The EMBO Journal vol.12 no.7 pp.2873-2879, 1993

Heterogeneity in glucose sensitivity among pancreatic β-cells is correlated to differences in glucose phosphorylation rather than glucose transport

Harry Heimberg, Anick De Vos, Annick Vandercammen[1], Emile Van Schaftingen[1], Daniel Pipeleers[2] and Frans Schuit[3]

Departments of Biochemistry and [2]Metabolism and Endocrinology, Diabetes Research Center, Vrije Universiteit Brussel and [1]Laboratory of Physiological Chemistry, Université Catholique de Louvain and International Institute of Cellular and Molecular Pathology, B-1200 Brussels, Belgium
[3]Corresponding author

Communicated by N.Glansdorff

Rat β-cells differ in their individual rates of glucose-induced insulin biosynthesis and release. This functional heterogeneity has been correlated with intercellular differences in metabolic redox responsiveness to glucose. The present study compares glucose metabolism in two β-cell subpopulations that have been separated on the basis of the presence (high responsive) or absence (low responsive) of a metabolic redox shift at 7.5 mM glucose. Mean rates of glucose utilization and glucose oxidation in high responsive β-cells were 2- to 4-fold higher than in low responsive β-cells, whereas their leucine and glutamine oxidation was only 10-50% higher. This heterogeneity in glucose metabolism cannot be attributed to differences in GLUT2 mRNA levels or in glucose transport. In both cell subpopulations, the rates of glucose transport (13-19 pmol/min/10^3 β-cells) were at least 50-fold higher than corresponding rates of glucose utilization. On the other hand, rates of glucose phosphorylation (0.3-0.7 pmol/min/10^3 β-cells) ranged within those of total glucose utilization (0.2-0.4 pmol/min/10^3 β-cells). High responsive β-cells exhibited a 60% higher glucokinase activity than low responsive β-cells and their glucokinase mRNA level was 100% higher. Furthermore, glucose phosphorylation via low K_m hexokinase was detected only in the high responsive β-cell subpopulation. Heterogeneity in glucose sensitivity among pancreatic β-cells can therefore be explained by intercellular differences in glucose phosphorylation rather than in glucose transport.

Key words: β-cells/glucokinase/glucose transporters/hexokinase/insulin

Introduction

Glucose homeostasis is regulated through the glucose sensitivity of pancreatic β-cells, which respond to the sugar by increasing the production and release of insulin (Grodsky *et al.*, 1963; Permutt and Kipnis, 1972). Rat islet β-cells have been found to differ in their individual glucose sensitivity (Van De Winkel and Pipeleers, 1983; Schuit *et al.*, 1988; Bosco and Meda, 1991; Hiriart and Ramirez-Mendelez, 1991; Kiekens *et al.*, 1992; Pipeleers, 1992; Van Schravendijk *et al.*, 1992a). Heterogeneity in the glucose-induced redox state of individual β-cells correlates with different thresholds for glucose-induced insulin biosynthesis (Kiekens *et al.*, 1992) and release (Van Schravendijk *et al.*, 1992a). The molecular basis for this cellular heterogeneity has not yet been clarified. Glucose responsiveness of β-cells is thought to result from their capacity to metabolize the sugar (MacDonald, 1990). Several sites in cellular glucose handling (glucose transport to mitochondrial events) may regulate cellular sensitivity to the sugar (Meglasson and Matschinsky, 1986; Thorens *et al.*, 1988; Liang *et al.*, 1990; MacDonald *et al.*, 1991; Unger, 1991). Both glucose transport and phosphorylation have been proposed as key steps in the regulation of β-cell functions (Meglasson and Matschinsky, 1986; Unger, 1991). The expression level of the glucose transporter GLUT2 was found to be reduced in animal models of diabetes (Johnson *et al.*, 1990a; Orci *et al.*, 1990; Thorens *et al.*, 1990), whereas transfection of the AtT-20 cell line with the *GLUT2* gene conferred glucose responsiveness to this cell type (Hughes *et al.*, 1992). Rates of glucose phosphorylation via the high K_m enzyme glucokinase (hexokinase IV, EC 2.7.1.2) paralleled the glucose sensitivity of isolated islets of Langerhans (Meglasson and Matschinsky, 1986). Glucose phosphorylation via low K_m hexokinases I-III (EC 2.7.1.1) may also play a role in the glucose sensor of β-cells as suggested by recent data in transgenic mice with expression of yeast hexokinase B in the β-cells (Epstein *et al.*, 1992).

The ability to purify rat β-cells on the basis of their metabolic sensitivity to glucose (Kiekens *et al.*, 1992), allows to test which of these proximal steps in cellular glucose handling is more closely associated with the previously observed heterogeneity in metabolic redox state and in glucose-induced insulin biosynthesis (Kiekens *et al.*, 1992) and release (Van Schravendijk *et al.*, 1992a). The present study measures glucose-induced proinsulin biosynthesis and overall glucose metabolism in two β-cell subpopulations that have been separated on the basis of high/low redox responsiveness to glucose and assesses a possible correlation with glucose transport and phosphorylation.

