**Cellular Origin of Hexokinase in Pancreatic Islets**

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Transgenic or tumoral pancreatic islet beta cells with enhanced expression of low $K_m$ hexokinases (HK) exhibit a leftward shift of the normal dose-response curve for glucose-induced insulin release. Furthermore, HK catalyzes roughly 50% of total glucose phosphorylation measured in extracts from freshly isolated rodent islets, suggesting that HK participates in the process of glucose sensing in beta cells. We previously observed that HK activity represents 20% of total glucose phosphorylation in purified rat beta cell preparations and that HK is not homogenously distributed over these cells. The present study provides several arguments for the idea that HK detected in freshly isolated rat islets or islet cell preparations originates mainly from contaminating exocrine cells. First, reverse transcription-polymerase chain reaction using isoform-specific primers allowed detection of hexokinase I and IV mRNA in rat beta cells, whereas the messenger levels encoding the hexokinase II and III isoforms were undetectably low. However, immunobots indicated that hexokinase I protein was 10-fold more abundant in freshly isolated islets and flow-sorted exocrine cells than in purified rat beta cell preparations. Second, comparison of HK activity in the different pancreatic cell types resulted in 15–25-fold higher values in exocrine than in endocrine cells (acinar cells: 21 ± 3 pmol of glucose 6-phosphate formed/h/ng of DNA; duct cells: 30 ± 8 pmol/h/ng of DNA; islet beta cells: 1.2 ± 0.2 pmol/h/ng DNA; alpha cells: 0.9 ± 0.4 pmol/h/ng of DNA). Since freshly purified beta cell preparations contain 3 ± 1% exocrine cells, at least 50% of their HK activity can be accounted for by exocrine contamination. Third, after 5 days of culture of purified islet beta cells, both HK activity and the proportion of exocrine cells decreased by more than 1 order of magnitude, while the ratio of glucokinase over hexokinase activity increased more than 10-fold. Finally, preincubating the cells with 50 mmol/liter 2-deoxyglucose did not affect glucose stimulation of insulin biosynthesis and release. In conclusion, the observation that pancreatic exocrine cells are responsible for a major part of HK activity in islet cell preparations cautions against the use of HK measurements in islet extracts in the study of these enzymes in glucose sensing by pancreatic beta cells.

Differentiated pancreatic beta cells isolated from both rodents (1) and man (2) possess the capacity to rapidly respond to changes in extracellular glucose concentration between 3 and 20 mmol/liter with adapted rates of proinsulin biosynthesis and insulin release. In addition to the rapid onset of these cellular processes, both the inactivity at basal glucose (below 3 mmol/liter) and the steep concentration-dependent activation at glucose levels between 5 and 10 mmol/liter are considered important aspects of the glucose-regulation of beta cells (3). It is well known that such regulatory properties of glucose proceed via metabolic steps in beta cells, comprising the uptake and phosphorylation of the sugar (4). Phosphorylation in islets is mediated by two classes of isoenzymes (5, 6), hexokinases I-III (HK) and glucokinase (GK), which can be distinguished biochemically by their molecular mass, enzyme kinetics, and allosteric properties (7). The concept that GK plays a crucial physiological role in glucose recognition by mammalian beta cells has been documented extensively (for review, see Ref. 3). Furthermore, genetically determined abnormalities in GK structure or protein expression levels are the cause of abnormal insulin secretion caused by abnormal threshold concentration for glucose-stimulated insulin release, both in transgenic animals (8) and man (9, 10). Depending on the shift of such threshold, these mutations can be the cause of diabetes (8, 9) or familial hyperinsulinism (10).

Strikingly, in extracts of mouse (5, 6) and rat (11) islets, at least 50% of total glucose phosphorylation seems to be catalyzed by HK. On basis of this high activity on the one hand and because of the observation that up-regulation of hexokinase expression in tumoral or transgenic beta cells causes a leftward shift of the normal concentration-dependent activation of glucose-induced insulin release on the other hand (12, 13), the possibility has been considered that low $K_m$ hexokinases influence the process of glucose sensing, for instance, by decreasing the threshold for glucose-induced insulin release or increasing insulin secretion at basal plasma glucose levels. Four studies may be interpreted along this view. First, Heimberg et al. (14) noted that hexokinase activity was associated with a rat beta cell subset with high sensitivity to glucose, prepared by fluorescence-activated cell sorting (FACS) on the basis of glucose-induced changes in NAD(P)H autofluorescence (15), which could indicate that low $K_m$ glucose phosphorylation sets the threshold point for glucose-induced beta cell activation. Second, Hosokawa et al. (16) observed that islets isolated from 90% pancreatectomized rats exhibited more than 2-fold up-regulation of hexokinase expression which was associated with a moderate shift to the left of the dose-response curve of glucose-induced insulin release. Third, Rabuazzo et al. (17) showed that 3-h exposure of rat islets to 16.7 mmol/liter glucose caused both translocation of islet-associated hexokinase I protein from a

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### References
1. The abbreviations used are: HK, hexokinases I, II, or III; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorting; Glu-6-P, glucose 6-phosphate; GK, glucokinase (GK); PCR, polymerase chain reaction.
cytosolic pool to the outer mitochondrial membrane and leftward shift of the concentration-dependent activation of glucose-induced insulin release. The correlation between the two events led to the suggestion that glucose-induced redistribution of hexokinase I in beta cells contributes to glucose regulation of insulin secretion (17). Fourth, in a model of conditional knock-out of the rat islet glucokinase gene, Piston et al. (18) recently observed some residual glucose-induced shift in NAD(P)H autofluorescence in the subset of islet beta cells that recombined both GK alleles, indirectly suggesting the participation of HK in glucose metabolism of these cells.

