Glucose Transport, Phosphorylation, and Utilization in Isolated Porcine Pancreatic Islets

Agata Maria Rabuazzo, Alberto M. Davalli, Massimo Buscema, Carlo Socci, Venera Caltabiano, Antonio E. Pontiroli, Valerio Di Carlo, Guido Pozza, Riccardo Vigneri, and Francesco Purrello

Porcine islets have been proposed as a donor source for human transplantation, mainly because of both structural and biological similarities of porcine and human insulin. However, the in vitro function of these islets is poorly characterized. In the present study, we first examined insulin release in response to glucose in static incubation experiments. Increasing glucose concentrations up to 8.3 mmol/L stimulated insulin release; however, this elevation was only twofold, and a paradoxical decline was observed at glucose concentrations higher than 8.3 mmol/L. In cultured porcine islets, a greater insulin secretion may be elicited by agents that increase intracellular cyclic adenosine monophosphate (cAMP) levels. To investigate the possible reasons for the porcine islet low response to glucose in vitro, we then evaluated in parallel experiments glucose transport, phosphorylation, and utilization. Glucose transport studies (using 3-*O*-methyl glucose uptake at 15°C for 15 seconds) indicated the presence of both a high-affinity (K_m, 1.2 ± 0.6 mmol/L) and a low-affinity (K_m, 11.8 ± 1.9 nmol/L, n = 5) component. Glucose phosphorylation, evaluated by measuring the rate of glucose-6-phosphate formation in a fluorimetric assay, indicated that glucokinase activity had a maximum (Vmax) of 7.97 ± 0.94 nmol/μg DNA/h and a K_m of 8.3 ± 0.9 mmol/L (mean ± SE, n = 8). Glucose utilization, determined by measuring ^3H_2O formation from (5-^{3}H)-glucose, increased from 1.79 ± 0.34 nmol/μg DNA/2 h at 5 mmol/L glucose to 5.97 ± 0.16 and 6.19 ± 0.29 at 20 and 30 mmol/L glucose, respectively, with a calculated Vmax of 6.9 ± 0.33 and a K_m of 7.0 ± 1.4 mmol/L (mean ± SE, n = 5). These data in porcine islets were similar to data previously obtained in rat islets using the same procedures. In conclusion, this study indicates that glucose transport, phosphorylation, and utilization are not altered in isolated porcine islets, and therefore, the in vitro low response of porcine islets to glucose is not due to alterations in glucose sensing or metabolism.

PORCINE ISLETS have been proposed as a future donor source for human transplantation, mainly because porcine insulin has a structure and biological activity similar to that of human insulin. Procedures for large-scale porcine islet isolation and purification are mainly based on either an automatic digestion-filtration method similar to the method used for the isolation of human islets,[1] a mild mechanical disruption,[2,3] or a continuous intraductal collagenase-elastase perfusion method.[4]

However, when cultured in vitro, porcine islets are very fragile and easily lose their morphological structure, dissociating in fragments or even in single cells.[1,3] Besides, their secretory function in vitro in response to glucose has been reported as low or absent[3-5] as compared with that of rat or mouse islets. An inherently low responsiveness of porcine islets to glucose has been hypothesized,[6] but data regarding the porcine perfused-pancreas response to glucose are conflicting: a threefold[7] or 10-fold[8] increase in insulin release has been reported in different studies. Contrasting results have been reported also with secretagogues other than glucose. The response to secretagogues that act mainly by amplifying the insulin response to glucose is also diminished,[5] but in contrast, the secretory response to agents that increase cyclic adenosine monophosphate (cAMP) levels (theophylline, isobutyl-methylxantine [IBMX]) has been reported as normal.[9]

In view of the potential role of porcine islets in human transplantation, it is important to investigate better the reason(s) for their low response to glucose after isolation. Therefore, in the present study we investigated whether the impaired response to glucose of isolated porcine pancreatic islets was associated with a damage of either glucose transport, phosphorylation, or utilization.

