Endogenous effectors of human liver glycogen phosphorylase modulate effects of indole-site inhibitors

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Phosphorylase activity is primarily regulated by phosphorylation of serine-14 in the NH2 terminus of the protein. The phosphorylated form, glycogen phosphorylase a (GPa), is active; the unphosphorylated form, glycogen phosphorylase b, is inactive at substrate concentrations in vivo (44). In addition to phosphorylation, allosteric effectors play a major role in regulating phosphorylase activity (8). A number of small-molecular-weight phosphorylase effectors have been identified by kinetic studies (7, 25, 26, 29, 42). These ligands bind to the following five effector sites: 1) the nucleotide activation site, which binds AMP, ATP, and glucose 6-phosphate (G-6-P; see Refs. 7 and 41); 2) the catalytic site, which binds glucose, UDP-glucose, and P, (12, 32); 3) the purine inhibitor site for which caffeine is the classical ligand studied. The endogenous purine-site ligand remains unknown (7, 24, 26). Caffeine inhibits enzymatic activity synergistically with glucose (10, 24, 26); 4) the indole inhibitor site, a new allosteric site that was identified by the binding of a synthetic inhibitor of human liver glycogen phosphorylase (GP) (36) (the natural ligand for this site is not known); and 5) finally, a separate, noncatalytic site termed the glycogen storage site has been identified on the enzyme (18).

We recently reported that, when added individually, AMP stimulated, whereas ADP, ATP, and glucose inhibited, human liver glycogen phosphorylase a (HLGPa) activity (9). UDP-glucose, G-6-P and fructose 1-phosphate (F-1-P) were only minor inhibitors. A combination of the above effectors at estimated physiological concentrations and under simulated in vivo conditions was inhibitory. However, we were surprised to find that glucose alone largely accounted for this inhibition, i.e., glucose cancelled the stimulatory effect of AMP and the inhibitory effects of ATP and ADP. This demonstrates that the activity of the enzyme is determined by the integration of signals from all the allosteric sites. Glucose also may regulate enzyme activity since it is the only allosteric effector whose concentration changes dramatically with feeding and fasting (9). Nevertheless, the enzymatic activity measured even at its most inhibited state was still two- to threefold higher than the rate of glycogenolysis in humans (40, 43, 47), implying the presence of additional inhibitors. Thus we were interested in determining whether indole-site ligands inhibited phosphorylase a to a degree that would explain the in vivo glycogenolysis rate in humans in the presence of other known regulators of the enzyme.
In the present study, we determined whether CP-403700 and other indole-site inhibitors of liver GP were still inhibitory in the presence of the known endogenous effectors of phosphorylase a and whether this inhibition remained glucose dependent. If so, such compounds would be especially attractive as therapeutic agents, since they could be less effective at a low physiological glucose concentration but would reduce glycogenolysis and decrease blood glucose exclusively under hyperglycemic conditions.

**Materials and Methods**

\(^{[32]P}\)I, was purchased from Amersham-Pharmacia-Biotech (Piscataway, NJ). \(^{[14]}\text{C}\)glucose 1-phosphate (G-1-P) was purchased from New England Nuclear. All chemicals were obtained from Sigma Chemical (St. Louis, MO). The rabbit liver glycogen used was purified by passage through a mixed-bed ion exchange resin (Amberlite MB-3; Mallinkrodt Laboratory Chemicals, Phillipsburg, NJ). 1-[N-(5-chloroindole-2-carbonyl)-L-phenylalanyl]azetidine-3-carboxylic acid (CP-403700; see Ref. 38), (3S,2R)-3-(5-chloroindole-2-carbonyl)-amino-2-hydroxy-4-phenylbutyric acid N-methyl-N-methoxyamide (CP-316819; see Ref. 21), and 1-[N-(5-chloroindole-2-carbonyl)-L-phenylalanyl]azetidine-3-ol (CP-308067; see Ref. 20) were synthesized at Pfizer Global Research and Development. CP-320626 1-[N-(5-chloroindole-2-carbonyl)-3-(4-fluorophenyl)-L-phenylalanyl]-4-hydroxypropyperidine (CP-320626) was synthesized at Pfizer Global Research and Development as described previously (30).

The Animal Care and Use Procedures for the animal experiments herein were reviewed and approved by the Institutional Animal Care and Use Committee (Pfizer) and were in compliance with the Animal Welfare Act and standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International.

