The effect of glucose on the potency of two distinct glycogen phosphorylase inhibitors

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Two distinct glycogen phosphorylase inhibitors, 5-chloro-1*H*-indole-2-carboxylic acid [1-(4-fluorobenzyl)-2-(4-hydroxypiperidin-1-yl)-2-oxoethyl]amide (CP-320,626) and 1,4-dideoxy-1,4-*D*-arabinitol (DAB), were characterized *in vitro* with respect to the influence of glucose on their potencies. CP-320,626 has previously been shown to bind to a newly characterized indole site, whereas DAB seems to act as a glucose analogue, but with slightly different properties from those of glucose. When analysed in pig liver glycogen phosphorylase a (GP_a) activity assays, the two inhibitors showed very different properties. When GP_a activity was measured in the physiological direction (glycogenolysis), DAB was the most potent inhibitor with an IC₅₀ value of 740 ± 9 nM compared with the IC₅₀ value for CP-320,626 of 2.39 ± 0.37 μM. There was no effect of glucose on the inhibitory properties of DAB, whereas a glucose analogue *N*-acetyl-β-*D*-glucopyranosylamine (1-GlcNAc) antagonized the effect of DAB. Likewise, there was no synergistic effect of CP-320,626 and glucose, whereas CP-320,626 and 1-GlcNAc inhibited GP_a in synergy. Moreover, the synergistic effect of glucose and CP-320,626 was GP_a-isoform-specific, since CP-320,626 and glucose

inhibited rabbit muscle GP_a in synergy when the GP_a activity was measured towards glycogenolysis. When GP_a activity was measured towards glycogen synthesis, CP-320,626 showed a synergistic effect with glucose, whereas the effect of DAB was slightly antagonized by glucose in this assay direction. Caffeine was included in the investigation as a control GP inhibitor, and both glucose and 1-GlcNAc potentiated the effect of caffeine independent of the assay direction. In primary cultured rat hepatocytes 1-GlcNAc and CP-320,626 inhibited basal and glucagon-induced glycogenolysis in synergy, whereas the ability of DAB to inhibit basal or glucagon-induced glycogenolysis was unaltered by 1-GlcNAc. Glucose had no effect on either CP-320,626 or DAB inhibition of glycogenolysis in cultured rat hepatocytes. In conclusion, the present study shows that the two GP inhibitors are kinetically very distinct and neither of the inhibitors demonstrates a physiologically relevant glucose dependence *in vitro*.

Key words: caffeine and cultured hepatocyte, CP-320,626, glucose analogue, 1,4-dideoxy-1,4-imino-*D*-arabinitol.

INTRODUCTION

In type 2 diabetes hepatic insulin resistance, hyperglucagonaemia and an increased level of gluconeogenic precursors are key factors for the increased hepatic glucose production seen both in the fasting and postprandial states [1,2]. In the liver, glucose is produced from two pathways: gluconeogenesis (GNG) and glycogenolysis (breakdown of glycogen). The relative contribution of glycogenolysis and GNG to the overall hepatic glucose production has been the focus of many studies in human. However, the studies have resulted in diverging estimates, dependent on the applied method and the length of fasting and the subjects studied [3–7]. Therefore it is not obvious whether inhibition of hepatic glycogenolysis can normalize the increased hepatic glucose production in type 2 diabetes. However, inhibition of glycogen phosphorylase (GP) (EC 2.4.1.1), the rate-controlling enzyme of glycogenolysis, has been shown to lower blood glucose levels in an animal model of type 2 diabetes [8–10], supporting the hypothesis that inhibition of GP could be a potential pharmacological target for lowering hyperglycemia in patients with type 2 diabetes. Furthermore, studies have shown that a substantial portion of the GNG appears to be cycled through the glycogen pool before efflux from the liver [11]. Also, in hepatocytes a GP inhibitor (CP-92,149) has been shown to decrease glucose production from lactate [8]. Therefore a gly-

cogen phosphorylase inhibitor could in principle inhibit glucose from both pathways.

Glucose is a physiological regulator of hepatic glycogen metabolism via its effects on both glycogen synthesis and glycogen degradation. Glucose, which binds to the active site of GP_a (the phosphorylated and active form of GP), inhibits GP_a in a competitive manner by promoting a conformational change of GP_a to the less active T-state [12,13]. The T-state is also a better substrate for protein phosphatase-1, the enzyme responsible for the inactivation of GP_a to GP_b by dephosphorylation. It has been shown that the glucose analogue *N*-acetyl-β-*D*-glucopyranosylamine (1-GlcNAc), which also binds to the glucose binding site, is a much more potent inhibitor of GP_a than glucose [14,15]. Moreover, glucose and 1-GlcNAc were both able to potentiate the inhibitory action of caffeine on GP_a, with 1-GlcNAc being more potent [14]. Another glucose analogue, 1,4-dideoxy-1,4-*D*-arabinitol (DAB), has recently been shown to be an inhibitor of GP_a both *in vitro* and *in vivo* [10,16]. From a mechanistic point of view, DAB possesses features other than those of glucose with respect to GP_a inhibition [10]. For example, DAB reverses the inhibitory effect of caffeine [10], whereas 1-GlcNAc and caffeine inhibit GP_a in synergy [15].