MATERIALS AND METHODS

Culture medium, fetal calf serum, and antibiotics were obtained from Seromed-Biochrom (Berlin, Germany). 3-*O*-[^{3}H]methyl-D-glucose ([^{3}H]-3OMG; specific activity, 2.74 Ci/mmol), [U-^{14}C]-sucrose (specific activity, 673 mCi/mmol), and [5-^{3}H]-glucose (specific activity, 15.7 Ci/mmol) were from Amersham (Amersham, UK).

Pig Islet Isolation and Culture

Islets were isolated from pancreases obtained at a local slaughterhouse from large white female pigs at a mean age of 24 ± 8 months and weighing 250 to 300 kg. Twelve separate isolation procedures were performed for these experiments.

The pancreases were removed from the animals during the exsanguination phase: warm ischemia time ranged from 5 to 8 minutes, and cold ischemia time was approximately 2 to 3 hours. After removal, the glands were transported to the islet-isolation facility in cold (4°C) Eurocollins solution (SIFRA, Verona, Italy).

The pancreatic duct was cannulated with a 20-gauge angiocatheter, and fat tissue, vessels, and membranes were carefully dissected and discarded. The gland was injected with Hanks solution containing 2 mg/dL collagenase (type V, Sigma, St Louis, MO) and then digested by the automated method for human islet isolation[10] with the modifications previously described in detail.[1]

From the Institute of Internal Medicine, Metabolism, and Endocrinology, University of Catania Medical School, Catania; and the Department of Medicine, Surgery, and Pathology, "Istituto Scientifico San Raffaele," Milano, Italy.

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Address reprint requests to Francesco Purrello, MD, Cattedra di Endocrinologia, Ospedale Garibaldi, Piazza S. Maria di Gesù, 95123 Catania, Italy.

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The digested preparation was purified from the exocrine tissue by centrifugation on discontinuous Ficoll gradient (Sigma) as previously described.[1] After purification, an average of 3,380 ± 679 islets per gram pancreas were obtained (mean ± SE of eight separate preparations). More than 80% of the cells were endocrine cells, as indicated by dithizone staining performed in islets that were first dissociated in single cells.

After isolation, pig islets were plated in untreated dishes and cultured at 37°C for 48 hours in CMRL 1066 culture medium containing 5.5 mmol/L glucose, 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate.

Immunohistochemistry

Aliquots of islet preparations were centrifuged (800 × *g* for 2 minutes) and the pellets fixed in Bouin's solution. Immunohistochemistry for insulin (1:2,000 guinea pig antiporcine insulin, Dako, Carpinteria, CA) was performed on 7-μm thick sections. Normal human pancreas served as a positive control, and omission of primary antibody was used to detect nonspecific staining.

Insulin Release

Insulin release in response to increasing concentrations of glucose was evaluated in static incubation conditions. Cultured islets (20 islets per well) were plated in multiwell plates (Transwell 24-plate, Costar, Cambridge, MA) and incubated in oxygenated Krebs-Ringer bicarbonate buffer (KRB) and 0.5% bovine serum albumin ([BSA] Sigma) in the presence of increasing concentrations of glucose (0 to 27.7 mmol/L). After 2 hours of incubation in air/5% CO_2 at 37°C, the two parts of the Transwell multiwell plate were separated. The lower part contained the incubation buffer, which was collected and stored at −20°C until assayed for insulin content. The upper part containing the islets was washed twice with KRB (0.5% BSA), dried (2 minutes over an absorbing paper), and then transferred into a new Transwell lower compartment containing 1 mL acid-alcohol, and used to measure islet insulin content.

Insulin was assayed by radioimmunoassay using a commercial kit (Immunonuclear, Stillwater, MN). In our experience, this radioimmunoassay has an intraassay and interassay coefficient of variation of 3.0% and 5.0%, respectively. The minimum sensitivity is 14.5 pmol/L; the recovery of added doses is 98% to 100%.

