Investigation on the mechanism by which fructose, hexitols and other compounds regulate the translocation of glucokinase in rat hepatocytes

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In isolated hepatocytes in suspension, the effect of sorbitol but not that of fructose to increase the concentration of fructose 1-phosphate and to stimulate glucokinase was abolished by 2-hydroxymethyl-4-(4-*N*,*N*-dimethylamino-1-piperazino)-pyrimidine (SDI 158), an inhibitor of sorbitol dehydrogenase. In hepatocytes in primary culture, fructose was metabolized at approximately one-quarter of the rate of sorbitol, and was therefore much less potent than the polyol in increasing the concentration of fructose 1-phosphate and the translocation of glucokinase. In cultures, sorbitol, commercial mannitol, fructose, D-glyceraldehyde or high concentrations of glucose caused fructose 1-phosphate formation and glucokinase translocation in parallel. Commercial mannitol was contaminated by approx. 1% sorbitol, which accounted for its effects. The effects of sorbitol, fructose and elevated concentrations of glucose were partly inhibited by ethanol, glycerol and glucosamine. Mannoheptulose increased translocation without affecting fructose 1-phosphate concentration. Kinetic studies performed with recombinant human β-cell glucokinase indicated that this sugar, in contrast with *N*-acetylglucosamine, binds to glucokinase competitively with the regulatory protein. All these observations indicate that translocation is promoted by agents that favour the dissociation of the glucokinase–regulatory-protein complex either by binding to the regulatory protein (fructose 1-phosphate) or to glucokinase (glucose, mannoheptulose). They support the hypothesis that the regulatory protein of glucokinase acts as an anchor for this enzyme that slows down its release from digitonin-permeabilized cells.

INTRODUCTION

Agius and Peak [1,2] have provided evidence for a role of a large series of compounds, mostly sugars and polyols, in the release of glucokinase from a binding site located in the matrix of liver cells in culture. Indeed, when cells that have been incubated in the presence of a low concentration of glucose (5 mM) are permeabilized with digitonin in a medium containing 5 mM $MgCl_2$, only minimal release of glucokinase occurs. When, in contrast, the cells have been incubated in the presence of fructose, sorbitol, mannitol, mannoheptulose or an elevated concentration of glucose, a much larger release of glucokinase is observed. These effects are counteracted by other agents such as ethanol, glycerol and glucosamine.

The sensitivity of glucokinase translocation to fructose and sorbitol, which is converted into fructose by sorbitol dehydrogenase, suggests that it is related to the formation of fructose 1-phosphate and therefore involves the glucokinase regulatory protein [3]. Fructose 1-phosphate does indeed bind to this regulator, and cancels its inhibitory effect on glucokinase [4] by preventing the association of the two proteins [5]. This interpretation, however, does not account for the fact that sorbitol was found to be severalfold more potent than fructose in causing translocation [2], a difference that was not observed for the ability of the two compounds to stimulate glucokinase in isolated hepatocytes [6]. Furthermore, it is not clear how mannitol acts, because this compound is generally thought not to be metabolized by mammalian tissues. The effect of high concentrations of glucose is also unexplained, because liver aldose reductase, the enzyme that could form sorbitol from glucose, was reported not to be active on this hexose in the liver [7].

The purpose of the present work was to investigate the mechanism by which all the compounds mentioned above affect the translocation of glucokinase. To perform these studies we took advantage of a recently developed assay to measure fructose l-phosphate in picomolar amounts [8]. We also used 2-hydroxymethyl-4-(4-*N*,*N*-dimethylamino-1-piperazino)-pyrimidine (SDI 158), a powerful inhibitor of sorbitol dehydrogenase described by Geisen et al. [9].

EXPERIMENTAL

Materials

Sorbitol, ATP and BSA were from Sigma (St. Louis, MO, U.S.A.). Fructose and digitonin were from Merck (Darmstadt, Germany). Glucose was from Janssen Chimica (Beerse, Belgium). Mannitol was from J. T. Baker Chemicals (Deventer, Holland), from Merck ('for biochemistry' grade) or from Sigma (ACS reagent). SDI 158 was a gift from Dr. Geisen (Hoechst, Frankfurt/Main, Germany). Collagenase and auxiliary enzymes were from Boehringer (Mannheim, Germany). Fructose-1-phosphate kinase was purified from *Clostridium difficile* as previously described [10].

Radiolabelled substrates were from Amersham International (Little Chalfont, Bucks., U.K.). [^{14}C]Mannitol was prepared by the reduction of [U-^{14}C]mannose with sodium borohydride and purified by gel filtration [11]. [2-^{3}H]Glucose was repurified by paper chromatography before use [12] to eliminate metabolizable impurities. Dowex AG 1-X8 (200–400 mesh) was from Bio-Rad (Richmond, CA, U.S.A.). Minimum essential medium was from Gibco–BRL (Paisley, Scotland, U.K.). Recombinant human

Abbreviation used: SDI 158, 2-hydroxymethyl-4-(4-*N*,*N*-dimethylamino-1-piperazino)-pyrimidine.

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β-cell glucokinase [13] and rat liver regulatory protein [5] were prepared as indicated.

isolation and incubation of hepatocytes

Hepatocytes were isolated from the liver of fed male Wistar rats weighing 300–340 g, by a modification [14] of the method of Seglen [15]. The cells were resuspended in a Krebs–Henseleit bicarbonate buffer at a final concentration of 4–10 mg/ml protein. Aliquots (2 ml) of this suspension were incubated at 37 °C in 20 ml vials equilibrated with an O_2/CO_2 (19:1) gas phase. Unless otherwise indicated, the substrates were added after a preincubation of 15 min in the presence of 5 mM glucose.

