Effects of C-1-substituted glucose analogue on the activation states of glycogen synthase and glycogen phosphorylase in rat hepatocytes

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A series of glucose-analogue inhibitors of glycogen phosphorylase *b* (GPb) has been designed, synthesized and investigated in crystallographic binding and kinetic studies. The aim is to produce a compound that may exert more effective control over glycogen metabolism than the parent glucose molecule and which could alleviate hyperglycaemia in Type-II diabetes. *N*-Acetyl-β-D-glucopyranosylamine (1-GlcNAc) has a K_i for muscle GPb in crude extracts of 30 μM, 367-fold lower than that of β-D-glucose [Board, Hadwen and Johnson (1995) Eur. J. Biochem. **228**, 753–761]. In the current work, the effects of 1-GlcNAc on the activation states of GP and glycogen synthase (GS) in cell-free preparations and in isolated hepatocytes are reported. In gel-filtered extracts of liver, which lack ATP for kinase activity, 1-GlcNAc produced a rapid and time-dependent inactivation of GP with a subsequent activation of GS. Effects of 1-GlcNAc on both enzymes were stronger than those of glucose, with 0.8 mM 1-GlcNAc being equipotent with 50 mM glucose. At 1 mM, 1-GlcNAc enhanced the dephosphorylation of exogenous GPa by liver extracts (600%) and by muscle extracts (75%). This represents an approximately 500-fold improvement on glucose for the liver activity and 40-fold for the muscle activity. In whole hepatocytes, 1-GlcNAc showed an approximately 5-fold enhancement of glucose effects for GP inactivation but failed to elicit activation of GS. Glucose-induced activation of GS in whole hepatocytes was reversed by subsequent addition of 1-GlcNAc. However, when GS activation was achieved via the adenosine analogue and kinase inhibitor, 5′-iodotubercidin (ITU), subsequent addition of 1-GlcNAc allowed continued activation of GS. Phosphorylation of 1-GlcNAc in rat hepatocytes was established using radiolabelled material. The rate of phosphorylation was 1.60 nmol/min per 10^6 cells at 20 mM 1-GlcNAc but was reduced by the presence of 50 μM ITU (0.775 nmol/min per 10^6 cells). It is suggested that the phosphorylated derivative of 1-GlcNAc formed in hepatocytes is 1-GlcNAc 6-phosphate and that the presence of this species is responsible for the failure of 1-GlcNAc to activate GS. The relative importance of the reduction in concentration of GPa versus increased glucose 6-phosphate levels for activation of GS is discussed.

INTRODUCTION

In muscle and liver, glycogen concentrations are regulated by the reciprocal activities of glycogen phosphorylase (GP) and glycogen synthase (GS). The regulation of these enzymes by phosphorylation/dephosphorylation reactions, stimulated by hormonal and neuronal signals and by allosteric effectors, has been the subject of investigation resulting in detailed understanding of the individual events involved. Activation of GP by phosphorylation is achieved by the action of a single enzyme, GP kinase, at a single site, Ser-14, but the reciprocal inhibition of GS via phosphorylation is effected by at least six different kinases acting on nine serine sites [1,2] with some differences between the muscle and liver [3–5]. The reverse reactions of inactivation of GP and activation of GS are achieved *in vivo* through the actions of protein phosphatases. Both liver protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) contribute to overall GP phosphatase activity whereas dephosphorylation of GS is largely due to the glycogen-bound species of PP1 (PP1-G). There is evidence to suggest that the co-ordination of glycogen synthesis and breakdown is achieved via control of phosphatase activity in response to glucose in the liver [6,7] and in response to insulin in muscle [8]. The regulation of PP1 differs in muscle and liver. Both forms have a catalytic subunit associated with a glycogen-binding subunit that locates the enzyme on the glycogen substrate together with the other enzymes of glycogen metabolism. In muscle, control is effected through phosphorylation of the glycogen-binding subunit in response to hormonal stimuli [9]. In liver, part of PP1 activity is targeted to glycogen by a different glycogen-binding subunit, and dephosphorylation of GS by this PP1-G is inhibited allosterically by extremely low concentrations of the active form of GP, GPa [9,10]. Such inhibition is relieved when hepatic GPa is converted into its inactive form, GPb.

The predominant role of liver glycogen is to supply glucose to the circulation for use by other tissues in response to the nutritional status of the body. The concept that glucose itself is an important regulator of hepatic glucose output [11] has been developed by Hers [6] and Stalmans [7]. The mechanism by which glucose causes a sequential inactivation of GP and activation of GS assumes that (i) glucose stimulates the dephosphorylation and inactivation of GP by binding to GPa and making it a better substrate for GP phosphatase and (ii) the conversion of GPa into GPb relieves inhibition of PP1-G, leaving it free to dephosphorylate GS. The demonstration that insulin augmented the glucose stimulation of GS and inhibition of GP [12] provided an important link between the roles of glucose and insulin in hepatic glycogen metabolism. Although there have been some suggestions that not all processes can be encompassed by this mechanism, the proposals have received substantial support from the discovery that GPa is a potent inhibitor of the GS phosphatase activity of liver PP1-G and that the interaction involves the glycogen-binding subunit which is distinct from its

Abbreviations used: 1-GlcNAc, *N*-acetyl-β-D-glucopyranosylamine; GP, glycogen phosphorylase; GS, glycogen synthase; ITU, 5′-iodotubercidin; PG, proglycosyn; PP1, protein phosphatase 1.