5-Chloro-1*H*-indole-2-carboxylic acid [1-(4-fluorobenzyl)-2-(4-hydroxypiperidin-1-yl)-2-oxoethyl]amide (CP-320,626), another potent inhibitor of GP, was found to bind to a new

Abbreviations used: CP-320,626, 5-chloro-1*H*-indole-2-carboxylic acid [1-(4-fluorobenzyl)-2-(4-hydroxypiperidin-1-yl)-2-oxoethyl]amide; DAB, 1,4-dideoxy-1,4-imino-*D*-arabinitol; 1-GlcNAc, *N*-acetyl-β-*D*-glucopyranosylamine; GNG, gluconeogenesis; GP, glycogen phosphorylase.

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allosteric site (the indole binding site) [17,18]. One of the interesting features of this class of compounds is that their potencies were found to be glucose dependent [8] in a way similar to caffeine [19]. These types of compounds could, in theory, therefore be more effective GP inhibitors in the presence of high plasma glucose compared with low plasma glucose. Thereby, treatment of type 2 diabetes with such compounds would diminish the risk of hypoglycaemia, a major concern of patients with type 2 diabetes.

In the light of the possible role of glucose as a modulator of the potency of GP inhibitors and the physiological relevance of this, the present investigation was undertaken to characterize this in further detail by examining the influence of glucose and 1-GlcNAc on the two GP inhibitors.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats were obtained from Møllegaard Breeding Center (Lille Skensved, Denmark). Female Yorkshire pigs were obtained from Ellegaard Breeding Center (Dalmose, Denmark). Tissue culture flasks and dishes were purchased from Nunc A/S (Roskilde, Denmark). Collagenase Type IV, collagen Type I (rat tail), glucose oxidase kit, rabbit muscle GP and all other enzymes and chemicals were purchased from Sigma and Boehringer Mannheim (Hørsholm, Denmark). Dexamethasone was purchased from MSD (Haarlem, The Netherlands). Medium 199, foetal calf serum, Trypan Blue, penicillin and streptomycin were purchased from Life Technologies (Roskilde, Denmark). Human insulin and glucagon were from Novo Nordisk A/S (Bagsvaerd, Denmark). Scintillation fluid Ultima Gold was from Packard Instrument Co. (Meriden, CT, U.S.A.). Protein Assay kit was from Bio-Rad (Copenhagen, Denmark). CP-320,626 was synthesized at Novo Nordisk A/S (Måløv, Denmark) according to procedures described by Hoover et al. [9]. DAB and 1-GlcNAc were synthesized at Novo Nordisk A/S as well.

GP activity

GP_a (GP_b < 5%) from pig liver was prepared as described previously by Fosgerau et al. [10]. Fed male Wistar rats (approx. 200 g) were anaesthetized with a freshly prepared mixture of Hypnorm (0.05 mg/ml fentanyl + 2.5 mg/ml flubazone) and Dormicum (1.25 mg/ml midazolam) (1:1) administered subcutaneously (1 ml/kg). After sedation, 50 µg of glucagon was administered intraperitoneally in order to activate GP_a; 4 min later the livers were perfused *in situ* through the vena porta with 60 ml ice-cold buffer (100 mM glycylglycine, 100 mM NaF and 1 mM EDTA), pH 7.4. The liver was excised and homogenized with a Polytron PT3000 in 30 ml buffer. The unbroken cells and cell debris were separated from the liver homogenate by centrifugation at 8000 *g* for 10 min at 2 °C. Then the supernatant was centrifuged at 40000 *g* for 120 min at 2 °C. After this step, a glycogen pellet containing GP_a was homogenized in 5 ml of 1 mM EDTA and stored at –80 °C until assayed.

