Diverse effects of two allosteric inhibitors on the phosphorylation state of glycogen phosphorylase in hepatocytes

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Two distinct allosteric inhibitors of glycogen phosphorylase, 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) and CP-91149 (an indole-2-carboxamide), were investigated for their effects on the phosphorylation state of the enzyme in hepatocytes *in vitro*. CP-91149 induced inactivation (dephosphorylation) of phosphorylase in the absence of hormones and partially counteracted the phosphorylation caused by glucagon. Inhibition of glycogenolysis by CP-91149 can be explained by dephosphorylation of phosphorylase *a*. This was associated with activation of glycogen synthase and stimulation of glycogen synthesis. DAB, in contrast, induced a small degree of phosphorylation of phosphorylase. This was associated with inactivation of glycogen synthase and inhibition of glycogen synthesis. Despite causing phosphorylation (activation) of phosphorylase, DAB is a very potent inhibitor of glycogenolysis in both the absence and presence of glucagon. This is explained by allosteric inhibition of phosphorylase *a*, which overrides the increase in activation state. In conclusion, two potent phosphorylase inhibitors exert different effects on glycogen metabolism in intact hepatocytes as a result of opposite effects on the phosphorylation state of both phosphorylase and glycogen synthase.

Key words: glycogen synthase, glycogen synthesis, Type II diabetes.

INTRODUCTION

Hyperglycaemia in Type 2 diabetes is due to impaired glucose disposal by the liver and periphery in the absorptive state and increased hepatic glucose production by gluconeogenesis and/or glycogenolysis in the post-absorptive state [1]. Inhibitors of phosphorylase have been proposed as a therapeutic strategy for improving glycaemic control in diabetes [2,3] and various studies have shown the efficacy of such compounds at lowering blood glucose in animal models of diabetes [4–6] or inhibiting liver glycogenolysis *in vivo* [7]. Novel inhibitors of phosphorylase have also proved to be very powerful tools for exploring the relative contributions of gluconeogenesis and glycogenolysis in hepatic glucose production [7,8] and for determining the control strength of phosphorylase on glycogen synthesis [9].

The active form of phosphorylase is a homodimer of 97 kDa subunits [10]. Phosphorylase has two conformational states, designated active R (relaxed) or inactive T (tense). The R-state is promoted by phosphorylation of a serine residue at the N-terminus and by allosteric activators, such as AMP. Glucose and purine analogues are allosteric inhibitors. They favour the T-state, which is also a better substrate for dephosphorylation. Glucose analogues bind at or near the catalytic site, whereas AMP binds at a distant allosteric nucleotide-activation site. The AMP-binding site also binds ATP and glucose 6-phosphate (glucose 6-*P*), which oppose the effects of AMP. In addition, there is a glycogen-binding site, which binds the enzyme to glycogen, a purine- or nucleoside-inhibitor site, which binds methylxanthines, and an additional inhibitory site, which binds novel inhibitors [2,11].

The phosphorylated and dephosphorylated forms of phosphorylase are designated *a* and *b* respectively [12]. Phosphorylase is conventionally assayed in either the physiological glycogenolytic direction, by coupling the glucose 1-phosphate (glucose 1-*P*) formed with phosphoglucomutase and glucose 6-*P* dehydrogenase, or, more commonly, in the reverse (glycogen synthesis) direction [13]. When assayed in the glycogenolytic direction, the *b* form of liver phosphorylase is essentially kinetically inactive at physiological concentrations of phosphate and allosteric activators and inhibitors [13]. It is assumed therefore that only phosphorylase *a* is involved in glycogenolysis in liver *in vivo* [12,13]. This contrasts with the muscle isoform of phosphorylase, which has different kinetic properties, and is also kinetically active in the *b* form at high physiological concentrations of AMP. Phosphorylase *a* is a potent allosteric inhibitor of glycogen synthase phosphatase by binding to the C-terminal domain of the glycogen-targeting protein designated G_L [12]. Glycogen synthase phosphatase is expressed predominantly in liver but also in skeletal muscle [14]. Activation of glycogen synthase by dephosphorylation in liver is dependent on glucose 6-*P*, which makes the enzyme a better substrate for the phosphatase [15], and on release of allosteric inhibition of the phosphatase by phosphorylase *a* [12]. Dephosphorylation of phosphorylase *a* in liver, therefore, has a dual effect on glycogen metabolism, involving a decrease in the catalytically active form of phosphorylase and a decrease in the concentration of the allosteric inhibitor of synthase phosphatase, which allows activation of glycogen synthase by glucose 6-*P*.

Several pharmacological inhibitors of phosphorylase have been described that can be broadly divided into five groups: glucose analogues that bind at or near the catalytic site and promote the conversion of phosphorylase *a* into *b* [16]; methylxanthines that bind to the nucleoside-inhibitor site and also promote dephosphorylation [17]; BAY W1807, a 1,4-dihydroxypyridine-2,3-dicarboxylate derivative that binds at the AMP-activation site [18]; indole 2-carboxamides that bind to a novel binding site [11,19]; and polyhydroxylated nitrogen-containing heterocylic compounds, such as 1,4-dideoxy-1,4-imino-D-arabinitol (DAB), that are thought to bind by a different mechanism on the basis of differences in kinetics [6,20]. Recent studies have

Abbreviations used: DAB, 1,4-dideoxy-1,4-imino-D-arabinitol; DMEM, Dulbecco's modified Eagle's medium; glucose 1-*P*, glucose 1-phosphate; glucose 6-*P*, glucose 6-phosphate; MEM, minimum essential medium.