Glucose Transport

Glucose transport was evaluated in dissociated pancreatic cells attached to culture dishes. Pancreatic cells were obtained from islets transferred to a Ca^{2+}-free Hanks balanced buffer (NaCl 136 mmol/L, $MgSO_4$ 0.8 mmol/L, KCl 5.4 mmol/L, Na_2HPO_4 0.35 mmol/L, KH_2PO_4 0.45 mmol/L, $NaHCO_3$ 4.2 mmol/L) at 30°C in the presence of trypsin (6.5 μg/mL) and DNAse (2 μg/mL).[11] The islet dissociation was stopped by adding Hanks balanced buffer containing 1.67 mmol/L calcium and 1% newborn calf serum when most cells were in groups of three to seven. Cells were then cultured in CMRL 1066 medium in 48-multiwell dishes (Costar) at approximately 300 × 10^3 cells per well. After 48 hours, cells were washed twice and incubated with Krebs-Ringer phosphate (KRP) buffer (140 mmol/L NaCl, 1.7 mmol/L KCl, 0.9 mmol/L $CaCl_2$, 1.47 mmol/L K_2HPO_4, 0.8 mmol/L $MgSO_4$, and 0.5% BSA, pH 7.4) for 30 minutes at 15°C. The buffer was then replaced with 0.4 mL fresh KRP containing increasing concentrations (0.1 to 60 mmol/L) of 3OMG at a constant specific activity of 0.06 μCi/mmol. After 15 seconds, the transport was stopped by washing the cells three times with ice-cold KRP containing 0.5% BSA, 400 mmol/L D-glucose, and 10 mmol/L phlorhizin, pH 7.4. Cells were then solubilized with 1 mL 0.1% sodium dodecyl sulfate, and the cell-incorporated radioactivity was counted and normalized to the DNA content.[12] [U-^{14}C]-Sucrose uptake was measured to correct for label in the extracellular space.

Glucose Phosphorylation

The glucose phosphorylation rate was determined by measuring the rate of glucose-6-phosphate formation in a fluorimetric assay.[13] Cultured pancreatic islets (~800) were homogenized in 300 μL ice-cold buffer, pH 7.7, containing K_2HPO_4 20 mmol/L, EDTA 1 mmol/L, KCl 110 mmol/L, and dithiothreitol 5 mmol/L. The islet homogenate was then centrifuged for 15 minutes at 12,000 × *g*, and the glucose phosphorylation activity was measured in the supernatant.

The assay volume contained 4 μL supernatant in 100 μL HEPES hydrochloride 50-mmol/L, pH 7.7, KCl 100 mmol/L, $MgCl_2$ 7.4 mmol/L, β-mercaptoethanol 15 mmol/L, NAD^+ 0.5 mmol/L, BSA 0.05%, glucose-6-phosphate dehydrogenase 2.5 μg/mL, adenosine triphosphate 5 mmol/L, and glucose concentrations ranging from 0.03 to 100 mmol/L. In a typical experiment, glucose was added at 0.03, 0.06, 0.12, 0.25, and 0.5 mmol/L to measure hexokinase activity, and at 5, 7.5, 10, 15, 20, 25, 50, 65, 80, and 100 mmol/L to measure glucokinase activity. The reaction was stopped after 1 hour at 30°C by adding 1 mL 500-mmol/L sodium bicarbonate buffer, pH 9.4. Fluorescence was then measured at 460 nm (excitation at 340 nm). A standard curve was obtained by incubating with the assay reagents 0.3 to 1.0 nmol glucose-6-phosphate and 1 nmol NADH.[13] In each assay, tissue blanks were also performed by incubating either 0.5 or 100 mmol/L glucose in the absence of adenosine triphosphate. Glucokinase activity was calculated by subtracting the hexokinase maximum rate (Vmax) from the activities measured at concentrations higher than 5.0 mmol/L glucose.[13]