Glycogen phosphorylase activity (GP_a) was measured in the direction of glycogenolysis by determination of NADPH in an assay coupled to phosphoglucomutase and glucose-6-phosphate dehydrogenase. The enzyme activity was assayed at pH 6.8 and 25 °C in a phosphate buffer containing KH₂PO₄ (18 mM), Na₂HPO₄ (27 mM), MgCl₂ (15 mM), EDTA (100 µM), NADP⁺ (340 µM), glucose 1,6-bisphosphate (4 µM), glucose-6-phosphate dehydrogenase (6000 units/l), phosphoglucomutase (800 units/l) and glycogen (2 mg/ml) [20]. DAB, CP-320,626, caffeine, glucose or 1-GlcNAc had no effect on the enzymes coupled to this assay.

Glycogen phosphorylase (GP_a) activity was also measured in the direction of glycogen synthesis using 0.5 mM glucose-1-phosphate as the substrate in a *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulphonic acid (50 mM) buffer at pH 6.8, containing glycogen (0.125%) and EDTA (1 mM) [21]. GP activity was determined at 25 °C by using purine nucleoside phosphorylase and 7-methyl-6-thioguanoside as the auxiliary enzyme and substrate respectively. The product of the reaction, 7-methyl-6-thioguanine, was measured spectrophotometrically at 360 nm. DAB, CP-320,626, caffeine, glucose or 1-GlcNAc had no effect on the coupling enzyme.

Isolation and culturing of hepatocytes

Male Sprague–Dawley rats (approx. 200 g) were anaesthetized with a freshly prepared mixture of Hypnorm (0.05 mg/ml fentanyl + 2.5 mg/ml flubazone) and Dormicum (1.25 mg/ml midazolam) (1:1) administered subcutaneously (1 ml/kg). Hepatocytes were isolated from rats fed *ad lib.* using a two-step perfusion technique essentially as described by Seglen [22]. Cell viability, assessed by Trypan Blue exclusion, was consistently greater than 80%. Cells were plated on to collagen-coated 12-well plates in basal medium (Medium 199 containing 5.5 mM glucose and 0.1 µM dexamethasone, 100 units/ml penicillin, 100 mg/ml streptomycin and 1 nM insulin) supplemented with 4% foetal calf serum at a cell density of 330 000 cells/well. The medium was replaced with a basal medium 1 h after initial plating in order to remove dead cells, and cells were cultured for 24 h. To study glycogenolysis, after the initial 24 h, the medium was replaced with basal medium supplemented with 9.5 mM glucose and 9 nM insulin to build up glycogen reserves, and experiments were performed 20 h later in the media defined below.

Protein content in the culture dishes was determined after solubilization of the cells in 0.1 M NaOH, using the Bio-Rad protein assay and human serum albumin as standard.

Glycogenolysis

After 20 h of glycogen synthesis, hepatocytes were washed twice with pre-warmed (37 °C) buffer A [117.6 mM NaCl/5.4 mM KCl/0.82 mM Mg₂SO₄/1.5 mM KH₂PO₄/20.0 mM Hepes/9.0 mM NaHCO₃/0.1% (w/v) human serum albumin/2.25 mM CaCl₂ (pH 7.4)] and incubated in buffer A containing increasing concentrations of CP-320,626 or DAB (range 0–20 µM) in the absence (basal conditions) or presence of 2 nM glucagon (stimulated conditions) for 180 min. To determine whether glucose could influence the potency of the GP inhibitors, a non-metabolizable glucose analogue (1-GlcNAc) was used as a surrogate for glucose. 1-GlcNAc is phosphorylated but not further metabolized [23]. Consequently, glucose released into the medium could be measured directly. Basal and glucagon-induced glycogenolysis were measured as glucose released into buffer A, by using a glucose oxidase kit from Sigma (510-A). Lactate in the medium was determined using the Sigma lactate reagent (735-10). Glycogen levels were determined after two rapid washes of cells with ice-cold 0.9% (w/v) NaCl by amyloglucosidase (exo-1,4- α -D-glucosidase) digest, as described by Gomez-Lechon et al. [24].

To determine the effect of glucose on the potency of the two inhibitors, hepatocytes were pre-incubated with 15 mM glucose traced with [U-¹⁴C]glucose (0.5 µM · Ci · ml^{–1}) and 10 nM insulin to build up labelled [¹⁴C]glycogen. After 20 h of incubation with radiolabelled glucose, cells were washed three times in pre-warmed 0.9% NaCl and incubated in the presence of increasing concentrations of CP-320,626 or DAB (range 0–25 µM) and

glucose (0–12.5 mM) in glucose-free medium 199 in the presence of 0.5 nM glucagon at 37 °C in 5% CO₂. After incubation for 90 min, the medium was transferred to scintillation vials and ¹⁴C radioactivity was determined by a scintillation counter.