In the experiment shown in Figure 1, the incubations were stopped at 10 and 20 min by mixing 1 ml of the cell suspension with 0.5 ml of 10% (w/v) perchloric acid and the resulting extracts were used for the measurement of fructose 1-phosphate and ^3H_2O [12] respectively. In the experiments shown in Figures 5 and 6, the cells were separated from the medium by centrifugation in tubes containing 0.5 ml of silicone oil on top of 0.5 ml of 10% (w/v) perchloric acid [16]. The superior phase was mixed with 0.5 vol. of 10% perchloric acid and used to determine the conversion of polyols and fructose into glucose and extracellular anions. The inferior phase was used to measure fructose 1-phosphate.

Hepatocyte culture

Hepatocytes were isolated as above and suspended in minimal essential medium containing 10% (v/v) foetal bovine serum and 100 nM dexamethasone. They were inoculated in six-well plates (well diameter 3.5 cm) at a cell density of approx. 8×10^4 cells/cm^2 and incubated at 37 °C in a humidified atmosphere consisting of air/CO_2 (19:1). After cell attachment (approx. 4 h), the medium was changed to a serum-free medium. Incubations with substrates were performed after 16–24 h of culture. Separate plates were used to determine glucokinase release and fructose 1-phosphate concentration.

Glucokinase release was determined essentially as described by Agius [2] in a medium (1.5 ml) containing 300 mM sucrose, 3 mM Hepes, 2 mM dithiothreitol, 5 mM $MgCl_2$ and 0.05 mM digitonin, pH 7.2, at 20 °C. The digitonin eluate was recovered; the residual cell matrix was maintained overnight at −20 °C and extracted with the same medium as for the permeabilization, but without digitonin, the sonication step [1] being omitted. The glucokinase activity in the digitonin eluate is expressed as a proportion of the total activity in the eluate and cell matrix. Incubations with substrates and metabolites did not affect the total activity of glucokinase.

For the assay of fructose 1-phosphate, the extracellular medium was aspirated and the cells were immediately mixed with 0.6 ml of 3.3% perchloric acid. In the experiment shown in Figure 4, the medium was mixed with 0.5 vol. of 10% (w/v) perchloric acid to determine the conversion of radioactive substrates.

Measurement of the ^{14}C-labelled metabolites

Perchloric acid extracts prepared from the extracellular medium of hepatocytes in culture or in suspension were neutralized, diluted 2-fold and applied on a Dowex AG 1-X8 Cl column (0.4 cm × 6 cm), which was washed with 1.5 ml of water (sugar and polyols) and 3 ml of 0.5 M NaCl (anionic metabolites). For cells incubated with [^{14}C]sorbitol, 1 ml of the water eluate was incubated for 30 min at 30 °C with 14 units of yeast hexokinase/5 mM $MgATP^{2-}$/25 mM Hepes (pH 7.5) in a final volume of 1.1 ml. The mixture (1 ml) was then applied on another column of Dowex AG 1-X8 Cl, which was successively eluted with 3 ml of water (unreacted polyol) and 3 ml of 0.5 M NaCl (fructose plus glucose as hexose 6-phosphates). For cells incubated with [^{14}C]fructose, the water eluate of the first column was incubated for 30 min at 30 °C with 50 m-units of fructokinase/5 mM $MgATP^{2-}$/25 mM Hepes (pH 7.5)/100 mM KCl in a final volume of 1.1 ml. The mixture (1 ml) was then applied on another column of Dowex AG 1-X8 Cl, which was successively washed with 3 ml of water (glucose) and 3 ml of 0.5 M NaCl (fructose as fructose 1-phosphate). These fractions were counted for radioactivity.

Other assays

Glucokinase activity was assayed in cells extracts as described [17] with 50 mM glucose. Fructose 1-phosphate was assayed either as described [10] (for Figures 1 and 6) or by stimulation of potato tuber PP_i-dependent phosphofructokinase after partial conversion into fructose 2,6-bisphosphate [8], the second method being approx. 100-fold more sensitive than the first.

For the measurement of fructokinase and of sorbitol dehydrogenase, hepatocyte pellets derived from suspensions or monolayers of hepatocyte, freed of medium, were maintained at −20 °C overnight and resuspended in 20 mM Hepes buffer, pH 7.1, containing 11 μg/ml leupeptin and 11 μg/ml antipain. The extracts were centrifuged for 10 min at 10000 ***g*** and the supernatants used for the determination of protein [18] and of enzymic activity. Sorbitol dehydrogenase activity was determined spectrophotometrically in a mixture containing 25 mM Hepes, pH 7.1, 0.6 mM NAD^+, 0.5 mM methylpyrrazole and different concentrations of sorbitol. Fructokinase was assayed as described [10] in extracts that had been heated at 71 °C for 5 min to destroy hexokinases and other interfering enzymes.

RESULTS

Suppression of the metabolic effects of sorbitol and mannitol by SDI 158

To confirm that the effects of sorbitol necessitated the conversion of this compound into fructose, and to explore the possibility that the same could be true for mannitol, we tested the effect of SDI 158, a powerful inhibitor of sorbitol dehydrogenase. Figure 1(B) shows that SDI 158 prevented, in a dose-dependent manner, the increase in the concentration of fructose 1-phosphate induced by 0.5 mM sorbitol or 10 mM mannitol in isolated hepatocytes in suspension, but that it barely affected the concentration of this phosphate ester in cells incubated in the presence of 0.5 mM fructose. Thus 0.5 mM sorbitol and 10 mM mannitol induced a stimulation of glucokinase, as estimated by detritiation of [2-^{3}H]glucose, similar to that induced by 0.5 mM fructose; SDI 158 antagonized their effects but not that of fructose (Figure 1A). These results suggest that SDI 158 was effective as an inhibitor of sorbitol dehydrogenase in isolated hepatocytes and confirm the notion that the effect of sorbitol and mannitol on detritiation was mediated by fructose 1-phosphate.

