Intracellular binding of glucokinase in hepatocytes and translocation by glucose, fructose and insulin

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The release of glucokinase from digitonin-permeabilized hepatocytes shows different characteristics with respect to ionic strength and [$MgCl_2$] from the release of other cytoplasmic enzymes. Release of glucokinase is most rapid at low ionic strength (300 mM sucrose, 3 mM Hepes) and is inhibited by increasing concentration of KCl [concn. giving half-maximal inhibition (I_{50}) 25 mM] or Mg^{2+} (I_{50} 0.5 mM). Release of phosphoglucoisomerase, phosphoglucomutase and glucose-6-phosphate dehydrogenase is independent of ionic strength, but shows a small inhibition by $MgCl_2$ (20%, versus > 80% for glucokinase). Lactate dehydrogenase release increases with increasing ionic strength [concn. giving half-maximal activation (A_{50}) 10 mM KCl] or [$MgCl_2$]. The rate and extent of glucokinase release during permeabilization in 300 mM sucrose, 5 mM $MgCl_2$ or in medium with ionic composition resembling cytoplasm (150 mM K^+, 50 mM Cl^-, 1 mM Mg^{2+}) depends on the substrate concentrations with which the hepatocytes have been preincubated. In hepatocytes pre-cultured with 5 mM glucose the release of glucokinase was much slower than that of other cytoplasmic enzymes measured. However, preincubation with glucose (10–30 mM) or fructose (50 μM–1 mM) markedly increased glucokinase release. This suggests that, in cells maintained in 5 mM glucose, glucokinase is present predominantly in a bound state and this binding is dependent on the presence of Mg^{2+}. The enzyme can be released or translocated from its bound state by an increase in [glucose] (A_{50} 15 mM) or by fructose (A_{50} 50 μM). The effects of glucose and fructose were rapid ($t_{\frac{1}{2}}$ 5 min) and reversible, and were potentiated by insulin and counteracted by glucagon. They were inhibited by cyanide, but not by cytochalasin D, phalloidin or colchicine. Mannose had a glucose-like effect (A_{50} ~ 15 mM), whereas galactose, 3-*O*-methyl-D-glucose and 2-deoxyglucose were ineffective. When hepatocytes were incubated with [2-^{3}H,U-^{14}C]glucose, the incorporation of $^3H/^{14}C$ label into glycogen correlated with the extent of glucokinase release. Since 2-^{3}H is lost during conversion of glucose 6-phosphate into fructose 6-phosphate, substrate-induced translocation of glucokinase from a Mg^{2+}-dependent binding site to an alternative site might favour the partitioning of glucose 6-phosphate towards glycogen, as opposed to phosphoglucoisomerase.

INTRODUCTION

Certain cytoplasmic enzymes reversibly partition between distinct intracellular locations or binding sites, depending on the metabolic status of the tissue, and have been described as 'ambiquitous' because they can occur in two distinct intracellular locations [1]. The mammalian low-K_m hexokinases (isoenzymes I, II, III) are one of the most extensively characterized examples of ambiquitous enzymes (for reviews see [2–4]). Hexokinase I is expressed in brain, kidney and several other tissues. It partitions between soluble and membrane-bound (mitochondrial or microsomal) states in different metabolic conditions [2–6]. The amino acid sequence responsible for binding of this isoenzyme has recently been assigned by site-directed mutagenesis to the first 15 amino acid residues at the N-terminus [7]. Hexokinase IV, commonly known as glucokinase, is expressed only in hepatocytes and in insulin-secreting pancreatic cells. It differs from the other three isoenzymes in its lower molecular mass (52 kDa compared with 100 kDa), its low affinity for glucose and its sigmoidal kinetics [8]. Hepatic glucokinase is assumed to be present in the free state in the cytoplasm. The lack of a hydrophobic cluster of amino acids characteristic of the N-terminus of the type I isoenzyme [9,10] has been taken as confirmatory evidence for the 'free' cytoplasmic location of glucokinase.

We report in the present paper that, when rat hepatocytes are cultured with 5 mM glucose, glucokinase is present predominantly in a bound state during permeabilization of hepatocytes with digitonin at physiological [Mg^{2+}] and is probably bound in association with the microfilament cytoskeleton. However, glucokinase can be released rapidly from permeabilized cells if these are preincubated with high concentrations of glucose or with low concentrations of fructose. We infer from these findings that hepatic glucokinase is an ambiquitous enzyme and that glucose and fructose are major determinants of translocation of glucokinase within the hepatocyte.

MATERIALS AND METHODS

Materials

Digitonin was from BDH Chemicals (Poole, Dorset, U.K.). Collagenase (type IV), glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*), hormones and substrates were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Culture media were from Gibco BRL (Paisley, Scotland, U.K.). [U-^{14}C]Glucose and [2-^{3}H]glucose were from Amersham International (Amersham, Bucks., U.K.). [2-^{3}H]Glucose was purified immediately before use [11].

Hepatocyte isolation and monolayer culture

Parenchymal hepatocytes were isolated by collagenase perfusion of the liver [12] from male Wistar rats (body wt. 170–270 g) fed on standard rat chow *ad libitum*. The hepatocytes were suspended in Minimum Essential Medium containing 5% (v/v) neonatal-calf serum and inoculated in 24-well plates (area of well 1.88 cm^2) at a cell density of 6×10^4 cells/cm^2 and incubated at 37 °C in a

Abbreviations used: I_{50} and A_{50}, concns. giving half-maximal inhibition or activation respectively.

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humidified atmosphere equilibrated with 5% CO_2/air [12,13]. After cell attachment (approx. 4 h) the medium was replaced by 500 μl of serum-free Minimum Essential Medium containing 10 nM dexamethasone. Incubations with digitonin for determination of enzyme release were performed after between 6 h and 30 h culture in serum-free medium.

Incubation of hepatocyte monolayers with substrates, hormones or inhibitors

In experiments in Figures 1–3 and Table 1, enzyme release during permeabilization with digitonin was performed on hepatocytes that had been cultured (6–30 h) in the standard culture medium containing 5 mM glucose and 10 nM dexamethasone. In other experiments hepatocyte monolayers were preincubated in standard medium supplemented with additional substrates, hormones or inhibitors for the time intervals indicated before permeabilization with digitonin.

Composition of digitonin-permeabilization media

Except in experiments in Figure 1, the [digitonin] used throughout this study was 50 μg/ml. All digitonin-permeabilization media were buffered with 3 mM Hepes (pH 7.2 at 20 °C). Media of low ionic strength contained 300 mM sucrose (or, where indicated, 300 mM raffinose or 300 mM mannitol). The ionic media contained 150 mM KCl or 150 mM NaCl or 150 mM potassium isethionate. Media of varying [K^+] were prepared by mixing appropriate ratios of 150 mM KCl/3 mM Hepes and 300 mM sucrose/3 mM Hepes. $MgCl_2$ or $CaCl_2$ was added at the final concentrations indicated. A medium with composition simulating cytosol contained: 50 mM KCl, 100 mM potassium isethionate, 1 mM $MgCl_2$, 3 mM Hepes and 50 μg/ml digitonin, pH 7.2. All permeabilization conditions were in duplicate in each experiment.

Permeabilization of hepatocyte monolayers with digitonin

The culture medium was aspirated and the monolayers were washed once with 2 ml of 150 mM NaCl (at 20 °C). Digitonin-permeabilization medium was then added (400 μl per well) and the plates were incubated at 20 °C under unstirred conditions for the time intervals indicated (2–8 min). Before termination of the timed incubation, the plates were swirled gently (about 5 s) and the digitonin eluate was transferred to tubes contained dithiothreitol (final concn. 4 mM) for immediate enzyme analysis. No cell detachment occurred during incubation with digitonin. Glucokinase activity in the digitonin eluate was assayed first (within 5 min), followed by glucose-6-phosphate dehydrogenase, phosphoglucomutase, phosphoglucoisomerase and lactate dehydrogenase (depending on the experiment). The plates containing the permeabilized cell matrix were stored at −30 °C for about 20–60 min before extraction and determination of enzyme activity.

Extraction of the cell matrix

In initial experiments similar to those in Figures 1 and 2, three extraction procedures for the cell matrix (after digitonin permeabilization) were compared: (1) with medium of the same composition as the digitonin-permeabilization medium (but without digitonin); (2) each matrix was extracted in 100 mM KCl, 50 mM Hepes, 7.5 mM $MgCl_2$, 4 mM dithiotreitol, pH 7.2 (Buffer A); (3) each matrix was extracted in Buffer A supplemented with 0.05% (w/v) Triton-X 100 (Buffer B). All were sonicated for 4 s at maximum intensity with a Mistral MSE sonicator with a 3 mm-diameter probe (at 0 °C). Detergent-free extracts (methods 1 and 2) were centrifuged at 15000 *g* for 20 min and activities were assayed on the supernatant, whereas extracts in Buffer B (method 3) were assayed in the whole extract. For lactate dehydrogenase, total activity recovered in the digitonin eluate + cell matrix was lower in the low-salt permeabilization (300 mM sucrose) compared with 150 mM KCl by 40–50% using method (1) and by 10–12% using method (2) or (3). Total glucokinase recovery was either similar by all three methods, or it was higher by 10–20% in the low-salt permeabilizations by the three methods. Buffer A was used for the extraction of the cell matrix in experiments in Figures 1 and 2 and Table 1, and buffer B in the rest of this study.

