

Subcellular Localization, Mobility, and Kinetic Activity of Glucokinase in Glucose-Responsive Insulin-Secreting Cells

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We investigated the subcellular localization, mobility, and activity of glucokinase in MIN6 cells, a glucose-responsive insulin-secreting β -cell line. Glucokinase is present in the cytoplasm and a vesicular/granule compartment that is partially colocalized with insulin granules. The granular staining of glucokinase is preserved after permeabilization of the cells with digitonin. There was no evidence for changes in distribution of glucokinase between the cytoplasm and the granule compartment during incubation of the cells with glucose. The rate of release of glucokinase and of phosphoglucoisomerase from digitonin-permeabilized cells was slower when cells were incubated at an elevated glucose concentration ($S_{0.5} \sim 15$ mmol/l). This effect of glucose was counteracted by competitive inhibitors of glucokinase (5-thioglucose and mannoheptulose) but was unaffected by fructose analogs and may be due to changes in cell shape or conformation of the cytoskeleton that are secondary to glucose metabolism. Based on the similar release of glucokinase and phosphoglucoisomerase, we found no evidence for specific binding of cytoplasmic digitonin-extractable glucokinase. The affinity of β -cells for glucose is slightly lower than that in cell extracts and, unlike that in hepatocytes, is unaffected by fructose, tagatose, or a high- K^+ medium, which is consistent with the lack of change in glucokinase distribution or release. We conclude that glucokinase is present in two locations, cytoplasm and the granular compartment, and that it does not translocate between them. This conclusion is consistent with the lack of adaptive changes in the glucose phosphorylation affinity. The glucokinase activity associated with the insulin granules may have a role in either direct or indirect coupling between glucose phosphorylation and insulin secretion. *Diabetes* 49:2048–2055, 2000

Glucokinase (hexokinase IV) is expressed in pancreatic islet cells, hepatocytes, and some rare neuroendocrine cells (1). In pancreatic β -cells, glucokinase functions as a glucose sensor or glucoreceptor, although there remains some debate

regarding the mechanism (2–4). In hepatocytes, glucokinase plays a role in the rapid metabolism of glucose in the absorptive state by regulation of glycogen synthesis and glycolysis by feed-forward activation (5,6). The affinity of hepatocytes for glucose phosphorylation is lower than the affinity of purified glucokinase because of binding of glucokinase to a 68-kDa regulatory protein in the nucleus at low glucose concentrations (7,8). Dissociation of glucokinase from its regulatory protein is induced by elevated glucose and by precursors of fructose 1-phosphate (9,10), and it involves translocation of both glucokinase and the regulatory protein from the nucleus to the cytoplasm (11). This mechanism accounts for the high control strength of hepatic glucokinase on glucose metabolism (6,12), and it also protects the enzyme from degradation (13).

Evidence for a glucokinase binding factor in β -cells is inconclusive. The glucokinase regulatory protein (7) was thought to be present in pancreatic β -cells based on the activation of glucokinase in islet extracts by fructose 1-phosphate (14,15). However, there is no direct evidence from immunostaining (8) or immunoblotting (16). The presence of an inhibitory protein distinct from the liver protein has been suggested (16). Immunostaining studies show marked intercellular heterogeneity of glucokinase expression among β -cells with apparent uniform distribution in the cytoplasm and negligible colocalization with GLUT2 in the plasma membrane (17,18). However, localization of glucokinase in a perinuclear zone under euglycemic conditions (19), in cytoplasmic regions close to the blood vessels (18), or in insulin granules (20) has been reported.

Understanding the function of the subcellular compartmentation of glucokinase in pancreatic β -cells requires parallel study of the kinetics of glucose phosphorylation in intact cells, imaging of the subcellular location of the protein, and determination of enzyme activity. Intact islets are not an ideal model for such studies because of the heterogeneity of the cells and the high activity of low- K_m hexokinase in nonendocrine islet cells (21). In this study, we used the MIN6 β -cell line to study glucose phosphorylation and glucokinase activity and subcellular localization, because this β -cell line expresses a similar sensitivity of insulin secretion to glucose as intact islets (22) and because glucokinase is the predominant glucose-phosphorylating activity in these cells (23).

RESEARCH DESIGN AND METHODS

Materials. Antibodies were from Chemicon (Temecula, CA) (guinea pig anti-insulin); Santa Cruz (Santa Cruz, CA) (goat affinity-purified anti-glucokinase human islet NH_2 -terminus, N-19, and blocking peptide), Sigma (St. Louis, MO) (rabbit anti-actin), or Jackson ImmunoResearch (West Grove, PA) (fluorescein isothiocyanate [FITC] or Texas Red-labeled donkey antibodies against goat,

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Received for publication 12 April 2000 and accepted in revised form 5 September 2000.

BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EBSS, Earle's balanced salt solution; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

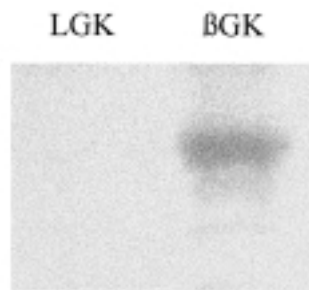


FIG. 1. Immunoreactivity to goat anti-N-19 β -cell glucokinase in extracts of hepatocytes overexpressing liver glucokinase (LGK) or β -cell glucokinase (BGK) isoforms.

rabbit, or guinea pig immunoglobulin). Sources of other reagents have been previously described (24).