In agreement with results obtained by Agius and Peak [1,2], incubation of cultured hepatocytes with sorbitol or fructose increased the fraction of glucokinase that could be released on

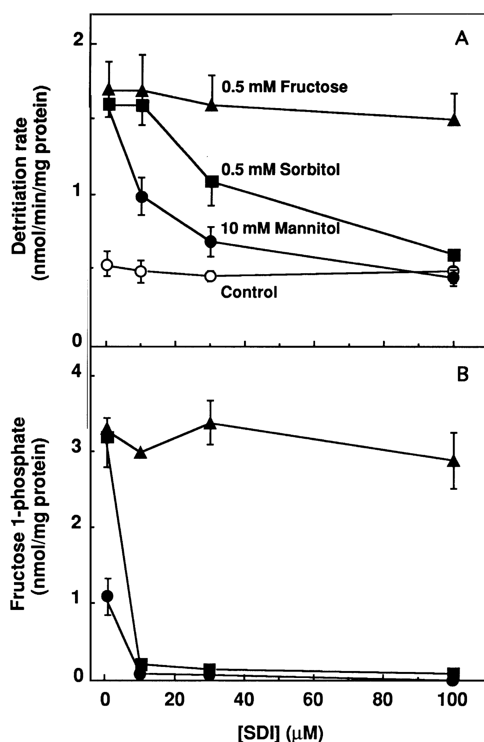

Figure 1 Effect of SDI 158 on the rate of detritiation of [2-^{3}H]glucose (A) and the concentration of fructose 1-phosphate (B) in suspensions of isolated hepatocytes incubated in the presence of 0.5 mM sorbitol, 0.5 mM fructose or 10 mM mannitol

The hepatocytes were preincubated for 30 min with 5 mM glucose and the indicated concentrations of SDI 158 before the addition of sorbitol, fructose or commercial mannitol (from Baker) together with a trace amount of [2-^{3}H]glucose. Values shown are means ± S.E.M. ($n = 3$).

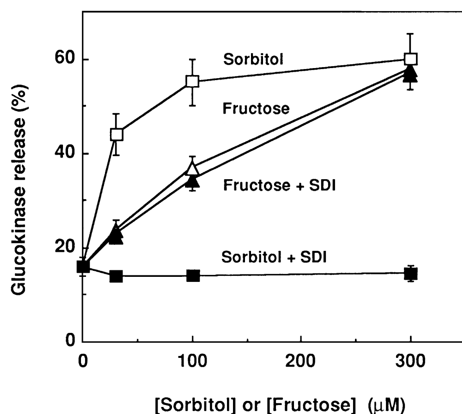

Figure 2 Effect of SDI 158 on the release of glucokinase induced by sorbitol or fructose in cultured hepatocytes

Hepatocyte monolayers were preincubated for 50 min with or without 1 mM SDI 158 before the addition of sorbitol or fructose at the indicated concentrations, and were permeabilized 30 min later. Values are means ± S.E.M. ($n = 4$).

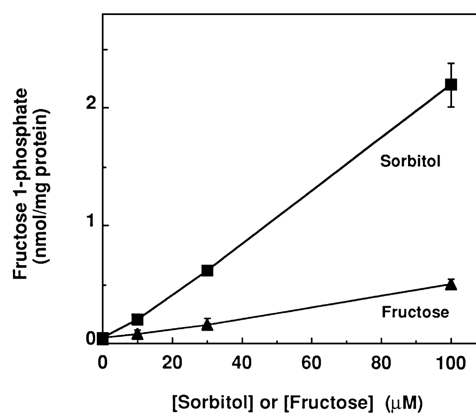

Figure 3 Effect of the concentration of fructose and sorbitol on the fructose 1-phosphate content of cultured hepatocytes

Sorbitol or fructose were added at the indicated concentrations and the incubations were arrested 10 min later. Values shown are means ± S.E.M. ($n = 3$).

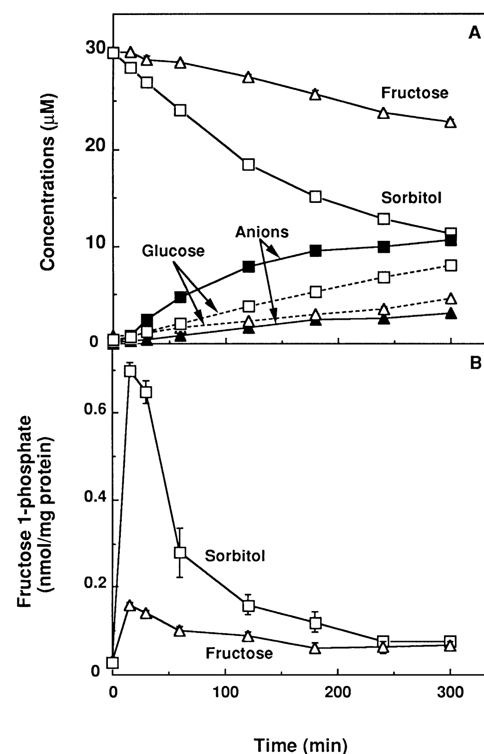

Figure 4 Time course of the disappearance of fructose and sorbitol (A) and of their conversion into fructose 1-phosphate (B), glucose and extracellular anions (A) in cultured hepatocytes

[^{14}C]-labelled fructose or sorbitol were added at a final concentration of 30 μM. Each dish contained 1.8 mg of protein in 1.2 ml of medium. (**A**) shows the conversion of radiolabelled substrates into extracellular glucose and anions. The radioactive anion concentration is expressed as C_6 equivalents. In (**B**), fructose 1-phosphate was assayed as in [8]. Values shown are means ± S.E.M. ($n = 3$). The calculated initial rates of disappearance were 67 ± 3 (sorbitol) and 17 ± 1 (fructose) pmol/min per mg of protein.

subsequent incubation with digitonin in the presence of 5 mM $MgCl_2$ (Figure 2); the polyol was approx. 5-fold more potent than the ketose. Remarkably, SDI 158 completely blocked the effect of sorbitol, whereas the effect of fructose was unchanged. These results show that the effect of sorbitol on glucokinase translocation, like that on glucokinase activity, necessitates its conversion into fructose and is therefore most probably mediated by fructose 1-phosphate.