Determination of enzyme activities

Glucokinase activity was assayed by coupling to NAD^+-linked glucose-6-phosphate dehydrogenase [14] as described in [15], except that dithiothreitol replaced dithioerythritol and the assay was performed routinely only with 100 mM glucose (without subtraction of activity with 0.5 mM glucose). The contribution of low-K_m hexokinases was determined by fractionation of hepatocyte extracts by f.p.l.c. on a Mono-Q Sepharose column eluted with a linear NaCl gradient (0–0.7 M). There were three peaks of low-K_m hexokinase activity, with a total activity amounting to 5% of the glucokinase activity. This low activity of low-K_m hexokinase agrees with previous findings in rat hepatocytes [16]. Phosphoglucomutase and phosphoglucoisomerase were assayed in 100 mM KCl, 50 mM Hepes, 5 mM $MgCl_2$, 0.25 mM NAD^+, 4 mM dithiothreitol, 2 units/ml glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*) and either 2 mM glucose 1-phosphate or 2 mM fructose 6-phosphate respectively. Fructokinase was assayed as in [17] and other enzymes were assayed as in [18].

Expression of enzyme activities

Enzyme activities in the digitonin eluate are expressed as a percentage of the total activity in the digitonin eluate plus cell matrix for each permeabilization condition (percentage enzyme release). For glucokinase, glucose-6-phosphate dehydrogenase and phosphoglucoisomerase the total activity recovered in the digitonin eluate + matrix for different permeabilization media within an experiment generally varied by less than 12%, and these differences were not statistically significant. Thus differences in enzyme activity in the digitonin eluate with different permeabilization or preincubation conditions were associated with inverse changes in activity in the cell matrix. For lactate dehydrogenase and phosphoglucomutase the total activities recovered in permeabilization at low ionic strength were significantly lower ($P < 0.01$) than at high salt (by 8–15% and 27% respectively). Throughout this study the total activity of glucokinase was between 7 and 13 m-units/mg of cell protein. The decline in total glucokinase activity in hepatocytes cultured in standard medium containing 10 nM-dexamethasone without insulin was about 15% in 24 h. These values agree with previous findings [19].

Determination of G-actin

G-actin in digitonin eluates and cell matrix extracts was determined by a DNAase-I inhibition assay based on ref. [20], except that the reaction was monitored from DNA fluorescence in the presence of ethidium bromide by using a centrifugal analyser. The assay involved mixing 50 μl of DNAase-I solution

(0.6 μg/ml in 50 mM Tris/0.01 mM phenylmethanesulphonyl fluoride/0.25 mM $CaCl_2$, pH 7.2) and sample (3–8 μl of digitonin eluate or cell matrix extract) for 10 s, followed by addition of 75 μl of DNA solution (120 μg/ml calf thymus DNA, 0.1 M Tris, 4 mM $MgSO_4$, 1 mM $CaCl_2$, 3 μM ethidium bromide). The fluorescence (excitation 540 nm, emission 590 nm) was monitored at 30 s intervals for 8.5 min. There was a lag phase of 30–60s, after which there was a linear decrease in fluorescence. Rates of DNAase activity were determined by a linear search. Higher concentrations of DNAase-I resulted in non-linear rates of decrease in fluorescence. The volume of sample (3–8 μl) was adjusted to give between 30% and 70% inhibition of DNAase. In some experiments extracts were pre-diluted (2–4 times). Each sample was assayed at either 2 or 3 assay dilutions. The G-actin content was determined from the percentage inhibition of the DNAase-I (assuming that 1.35 μg of G-actin inhibits 1 μg of DNAase-1 [20]) and was expressed as μg of G-actin per ml of extract or mg of total cell protein. The G-actin and F-actin contents were also determined on hepatocyte monolayers that were not permeabilized with digitonin. The monolayers were washed and sonicated in 150 mM NaCl/5 mM KH_2PO_4/2 mM $MgCl_2$/0.5% (w/v) Triton X-100/0.2 mM dithiothreitol/0.2 mM ATP, and G-actin was determined as described above. The samples were then diluted with an equal volume of 1.5 M guanidinium chloride/1 M sodium acetate/50 mM Tris/1 mM $CaCl_2$/1 mM ATP and incubated at 0 °C for 20 min (for conversion of F-actin into G-actin), and G-actin was determined after appropriate dilution of the samples.

Incorporation of [2-^{3}H,U-^{14}C]glucose into glycogen

Hepatocyte monolayers were incubated in Minimum Essential Medium containing [2-^{3}H,U-^{14}C]glucose (9 μCi/ml ^{3}H, 3 μCi/ml ^{14}C) and supplemented with glucose, insulin or fructose as indicated. The incubations were terminated after 3 h and the incorporation of radioactivity into glycogen was determined by ethanol precipitation of the glycogen [12]. The conversion of glucose into glycogen was determined from the incorporation of ^{14}C label into glycogen, and is expressed as nmol of glucose incorporated/3 h per mg of cell protein. During incubation with increasing [glucose] the incorporation of [^{14}C]glucose into glycogen correlates with glycogen deposition determined enzymically [13]. The ^{3}H/^{14}C radioactivity incorporated into glycogen is expressed as a percentage of the ^{3}H/^{14}C ratio of the glucose in the medium [13]. To establish whether ^{3}H label incorporated into glycogen was in the 2-position of glucosyl residues, the glycogen was first hydrolysed with amyloglucosidase (5 units/ml in 0.1 M sodium acetate, pH 4.8, for 90 min) and then incubated with hexokinase and phosphoglucoisomerase (2 units/ml) in 200 mM Tris/10 mM $MgCl_2$/0.6 mM ATP/0.05 mM glucose (carrier), pH 7.5, for 60 min at 37 °C. The samples were then freeze-dried to remove ^3H_2O and the radioactivity was determined. ^{3}H radioactivity was below detectable limits, confirming that it was present in the 2-position. Cell protein was determined by an automated Lowry method [21].

RESULTS

Effects of [digitonin] on release of glucokinase and lactate dehydrogenase

Digitonin permeabilizes cell membranes by binding to membrane cholesterol, and at low concentrations causes selective permeabilization of the plasma membrane with little damage to intracellular membranes [22]. Figure 1 shows the effects of [digitonin] on the release of glucokinase and lactate dehydrogenase in media of either low ionic strength (300 mM sucrose) or high ionic strength (75 mM or 150 mM KCl or 150 mM NaCl) each buffered with 3 mM Hepes. At low [digitonin] (0.05–0.1 mg/ml), the release of glucokinase was greater in 300 mM sucrose than in 150 mM KCl or 150 mM NaCl, whereas release of lactate dehydrogenase was higher in the ionic media (Figure 1). Enzyme release was unchanged when KCl was replaced by potassium isethionate or when sucrose was replaced by raffinose or mannitol (results not shown). Ionic strength, rather than ion composition, therefore appears to be the main determinant for diminished release of glucokinase and enhanced release of lactate dehydrogenase in these ionic media. Increasing [digitonin] up to 1 mg/ml resulted in total release of both glucokinase and lactate dehydrogenase in 150 mM KCl. However, release of lactate dehydrogenase was incomplete in sucrose even at 1 mg/ml. Because total release of glucokinase in 300 mM sucrose occurred at 0.05–0.1 mg/ml digitonin, these concentrations presumably cause complete permeabilization of the plasma membrane. However, at these concentrations release of lactate dehydrogenase was incomplete (70–75% at 0.05 mg/ml and 85–87% at 0.1 mg/ml) in the ionic media. This residual lactate dehydrogenase that required high [digitonin] (0.5–1 mg/ml) for total release may represent membrane-bound or intra-organelle isoenzymes as reported previously [23,24]. There was small but significant release of glutamate dehydrogenase at 0.1 mg/ml digitonin both in 300 mM sucrose (6.9 ± 0.8%, means ± S.E.M., $n = 3$) and in 150 mM KCl (3.7 ± 0.5%) and a progressive increase in release of glutamate dehydrogenase at increasing [digitonin] (> 0.1 mg/ml), indicating mitochondrial damage at concentrations > 0.1 mg/ml. In the rest of this study we used a [digitonin] of 0.05 mg/ml to determine the effects of medium composition on the release of cytoplasmic enzymes.