A number of observations are more difficult to reconcile with the idea that HK is important in the process of glucose recognition by beta cells. First, low \( K_m \) glucose phosphorylation in islet extracts occurs much more rapidly than low \( K_m \) glucose utilization in whole islets, suggesting that intracellular mediators such as glucose 6-phosphate and glucose 1,6-bisphosphate repress most islet HK allosterically (19). Second, HK activity on the one hand and insulin release on the other could be dissociated from each other by maintaining rat islets in tissue culture, the former disappearing almost completely after 5 days of culture, whereas the latter could be well preserved (20, 21). Third, HK is undetectably low (14) in the subset of the beta cells that was flow-sorted on the basis of a low glucose-induced shift in NAD(P)H autofluorescence at 7.5 mM glucose (approximately 50% of the cells, Ref. 15).

Altogether, the concept that HK is required together with GK for normal glucose regulation is controversial and still insufficiently documented. We have therefore assessed in the present study mRNA expression of different hexokinase isoforms as well as hexokinase I protein abundance and HK enzymatic activity in FACS-purified rat pancreatic cell populations. Our results show that both acinar cells and ducts of the exocrine pancreas which are co-isolated with the islets of Langerhans and contaminate to a minor extent the FACS-purified beta cells, express hexokinase-I at very high levels. As a consequence, when this factor is not taken into account, major artifacts are introduced in the study of HK and its role in glucose regulation of beta cells.

**EXPERIMENTAL PROCEDURES**

**Cell Isolation and Purification**—Pancreata were isolated from adult male Wistar rats (proeidencerium Heverlee, Belgium) which were bred according to Belgian regulations of animal welfare. Islets of Langerhans were prepared in isolation medium (123 mmol/liter NaCl, 5.4 mmol/liter KCl, 1.8 mmol/liter CaCl\(_2\), 4.2 mmol/liter NaHCO\(_3\), 0.8 mmol/liter MgSO\(_4\), 10 mmol/liter HEPES, 5.6 mmol/liter glucose, 0.1% kanamycin, and 0.25% bovine serum albumin (BSA), pH 7.4) using a modified collagenase technique which has been described (22). The freshly isolated islets were dissociated into dispersed islet cells (23) using dissociation medium (123 mmol/liter NaCl, 5.4 mmol/liter KCl, 4.2 mmol/liter NaHCO\(_3\), 0.8 mmol/liter MgSO\(_4\), 10 mmol/liter HEPES, 5.6 mmol/liter glucose, 0.1% kanamycin, and 0.25% bovine serum albumin (BSA), pH 7.4) containing trypsin (final concentration 25 \( \mu \)g/ml) and DNase (2 \( \mu \)g/ml). Islet beta cells were FACs purified from endocrine non-beta cells via autofluorescence-activated cell sorting at 2.8 mmol/liter glucose (22) on the basis of FAD/scatter using an argon laser (Spectra Physics, Mountain View, CA) at 488 nm (excitation) and 510–550 nm (emission). In some experiments, the flow-sorted total beta cell population was further subdivided into beta cell subsets on basis of low and high NAD(P)H autofluorescence at 2.8 mmol/liter glucose (15), using a UV-laser (351–363 nm excitation/400–470 nm emission; Spectra Physics). Analysis of the cellular composition of the sorted or non-sorted islet cell preparations was performed by electron microscopy and immunocytochemistry for glucagon and insulin. Immunocytochemistry was described previously (12). For the determination of the percentage of exocrine cells in the purified beta cell preparations, approximately 500 cells/sample were counted in the electron microscopical analysis. To assess the effect of tissue culture on HK associated with flow-sorted rat beta cells, the cells were cultured for 5 days in serum-free Ham’s F-10 medium (Life Technologies, Inc., Stratthclyde, United Kingdom) containing 0.075 mg/ml penicillin, 0.1 mg/ml streptomycin, 50 \( \mu \)g/ml 3-isobutyl-1-methylxanthine, 1% (v/v) charcoal-extracted BSA (fraction V, RIA grade, Sigma), 2 mmol/liter glutamine, and 10 mmol/liter glucose (24).