Statistical analyses

Statistical analyses were performed using a one- or two-tailed paired Student's *t* test (homoscedastic/heteroscedastic, depending upon variance). Statistical significance was evaluated and denoted as follows: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Replicates (*n*) were defined as separate hepatocyte preparations and for the enzyme assays as separate determinations. Results are presented as means ± S.E.M.

RESULTS

The roles of glucose and 1-GlcNAc on the inhibitory actions of DAB, CP-320,626 and caffeine were investigated when GP_a was analysed in the direction of glycogenolysis. The IC₅₀ values

Figure 1 Inhibition of pig liver GP_a by DAB, CP-320,626 and caffeine measured in the direction of glycogenolysis

Pig liver GP_a activity was measured in the direction of glycogenolysis with increasing concentrations of DAB (A), CP-320,626 (B) and caffeine (C) with no additives (■), with 5 mM glucose (▲) and with 5 mM 1-GlcNAc (◆). Results are means ± S.E.M. for three independent experiments.

Figure 2 Potency of DAB, CP-320,626 and caffeine on the GP_a activity in the presence of glucose or 1-GlcNAc measured in the direction of glycogenolysis

Pig liver GP_a activity was measured in the direction of glycogenolysis. (A) The effect of glucose on the potencies of DAB (■), CP-320,626 (▲) and caffeine (▼) was determined at various glucose concentrations. (B) The effect of 1-GlcNAc on the potency of DAB (■), CP-320,626 (▲) and caffeine (▼) was determined at various 1-GlcNAc concentrations; 100% corresponds to 740 ± 9 nM (DAB), 2.39 ± 0.37 μM (CP-320,626) and 20.33 ± 0.93 mM (caffeine). The calculated IC₅₀ values were normalized to 100% by dividing the IC₅₀ values with the IC₅₀ values obtained in the absence of glucose or 1-GlcNAc. The IC₅₀ values are plotted as a function of glucose or 1-GlcNAc concentrations. Results are means ± S.E.M. for three independent experiments.

without effectors were calculated from Figure 1 to be 740 ± 9 nM (DAB), 2.39 ± 0.37 μM (CP-320,626) and 20.33 ± 0.93 mM (caffeine).

The effect of DAB on GP_a activity measured in the presence of glucose (5 mM) or 1-GlcNAc (5 mM) is shown in Figure 1(A). Glucose had no effect on the potency of DAB, whereas 1-GlcNAc decreased the potency by half at 5 mM. There was no synergistic effect of glucose on the potency of CP-320,626 (Figure 1B), whereas 1-GlcNAc was able to increase the potency of CP-320,626 by 10-fold at 5 mM. To verify the results obtained on GP_a activity, when measured in the direction of glycogenolysis, caffeine was used as control inhibitor. Caffeine showed synergy with both glucose and 1-GlcNAc in this experimental set-up (Figure 1C). In the absence of DAB, CP-320,626 and caffeine, glucose (5 mM) and 1-GlcNAc (5 mM) inhibited GP_a by 5 and 50% respectively. It is therefore not possible to calculate an IC₅₀ value for GP_a inhibition by glucose, whereas the IC₅₀ for 1-GlcNAc must be close to 5 mM.

Figure 2(A) shows the relative IC₅₀ values for DAB, CP-320,626 and caffeine in the presence of increasing concentrations of glucose. There was no effect of glucose on the potency of DAB or CP-320,626, whereas the effect of caffeine was potentiated

Table 1 IC_{50} values for inhibition of rabbit muscle GPa by CP-320,626 and caffeine

Rabbit muscle GPa activity was measured in the direction of glycogenolysis. Results are means $\pm$ S.E.M. for three independent experiments.

	Glucose (mM)		
	0	5	15
CP-320,626	590 $\pm$ 30 nM	305 $\pm$ 30 nM	82 $\pm$ 10 nM
Caffeine	727 $\pm$ 25 μ M	180 $\pm$ 9 μ M	83 $\pm$ 2 μ M

Figure 3 Potency of DAB, CP-320,626 and caffeine on GPa activity in the presence of glucose or 1-GlcNAc measured in the direction of glycogen synthesis

Pig liver GPa activity was measured in the direction of glycogen synthesis. (A) The effect of glucose on the potencies of DAB (■), CP-320,626 (▲) and caffeine (▼) was determined at various glucose concentrations. (B) The effect of 1-GlcNAc on the potencies of DAB (■), CP-320,626 (▲) and caffeine (▼) was determined at various 1-GlcNAc concentrations; 100% corresponds to 233 $\pm$ 6 μ M (DAB), 465 $\pm$ 1 nM (CP-320,626) and 642 $\pm$ 7 nM (caffeine). The calculated IC_{50} values were normalized to 100% by dividing the IC_{50} values by the IC_{50} values obtained in the absence of glucose or 1-GlcNAc. The IC_{50} values are plotted as a function of glucose or 1-GlcNAc concentrations. Results are means $\pm$ S.E.M. for three independent experiments.