Culture of MIN6 cells. MIN6 cells (passage 16–27) were grown in 25-cm² flasks in Dulbecco's modified Eagle's medium (DMEM) (25 mmol/l glucose) supplemented with fetal bovine serum (15% vol/vol), β -mercaptoethanol (5 μ l/l), 75 mg/l penicillin, and 50 mg/l streptomycin. For immunostaining, MIN6 cells were cultured on 13-mm glass coverslips coated with 1% (wt/vol) gelatin, and for determination of glucokinase activity and glucose phosphorylation, they were cultured in 24-well plates.

Immunostaining and immunoblotting. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 30 min and washed in PBS. They were treated for 10 min each with 1 mg/ml NaBH₄, 0.2% Triton X-100, and 0.2% Triton X-100/1% bovine serum albumin (BSA) (all in PBS). Incubation with the primary antibodies was overnight at 4°C. Dilutions were 1:40 for rabbit anti-actin, 1:100 for guinea pig anti-insulin, and 1:20 for goat anti-glucokinase in 0.1% Triton X-100/1% BSA/PBS. For blocking, goat anti-glucokinase IgG 33 μ g/ml was preincubated overnight at 4°C with 166 μ g/ml antigenic peptide. Coverslips were washed three times in PBS (10 min) and incubated for 1 h at room temperature with the appropriate secondary antibody (FITC donkey anti-goat immunoglobulin and Texas Red donkey anti-guinea pig or anti-rabbit immunoglobulin) 1:50 dilution in 0.1% Triton X-100/1% BSA. They were then washed in PBS as described above, once briefly in water and then in 100% ethanol. After drying, they were mounted onto slides with Mowiol containing 2.5% diazabicyclo[2.2.2]octane. There was no detectable cross-reactivity of the secondary antibodies (donkey anti-guinea pig and donkey anti-goat) used in dual-staining. Images of FITC and Texas Red staining for glucokinase and insulin were obtained using a Bio-Rad MRC-600 confocal laser scanning microscope (60 \times oil immersion with zoom factor 2 for Fig. 3). Images for actin were obtained using either Bio-Rad MRC-600 confocal microscope or a Zeiss Axioplan 2 microscope (40 \times objectives) fitted with an imaging camera. Immunoblotting of rat hepatocytes overexpressing either the liver isoform or the islet isoform of glucokinase (25) was conducted as previously described (6).

Cell area determination. Actin staining was used for determination of cell area. Digital images of cells with clearly defined borders were obtained. Cell area was determined using ScionImage software. Samples of 10–15 cells were imaged for each condition per experiment, and results are expressed as percentage of controls.

Enzyme release from digitonin-permeabilized cells. For determination of the effects of glucose and glucokinase inhibitors on glucokinase binding, the cells were incubated in medium with the concentrations of glucose and inhibitors indicated at 37°C and were equilibrated with 5% CO₂/95% O₂. On termination of the incubations, the medium was removed, and the cells were washed in 150 mmol/l NaCl and incubated with the permeabilization medium (200 μ l/well) for the time indicated. Unless otherwise indicated, the permeabilization medium contained 25 μ g/ml digitonin, 2 mmol/l dithiothreitol (DTT), 150 mmol/l KCl, 1 mmol/l MgCl₂, and 3 mmol/l HEPES, pH 7.2. After 4 min, the medium was removed for assay of enzyme activity and replaced with 200 μ l of second extraction buffer (as described above but with 40 μ g/ml digitonin). The cells were incubated for a further 20 min before the buffer was removed for assay. For studies with varying glucose concentration, to ensure complete extraction of enzymes from the MIN6 cells, a third extraction was performed by sonicating in 200 μ l medium containing 150 mmol/l KCl, 2 mmol/l DTT, and 3 mmol/l HEPES, pH 7.2. Enzyme activity in the first digitonin permeabilization medium is expressed as percentage of total extracted activity.

Enzyme activity determination. Glucokinase and low- K_m hexokinase activity were determined by the glucose 6-phosphate dehydrogenase (*Leuconos-*

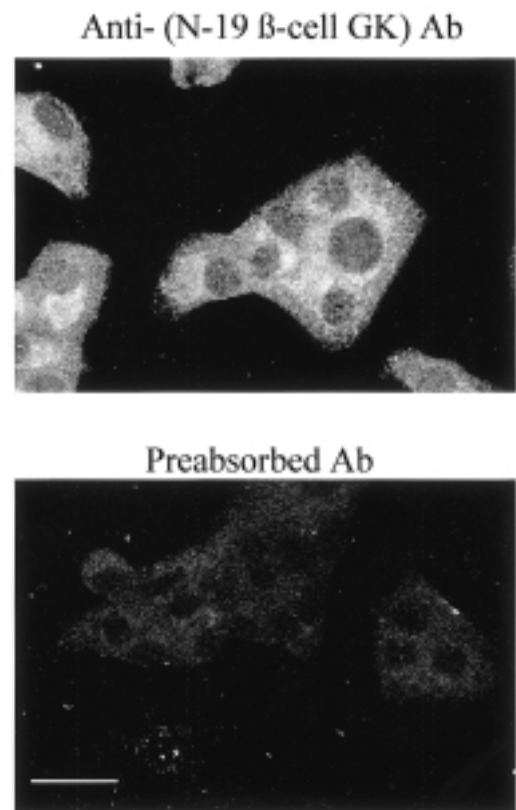


FIG. 2. Immunostaining of MIN6 cells with anti-N-19 β -cell glucokinase without (top panel) or with (bottom panel) preabsorption with N-19 peptide. Scale bar, 25 μ m.

toc mesenteroides) coupled assay (6). The glucose concentration was 0.5 mmol/l for the low- K_m hexokinase activity and 100 mmol/l for total phosphorylating activity. Glucokinase activity was determined from the difference in activity between 100 and 0.5 mmol/l glucose. Phosphoglucosomerase and lactate dehydrogenase were determined as previously described (9).