**Expression of Recombinant Luman Liver GP**

Recombinant HLGPa was used in all in vitro experiments. It was expressed and purified from baculovirus-infected cells, as described previously (37). Phosphorylase a represented 84% of the total activity present, as determined in the direction of glycogen synthesis (17, 44).

**Phosphorylase a Activity Assays**

The IC\(_50\) values for the indole-site ligands were determined in the direction of glycogen synthesis (30) and/or glycogenolysis, as described previously (7, 16). In the latter assay, phosphorylase a activity is determined by incubating purified phosphorylase in the presence of glycogen and \(^{[32]}\text{P}\)I. The radioactivity incorporated into G-1-P is measured. A unit represents 1 \(\mu\)mol of product produced/min at 37°C under the conditions of the assay. The glycogen concentration was saturating. Specific activity assayed in the direction of glycogenolysis (7, 16) was 34 U/mg protein.

Results are expressed as percent control. The mean phosphorylase a activities, i.e., control activities in the absence of effectors and thus representing 100%, were 1.2 \(\pm\) 0.05 and 3.4 \(\pm\) 0.3 \(\mu\)mol G-1-P produced/min \(-1\) g protein at 1 and 5 mM P, concentrations, respectively.

Phosphorylase activity was stable at 37°C; the velocity of the reaction was linear with time and the amount of phosphorylase added. Over the 3-min incubation time period used in the assay, only 0.03% of the substrate was converted into product. Thus the conditions approximated an initial velocity (data not shown). The determined Michaelis constant for P, was 5 mM.

Protein was determined spectrophotometrically by measuring the absorbance at 280 nm using an extinction coefficient.

**Estimation of Intracellular Concentrations of Effectors**

Approximately 55% of the total liver weight is represented by intracellular water. Therefore, intracellular concentrations of the effectors were estimated by dividing their measured concentrations in a liver extract by 0.55 (34). P, concentrations of 1 and 5 mM were used since they are likely to represent the range of free P, concentrations in the liver (2, 33).

**Glycogenolysis Inhibition in Forskolin-Stimulated SK-HEP-1 Cells**

Cell-based IC\(_50\) values for CP-316819 and CP-320626, and CP-380867 for inhibition of glycogenolysis, were determined in human liver-deriv SK-HEP-1 cells (22). SK-HEP-1 cells were grown to confluence in six-well cluster dishes in DMEM (5 mM glucose) supplemented with 10% FBS. The medium was aspirated and replaced with serum-free DMEM for 18 h, and then the cells were incubated with 5 mM glucose (0.2 \(\mu\)Ci/\(\mu\)l) in 2 ml of DMEM-5% FBS plus 1 \(\mu\)M insulin for 4 h to label the intracellular glycogen pool. The labeling medium then was removed, and the cells were washed one time with PBS. Cells then were incubated in DMEM containing 10 mM glucose for 2 h in the absence (basal) or presence (stimulated) of 50 \(\mu\)M forskolin to stimulate glycogenolysis plus the indole-site glycogen phosphorylase inhibitor (GPI) compounds at concentrations 0–30 \(\mu\)M. The assay was terminated by aspirating the medium, washing the monolayer three times with PBS, and solubilizing cells with 1 ml of 1 N NaOH for 10 min at 60°C. Radiolabeled glycogen then was isolated by adding a solution of carrier glycogen (1 mg) to each tube. Total glycogen was precipitated from solution by the addition of 2.5 ml of 100% ethanol (EtOH), incubation at −20°C for 1 h, and centrifugation at 700 g for 20 min at 4°C. The glycogen pellet was washed one time with 2.5 ml of 60% EtOH, dissolved in 0.5 ml water, mixed with 5 ml of Beckman Ready-Safe scintillant, and counted in a Wallac 1409 Liquid Scintillation Counter. Glycogenolysis was calculated as the difference in \(^{14}\text{C}\)glycogen (disintegrations/min) content between basal and forskolin-treated conditions, and the results are expressed as percent inhibition of the forskolin-induced glycogenolysis in the cells. The results are the mean of triplicate determinations from two or more independent experiments.