significantly by glucose. Nevertheless, both CP-320,626 and caffeine inhibited rabbit muscle GPa in synergy with glucose when GPa was analysed in the direction of glycogenolysis (Table 1). Figure 2(B) shows that 5 mM 1-GlcNAc antagonized the effect of DAB, whereas 1 mM 1-GlcNAc had no effect. The IC_{50} value increased by approx. 1.8-fold at 5 mM 1-GlcNAc. On the other hand, CP-320,626 and caffeine inhibited GP in synergy with 1-GlcNAc at both 1 and 5 mM.

Figure 4 Effect of DAB on basal and glucagon-stimulated glycogenolysis in the presence or absence of 1-GlcNAc in primary rat hepatocytes

Hepatocytes were incubated in a glucose-free buffer for 3 h as described in the Methods section. Open symbols indicate experiments performed in the absence of 1-GlcNAc and closed symbols correspond to experiments performed in the presence of 1 mM 1-GlcNAc. (A) Subsequently, basal (□, ■) and glucagon-stimulated (○, ●) glucose production was measured. 100% corresponds to basal glucose production in the absence of DAB and 1-GlcNAc and is equal to 543 $\pm$ 117 nmol glucose/mg protein. (B) Basal (□, ■) and glucagon-stimulated (○, ●) lactate production was measured; 100% corresponds to basal lactate production in the absence of DAB and 1-GlcNAc and is equal to 2323 $\pm$ 264 nmol of lactate/mg of protein. (C) After glycogenolysis, glycogen levels after basal (□, ■) and glucagon-stimulated (○, ●) glycogenolysis were determined; 100% corresponds to glycogen retained after basal glucose production in the absence of DAB and 1-GlcNAc and is equal to 2140 $\pm$ 303 nmol of glycosyl unit/mg of protein. Glycogen level before the start of the experiment was 5917 $\pm$ 895 nmol of glycosyl unit/mg of protein. Results are means $\pm$ S.E.M. for three independent experiments.

The potency of DAB has been described previously to be dependent on the assay direction of GPa [10]. DAB was less potent when GPa activity was analysed in the direction of glycogen synthesis. The IC_{50} value increased by two orders of magnitude and was calculated to be 233 $\pm$ 6 μ M in the present study. By contrast, CP-320,626 was approx. 5 times more potent, with an IC_{50} of 465 $\pm$ 1 nM, which was also the case for caffeine, glucose and 1-GlcNAc, with IC_{50} values calculated to be

Figure 5 Effect of CP-320,626 on basal and glucagon-stimulated glycogenolysis in the presence or absence of 1-GlcNAc in primary rat hepatocytes

Hepatocytes were incubated in a glucose-free buffer for 3 h as described in the Methods section. Open symbols indicate experiments performed in the absence of 1-GlcNAc and closed symbols correspond to experiments performed in the presence of 1 mM 1-GlcNAc. (A) Subsequently, basal (□, ■) and glucagon-stimulated (○, ●) glucose production was measured; 100% corresponds to basal glucose production in the absence of CP-320,626 and 1-GlcNAc and equals 846 ± 86 nmol of glucose/mg of protein. (B) Basal (□, ■) and glucagon-stimulated (○, ●) lactate production was measured; 100% corresponds to basal lactate production in the absence of CP-320,626 and 1-GlcNAc and is equal to 2558 ± 687 nmol of lactate/mg of protein. (C) After glycogenolysis, glycogen levels after basal (□, ■) and glucagon-stimulated (○, ●) glycogenolysis were determined; 100% corresponds to glycogen retained after basal glucose production in the absence of CP-320,626 and 1-GlcNAc and is equal to 1900 ± 418 nmol of glycosyl unit/mg of protein. Glycogen level before the start of the experiment was 6464 ± 1396 nmol of glycosyl unit/mg of protein. Results are means $\pm$ S.E.M. for three independent experiments.

642 ± 7 nM, 11.7 ± 0.7 mM and 160 ± 4.8 μ M respectively. Since the potency of the compounds depends on the assay direction, the effect of glucose and 1-GlcNAc on the two inhibitors was studied in the direction of glycogen synthesis. Figure 3(A) shows the relative IC_{50} values for DAB, CP-320,626 and caffeine in the presence of increasing concentrations of glucose. The potency of DAB significantly decreased with glucose concentrations of 3.5, 5 and 10 mM. The IC_{50} value for DAB increased by approx. 2-

Table 2 IC_{50} values in μ M for inhibition of basal and glucagon-induced glycogenolysis in primary rat hepatocytes by DAB and CP-320,626

Hepatocytes were incubated with increasing concentrations of DAB and CP-320,626 in the presence or absence of 2 nM glucagon as indicated in the Table. The IC_{50} values were calculated from Figures 4 and 5. The IC_{50} values for inhibition of glycogenolysis with and without addition of 1 mM 1-GlcNAc are statistically compared. Results are means $\pm$ S.E.M. of three independent experiments.