Pancreatic acinar cells were prepared from the total collagenase digest of the pancreas and enriched by countercurrent elutriation (23). Remaining cellular fractions of 100 mmol/liter EDTA dissociated cell clumps were washed by two sedimentations, resuspended in isolation medium and then cleared from debris and dead cells via sedimentation through a Percoll layer of density 1.04. After one wash in isolation medium and two washes in dissociation medium (250 g for 3 min), the cells were preincubated for 10 min at 30 °C in dissociation medium without continuous shaking. Enzymatic dissociation was started by addition of trypsin (final concentration 25 \( \mu \)g/ml) and DNase (2 \( \mu \)g/ml) and followed under a phase-contrast microscope. Dissociation was stopped (median required time of 30 min) when 50–60% of the cells occurred as single units. After three washes in isolation medium (250 g for 3 min) the cells were filtered through a 5-\( \mu \)m nylon filter to remove undigested material and finally submitted to autofluorescence-activated cell sorting at 2.8 mmol/liter glucose in order to remove dead cells and cell debris. This isolation procedure yielded a FACS-purified cell preparation with >90% acinar cells and viability exceeding 95%.

Preparation of ducts from the countercurrent elutriation fraction with particle size below 100 \( \mu \)m was performed according to a previously published method (25). Isolated ducts were cultured for 7 days in Ham’s F-10 basal medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 6 mmol/liter glucose, 2 mmol/liter l-glutamine, 1% BSA (fraction V, RIA grade, Sigma), 0.075 mg/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma), and 5% heat-inactivated fetal calf serum. The cell density was 15 ducts per 3 ml in a bacteriological Petri dish.

**Analysis of Amplified cDNA**—Total RNA (0.5 \( \mu \)g) was extracted from beta cells and control tissues (brain, liver, and muscle) reverse transcribed and amplified as described below (26) with appropriate blanks in each assay. Specific primer sequences used for PCR were as follows: hexokinase-I (5'-codoon 17–23: GCAAGCTCAAGAAGTTAGGA, hexokina-se-I (3'-codoon 95–102): TCTTCTCGTGGTTCCACCTGC, yielding an amplified fragment of 256 base pairs; hexokinase-II (5'-codoon 18–24): 5'-CAAGTCAGAAGGTTTCAAGC, hexokinase-II (3'-codoon 18–24): 5'-CTCTAGAGGGGCTTGTGCTT (259-base pair fragment); hexokinase-III (5'-codoon 12–18): 5'-GACAAGAGGATACCGTGC, hexokinase-III (3'-codoon 106–112): 5'-CCGGTCAGTTGTACCCACAAA (300-base pair fragment); glucokinase beta cell-specific variant (see Ref. 27, 5'-codoon 9–15): 5'-AGGCCACCAAAGGAGGAAAG, glucokinase beta cell variant (3'-codoon 97–104): 5'-TTGTCTCAGTTCCTACCAGTCC (288-base pair fragment). As a control for the quality and quantity of the RNA, a priming reaction (equimolar concentrations) recognizing six nucleotide transporters GLUT1, GLUT2, and GLUT4 was used; the sequences of these primers were as described before (26) yielding an amplified fragment of 540 base pairs. The cycling profile for each of the PCR experiments was as follows: 2.5 min at 95 °C followed by 1 min at 94 °C, 1.5 min at 65 °C, and 1.5 min at 72 °C for 10 cycles and 0.5 min at 94 °C, 1 min at 60 °C, and 1.5 min at 72 °C for 20 cycles, bringing the total number of cycles to 30. PCR products were resolved in 1% agarose gels (20–40% acinar cells and viability exceeding 95%).

**Immunoblotting of Hexokinase-I, GK, and β-Actin**—Cell and tissue samples were washed twice in phosphate-buffered saline and homogenized by sonication in lysis buffer containing 80 mmol/liter Tris-Cl (pH 6.8), 5 mmol/liter EDTA, 5% SDS, 5% β-mercaptoethanol, and 10% glycerol in the presence of 1 mmol/liter phenylmethylsulfonyl fluoride. Aliquots were taken for protein determination using the BCA protein assay kit (Pierce, Rockford, IL) using BSA as standard. Homogenates (20–40 \( g \) of total cellular protein per lane) were separated on a 10% SDS-polyacrylamide gel (Mini-Protein, Bio-Rad) and electroblotted overnight onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Blotting efficiency as well as the position of the protein standards was assessed by Ponceau staining. The blots were blocked for 1 h at room temperature in 5% non-fat dry milk (pH 7.6). Hexokinase-I was detected using a monoclonal anti-hexokinase-I antibody (Chemicon, Temakula, CA) at 1/1,000 dilution (16). The second antibody (sheep anti-mouse peroxidase, 1/2000, Amersham Pharmacia Biotech) was incubated at room temperature for 50 min. Peroxidase activity was visualized by a chemiluminescence system (ECL, Amersham, Arlington Heights, IL). The intensity of the signals was quantified via laser densitometry. Striped blots were re-exposed subsequently to sheep polyclonal anti-β-GK antibody at 1/4000 dilution (kindly donated by Dr. H. Seitz, University of Hamburg, Germany) and goat polyclonal antibody against β-actin at 1/1000 dilution (Santa Cruz, San Diego, CA), the latter to validate quality and quantity of the loaded protein. Exposure times
were 20 min for hexokinase-I, 1 min for GK, and 1 min for β-actin.