To calculate the ratio between glucose transport and phosphorylation activity at the different glucose concentrations tested, we assumed a Q_{10} of 2 to adjust for the different temperature used for measuring glucose transport (15°C) and phosphorylation (30°C); Q_{10} is a correction factor for increasing temperature.[14]

Glucose Utilization

The utilization of glucose was determined by measuring the formation of ^3H_2O from (5-^{3}H)-glucose.[15] Cultured islets were incubated in 40 μL KRB buffer (NaCl 118 mmol/L, KCl 4.8 mmol/L, $CaCl_2$ 2.5 mmol/L, $MgSO_4$ 1.2 mmol/L, KH_2PO_4 1.2 mmol/L, $NaHCO_3$ 25 mmol/L) supplemented with 10 mmol/L HEPES, pH 7.4, containing 2 μCi D-(5-^{3}H)-glucose, and glucose concentrations ranging from 5 to 30 mmol/L. In a typical experiment, glucose was added at 5, 7.5, 10, 15, 20, and 30 mmol/L. The incubation was performed in 1-mL glass vials inside an airtight, sealed, 20-mL glass scintillation vial that contained 500 μL distilled water. After 2 hours at 37°C, the reaction was stopped by adding 0.5 mol/L HCl (100 μL injected through the rubber seal). The scintillation vials were then incubated overnight at 37°C, and the water radioactivity was measured. Blanks without islets were also performed. Under these experimental conditions, the recovery from known amounts of ^3H_2O was fairly constant, ranging from 50% to 60%.

RESULTS

Insulin Secretion Studies

In static incubation conditions, insulin release in the medium progressively increased in the presence of increasing glucose concentrations from 0 to 8.3 mmol/L. A significant increase was observed at 5.0 mmol/L glucose, and maximal insulin release was obtained at 8.3 mmol/L glucose (Fig 1). However, the maximum release was only

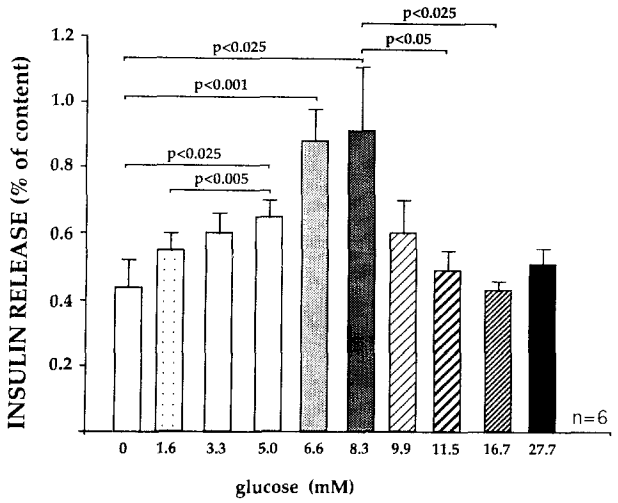

Fig 1. Insulin release by isolated porcine islets during static incubations in the presence of increasing concentrations of glucose. Islets were cultured in CMRL 1066 medium containing glucose 5.5 mmol/L. After 48 hours, culture medium was replaced with KRB containing increasing concentrations of glucose (0 to 27 mmol/L). The amount of insulin released during the following 2-hour incubation was measured and expressed as the percentage of total islet insulin content. Data are the mean ± SE of six separate experiments. V/S, velocity/substrate.

twofold greater than the value observed in islets exposed to 1.6 mmol/L glucose. A paradoxical progressive decline in insulin release was observed when islets were incubated at glucose concentrations higher than 8.3 mmol/L (Fig 1). However, porcine islets were able to increase further the insulin secretion when either 0.1 mmol/L IBMX or 1 mmol/L db-cAMP were added in addition to glucose 8.3 mmol/L (Table 1). Similar data were obtained in freshly isolated islets.