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shown that both BAY W1807 [21] and the indole 2-carboxamides (CP-91149 and CP-320626) [9,22] promote conversion of phosphorylase *a* into phosphorylase *b* and activation of glycogen synthase in intact hepatocytes. DAB is a very potent inhibitor of phosphorylase *a* when the enzyme is assayed in the physiological direction [6,20]. However, the effects of DAB on the phosphorylation state of phosphorylase have not been reported.

In the present study, we have investigated the effects of DAB on the activation state of phosphorylase in hepatocytes. We demonstrate that, in contrast with the other phosphorylase inhibitors tested to date, DAB promotes phosphorylation rather than dephosphorylation of phosphorylase, with concomitant inactivation of glycogen synthase. To our knowledge, this is the first report of a very potent inhibitor of phosphorylase that promotes enzyme phosphorylation. The overall effects of DAB on glycogen metabolism in hepatocytes are the combined effects of the high potency of this compound as an allosteric inhibitor of phosphorylase and its effects on the phosphorylation state of both phosphorylase and glycogen synthase.

EXPERIMENTAL

Materials

DAB was synthesized by Novo-Nordisk [6], CP-91149 [4] was generously provided by Pfizer Global Research and Development (Groton, CT, U.S.A.). UDP-[6-^{3}H]glucose was from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Glucagon was from Sigma (Poole, Dorset, U.K.). Sources of other reagents were as described previously [9].

Hepatocyte monolayer culture

Hepatocytes were isolated by collagenase perfusion of the liver [23] from male Wistar rats (body weight 240–340 g; B&K Universal, Hull, East Yorkshire, U.K.). The hepatocytes were suspended in minimum essential medium (MEM) containing 7 % (v/v) newborn-calf serum and seeded into multi-well plates. After cell attachment (approx. 4 h), the medium was replaced with serum-free MEM containing 10 nM dexamethasone phosphate and either 5 or 25 mM glucose in the absence or presence of 10 nM insulin for 16–18 h as indicated and as described previously [9].

Metabolic studies

For the analytical determination of glycogenolysis, hepatocytes were precultured with MEM containing 25 mM glucose and 10 nM insulin to replete glycogen stores. The monolayers were then washed twice in saline and incubated for 1 h in glucose-free Dulbecco's modified Eagle's medium (DMEM). The medium was then collected for determination of glucose [24]. For radiochemical determination of glycogenolysis, incubation conditions were as above, except that [U-^{14}C]glucose (2 μCi/ml) was included in the medium during the 16–18 h preculture and, at the end of the 1 h incubation in glucose-free DMEM, the hepatocyte monolayers were washed with 150 mM NaCl and extracted in 0.1 M NaOH. Incorporation of the ^{14}C label into glycogen was determined by ethanol precipitation [23]. For determination of glycogen synthesis, hepatocytes were precultured in MEM containing 5 mM glucose and then incubated in MEM containing [U-^{14}C]glucose (2 μCi/ml) and the glucose concentrations indicated for 3 h. Incorporation of ^{14}C label into glycogen was determined by ethanol precipitation [23], and rates of glycogen synthesis are expressed as nmol of glucose incorporated/3 h per mg of cellular protein. Glucose phosphorylation and glycolysis were determined from detritiation of [2-^{3}H]glucose and [3-^{3}H]glucose [25].

Enzyme activity determination

After incubation of hepatocytes with inhibitors, the medium was aspirated and the plates were snap-frozen in liquid nitrogen and stored at −80 °C until assay. For phosphorylase assays, hepatocyte monolayers in 24-well plates were extracted in 250 μl/well of extraction buffer [150 mM KF, 20 mM Hepes, 0.5 mM EDTA, 1 mM benzamidine, 1 mM PMSF (pH 7.4)]. The plates were then centrifuged for 10 min at 13000 ***g***. Phosphorylase *a* was determined spectrometrically in the supernatant in the glycogenolytic direction from the phosphate-induced hydrolysis of glycogen coupled to phosphoglucomutase and glucose 6-*P* dehydrogenase as described previously [26]. The protein concentration in the final assay was approx. 0.05 mg/ml. This was the maximum dilution that allowed spectrometric determination of phosphorylase *a* with minimal interference of DAB in the final assay. At this sample dilution, the concentration of inhibitor in the final assay was estimated at approx. 0.7 % of that in the incubation medium. This estimate was based on labelling with ^3H_2O and assumes an equal concentration of inhibitor in the intracellular space as in the extracellular medium. The activity of phosphorylase *a* was expressed as m-units/mg of protein, where 1 m-unit is the amount of enzyme that forms 1 nmol of glucose 1-*P*/min.

Glycogen synthase was determined as described previously [27] with the following modifications. The final UDP-glucose concentration in the assay was 1.0 mM and the specific radioactivity of the UDP-[6-^{3}H]glucose was 3 Ci/mol. Assays were performed in a final volume of 60 μl in the absence or presence of 6.6 mM glucose 6-*P* for the determination of active and total glycogen synthase respectively. On termination of the incubation, the extracts were precipitated with 20 μl of 60 % trichloroacetic acid and, after centrifugation for 5 min at 13000 ***g***, the supernatant (50 μl) was spotted on to 3MM Chr paper (Labsales Co., Over, Cambs., U.K.) and the radioactivity was determined. Total glycogen synthase assayed in the presence of glucose 6-*P* is expressed as m-units/mg of protein and active glycogen synthase is expressed as a ratio of total glycogen synthase (activity ratio).