Effects of [KCl] and [Mg^{2+}]

The [KCl] causing half-maximum inhibition (I_{50}) of glucokinase release during permeabilization with digitonin (0.05 mg/ml) was 25 mM and that causing half-maximum increase (A_{50}) in lactate dehydrogenase release was 10 mM (Figure 2a). Total recovery of glucokinase at varying KCl concentration (in the digitonin eluate + cell matrix) was constant, but recovery of lactate dehydrogenase was lower (by 15%) during permeabilization at low salt. Mg^{2+} inhibited glucokinase release in 300 mM sucrose and in 150 mM KCl, but it increased lactate dehydrogenase release in sucrose, whereas Ca^{2+} inhibited glucokinase release in sucrose, but not in KCl (Table 1). The [$MgCl_2$] causing half-maximum inhibition of glucokinase release was 0.5 mM and that causing half-maximum increase in lactate dehydrogenase release was 1–2 mM (Figure 2b).

Release of phosphoglucomutase, phosphoglucoisomerase and glucose-6-phosphate dehydrogenase

Because glucokinase and lactate dehydrogenase showed opposite release patterns in responses to ionic strength and Mg^{2+} (Figures 1 and 2) we compared the rates of release of these enzymes with phosphoglucomutase, phosphoglucoisomerase and glucose-6-phosphate dehydrogenase (Figure 3). These enzymes were selected because they catalyse reactions using the product of the glucokinase reaction and are therefore metabolically proximal to glucokinase. We used three permeabilization media, (i) 300 mM sucrose, (ii) 300 mM sucrose + 5 mM $MgCl_2$ and (iii) 150 mM KCl, that cause either maximum or minimum release of glucokinase and lactate dehydrogenase (see Figure 2). The recovery of total activity (digitonin eluate + cell matrix) was not significantly different in the three media for glucokinase (i, 9.6 ± 0.4; ii,

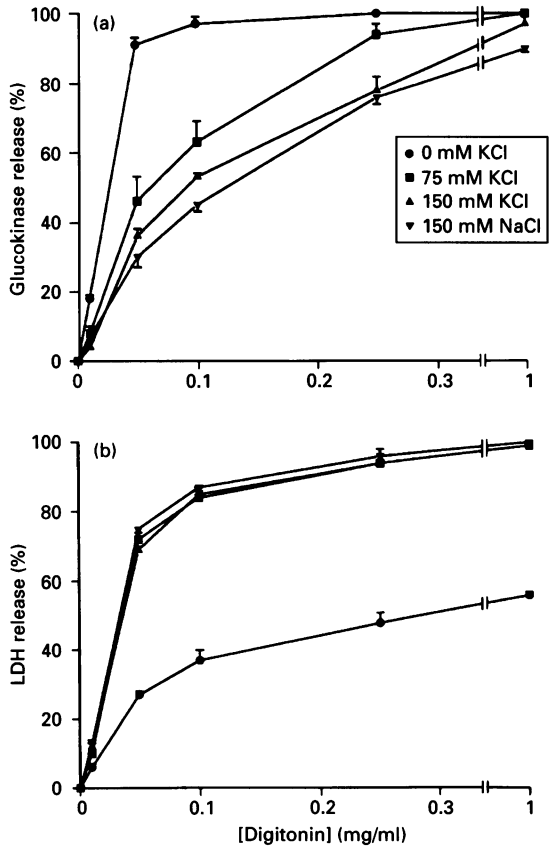

Figure 1 Effects of [digitonin] on the release of glucokinase and lactate dehydrogenase

Hepatocyte monolayers were permeabilized for 6 min with the digitonin concentrations indicated in 300 mM sucrose (●), 75 mM KCl/150 mM sucrose (■), 150 mM KCl (▲) or 150 mM NaCl (▼), each containing 3 mM-Hepes. Glucokinase (**a**) and lactate dehydrogenase (LDH) (**b**) activities were determined in the digitonin eluate and residual cell matrix, and the activity in the digitonin eluate is expressed as a percentage of total activity. Values are means ± S.E.M. for 3 experiments.

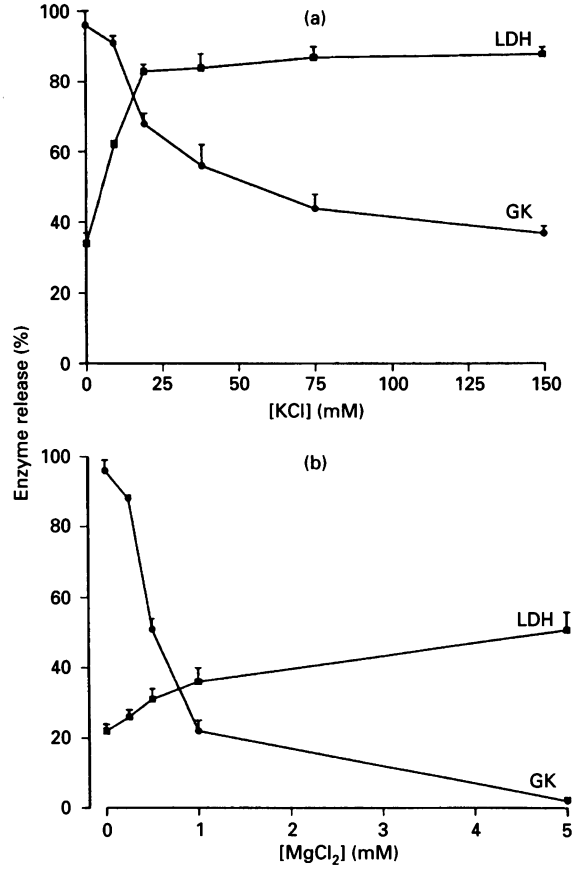

Figure 2 Effects of [KCl] and [$MgCl_2$] on glucokinase and lactate dehydrogenase release

The procedure was as described in Figure 1, except that [digitonin] was 0.05 mg/ml. In (**a**) the KCl concentration was varied by mixing appropriate volumes of 150 mM KCl and 300 mM sucrose (+3 mM Hepes). In (**b**) the effect of [$MgCl_2$] was determined in the presence of 300 mM sucrose and 3 mM Hepes. Activities of glucokinase (GK; ●) and lactate dehydrogenase (LDH; ■) in the digitonin eluate are expressed as a percentage of total activity. Values are means ± S.E.M. for 4 experiments.

9.4±0.1; iii, 8.8±0.4; means±S.E.M., $n = 12$; m-units/mg of protein), glucose-6-phosphate dehydrogenase (i, 5.2±0.3; ii, 5.8±0.3; iii, 5.7±0.3 m-units/mg of protein) and phosphoglucoisomerase (i, 299±10; ii, 310±8; iii, 315±10 m-units/mg of protein). However, there was lower ($P < 0.05$) recovery of activity in 300 mM sucrose for both phosphoglucomutase (i, 339±11; ii, 464±8; iii, 389±11) and lactate dehydrogenase (i, 2226±29; ii, 2413±32; iii, 2442±45, m-units/mg). Rates of release of phosphoglucomutase, phosphoglucoisomerase and glucose-6-phosphate dehydrogenase were similar in 150 mM KCl and in 300 mM sucrose, but slightly lower in the additional presence of 5 mM $MgCl_2$ (Figure 3). Release of lactate dehydrogenase was highest in 150 mM KCl and lowest in 300 mM sucrose without Mg^{2+}. The rate of glucokinase release was higher in 300 mM sucrose than in 150 mM KCl, and there was no detectable glucokinase release in the presence of 5 mM $MgCl_2$. Throughout this study, release of glucokinase in the presence of 5 mM Mg^{2+} varied between 0 and 25%, depending on the age of the culture (see below).