Metabolic Studies

Glucose transport, phosphorylation, and utilization were investigated in isolated porcine islets in parallel experiments.

Glucose transport. Glucose transport in porcine pancreatic cells increased with increasing glucose concentrations up to 20 mmol/L (Fig 2A). In agreement with previous studies in rat pancreatic islets,[16,17] kinetic characteristics of

Table 1. Insulin Secretion From Isolated Porcine Islets

	Insulin Release (% of islet insulin content)
Glucose O mmol/L	0.42 ± 0.1
Glucose 8.3 mmol/L	0.89 ± 0.2
Glucose 8.3 mmol/L + IBMX 0.1 mmol/L	2.96 ± 0.4
Glucose 8.3 mmol/L + db-cAMP 1 mmol/L	2.25 ± 0.4

NOTE. Islets were cultured in CMRL 1066 medium containing glucose 5.5 mmol/L. After 48 hours, culture medium was replaced with KRB containing either glucose 8.3 mmol/L or glucose plus 0.1 mmol/L IBMX or 1 mmol/L db-cAMP. The amount of insulin released during the following 2-hour incubation was measured and expressed as the percentage of total islet insulin content. Data indicate the mean ± SE of six separate experiments.

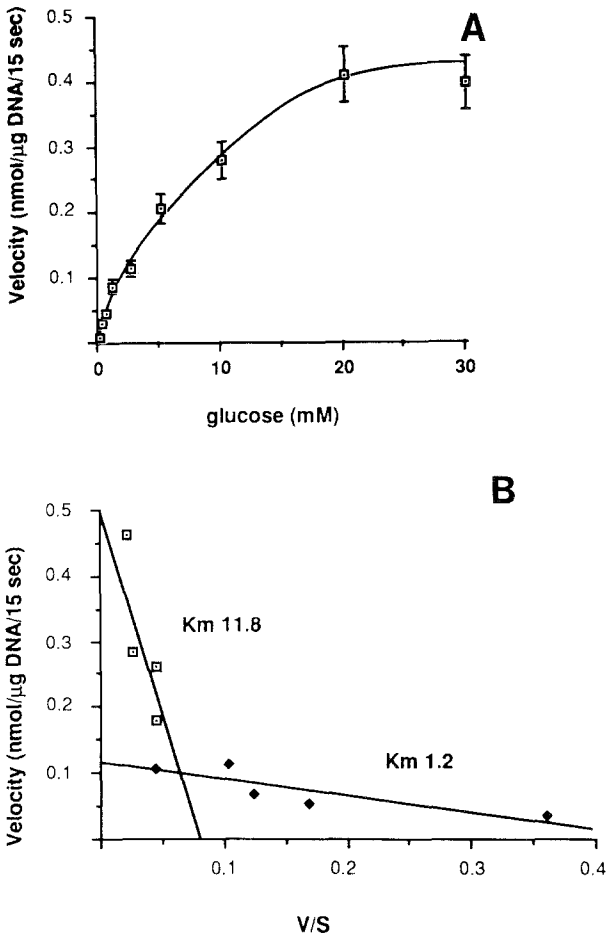

Fig 2. Glucose transport kinetics in porcine pancreatic islet cells. Porcine islets were dissociated in groups of three to seven cells and cultured in CMRL 1066 medium containing 5.5 mmol/L glucose in multiwell dishes. After 48 hours, cells were washed twice and incubated in KRP buffer for 30 minutes at 15°C. To measure [^{3}H]-3OMG transport, the buffer was replaced with 0.4 mL fresh KRP containing different concentrations of [^{3}H]-3OMG (range, 0.1 to 60 mmol/L). After 15 seconds, the transport was stopped by washing the cells with ice-cold KRP containing 0.5% BSA, 400 mmol/L D-glucose, and 10 mmol/L phlorhizin, pH 7.4. Counts were normalized for DNA content. Results are presented as 3OMG transported in the presence of increasing extracellular glucose concentrations (A) or according to the Eadie-Hofstee plot (B). A representative of five separate experiments is shown. V/S, velocity/substance.