Glucose phosphorylation. MIN6 cells were incubated for 3 h in minimum essential medium containing the glucose concentrations and other additions indicated and [2-³H]glucose (2 μ Ci/ml). Additional experiments were performed in Earle's balanced salt solution (EBSS) or in EBSS in which NaCl was replaced by KCl. Radioactivity incorporated into ³H₂O was determined as described previously (26). Rates of glucose phosphorylation determined from formation of ³H₂O are expressed as nanomoles of glucose per 3 h per milligram of cell protein. The affinity for glucose ($S_{0.5}$ and Hill's coefficient) was determined from Hill plots (FigP, Biosoft software). Results are expressed as means \pm SE for the number of experiments indicated. Statistical analysis was performed with Student's paired *t* test.

RESULTS

Immunostaining of glucokinase in MIN6 β -cells. The specificity of the anti-glucokinase antibody for the β -cell isoform of glucokinase was confirmed by Western blotting of extracts of hepatocytes overexpressing either liver or islet glucokinase by approximately fivefold above endogenous activity. This showed immunoreactivity only to the latter extracts (Fig. 1). The specificity for immunofluorescence was checked by staining MIN6 cells with or without preabsorption of the antibody with the immunizing peptide. There was a perinuclear granular staining that was blocked by the immunizing peptide (Fig. 2). When COS-1 cells were stained using the same procedure as described in Fig. 2, they did not show any distinct staining (data not shown).

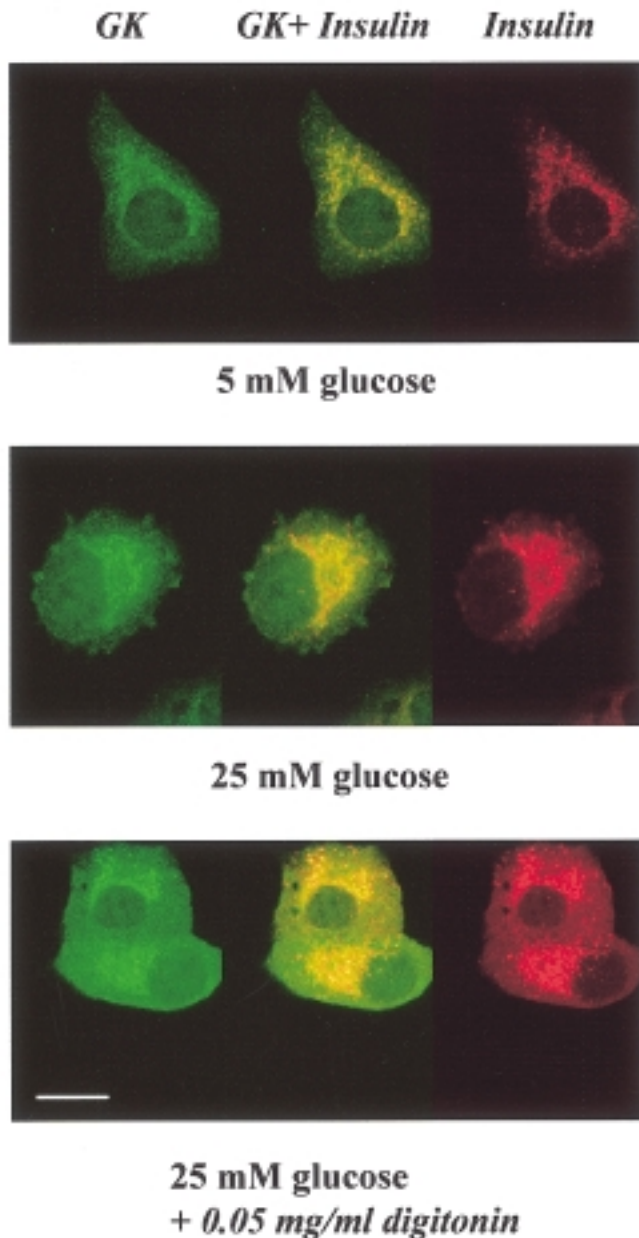


FIG. 3. Immunostaining of glucokinase and insulin in MIN6 β -cells. Cells were cultured for 12 h in medium with either 5 or 25 mmol/l glucose and were either untreated or treated with digitonin (50 μ g/ml for 5 min) before fixation. They were dual stained for glucokinase (green) and insulin (red). The middle panel shows the superimposed image. Yellow color indicates colocalization. Scale bar, 10 μ m.

Figure 3 shows glucokinase and insulin immunofluorescence in MIN6 cells incubated in medium containing either 5 or 25 mmol/l glucose. Both glucokinase and insulin show cytoplasmic staining with a punctate or granular pattern. Some of the granules show colocalization of glucokinase and insulin. Although there appear to be fewer glucokinase than insulin granules, this may be due to a lower sensitivity for glucokinase. There was no apparent difference in the staining pattern between 5 and 25 mmol/l glucose. This could be due to intercellular heterogeneity in the number and intensity of granules. Staining of granules in MIN6 cells was still seen after treatment with 50 μ g/ml digitonin (Fig. 3, bottom panel).