Results

Total protein and proinsulin biosynthesis in low and high responsive β-cells

Purified β-cells with low and high responsiveness to glucose were separated by autofluorescence-activated cell sorting using cellular NAD(P)H fluorescence as an index for cellular glucose recognition (Van De Winkel and Pipeleers, 1983; Kiekens *et al.*, 1992). During a subsequent 2 h incubation, proinsulin biosynthesis in the high responsive β-cells proceeded at significantly higher rates than in the low responsive β-cells (Figure 1). This difference in biosynthetic activity was noted at all glucose concentrations tested but was most marked at the lower levels, being >3-fold at 2.5

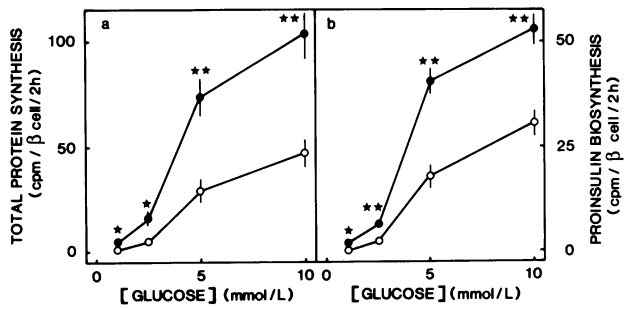

Fig. 1. Effect of glucose upon total protein synthesis and proinsulin biosynthesis by low (○) and high (●) responsive β-cells. Results are expressed as c.p.m. per β-cell and represent mean values ± SEM (n = 4). Significance of differences between the low and high responsive β-cells was calculated by using the unpaired Student's t-test. *, $P < 0.05$; **, $P < 0.005$.

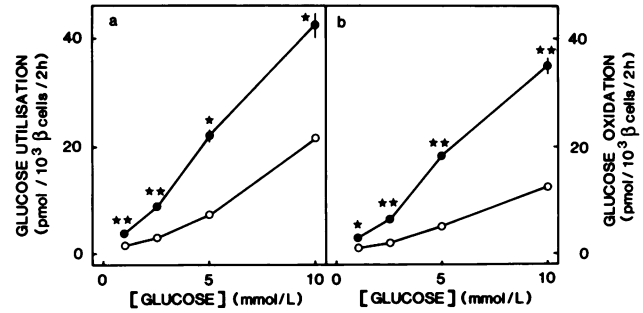

Fig. 2. Glucose utilization and oxidation by low (○) and high (●) responsive β-cells. Glucose utilization was measured as ^3H_2O production from [5-^{3}H]D-glucose and oxidation as $^{14}CO_2$ output from [U-^{14}C]D-glucose. Results are expressed as picomoles of glucose utilized or oxidized/10^3 β-cells; data represent mean values ± SEM (n = 3 for utilization, n = 4 for oxidation). Significance of differences between the two subpopulations was calculated by using the unpaired Student's t-test. *, $P < 0.005$; **, $P < 0.001$.

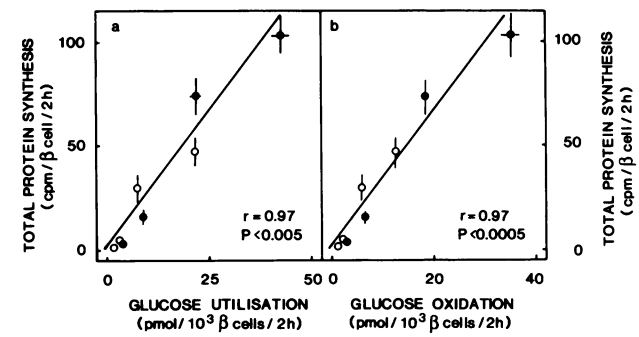

Fig. 3. Correlation between glucose metabolism and total protein synthesis by low (○) and high (●) responsive β-cells. Data represent mean values ± SEM of 3–4 experiments.

mM glucose. Insulin biosynthesis was preferentially stimulated over total protein synthesis in the β cells exposed to glucose: ratios of insulin biosynthesis over total protein synthesis increased from 0.34 ± 0.06 (low β-cells) and 0.30 ± 0.04 (high β-cells) at 1 mM glucose to 0.67 ± 0.07 (low β-cells) and 0.53 ± 0.08 (high β-cells) at 20 mM glucose (mean ± SEM, n = 4, low versus high $P > 0.05$).

Glucose utilization and oxidation in low and high responsive β-cells

Raising the glucose concentration from 1 to 10 mM resulted in a dose-dependent increase of glucose utilization in both low and high responsive β-cells. At each tested glucose level, the rate of glucose utilization by the high responsive β-cells was >2-fold higher than that by the low responsive β-cells (Figure 2). The rate of glucose utilization by high responsive β-cells at 5 mM (21.9 ± 1.9 pmol/10^3 β-cells) was comparable with that by low responsive β-cells at 10 mM (21.6 ± 1.7 pmol/10^3 β-cells) (Figure 2). Glucose oxidation in high responsive β-cells also occurred significantly more rapidly than in low responsive β-cells (Figure 2). When the rates of protein biosynthesis were plotted against the corresponding rates of glucose utilization and oxidation (Figure 3), all data fitted onto the same regression line, expressing a strong correlation between functional response (protein synthesis) and metabolic activity (glucose utilization and glucose oxidation).