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catalytic subunit [13–15]. A rationalization of the effects of glucose on GP has come from the X-ray crystal structure of the active and inactive forms of rabbit muscle GP [16–18]. Glucose is an inhibitor which binds to the catalytic site in competition with substrate but also stabilizes the T-state (less active) form of the enzyme (nomenclature of Monod et al. [19]) by making specific interactions with a loop of chain that blocks access to the catalytic site. T-state GP is a better substrate for PP1 [20,21]. Hence glucose acts not only to inhibit GPa, but also to stimulate (or enhance) the dephosphorylation (or inactivation) of GPa. A systematic analysis of glucose-analogue inhibitors has been carried out using knowledge of the T-state structure of rabbit muscle GPb as a model [18,22,23]. The N-linked C-1 derivative of β-D-glucose, *N*-acetyl-β-D-glucopyranosylamine (1-GlcNAc), exhibits a K_i for the purified rabbit muscle enzyme of 32 μM, a value that is 367-fold lower than the corresponding K_i for β-D-glucose [23].

The present work extends these results and examines the effects of 1-GlcNAc on the co-ordinated regulation of GP and GS in whole hepatocytes. The effects of acutely increasing the glucose concentration on the activation states of GP and GS have been established [12,24] and result in rapid inactivation of GP accompanied, after a short time lag, by activation of GS. Results of similar experiments on whole hepatocytes and gel-filtered liver extracts in which glucose is replaced by 1-GlcNAc are reported here. It is established that 1-GlcNAc is an effective regulator of both muscle and liver GP and promotes the dephosphorylation of GPa, as predicted. The effects on GS are more complex but may be partially explained by the phosphorylation of 1-GlcNAc in the hepatocyte. This modification does not occur in gel-filtered extracts. The results allow further conclusions to be drawn about the effects of the analogue on the enzymes involved in hepatic glycogen metabolism and the relative importance of regulation by kinase/phosphatase activity versus that by metabolites such as glucose 6-phosphate.

MATERIALS AND METHODS

ATP, pyruvate, phosphocreatine and rabbit muscle GPb were purchased from Boehringer-Mannheim (Lewes, Sussex, U.K.) and bovine liver glycogen, dithiothreitol, creatine kinase, glycylglycine hydrochloride and PMSF from Sigma (Poole, Dorset, U.K.). Lactic acid and dimethoxymethane came from Janssen Chimica, Brussels, Belgium, and all salts and buffers from British Drug Houses (Merck Ltd., Poole, Dorset, U.K.). 5′-Iodotubercidin (ITU) was purchased from Research Biochemicals International [Semat Technical (U.K.) Ltd., St. Albans, Herts., U.K.]. D-[U-^{14}C]glucose, D-[U-^{14}C]glucose 1-phosphate, D-[U-^{14}C]UDP-glucose, [^{14}C]inulincarboxylic acid and [γ-^{32}P]ATP were purchased from Amersham International (Amersham, Bucks., U.K.), and Optiphase Hisafe 3 from Wallac Scintillation Products (Leicester, Leics., U.K.). Whatman (Maidstone, Kent, U.K.) supplied 3 MM filter paper for binding glycogen and DE81 ion-exchange discs. Pharmacia LKB (Uppsala, Sweden) supplied Sephadex G-25.

The syntheses of 1-GlcNAc and radiolabelled 1-[^{14}C]GlcNAc, in which the label was incorporated at the methyl group of the *N*-acetyl substituent, are described below.

Preparation of hepatocytes

Hepatocytes were prepared as detailed in [25] from male Wistar rats fed *ad libitum*. Resulting cell suspensions contained about 1×10^8 cells/g of cell pellet after centrifugation.

Incubation of hepatocytes

Cells, at a final density of 5×10^6–10×10^6/ml in a final volume of approx. 1 ml, were incubated essentially by the method of Bollen et al. [25] in Krebs–Henseleit buffer, pH 7.4, containing 13.5 mM lactate, 1.5 mM pyruvate and 0.16 mM glycerol under an atmosphere of 95% O_2/5% CO_2 in a shaking (100/min) water bath at 37 °C. A 10 min preincubation with 10 mM glucose was followed by addition of 1-GlcNAc or glucose at $t = 0$. Samples were taken at various time points and immediately added to a buffer (10 mM glycylglycine, 0.5% glycogen, 20 mM EDTA and 100 mM NaF) designed to prevent kinase and phosphatase activities, with quick freezing in liquid nitrogen. Samples were thawed and assays of enzyme activity were performed up to 24 h after freezing. Such treatment yields similar enzyme activities to those obtained after homogenization in a Potter–Elvehjem tube [26].

Measurement of viability

The percentage of living cells in a hepatocyte population before and after an incubation of up to 2 h in the presence of 50 mM 1-GlcNAc was measured by Trypan Blue exclusion. Under the conditions of incubation outlined above, viability (85–90%) was not affected by the presence of 1-GlcNAc.

Measurement of GP activity

GP activity was measured in the direction of glycogen synthesis by the method of Hue et al. [27] as the liberation of phosphate from glucose 1-phosphate. Measurements of total GP activity (a + b) [28] were made by incubating a 15 μl sample in a medium containing 1% glycogen, 50 mM glucose 1-phosphate, 100 mM NaF, 0.1% BSA, 2 mM AMP, 20 mM Pipes, pH 6.8, and 5% dimethoxymethane in a volume of 200 μl. The assay mixture was incubated at 25 °C for 40 min. This was followed by the addition of 0.5 ml of ice-cold 10% trichloroacetic acid, cooling on ice, dilution with 7 vol. of distilled water and centrifugation for 5 min at 3600 *g*. P_i was measured by the Fiske–Subbarow [29] procedure [27].

For measurement of GPa activity, a similar procedure was followed but the sample was incubated in a medium containing 1% glycogen, 50 mM glucose 1-phosphate, 100 mM NaF, 0.5 mM caffeine, 0.1% BSA and 20 mM Pipes, pH 6.8. It is considered that the presence of caffeine will result in substantial inhibition of GPb whereas activity of GPa will be unaffected [27,30].