G-actin release during permeabilization with digitonin

Hepatocytes permeabilized with 0.05 mg/ml digitonin stain with Trypan Blue, but do not show obvious changes in cell morphology as viewed by light microscopy. Lack of morphological changes was reported during permeabilization of hepatocyte monolayers with 0.8 mg/ml digitonin at low salt for 5 min [25]. We determined the monomeric actin (G-actin) content of digitonin eluates to examine whether the composition of the permeabilization medium affects depolymerization of microfilaments. The G-actin content in the digitonin eluate and also the total G-actin in the eluate plus cell matrix were higher during permeabilization with 300 mM sucrose or 300 mM mannitol without bivalent cations as compared with media supplemented with $MgCl_2$ or $CaCl_2$ or in 150 mM KCl (Table 1). The [$MgCl_2$] causing half-maximum inhibition of G-actin release in mannitol or sucrose was 0.4–0.5 mM. During 2–8 min permeabilization in 300 mM sucrose plus 0.05 mg/ml digitonin, the G-actin content of the digitonin eluate increased (2 min, 5.9±0.8; 4 min, 9.9±1.1; 6 min, 11.6±1.9; 8 min, 13.6±1.9; means±S.E.M., $n = 3$; μg/mg of protein), whereas that in the cell matrix decreased (2 min, 6.6±1.9; 4 min, 5.3±1.3; 6 min, 3.5±0.5; 8 min, 4.4±1.2) with a significant increase ($P < 0.05$) in total G-actin between 2 and 8 min (2 min, 12.5±1.2; 4 min, 15.2±0.4; 6 min, 15.0±0.6; 8 min 18.0±0.6 μg of G-actin/mg of protein). The G- and F-actin contents determined in separate experiments on

Table 1 Effects of Mg^{2+} and Ca^{2+} on release of glucokinase, lactate dehydrogenase and G-actin

Hepatocyte monolayers were permeabilized for 4–6 min with digitonin (0.05 mg/ml) either in 300 mM sucrose in 150 mM KCl and supplemented with the concentrations of $MgCl_2$ or $CaCl_2$ indicated and containing 3 mM Hepes. Glucokinase, lactate dehydrogenase and G-actin were determined in the digitonin eluate and cell matrix, and activity in the digitonin eluate is expressed as a percentage of total activity. The total enzyme activity recovered in incubations with KCl and/or bivalent cations is expressed as a percentage of total activity in incubations with 300 mM sucrose without bivalent cations. Data for G-actin are from separate experiments from the enzyme data. In some of these experiments mannitol was used in place of sucrose. G-actin is expressed as μg/mg of cell protein. Total G-actin represents the sum of that released and that recovered from the cell extract. Values are means ± S.E.M. for the numbers of experiments indicated: $^*P < 0.05$, $^{**}P < 0.01$ relative to values with 300 mM sucrose. ND, not determined.

	Lactate dehydrogenase		Glucokinase		G-actin (μg/mg of protein)	
Medium	(% release)	(total)	(% release)	(total)	(release)	(total)
300 mM sucrose (10)	20±1	100	90±4	100	12.1±1.1 (10)	17.0±1.1
+0.5 mM $MgCl_2$ (8)	25±2*	101±2	42±4**	90±5	6.7±0.7** (9)	13.2±0.7*
+5 mM $MgCl_2$ (8)	47±4**	106±4	7±2**	91±6	4.1±1.0** (3)	9.4±1.4**
+0.5 mM $CaCl_2$ (3)	19±4	106±6	33±16**	96±2	7.3±0.8* (3)	ND
150 mM KCl (6)	69±4**	115±2	29±5	106±5**	6.9±0.6** (7)	11.7±0.6**
+0.5 mM $MgCl_2$ (3)	67±6**	117±2	21±6**	105±3	7.3±1.3* (3)	ND
+5 mM $MgCl_2$ (3)	34±6**	108±11	10±3**	110±6	ND	
+0.5 mM $CaCl_2$ (3)	48±16**	115±4	31±17**	110±7	7.9±1.4 (3)	ND

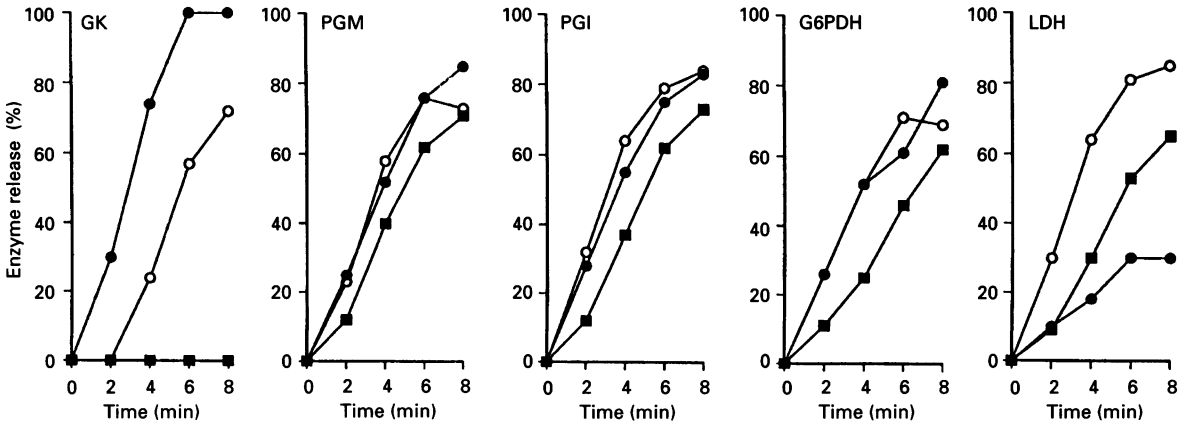

Figure 3 Time course of enzyme-activity release during permeabilization with digitonin

Hepatocyte monolayers were permeabilized for the time intervals indicated with 0.05 mg/ml digitonin in 300 mM sucrose (●), 300 mM sucrose/5 mM $MgCl_2$ (■) or 150 mM KCl (○). Activities of glucokinase (GK), phosphoglucomutase (PGM), phosphoglucoisomerase (PGI), glucose-6-phosphate dehydrogenase (G6PDH) and lactate dehydrogenase (LDH) released are expressed as a percentage of total activity. Values are means of duplicate determinations; similar results were obtained in a further experiment.

hepatocytes not treated with digitonin were 12.0±0.8 and 36.1±1.8 μg/mg of protein respectively.

Effects of cytochalasin D and phalloidin

To investigate whether the microfilament cytoskeleton is involved in binding of glucokinase to the cell matrix, we determined the effects of pre-treatment of hepatocytes with cytochalasin D and phalloidin which cause rapid changes in hepatocyte morphology by redistribution of microfilaments [26]. Cytochalasin D caps the barbed ends of microfilaments and inhibits subunit association and dissociation and filament growth [27], whereas phalloidin binds to actin filaments more tightly than to actin monomers and shifts equilibrium between filaments and monomers towards filaments [27]. When hepatocytes were incubated without or with 20 μM cytochalasin D for 2 h and then permeabilized for 6 min with 0.05 mg/ml digitonin at varying [KCl] (as in Figure 2), the cytochalasin-D-treated cells showed increased release of glucokinase in 150 mM KCl (treated, 51±2%, versus control, 39±1%; $P < 0.05$), but not at lower concentrations ($P > 0.05$) of KCl (75 mM KCl, 53±1 versus 47±2%; 38 mM KCl, 64±2 versus 63±2%; 19 mM KCl, 75±3 versus 70±1%; 10 mM KCl, 86±2 versus 91±2%; 0 KCl, 97±2 versus 100%; means ± S.E.M., $n = 4$). The time course of exposure to 20 μM cytochalasin D (10, 30, 60 and 240 min) showed a maximum effect on glucokinase release (in 150 mM KCl plus 0.05 mg/ml digitonin) after 60 min exposure to cytochalasin D (control, 47%; 10 min, 48%; 30 min, 55%; 60 min, 60%; 4 h, 58%). Increasing [digitonin] from 0.05 to 0.15 mg/ml did not affect the increment in glucokinase release by cytochalasin D (results not shown).

We determined the effects of pre-treatment with cytochalasin D or phalloidin on glucokinase release in ionic conditions that favour either microfilament stabilization (150 mM KCl) or depolymerization (0–10 mM or 1 M KCl) [28]. G-actin release was higher in 0–10 mM KCl than in 150 mM KCl (Table 2). However, G-actin could not be determined in the presence of 1 M KCl because of inhibition (50%) of DNAase-I by 1 M KCl.

Table 2 Effects of pre-treatment with cytochalasin D and phalloidin on enzyme release during permeabilization with digitonin

Hepatocyte monolayers were incubated for 4 h without or with 20 μM cytochalasin-D (Cyt-D) or 5 μM phalloidin (Phall). They were then permeabilized for 6 min with digitonin (0.05 mg/ml) in 0 mM KCl/300 mM sucrose, 10 mM KCl/300 mM sucrose, or 150 mM KCl or 1 M KCl each containing 3 mM Hepes. Enzyme activities released with digitonin are expressed as a percentage of total activity, and G-actin released in the digitonin eluate is expressed as μg/mg of total cell protein. Values are means ± S.E.M. for 3 experiments: $^*P < 0.05$, $^{**}P < 0.01$. ND, not determined; ud, undetectable.