Glucose transporter gene expression in low and high responsive β-cells

Since the glucose transporter GLUT2 was proposed to be part of the glucose sensor in β-cells (Unger, 1991), we compared the activity and expression of glucose transporters in low and high responsive β-cells. In both subpopulations the uptake of the non-metabolizable glucose analog 3-*O*-methyl glucose (3-OMG) reached equilibrium after 3 min at 12°C and 1 mM substrate concentration (Figure 4). The kinetics and the final 3-OMG space were similar in both preparations. At 37°C and 7.5 mM substrate level, the initial rates of 3-OMG uptake were comparable in both populations: 3.2 ± 0.09 and 3.5 ± 0.08 mmol/10 s/l space in the low and high responsive cells respectively ($P > 0.05$). Cellular rates of glucose uptake were therefore at least 50-fold higher than the corresponding rates of glucose utilization (Table I). Northern blot analysis of the low and high responsive β-cells revealed two transcripts of 2.8 and 3.9 kb that hybridized with GLUT2 cDNA (Figure 5A). Autoradiographic band intensity values were normalized for β-actin mRNA. Mean intensities of the 2.8 kb GLUT2 mRNA were statistically similar in low and high responsive β-cells (Figure 5B). The 3.9 kb transcript hybridized specifically with the GLUT2 probe and was only detected in pancreatic β-cells (Figure 5A). The abundance of this mRNA was 2-fold higher in high responsive than in low responsive β-cells (Figure 5B). After autoradiographic exposure of 20 days, a weak GLUT1 mRNA signal of the same intensity was detected in low and high responsive β-cells (Figure 5). No GLUT4 mRNA was detected in β-cells (Figure 5A). Western blots of low and high responsive β-cells that were incubated with an anti-GLUT2 antibody showed a GLUT2-specific band of the same intensity in both subpopulations (data not shown).

Hexokinase and glucokinase in low and high responsive β-cells

Glucose phosphorylation was measured both at 1 and 20 mM substrate concentration either in the absence or presence of 2.5 mM glucose 6-phosphate (Glc6-P) in order to distinguish between low K_m Glc6-P-suppressible hexokinases and high K_m Glc6-P-insensitive glucokinase. In contrast to the rates of glucose uptake, those of glucose phosphorylation ranged within the levels of glucose utilization (Table I) and were at 20 mM glucose two times higher in the high β-cells than

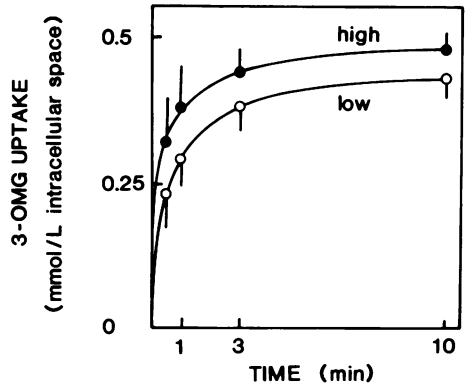

Fig. 4. Time-course of zero-*trans* 3-*O*-methyl-D-glucose uptake in low (○) and high (●) responsive β-cells. Uptake was measured at 12°C in low and high β-cells. External 3-*O*-methyl-D-glucose concentration at time zero was 1 mM. Data represent mean values ± SEM from four independent experiments. Uptake in low and high β-cells was statistically similar at all time points.

Table I. Rates of glucose uptake, phosphorylation and utilization in low- and high responsive β-cells

Metabolic site	Metabolic flux		*n*	*P*
	Low β-cells (pmol/min/10^3 β-cells)	High β-cells (pmol/min/10^3 β-cells)		
Glucose uptake	13.3 ± 1.5	19.1 ± 0.8	4	<0.05
Glucose phosphorylation	0.32 ± 0.03	0.67 ± 0.11	5	<0.005
Glucose utilization	0.18 ± 0.02	0.36 ± 0.05	4	<0.005

Data represent mean values ± SEM of *n* independent measurements. Statistical significance of differences between low and high responsive β-cells was calculated with the unpaired two-tailed Student's *t*-test. Glucose uptake was measured as initial (0–10 s) 3-OMG uptake rates at 7.5 mM substrate level; glucose utilization and glucose phosphorylation at 10 and 20 mM glucose respectively, over 1–3 h incubations as described in Materials and methods.

in the low β-cells. No glucose phosphorylation via hexokinase was measured in low β-cells (Figure 6a). On the contrary, hexokinase activity in high β-cell homogenates accounted for 82% of total glucose phosphorylation at 1 mM substrate level and for 18% at 20 mM glucose. The rate of glucose phosphorylation via glucokinase was 60% more elevated in the high responsive β-cells than in the low responsive β-cells ($P < 0.005$, Figure 6b). Differences between low and high responsive β-cells were also detected at the level of mRNA abundance (Figure 5). As previously described (Iynedjian *et al.*, 1986, 1989; Magnuson and Shelton, 1989), β-cells contained a larger glucokinase transcript (2.6 kb) compared with liver (2.4 kb). The same transcript was found in low and high responsive β-cells (Figure 5A); autoradiographic band intensity of glucokinase mRNA was 2-fold more elevated in the high responsive β-cells than in the low responsive β-cells ($P < 0.05$) (Figure 5B).

Amino acid oxidation, glyceraldehyde 3-phosphate dehydrogenase, pyruvate dehydrogenase and glutamate dehydrogenase activities

To investigate whether increased rates of glucose metabolism in high responsive β-cells is caused by a non-specific increase in β-cell metabolism or enzyme biosynthesis, the oxidation of leucine and glutamine and the activities of several other catabolic enzymes were measured. In contrast to the observed differences in glucose oxidation rates, high and low responsive β-cells exhibited similar catabolic rates for leucine or glutamine at 1 mM substrate concentration (Table II). At 10 mM substrate levels, high responsive β-cells oxidized only 50% more leucine than low responsive β-cells (11.8 ± 0.7 versus 7.8 ± 1.1 pmol/2 h/10^3 cells; $P < 0.05$), whereas their corresponding rates of glucose oxidation were 180% higher (35.2 ± 2.5 versus 12.5 ± 0.8 pmol/2 h/10^3 cells; $P < 0.001$). Glyceraldehyde 3-phosphate dehydrogenase activity was 30% higher in the high responsive β-cells compared with the low responsive ones ($P < 0.05$); metabolic flux through this glycolytic enzyme exceeded maximal flux through glucokinase by at least two orders of magnitude. Finally, the activity of pyruvate dehydrogenase and of glutamate dehydrogenase was similar in both subpopulations (Table II).