Immunoblotting for phosphorylase *a*

Samples were extracted as described above for determination of phosphorylase activity. After centrifugation at 13000 ***g*** (10 min), the pellet was suspended in 30 % of the volume of the original homogenate. The homogenate, supernatant and suspended pellet were diluted with 0.25 vol. of SDS-loading buffer [250 mM Tris/HCl (pH 6.8), 50 mM dithiothreitol, 10 % (v/v) SDS, 0.5 % (w/v) Bromophenol Blue and 50 % (v/v) glycerol] and denatured at 100 °C for 3 min. Equal volumes of homogenate, supernatant and pellet (maximum protein, 12 μg) were loaded on to a 3 % (w/v) stacking and 8 % (w/v) resolving polyacrylamide gel and subjected to 45 min of electrophoresis at 180 V. The protein was then transferred electrophoretically on to nitrocellulose membranes (Hybond N; Amersham Biosciences) by semi-dry transfer (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.) for 50 min at 15 V. Membranes were stained with Ponceau Red to verify protein transfer and blocked overnight at 4 °C in PBS containing 5 % (w/v) BSA and 0.05 % (v/v) Tween 20. After incubation, membranes were probed with a chicken anti-phos-

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phorylase *a* antibody raised against residues EKRRQIS(P)IRGI [where single-letter amino-acid notation has been used and (P) represents phosphorylation]. After washing, membranes were probed with horseradish peroxidase-linked anti-(chicken IgG) (Jackson Immunoresearch, West Grove, PA, U.S.A.). Bound antibody was detected using an enhanced chemiluminescence kit (Amersham Biosciences), and the signal on the X-ray film was quantified by densitometry.

Results are expressed as means ± S.E.M. for the numbers of experiments indicated. Statistical analysis was performed using the paired Student's *t*-test.

RESULTS

Effects of CP-91149 on the activity of phosphorylase *a* and glycogenolysis

Previous studies [9,22] have shown that incubating hepatocytes with CP-91149 or CP-320626, as with incubation with high glucose, causes inactivation of phosphorylase within approx. 30 min. Figure 1(A) shows that, in the absence of glucagon, CP-91149 caused a dose-dependent decrease in the activity of phosphorylase *a*, as shown previously [9]. The present study also shows that CP-91149 partially counteracted the activation of phosphorylase by glucagon. At an intermediate concentration of the inhibitor (2.5 μM), the fractional inactivation of phosphorylase was 61 ± 8 % in the absence of glucagon and 28 ± 4 and 22 ± 4 % in the presence of 2 or 10 nM glucagon respectively. Inhibition of glucose production by CP-91149 showed a similar trend, and the fractional inhibition by 2.5 μM CP-91149 was greater in the absence (42 ± 7 %) than in the presence of 2 or 100 nM glucagon (30 ± 7 and 24 ± 7 % respectively; Figure 2A). To test whether the inhibition of glucose production by

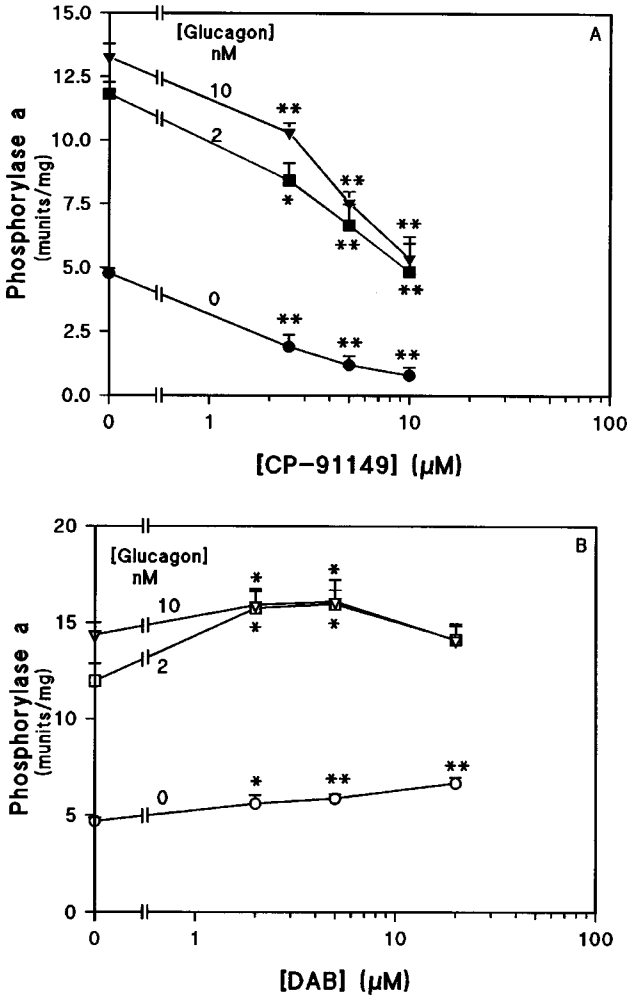

Figure 1 Effects of CP-91149 (A) and DAB (B) on inactivation of phosphorylase

Hepatocytes were incubated for 30 min with 5 mM glucose and the indicated concentrations of inhibitor [CP-91149 (**A**) and DAB (**B**)] in either the absence (●, ○) or presence of 2 nM (■, □) or 10 nM (▼, ▽) glucagon. The activity of phosphorylase *a* is expressed in m-units/mg of protein. Values are means ± S.E.M., $n = 3$. $^{*}P < 0.05$ and $^{**}P < 0.005$ compared with no inhibitor.