Evidence for a more rapid utilization of sorbitol than of fructose in cultured cells

The fact that sorbitol was more potent than fructose in causing glucokinase translocation suggested that the polyol increased the concentration of fructose 1-phosphate more than did the ketose. Figures 3 and 4 show that this was indeed true. Both compounds increased the concentration of fructose 1-phosphate almost proportionally to their concentration in the medium, but sorbitol was approx. 5-fold more potent than fructose in this respect (Figure 3). As shown in Figure 4, this difference was related to a slower uptake of fructose than of sorbitol associated with a slower conversion into glucose and lactate. Calculations showed that, at a concentration of 30 μM, fructose was consumed at approx. one-quarter of the rate of sorbitol (17 compared with 67 pmol/min per mg of protein).

In contrast, almost no difference was observed in the rates of consumption of fructose and sorbitol (166 and 196 pmol/min per mg of protein respectively) and in the formation of fructose 1-phosphate and radioactive products in hepatocytes in suspension (Figure 5).

Effect of mannitol

We have shown in Figure 1 that a commercial preparation of mannitol gave rise to fructose 1-phosphate and stimulated glucokinase in isolated hepatocytes and that these effects were completely blocked by the addition of SDI 158, indicating that they were mediated by sorbitol dehydrogenase. Similar results were obtained with two other commercial preparations of mannitol. We also confirmed the observation made by Agius [2], that commercial mannitol stimulates the translocation of glucokinase (results not shown), being approx. one-hundredth as potent as sorbitol in this respect. That these effects were actually due to contaminating sorbitol was indicated by two pieces of evidence. (1) A ^{13}C-NMR spectrum of commercial mannitol (performed as in [19]) showed, in addition to the three mannitol peaks (at 63.5, 69.6 and 71.2 p.p.m.), six smaller peaks at values (62.8, 63.2, 70.0, 71.4, 71.5 and 73.3 p.p.m.) that exactly matched those of sorbitol. This allowed us to calculate that the mannitol used contained about 1 % sorbitol. (2) Addition of commercial mannitol at a concentration of 30 mM to a suspension of hepatocytes led to a transient increase in the concentration of fructose 1-phosphate that lasted only approx. 30 min and was comparable, both in its time-course and in its amplitude, with that elicited by the addition of 0.3 mM sorbitol (Figure 6). At the end of the experiment, approx. 75 % of the [^{14}C]sorbitol had been converted into extracellular anions, but there was no detectable conversion of [^{14}C]mannitol (results not shown).

Effects of other agents

We have also explored the effect of various compounds reported to increase (glucose at elevated concentrations, dihydroxyacetone, mannoheptulose) or to decrease (ethanol, glycerol, glucosamine) the translocation of glucokinase, or to affect the concentration of fructose 1-phosphate (D-glyceraldehyde), to see whether there was a correlation between these two parameters (Table 1). Glucose at elevated concentrations increased both the translocation of glucokinase and the concentration of fructose 1-phosphate; these effects were largely decreased by glucosamine and to a smaller extent by ethanol and glycerol, which also decreased the concentration of fructose 1,6-bisphosphate (resultes not shown), but not by SDI 158. Ethanol and glycerol also decreased the effects of fructose and of sorbitol on translocation and on the concentration of fructose 1-phosphate, whereas SDI 158 inhibited only the action of sorbitol, in confirmation of results shown above. D-Glyceraldehyde caused a remarkable effect on the concentration of fructose 1-phosphate and on translocation, whereas, in marked contrast with other

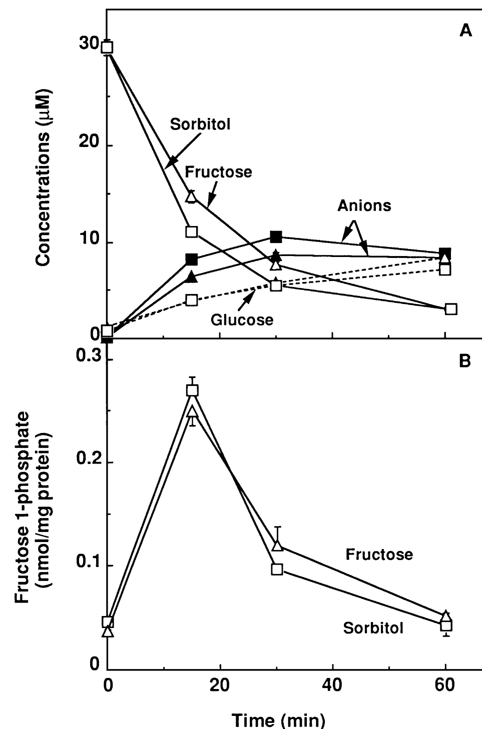

Figure 5 Time course of the disappearance of fructose and sorbitol (A) and of their conversion into fructose 1-phosphate (B), glucose and extracellular anions (A) in suspensions of hepatocytes

^{14}C-labelled fructose or sorbitol were added at a final concentration of 30 μM. The protein concentration of the suspension was 6.2 mg/ml. Results are means $\pm$ S.E.M. ($n = 3$). The presentation of data is the same as in Figure 4. The calculated rates of disappearance during the first 15 min were 196 $\pm$ 1 (sorbitol) and 166 $\pm$ 3 (fructose) pmol/min per mg of protein.