Measurement of Glucose phosphorylation—Glucose phosphorylation was measured by a radiochemical assay (28). Cells or ducts (2 × 10^6 cells/μl (beta cells) or 5 ng of DNA/μl (acinic cells and ducts)) were homogenized via sonification in 20 mmol/liter HEPEs buffer (pH 7.1) containing 50 mmol/liter KCl, 1 mmol/liter dithiothreitol, 0.5 mmol/liter EDTA, 20 μg/ml antipain, and 20 μg/ml leupeptin. The phosphoryla-
tion assay was started by addition of 12.5 μl of cell homogenate to 12.5 μl of HEPEs (40 mmol/liter buffer (pH 7.1), containing 125 mmol/liter KCl, 1.5 mmol/liter dithiothreitol, 40 mmol/liter potassium fluoride, 0.75 mmol/liter EDTA, 10 mmol/liter Mg-ATP, 30 μg/ml antipain, 30 μg/ml leupeptin, 0.2 μg/ml BSA, and 0.125 μCi of (U-14C)glucose in the presence of 0.5 or 20 mmol/liter glucose. Glucose 6-phosphate (Glu-6-P, final concentration = 2.5 mmol/liter) was absent or present in order to distinguish between low Km hexokinase (Glu-6-P-sensitive) and GK which is Glu-6-P-insensitive and which exhibits a high k_m value for glucose (3). After 90 min incubation at 37 °C, the amount of reaction product was measured by spotting duplicates of 10 μl of the incubation medium on DE-81 paper. Non-phosphorylated glucose was removed by three washes in water followed by one wash in ethanol and a final wash in ether. Radioactivity bound to the paper was counted after mixing the dried papers with liquid scintillation mixture (OptiPhase “Hisafe” II, Wallac, Turku, Finland).

Insulin Biosynthesis and Release—FACS-purified beta cells were reaggregated for 1 h at 37 °C in Ham’s F-10 medium supplemented with 2 mmol/liter lactate-glutamine, 1% BSA (fraction V, RIA grade), 0.075 μg/ml penicillin (Continental Pharma, Brussels, Belgium), 0.1 mg/ml streptomycin (Laboratories Diamant, Puteau, France), and 2 mmol/liter CaCl₂ (basal medium) supplemented with 10 mmol/liter glucose. Insulin release and proinsulin biosynthesis were measured after 30 min preincubation in basal medium plus 1 mmol/liter glucose (control condition) or basal medium supplemented with 1 mmol/liter glucose and 50 mmol/liter 2-deoxyglucose which is converted in the cells to 2-deoxyglucose 6-phosphate to inhibit HK (29). After washing the cells three times in basal medium, batches corresponding to 5 × 10⁶ cells per condition were incubated for 1 h at 37 °C in 200 μl of basal medium with 0, 1, or 20 mmol/liter glucose either with or without 20 mmol/liter mannoheptulose and 50 μCi of [U-3H]tyrosine (Amersham Pharmacia Biotech), resulting in a final specific activity of 16.7 Ci/mmol and a tyrosine concentration of 15 μmol/liter, in order to measure release and biosynthesis from the same cells (24, 30).

DNA Measurements—DNA was measured using Hoechst 33258 (22). Duct cells and acinar cells were washed in calcium-free buffer containing 150 mmol/liter NaCl, 15 mmol/liter citrate, and 3 mmol/liter EDTA (pH 7.0) and extracted for 15 min at 37 °C in 100 mmol/liter NaCl, 10 mmol/liter Tris, and 10 mmol/liter EDTA (pH 7.0). The fluorometric assay at 355 nm (excitation)/455 nm (emission) was carried out after addition of Hoechst 33258 solution (100 nm/gl) to the samples.

Statistical Analysis—Data are expressed as mean ± S.E. of n independent experiments. Unless stated otherwise, the significance of differences between conditions was tested by unpaired two-tailed Students’ t test.

RESULTS

Amplification of Different Hexokinase Transcripts from Flow-sorted Rat Pancreatic Cells—We first assessed the abundance of various hexokinase transcripts in FACS-purified beta cells, using isoform-specific primer sets and taking total RNA extracted from rat brain, liver, and muscle as positive controls. The amplification reaction utilizing the GK-primer set from which the 5’-primer is directed against beta cell-specific codons 9–15 of GK-mRNA, resulted in the expected (27, 31) 288-base pair fragment when RNA was used from flow-sorted rat beta cells (Fig. 1). Moreover, the same primers gave negative results starting from rat liver RNA (Fig. 1). RNA extracted from FACS-purified rat beta cell preparations appeared also positive for hexokinase-I messenger, since with hexokinase-I-specific primers a PCR product of the same length and nucleotide sequence could be amplified as when the reaction was performed with rat brain RNA. On the contrary, amplification of hexokinase-II or -III cDNA fragments was negative when starting from rat beta cell RNA, while rat muscle and liver, respectively, gave positive results.

Detection of Hexokinase-I and GK Protein in Rat Pancreatic Islets

FIG. 1. Reverse transcription polymerase chain reaction of different hexokinase transcripts in FACS-purified beta cells. Total RNA was reverse transcribed and amplified using hexokinase-specific primers. A set of primers that recognize the glucose transporter isoforms 1, 2, and 4 (Glut1–2–4) was used as a control for the integrity and quantity of amplified RNA (26). Amplification of cDNA from purified beta cells generated a PCR fragment with the hexokinase type I and hexokinase type IV β (beta cell-specific variant of glucokinase; Ref. 27) primers but not with the hexokinase type II- or III-specific primers. The Glut1–2–4 signal was similar in all cell and tissue types under examination. Blank PCR conditions contained water instead of added cDNA. The results in this figure are representative for three experiments.