Measurement of GS Activity

GS activity was measured largely as described by Thomas et al. [31], incorporating the modification of Witters and Avruch [12] where the more active GSa (I) form is measured in the presence of Na_2SO_4 and total GS [(a + b) or (I + D)] in the presence of glucose 6-phosphate. Activity is measured as the incorporation of ^{14}C label from [U-^{14}C]UDP-glucose into glycogen. For assay of total GS, 10 μl aliquots of sample were incubated with buffer {10 mg/ml glycogen, 70 mM EDTA, 70 mM Tris/HCl, pH 7.8, 10 mM UDP-glucose (containing 2.5 μCi/ml [U-^{14}C]UDP-glucose), 10 mM glucose 6-phosphate} in a total volume of 100 μl at 25 °C for 30 min. The procedure was similar for measurements of GSa activity except that glucose 6-phosphate was replaced with 10 mM Na_2SO_4. The reaction was stopped by spotting aliquots of the reaction mixture on to filter paper and washing with ice-cold 66% ethanol. Further washing at room temperature with ethanol and acetone completed the procedure.

Preactivation of GS

GS activity can be activated in whole hepatocytes by the presence of ITU, an adenosine analogue, which has been shown to inhibit GS kinases and GP kinase [32]. The presence of proglycosyn (PG), which promotes glycogen synthesis [33], is assumed to result in further activation of GS. During experiments involving GS activation in whole hepatocytes in the present work, this was achieved by addition of either (i) 50 mM glucose or (ii) 50 μM ITU plus 10 μM PG. In both cases, preactivation of GS was followed, after 30 min, by the addition of 10 mM 1-GlcNAc.

Measurement of GP phosphatase activity

A ^{32}P-labelled GPa substrate was prepared from purified rabbit muscle GPb by incubation with phosphorylase kinase as described by Antoniw et al. [34] with the modifications of Nimmo and Cohen [35]. Crude extracts of rat liver and soleus muscle tissue were prepared by homogenizing the tissue in a Polytron homogenizer in approx. 5 vol. of an extraction buffer containing 20 mM Mops, pH 7.5, 5 mM EDTA, 0.5 mM EGTA, 5 mM NaN_3, 1 mM dithiothreitol and 0.15 mM PMSF. The homogenate was centrifuged at 2100 *g* for 15 min in a Beckman GS-6R centrifuge, and the supernatant used for assay of enzyme activity. GP phosphatase activity was measured as the release of radiolabelled [^{32}P]P_i from GPa. The method of Nyomba et al. [36] was used for assay in the presence or absence of appropriate concentrations of glucose or 1-GlcNAc.

Preparation of a gel-filtered extract

Livers were homogenized in a Polytron homogenizer in 2 vol. of buffer (50 mM glycylglycine, 0.5 mM dithiothreitol and 0.25 M sucrose, pH 7.4) on ice. The homogenate was centrifuged at 8000 *g* for 10 min, and the supernatant filtered through glass wool and then through a 10–15 ml column of Sephadex G-25 (equilibrated with the same buffer) at 4 °C.

Measurement of phosphorylation of 1-GlcNAc by hepatocytes

Hepatocytes were prepared and incubated as detailed above with 10, 20 or 50 mM concentrations of 1-GlcNAc (containing approx. 1.4 μCi/10 μmol 1-[^{14}C]GlcNAc) or glucose (containing approx. 1 μCi/10 μmol D-[U-^{14}C]glucose). The incubation medium also contained 50 μM ITU where indicated. At time points 0, 10, 20 and 60 min, 0.2 ml aliquots of cell suspension were removed from each flask and added to 40 μl of 25% $HClO_4$ and cooled on ice. The deproteinized medium was quickly brought to neutral pH using a 40% solution of KOH. Phosphorylated glucose or 1-GlcNAc was separated from the native sugar by spotting aliquots of the neutralized medium on to DEAE-cellulose ion-exchange discs followed by washing with approx. 4 litres of doubly distilled water per 50 discs. Radioactivity in the discs was measured by adding 1 ml of water per disc plus aqueous scintillation fluid and measuring the d.p.m. in a Beckman LS1701 scintillation counter.

Measurement of rate of entry of 1-GlcNAc into the hepatocyte

Hepatocytes were incubated in the usual way (see above) with final concentrations of 0, 10, 20 or 50 mM 1-GlcNAc containing a trace of 1-[^{14}C]GlcNAc. At time points, 0, 10 and 60 min, 1 ml of an ice-cold solution of 1 M glucose was added to the cell suspension. The presence of a high concentration of glucose in the extracellular medium is considered to minimize extrusion via the near-equilibrium GLUT2 glucose transporter and therefore will minimize loss of 1-[^{14}C]GlcNAc from the intracellular compartment. The suspension was centrifuged at 270 *g* at 4 °C for 3 min. The medium was removed and radioactivity measured in both cells and medium. [^{14}C]Inulincarboxylic acid, which does not enter the cell, was used by a similar procedure to estimate the volume of the extracellular space in the cell pellet.

Synthesis of 1-[^{14}C]GlcNAc

Acetic anhydride (11 M) was added to a stirred suspension of β-D-glucopyranosylamine (820 mM) [37] in dry dimethyl fluoride at 0 °C and the mixture allowed to reach room temperature. After 5 min, the glycosylamine dissolved, and the product began to crystallize out 5 min later. After 30 min, the solvent was removed under reduced pressure and the residue recrystallized from water/ethanol several times to give 1-GlcNAc.

The radiolabelled compound was synthesized by dissolving acetic anhydride with a trace of ^{14}C label (83 nCi/μmol) in dimethyl fluoride and adding it to β-D-glucopyranosylamine (820 nM in dimethyl fluoride). The resulting solution was stirred under N_2 for 22 h, after which the solvent was removed *in vacuo* and the product recrystallized from water/ethanol. The resulting compound is 1-GlcNAc with a ^{14}C label incorporated into the methyl group of the N-linked substituent at carbon 1.