	Glucagon	DAB	DAB + 1-GlcNAc	CP-320,626	CP-320,626 + 1-GlcNAc
Glucose	—	1.27 ± 0.44	1.55 ± 0.42	0.25 ± 0.10	0.08 ± 0.06
Glucose	+	1.20 ± 0.22	1.56 ± 0.11	1.08 ± 0.63	$0.23 \pm 0.03^*$
Lactate	—	1.94 ± 0.37	2.24 ± 0.39	0.68 ± 0.27	$0.21 \pm 0.14^*$
Lactate	+	1.42 ± 0.30	1.58 ± 0.24	0.81 ± 0.56	$0.19 \pm 0.10^*$
Glycogen	—	0.97 ± 0.21	$3.90 \pm 1.07^*$	0.48 ± 0.27	$0.21 \pm 0.05^*$
Glycogen	+	1.01 ± 0.07	1.15 ± 0.46	1.05 ± 0.29	$0.35 \pm 0.25^*$

* $P < 0.05$.

fold to 488 ± 75 μ M in the presence of 10 mM of glucose. On the other hand, at all tested concentrations of glucose, CP-320,626 and caffeine showed synergy with glucose. The maximal synergistic effect of glucose was obtained at approx. 5 mM, and the IC_{50} values for CP-320,626 and caffeine at this concentration of glucose were calculated to be 110 ± 41 and 232 ± 14 nM respectively; approx. 3 times lower than the value in the absence of glucose in each case. Also, when the glucose analogue 1-GlcNAc was used, the same profiles for the three inhibitors were seen (Figure 3B). The concentration of 1-GlcNAc used in the present study was 10 times lower than the concentration of 1-GlcNAc used in the assay where GP activity was measured towards glycogenolysis, due to the increased potency of 1-GlcNAc. The maximal synergistic effect of 1-GlcNAc on CP-320,626 and caffeine was observed at approx. 100 μ M 1-GlcNAc, which is almost 50 times lower than that seen for glucose, in agreement with published results [13,14].

To characterize the effects of glucose on the two compounds in a functional model system, the inhibitors were tested in primary cultured rat hepatocytes. The hepatocytes were incubated with 1-GlcNAc as a surrogate for glucose, which allowed measurement of endogenous glucose released into the medium. The maximal effect of 1-GlcNAc on glycogenolysis in hepatocytes was reached at 1 mM 1-GlcNAc (results not shown), and therefore this concentration of 1-GlcNAc was used throughout the study. At this concentration of 1-GlcNAc, basal and glucagon-induced glycogenolysis were inhibited by approx. 25% (Figures 4 and 5). Basal and glucagon-stimulated (2.0 nM glucagon) glucose production (A), lactate production (B) and retained glycogen levels (C) after incubation with increasing concentrations of DAB in the presence or absence of 1 mM 1-GlcNAc are shown in Figure 4. The IC_{50} values for inhibition of glycogenolysis by DAB did not change significantly by the addition of 1 mM 1-GlcNAc. The IC_{50} values for inhibition of glucagon-induced glycogenolysis in the absence or presence of 1 mM 1-GlcNAc (Figure 4A) were calculated to be 1.20 ± 0.22 and 1.56 ± 0.11 μ M respectively. Furthermore, the IC_{50} values calculated from Figures 4(B) and 4(C) for basal and glucagon-induced conditions were not significantly different from the IC_{50} values obtained from Figure 4(A), substantiating that 1-GlcNAc has no effect on the potency of DAB in hepatocytes. Basal and glucagon-stimulated (2.0 nM glucagon) glucose production (A), lactate production (B) and retained glycogen levels (C) after incubation with increasing concentrations of CP-320,626 in the presence or absence of 1 mM 1-GlcNAc are shown in Figure 5. The IC_{50} values for

Figure 6 Effect of DAB and CP-320,626 on glucagon-stimulated glycogenolysis in the presence of increasing glucose concentrations in primary rat hepatocytes