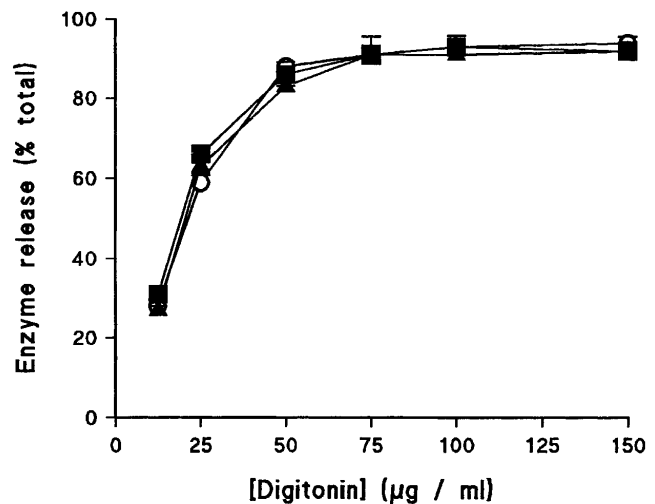


FIG. 4. Effects of digitonin concentration on the fractional release of enzyme activity. MIN6 cells cultured in medium with 25 mmol/l glucose were permeabilized for 5 min with the digitonin concentrations indicated, as described in RESEARCH DESIGN AND METHODS. The activity of glucokinase (\circ), phosphoglucosomerase (\blacksquare) and lactate dehydrogenase (\blacktriangle) released in the digitonin medium are expressed as a percentage of total activity. Values are means and SE for three experiments.

This suggests that glucokinase immunoreactivity associated with the granules is not released into the digitonin permeabilization medium. This digitonin concentration releases >85% of the activity of phosphoglucosomerase, lactate dehydrogenase, and glucokinase (Fig. 4).

Extraction of glucokinase during permeabilization with digitonin.

To investigate whether there is evidence for partitioning of glucokinase between a free and bound state, like the mechanism in liver cells (9), MIN6 cells were preincubated with either 5 or 25 mmol/l glucose and then permeabilized with a concentration of digitonin that causes only partial release of glucokinase activity using media of different ionic composition (Table 1). The fractional release of glucokinase during permeabilization for 4 min was lower when Mg^{2+} was present in the permeabilization medium, and it was also lower in cells that were preincubated with 25 vs. 5 mmol/l glucose. The latter effect was more pronounced in medium containing 150 mmol/l KCl (Table 1). It is worth noting that these differences in fractional release of glucokinase with either Mg^{2+} in the permeabilization medium or glucose in the preincubation are due to differences in the rate of glucokinase release, because, during prolonged incubation, there was similar release of enzyme activity in all conditions.

Effects of glucose on the rate of glucokinase release. To determine whether the effect of glucose on glucokinase release is direct or secondary to glucose metabolism, we used the glucokinase inhibitors, mannoheptulose and 5-thiogluconate (24). 5-Thiogluconate is a more potent inhibitor of glucose phosphorylation than mannoheptulose in MIN6 cells (Fig. 5A and B), as was also shown in hepatocytes (24). Mannoheptulose and 5-thiogluconate counteracted the effect of 25 mmol/l glucose on the rate of glucokinase release but had no effect at 5 mmol/l glucose (Fig. 5C and D), and the inhibitors abolished the difference between 25 and 5 mmol/l glucose. Together, these results establish that the effect of glucose on glucokinase release is secondary to glucose metabolism.

TABLE 1

Effects of glucose in the preincubation and Mg^{2+} during permeabilization on the fractional release of glucokinase during permeabilization with digitonin

Permeabilization conditions			Preincubation conditions	
Medium	<i>n</i>	Mg^{2+} (mmol/l)	5 mmol/l glucose	25 mmol/l glucose
300 mmol/l sucrose	3	0	58 ± 1	47 ± 6
300 mmol/l sucrose	3	2	20 ± 1*	14 ± 1†
300 mmol/l sucrose	3	5	19 ± 2*	16 ± 2
150 mmol/l KCl	4	0	56 ± 4	37 ± 5†
150 mmol/l KCl	4	2	45 ± 3*	30 ± 4†
150 mmol/l KCl	4	5	36 ± 8*	22 ± 5‡

Data are means ± SE. MIN6 cells were preincubated for 3 h in medium with either 5 or 25 mmol/l glucose and were then permeabilized with digitonin (25 µg/ml for 4 min) in medium with the composition indicated and the additional presence of 2 mmol/l DTT and 3 mmol/l HEPES, pH 7.2. The activity released was expressed as a percentage of total extractable activity. * $P < 0.05$ vs. absence of Mg^{2+} ; † $P < 0.05$; ‡ $P < 0.005$ vs. 5 mmol/l glucose.

Comparison of release of glucokinase, hexokinase, and phosphoglucosomerase. Figure 6 shows the fractional rates of release of glucokinase compared with low- K_m hexokinase activity and phosphoglucosomerase after preincubation with varying glucose concentration. Glucokinase and phosphoglucosomerase showed very similar fractional release as a function of glucose concentration, whereas release of low- K_m hexokinase was unaffected by glucose. Lactate dehydrogenase showed changes in relation to glucose concentration similar to those of glucokinase and phosphoglucosomerase (data not shown).