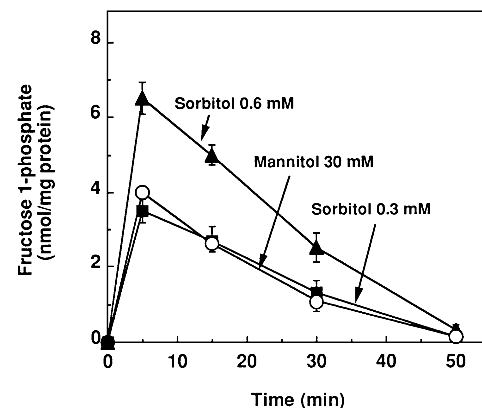

Figure 6 Time course of the changes in the concentration of fructose 1-phosphate after the addition of 0.3 or 0.6 mM sorbitol or 30 mM commercial mannitol to a suspension of hepatocytes

Perchloric acid extracts were prepared at the indicated times after the additions. Values shown are means $\pm$ S.E.M. ($n = 3$).

Table 1 Effect of different substrates and inhibitors on the translocation of glucokinase and the concentration of fructose 1-phosphate in cultured hepatocytes

Cells were incubated for 30 min in the presence of the indicated substrates and inhibitors.

Substrates	Inhibitor	Glucokinase release (% of total glucokinase)	[Fructose 1-phosphate] (nmol/mg of protein)
5 mM glucose		22 ± 0.7	0.07 ± 0.01
+ 0.5 mM D-glyceraldehyde		65 ± 1.2	5.00 ± 0.10
+ 20 mM mannoheptulose		65 ± 1.5	0.10 ± 0.01
+ 1 mM mannitol		53 ± 0.3	1.33 ± 0.10
+ 0.05 mM sorbitol		58 ± 2.6	1.40 ± 0.10
	0.1 mM SDI 158	21 ± 1.0	0.10
	10 mM ethanol	31 ± 3.5	1.00 ± 0.10
	5 mM glycerol	26 ± 2.0	0.88 ± 0.09
	40 mM glucosamine	36 ± 4.7	0.51 ± 0.05
+ 0.2 mM fructose		58 ± 2.6	1.15 ± 0.05
	0.1 mM SDI 158	56 ± 1.5	1.0
	10 mM ethanol	36 ± 1.2	0.97 ± 0.02
	5 mM glycerol	30 ± 2.3	0.88 ± 0.05
	40 mM glucosamine	40 ± 5.0	0.56 ± 0.07
15 mM glucose		47 ± 2.0	0.29 ± 0.03
	10 mM ethanol	35 ± 2.7	0.25 ± 0.05
	5 mM glycerol	24 ± 2.8	0.18 ± 0.01
	5 mM dihydroxyacetone	56 ± 1.5	0.52 ± 0.05
45 mM glucose		65 ± 3	0.80 ± 0.03
	0.1 mM SDI 158	63 ± 1.5	0.75 ± 0.08
	10 mM ethanol	49 ± 1.5	0.55 ± 0.07
	5 mM glycerol	29 ± 2.4	0.38 ± 0.05
	40 mM glucosamine	36 ± 1.7	0.12 ± 0.01

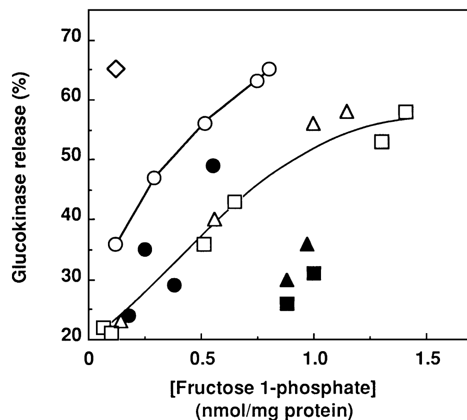

Figure 7 Plot of glucokinase release against the concentration of fructose 1-phosphate

Data shown are from Table 1 and Figures 2 and 4 (fructose and sorbitol at 30 μM). Symbols: ○, elevated concentration of glucose (15 or 45 mM) with or without dihydroxyacetone or glucosamine; ●, elevated concentrations of glucose with ethanol or glycerol; △, fructose at different concentrations, with or without SDI 158 or glucosamine; ▲, fructose plus ethanol or glycerol; □, sorbitol at different concentrations, with or without SDI 158 or glucosamine; ■, sorbitol plus ethanol or glycerol; ◇, mannoheptulose.

agents, mannoheptulose increased translocation without affecting the fructose 1-phosphate content.

The results are also presented in Figure 7 as a plot of translocation against the fructose 1-phosphate content. The same function seemed to apply for the effects of different concentrations of fructose and of sorbitol with or without glucosamine, the values observed with ethanol or glycerol falling clearly to the right of the curve. A different function, more to the left, was observed for the effect of elevated concentrations of glucose with or without glucosamine or dihydroxyacetone; here again the data obtained with glycerol and ethanol fell to the right of the curve. The point corresponding to mannoheptulose did not fit on any of the curves.

Activities of sorbitol dehydrogenase and of fructokinase in hepatocytes in culture and in suspension

Enzymic activities were measured in extracts of hepatocytes in suspensions incubated for 30 min or in culture for 18–24 h. The V_{max} of sorbitol dehydrogenase amounted to 6 nmol/min per mg of protein at pH 7.1 and 30 °C, and its K_m for sorbitol was 1.5 mM. For fructokinase the V_{max} was 24 nmol/min per mg of protein and the K_m was 0.35 mM for both cultures and fresh suspensions.