Studies with hepatocytes showed no increase in acetate production on incubation with 1-GlcNAc. It is assumed therefore that the presence of the ^{14}C label indicates the presence of the intact carbon skeleton of 1-GlcNAc.

RESULTS

The effects of glucose and 1-GlcNAc on the activity of GPa [expressed as a percentage of total GP (a + b)] in hepatocytes are shown in Figure 1(a). Glucose, at a concentration of 50 mM, produced a rapid inactivation of GPa, so that after 30 min the percentage of GPa was reduced from 96 to 14, whereas with no addition the percentage of GPa was about 35 at 30 min. These results are in agreement with those reported by Witters and Avruch [12]. Similar effects to those produced by 50 mM glucose were achieved with 1-GlcNAc at a concentration of 10 mM. The change in activation state (i.e. decrease in GPa) produced by either glucose or 1-GlcNAc shows that the enzymes catalysing interconversion between the a and b forms of GP are affected. The role of 1-GlcNAc as a competitive inhibitor of GP has already been established [38]. Glucose produced the expected increase in the activation state of GS (Figure 1b) in which the percentage of GSa rose from 14 to 90 within 40 min. 1-GlcNAc did not elicit any detectable activation of GS during the incubation of whole hepatocytes, and, at high concentration (10 mM), there was a slight reduction in the activation state after 30–40 min.

Figures 2(a) and 2(b) compare the effects of glucose and 1-GlcNAc on GP phosphatase activity in crude extracts of muscle and liver. Muscle GP phosphatase showed an increase in activity of approx. 75% in the presence of either 50 mM glucose or 1 mM 1-GlcNAc. However, the effects of both glucose and the analogue on liver GP phosphatase were more dramatic. In the presence of 50 mM glucose, activity increased by 200% above basal and with 1 mM 1-GlcNAc by 600%.

Measurement of rates of uptake of 1-GlcNAc and glucose by whole hepatocytes using ^{14}C-labelled substrates showed that 1-GlcNAc entered the cell at lower rates than glucose at all concentrations tested. At 10 mM, glucose was taken up at the rate of 22 nmol/min per 10^6 cells whereas 1-GlcNAc was taken up at 13 nmol/min per 10^6 cells or 60% of the rate of glucose uptake (for a 10 min incubation). At 50 mM concentration of

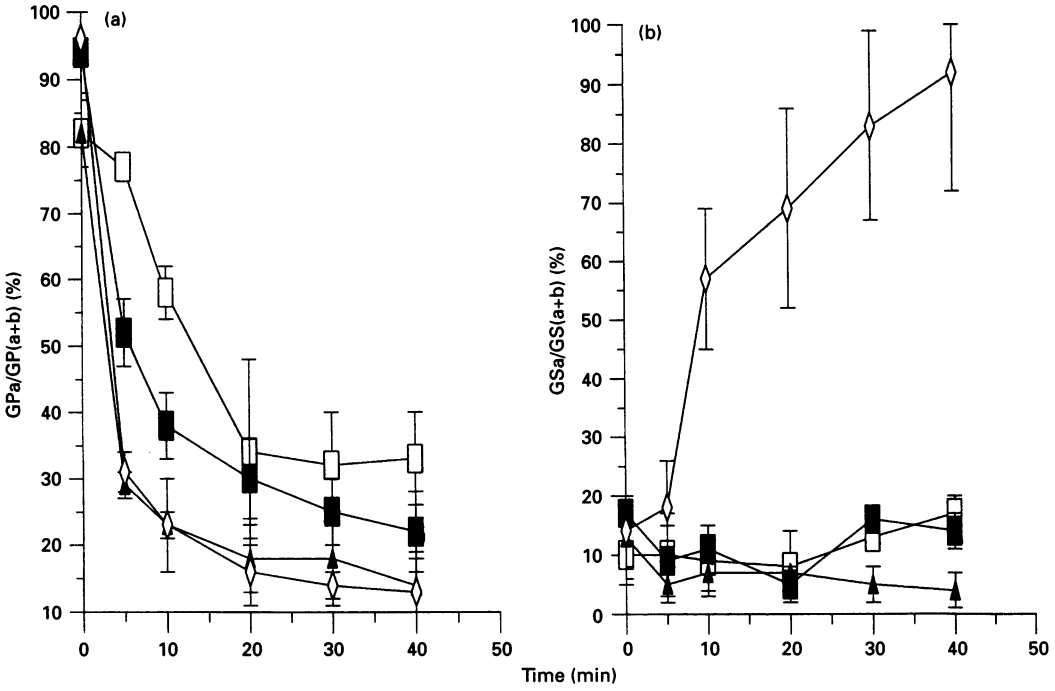

Figure 1 GP and GS activities in hepatocytes

(**a**) GP activity in hepatocytes represented by GPa activity as a percentage of total GP (a + b); (**b**) GS activity in hepatocytes represented by GSa activity as a percentage of total GS (a + b) activity. Data were obtained from a 40 min incubation of hepatocytes isolated from rats fed *ad libitum*. Cells were perfused with glucagon during preparation. Data points are means ± S.E.M. for at least four hepatocyte preparations. □, 0 mM 1-GlcNAc; ■, 2 mM 1-GlcNAc; ▲, 10 mM 1-GlcNAc; ◇, 50 mM glucose