Glycogenolysis was measured as c.p.m. released into the medium as described in the Methods section. (A) Hepatocytes were incubated with increasing concentrations of DAB. 100% corresponds to c.p.m. released into the medium in the absence of DAB and glucose and is equal to 5614 ± 2445 c.p.m./well. (B) Hepatocytes were incubated with increasing concentrations of CP-320,626. 100% corresponds to c.p.m. released into the medium in the absence of CP-320,626 and glucose and is equal to 5365 ± 2408 c.p.m./well. The inhibitory effect of DAB and CP-320,626 was measured in the presence of 0 mM glucose (■), 5.5 mM glucose (●), 8.5 mM glucose (▲) and 12.5 mM glucose (▼). Results are means $\pm$ S.E.M. for three independent experiments.

inhibition of glucagon-induced glycogenolysis by CP-320,626 were significantly ($P < 0.05$) decreased when hepatocytes were incubated with 1 mM 1-GlcNAc. IC_{50} values for the inhibition of glucagon-induced glycogenolysis in the absence or presence of 1-GlcNAc were calculated from Figure 5(A) to be 1.08 ± 0.63 and 0.24 ± 0.03 μ M respectively. The same pattern was seen in Figures 5(B) and 5(C), where the IC_{50} values calculated for basal and glucagon-induced conditions were significantly different from the IC_{50} values calculated under the same conditions in the presence of 1-GlcNAc. The IC_{50} values calculated from Figures 4 and 5 are summarized in Table 2.

To further elucidate the effects of glucose on the potency of DAB and CP-320,626 in hepatocytes, cellular glycogen levels were radiolabelled with [$U-^{14}C$]glucose before glycogenolysis. After extensive washes of the hepatocytes, counts released into the medium will mainly be a mixture of [$U-^{14}C$]glucose, [$U-^{14}C$] lactate and, to a minor extent, other carbon-containing metabolites [25]. The amount of glucose and lactate released into the medium has been shown to be a valid measure of glycogenolysis [15]. The counts released into the medium were furthermore inhibited to the same extent each time the experiments were performed, indicating full inhibition of glycogenolysis. Incu-

bation of hepatocytes with increasing concentrations of DAB (A) and CP-320,626 (B) in the presence of increasing glucose concentrations is shown in Figure 6. The increasing concentrations of glucose did not affect the potency of DAB or CP-320,626. The IC_{50} values for DAB in the absence or presence of 12.5 mM glucose were calculated to be 0.97 ± 0.42 and 0.97 ± 0.82 μ M respectively from Figure 6(A). The IC_{50} values for CP-320,626 in the absence or presence of 12.5 mM glucose were calculated to be 0.60 ± 0.06 and 0.76 ± 0.31 μ M respectively from Figure 6(B). Glucose inhibited glycogenolysis in a dose dependent manner, by approx. 30% at 12.5 mM of glucose.

DISCUSSION

Allosteric effectors alter GP_a activity by stabilizing either the active (R state) or the inactive state (T state) [12]. Therefore the presence of different allosteric effectors in the two different assays (glycogenolysis and glycogen synthesis) has a major influence on the kinetics of the two inhibitors DAB and CP-320,626. Nevertheless, the results obtained when GP_a was analysed in the physiological direction (glycogenolysis) correlates with the results obtained in the primary hepatocytes and emphasis should be put on these results.

The kinetics of DAB was influenced by the direction in which the GP_a activity was measured. Whereas glucose had no effect on DAB when GP_a activity was measured in the direction of glycogenolysis, glucose antagonized effects of DAB when GP_a activity was measured towards glycogen synthesis. One possible explanation for this difference could be related to the lack of potency of DAB in the GP_a activity assay measured towards glycogen synthesis. The potency of DAB increased by more than two orders of magnitude when GP_a activity was measured in the direction of glycogenolysis, and in this assay glucose had no effect on the potency of DAB. The presence of inorganic phosphate (P_i) in the assay buffer when GP_a activity was measured towards glycogenolysis may be of significant importance in terms of potency of DAB (N. Westergaard and B. Andersen, unpublished work).

The effect of glucose on the potency of CP-320,626 was also dependent on the direction in which GP_a activity was measured. No synergy was observed between CP-320,626 and glucose when GP_a activity was measured towards glycogenolysis, whereas synergy was found when GP_a activity was measured in the other direction. However, when the kinetics of caffeine was investigated, synergy with glucose was observed in both assay directions. Difference in the behaviour of CP-320,626 and caffeine is probably a result of their different binding sites [16,17]. The observed synergistic effect between CP-320,626 and glucose when GP_a activity was measured in the direction of glycogen synthesis is in agreement with published results on a structural analogue of CP-320,626 [8]. Also, the synergistic effect of caffeine and glucose is well described [19,26].