Discussion

It was previously demonstrated that individual pancreatic β-cells display heterogeneity in glucose-induced proinsulin biosynthesis (Schuit *et al.*, 1988) and that such cell-to-cell variation is linked to heterogeneity in the glucose-induced metabolic redox state (Kiekens *et al.*, 1992). This correlation allowed us to separate isolated β-cells into two subpopulations composed of low responsive or high responsive β-cells differing in their respective rates of glucose-induced proinsulin biosynthesis (Kiekens *et al.*, 1992) and insulin release (Van Schravendijk *et al.*, 1992a). β-cell heterogeneity appears to result from relatively stable differences in the expression level and/or intrinsic activity of a regulator that influences the cellular sensitivity for glucose (Giordano *et al.*, 1991; Kiekens *et al.*, 1992; Van Schravendijk *et al.*, 1992a). Our search for this regulator is based on the hypothesis that glucose-induced β-cell stimulation is generated through catabolic flux of the sugar (MacDonald, 1990). Compatible with this concept is the fact that the elevated rates of glucose-induced proinsulin and total protein synthesis in high responsive β-cells are closely paralleled by elevated rates of higher glucose utilization and glucose oxidation. The correlation between glucose metabolism and protein synthesis may, in theory, indicate a cause–effect relationship in either way. However, metabolic heterogeneity was found to persist in subpopulations pretreated with cycloheximide (data not shown) so that the high rate of glucose utilization in high responsive β-cells should not be considered as a consequence of a higher rate of protein synthesis. A general metabolic defect in low responsive β-cells is also rendered unlikely by the measurements of leucine and glutamine oxidation. The catabolic rates for both non-glucose metabolites differed indeed only moderately between low and high responsive β-cells; glutamine dehydrogenase activity was also identical in both subpopulations. In line with these data, the leucine-induced secretion of glucose unresponsive β-cells was equal to that of glucose responsive cells (Van Schravendijk *et al.*, 1992b). Taken together, these data suggest that the basis for the functional heterogeneity of β-cells resides in one or more steps of glucose catabolism. The measurement of similar pyruvate dehydrogenase activity in low and high responsive

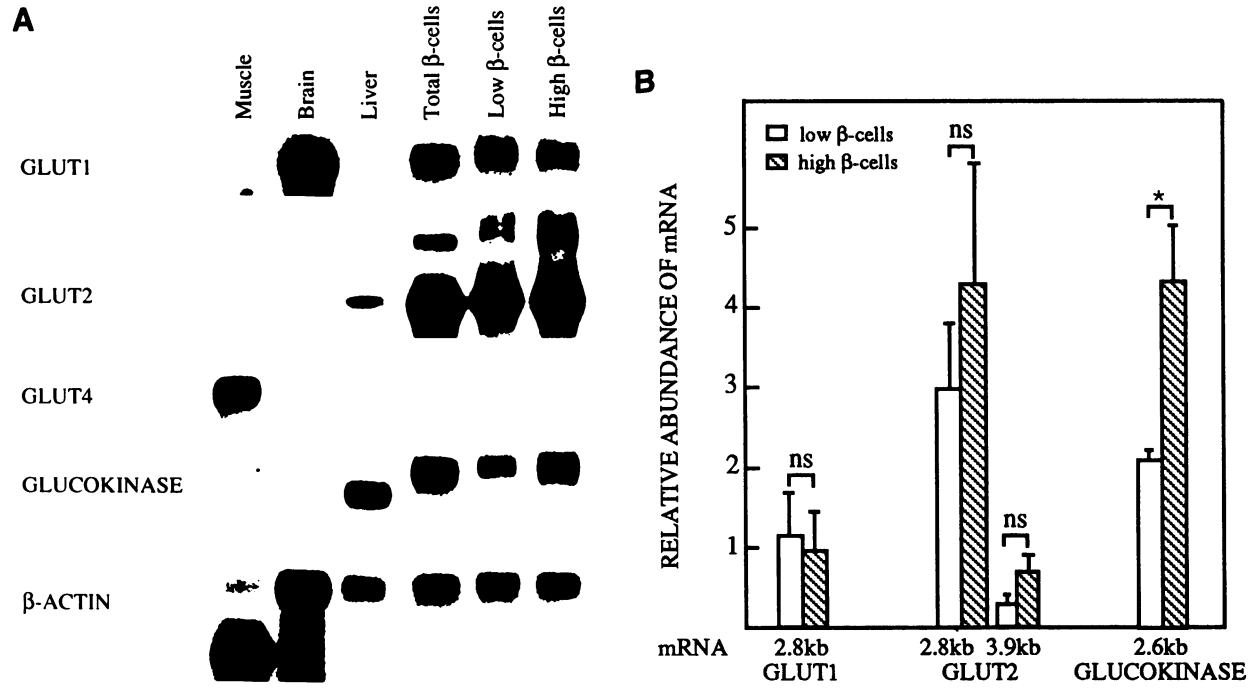

Fig. 5. Northern analysis of low and high responsive β-cells. (**A**) 10 μg of total RNA were separated on a 1.5% formaldehyde–agarose gel, transferred to nylon membranes, UV-cross-linked and successively hybridized with cDNA probes specific for GLUT1, GLUT2, GLUT4, glucokinase and β-actin. Autoradiographic exposure times required to generate the signals that are shown were 1 day for β-actin, 2 days for GLUT2, 4 days for GLUT4, 10 days for glucokinase and 20 days for GLUT1. RNA from β-cells and control tissues was extracted from the same animals. (**B**) The intensity of the autoradiographic signals was scanned on a laser densitometer using less exposed films than the ones shown here. Of three independent experiments, mean values ± SEM are expressed per β-actin mRNA. ns, not significant; *, $P < 0.05$.