		Permeabilization medium			
Protein release	Culture	0 mM	10 mM KCl	150 mM KCl	1 M KCl
Glucokinase	Control	88 ± 2	78 ± 1	25 ± 1	70 ± 2
	Cyt-D	83 ± 2	75 ± 2	33 ± 2*	62 ± 2*
	Phall	82 ± 4	75 ± 2	49 ± 2*	75 ± 2**
Lactate dehydrogenase	Control	14 ± 1	39 ± 1	53 ± 4	71 ± 4
	Cyt-D	17 ± 2	42 ± 2	48 ± 5	69 ± 4
	Phall	18 ± 2	35 ± 2	62 ± 4	73 ± 3
G-actin	Control	9.6 ± 0.2	6.7 ± 0.4	3.5 ± 0.5	ND
	Cyt-D	10.8 ± 0.4**	8.8 ± 0.3**	4.7 ± 0.4*	ND
	Phall	ud	ud	ud	ND

G-actin release was below detectable limits in cells pre-treated with phalloidin, but was significantly increased in cells pre-treated with cytochalasin D. Lactate dehydrogenase release increased with increasing salt, whereas glucokinase release was biphasic and was lower at 150 mM KCl than at either 0–10 mM or 1 M KCl. Both these conditions favour depolymerization of microfilaments [28]. Cytochalasin D and phalloidin increased glucokinase release during permeabilization in 150 mM KCl, but not at low salt, suggesting that increased glucokinase release by compounds that interfere with microfilament structure is only observed under permeabilization conditions that favour microfilament stabilization. Similar effects of cytochalasin D and phalloidin on glucokinase release were observed during permeabilization in 300 mM sucrose and 5 mM $MgCl_2$ (control, 5.0 ± 1.6; cytochalasin, 8.4 ± 0.7; phalloidin, 11.7 ± 2.0 % release; means ± S.E.M., $n = 4$) or 2 mM $MgCl_2$ (control, 10.5 ± 1.4; cytochalasin, 19.5 ± 1.3; phalloidin, 18.1 ± 2.5 %). Colchicine (20 μM), unlike cytochalasin D or phalloidin, did not affect glucokinase release, suggesting that changes in the conformation of the microfilaments, but not the microtubules, affect glucokinase binding.

Effects of incubation of hepatocytes with glucose and fructose on glucokinase release

In the above experiments, hepatocytes were cultured in medium containing 5 mM glucose before permeabilization with digitonin. There was a much higher rate of glucokinase release when hepatocytes were preincubated for 20 min with a high [glucose] (10–35 mM) or with fructose and subsequently washed and permeabilized in 5 mM $MgCl_2$/300 mM sucrose/3 mM Hepes/0.05 mg/ml digitonin (Figure 4). Increasing [glucose] (10–35 mM) increased glucokinase release from 1 % to 57 %, and mannose (5–30 mM) in the presence of 5 mM glucose had a similar effect (Figure 4), whereas galactose, 2-deoxyglucose and 3-*O*-methylglucose (10–30 mM, in the presence of 5 mM glucose) had no effect ($n = 3$–5; results not shown). Fructose increased glucokinase release at much lower concentrations (0.05–1 mM) than either glucose or mannose, with half-maximum increase at 50 ± 6 μM (means ± S.E.M., $n = 10$). At higher concentrations of fructose (10–30 mM), the effect was biphasic and release of glucokinase was lower at 30 mM compared with 1 mM fructose (40 ± 3 % versus 66 ± 4 %; means ± S.E.M., $n = 4$; $P < 0.01$).

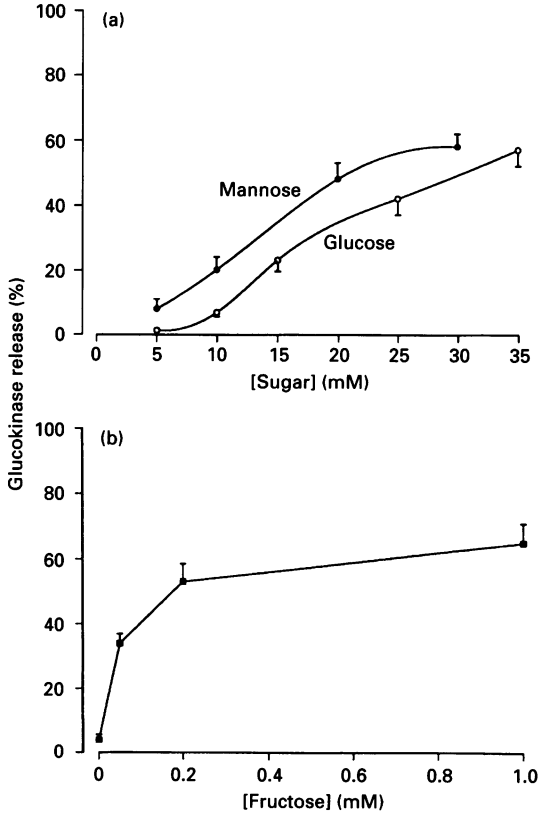

Figure 4 Effects of hepatocyte preincubation with glucose, mannose or fructose on glucokinase release during permeabilization of heptocytes with digitonin

Hepatocytes were incubated for 20 min with the concentrations of glucose and mannose (**a**) or fructose (**b**) indicated. The mannose and fructose incubations were in the presence of 5 mM glucose. The cells were then washed and permeabilized for 6 min with digitonin (0.05 mg/ml) in 300 mM sucrose/5 mM $MgCl_2$/3 mM Hepes. Glucokinase activity in the digitonin eluate is expressed as a percentage of total activity. Values are means ± S.E.M. for 3 (**a**) or 10 (**b**) experiments.

This decline in glucokinase release at 30 mM fructose may be due to depletion of ATP [29]. Total glucokinase recovery in the digitonin eluate plus cell matrix was not affected by glucose,

mannose or fructose at any of the substrate concentrations tested.

Effects of permeabilization medium on the fructose effect

We determined whether the concentration of fructose that causes half-maximum effect on glucokinase release during subsequent digitonin permeabilization depends on the composition of the digitonin-permeabilization medium. Hepatocytes preincubated for 20 min with fructose (0.05–1 mM) were permeabilized with digitonin (0.05 mg/ml) either in 300 mM sucrose with 5 mM $MgCl_2$ or 1 mM $MgCl_2$, or in 150 mM KCl. Although the basal release of glucokinase in untreated cells was higher in 150 mM KCl or with 1 mM $MgCl_2$ compared with 5 mM $MgCl_2$, the incremental increase in release caused by pre-treatment with fructose and the concentration of substrate that caused half-maximum release were similar in the different media (results not shown).

Time course, stability and reversibility of the fructose effect

Figure 5 shows the effects of incubation time with 20 mM glucose or 2 mM fructose on glucokinase translocation. Half-maximum effect was observed after about 5 min. In experiments ($n = 2$) comparing the effects of 0.2 mM and 1 mM fructose after 20 min, 40 min or 3 h, the effect of fructose was stable for at least 3 h. The reversibility of the effect of fructose was determined in experiments where cells were preincubated with 50 μM or 200 μM fructose for 60 min and then incubated in fructose-free medium for 10–40 min. There was little or no reversal after 10 min, partial reversal after 20 min (30–50 %) and total reversal of the fructose effect after 40 min in fructose-free medium.

KCN suppresses the effects of glucose and fructose on glucokinase release

When hepatocytes were incubated in medium supplemented with 4 mM KCN, to lower cellular ATP levels, the effects of glucose and fructose on glucokinase release were diminished by 50 % relative to controls with 4 mM KCl (Table 3). The effects of 4 mM KCN were partially reversible when KCN was present during the 15 min preincubation and then removed during the substrate incubation. Surface blebbing was evident by light microscopy after 30 min incubation with 4 mM KCN. In experiments ($n = 3$) with lower concentrations of KCN, the inhibition by 1 mM, 2 mM and 3 mM KCN of fructose-induced glucokinase release was respectively 26 %, 68 % and 82 % of that with 4 mM KCN.

Lack of effect of cytochalasin D, phalloidin or colchicine on substrate-induced glucokinase release

We used cytochalasin D (0.2, 2 and 20 μM) and phalloidin (5 μM) to determine whether dynamic changes in the microfilament cytoskeleton are involved in substrate-induced glucokinase release. Preincubation with cytochalasin D or phalloidin for 40 min ($n = 2$) or 3 h ($n = 3$) did not suppress the fructose-induced (1 mM, 30 min) or glucose-induced (10–20 mM, 30 min) glucokinase release. Preincubation for 2 h with 20 μM or 50 μM colchicine ($n = 2$), which interferes with microtubule dynamics, also did not suppress the fructose effect.

Lack of effect of substrates added to permeabilized hepatocytes

We investigated whether glucokinase release from hepatocytes that were pre-cultured with 5 mM glucose could be increased if phosphorylated forms of glucose or fructose are added during permeabilization with digitonin (0.05 mg/ml) in 5 mM $MgCl_2$/300 mM sucrose/3 mM Hepes. Additions including 2 mM fructose or 20 mM glucose in the absence or presence of ATP (0.2 mM, 1 mM or 3 mM), or glucose 6-phosphate, fructose 1-phosphate, fructose 6-phosphate or fructose 1,6-bisphosphate (0.02, 0.2 mM, 1 mM), did not affect glucokinase release, determined from the activity remaining in the cell matrix. In these experiments, glucokinase could only be determined in the residual cell matrix, because many of the additions to the digitonin medium interfered with the glucokinase assay.