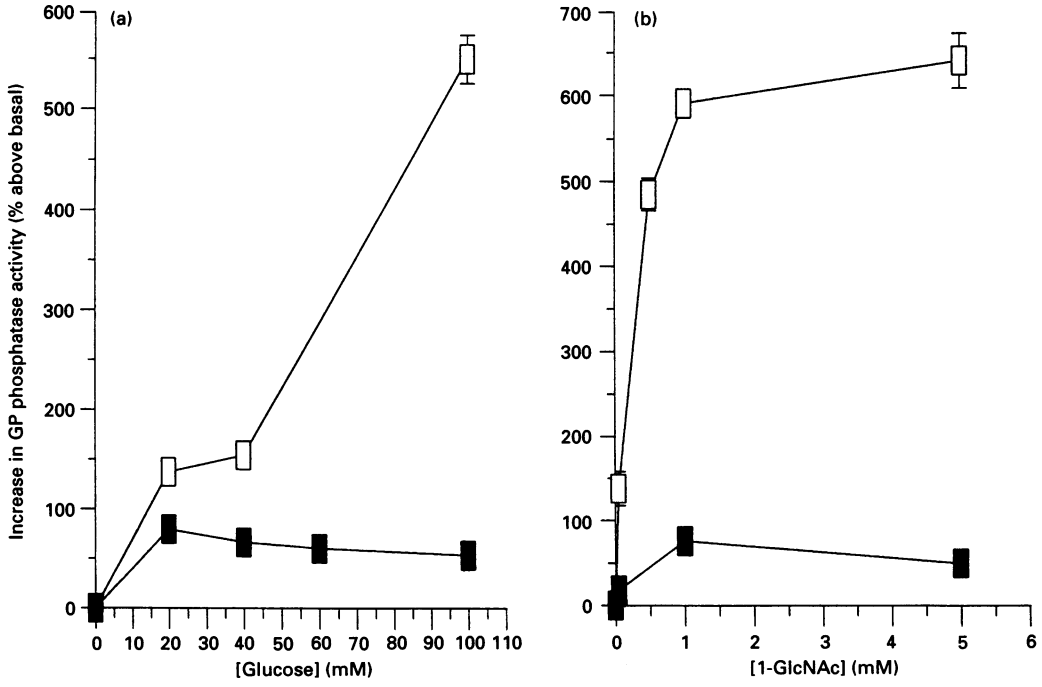

Figure 2 Percentage activation of GP phosphatase activity by (a) glucose and (b) 1-GlcNAc in extracts of liver and muscle

Enzyme activity was measured using muscle GPa as a substrate. Data points are means ± S.E.M. for at least four preparations of extract. □, Liver extract; ■, muscle extract.

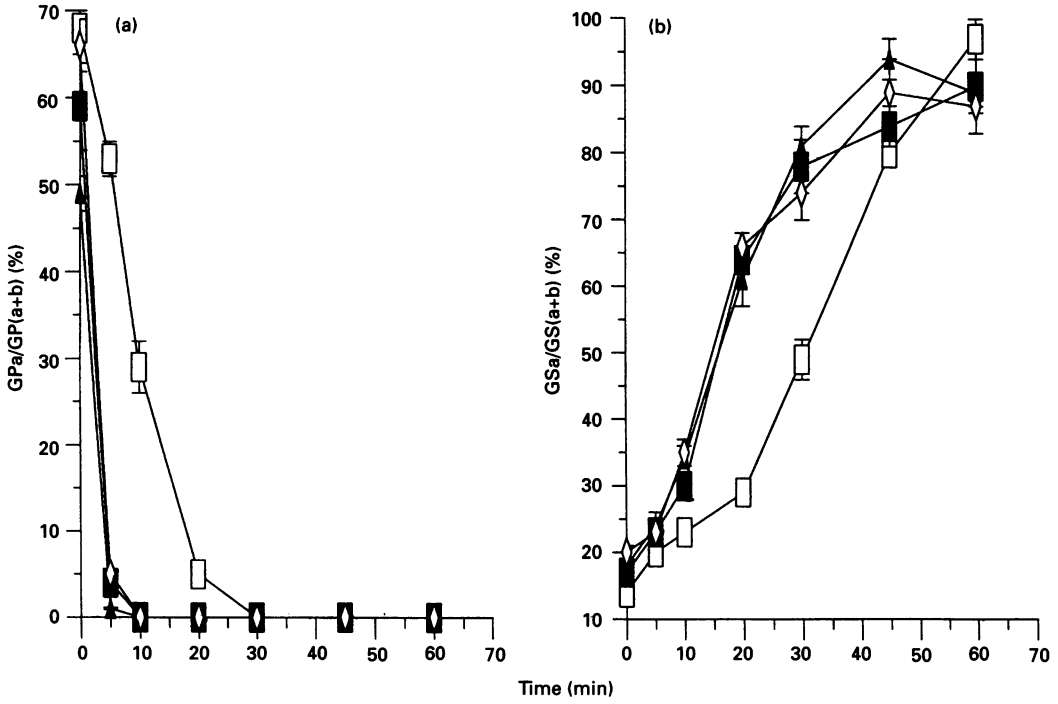

Figure 3 GP and GS activities in liver extracts

(**a**) GP activity in gel-filtered liver extracts represented by GPa activity as a percentage of total GP (a + b); (**b**) GS activity in gel-filtered liver extracts represented by GSa activity as a percentage of total GS (a + b). Data were obtained from a 60 min incubation of liver extract that had been filtered through Sephadex G-25. Further experiments showed that under similar conditions, 0.8 mM 1-GlcNAc and 50 mM glucose were equipotent (not shown). Rats fed *ad libitum* were treated with glucagon 10 min before death. Symbols are as for Figure 1.

substrates, uptake of glucose was 67 nmol/min per 10^6 cells and that of 1-GlcNAc 49 nmol/min per 10^6 cells or 73% of the glucose rate. The lower rates of uptake of 1-GlcNAc compared with glucose may indicate that the specificity of the GLUT-2 glucose transporter present in hepatocytes, although broad, does not extend to carrying 1-GlcNAc as easily as glucose. Alternatively, it may be that 1-GlcNAc fails to mimic the action of glucose in increasing the density of GLUT2 glucose transporters [39]. It is possible that rates of uptake limit the intracellular concentration of 1-GlcNAc by failing to equilibrate extra- and intra-cellular pools.

