNA levels, the mitochondrial substrate α-ketoisocaproic acid had no effect on glucokinase enzyme activity. The decrease of β-cell glucokinase activity by the competitive inhibitor mannoheptulose is further strong support for glucose regulation of glycolytic flux through a direct interaction with the enzyme.

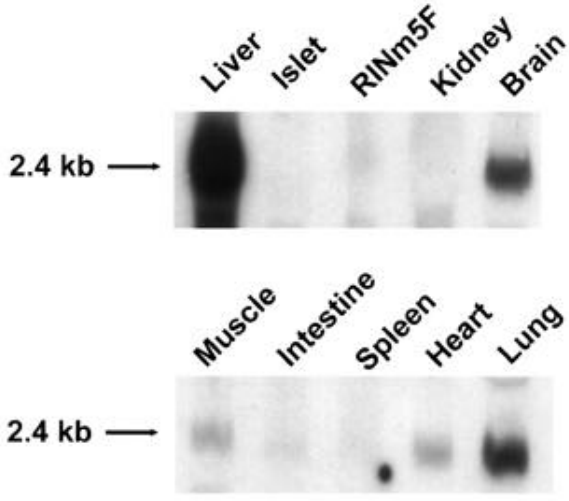

FIG. 8. Northern blot analysis of glucokinase regulatory protein mRNA expression in various tissues from rats. Total RNA (10 µg per lane) was loaded, and blots were probed with antisense cRNA coding for rat glucokinase regulatory protein by nonradioactive hybridization. Shown is a representative blot from three independent experiments.

TABLE 3
Effects of glucose on glucokinase and hexokinase enzyme activities released from RINm5F-GK cells after permeabilization with digitonin

	Digitonin (μg/ml)	Glucose (mmol/l)	
		1	10
Glucokinase			
(% of total activity)	3	44 ± 4 (7)	64 ± 4 (7)*
	5	70 ± 3 (7)	80 ± 2 (7)*
	10	79 ± 3 (7)	86 ± 2 (7)
Hexokinase			
(% of total activity)	3	10 ± 2 (7)	11 ± 2 (6)
	5	15 ± 2 (7)	17 ± 2 (6)
	10	18 ± 1 (7)	20 ± 1 (7)

Data are means ± SE (number of experiments). Cells (4×10^5) were incubated at 1 or 10 mmol/l glucose for 24 h in RPMI-1640 medium. Cells were permeabilized by 3, 5, or 10 μg/ml digitonin in glucokinase assay buffer (7.5 mmol/l $MgCl_2$) for 5 min at room temperature. Thereafter the cell matrix was extracted by 0.5% Triton X-100 in glucokinase assay buffer for 5 min at room temperature. Glucokinase and hexokinase enzyme activities were measured by a spectrophotometric assay in the eluates and the matrix extracts. The activities in the eluates are expressed as percent of total activity in eluate plus matrix extract. *$P < 0.05$ compared with 1 mmol/l glucose.

Although the basal glucose concentration of 2 mmol/l, alone and in combination with 10 mmol/l mannoheptulose, reflects a nonphysiologic situation, it proved suitable to characterize possible mechanisms by which glucokinase enzyme activity is regulated in islets in response to glucose. The kinetic properties of glucokinase allow the conclusion that a self-regulatory function of this enzyme is presumably also effective in the physiologic glucose concentration range.

In the liver a glucokinase regulatory protein participates in the posttranslational regulation of this enzyme (31–34). Other concepts of posttranslational regulation of glucokinase that have been considered are the intracellular translocation of the glucokinase protein (55), conformational changes induced by glucose (56,57), and changes of the sulfhydryl redox status of the enzyme (40,58). In this study, we focused our attention on the posttranslational regulation of glucokinase in insulin-secreting cells through intracellular binding. Permeabilization of RINm5F-GK cells with digitonin (29,30), which opened the cytoplasmic compartment of the cells, showed that more than 90% of the glucokinase enzyme activity was released in a concentration-dependent manner into the incubation medium. In contrast, most of the hexokinase remained bound to mitochondria, confirming earlier observations (59–61). Calculating the specific activities of glucokinase in relation to the cellular glucokinase protein content revealed lower specific activities for the intracellularly bound glucokinase fraction than for the diffusible glucokinase fraction. The lower specific activity of bound glucokinase after solubilization by Triton X-100 was observed only in a cellular environment. Triton X-100 affected neither the activity of recombinant β-cell glucokinase in control experiments nor the activity of the low K_m hexokinase isoenzymes. Thus, a cellular factor that is released by Triton X-100 specifically reduced the activity of the glucokinase enzyme, because simple denaturation of the glucokinase protein by the non-ionic detergent would have also inhibited the recombinant glucokinase enzyme activity. This conclusion is further supported by the inhibition of recombinant β-cell glucokinase added to a cellular extract of RINm5F cells in the presence of Triton X-100. The inhibition of recombinant glucokinase in a cellular Triton X-100 extract was abolished through pretreatment of the cellular extract by immobilized proteinase K or by heat inactivation. These results provide evidence for the existence of a hitherto unknown protein factor in insulin-secreting cells that interacts with the glucokinase protein. Northern blot analyses of rat pancreatic islets and RINm5F cells indicate that this factor is not identical to the well-characterized glucokinase regulatory protein in liver (24,38). Although this liver glucokinase regulatory protein has also been believed to exist in pancreatic β-cells (62), our studies do not support that assumption.