Cell Preparations—To determine whether the hexokinase-I mRNA that was detected in flow-sorted rat beta cells is translated, we performed immunoblotting starting from total protein from collagenase-isolated rat islets as well as from purified beta cells and exocrine acinar cells (Fig. 2). In freshly purified beta cells, the hexokinase-I abundance appeared low, at least 10-fold less abundant than in freshly isolated islets of Langerhans: the OD ratios normalized for β-actin were 0.4 (islets) and 0.02 (beta cells). The cellular origin of the detected hexokinase-I protein in whole islets is suggested by high hexokinase-I expression in exocrine pancreatic cells (virtually as abundant as in rat brain). In contrast to the large difference in hexoki-

Glucose Phosphorylation in Endocrine and Exocrine Pancreas—To assess the functional integrity of HK and GK protein, we measured glucose phosphorylation in FACS-purified beta and alpha cells as well as in purified pancreatic acinar and ductal cells (Table I). In agreement with the protein blots, HK activity, when expressed per microgram of cellular DNA, was at least 1 order of magnitude higher in exocrine pancreas (both ducts and acinar cells) than in flow-sorted islet beta and alpha cells. Since the examined beta cell preparations were contami-

nated by 2.6 ± 0.7% acinar cells (mean ± S.E.; n = 15) and 0.6 ± 0.2% non-granulated cells (mostly duct cells), the calculated activity in beta cells after correction for the exocrine cell contamination becomes 0.5 ± 0.1 pmol/h/ng DNA (mean ± S.E.; n = 15). Therefore, about 60% of the HK activity associated with FACS-purified beta cells can be accounted for by exocrine
cellular contamination rather than genuine HK expression in the insulin-producing cells. This interpretation is further strengthened by the linear correlation between the amount of HK activity associated with 31 individual beta cell or islet cell preparations on the one hand and the percentage contaminating acinar cells in these preparations on the other hand (Fig. 3; r = 0.94; p < 0.0001 in F-test). On the contrary, no such correlation was observed between GK activity associated with beta cells and acinar cell contamination (data not shown). By sorting the total rat beta cell population on the basis of low and high NAD(P)H autofluorescence at a particular glucose concentration (15), much higher HK activity is found in the cellular preparation corresponding to high NAD(P)H autofluorescence than in the low beta cells which contained virtually no acinar cells (Fig. 3). Please note that dissociated rat islet cells, analyzed before flow sorting, contained 30 µg of total protein/lane extracted from rat brain, exocrine pancreas, pancreatic islets, liver, or total protein from 2 × 10^6 flow sorted rat pancreatic beta cells. Blotting efficiency was established by Ponceau staining. Exposure times were 20 min for hexokinase-I, 1 min for GK, and 1 min for β-actin. The blot shown here is representative for three experiments.

To further investigate the cellular origin of HK in flow-sorted rat beta cells, we compared freshly isolated and 5-day cultured beta cell preparations in terms of hexokinase activity and acinar/duct cell contamination. As was previously reported for whole collagenase-isolated islets of Langerhans (20, 21), hexokinase activity dropped dramatically, by roughly 95%, during tissue culture (Table II), while the HK content of the cells decreased nonsignificantly. Consequently, the GK/hexokinase ratio in 5-day cultured beta cell preparations was 1 order of magnitude higher than in freshly isolated FACS-purified beta cells. This severe reduction in HK was paralleled by a more than 95% decrease in the number of surviving acinar cells (Table II).

In summary, these data suggest that the measured HK activity in collagenase-isolated islets is influenced to a large extent by adherent acinar cells. Even in FACS-purified rat beta cells, with acinar cell contamination of less than 10%, at least half of the low $K_m$ glucose phosphorylation can be attributed to this artifact.

**Effect of 2-Deoxyglucose and Mannoheptulose on Glucose-Induced Insulin Release and Proinsulin Biosynthesis**—To examine the possible functional relevance of any low $K_m$ glucose phosphorylation that is present in these cells, we assessed the effect of two well known inhibitors of HK on glucose-induced proinsulin biosynthesis and insulin release. Mannoheptulose is known to block competitively all hexokinases including GK (6). In contrast, 2-deoxyglucose inhibits only HK after the sugar is phosphorylated by the cells to 2-deoxyglucose-6-phosphate (29).

In control cells, glucose-induced proinsulin biosynthesis and insulin release were not responsive to 1 mmol/liter glucose, but were stimulated 15 ± 4 and 75 ± 15-fold, respectively, when medium glucose was raised from 1 to 20 mmol/liter (Table III). Furthermore, the potent stimulation by 20 mmol/liter glucose could not be prevented by preincubating the cells to 2-deoxyglucose. On the contrary, the addition of mannoseheptulose blocked the stimulatory effect of glucose upon insulin and proinsulin synthesis by more than 98% (Table III). Therefore, these data indicate that if HK contributes to glucose metabolism in rat beta cells, its enzymatic role in glucose regulation of proinsulin biosynthesis and insulin secretion is undetectably low, at least in the experimental conditions that were tested.