glucose uptake in porcine pancreatic cells calculated according to the Eadie-Hofstee plot indicated the presence of two kinetically distinct components. The low-affinity component had an apparent K_m of 11.8 ± 1.9 mmol/L (n = 5), and the high-affinity component had an apparent K_m of 1.2 ± 0.6 mmol/L (Fig 2B). The low-affinity glucose transport function is believed to represent the GLUT-2 transporter, a glucose carrier unique to β cells among all pancreatic cells. The maximal capacity of this transporter was 0.48 ± 0.08 nmol/µg DNA/15 s. The relative capacity of the high-affinity component was 15% to 20% of the total capacity.

Glucose phosphorylation. Glucose-6-phosphate formation was used to calculate porcine islet hexokinase and glucokinase activity Vmax and K_m according to the Eadie-

Hofstee plot. In porcine islets cultured at 5.5 mmol/L glucose, glucokinase activity measured at glucose concentrations ranging from 5 to 100 mmol/L showed a Vmax of 7.97 ± 0.94 nmol/μg DNA/h and a K_m of 8.3 ± 0.9 mmol/L (mean ± SE, n = 8; Fig 3). The calculated ratio of glucose transport to glucose phosphorylation varied at the various glucose concentrations tested, decreasing from 22 at 20 mmol/L glucose to 7 at 5 mmol/L glucose (Table 2).

Glucose utilization. In porcine islets cultured at glucose 5.5 mmol/L, glucose utilization increased from 1.79 ± 0.34 nmol/μg DNA/2 h at glucose 5 mmol/L to 5.97 ± 0.16 and 6.19 ± 0.29 at glucose 20 and 30 mmol/L, respectively (Fig 4), with a calculated Vmax of 6.9 ± 0.33 and a K_m of 7.0 ± 1.4 mmol/L (mean ± SE, n = 5).

Values for glucose transport, phosphorylation, and utilization in porcine islets were then compared (Table 3) with values obtained in previous experiments in rat islets using the same procedures.[16] No significant difference was present except in the glucose transport K_m ($P < .05$).

DISCUSSION

The present study confirms that the in vitro glucose-induced insulin release of porcine islets is considerably less (~two times over basal) than that observed in rat or mouse islets (~10 times over basal). Under our experimental conditions, other differences between porcine and rat islets were observed: porcine islets have a lower threshold for insulin secretion in response to glucose, and show a decrease of insulin secretion when islets are stimulated with glucose concentrations higher than 8.3 mmol/L. This paradoxical response is a novel observation, because a detailed analysis of the dose-response curve for insulin release in response to glucose has never been assessed in porcine islets. Crowther et al[5] have already reported that porcine islets have a low threshold for insulin release in response to glucose (between 2.8 and 4.2 mmol/L glucose), but in their study glucose concentrations higher than 8.3 mmol/L were not used. Other investigators studied only the response to a single glucose concentration in static or perifusion systems.[7,17]

To investigate whether abnormalities of glucose transport, phosphorylation, or utilization were responsible for the low secretory response to glucose, we studied these functions in porcine islets. Our data indicate that no alteration of either glucose transport, phosphorylation, or utilization was present in isolated porcine islets. All these functions appeared both qualitatively and quantitatively similar to those previously observed in rat islets. In porcine islets, as in rat islets,[16] glucose transport exceeds glucose phosphorylation, in particular at high glucose concentrations. Two kinetically distinct transport components are present.[18] The high-affinity (K_m, 1.2 mmol/L), low-capacity

Table 2. Ratio of Glucose Transport to Glucose Phosphorylation at Different Glucose Concentrations in Porcine Islets Cultured at 5.5 mmol/L Glucose

Glucose (mmol/L)	Transport (nmol/μg DNA/h)	Phosphorylation (nmol/μg DNA/h)	Ratio
5	35.9	4.8	7
10	89.7	6.9	13
20	187.4	8.5	22

NOTE. To calculate the ratio of glucose transport *v* glucokinase activity at the different glucose concentrations tested, we assumed a correction factor (Q_{10}) of 2 to adjust for the different temperatures used for measuring glucose transport (15°C) and phosphorylation (30°C).