Van Schaftingen and co-workers (34,38) have extensively tested nonliver tissues for the existence of the fructose-regulated liver glucokinase regulatory protein both functionally

TABLE 4
Effects of $MgCl_2$ on glucokinase and hexokinase enzyme activity release from RINm5F-GK cells after permeabilization with digitonin

	Digitonin (μg/ml)	$MgCl_2$ (mmol/l)		
		0	2	7.5
Glucokinase (% of total activity)	3	71 ± 7 (8)	59 ± 5 (7)*	44 ± 4 (7)*
	5	84 ± 3 (9)	75 ± 5 (7)*	70 ± 3 (7)*
	10	86 ± 3 (9)	83 ± 6 (7)	79 ± 3 (7)*
Hexokinase (% of total activity)	3	14 ± 2 (9)	10 ± 1 (7)	10 ± 2 (7)
	5	18 ± 2 (9)	15 ± 2 (7)	15 ± 2 (7)
	10	22 ± 1 (9)	23 ± 3 (7)	18 ± 1 (7)

Data are means ± SE (number of experiments). Cells (4×10^5) were incubated in RPMI-1640 medium at 1 mmol/l glucose. Cells were permeabilized by 3, 5, or 10 μg/ml digitonin in glucokinase assay buffer containing 0, 2, or 7.5 mmol/l $MgCl_2$ for 5 min at room temperature. Thereafter the cell matrix was extracted by 0.5% Triton X-100 in the same glucokinase assay buffer for 5 min at room temperature. Glucokinase and hexokinase enzyme activities were measured by a spectrophotometric assay in the eluates and the matrix extracts. The activities in the eluates are expressed as percent of total activity in eluate plus matrix extract. *$P < 0.05$ compared with 0 mmol/l $MgCl_2$.

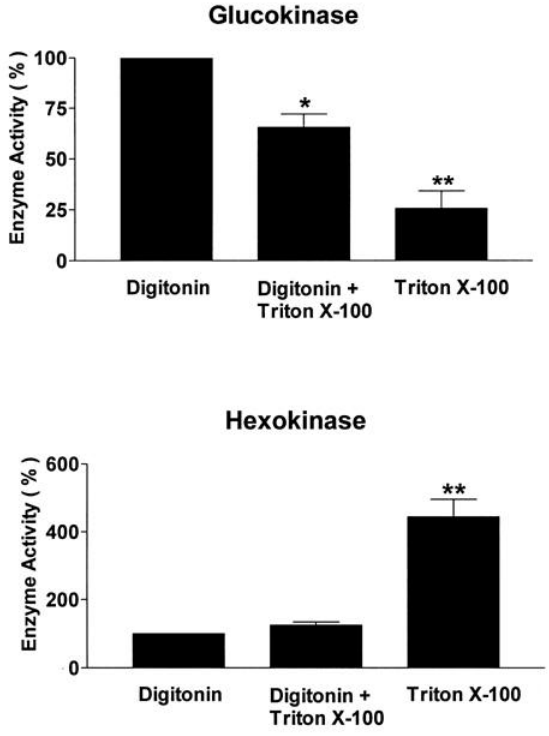

FIG. 9. Effects of digitonin and Triton X-100 on glucokinase and hexokinase activity release from *ob/ob* mouse islet cells. Dispersed islet cells equivalent to 250 islets were exposed to 10 µg/ml digitonin or 0.5% Triton X-100 in 150 µl glucokinase assay buffer. In experiments with combined digitonin and Triton X-100, cells were first permeabilized with digitonin followed by the addition of Triton X-100 to the eluate. Enzyme activities were measured by a spectrophotometric assay. Data are expressed in % of the enzyme activities measured in eluates after permeabilization with digitonin. Shown are means ± SE from 6 to 10 experiments. *$P < 0.05$, **$P < 0.01$ compared with enzyme activities in digitonin eluates.