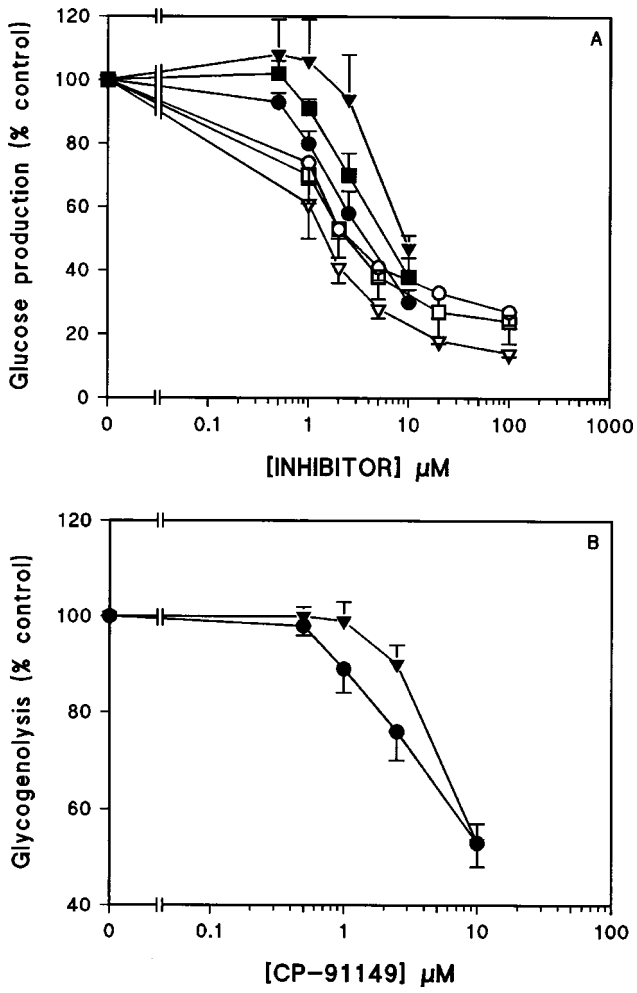

Figure 2 Inhibition of glucose production (A) and glycogenolysis (B)

(**A**) Hepatocytes were precultured with 25 mM glucose and 10 nM insulin. Cells were then incubated in glucose-free DMEM containing the indicated concentrations of CP-91149 (closed symbols) or DAB (open symbols) in either the absence (●, ○) or presence of 2 nM (■, □) or 100 nM (▼, ▽) glucagon. Accumulation of glucose in the medium was determined analytically [control, 133 ± 11; 2 nM glucagon, 266 ± 27; 10 nM glucagon, 274 ± 35 (results not shown); 100 nM glucagon, 350 ± 32 nmol/h per mg). (**B**) Hepatocytes were pretreated as in (**A**), except that [U-^{14}C]glucose was also included in the preculture medium prior to treatment with the indicated concentrations of CP-91149 in the absence (●) or presence (▼) of 100 nM glucagon. Glycogen depletion was determined radiochemically. Results are expressed as a percentage of the respective controls without inhibitor and are means ± S.E.M., $n = 3$–5.

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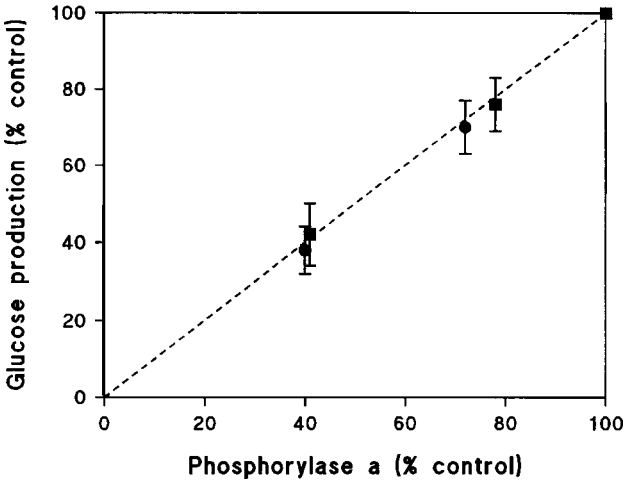

Figure 3 Inhibition of glucose production by CP-91149 correlates with inactivation of phosphorylase

Hepatocytes were incubated in the absence (100%) or presence of 2.5 μM or 10 μM CP-91149 with either 2 nM (●) or 10 nM (■) glucagon. Results are from the experiments described in Figures 1 and 2. Values are means ± S.E.M., $n = 3$.

CP-91149 could be explained by inhibition of glycogenolysis, this was determined radiochemically by prelabelling glycogen with [U-^{14}C]glucose and measuring the glycogen depletion during a subsequent 1 h incubation in the absence or presence of glucagon or inhibitor. Glucagon (100 nM) caused a 2.6-fold stimulation of glucose production (Figure 2A) and a 2-fold stimulation of glycogenolysis (204 ± 30%). The fractional inhibition of glycogenolysis by 2.5 μM CP-91149 determined radiochemically was lower in the presence of glucagon (Figure 2B), indicating that the smaller inhibition of glucose production by CP-91149 in the presence of glucagon (Figure 2A) can be explained by the effect of the inhibitor on glycogenolysis. The fractional inactivation of phosphorylase by CP-91149 was similar to the fractional inhibition of glucose production (Figure 3), suggesting that inactivation of phosphorylase can account for the inhibition of glucose production under these conditions.