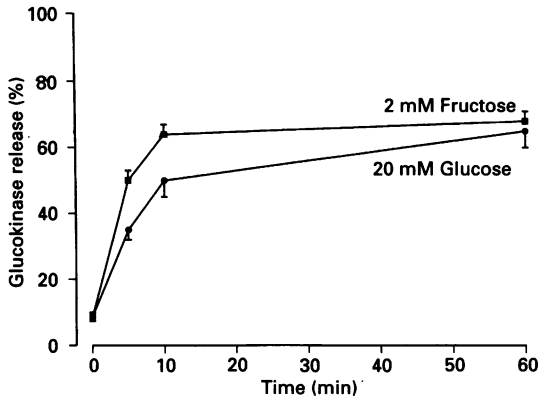

Figure 5 Time course of exposure to glucose or fructose on glucokinase release

Hepatocyte monolayers were incubated for the time intervals indicated with 20 mM glucose (●) or 2 mM fructose (■). They were then washed and permeabilized as described in Figure 4. Glucokinase activity in the digitonin eluate is expressed as a percentage of total activity. Values are means ± S.E.M. for 3 experiments.

Table 3 Effects of KCN and KCl on glucose- and fructose-induced glucokinase release

Hepatocyte monolayers that had been pre-cultured for 28 h were incubated for 15 min with the substrates indicated in the absence or presence of 4 mM KCl or 4 mM KCN, which were added 15 min before the addition of substrates. In a further set of incubations 4 mM KCN was added during the 15 min preincubation and removed during the incubation with substrates. On termination of the incubations, the hepatocytes were washed and permeabilized as described in Figure 4. Glucokinase activity in the digitonin eluate is expressed as a percentage of total activity. Values are means ± S.E.M. of 3 experiments: $^*P < 0.05$ relative to KCl.

	Release (%)			
Preincubation (15 min)...		KCl	KCN	KCN
Incubation (15 min)...	Control	KCl	KCN	—
Substrate				
None	0	0	0	0
+20 mM glucose	49 ± 6	41 ± 4	19 ± 3*	33 ± 7
+1 mM fructose	57 ± 3	53 ± 2	25 ± 4*	45 ± 4

Effects of insulin and glucagon on glucokinase release

The effects of insulin (10 nM) and glucagon (100 nM) on glucokinase release were determined during 60 min incubation of hepatocytes with varying [glucose] or [fructose] (Figure 6). Insulin potentiated the stimulatory effects of increasing [glucose], whereas glucagon had the converse effect. In the presence of 5 mM

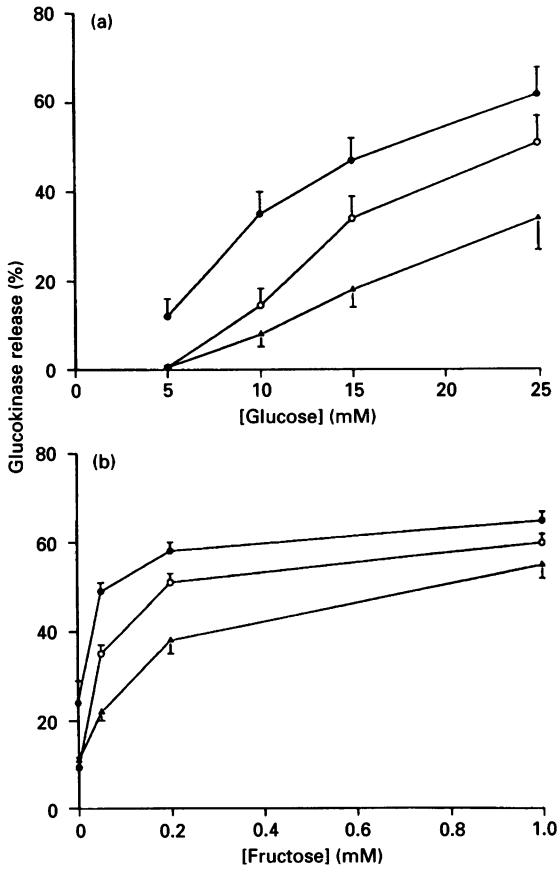

Figure 6 Effects of insulin and glucagon on glucokinase release

Hepatocytes were incubated for 60 min without hormones (○) or with 10 nM insulin (●) or 100 nM glucagon (▲) at the concentrations of glucose (**a**) or fructose (**b**) indicated. They were then washed and permeabilized as described in Figure 4. Glucokinase activity in the digitonin eluate is expressed as a percentage of total activity. Values are means ± S.E.M. for 6 (**a**) or 7 (**b**) experiments.

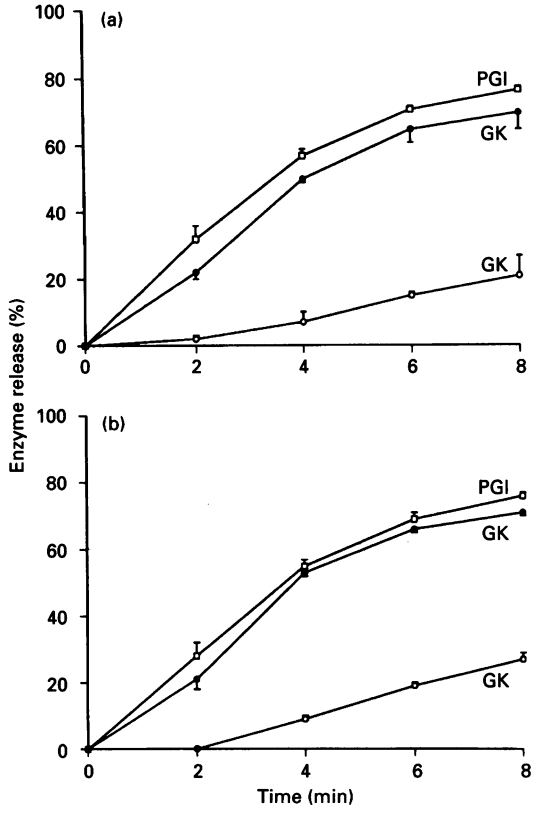

Figure 7 Rates of enzyme activity release in 'cytosol-like' permeabilization medium

Hepatocytes were incubated for 30 min without or with 1 mM fructose. They were then washed and permeabilized with digitonin (0.05 mg/ml) for the time intervals indicated, either in (**a**) 300 mM sucrose/5 mM $MgCl_2$/3 mM Hepes, or (**b**) 'cytosol-like' medium (150 mM K^+/ 50 mM Cl^-/100 mM isethionate/1 mM $MgCl_2$/3 mM Hepes. Release of enzyme activity is expressed as a percentage of total activity. ○, Glucokinase (GK) release from untreated cells; ●, glucokinase from fructose-treated cells; □, phosphoglucoisomerase (PGI) in control or fructose-treated cells. Values are means ± SEM of triplicate incubations from each of two experiments.

glucose, the stimulation by insulin was additive with that by 50 μM fructose, but not with 1 mM fructose. Insulin decreased the [fructose] causing half-maximum stimulation (control, 51.4 ± 5.6 μM; insulin, 31.4 ± 3.0 μM; means ± S.E.M., $n = 7$). Glucagon suppressed glucokinase release ($P < 0.05$) in the presence of 50 μM and 200 μM fructose, but not 1 mM fructose, and increased the [fructose] causing half-maximum effect (control, 48.0 ± 4.0 μM; glucagon, 142 ± 7 μM; means ± S.E.M., $n = 9$). Total recovery of activity in the eluate plus cell matrix was not affected by either insulin or glucagon in any of the substrate conditions.

Rates of enzyme-activity release during digitonin permeabilization in 'cytosol-like' medium

In the above experiments the effects of substrates and hormones on glucokinase release during digitonin permeabilization were determined in 300 mM sucrose/5 mM $MgCl_2$/3 mM Hepes, because this medium resulted in maximum difference in percentage glucokinase release between control and fructose-treated cells. In additional experiments we compared rates of enzyme activity release from cells permeabilized in 300 mM sucrose/ 5 mM $MgCl_2$ with a medium with composition simulating cytosol (containing 150 mM K^+, 50 mM Cl^- and 1 mM Mg^{2+}). Rates of release of phosphoglucoisomerase were identical in fructose-treated and control cells, and data are pooled (Figure 7). Rates of glucokinase release were higher in fructose-treated than in control cells and similar in the two media, suggesting that the slower rate of glucokinase release from control compared with fructose-treated cells probably represents enzyme binding in the intact cell. The release profiles of several other enzymes, including phosphoglucomutase, lactate dehydrogenase and the cytoplasmic forms of malate dehydrogenase, aspartate aminotransferase and alanine aminotransferase during permeabilization in the 'cytosol-like' medium were similar to the release of phosphoglucoisomerase (Figure 7), and likewise were unaffected by incubation with fructose (results not shown). Fructokinase release in 300 mM sucrose/5 mM $MgCl_2$ was also not affected by fructose (results not shown).