**DISCUSSION**

From a theoretical standpoint, the low $K_m$ hexokinase isoforms are poorly adapted to participate in the process of glucose sensing in differentiated mammalian beta cells: both their kinetic and allosteric properties preclude any role in the glucose-induced metabolic flux control operating within the physiological range of substrate concentrations. Because islet blood flow

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**Fig. 2.** Analysis of pancreatic cell preparations for the presence of hexokinase-I and glucokinase. As positive control tissues for the expression of hexokinase-I and glucokinase we used, respectively, rat brain and liver. Western blots were carried out with 20 µg of total protein/lane extracted from rat brain, exocrine pancreas, pancreatic islets, liver, or total protein from 2 × 10^6 flow sorted rat pancreatic beta cells. Blotting efficiency was established by Ponceau staining. Exposure times were 20 min for hexokinase-I, 1 min for GK, and 1 min for β-actin. The blot shown here is representative for three experiments.

**Fig. 3.** Low $K_m$ hexokinase in function of the percent of acinar cells in rat pancreatic islet cell preparations. Measured activities of HK and % acinar cells are plotted for: ●, non-sorted dissociated islet cells (n = 7); ○, FAD/scatter flow-sorted beta cells (n = 15); ■, flow-sorted beta cells with high NAD(P)H autofluorescence at 2.8 mM glucose (n = 4); ◆, flow-sorted beta cells with low NAD(P)H autofluorescence at 2.8 mM glucose (n = 4).

**TABLE I**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Enzymatic activity (pmol/h/mg DNA⁻¹)</th>
<th>n</th>
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<tbody>
<tr>
<td>Beta cells</td>
<td>1.2 ± 0.2</td>
<td>15</td>
</tr>
<tr>
<td>Alpha cells</td>
<td>0.9 ± 0.4</td>
<td>4</td>
</tr>
<tr>
<td>Acinar cells</td>
<td>21 ± 1</td>
<td>7</td>
</tr>
<tr>
<td>Ducts</td>
<td>30 ± 8</td>
<td>4</td>
</tr>
</tbody>
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Mannoheptulose 0.1. 6
2-Deoxyglucose 0.1. 3

such as MIN6 (13), INS-1 (34), HIT-T15 (35), and unresponsive between 1 and 20 mmol/liter glucose, whereas in agreement with this idea, the beta cell line RINm5F, which exhibits a relatively high GK over HK expression ratio. Furthermore, up-regulation of HK in MIN6-cells via gene transfer (31) shifts the dose-response curve for glucose-induced insulin release to the left, similar to what has been observed in vivo by expressing the yeast hexokinase transgene in mouse islet beta cells (37). In view of these results it has been difficult to reconcile the large amounts of HK activity in extracts of whole islets (5, 6, 11) with the virtual absence of a secretory response of islet beta cells to glucose concentrations between 0 and 3 mmol/liter. One possible explanation has been suggested by Girot et al. (19) who proposed on the basis of Glu-6-P and glucose 1,6-bisphosphate content of rat islets and flow-sorted beta cells, that allosteric inhibition of hexokinase in situ reduces enzymatic activity below 25% of what is measured in extracts. This line of reasoning needs, however, some revision in the context of the biochemical association of HK with the outer mitochondrial membrane (17), a subcellular localization which renders the enzyme less sensitive to allosteric inhibition by Glu-6-P (38). Importantly, it should be emphasized that direct evidence for the presence of low $K_m$ hexokinase(s) in any $\beta$-subcellular compartment, e.g., using ultrastructural or laser confocal microscopic analysis, has not been published so far.

The results presented in this study provide direct support for the idea that most of the measured islet HK gene expression, either in the form of hexokinase-I protein abundance or HK enzymatic activity, does not reflect events occurring inside beta cells but represent protein activity associated with a low percentage of other cells contaminating the beta cell preparations. One potential source could be glucagon-producing alpha cells, which normally amount to up 5% of FACS-purified beta cells (22). However, since the measured HK in FACS-purified alpha cells was lower than in beta cells and rat alpha cells were reported to express GK (31), we propose that the few contaminating alpha cells are not responsible for the measured HK activity in flow-sorted beta cell preparations. Our data rather suggest that a large fraction of islet-associated hexokinase is not localized in endocrine pancreatic cells but originates from acinar or ductal cells which are co-purified with the collagenase-isolated rat islets and, consequently, contaminate the flow-sorted beta cells to a minor degree. Since HK enzymatic activity in exocrine pancreas appears at least 1 order of magnitude higher than in the FACS-purified beta cells (with impurities included) it is easy to understand why even flow-sorted beta cells contain detectable amounts of HK. Freshly isolated rat islets using collagenase and handpicking under a stereo dissecting microscope usually still contain acinar cells which adhere to the islet collagen capsule. Surprisingly little information on quantitative aspects of this subject can be found in the literature. Since the procedure of handpicking involves a phenomenon of selection, the degree of exocrine co-purification will probably vary from batch to batch and from laboratory to laboratory. We noted that during a 5-day culture period most acinar cells die, while the functional integrity of endocrine beta cells can be preserved (24). Our observation fits well with the reproducible, but hitherto unexplained, observation that HK activity of isolated islets decreases profoundly during culture, while the concentration-response curve of glucose-induced insulin release is very well preserved (20, 21). The current data also explain our previous observation that HK activity is only detected in one particular subset of flow-sorted beta cells (14). Indeed, our present data show that the rare acinar cells contaminating the total beta cell preparation are preferentially sorted into the high NADPH cellular subset. Some biochemical parameters measured in non-sorted islet cells, e.g., the