β-cells argues against a key role of this enzyme in the metabolic heterogeneity of the β-cells. Studies on isolated rat islets had indicated that this enzyme may function as a regulator of the glucose sensor in β-cells (MacDonald *et al.*, 1991).

The expression level of the glucose transporter GLUT2, a recently proposed candidate for the regulation of β-cell functions (Thorens *et al.*, 1988; Unger, 1991), was similar in low and high responsive β-cells, both at the level of mRNA abundance and of immunoreactive protein (data not shown). Homogeneous immunostaining for GLUT2 was already reported in islet sections (Thorens *et al.*, 1988; Jetton and Magnuson, 1992). More importantly, initial rates of glucose uptake in both β-cell subpopulations largely exceeded the rates of glucose utilization and oxidation. It is therefore unlikely that the level of *GLUT2* expression plays a major role in setting the glucose sensor of normal β-cells. A dramatic reduction of *GLUT2* expression would be required to make glucose uptake the rate-limiting step for glucose catabolism by the β-cells. It is not clear whether this is indeed the case in those conditions where a decrease in GLUT2 levels was proposed as cause for a state of non-insulin dependent diabetes (Johnson *et al.*, 1990a; Orci *et al.*, 1990; Thorens *et al.*, 1990). We noticed that high responsive β-cells contained 2-fold more of a 3.9 kb transcript that strongly cross-hybridizes with the GLUT2 cDNA-probe. cDNA sequencing will be required to find the relationship of this transcript to the glucose transporter family.

Glucose phosphorylation by glucokinase has also been proposed as a possible glucose sensor given its rate limiting influence on glucose utilization in unpurified β-cells (Meglasson and Matschinsky, 1986; Liang *et al.*, 1990). Translational regulation of glucokinase was found in islets

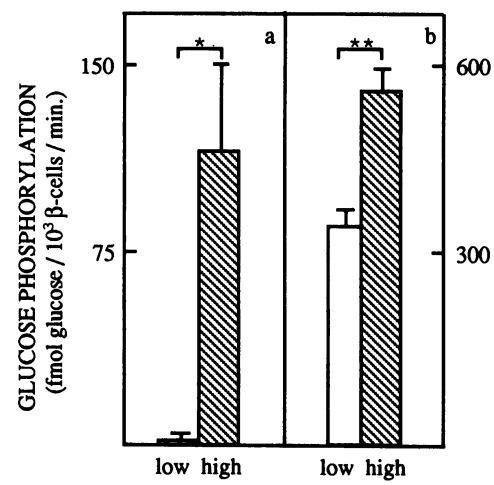

Fig. 6. Hexokinase and glucokinase activities in low β-cells and high β-cells. Glucose phosphorylation was measured as described in Materials and methods. Hexokinase activity (**a**) was determined as Glc6-P suppressible activity at 1 mM glucose; glucokinase activity (**b**) represents glucose phosphorylation at 20 mM substrate level in the presence of 2.5 mM Glc6-P. Data represent means ± SEM of five experiments. *, $P < 0.05$; **, $P < 0.005$.

that were kept in culture for 10 days (Liang *et al.*, 1992). In purified β-cells glucokinase activity also ranges within the values of cellular glucose utilization. Furthermore, we observed significant heterogeneity in both glucokinase activity and mRNA abundance, with high responding cells containing 1.6-fold more enzyme activity and 2-fold more mRNA than low β-cells. The data indicate that the heterogeneous distribution of glucokinase arises at the level

Table II. Leucine and glutamine oxidation and control enzyme activities in low and high responsive β-cells

	Low β-cells (pmol/min/10^3 β-cells)	High β-cells (pmol/min/10^3 β-cells)	n	P
Metabolism in intact cells				
Leucine oxidation	0.04 ± 0.01	0.05 ± 0.01	4	ns
Glutamine oxidation	0.10 ± 0.01	0.12 ± 0.01	4	ns
Enzyme activities in β-cell extracts				
Glyceraldehyde 3-phosphate dehydrogenase	111 ± 6	146 ± 9	3	<0.05
Pyruvate dehydrogenase	1.18 ± 0.14	0.82 ± 0.10	3	ns
Glutamate dehydrogenase	45 ± 5	46 ± 7	3	ns

Data represent mean values ± SEM of n independent measurements. Statistical significance of differences between low and high responsive β-cells was calculated with the unpaired two-tailed Student's t-test (ns, not significant). Leucine and glutamine oxidation were measured at 1 mM substrate levels.

of transcription and/or mRNA stability. Transcriptional regulation of glucokinase was reported in liver but not in pancreatic islets (Iynedjian *et al.*, 1986). The difference in glucokinase gene expression between the two β-cell subpopulations is 1.6- to 2-fold and parallels the differences in mean glucose utilization and in mean glucose-induced biosynthetic activity. Since each β-cell subpopulation comprises subgroups with different thresholds for glucose recognition (Kiekens *et al.*, 1992) (below 7.5 mM for the high responsive β-cells and above 7.5 mM for the low responsive β-cells), it is expected that larger differences for each of these variables would be found if more extreme subgroups were compared. Intercellular differences in β-cell glucokinase immunoreactivity (Jetton and Magnuson, 1992) indeed suggests that such large cell-to-cell variations may exist. Even larger intercellular differences are expected to exist for the low K_m hexokinases, which were not detectable in the low β-cell homogenate but which significantly contributed to glucose phosphorylation in the high β-cell homogenate. The >90% purity of both cell preparations renders selective contamination of the high β-cell subpopulation by another hexokinase-expressing cell type an unlikely explanation for the reported difference. Since the K_m of hexokinase for glucose is <1 mM, a high hexokinase expression level in certain β-cells may coincide with biological responsiveness to 1 mM glucose. That such β-cells exist has been shown in our previous autoradiographic experiments on glucose-induced proinsulin biosynthesis (Schuit *et al.*, 1988; Kiekens *et al.*, 1992). In agreement with this idea is the recent report of increased glucose-sensitivity in transgenic β-cells with expression of yeast hexokinase B (Epstein *et al.*, 1992). It could, however, be argued that in contrast to yeast hexokinase B, accumulation of Glc6-P in intact β-cells will restrict phosphorylation through endogenous hexokinases. Localization of hexokinase mRNA or protein in individual β-cells recruited to proinsulin biosynthesis at low glucose may be required to clarify this issue further.