Effects of age of culture on glucokinase release in response to substrates and insulin

Throughout this study hepatocytes were cultured in monolayer for between 6 h and 30 h before determination of substrate or hormone-induced glucokinase release. Table 4 shows the effects

Table 4 Effects of age of culture on glucokinase release in response to fructose, glucose and insulin

Hepatocytes were pre-cultured for the time intervals indicated (with 5 mM glucose) and then incubated for 20 min with fructose, glucose or insulin as indicated. They were then washed and permeabilized as described in Figure 4. Glucokinase activity released into the digitonin eluate is expressed as a percentage of total activity.

	Glucokinase release (%)			
Time in culture... Substrate	4 h	9 h	24 h	32 h
Control	25	15	9	0
+50 μM fructose	48	40	19	11
+200 μM fructose	62	57	33	28
+1 mM fructose	74	65	43	41
+10 mM glucose	54	41	32	21
+50 μM fructose	73	66	37	36
+200 μM fructose	78	73	46	41
+1 mM fructose	79	75	53	49
10 nM insulin	32	26	15	8
+50 μM fructose	58	51	26	25
+200 μM fructose	72	58	34	34
+1 mM fructose	75	65	44	42

of age of culture on glucokinase release from cells cultured in standard culture medium and after incubation for 20 min with fructose (50 μM–1 mM), glucose (+10 mM) or insulin. Glucokinase release in cells maintained in 5 mM glucose declined progressively with the age of culture from 25% to 0%. This decline in percentage release could not be accounted for by a decrease in total activity, and particularly during the early stages of culture when the decrease in total activity was negligible compared with the decrease in activity released. Stimulation of glucokinase release by +10 mM glucose, fructose (50 μM–1 mM) or insulin was observed at all stages of culture (Table 4).

Incorporation of [2-^{3}H,U-^{14}C]glucose into glycogen

The above experiments show that, during permeabilization in 'cytosol-like' medium or in 300 mM sucrose+5 mM $MgCl_2$, glucokinase is released slowly from cells preincubated with 5 mM glucose, but much more rapidly from cells pre-treated with fructose, high [glucose] or insulin. We infer that glucokinase is present predominantly in a bound state in cells maintained in 5 mM glucose, but, after exposure to fructose, high [glucose] or insulin it translocates either to the free state in the cytoplasm or to a loosely bound state with different ionic interactions. This translocation mechanism might involve: (i) a change in activity of glucokinase if it is bound to the matrix through its regulatory (inhibitory) protein [30]; (ii) transfer from a central location [31] to a more peripheral site, where it would be closer to the glucose transporters on the plasma membrane; (iii) translocation to favour selective partitioning of the product (glucose 6-phosphate) towards one of the three alternative routes of metabolism (glycogen synthesis, glycolysis or pentose phosphate pathway). We tested the last possibility by determining the incorporation of [2-^{3}H,U-^{14}C]glucose into glycogen. The 2-^{3}H is lost during conversion of glucose 6-phosphate into fructose 6-phosphate via phosphoglucoisomerase [32]. Because phosphoglucoisomerase is presumed to maintain glucose 6-phosphate in equilibrium with fructose 6-phosphate, loss of ^3H_2O from [2-^{3}H]glucose has been used to estimate the rate of flux through glucokinase [32]. However, loss of 2-^{3}H is incomplete, and a substantial proportion of the 2-^{3}H is incorporated into glycogen [33]. If glucose 6-phosphate is converted into glycogen without equilibrating with phosphoglucoisomerase, the proportion of 2-^{3}H incorporated into glycogen relative to ^{14}C would increase. We therefore determined the incorporation of ^{3}H/^{14}C label into glycogen during incubation of hepatocytes with [2-^{3}H,U-^{14}C]glucose as a qualitative measure of the proportion of glucose 6-phosphate that partitions towards glycogen synthesis without equilibration with phosphoglucoisomerase. These experiments were performed with concentrations of glucose, insulin and fructose that cause translocation of glucokinase. This was determined in parallel experiments with unlabelled glucose (Figures 8 and 9). Fructose increased the incorporation of [^{14}C]glucose into glycogen (A_{50} 50 μM), and it increased the ratio of ^{3}H/^{14}C-label incorporation from 37% to 47% (Figure 8). Glucose conversion into glycogen increased with increasing [glucose], and the ^{3}H/^{14}C ratio increased from 35% to 56% in the absence of insulin and to 62% in its presence (Figure 9). The ratio of ^{3}H/^{14}C incorporated into glycogen during incubation with glucose and

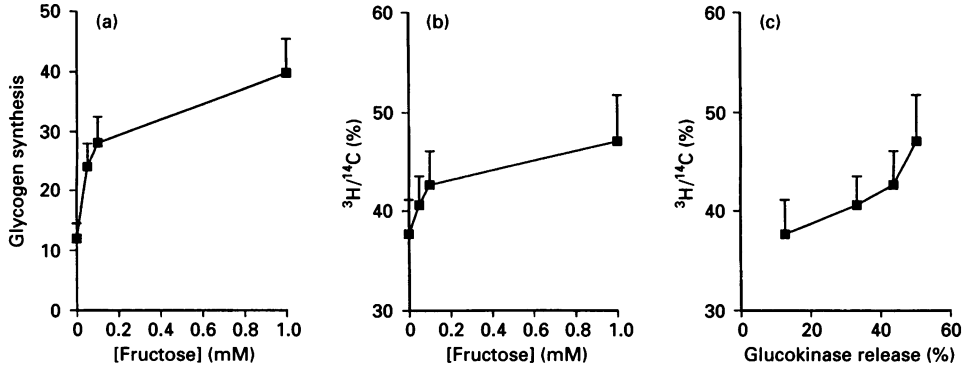

Figure 8 Effects of fructose on the incorporation of [2-^{3}H,U-^{14}C]glucose into glycogen

Hepatocytes were incubated for 3 h with 10 mM [2-^{3}H,U-^{14}C]glucose and the [fructose] indicated. Glucokinase release was determined as described in the legend to Figure 4 in parallel 1 h incubations with unlabelled substrate. (**a**) Glycogen synthesis represents the incorporation of [^{14}C]glucose into glycogen, expressed as nmol of glucose incorporated/3 h per mg of protein. (**b**) The ratio of ^{3}H/^{14}C label incorporated into glycogen as a percentage of the ratio of ^{3}H/^{14}C label in glucose in the medium. (**c**) represents the ^{3}H/^{14}C (%) ratios in glycogen (**b**) plotted against the respective percentage release of glucokinase. Results are means ± S.E.M. of 3 experiments.

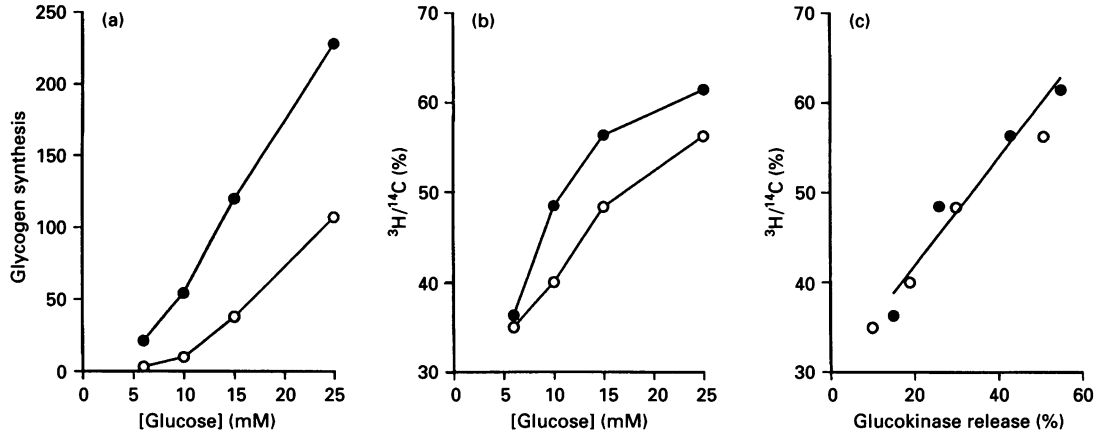

Figure 9 Effects of [glucose] and insulin on the incorporation of [2-^{3}H,U-^{14}C]glucose into glycogen

Hepatocyte monolayers were incubated for 3 h with [2-^{3}H,U-^{14}C]glucose at the [glucose] indicated in either the absence (○) or presence (●) of 10 nM insulin. Glucokinase release was determined as described in the legend to Figure 4 in parallel 1 h incubations with unlabelled substrate. (**a**) Glycogen synthesis represents the incorporation of [^{14}C]glucose into glycogen, expressed as nmol of glucose incorporated/3 h per mg of protein. (**b**) The ratio of $^3H/^{14}C$ label incorporated into glycogen as a percentage of the ratio of $^3H/^{14}C$ label in glucose in the medium. (**c**) represents the $^3H/^{14}C$ (%) ratios (**b**) plotted against the respective percentage release of glucokinase. Results are means of two experiments.

insulin correlated with the proportion of glucokinase released during permeabilization with digitonin (Figure 9). These results show that the ratio of incorporation of $^3H/^{14}C$ from [2-^{3}H,U-^{14}C]glucose into glycogen is not constant and correlates with the proportion of glucokinase released, and presumably therefore with the location of glucokinase.