Effects of glucose concentration on cell area. The aforementioned results suggest that cytoplasmic glucokinase shows mobility (measured as diffusion from permeabilized cells) similar to that of phosphoglucosomerase, which in hepatocytes shows negligible binding (9,27). The lower fractional release of glucokinase during permeabilization of MIN6 with digitonin in the presence of Mg^{2+} (Table 1) could be explained by inhibition of depolymerization of the actin filaments by Mg^{2+} (9). Previous studies on pancreatic β -cells have shown that high glucose concentrations increase the polymerization state of F-actin (28). The slower release of glucokinase and phosphoglucosomerase at high glucose concentrations might therefore be due to an increase in the poly-

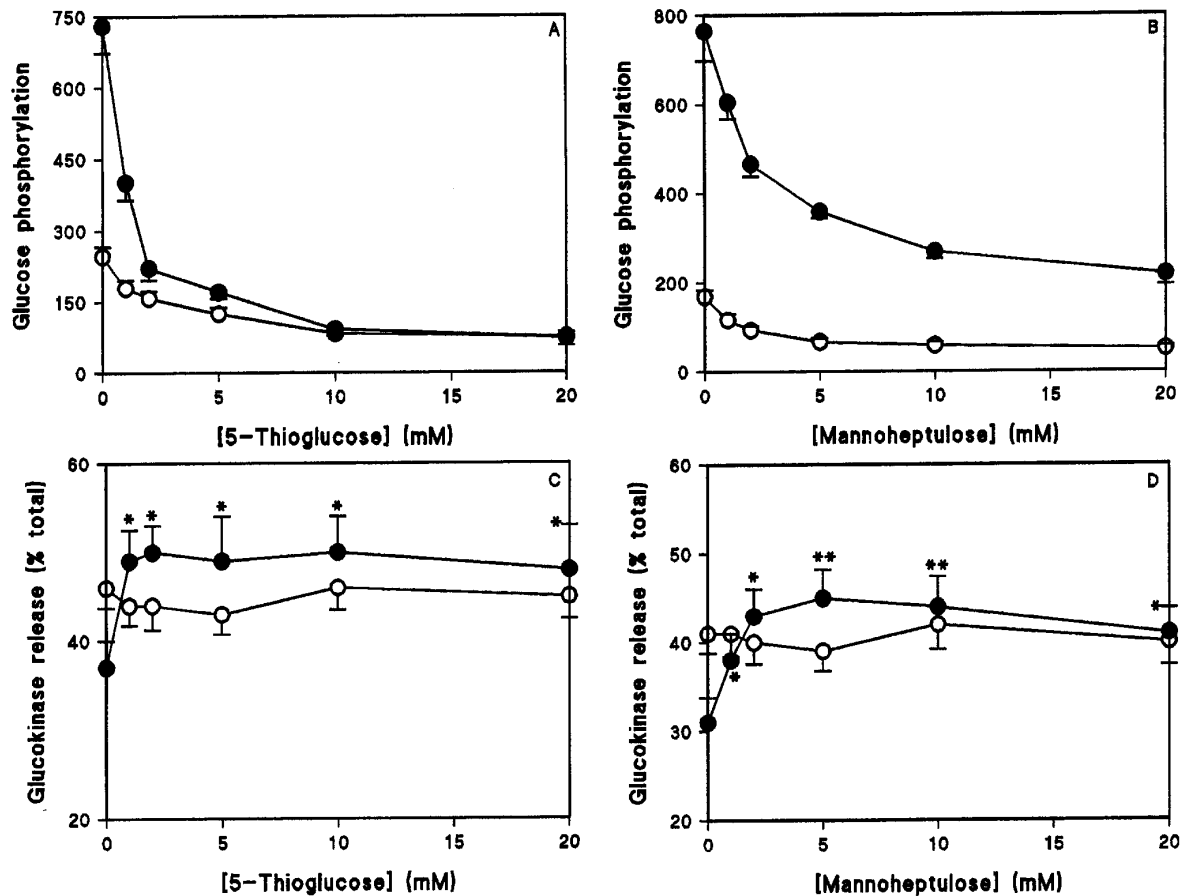


FIG. 5. Effects of glucokinase inhibitors on glucose phosphorylation and rates of glucokinase release from digitonin-permeabilized cells. MIN6 cells were incubated for 3 h in medium with either 5 (○) or 25 (●) mmol/l glucose and the concentrations of inhibitors indicated and [$2\text{-}^3\text{H}$]glucose for determination of glucose phosphorylation (A and B) expressed as nanomoles of glucose metabolized per 3 h per milligram cell protein. For determination of glucokinase release (C and D), monolayers were washed and incubated with digitonin (25 µg/ml for 4 min), as described in RESEARCH DESIGN AND METHODS. The glucokinase activity released during the first extraction is expressed as a percentage of total extracted glucokinase activity. Values are means ± SE for four to five experiments. * $P < 0.02$; ** $P < 0.002$ relative to the corresponding controls.

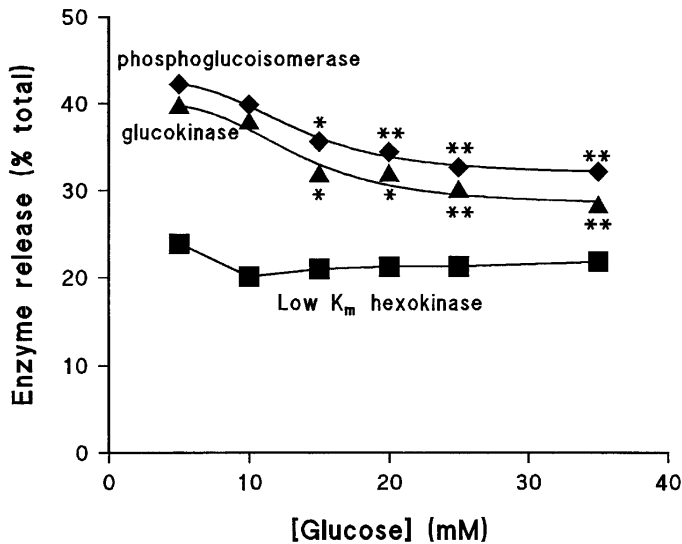


FIG. 6. Effects of glucose concentration on release of glucokinase, phosphoglucosomerase, and low- K_m hexokinase from digitonin-permeabilized cells. MIN6 cells were incubated in medium with 5–35 mmol/l glucose for 4–6 h. They were then permeabilized with digitonin (25 μ g/ml for 4 min), and enzyme release (% total activity) was determined as in Fig. 3. Values are means of 10–11 experiments for glucokinase (\blacktriangle), low- K_m hexokinase (\blacksquare), and phosphoglucosomerase (\blacklozenge); SEs were <8%. * $P < 0.01$; ** $P < 0.001$ relative to 5 mmol/l glucose.