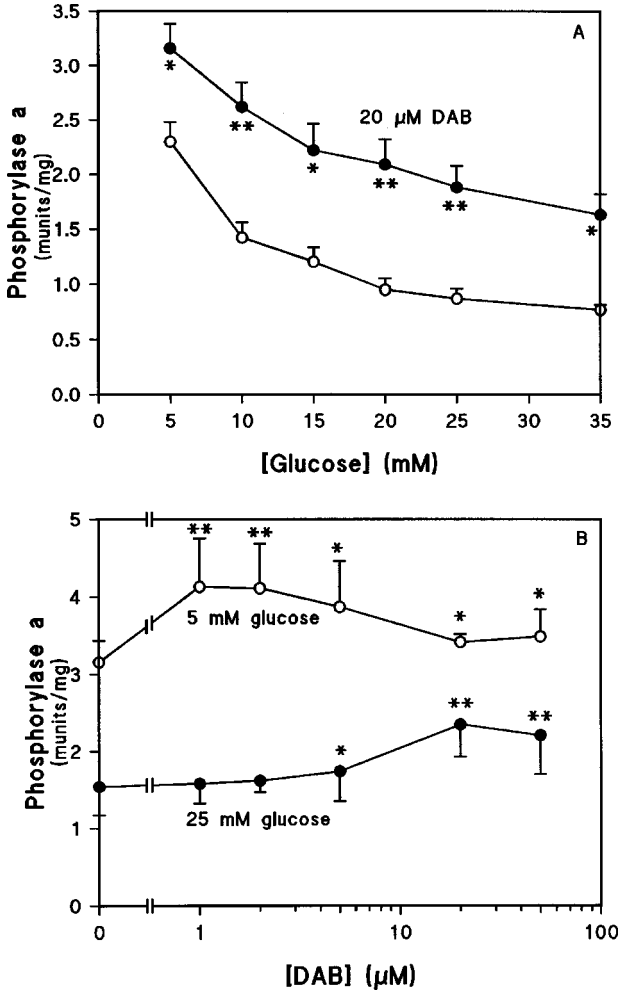

Figure 4 Effects of DAB and glucose on phosphorylase *a* activity

(**A**) Hepatocytes were preincubated for 60 min with 5 mM glucose in the absence (○) or presence (●) of 20 μM DAB. Cells were then incubated for 15 min with the glucose concentration indicated. (**B**) Hepatocytes were precultured in medium with either 5 mM (○) or 25 mM (●) glucose and then incubated for 60 min with the concentrations of DAB indicated. The activity of phosphorylase *a* is expressed as m-units/mg of protein. Results are means ± S.E.M. for three (**A**) or six (**B**) hepatocyte preparations. $^{*}P < 0.05$ and $^{**}P < 0.005$ compared with the respective controls.

Effects of DAB on the activity of phosphorylase *a* and glycogenolysis

Preliminary dose–response curves of the effects of DAB on the activity of phosphorylase *a* in hepatocytes showed an apparent biphasic effect on phosphorylase *a* activity, involving stimulation at low and inhibition at high concentrations (control, 9.0 ± 0.9; 1 μM DAB, 12.8 ± 2.1; 2.5 μM DAB, 13.0 ± 2.1; 5 μM DAB, 11.3 ± 2.3; 20 μM DAB, 5.7 ± 0.8; and 50 μM DAB, 5.7 ± 0.3 m-units/mg of protein; values are means ± S.E.M., $n = 4$). The time course of the effect of 20 μM DAB showed an immediate decrease in activity followed by a significant increase ($P < 0.05$) between 5 and 30 min before reaching a plateau (control, 9.5 ± 2.1; 5 min, 4.8 ± 1.0; 30 min, 5.8 ± 1.1; 60 min, 5.9 ± 0.8; and 120 min, 5.7 ± 1.3 m-units/mg of protein; values are means ± S.E.M., $n = 4$). Assays at varying sample dilutions showed that the lower activity of phosphorylase *a* in extracts from incubations with 20 and 50 μM DAB was due to allosteric inhibition by 'residual' DAB in the extracts. Subsequently, samples were assayed at the maximum dilution (see the Experimental section) that allowed spectrometric determination of phosphorylase *a* with minimum allosteric inhibition by residual inhibitor in the final assay (< 1% of the concentration in the incubation). Under these conditions, there is negligible allosteric inhibition by residual inhibitor in incubations with CP-91149 up to 10 μM, but there is some allosteric inhibition by DAB at concentrations of 20–50 μM.

DAB incubated in the absence or presence of glucagon, in contrast with CP-91149, caused a small increase in the activity of phosphorylase *a* (Figure 1B). Despite the increase in the activity of phosphorylase *a*, DAB is a very potent inhibitor of glycogenolysis and glucose production (Figure 2A), with half-maximal inhibition at approx. 2 μM, in agreement with previous findings [28]. The fractional inhibition of glucose production by DAB was not affected by glucagon and it can only be explained by allosteric inhibition.

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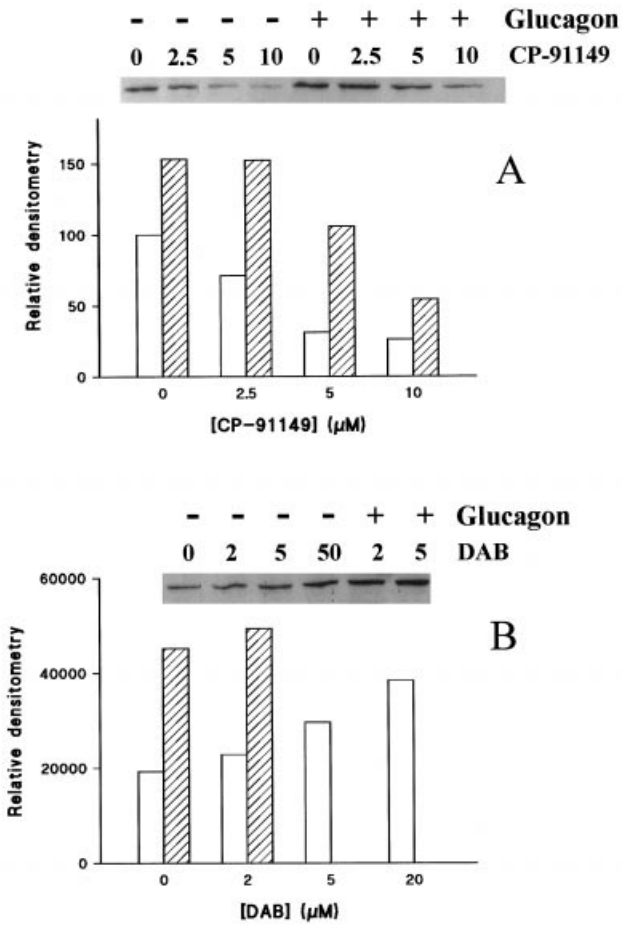