Effects of glucose and 1-GlcNAc on gel filtrates of homogenized tissue, rather than whole cells, were more dramatic for both GP inactivation and GS activation (Figures 3a and 3b). At a 2 mM concentration, 1-GlcNAc produced an inactivation of GPa that was approximately as effective as that produced by 50 mM glucose and inactivation by both effectors was complete within 10 min (Figure 3a). In the whole hepatocytes, similar inactivation was only achieved after 20–40 min. The fact that a response is seen more quickly with the gel-filtered extracts is probably at least partly due to the removal of the cell membrane and the process of transport. In addition, the action of GP phosphatase is opposed by that of GP kinase in the whole hepatocytes but not in the gel-filtered extracts and this may contribute to the accelerated inactivation of GP in the cell-free system. In contrast with the effects in whole hepatocytes, when gel-filtered extracts were incubated with 1-GlcNAc, activation of GS was observed (Figure 3b). Both 2 mM 1-GlcNAc and 50 mM glucose produced an approx. 80% activation within 30 min. Further studies established that, both in terms of inactivation of GP and activation of GS, 0.8 mM 1-GlcNAc and 50 mM glucose were equipotent (results not shown). This indicates an approx. 60-fold improvement and is presumably due to the increased efficiency with which the analogue binds to GPa and stimulates the GP phosphatase reaction compared with glucose.

1-GlcNAc has been shown to be a weak inhibitor of both rat liver hexokinase and glucokinase when enzyme activity is assayed using a glucose substrate [38]. This raises the possibility that 1-GlcNAc itself may be a substrate for either or both of these kinases. However, hexokinase activity cannot be detected in isolated hepatocytes [40] and further studies confirmed that only glucokinase (and not hexokinase) activity could be detected in the cell preparations of the present study (results not shown). Thus, if 1-GlcNAc is phosphorylated in hepatocytes, it is probably because it is a substrate for glucokinase. Experiments in which radiolabelled 1-GlcNAc was incubated with whole hepatocytes followed by separation of products by ion-exchange DEAE-cellulose chromatography showed that a negatively charged derivative of 1-GlcNAc accumulated in a concentration- and time-dependent manner (Figures 4a–4c). 1-GlcNAc 6-phosphate is the only possible negatively charged metabolite of 1-GlcNAc. Indeed, further metabolism of 1-GlcNAc 6-phosphate (except dephosphorylation) is not possible, as the C-1 position is blocked by the *N*-acetyl substituent and hence the pyranose ring cannot be opened. This precludes the possibility that the 1-GlcNAc 6-phosphate could follow any of the fates of glucose 6-phosphate, such as conversion into the 1-phospho derivative by phosphoglucomutase, conversion into the 6-phosphoketohexose by glucose-6-phosphate isomerase or entry into the pentose phosphate pathway through the irreversible (via glucose-6-phosphate dehydrogenase) or reversible (via transketolase/transaldolase) routes.

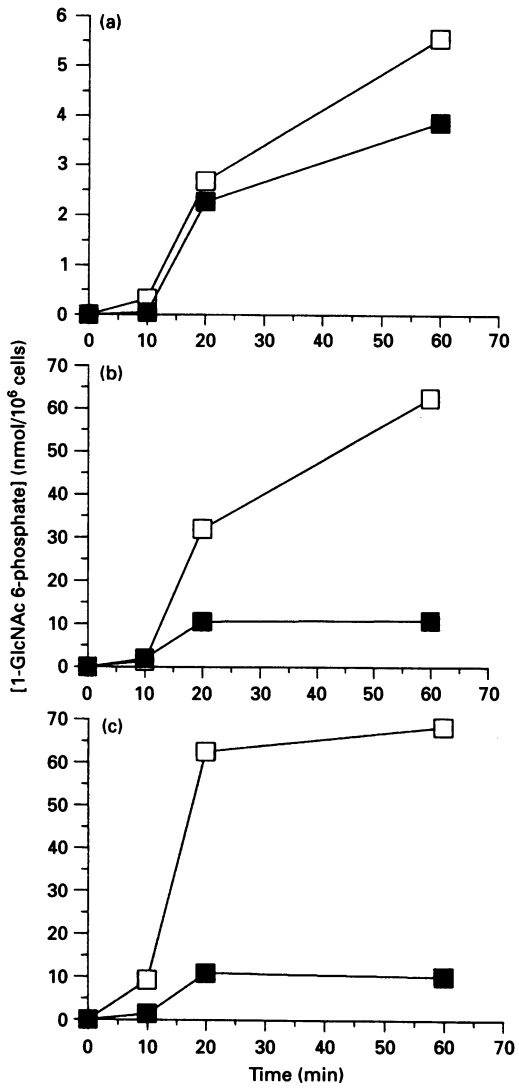

Figure 4 Time-dependent accumulation of 1-GlcNAc 6-phosphate by isolated hepatocytes

Hepatocytes were incubated with 1-GlcNAc containing a trace of 1-[^{14}C]GlcNAc and amounts of 1-GlcNAc 6-phosphate measured at the time points indicated (see the Materials and methods section for full details). Data points are means ± S.E.M. for incubation of at least three separate preparations. S.E.M. values ranged from 0.01 to 0.5 nmol/10^6 cells and are, in most cases, obscured by the symbols. (**a**) 10 mM 1-GlcNAc ± 50 μM ITU; (**b**) 20 mM 1-GlcNAc ± 50 μM ITU; (**c**) 50 mM 1-GlcNAc ± 50 μM ITU. □, No ITU; ■, 50 μM ITU.

Phosphorylation reached maximal rates at 20 min and thereafter declined, indicating either that saturating levels of 1-GlcNAc phosphate had been reached or that it is a substrate for glucose-6-phosphatase. The kinase inhibitor, ITU [32], inhibited the phosphorylation of 1-GlcNAc by isolated hepatocytes at all concentrations of 1-GlcNAc tested (Figures 4a–4c). The severity of inhibition increased with concentration. Amounts of phosphorylated analogue present after 20 min were 16% lower at 10 mM 1-GlcNAc, 52% lower at 20 mM and 82% lower at 50 mM. After 60 min, effects of ITU were even more pronounced, with amounts of 1-GlcNAc phosphate being 30% lower at 10 mM, 83% lower at 20 mM and 85% lower at 50 mM.