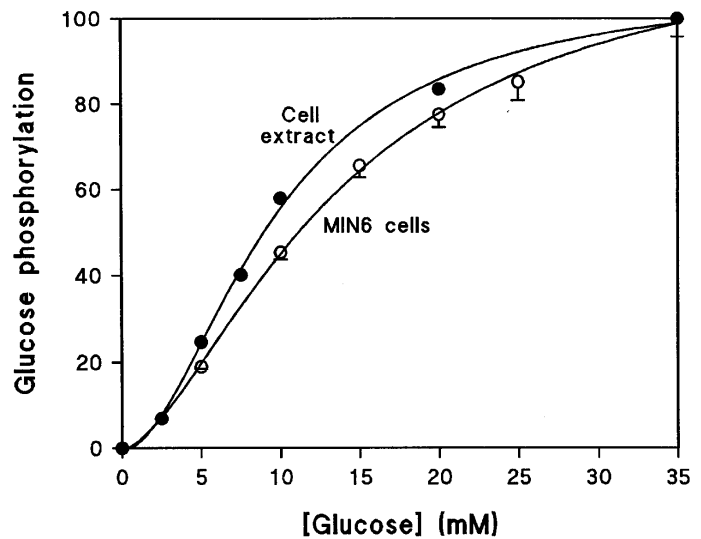


FIG. 7. Glucose phosphorylation activity in MIN6 cells and cell extracts. Glucokinase activity in cell extracts (13,000g supernatants) was determined spectrometrically (\bullet), and glucose phosphorylation in MIN6 cells (\circ) was determined radiochemically using [2- 3 H]glucose as described in RESEARCH DESIGN AND METHODS. Results are expressed as percentage of the values at 35 mmol/l glucose. Values for intact cells are means \pm SE for seven experiments; for cell extracts, they are representative of four experiments summarized in Table 2.

merization state of F-actin, which restricts the release of cytosolic enzymes. To test this possibility, we determined cell area from actin staining. The area of MIN6 cells incubated in medium containing 5 mmol/l glucose for 2 or 4 h (92 ± 4 and $86 \pm 5\%$ relative to DMEM control) was lower than that for incubations at 25 mmol/l glucose (103 ± 6 and $103 \pm 7\%$, $n = 5-7$). The difference was significant at 4 h ($P < 0.05$).

Glucose phosphorylation kinetics in MIN6 cells and cell extracts. Phosphorylation of glucose in MIN6 β -cells and cell extracts (13,000g supernatant) is sigmoidal (Fig. 7 and Table 2) with a Hill coefficient of ~ 1.5 and a slightly lower affinity (higher $S_{0.5}$) for glucose in the intact cells than in extracts (14 vs. 11 mmol/l). This difference in $S_{0.5}$ between β -cells and extracts is much smaller than that for rat hepatocytes (20–30 mmol/l for intact cells, 8–10 mmol/l for cell extracts) (7,10). The $S_{0.5}$ for glucose in hepatocytes is

decreased by precursors of fructose 1-phosphate or by replacement of Na^+ by K^+ , which is associated with an increase in cytoplasmic Cl^- . These effects are explained by the lower affinity of the regulatory protein for glucokinase in the presence of fructose 1-phosphate or elevated Cl^- (7,10).

To investigate whether the higher $S_{0.5}$ for glucose in MIN6 cells can be explained by an analogous mechanism to that of hepatocytes, we determined the effects of precursors of fructose 1-phosphate. There was no effect of fructose or sorbitol (0.1 and 1 mmol/l) on glucose phosphorylation in MIN6 cells (data not shown). Because fructose 1-phosphate is formed from fructose by fructokinase and is further metabolized by aldolase, the lack of effect of fructose or sorbitol may be due to a low concentration of fructose 1-phosphate because of a low activity of fructokinase relative to aldolase. To circumvent this problem, we used tagatose, which is phosphorylated by

TABLE 2
Glucose phosphorylation in MIN6 cells and glucokinase activity in cell extracts

	Experiments	$S_{0.5}$ glucose (mmol/l)	Hill coefficient
Glucose phosphorylation in MIN6 cells			
Control incubations	30	14.2 ± 0.4	1.51 ± 0.04
+ 2 mmol/l tagatose	3	16.3 ± 1.3	1.40 ± 0.08
+ 20 μ mol/l Zn^{2+}	10	15.1 ± 0.8	1.20 ± 0.12
Replacement of Na^+ by K^+	3	15.9 ± 0.3	1.60 ± 0.06
Glucokinase activity in MIN6 cell extracts			
Control	4	$11.4 \pm 0.4^*$	1.61 ± 0.08
+ 20 μ mol/l Zn^{2+}	3	$26.9 \pm 2.2^\dagger$	1.88 ± 0.3