The IC_{50} values of DAB, CP-320,626 and caffeine obtained when GP_a activity was measured in the two assay directions cannot be directly compared. However, the relative potency of the compounds can be compared. The potency of GP inhibitors are, as shown, highly dependent on the assay direction and conditions. Therefore caution must be exercised when the potency of GP inhibitors described in the literature is compared.

The present study also showed that different isoforms of GP_a behave differently in the presence of glucose. As shown in Table 1, CP-320,626 and caffeine inhibit rabbit muscle GP_a in synergy with glucose when GP_a activity was measured towards glycogenolysis, opposite results being obtained with pig liver GP_a. However, since skeletal muscle has very limited intracellular

concentrations of glucose, the increased potency of CP-320,626 on muscle GP in the presence of glucose has no physiological relevance.

DAB and CP-320,626 have previously been tested in hepatocytes. DAB was found to inhibit hepatic glycogenolysis in primary rat hepatocytes with an IC_{50} value of approx. $1.1 \mu\text{M}$ [15], and CP-320,626 inhibited glycogenolysis in human hepatocytes with an IC_{50} value of $2.7 \mu\text{M}$ [9]. The IC_{50} values for inhibition of glycogenolysis by DAB found in the present study are in accordance with previous reported results [15]. The IC_{50} value for inhibition of glucagon-induced glycogenolysis by CP-320,626 was approx. 3-fold lower in the present study compared with literature results. However, primary rat hepatocytes were used in the present study compared with primary human hepatocytes used in a previous publication [9]. The two inhibitors inhibited glucose and lactate production to the same extent. However, approx. 20% ($260 \pm 6\%$ compared with $210 \pm 7\%$ of the basal value) more glycogen was retained in hepatocytes treated with $20 \mu\text{M}$ CP-320,626 compared with hepatocytes treated with $20 \mu\text{M}$ DAB. A compound structurally related to CP-320,626 was recently found to activate glycogen synthase and thereby to stimulate glycogen synthesis in primary hepatocytes [27]. The stimulatory effect of glycogen synthase has not been observed with DAB ([15]; T. Latsis, B. Andersen and L. Agius, unpublished results). Therefore the higher glycogen level retained after treatment with CP-320,626 might be due to a stimulatory effect on glycogen synthesis by CP-320,626.

The effects of 1-GlcNAc were also studied in hepatocytes in order to compare the results obtained for the enzyme with those from a more physiological system. The potency of CP-320,626 but not DAB was affected by 1 mM 1-GlcNAc. The potency of CP-320,626 was increased by approx. 3-fold in the presence of 1 mM 1-GlcNAc in hepatocytes. This correlates well with the results obtained in the enzyme assay (glycogenolysis), where 1 mM 1-GlcNAc increased the potency of CP-320,626 by 5-fold. The results obtained with 1-GlcNAc and DAB also correlate well, since 1 mM 1-GlcNAc had no effect on DAB in the enzyme assay or in the hepatocytes. According to the results for the enzyme in the present study and the literature, 1-GlcNAc is approx. 50-fold more potent than glucose [22]. Therefore CP-320,626 will probably only inhibit glycogenolysis in synergy with glucose at extremely high concentration of glucose (50 mM) in hepatocytes.

In the second cellular assay, utilizing radiolabelled glucose, neither CP-320,626 nor DAB was affected by glucose, at glucose concentrations up to 12.5 mM glucose. This supports the hypothesis that extremely high concentrations of glucose (50 mM) are required to obtain a synergistic effect of CP-320,626 and glucose. The IC_{50} values calculated using this assay are not significantly different from the IC_{50} values calculated from Figures 4 and 5. It has not been possible to obtain reliable results on glycogen levels after glycogenolysis in this assay since the presence of varying glucose concentrations in this assay has an effect on the glycogen level.

The role of GP inhibitors in the treatment of hyperglycemia in type 2 diabetes is yet to be determined. However, a considerable amount of data suggest *in vivo* efficacy of GP inhibitors in animal models of diabetes [8–10]. Also, first results for human models have been published [28] and new results also confirm that inhibition of GP might have a positive effect on hepatic glycogen synthesis [27] and thereby glucose disposal. Furthermore, a glucose-dependent GP inhibitor could be quite important in terms of avoiding hypoglycemia; however, CP-320,626 showed no synergy with glucose either when GP activity was measured in the direction of glycogenolysis or in cultured hepatocytes.

Likewise, the potency of DAB was unaffected by glucose when GP activity was measured in the direction of glycogenolysis and in cultured hepatocytes. In conclusion, neither CP-320,626 nor DAB is affected by physiologically relevant concentrations of glucose in a functional model system.

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