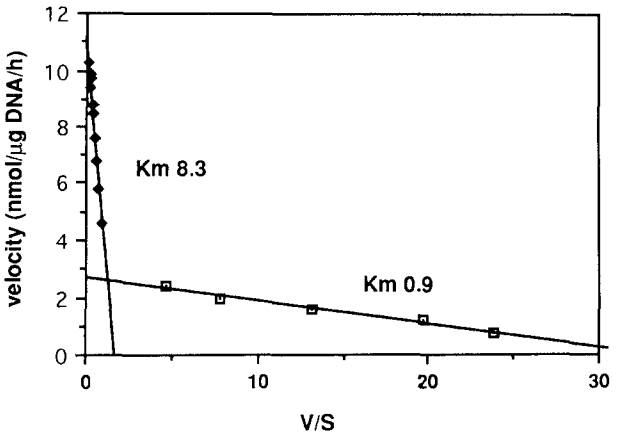

Fig 3. Glucose phosphorylation kinetics in porcine pancreatic islets. Islets (~800) cultured in medium containing 5.5 mmol/L glucose were homogenized centrifuged, and glucose phosphorylation activity was measured in the supernatant by evaluating the rate of glucose-6-phosphate formation in a fluorimetric assay. Data are presented according to the Eadie-Hofstee plot. A representative of five separate experiments is shown. In a typical experiment, the following glucose concentrations were used: 0.03, 0.06, 0.12, 0.25, and 0.5 mmol/L to measure hexokinase activity, and 5, 7.5, 10, 15, 20, 25, 50, 65, 80, and 100 mmol/L to measure glucokinase activity. To calculate glucokinase Vmax and K_m, the Vmax for hexokinase was subtracted from the activity measured at glucose concentrations higher than 5.0 mmol/L.

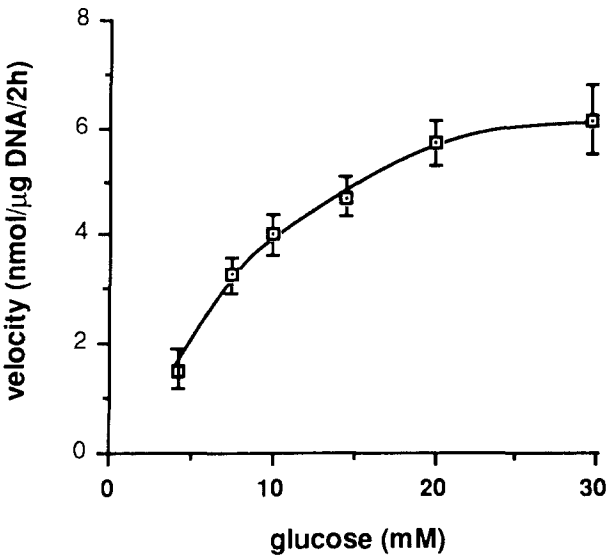

Fig 4. Glucose utilization in porcine pancreatic islets. Islets cultured in CMRL 1066 medium for 48 hours were incubated in 40 μL KRB buffer containing 10 mmol/L HEPES, 2 μCi D-(5-^{3}H)-glucose, and different glucose concentrations (5, 7.5, 10, 15, 20, and 30 mmol/L). The utilization of glucose was determined by measuring the formation of ^3H_2O from (5-^{3}H)-glucose. Data represent the mean ± SE of five separate experiments.