Data are n or means \pm SE. Glucose phosphorylation in MIN6 cells was determined in medium containing 5–35 mmol/l glucose from the detritiation of [2- 3 H]glucose as described in RESEARCH DESIGN AND METHODS. Glucokinase activity in cell extracts was determined spectrometrically. The $S_{0.5}$ for glucose and the Hill coefficient were determined from Hill plots. * $P < 0.005$ relative to control incubations in MIN6 cells; $^\dagger P < 0.005$ relative to no Zn^{2+} .

fructokinase to tagatose 1-phosphate, which mimics the effects of fructose 1-phosphate (29) and accumulates at high concentrations because it is not further metabolized. There was no effect of tagatose on glucose phosphorylation in MIN6 cells (Table 2) or on the rate of glucokinase release during permeabilization with digitonin (data not shown). Another possibility is that β -cells contain a protein that shares some similarities with rat liver regulatory protein without being responsive to fructose 1-phosphate, as is the case for *Xenopus* liver regulatory protein, which lacks a fructose 1-phosphate binding site (30). However, there was no effect of replacement of extracellular Na^+ by K^+ (Table 2), which in hepatocytes increases the affinity for glucose by dissociation of glucokinase from the regulatory protein (10). We found no immunoreactivity by Western blotting in MIN6 extracts using antiserum against rat liver regulatory protein (31).

Another protein that binds glucokinase is the 11.5-kDa Zn^{2+} -binding protein, also known as parathymosin (32). We determined the effects of micromolar concentrations of Zn^{2+} on glucose phosphorylation in MIN6 cells and on glucokinase activity in cell extracts. Zn^{2+} (20 $\mu\text{mol/l}$) markedly inhibited glucokinase activity in cell extracts but had no effect on intact cells when added to the medium during incubation with [$2\text{-}^3\text{H}$]glucose (Table 2).

DISCUSSION

The subcellular compartmentation of glucokinase in insulin-secreting cells has been investigated previously by either immunostaining or cell permeabilization techniques. In pancreatic β -cells, unlike liver cells, glucokinase is not detectable in the nucleus but is confined to the cytoplasm and shows no colocalization with GLUT2 in the plasma membrane (17,19). Noma et al. (22) found a perinuclear localization of glucokinase at euglycemia but not at hyperglycemia, and Jorns et al. (18) reported a polarized distribution of glucokinase in islets from fed but not from fasted rats. Neither of these studies tested for colocalization with insulin. Toyoda et al. (20) reported a granular pattern of glucokinase staining in islet β -cells with most of the immunoreactivity colocalizing with insulin. Using a digitonin cell permeabilization technique on RINm5F cells overexpressing glucokinase, Tiedge et al. (16) found that digitonin released 50% of the activity as determined by Western blotting and that the residual immunoreactive protein was largely kinetically inactive. In the present study, we combined immunostaining, a digitonin-permeabilization technique, and glucose phosphorylation in intact cells to investigate the kinetic activity and subcellular location of endogenous glucokinase activity in the glucose-responsive MIN6 cell line.

The combined results of the immunostaining and the digitonin-permeabilization technique suggest that glucokinase is present mainly in two compartments, the cytoplasm and granule compartments. The resolution of the imaging technique does not allow us to determine what proportion of total glucokinase is associated with granules. However, as suggested by the finding that the granular staining pattern is preserved after treatment of the cells with a digitonin concentration that releases >85% of phosphoglucoisomerase, we infer that the glucokinase activity released represents mainly the cytoplasmic fraction. Phosphoglucoisomerase was selected as a reference because it is present in very high activity and does not show specific binding in other cells (27). Also, unlike gluco-

kinase and various glycolytic enzymes, it does not bind to the 11.5-kDa zinc-binding protein (32).

We found no evidence for rapid changes in the distribution of glucokinase between the cytoplasm and granule compartment from either the digitonin-release assay or immunostaining. The latter technique is not sensitive because of intercellular heterogeneity and also because the limits of resolution do not allow estimation of the distribution of fluorescence intensity between individual granules and background cytoplasm. However, digitonin permeabilization is a fairly sensitive technique (9). If there were rapid changes in the distribution of glucokinase between granules and the cytoplasm in response to changes in glucose concentration, these changes should have been detectable in terms of the total glucokinase activity extracted in the digitonin-release assay. The lack of rapid changes in free glucokinase in the digitonin extractable fraction rules out acute changes in glucokinase activity in the cytoplasm, as is the case in hepatocytes (10).

The digitonin-release assay showed that the rate of release of glucokinase from the permeabilized cells was significantly slower when the cells were preincubated at higher glucose concentrations. This effect was similarly observed for rates of release of phosphoglucoisomerase and lactate dehydrogenase, which indicates that the effect is not a result of specific binding of glucokinase. Based on measurements of cell area from actin staining and the finding that the effect of glucose was counteracted by competitive inhibitors of glucokinase, we infer that the slower release of cytoplasmic enzymes in cells cultured at high glucose concentration is most likely due to differences in cell shape and/or conformation of the cytoskeleton that are secondary to glucose metabolism (28) or to differences in organization of cytoplasmic enzymes that are not specific for glucokinase. The difference in the perinuclear localization of glucokinase reported previously between euglycemia and hyperglycemia (19) or between fed and fasted rats (18) may be due to changes in the location of granules caused by changes in cell morphology or conformation of the cytoskeleton rather than redistribution of glucokinase between the granules and cytoplasm.

Three sets of evidence support the conclusion that a regulatory protein analogous to liver regulatory protein does not exist in insulin-secreting β -cells. First, there was no effect of fructose, sorbitol, or tagatose on glucose phosphorylation. Second, replacement of extracellular Na^+ by K^+ , which in hepatocytes causes dissociation of glucokinase from the regulatory protein through an increase in cytoplasmic Cl^- , also had no effect on glucose phosphorylation kinetics. Third, there was no evidence for immunoreactivity by Western blotting to antiserum against the liver regulatory protein.