TABLE II

Effect of culture on hexokinase/glucokinase activity and exocrine cell contamination of flow-sorted rat beta cells

<table>
<thead>
<tr>
<th>Glucose phosphorylation</th>
<th>Hexokinase/glucokinase</th>
<th>Exocrine cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose phosphorylation</td>
<td>Hexokinase</td>
<td>Glucokinase</td>
</tr>
<tr>
<td>Freshly isolated beta cells</td>
<td>26 ± 5</td>
<td>41 ± 7</td>
</tr>
<tr>
<td>5-Day culture</td>
<td>1.8 ± 0.4*</td>
<td>28 ± 6</td>
</tr>
</tbody>
</table>

* $p < 0.02$

**p < 0.001.

$^p < 0.05.$

TABLE III

Effect of 2-deoxyglucose and mannoheptulose on insulin release and proinsulin biosynthesis from rat beta cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose phosphorylation</th>
<th>Insulin release</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Glucose] mmol/liter</td>
<td>cpm h$^{-1}$ ng insulin content$^{-1}$</td>
<td>pg h$^{-1}$ ng insulin content$^{-1}$</td>
</tr>
<tr>
<td>Control</td>
<td>0  0.9 ± 0.1</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>1  1.5 ± 0.2</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>2-Deoxyglucose</td>
<td>20 114 ± 28</td>
<td>56 ± 8</td>
</tr>
<tr>
<td></td>
<td>0  1.3 ± 0.1</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>1  2.9 ± 0.5</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>Mannoheptulose</td>
<td>20 192 ± 25</td>
<td>65 ± 12</td>
</tr>
<tr>
<td></td>
<td>0  1.0 ± 0.2</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1  2.3 ± 1.0</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>20 3.0 ± 0.8*</td>
<td>4.6 ± 0.2*</td>
</tr>
</tbody>
</table>

* $p < 0.05.$

**p < 0.01.

is very high, islet capillaries are fenestrated, and glucose uptake in beta cells is rapid, it can be assumed (32) that both interstitial and intracellular glucose concentrations equilibrate within seconds to physiologic changes in plasma glucose which range normally between 3 and 10 mM. Unlike glucokinase, which requires approximately 10 mM glucose for half-maximal activity (3), the low $K_m$ hexokinase isoforms are characterized by $K_m$ values well below 1 mM/liter, so they are fully saturated over the range of physiological blood glucose concentrations when glucose transport into the cell is not rate-limiting. Moreover, these enzymes are negatively regulated by Glu-6-P, causing a buffering effect on the Glu-6-P-concentration within the cell (32).

In rodent (14) and human (33) beta cells, sugar transport appears not to be rate-limiting for glucose metabolism, so that the $K_m$ of the glucose phosphorylation step seems directly related to the $K_m$ of glucose-stimulated insulin release. In agreement with this idea, the beta cell line RINM5F, which expresses HK and almost no GK (29, 34, 35), is virtually glucose unresponsive between 1 and 20 mmol/liter glucose, whereas beta cell lines with a relatively good glucose responsiveness such as MIN6 (13), INS-1 (34), HIT-T15 (35), and b1C7 (36) exhibit a relatively high GK over HK expression ratio. Furthermore, up-regulation of HK in MIN6-cells via gene transfer (13) shifts the dose-response curve for glucose-induced insulin release to the left, similar to what has been observed in vivo by expressing the yeast hexokinase transgene in mouse islet beta cells (37). In view of these results it has been difficult to reconcile the large amounts of HK activity in extracts of whole islets (5, 6, 11) with the virtual absence of a secretory response of islet beta cells to glucose concentrations between 0 and 3 mmol/liter. One possible explanation has been suggested...
cellular (ATP)/(ADP) ratio or total glucose utilization (39), reflect quite well the situation present in beta cells which occupy the largest part of the islet volume. For HK, however, the situation seems to be quite different. The expression level of HK in acinar and duct cells is at least 1, perhaps 2, orders of magnitude higher than in flow-sorted endocrine beta and non-beta cells. Therefore, HK values from islet cell preparations with only a few percent of acinar cells do not reflect glucose phosphorylation in beta cells. Since most acinar cells are known to die during tissue culture, it seems indicated to conduct further studies of glucose phosphorylation in non-tumoral beta cells in models where the islets or beta cells were cultured for at least several days. Therefore, although experiments in transfected or transgenic beta cells clearly show that the presence of HK has an effect on insulin secretion at basal glucose concentrations (12, 13, 36, 37, 40), the present study did not support the idea that HK together with GK sets the threshold point of glucose sensing in rat adult differentiated beta cells. This would imply that cells of the endocrine pancreas (at least both alpha and beta cells) expresses GK only, while the exocrine pancreas expresses low \(K_m\) hexokinases, analogous to the clear distinction between parenchymal hepatic versus non-parenchymal cells (41). Interestingly, mathematical modeling data of glycolysis in the beta cell compartment of rodent islets (42) are in close agreement with the experimental results presented in this present paper.