Effect of the regulatory protein and mannoheptulose on glucokinase activity

In the experiment shown in Figure 8 we investigated the effect of pairs of inhibitors (regulatory protein plus mannoheptulose or N-acetylglucosamine) on the activity of human β-cell glucokinase, which is as sensitive to the regulatory protein as its liver counterpart [13]. The results were plotted as the reciprocal of the velocity as a function of the concentration of one inhibitor. Lines converging on the x-axis are expected for inhibitors that can bind simultaneously to the enzyme, whereas parallel lines are expected for inhibitors that cannot bind simultaneously [20]. The results confirm the data obtained previously [21] for N-acetylglucosamine with the rat liver enzyme, indicating that this inhibitor can bind independently of the regulatory protein (Figure 8, lower panel). The plots obtained with mannoheptulose (Figure 8, upper panel) indicate that the binding of this sugar to glucokinase is competitive with that of the regulatory protein.

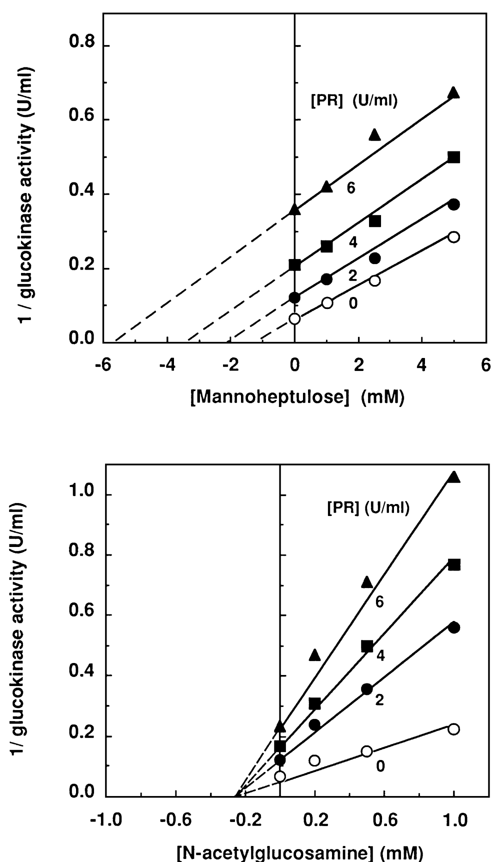

Figure 8 Inhibition of human glucokinase by the regulatory protein together with mannoheptulose or with *N*-acetylglucosamine

Human recombinant glucokinase was assayed in the presence of 5 mM glucose, 50 μM sorbitol 6-phosphate and the indicated concentrations of rat regulatory protein (PR) and of *N*-acetylglucosamine or mannoheptulose. The reciprocal of the activity was plotted against the concentration of mannoheptulose (upper panel) or of *N*-acetylglucosamine (lower panel).

DISCUSSION

Modulation of the interaction between glucokinase and its regulatory protein

The results presented in this paper strongly support the view that the interaction of glucokinase with its regulatory protein plays a pivotal role in the translocation phenomenon. This interaction can be prevented by compounds that bind either to the regulatory protein or to glucokinase.

The first class comprises mostly fructose 1-phosphate, fructose 1-phosphate analogues and P_i; the effect of these compounds is antagonized by fructose 6-phosphate. It has been shown that the concentration of fructose 1-phosphate required to achieve a half-maximal effect on glucokinase activity increases as a function of the concentrations of fructose 6-phosphate and regulatory protein [11]. The fact that approx. 60-fold higher concentrations of fructose 1-phosphate are needed to increase glucokinase translocation in intact hepatocytes (approx. 0.25 mM, assuming an intracellular water content of 2 μl/mg of protein; see Figure 7) than to stimulate purified glucokinase (4.4 μM) [11] is most probably due to the presence of much higher concentrations of regulatory protein in the liver (approx. 700 pmol/g of liver) [17] than in assays performed with purified proteins (typically 10 pmol/ml) [11].

The second class comprises glucose and the glucose analogue mannoheptulose, but not *N*-acetylglucosamine ([21], and this study). The reason for the different behaviours of the glucose analogues could be that they bind differently to the catalytic site [22]. Recent evidence indicates that part of binding site for the regulatory protein is in close vicinity to the catalytic site [13,23]; competition between mannoheptulose and the regulatory protein could therefore be the result of steric hindrance.

As discussed below, the action of the various compounds reported by Agius and co-workers to affect glucokinase translocation can be understood by one mechanism or a combination of them both.

The effect of fructose, sorbitol and tagatose

The positive effect of sorbitol and fructose on glucokinase translocation is easily explained by the well-known conversion of sorbitol into fructose and of fructose into fructose 1-phosphate. The complete inhibition of the sorbitol effect by SDI 158 shows that this effect is entirely explained by the conversion of sorbitol into fructose.

We have confirmed that fructose, at low concentration, causes a much lower increase in glucokinase translocation than does sorbitol in hepatocytes in culture. This difference can now be explained by our observation that the metabolism of both fructose and sorbitol was markedly slower in cultured cells than in hepatocytes in suspension, the difference being about 3-fold for sorbitol, but as much as 10-fold for fructose. Because the activities of fructokinase and of sorbitol dehydrogenase were unchanged and because the limiting step of fructose metabolism is at the level of its intracellular transport [24,25], the slow utilization of both compounds is best explained by a decrease in the rate of their transport, assuming that fructose and sorbitol are transported by different mechanisms. It is generally admitted that fructose transport in the liver is mediated by GLUT-2 [26], but Okuno and Gliemann [25] found kinetic evidence for two components with different affinities. The decrease of the transport of fructose is most probably not due to a decrease in the amount of the GLUT-2 protein, which apparently has a half-life of several days [27]. It could be caused by a change in the accessibility of the transporter as a result of internalization or by concentration of the transporter in membrane domains that are not in direct contact with the medium. The mechanism by which sorbitol is transported in liver cells has not been investigated until now. In astrocytes, sorbitol seems to be taken up by diffusion through a proteinaceous, channel-like structure [28].