The process of gel filtration removes ATP from liver extracts, thereby preventing kinase, including glucokinase, activity. Thus 1-GlcNAc must remain in its native dephosphorylated state in gel-filtered extracts and, under these conditions, activation of GS is rapid and potent (Figure 3b). The formation of 1-GlcNAc 6-phosphate in incubated hepatocytes is the most likely explanation for the failure to activate GS in whole cells (Figure 1b; discussed below).

The results of preactivation of GS using 50 mM glucose followed by addition of 10 mM 1-GlcNAc after 30 min are shown in Figure 5(a). Under these conditions, it was found that 1-GlcNAc reversed the glucose-induced activation of GS. An alternative means of preactivation used during the present work is through ITU, an adenosine analogue that causes activation of GS and inactivation of GP [41], apparently by inhibiting GS kinase and phosphorylase kinase [32], plus PG, a promoter of glycogen synthesis [33]. In the presence of ITU, subsequent addition of 1-GlcNAc had no effect on the activation state of GS (Figure 5b).

The enzyme-inhibitory action of ITU on glucokinase [32] may explain the difference in effects of subsequent addition of 1-GlcNAc seen in Figures 5(a) and 5(b). Further studies established that there was a 5-fold difference in amounts of 1-GlcNAc 6-phosphate present at the $t = 60$ min time point of Figure 5(a) (5.2 nmol/10^6 cells) compared with amounts at the $t = 60$ min time point of Figure 5(b) (1 nmol/10^6 cells). It appears probable that addition of the analogue after glucose-stimulated activation of GS resulted in formation of 1-GlcNAc 6-phosphate by the action of glucokinase, and 1-GlcNAc 6-phosphate then reversed the glucose-induced activation of GS (as seen in Figure 5a). Formation of 1-GlcNAc 6-phosphate may also explain the observations in Figures 1(a) and 1(b) where the presence of 1-GlcNAc achieved potent inactivation of GP, but no activation of GS. In Figure 5(b), addition of 1-GlcNAc had no effect on the ITU-induced activation of GS and under these conditions formation of 1-GlcNAc 6-phosphate was severely inhibited. A similar explanation may be proposed for the data presented in Figures 3(a) and 3(b) where filtration of the liver extract ensures that ATP-dependent glucokinase activity cannot occur. Thus the different responses of GS in whole hepatocytes and gel-filtered extracts may be due to the presence or absence of 1-GlcNAc 6-phosphate.

DISCUSSION

The results of the present study show that 1-GlcNAc is an effective regulator of the activation state of GP, both in whole hepatocytes and cell extracts, and promotes the dephosphorylation of GPa to GPb. Previous work had established that 1-GlcNAc is a more potent competitive inhibitor of GP than glucose [23,38], and results of the present work demonstrate that a more potent inhibitor of GP constitutes a more potent activator of GP phosphatase activity. GP has a K_i of 30 μM for 1-GlcNAc which is 367-fold lower than that for glucose and, at 1 mM, 1-GlcNAc causes 600% activation of hepatic GP phosphatase activity, 500-fold more potent than the effects of glucose. A likely mechanism, supported by the present results which demonstrate enhanced GP phosphatase activity in the presence of 1-GlcNAc, would be that 1-GlcNAc binds tightly to GPa, promoting the R- to T-state transition and rendering GPa a better substrate for its phosphatase. These effects on GP phosphatase and GP are consistent with the sequential mechanism proposed by Hers [6] and Stalmans [7] for glucose regulation of glycogen metabolism.

The effects of 1-GlcNAc on GS activation are complex. In gel-filtered liver extracts, 1-GlcNAc promoted GS activation after a lag period, with 0.8 mM 1-GlcNAc being approximately

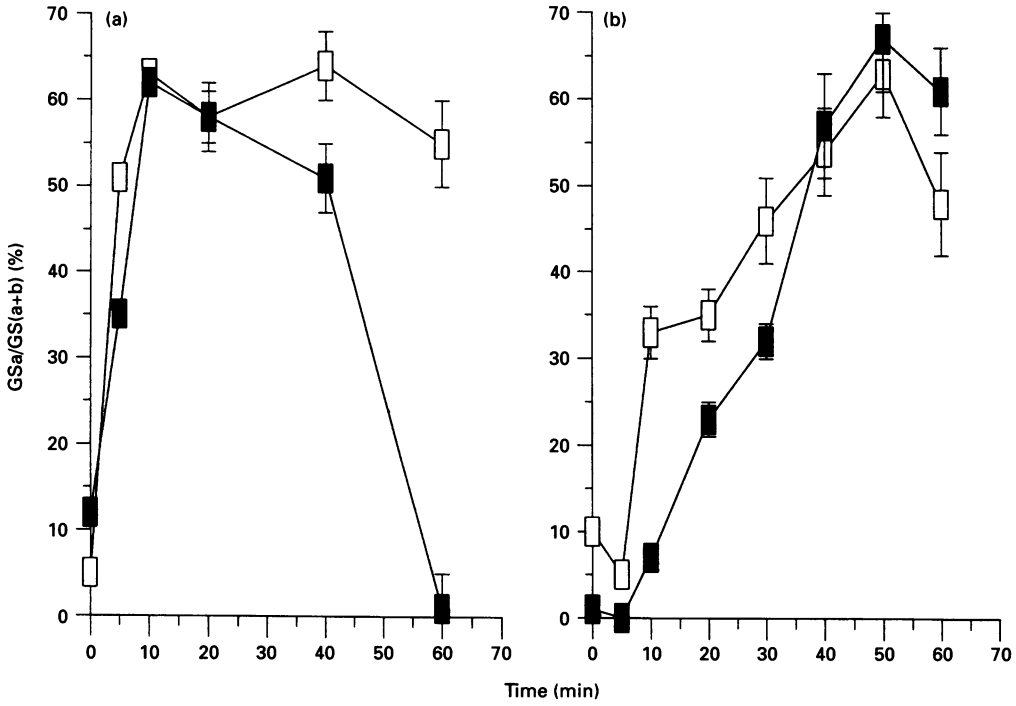

Figure 5 Effects of 1-GlcNAc after prior activation of GS in hepatocytes

GS was preactivated with (**a**) 50 mM glucose or (**b**) 50 μM ITU/10 μM PG. 1-GlcNAc (10 mM) was added after 30 min. □ No 1-GlcNAc; ■ 10 mM 1-GlcNAc