Figure 5 Effects of inhibitors on the phosphorylation state of phosphorylase

Experimental conditions were as described in the legend for Figure 1. Hepatocytes were incubated with the concentrations of CP-91149 (**A**) or DAB (**B**) indicated in the absence (−; open bars) or presence (+; hatched bars) of 100 nM glucagon. Samples were fractionated by SDS/PAGE and immunoblotted with an antibody to phosphorylase *a*, as described in the Experimental section. The histogram shows the intensity expressed in relative densitometry units. Results shown are representative of two experiments.

Interactions of DAB and glucose on the activity of phosphorylase *a*

When hepatocytes were preincubated with 20 μM DAB and subsequently incubated with varying glucose concentrations, the activity of phosphorylase *a* was significantly higher in cells pretreated with DAB at all glucose concentrations tested (Figure 4A). DAB impaired, but did not abolish, the inhibition caused by increasing glucose concentrations. Conversely, when hepatocytes were preincubated with either 5 or 25 mM glucose and then incubated with DAB, lower concentrations of DAB were effective at increasing phosphorylase *a* at 5 mM compared with 25 mM glucose (Figure 4B), suggesting that the effect of DAB may be competitive either with glucose itself or with a metabolite of glucose.

Effects of inhibitors on the phosphorylation state of phosphorylase

To confirm that the changes in the activity of phosphorylase *a* in cells treated with CP-91149 and DAB (Figure 1) are due to decreased and increased phosphorylation of the enzyme respectively, we determined the amount of phosphorylase *a* by immunoblotting using an antibody to the phosphorylated N-terminus. CP-91149 caused a concentration-dependent decrease

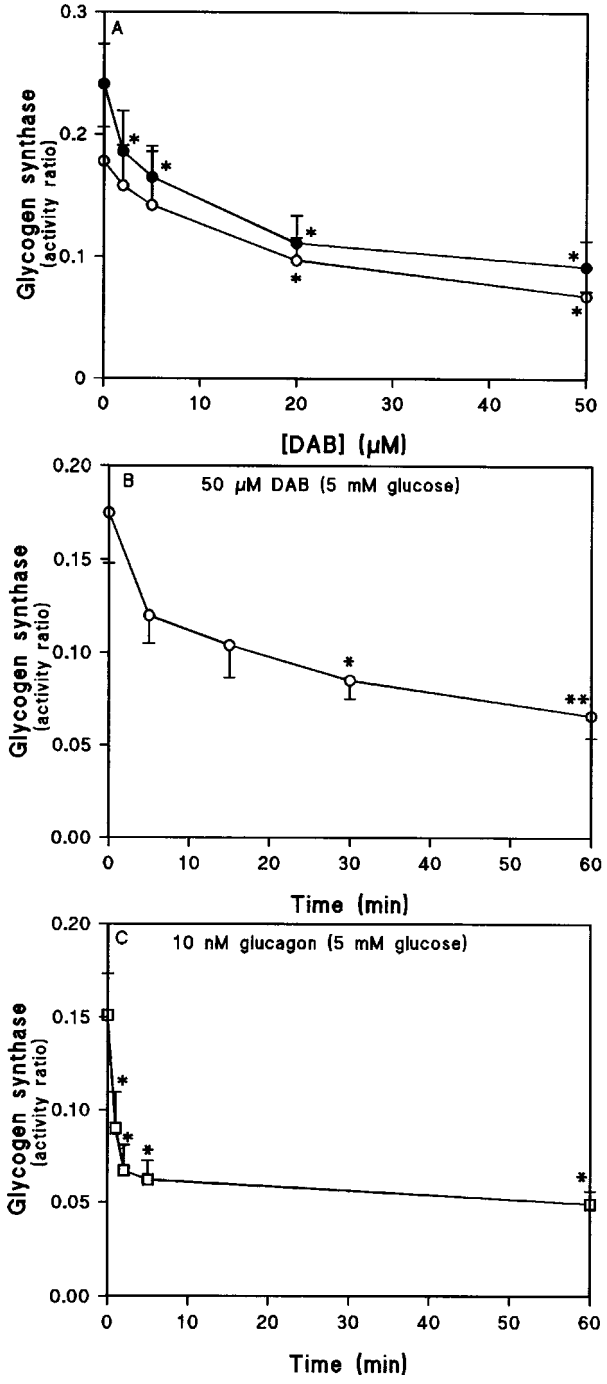

Figure 6 Inactivation of glycogen synthase by DAB in intact hepatocytes

(**A**) Hepatocytes were incubated for 60 min with varying DAB concentrations with either 5 mM (○) or 15 mM (●) glucose and glycogen synthase activity determined. (**B**) Time course of glycogen synthase activity with 50 μM DAB and 5 mM glucose. (**C**) Time course of glycogen synthase activity with 10 nM glucagon and 5 mM glucose. The activity of glycogen synthase was determined in the trichloroacetic acid supernatant in the absence or presence of glucose 6-*P* and is expressed as the activity ratio (±glucose 6-*P*). Values are means ± S.E.M., $n = 3$–5. $^{*}P < 0.05$ and $^{**}P < 0.005$, compared with respective controls.