Tagatose, which is converted into tagatose 1-phosphate by fructokinase [29], is known to favour glucokinase translocation with the same efficiency as fructose [2], whereas tagatose 1-phosphate is a ligand of the regulatory protein but with one-fifth of the affinity of fructose 1-phosphate [11]. This apparent contradiction can be resolved by the fact that tagatose 1-phosphate accumulates in the liver at higher concentrations than fructose 1-phosphate, particularly at low concentrations of the ketoses, presumably because of a slow utilization by aldolase [30].

The effect of mannitol

Our results indicate quite clearly that the effect of several commercial preparations of mannitol to stimulate the translocation of glucokinase is due to contaminating sorbitol. It has been claimed that sorbitol dehydrogenase acts with low affinity on mannitol [31]. Because the three mannitol samples that we tested were contaminated with sorbitol, it is most likely that the activity observed by Lindstad et al. [31] was due to substrate

contamination. This fits with the concept that dehydrogenases such as L- and D-lactate dehydrogenase and D-glycerate dehydrogenase display a high degree of stereoselectivity.

The effects of D-glyceraldehyde, dihydroxyacetone, ethanol, glycerol and glucosamine

The effect of D-glyceraldehyde in increasing glucokinase translocation is easily explained by its conversion into fructose 1-phosphate through aldolic condensation with dihydroxyacetone phosphate [10]. The physiological role of aldolase is, however, to split fructose 1-phosphate into D-glyceraldehyde and dihydroxyacetone phosphate; this reaction occurs more readily when the concentration of dihydroxyacetone phosphate is low [10]. This explains the positive effect of dihydroxyacetone and the negative effect of ethanol (which favours the conversion of dihydroxyacetone phosphate into glycerol 3-phosphate) on the concentration of fructose 1-phosphate [10] and most probably also of glycerol and of glucosamine. Glycerol is indeed converted into glycerol 3-phosphate, which causes a decrease in the concentration of fructose 2,6-bisphosphate owing to its effect on phosphofructokinase 2 and fructose-2,6-bisphosphatase [32,33]; the decrease in the concentration of fructose 2,6-bisphosphate facilitates the utilization of fructose 1,6-bisphosphate by fructose-1,6-bisphosphatase. Glucosamine is a powerful inhibitor of glucokinase [34], which decreases the concentration of hexose 6-phosphates [35] and of fructose 1,6-bisphosphate (this study). The near-absence of effect of *N*-acetylglucosamine compared with glucosamine is most probably due to its poor penetration in liver cells [35].

However, the fact that glycerol and ethanol induced a more pronounced decrease in translocation than expected from the decrease in fructose 1-phosphate concentration indicates that other metabolites could play a role. Glycerol 3-phosphate does not seem to be an effector of the regulatory protein of glucokinase [11]. The potential role of changes in the redox state induced by ethanol and glycerol has previously been discussed by Agius [2,36].

The effect of high concentrations of glucose and mannoheptulose

The results shown in Table 1 confirm that a high concentration of glucose induces a translocation of glucokinase in cultured cells and shows that this effect was associated with the formation of fructose 1-phosphate. However, these two effects were not affected by SDI 158 and therefore did not involve an intermediary formation of sorbitol. This is in agreement with the lack of activity of liver aldose reductase on glucose [7].

Beaufay and de Duve [37] reported that glucose-6-phosphatase, as assayed in intact microsomes, is not completely specific for glucose 6-phosphate, but also acts on several other phosphoric esters, including fructose 6-phosphate, the latter being hydrolysed at approx. one-fifth of the rate of glucose 6-phosphate. Re-investigations of the kinetic properties of microsomes that had been extensively washed to remove phosphoglucose isomerase confirmed this relative lack of specificity and permitted the calculation that, with concentrations of fructose 6-phosphate and glucose 6-phosphate in a physiological 1:3 ratio, the formation of fructose would be about 1% of that of glucose [38]. Considering the high K_m of glucose-6-phosphatase for all its substrates, it is expected that fructose formation would be favoured at high concentrations of hexose 6-phosphates, as were observed, in a dose-dependent fashion, in hepatocytes incubated in the presence of very high concentrations of glucose [33].

The effect of glucose is, however, not mediated only by the formation of fructose 1-phosphate, the action of which is partly neutralized by the increase in the concentration of fructose 6-phosphate, but can also result from the direct interaction of the hexose with glucokinase. This is best indicated by the fact that, for the same concentration of fructose 1-phosphate, more translocation is observed in the presence of an elevated concentration of glucose (15 or 45 mM) than in the presence of fructose or sorbitol at a low concentration of glucose (see Figure 7). The case is even apparently stronger for mannoheptulose, which caused a marked increase in translocation without a change in the fructose 1-phosphate concentration. Mannoheptulose has been reported to be a substrate for fructokinase with a K_m more than 100 times that of fructose [29]. We cannot therefore exclude the possibility that mannoheptulose acts through mannoheptulose 1-phosphate; it is currently not known whether this compound is an effector of the regulatory protein.