Because individual β-cells respond to glucose over a narrow concentration range (Schuit *et al.*, 1988), their heterogeneity in glucose sensitivity may be important to allow the endocrine pancreas to respond gradually over wide variations in the glycemic level. The present work shows that the heterogeneity in glucokinase and hexokinase expression may qualify as molecular basis for the heterogeneity in glucose sensitivity. Mutations in the glucokinase gene have recently been shown to be implicated in the development of maturity-onset diabetes of the young (MODY), a rare form of type II diabetes (Vionnet *et al.*, 1992). A corollary of the present study is that mutations that affect the expression of this enzyme are expected to lower insulin secretion by causing a shift in the distribution of individual sensitivities of the β-cells towards higher glucose thresholds.

Materials and methods

Materials

The radioactively labelled compounds were purchased from Amersham International [[5-^{3}H]D-glucose (21 Ci/mmol), [U-^{14}C]D-glucose (286 mCi/mmol), [U-^{14}C]L-glutamine (270 mCi/mmol), [U-^{14}C]L-leucine (308 mCi/mmol), [3,5-^{3}H]L-tyrosine (50 Ci/mmol), [1-^{14}C]pyruvate (29 mCi/mmol) and [α-^{32}P]dCTP (>400 Ci/mmol)] or from New England Nuclear–Dupont [[1-^{3}H]L-glucose (20 Ci/mmol), [^{14}C]urea (56 mCi/mmol) and [^{3}H]3-OMG (79 Ci/mmol)].

Purification of islet β-cells

Islet β-cells were purified from adult male Wistar rats using previously published methods (Van De Winkel and Pipeleers, 1983; Kiekens *et al.*, 1992). The isolated β-cells (purity and viability >90%) were further distributed according to their metabolic redox state at 7.5 mM glucose (Kiekens *et al.*, 1992). After 15 min incubation at 7.5 mM glucose (37°C), ~50% of the cells exhibited higher than basal NAD(P)H levels; this subpopulation of 'high responsive' β-cells was sorted from 'low responsive' β-cells that were recovered at basal NAD(P)H levels (Kiekens *et al.*, 1992). Viability of both cell preparations was identical, 94 ± 1% low β-cells versus 95 ± 1% high β-cells staining positively with the vital dye neutral red (mean ± SEM; n = 12). Low and high responsive β-cells differ in their intracellular space by a factor of 1.2 (755 ± 11 and 940 ± 31 fl/cell, respectively, mean ± SEM; n = 7; P < 0.001).

Total protein and (pro)insulin biosynthesis

Low and high responsive β-cells were labelled with [^{3}H]tyrosine at various glucose levels for measuring total protein and (pro)insulin biosynthesis (Schuit *et al.*, 1991). Batches of 2×10^4 cells were incubated for 2 h at 37°C in 0.2 ml Earle's–HEPES buffer containing 50 μCi [3,5-^{3}H]L-tyrosine (50 Ci/mmol), washed with Earle's–HEPES buffer containing 1 mM unlabelled tyrosine and extracted in 2 M acetic acid containing 0.25% (w/v) BSA. Total protein synthesis was determined in acid extracts by trichloroacetic acid precipitation and ^{3}H-labelled (pro)insulin by immunoprecipitation after incubation with excess guinea pig antiporcine insulin serum (Schuit *et al.*, 1988).

Glucose and amino acid metabolism

Glucose and amino acid metabolism was measured in batches of 5×10^4 low and high responsive β-cells over 2 h incubations at 37°C. Glucose utilization was derived from the conversion of [5-^{3}H]D-glucose into tritiated water (De Vos *et al.*, 1991). Oxidation of glucose, leucine and glutamine was measured as $^{14}CO_2$ production from uniformly ^{14}C-labelled tracers (Gorus *et al.*, 1984). For measurements of glucose utilization and oxidation, cells were incubated in 100 μl Earle's–HEPES buffer containing the indicated concentrations of [5-^{3}H]D-glucose at specific activities 10–100 μCi/μmol and 50 μCi/ml [U-^{14}C]D-glucose (5–50 μCi/μmol). At the end of the incubation, cellular metabolism was stopped by the addition of 20 μl poison mixture (5 mM KCN, 10μM antimycine, 10 μM rotenone in 0.4 M citrate, pH 4.9). Hydroxyhyamine (Hewlett Packard) was added to capture the produced $^{14}CO_2$. The oxidation rates of [U-^{14}C]L-leucine (50 μCi/ml; 50 μCi/μmol) and [U-^{14}C]L-glutamine (25 μCi/ml; 25 μCi/μmol) were determined under similar conditions, with 1 mM unlabelled glucose in the medium.