(rat brain, heart, skeletal muscle, kidney, and spleen) (34) and through Northern blot analysis (muscle, brain, heart, testis, intestine, and spleen) under different conditions (38). These authors did not obtain evidence for expression in nonliver tissues. This finding could be confirmed in the present study where, under high-stringency conditions, hybridization signals were also seen only in liver, but not in other tissues.

The regulation of glucokinase enzyme activity in insulin-secreting cells by glucose correlates with the increase of the diffusible portion of the total glucokinase enzyme activity when RINm5F-GK cells had been incubated for 24 h with 10 mmol/l glucose compared with 1 mmol/l, which we observed in the present study after permeabilization of the cells with digitonin. This diffusible portion represents a glucokinase protein state of high specific activity in contrast with the bound glucokinase, which is inhibited through interaction with a Triton X-100 extractable intracellular protein factor. Thus the intracellular distribution of glucokinase may be of functional relevance for posttranslational regulation of glucokinase activity in insulin-secreting cells. This long-term adaptation of the diffusible glucokinase portion must be distinguished from the short-term effects of fructose in liver, which can be observed within 1 h (29,31). In our RINm5F-GK cells, neither fructose nor glucose exhibited significant effects on glucokinase release after a short-term incubation of 30 min (M.T., unpublished observations). On the other hand Mg^{2+}, a well-characterized regulator of glucokinase binding in hepatocytes (29), proved to be a cofactor that also modulates intracellular glucokinase binding in insulin-secreting cells within minutes. In dispersed rat islet cells, differences were also not observed in the intracellular glucokinase distribution at low and high glucose concentrations during a 1-h incubation period (48). Thus it is unlikely that short-term effects of glucose on glucokinase distribution can be detected in permeabilization experiments using the technique developed by Agius and co-workers (25,29,30) that allowed the successful characterization of fructose-dependent glucokinase release from hepatocytes.

Tissue-specific differences between glucokinase regulation in liver and pancreatic β-cells have also been evident in immunohistochemical studies. In liver, the translocation of glucokinase between nucleus and cytoplasm is mediated by the glucokinase regulatory protein functioning as a docking protein (26–28,63). Such an intranuclear localization of glucokinase has not been observed in pancreatic β-cells (55,64). However, short-term stimulation of β-cells by glucose revealed a translocation of glucokinase from the perinuclear region to the cytoplasm (55). This observation provides evidence for the existence of a β-cell–specific glucokinase binding protein, which may also mediate the regulation of β-cell glucokinase activity under conditions of long-term adaptations to starvation and refeeding (55). Such a glucokinase binding protein may affect glucokinase function in pancreatic β-cells not only through intracellular translocation into compartments that favor the metabolic flux through glycolytic channeling (65,66) or through interaction with the GLUT2 glucose transporter (67), but also through conformational changes of the glucokinase protein (40,56–58). The extraordinary instability of glucokinase enzyme activity in cellular extracts from β-cells is a key characteristic of this enzyme (7,68). In particular, structural instabilities have been demonstrated for distinct human glucokinase mutants with an impaired catalytic function (69,70). In contrast, mutations that result in an activated glucokinase protein were recently detected in patients suffering from familial hyperinsulinism (71). Furthermore, the redox status of the sulfhydryl groups of the enzyme protein significantly affected the stability of the enzyme (40,58,68).

In conclusion, the results of this study emphasize the extraordinary complexity of glucokinase regulation in insulin-secreting cells. In contrast with glucokinase mRNA regulation, which is determined by β-cell metabolism, glucose alone proved to be the crucial regulatory factor on the posttranslational level. Most importantly, the results provide evidence for the existence of a protein factor with glucokinase regulatory function in insulin-secreting cells with characteristics different from the liver glucokinase regulatory protein. This so-far-unknown protein factor, the exact chemical nature of which remains to be elucidated, may have the function of a glucokinase regulatory protein in the pancreatic β-cell, which physiologically determines the availability of glucokinase in insulin-secreting cells in a glucose-dependent manner. It may help to fill the existing gap between theoretical concepts and experimental realities in glucokinase enzyme kinetics (72). A better understanding of the intracellular interactions of glucokinase with other specific binding structures may also provide new insights into the mechanisms of defective stimulus-secretion coupling in pancreatic β-cells in the diabetic state.

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