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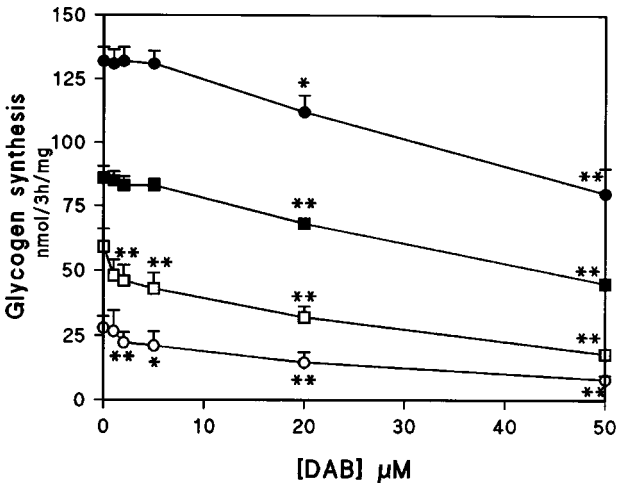

Figure 7 Inhibition of glycogen synthesis by DAB in intact hepatocytes

Glycogen synthesis was determined from the incorporation of [U-^{14}C]glucose during the incubation of various concentrations of DAB with 10 mM (○), 15 mM (□), 35 mM (●) glucose or 15 mM glucose + 5 mM dihydroxyacetone (■). Results are expressed as nmol of glucose incorporated/3 h per mg of cellular protein. Values are means ± S.E.M., $n = 4$–5 hepatocyte preparations. $^{*}P < 0.05$ and $^{**}P < 0.005$ compared with no inhibitor.

in phosphorylase *a*, which was lower in the presence of glucagon (Figure 5A), whereas DAB caused a 2-fold increase in phosphorylase *a* in the absence of glucagon (Figure 5B). When the immunoreactivity to phosphorylase *a* was determined in the 13000 ***g*** supernatant and pellet fraction of hepatocyte homogenates, the amount of phosphorylase *a* in the supernatant fraction accounted for more than 80 % of the total, and there was no evidence for inverse effects of DAB on the distribution of phosphorylase *a* between supernatant and pellet fractions (results not shown).

Effects of inhibitors on glycogen synthase activity in hepatocytes

DAB had no effect on the total activity of glycogen synthase assayed in the presence of glucose 6-*P* (control, 0.41 ± 0.03; 2 μM DAB, 0.40 ± 0.03; 5 μM DAB, 0.41 ± 0.03; 20 μM 0.42 ± 0.04; 50 μM DAB, 0.41 ± 0.04 m-unit/mg of protein; values are means ± S.E.M., $n = 5$). However, it caused a concentration-dependent decrease in the activity ratio (± glucose 6-*P*), indicating inactivation of glycogen synthase at both 5 and 15 mM glucose (Figure 6A). Time-course experiments showed that the inactivation by DAB was slower than by glucagon (compare Figure 6B with Figure 6C). The activity ratio of glycogen synthase was increased by approx. 2-fold by 2.5 μM CP-91149 (results not shown), in agreement with previous findings [9].

Effects of inhibitors on glycogen synthesis

CP-91149 caused a concentration-dependent increase in the rate of glycogen synthesis in hepatocytes incubated with 10 mM glucose (control, 28 ± 3; 1 μM, 44 ± 1; 2.5 μM, 86 ± 9; and 10 μM, 125 ± 14 nmol/3 h per mg of protein; values are means ± S.E.M., $n = 4$), in agreement with previous findings [9]. However, DAB (5–50 μM) inhibited glycogen synthesis at 10–15 mM glucose (Figure 7). The fractional inhibition by 50 μM DAB was lower at 35 mM glucose (40 %) than at 10–15 mM (70 %). In the additional presence of 5 mM dihydroxyacetone, which causes a 2-fold increase in the cellular content of glucose 6-*P* (0.48 ± 0.08 to 0.91 ± 0.09 nmol/mg of protein) and a 50 % increase in glycogen synthesis from labelled glucose, the fractional inhibition by DAB was lower than in the absence of dihydroxyacetone (50 % compared with 70 %). The concentration of DAB that caused half-maximal inhibition of glycogen synthesis was approx. 10-fold higher than that for inhibition of glycogenolysis ($IC_{50} > 20$ μM compared with 2 μM). The fractional inhibition of glycogen synthesis by DAB was not affected by the presence of insulin (10 nM) added either during preculture or the final incubation with [U-^{14}C]glucose (results not shown), and it was decreased, but not abolished (30 % compared with 70 %), by preculture of the hepatocytes with 20 mM glucose to increase glycogen stores (results not shown).

To check whether inhibition of glycogen synthesis can be explained by proximal effects on glucose metabolism, we tested the effects of DAB on the rates of detritiation of [2-^{3}H]glucose and [3-^{3}H]glucose and on glucokinase translocation. However, there was no effect of DAB (2–50 μM) on the above parameters (results not shown), indicating that DAB does not affect glucose phosphorylation or glycolysis.

DISCUSSION

Several phosphorylase inhibitors have been shown to promote the conversion of phosphorylase *a* into *b* and the dephosphorylation of glycogen synthase *b*. These include glucose analogues, which bind at or near the substrate/product-binding site [16], methylxanthines, which bind the purine nucleoside-binding site [17], 1,4-dihydropyridine 2,3-dicarboxylate derivatives, which bind the AMP site [21], and indole 2-carboxamides, which bind at a distinct inhibitory site [9,22]. In the present study, we demonstrate that DAB, in contrast with the above-mentioned inhibitors, promotes the conversion of phosphorylase *b* into *a*. This is based on two sets of evidence: (i) a kinetic assay for phosphorylase *a* in the glycogenolytic direction; and (ii) immunoblotting using an antibody to the phosphorylated N-terminus of liver phosphorylase. Qualitatively, similar results were obtained by the two assays; however, the magnitude of the increase in phosphorylase *a* caused by DAB was apparently greater when determined by immunoblotting compared with the kinetic assay. In the kinetic assay, DAB is a very potent allosteric inhibitor of activity and we cannot exclude the possibility that the measured activity of phosphorylase *a* in cell extracts from incubation with 20–50 μM DAB is an underestimate, due to allosteric inhibition of phosphorylase *a* by the low residual concentrations of DAB in the extracts.