Glucose uptake measurements

5×10^4 β-cells were preincubated for 20 min at 37°C in glucose-free Earle's–HEPES buffer containing 2 mM [^{14}C]urea (2.5 mCi/mmol) as intracellular space marker (Gorus *et al.*, 1984; Johnson *et al.*, 1990b). Equal

volumes of cell suspension were then mixed with Earle's–HEPES buffer containing 2 mM [^{14}C]urea (2.5 mCi/mmol) and 2 mM [^{3}H]3-OMG (37.5 mCi/mmol) or 2 mM [^{3}H]L-glucose (37.5 mCi/mmol) as an extracellular volume marker. Cellular uptake of label was measured at 12°C after 0.5, 1, 3 and 10 min. Initial glucose uptake rates were determined for 10 s at 37°C in the presence of 7.5 mM [^{3}H]3-OMG (2.5 mCi/mmol). At each time point, uptake was stopped by the addition of 150 μl ice-cold Earle's–HEPES buffer containing 50 mM glucose and 2 mM $HgCl_2$; this mixture was immediately layered on top of 50 μl 1 M glucose, 0.1% SDS and 10 mM EDTA and covered by 150 μl oil phase (4:1 dibutyl–dinonylphtalate). After spinning for 30 s in a microfuge (Beckman Instruments), the bottom phase of the tubes was counted in liquid scintillation cocktail (OptiPhase 'Hisafe'II, LKB). 3-OMG uptake data were expressed per intracellular volume ([^{14}C]urea space) after correction for the extracellular [^{3}H]L-glucose space.

mRNA analysis

Total RNA was isolated from 10^6 cells by a microadaptation of the guanidinium isothiocyanate–cesium chloride method (Rappolee *et al.*, 1988), separated according to its size on 1.5% agarose gels containing formaldehyde and transferred by capillary blot to nylon membranes (GeneScreen, NEN). Control RNAs were taken from tissues (brain, liver and muscle) of the same male adult Wistar rats as were used for β-cell preparation. The blots were UV-cross-linked and hybridized. cDNA probes were ^{32}P-labelled using the Megaprime system (Amersham International). Two stringent washes were done for 30 min in 0.2×SSC and 2.5% SDS at 65°C. Autoradiographic exposure times required to generate the indicated signals were 1 day for β-actin, 2 days for GLUT2, 4 days for GLUT4, 10 days for glucokinase and 20 days for GLUT1. The intensity of the signals on autoradiographic films were scanned on an Ultroscan XL densitometer (Pharmacia). Values used for calculations were deduced from the integrated area under the curve.

Determination of hexokinase and glucokinase activities

Glucose phosphorylation was measured by a radiochemical assay (Van Schaftingen, 1989). Low and high β-cells were homogenized by freeze–thawing in 20 mM HEPES buffer pH 7.1 containing 50 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, 20 μg/ml antipaine and 20 μg/ml leupeptin (10 μl/5×10^4 cells). The phosphorylation assay was started by addition of 10 μl cell homogenate to 50 μl medium containing 25 mM HEPES (pH 7.1), 50 mM KCl, 1 mM dithiothreitol, 20 mM KF, 0.5 mM EDTA, 5 mM Mg-ATP, 10 μg/ml antipain, 10 μg/ml leupeptin, 0.2 mg/ml BSA and 2.5 $\times10^5$ c.p.m. [U-^{14}C]glucose, either at 1 mM or 20 mM glucose concentration. Glc6-P (2.5 mM) was absent or present in order to distinguish between low K_m hexokinase (Glc6-P-sensitive) and high K_m glucokinase (Glc6-P-insensitive). After 0, 1, 2 and 3 h of incubation at 30°C, the amount of reaction product was measured by spotting 10 μl of the incubation medium on DE-81 paper. Non-phosphorylated glucose was removed by three washes in water and bound radioactivity was counted after mixing the dried papers with liquid scintillation cocktail (OptiPhase 'Hisafe'II, LKB).

Determination of other enzymes

Glyceraldehyde 3-phosphate dehydrogenase activity was measured as described by Trus *et al.* (1981). The formation of NADH was determined spectrophotometrically at 340nm using a centrifugal analyser (Cobas Bio, Roche). Pyruvate dehydrogenase activity was measured by the method described by MacDonald *et al.* (1991), with some minor modifications. Homogenates of β-cells were mixed with an equal volume of enzyme reaction mixture containing 1 mM pyruvate, 0.1 mCi [1-^{14}C]pyruvate (4 mCi/mmol), 4 mM NAD, 1 mM thiamine pyrophosphate, 1 mM coenzyme A, 30 U/ml porcine heart lipoamide dehyrogenase, 2 mM dithiothreitol, 0.1% Triton X-100, 5 mM $MgCl_2$ and 50 mM HEPES buffer pH 8.0. The enzyme reaction was carried out at 37°C for 2 h. The activity of glutamate dehydrogenase was determined spectrophotometrically as the decrease in NADH concentration (absorption at 340 nm) caused by the amination of α-ketoglutarate during the first 10 min of the reaction (Schmidt, 1974). The reaction was performed automatically by a centrifugal analyser (Cobas Bio, Roche) following the instructions from a commercial kit (Boehringer Mannheim).

Statistical analysis

Results are expressed as mean ± SEM for the number of experiments stated. The statistical significance of the differences between experimental groups was assessed by the unpaired two-tailed Student's *t*-test.