Table 3. Comparison of Glucose Transport, Phosphorylation, and Utilization Between Rat and Pig Pancreatic Islets

	Transport		Phosphorylation		Utilization	
	K_m (mmol/L)	Vmax (nmol/μg DNA/15 min)	K_m (mmol/L)	Vmax (nmol/μg DNA/h)	K_m (mmol/L)	Vmax (nmol/μg DNA/2 h)
Pig islets	11.8 ± 1.9*	0.48 ± 0.08	8.3 ± 0.9	7.97 ± 0.94	7.7 ± 1.4	6.90 ± 0.33
Rat islets	16.6 ± 2.4	0.32 ± 0.04	9.7 ± 1.0	8.70 ± 0.79	8.9 ± 0.4	7.60 ± 0.28

NOTE. Data for rat islets are from Purrello et al.[16]

*$P < .05$ in comparison to rat islets.

component has characteristics similar to those of the GLUT-1 transporter. Under our experimental conditions (mixed pancreatic cells), it was impossible to distinguish whether this transporter is present in all the endocrine cells or expressed only in the non-β cells. The low-affinity (K_m, 11.8 mmol/L), high-capacity glucose transport function is believed to represent the GLUT-2 transporter, a glucose carrier specific to the β cells among all pancreatic cells. The calculated K_m of this transporter in porcine islets (11.8 mmol/L) is lower than the value observed in rat islets (16 to 20 mmol/L).[16,18] This difference might be either due to differences in the primary sequence of the porcine versus rat GLUT-2 protein, or due to posttranslational changes, since an alteration in the GLUT-2 affinity has recently been associated with changes in its glycosylation.[19] Finally, we cannot exclude that the islet-isolation procedure or the culture conditions might have affected the glucose transporter affinity. However, the difference in the low-affinity glucose transporter K_m cannot explain the reduced porcine islet responsiveness to glucose in comparison to that of rat islets.

In porcine islets, glucose phosphorylation shows two kinetically different components. The high-affinity component is probably due to the enzyme hexokinase,[13] whereas the low-affinity one is probably due to the enzyme glucokinase,[13] an enzyme that is specific to β cells among all pancreatic cells. In porcine islets glucose utilization also is similar to that found in rat islets. In particular, at low glucose concentrations the rate of glucose utilization in intact islets is lower than the rate of glucose phosphorylation in cell homogenates, thus suggesting that, as in rat islets and at variance with tumoral insulinoma cells, hexokinase is mostly inhibited by endogenous glucose-6-phosphate. Since values for glucose transport, phosphorylation, and utilization in porcine islets are similar to those found in rat islets, it is unlikely that the low response to glucose observed in vitro with porcine islets is due to differences in the β-cell glucose-sensing mechanism, due to either species characteristics or a functional damage during the isolation procedure. However, our data cannot exclude that an impairment in glucose metabolism beyond the level of triose phosphates may be responsible for the low secretory response to glucose. Finally, in our experimental conditions the insulin-storage and -secretory mechanisms of porcine islets were not damaged during the isolation procedure, since a good insulin-secretory response of porcine islets to secretagogues able to increase cAMP levels (IBMX, db-cAMP) was observed. Therefore, this was not the reason for the low response to glucose.

In conclusion, the present study suggests that isolated porcine islets have a normal glucose-sensing mechanism and a normal insulin-storage and -secretory machinery. However, the porcine islets' blunted insulin secretion in response to glucose may adversely affect their use for human transplantation. Of course, it remains to be investigated whether the low response to glucose observed in vitro persists in vivo after transplantation. A certain functional recovery is theoretically possible after transplantation, since reaggregation of dispersed pancreatic cells into islet cell clusters with a peripheral α-cell mantle has been reported after implantation in immunosuppressed rats.[20] Further studies are therefore required to ascertain that an adequate functional capacity can be obtained from porcine islets before they are considered a convenient source of tissue for transplantation in diabetic patients.

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