In conclusion, all these results indicate that, as suggested by Agius et al. [3], the regulatory protein acts as an anchor for glucokinase, to maintain it in an intracellular structure. The finding [39–41] that glucokinase and its regulatory protein are both in the nucleus in rat livers perfused with 5 mM glucose, but in the cytoplasm when the glucose concentration is elevated to 30 mM, suggests that this intracellular site is the nucleus. This intracellular localization is, however, quite surprising, unless glucokinase or its regulatory protein have some role to play in the control of gene expression.

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REFERENCES

1 Agius, L. and Peak, M. (1993) Biochem. J. **296**, 785–796
2 Agius, L. (1994) Biochem. J. **298**, 237–243
3 Agius, L., Peak, M. and Van Schaftingen, E. (1995) Biochem. J. **309**, 711–713
4 Van Schaftingen, E. (1989) Eur. J. Biochem. **179**, 179–184
5 Vandercammen, A. and Van Schaftingen, E. (1990) Eur. J. Biochem. **191**, 483–489
6 Van Schaftingen, E. and Vandercammen, A. (1989) Eur. J. Biochem. **179**, 173–177
7 Hers, H. G. (1960) Biochim. Biophys. Acta **37**, 120–126
8 Niculescu, L., Veiga-da-Cunha, M. and Van Schaftingen, E. (1996) Anal. Biochem. **235**, 243–244
9 Geisen, K., Utz, R., Grötsch, H., Lang, H. J. and Nimmesgern, H. (1994) Arzneim.-Forsch./Drug Res. **44**, 1032–1043
10 Davies, D. R., Detheux, M. and Van Schaftingen, E. (1990) Eur. J. Biochem. **192**, 283–289
11 Detheux, M., Vandercammen, A. and Van Schaftingen, E. (1991) Eur. J. Biochem. **200**, 553–561
12 Bontemps, F., Hue, L. and Hers, H. G. (1978) Biochem. J. **174**, 603–611
13 Veiga-da-Cunha, M., Xu, L. Z., Lee, Y. H., Marotta, D., Pilkis, S. J. and Van Schaftingen, E. (1996) Diabetologia, **39**, 1173–1179
14 Bartrons, R., Hue, L., Van Schaftingen, E. and Hers, H. G. (1983) Biochem. J. **214**, 829–837
15 Seglen, P. O. (1973) Exp. Cell Res. **82**, 391–398
16 Van Schaftingen, E., Opperdoes, F. R. and Hers, H. G. (1987) Eur. J. Biochem. **166**, 653–661
17 Vandercammen, A. and Van Schaftingen, E. (1993) Biochem. J. **294**, 551–556
18 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
19 Veiga-da-Cunha, M., Firme, P., San Romao, M. V. and Santos, H. (1992) Appl. Environ. Microbiol. **58**, 2271–2279
20 Segel, I. H. (1975) Enzyme Kinetics, Wiley and Sons, New York
21 Vandercammen, A. and Van Schaftingen, E. (1991) Eur. J. Biochem. **200**, 545–551
22 Xu, L. Z., Weber, I. T., Harrison, R. W., Gidh-Jain, M. and Pilkis, S. J. (1995) Biochemistry **34**, 6083–6092
23 Veiga-da-Cunha, M., Courtois, S., Michel, A., Gosselain, E. and Van Schaftingen, E. (1996) J. Biol. Chem. **271**, 6292–6297
24 Sestof, L. and Fleron, P. (1974) Biochim. Biophys. Acta **345**, 27–38
25 Okuno, Y. and Gliemann, J. (1986) Biochim. Biophys. Acta **862**, 329–334

26 Colville, C. A., Seatter, M. J., Jess, T. J., Gould, G. W. and Thomas, H. M. (1993) Biochem. J. **290**, 701–706
27 Weinstein, S. P., O'Boyle, E., Fisher, M. and Haber, R. S. (1994) Endocrinology (Baltimore) **135**, 649–654
28 Stahl, B., Wiesinger, H. and Hamprecht, B. (1989) J. Neurochem. **53**, 665–671
29 Raushel, F. M. and Cleland, W. W. (1971) Biochemistry **16**, 2169–2175
30 Van den Berghe, G. (1975) in Normal Development of Energy Metabolism (Hommes, F. A. and Van den Berg, C. J., eds.), pp. 211–228, Academic Press, London
31 Lindstad, R. I., Hermansen, L. F. and McKinley-McKee, J. S. (1992) Eur. J. Biochem. **210**, 641–647
32 Claus, T. H., Schlumpf, J. R., El-Maghrabi, M. R. and Pilkis, S. J. (1982) J. Biol. Chem. **257**, 7541–7548
33 Van Schaftingen, E., Bartrons, R. and Hers, H. G. (1984) Biochem. J. **222**, 511–518
34 Salas, J., Salas, M., Viñuela, E. and Sols, A. (1965) J. Biol. Chem. **240**, 1014–1018
35 Van Schaftingen, E. (1995) Biochem. J. **308**, 23–29
36 Agius, L. (1994) Biochem. J. **303**, 841–846
37 Beaufay, H. and de Duve, C. (1954) Bull. Soc. Chim. Biol. **36**, 1525–1537
38 Van Schaftingen, E., Hue, L. and Hers, H. G. (1980) Biochem. J. **192**, 263–271
39 Miwa, I., Mitsuyama, S., Toyoda, Y., Nonogaki, T., Aoki, S. and Okuda, J. (1990) Biochem. Int. **22**, 759–767
40 Toyoda, Y., Miwa, I., Kamiya, M., Ogiso, S., Nonogaki, T., Aoki, S. and Okuda, J. (1994) Biochem. Biophys. Res. Commun. **204**, 252–256
41 Toyoda, Y., Miwa, I., Satake, S., Anai, M. and Oka, Y. (1995) Biochem. Biophys. Res. Commun. **215**, 467–473

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