equipotent with 50 mM glucose. The lack of ATP in the extracts precludes both protein kinase activity, so that the action of PP1-G is unopposed by that of GS kinase, and also glucokinase activity. Thus there can be no formation of hexose 6-phosphate whether GS activation is achieved in the presence of glucose or 1-GlcNAc (Figures 3a and 3b). Under these conditions, activation of GS is closely correlated with inactivation of GP after the initial lag period. This observation is consistent with the Hers–Stalmans hypothesis which predicts that the removal of GPa by stimulation of GP phosphatase relieves the GPa-induced inhibition of PP1-G. Thus PP1-G is made available to dephosphorylate and activate GS.

However, no activating effect of 1-GlcNAc on GS was observed in whole hepatocytes despite rapid inactivation of GPa (Figures 1a and 1b). Massillon et al. [42] report similar results with the metabolically inert GP inhibitor 2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride which elicited potent inactivation of GP but no change in the activation state of GS. These authors concluded that inactivation of GP is not, in itself, sufficient to elicit activation of GS in whole cells. Further insight into the mechanisms of GS activation has been provided by other work. Using a series of 6-phosphorylatable and non-phosphorylatable glucose analogues, Carabaza et al. [43] were able to demonstrate that only the former resulted in GS activation in hepatocytes. Observations on the effects of phosphorylatable glucose analogues have led to the conclusion that glucose itself must be converted into glucose 6-phosphate before activation of GS may be achieved [43] and levels of glucose 6-phosphate appear to correlate with the activation state of GS [42].

The present work demonstrates that 1-GlcNAc is modified in whole hepatocytes and that the product must be 1-GlcNAc 6-phosphate. Under conditions in which 1-GlcNAc 6-phosphate is formed, activation of GS may not be achieved. This observation may explain why addition of 1-GlcNAc to hepatocytes in which GS had been previously activated with glucose resulted in reversal of this activation but if activation had been achieved via ITU, 1-GlcNAc had no effect. Levels of 1-GlcNAc 6-phosphate present after 60 min in the incubation with glucose were 5-fold greater (5.2 nmol/10^6 cells) than in the incubation with ITU (1 nmol/10^6 cells) because of inhibition of glucokinase under the latter conditions. Thus reversal of GS activation appears to be correlated with 1-GlcNAc 6-phosphate formation. Such a reversal of GS activation is not observed with the non-phosphorylatable 2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride [42]. In conclusion, it appears that 1-GlcNAc 6-phosphate antagonizes the glucose 6-phosphate-led activation of GS.

The molecular basis for this phenomenon is speculative but could involve either an effect on one of the interconverting activities or a direct effect on the GS enzyme molecule. It may be that 1-GlcNAc 6-phosphate has an activating effect on GS kinases although the present results, which show dramatic reductions in GPa in the presence of either 1-GlcNAc or 1-GlcNAc 6-phosphate, indicate no activation of GP kinase which is one of the kinases involved. Similarly, the analogue may inhibit PP1-G despite its potent activating effect on GP phosphatase. An alternative explanation involves a direct interaction between 1-GlcNAc 6-phosphate and GS. 1-GlcNAc 6-phosphate antagonizes the glucose 6-phosphate-led activation of GS and this may be the result of a competitive effect if both glucose 6-phosphate and 1-GlcNAc 6-phosphate bind to the same site on GS. In contrast with the results obtained with glucose incubations, subsequent addition of 1-GlcNAc had no effect on the ITU-induced activation of GS. Under these conditions, rates of formation of 1-GlcNAc 6-phosphate are much reduced. Thus

if competition between 1-GlcNAc 6-phosphate and glucose 6-phosphate is responsible for the reversal of the glucose-stimulated activation of GS, such an effect would not be observed on incubation with ITU. When hepatocytes are incubated with 1-GlcNAc, inactivation of GP is observed, indicating that either both 1-GlcNAc and 1-GlcNAc 6-phosphate can cause inactivation or, at least, the presence of 1-GlcNAc 6-phosphate does not interfere with the 1-GlcNAc-induced inactivation of GP.

The present work demonstrates that activation of GS may be separated from inactivation of GP and is in agreement with previous work [42,43]. Mechanisms of activation of GS may be more complex than the sequential hypothesis implies and may represent a balance between the disappearance of GPa and the level of glucose 6-phosphate in the intact cell. Despite the failure to achieve activation of GS in the presence of 1-GlcNAc 6-phosphate, further work has shown that the reduction in rates of glycogen breakdown brought about by inhibiting GP may be sufficient to protect glycogen stores and stimulate glucose utilization. 1-GlcNAc stimulates rates of glycogen synthesis by isolated hepatocytes and has a conservative effect on glycogen stored by incubated cells [44]. The positive effect on glycogen storage contributes to stimulation by 1-GlcNAc of rates of uptake of exogenous glucose by isolated hepatocytes (47% at 20 mM 1-GlcNAc and 30 mM glucose). This indicates that there is a potential for use of compounds such as 1-GlcNAc for the control of hyperglycaemia in Type-II diabetes.

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