The absence of rapid changes in distribution of glucokinase between the cytoplasm and granules does not rule out other mechanisms of control. The present study shows that Zn^{2+} is a potent inhibitor of glucokinase activity at micromolar concentrations. Consequently, changes in cytoplasmic Zn^{2+} could regulate glucose phosphorylation. In pancreatic β -cells, Zn^{2+} is concentrated at levels >50 times blood levels and is present in both the protein core of the insulin granule and in an extragranular compartment (33). Because exposure of islet cells to glucose decreases the content of labile zinc (34), the slightly lower affinity of glucose phosphorylation in intact cells compared with extracts could be explained by greater inhibition by Zn^{2+} at 5 mmol/l glucose than that at

higher glucose concentrations. The lack of acute effect of extracellular Zn^{2+} on intact cells is consistent with the slow kinetics of uptake of Zn^{2+} by β -cells (35). In hepatocytes, rapid changes in cytoplasmic free Zn^{2+} in response to glucagon could contribute to inhibition of glycolysis (35). Thus, the potential role for changes in cytoplasmic free Zn^{2+} in regulating glucokinase activity and glycolysis in the pancreatic β -cell warrants further study.

A key issue concerns the function of the association of glucokinase with insulin granules. Two possibilities can be considered. Glucokinase associated with the granules could be in an inactive state, and this could be a mechanism for protecting glucokinase against degradation, analogous to the proposed stabilization of the hepatic enzyme by the regulatory protein in the nucleus (13). This mechanism seems unlikely, however, because we found no evidence for translocation of glucokinase between the granule and cytoplasmic compartment. An alternative possibility is that glucokinase associated with the granules is involved in direct or indirect coupling between glucose (or glucose 6-phosphate) and ionic events involved in insulin secretion.

Glucose causes biphasic stimulation of insulin secretion. This consists of initial rapid secretion (within 15 min) of a readily releasable pool of granules comprising <1% of the total granule pool, followed by a slower sustained release, which is thought to represent replenishment of the readily releasable granules by the reserve pool (37). The latter process is thought to involve glucose metabolism and ATP hydrolysis by a granular proton pump, which causes acidification of the granule core. Conversion of proinsulin to insulin occurs in vesicles that become more acidic as the granules mature (38). Because colocalization of glucokinase and insulin is >1% of the insulin granule pool, it is unlikely that glucokinase is confined to the readily releasable pool of granules.

Insulin granules have an acidic core and a membrane potential that is positive inside (39). Glutamate uptake by insulin granules is a component of the mechanism that contributes to granule acidification (40). Glutamate uptake opposes membrane potential, thereby allowing a larger pH gradient to develop by the H^+ -ATPase. A key question concerns whether glucokinase associated with the insulin granule plays a role in the ionic events involved in acidification or accumulation of Ca^{2+} . Hexokinases are conventionally regarded as irreversible enzymes in physiological conditions, because the equilibrium constant favors the product ($K_{eq} 2 \times 10^3$). However, hexokinases can function as ATP-generating systems (from glucose 6-phosphate and ADP) if coupled to ATP-utilizing enzymes with a very high affinity for ATP, such as H^+ -ATPases or Ca^{2+} -ATPases (41,42) (review provided by Montero-Lomeli and De Meis [43]). An analogous mechanism could occur for glucokinase in association with insulin granules. Two possibilities can be considered. Glucokinase in association with the granule may utilize cytoplasmic glucose 6-phosphate and ADP to generate ATP for either the H^+ -ATPase, which would result in acidification of the granule or for Ca^{2+} uptake by the Ca^{2+} -ATPase. This would involve coupling to cytoplasmic glucose 6-phosphate. Alternatively, glucokinase in association with the granule may be structurally organized so that the enzyme facing the cytoplasmic side converts glucose and ATP to glucose 6-phosphate and ADP. The latter products may be utilized by glucokinase that is not exposed to the cytoplasmic surface for the reverse reaction so that the

formed ATP is coupled to the H^+ -ATPase. In the latter case, granule acidification would be coupled to cytoplasmic glucose. In either case, glucokinase activity associated with the insulin granule would be apparently inactive if assayed using glucose 6-phosphate dehydrogenase as an auxiliary enzyme. The kinetic function of glucokinase in association with the insulin granule warrants detailed study.

In summary, glucokinase in glucose-responsive MIN6 β -cells is present in two compartments: a cytoplasmic compartment, in which the enzyme has a similar mobility as phosphoglucosomerase, and a granule compartment that shows colocalization with the insulin granules. There is no evidence for rapid glucose-induced changes in distribution of glucokinase between these two compartments, similar to the mechanism that operates in liver (9,10) or for changes in the affinity of glucose phosphorylation for glucose. This lack of adaptive changes is in marked contrast to the hepatocyte, in which the affinity for glucose is regulated acutely through changes in concentrations of fructose 1-phosphate (7) and chronically through changes in the ratio of expression of glucokinase and its regulatory protein (7,12). The lack of adaptive change in affinity of the β -cell for glucose is consistent with the role of pancreatic β -cell glucokinase as a glucose sensor or glucoreceptor (1,2). The function of the liver enzyme is not as a glucose sensor but for rapid metabolism of glucose and feed-forward activation of glycogen synthesis and glycolysis in the absorptive state (5,6).

ACKNOWLEDGMENTS

This work was supported by the British Diabetic Association. We thank Prof. Jun-ichi Miyazaki for the kind gift of MIN6 cells.

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