Acknowledgements

We thank Willy Malaisse for helpful comments, Erik Quartier for technical assistance, Rita Kiekens for sorting the low and high β-cells, Annick Smismans for measuring glutamate dehydrogenase and the technical staff of the department of Endocrinology and Metabolism for β-cell preparation. We gratefully acknowledge Drs M.Mueckler and D.James [Washington University Medical School (rat GLUT4 cDNA and rat GLUT2 antiserum), Dr B.Thorens [Institut de Pharmacologie, Université de Lausanne (rat GLUT2 cDNA and antiserum)], Dr M.Birnbaum [Harvard Medical School (rat GLUT1 cDNA)] and Dr M.Magnuson [(Vanderbilt University Medical School (rat β-cell glucokinase cDNA)]. This study was supported by grants from the Belgian Fund for Medical Scientific Research (FGWO grants 3.0013.89 and 3.0075.88), the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State (IUAP 15), the Flemish Community (Concerted Action 92/97-1807) and the Juvenile Diabetes Foundation International.

References

Bosco,D. and Meda,P. (1991) *Endocrinology*, **129**, 3157–3166.
De Vos,A., Schuit,F.C. and Malaisse,W.J. (1991) *Biochem. Int.*, **24**, 117–121.
Epstein,P.N., Boschero,A.C., Atwater,I., Cai,X. and Overbeek,P. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 12038–12042.
Giordano,E., Bosco,D., Cirulli,V. and Meda,P. (1991) *J. Clin. Invest.*, **87**, 2178–2185.
Gorus,F.K., Malaisse,W.J. and Pipeleers,D.G. (1984) *J. Biol. Chem.*, **259**, 1196–1200.
Grodsky,G.M., Batts,A.A., Bennet,L.L., Vcella,C., McWilliams,N.B. and Smith,D.F. (1963) *Am. J. Physiol.*, **205**, 638–644.
Hiriart,M. and Ramirez-Mendelez,M.C. (1991) *Endocrinology*, **128**, 3193–3198.
Hughes,S.D., Johnson,J.H., Quaade,C. and Newgard,C.B. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 688–692.
Iynedjian,P.B., Möbius,G., Seitz,H.J., Wollheim,C.B. and Renold,A.E. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 1998–2001.
Iynedjian,P.B., Pilot,P.-R., Nouspikel,T., Milburn,J.L., Quaade,C., Hughes,S., Ucla,C. and Newgard,C.B. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 7837–7842.
Jetton,T.L. and Magnuson,M.A. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 2619–2623.
Johnson,J.H., Ogawa,A., Chen,L., Orci,L., Newgard,C.B., Alam,T. and Unger,R.H. (1990a) *Science*, **250**, 546–549.
Johnson,J.H., Newgard,C.B., Milburn,J.L., Lodish,H.F. and Thorens,B. (1990b) *J. Biol. Chem.*, **265**, 6548–6551.
Kiekens,R., In't Veld,P.A., Mahler,T., Schuit,F.C., Van De Winkel,M. and Pipeleers,D.G. (1992) *J. Clin. Invest.*, **89**, 117–125.
Liang,Y., Najafi,H. and Matschinsky,F.M. (1990) *J. Biol. Chem.*, **265**, 16863–16866.
Liang,Y., Najafi,H., Smith,R.M., Zimmerman,E.C., Magnuson,M.A., Tal,M. and Matschinsky,F.M. (1992) *Diabetes*, **41**, 792–806.
MacDonald,M.J. (1990) *Diabetes*, **39**, 1461–1466.
MacDonald,M.J., Kaysen,J.H., Moran,S.M. and Pomije,C.E. (1991) *J. Biol. Chem.*, **266**, 22392–22397.
Magnuson,M.A. and Shelton,K.D. (1989) *J. Biol. Chem.*, **264**, 15936–15942.
Meglasson,M.D. and Matschinsky,F.M. (1986) *Diabetes Metabolism Rev.*, **2**, 163–214.
Orci,L., Ravazolla,M., Baetens,D., Inman,L., Amherdt,M., Peterson,R.G., Newgard,C.B., Johnson,J.H. and Unger,R.H. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 9953–9957.
Permutt,M.A. and Kipnis,D.M. (1972) *J. Biol. Chem.*, **25**, 1194–1199.
Pipeleers,D. (1992) *Diabetes*, **41**, 777–781.
Rappolee,D.A., Brenner,C.A., Schultz,R., Mark,D. and Werb,Z. (1988) *Science*, **241**, 1823–1825.
Schmidt,E. (1974) In Bergmeyer,H.U. (ed.), *Methods of Enzymatic Analysis*. Verlag Chemie Weinheim, Academic Press, NY Vol. 2, pp. 650–656.
Schuit,F.C., In't Veld,P.A. and Pipeleers,D.G. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 3865–3869.
Schuit,F.C., Kiekens,R. and Pipeleers,D.G. (1991) *Biochem. Biophys. Res. Commun.*, **178**, 1182–1187.

Thorens,B., Sarkar,H.K., Kaback,H.R. and Lodish,H.F. (1988) *Cell*, **55**, 281–290.
Thorens,B., Weir,G.C., Leahy,J.L., Lodish,H.F. and Bonner-Weir,S. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 6492–6496.
Trus,M.D., Zawalich,W.S., Burch,P.T., Berner,D.K., Weill,V.A. and Matschinsky,F.M. (1981) *Diabetes*, **230**, 911–922.
Unger,R.H. (1991) *Science*, **251**, 1200–1205.
Van De Winkel,M. and Pipeleers,D.G. (1983) *Biochem. Biophys. Res. Commun.*, **114**, 835–842.
Van Schaftingen,E. (1989) *Eur. J. Biochem.*, **179**, 179–184.
Van Schravendijk,C., Kiekens,R. and Pipeleers,D. (1992a) *J. Biol. Chem.*, **267**, 21345–21348.
Van Schravendijk,C., Kiekens,R. and Pipeleers,D. (1992b) *Diabetologia*, **35**.
Vionnet,N. *et al.* (1992) *Nature*, **356**, 721–722.

Received on February 11, 1993; revised on March 29, 1993