As was demonstrated in this paper, only a tiny fraction of HK remained “associated” with beta cells after correction for the contaminating acinar and duct cells. Our experiments with 2-deoxyglucose, the metabolic precursor of a potent HK inhibitor (29), do not support the idea that this low amount of HK, which might or might not be present in normal rat beta cells, contributes functionally to glucose regulation of these cells, since glucose-induced insulin biosynthesis and release were completely preserved after preincubation with 2-deoxyglucose. One possible caveat of these observations is that the accumulated 2-deoxyglucose-6-phosphate, a potent inhibitor of HK (29), could have been degraded in the cells during the washing period after 2-deoxyglucose loading. Direct measurements of 2-deoxyglucose-6-phosphate content in beta cells after the preincubation period would perhaps provide an answer but, given the limited supply of FACS-purified cells, this appears to be technically difficult and demanding. Furthermore, given the shortness of the washing period (a few minutes), the low cellular density during incubation (2.5 \( \times 10^5 \) cells/ml) and the fact that the used 2-deoxyglucose concentration was high (50 mmol/liter), we propose it is reasonable to interpret our observations as evidence for the lack of functional amounts of hexokinases I, II, or III in adult rat beta cells.

This interpretation might be of physiological importance, since it underlines the idea that the presence of extremely low amounts of hexokinases I, II, or III is a hallmark of differentiated adult beta cells, very similar to what was previously demonstrated for adult differentiated hepatocytes (41). In physiological terms this makes sense, since co-expression of HK and GK would not only shift the glucose sensitivity of insulin-secreting cells to inappropriately low values, but also because of the allosteric properties of HK which would buffer glucose 6-phosphate content in the cells, thereby limiting the range of metabolic control by glucose (32). An important question to be answered in future work is whether or not de-differentiation of animal and human beta cells, either as a consequence of chronic stress or because of DNA mutations associated with beta cell immortality, is a major cause of up-regulation of hexokinase I gene expression on the one hand and loss of normal glucose-induced insulin release on the other. In other words, could a rise in HK over GK in human beta cells be one of the mechanisms by which diabetes is caused? A series of recent studies in rodent islets of Langerhans (16, 18, 43–47) seem to suggest indirectly that chronic stress indeed up-regulates HK activity and loss of the normal beta cell phenotype in parallel. In a model of chronic stress imposed upon remaining beta cells after 85–95% pancreatectomy, Hosakowa et al. (16) observed more than 2-fold up-regulation of HK activity in isolated islets from these animals, while Jonas et al. (43) recently described multiple abnormalities in gene expression in these islets, comprising a clear increase in the abundance of hexokinase I mRNA, an animal model of obesity and hyperinsulinism, a parallel increase in HK activity and alterations in the glucose-induced function of isolated islets has been observed (44). It is possible that these abnormalities are caused by increased concentrations of free fatty acids which can induce similar perturbations after in vitro exposure of normal islets (45, 46). Cellular stress may also be induced by the homozygous null mutation of the glucokinase gene, for instance, after deletion of the beta cell-specific GK promoter (8), or via a strategy including conditional activation of Cre-recombinase in islet beta cells (18). Following the latter approach, Piston et al. (18) suggested the presence of low \(K_m\) glucose metabolism in those islet beta cells which underwent recombination of the glucokinase gene, indicating that HK can be up-regulated under such conditions (18). However, it should be mentioned that the interpretation in this paper was indirect, so that HK gene expression should still be assessed in GK−/− cells, either by mRNA or protein analysis. Furthermore, the functional consequence of up-regulated HK expression in beta cells defective in GK expression is still uncertain, since Sakura et al. (47) did not observe any functional beta cell response in terms of \(K_m\), channel activity between 0 and 1 mm glucose in islets isolated from homozygous HK knockout mice. Nevertheless, the concept that up-regulation of HK perturbs the normal phenotype of differentiated beta cells which are virtually devoid of low \(K_m\) glucose phosphorylation, and hence contributes to a diabetic state, is interesting and deserves further analysis.

In summary, our observations strongly indicate that differentiated adult rat beta cells express extremely low amounts of low \(K_m\) hexokinases, a characteristic which can contribute to the powerful glucose regulation of these cells within the millimolar concentration range. Our data also draw attention to potential pitfalls in the interpretation of biochemical data obtained from mixed cell populations as is illustrated by the fact that most HK associated with rat islets of Langerhans originates from non-endocrine pancreatic cells. This methodological concern seems equally relevant for any other enzyme or molecule which is expressed more abundantly in exocrine pancreas than in endocrine beta cells.

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