The conversion of phosphorylase *b* into *a* by DAB is best explained by DAB counteracting by the effects of endogenous ligands that promote the conversion of phosphorylase *a* into *b*. This is based on two sets of observations: (i) the effect of DAB on conversion of phosphorylase *b* into *a* is smaller than the effect of glucagon; and (ii) lower concentrations of DAB are effective in activating phosphorylase at 5 mM compared with 25 mM glucose. This suggests that the mechanism of action of DAB on phosphorylase activation may involve competition with glucose for the active site or competition with glucose 6-*P* for the AMP site. Alternatively, DAB may compete with endogenous inhibitors that interact with the nucleoside-binding site [29].

The inhibition of glycogen synthesis by DAB cannot be explained by non-specific effects on glucose phosphorylation, glycolysis or glucokinase compartmentation. Inactivation of

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glycogen synthase is the most likely explanation for the inhibition of glycogen synthesis. This is probably, in part, due to the increase in phosphorylase *a*, which is a potent inhibitor of synthase phosphatase. However, the lower potency of DAB in inactivating of glycogen synthase (and inhibition of glycogen synthesis) compared with inhibition of glycogenolysis and activation of phosphorylase is suggestive of additional mechanisms. Since binding of glucose 6-*P* to glycogen synthase promotes activation of synthase by making the enzyme a better substrate for the synthase phosphatase [15], competition of DAB with glucose 6-*P* may be involved in the inactivation of the synthase. The lower fractional inhibition of glycogen synthesis by DAB in incubations with dihydroxyacetone, which causes accumulation of glucose 6-*P*, is consistent with such a mechanism. However, a possible additional allosteric effect of DAB on glycogen synthase cannot be excluded.

The inhibition of glycogenolysis by BAY R3401, via its active metabolite BAY U6751, is attributed to the combined effects of partial conversion of phosphorylase *a* into *b* and allosteric inhibition of the residual phosphorylase *a*, because the fractional inhibition of glycogenolysis by BAY R3401 was greater than the fractional conversion of phosphorylase *a* into *b* [21]. Similar considerations would apply in the case of inhibition of glycogenolysis by indole 2-carboxamides (e.g. CP-91149). In the latter case, the conversion of phosphorylase *a* into *b* is probably the predominant mechanism, because the fractional inhibition of glucose production by CP-91149 paralleled the conversion of phosphorylase *a* into *b*. The lower fractional inhibition by CP-91149 in the presence of glucagon of both glycogenolysis and inactivation of phosphorylase *a* also supports the role of phosphorylase dephosphorylation in the mechanism by which CP-91149 inhibits glycogenolysis.

Despite the fact that DAB does not induce dephosphorylation of phosphorylase *a* but causes conversion of phosphorylase *b* into *a*, it is a very potent inhibitor of glycogenolysis in rat hepatocyte monolayers and its potency is not diminished by glucagon. Accordingly, the inhibition of glycogenolysis by DAB is due exclusively to allosteric inhibition, which totally overrides the increase in the activation state of phosphorylase *a*. This is consistent with the high potency of DAB as an allosteric inhibitor of phosphorylase *a* when assayed in the physiological (glycogenolytic) assay [20].

DAB is a less potent inhibitor of glycogen synthesis than of glycogen degradation by approximately an order of magnitude. From a theoretical standpoint, low concentrations of DAB that are inhibitory for glycogen degradation, but not synthesis ($< 2\ \mu$M), would be expected to cause apparent stimulation of glycogen synthesis if there was significant substrate cycling between glycogen and glucose 1-*P* [30,31]. However, we found no evidence for stimulation of glycogen synthesis by low concentrations of DAB ($< 2\mu$M) in hepatocyte cultures. This is consistent with previous studies [8,32] that demonstrated lack of cycling between glycogen synthesis and degradation in hepatocyte cultures and contrasts with freshly isolated hepatocytes from fasted rats [16], which show a high apparent rate of cycling. In humans, cycling between glycogen and glucose 1-*P* is thought to be negligible in the post-absorptive state. However, it is elevated in Type II diabetes or after an oral glucose load [30,31]. At the minimal concentrations of DAB that are effective at inhibiting glycogenolysis, DAB is a potential tool to study cycling between glucose 1-*P* and glycogen. The possibility that DAB may increase glycogen synthesis in situations associated with high cycling, as in Type II diabetes, remains to be tested [8].

Various studies [4–6] have shown the efficacy of novel allosteric inhibitors of phosphorylase at correcting hyperglycaemia in animal models of diabetes. Inhibitors that promote dephosphorylation of phosphorylase cause reciprocal activation of glycogen synthase [9,21,22] with consequent stimulation of glycogen synthesis [9,22]. The present finding that DAB, in contrast with most other phosphorylase inhibitors described to date, does not stimulate glycogen synthesis shows that inhibition of hepatic glucose production can be achieved without simultaneous stimulation of glycogen synthesis.

We thank Dr Judith Treadway and Dr Mar Garcia Rocha for generously providing CP-91149 and phosphorylase antibody respectively, and for helpful advice. We thank Dr Viggo Diness for useful discussions, and Diabetes U.K. for the award of an equipment grant.

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Received 8 July 2002/16 August 2002; accepted 19 August 2002
Published as BJ Immediate Publication 19 August 2002, DOI 10.1042/BJ20021070

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