DISCUSSION

Glucokinase binding to the cell matrix in cells cultured in 5 mM glucose

The slow rate of glucokinase release compared with that of various cytoplasmic enzymes, including phosphoglucoisomerase and lactate dehydrogenase, from hepatocytes permeabilized in medium of composition simulating the cytosol suggests that in the intact hepatocyte a high proportion of glucokinase is present in a bound state. The similarity in rates of glucokinase release when hepatocytes are permeabilized in 300 mM sucrose + 5 mM $MgCl_2$ compared with medium simulating cytosol suggests that Mg^{2+} rather than ionic strength is the main determinant responsible for glucokinase binding to the cell matrix in the 'cytosol-like' medium. The rapid rate of glucokinase release during permeabilization of hepatocytes at low ionic strength in the absence of bivalent cations (Figures 1–3) could be explained by two types of mechanism: first, that glucokinase is attached to its binding site by ionic interactions that can be dissociated in the absence of Mg^{2+} at low ionic strength; secondly, that media of low ionic strength without bivalent cations cause an irreversible disruption of a subcellular structure containing the bound glucokinase. The greater release and total recovery of monomeric G-actin during permeabilization in 300 mM sucrose indicate depolymerization of the microfilament cytoskeleton and support the second hypothesis. The increase in glucokinase release from cells pre-treated with cytochalasin D or phalloidin when these are permeabilized in conditions that minimize depolymerization of the microfilament cytoskeleton (150 mM KCl or 5 mM $MgCl_2$) is consistent with an association of glucokinase with the microfilament cytoskeleton. Such an association might be indirect, and glucokinase may be bound to the microfilaments by protein–protein interaction, as proposed for the microtrabecular lattice [34,35]. Permeabilization of hepatocytes in medium without Mg^{2+} may accelerate disruption of this cytoskeletal structure [35]. Although glucokinase remains bound to the cell matrix during permeabilization of hepatocytes in the presence of Mg^{2+} (Figure 2), the enzyme can be 'solubilized' during sonication in a Mg^{2+}-containing medium (it is recovered from the cytosolic fraction), suggesting that sonication either releases glucokinase from its binding site or disrupts or solubilizes the matrix to which it is attached. Disruption of the microtrabecular lattice by cooling and sonication is consistent with this interpretation [34,35].

Substrate-induced translocation of glucokinase

The increased rate of glucokinase release when hepatocytes are incubated with fructose, glucose or insulin and then permeabilized either in the 'cytosol-like' medium or in sucrose and 5 mM $MgCl_2$ suggests that glucokinase translocates from its bound state to either an alternative binding site (with different ionic properties) or to the free state in the cytoplasm. Since fructose treatment did not affect the release of several cytoplasmic enzymes, including fructokinase, the increased release of glucokinase is not due to a non-specific mechanism. A recent study reported that glycogen synthase in hepatocyte suspensions translocates in response to glucose, galactose, fructose and 2-deoxyglucose [36,37]. The present results differ from the glycogen synthase study insofar as, although mannose mimicked the effect of glucose on glucokinase translocation, both galactose and 2-deoxyglucose were ineffective. Mannose and 2-deoxyglucose are both substrates for glucokinase. However, 2-deoxyglucose is not metabolized further beyond phosphorylation. Thus the glucose- and mannose-induced translocation of glucokinase could be due to a metabolic step/sequence beyond substrate phosphorylation. The inhibition by cyanide of the glucose- or fructose-induced translocation and the biphasic effect of fructose at high concentration are both consistent with an ATP-dependent mechanism, since high fructose concentrations deplete ATP [29].

Recent studies have identified a 62 kDa regulatory protein in rat liver that binds to glucokinase and decreases the affinity of the enzyme for glucose [30]. This binding is potentiated by

fructose 6-phosphate and counteracted by fructose 1-phosphate. Because low concentrations of fructose (A_{50} 50 μM) induce translocation of glucokinase from the Mg^{2+}-dependent binding site, it is possible that the 62 kDa regulatory protein may be involved in the binding of glucokinase to the cytoskeleton. It could be speculated that, during incubation with fructose, the increase in fructose 1-phosphate [38] may cause the dissociation of the glucokinase from its binding protein.

Control of translocation by insulin and glucagon

The potentiation by insulin and counteraction by glucagon of the glucose-induced or fructose-induced translocation of glucokinase are intriguing. If the glucose effect involved a mechanism whereby glucose 6-phosphate, the product of the glucokinase reaction, caused the dissociation of glucokinase from its binding site, then the hormonal effects would be suggestive of acute activation of glucokinase by insulin and inhibition by glucagon. However, the total activity of glucokinase recovered from the digitonin eluate and cell matrix was not affected by hormones. An alternative possibility is that glucose-induced translocation is mediated not by glucose 6-phosphate but by a different metabolic product of glucose, the formation of which is regulated by hormones. A further possibility is that the effects of insulin and glucagon are due not to secondary effects on glucose or fructose metabolism, but to independent mechanism(s) that converge on the glucokinase binding site.

Implications of the variable ratio of $^3H/^{14}C$ incorporation into glycogen from [2-^{3}H,U-^{14}C]glucose

^3H in the 2-position of glucose is lost at the level of phosphoglucoisomerase [32]. The flux through glucokinase in hepatocytes is conventionally determined from the rate of formation of ^3H_2O from [2-^{3}H]glucose. This assumes that glucose 6-phosphate is in equilibrium with fructose 6-phosphate. However, during metabolism of [2-^{3}H,U-^{14}C]glucose a significant proportion (about 40%) of ^3H is incorporated into glycogen [33]. Because detritiation is incomplete, most studies apply a correction factor in the estimation of glucokinase flux. This factor is generally assumed to be constant. However, Katz and Rognstad [32] showed that the ratio of $^3H/^{14}C$ incorporated into glycogen from [2-^{3}H,U-^{14}C]glucose is influenced by various substrates. Our results, in agreement with those of Katz and Rognstad [32], show an increase in $^3H/^{14}C$ ratio with fructose and with increasing [glucose]. In addition, we show that insulin increases the $^3H/^{14}C$ ratio incorporated into glycogen. If glucokinase flux is determined from rates of formation of ^3H_2O from [2-^{3}H]glucose, assuming a constant ratio for retention of ^3H in glucose, estimates for glucokinase flux in the presence of insulin would be underestimates. Thus the effect of insulin on glucokinase flux in hepatocytes needs to be re-evaluated, taking into account the increased retention of 2-^{3}H.

Physiological implications of translocation

Several enzymes are known to translocate from a central to a peripheral location in cells in response to external stimuli. Hexokinase in macrophages translocates from a central to a peripheral location in response to phorbol ester [39]. A histochemical study on rat liver suggested that glucokinase is localized in the nucleus [31]. The possibility that the Mg^{2+}-dependent binding site of glucokinase in cells maintained at low [glucose] in the absence of fructose or insulin may represent a central or perinuclear location needs to be considered. We postulate three possible mechanisms whereby translocation of glucokinase might affect glucose metabolism in the liver cell: first, by altering the kinetic properties from an inactive (bound) to an active (deinhibited/translocated) state, by analogy with the binding characteristics of the 62 kDa regulatory protein [30]; secondly, if the bound state is in a central/perinuclear location, then translocation to the periphery would increase the proximity of the enzyme to the glucose transporters on the plasma membrane; thirdly, by enabling the selective partitioning (channelling?) of the product, glucose 6-phosphate, towards glycogen synthesis as opposed to glycolysis. Glycogen synthesis is an important route of disposal of glucose in the liver cell, particularly at high glucose concentrations [13]. Translocation of glucokinase to a site close to the glycogen particle may favour increased partitioning of glucose 6-phosphate to glycogen as compared with glycolysis. The correlation between the $^3H/^{14}C$ ratio incorporated into glycogen from [2-^{3}H,U-^{14}C]glucose and the proportion of glucokinase release suggests that substrate-induced translocation of glucokinase may be associated with increased partitioning of glucose 6-phosphate towards glycogen as opposed to phosphoglucoisomerase. Several studies have suggested inhomogeneity of the glucose 6-phosphate pool(s) in the liver cell [40–42]. Translocation of glucokinase in response to substrates and hormones may be important in the generation of glucose 6-phosphate in specific locations in the cell.

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