**Rat Liver Perfusion to Measure Glucagon-Stimulated Glycogenolysis and Glucose Production**

Normal fed, male Sprague-Dawley rats (300 g) were anesthetized with pentobarbital sodium (50 mg/kg ip) and then surgically prepared and cannulated to limit liver blood flow to the portal vein (in) and inferior vena cava (out). This perfusion technique is based on a modification of the method described by Hemmerle et al. (19). The preparation was then perfused at a rate of 30 ml/min in medium containing 118 mM NaCl, 4.74 mM KCl, 1.18 mM KH2PO4, 1.18 mM MgSO4, 25 mM NaHCO3, 2.54 mM CaCl2, and 1% gelatin (Difco Bacto-Gelatin) for a 100-ml wash-out period, followed by conversion to a recirculating system. After a 30-min baseline period, 3 mM glucagon was added to the medium to stimulate glycogenolysis, followed by addition of the indole-site ligands at 10–30 \(\mu\)M. The preparation continued to be perfused to 120 min; the medium was sampled for glucose determination (Abbott CCX Chemistry Analyzer) every 10 min throughout the experimental period. Results are expressed as micromoles glucose produced per gram liver weight.

**Acute Plasma Glucose Lowering by CP-316819 in the ob/ob Mouse**

The acute hypoglycemic activity of the CP-316819 was determined using 5- to 8-wk-old male C57BL/6j ob/ob mice (Jackson Laboratory, Bar Harbor, ME) housed five per cage under standard animal care practices. The animals were normal fed. After a 1-wk acclimation period, the animals were weighed, and 25 \(\mu\)l of cage under standard animal care practices. The animals were normal fed. After a 1-wk acclimation period, the animals were weighed, and 25 \(\mu\)l of saline containing 0.025% sodium heparin and held on ice for metabolite analysis. Animals were assayed...
signed to treatment groups such that each group has a similar mean for plasma glucose concentration, and then animals were dosed orally each day for 4 days with the vehicle consisting of 10% DMSO/0.1% Pluronic P105 Block Copolymer Surfactant (BASF, Parsippany, NJ) in 0.1% saline without pH adjustment. On day 5, the animals were weighed again and then dosed orally with CP-316819 or the vehicle alone. Mice were then bled from the retroorbital sinus 3 h later for determination of plasma glucose levels. The freshly collected samples were centrifuged for 2 min at 10,000 g at room temperature. The supernatant was analyzed for glucose by the Abbott VP (Abbott Laboratories, Diagnostics Division, Irving, TX) and VP Super System Test reagent system (Abbott Laboratories). Hypoglycemic activity of the test compounds was determined by statistical analysis (unpaired t-test) of the mean plasma glucose concentration between the test compound group and vehicle-treated group on day 5.

Determination of Drug Concentrations in the Liver, the Portal Vein, and the Vena Cava

In a separate series of experiments, normal fed ob/ob mice (n = 4/time point) received CP-316819 or CP-320626 at 5 mg/kg in the 10% DMSO/0.1% Pluronic P105 vehicle and then were killed with CO2 for blood collection from the portal vein and vena cava at 0.17, 0.5, 1, 2, and 4 h postdose; a liver biopsy was also obtained at each time point. Samples were stored at −20°C until assay of compound concentration. For plasma, 100 µl were added to 50 µl of an internal standard (2 µg/ml CP-89816 in methanol), 5 ml of methyl tert-butyl ether, and 1 ml of 0.5 M sodium carbonate (pH 9). After vigorous mixing and centrifugation, the ether layers were collected and evaporated to dryness, and the resulting solid was reconstituted with 75 µl of mobile phase [45% acetonitrile, 55% of 50 mM sodium phosphate monobasic, and 30 mM triethylamine (pH 3)]. Aliquots (30 µl) of reconstituted samples were injected on a 4-µm Waters Nova-Pak C18 reverse-phase column (3.9 × 150 mm) with a mobile phase flow rate of 1 ml/min. For liver, samples were homogenized and extracted with acetonitrile as described previously (35), and the extract was then injected directly on to the column. CP-320626 or CP-316819 and internal standard were detected by fluorescence (excitation at 290 nm and emission at 348 nm). The linear dynamic range was between 0.1 µg/ml (lower limit of quantification) and 1 µg/ml (upper limit of quantification). Cmax was the concentration in the blood sample in which the highest plasma concentration was measured. The area under the plasma concentration time curve from 0 to tlast was calculated using a linear trapezoidal approximation, where tlast is the time point of the last quantifiable plasma concentration.

Data are presented as means ± SE. Statistics were done by two-way ANOVA. When the number of observations taken within each treatment was different, ANOVA was done for an “unbalanced” design. Comparison of treatment means with the control was done by Dunnett’s procedure. P < 0.05 was the criterion for statistical significance. The Student’s t-test was used where appropriate.

RESULTS

Kinetics of Inhibition of HLGPa by the Indole-Site Inhibitor CP-403700

GPI CP-403700 inhibited activity with an IC50 of 0.1 µM at a 1 mM Pi concentration (Fig. 1). Thus the indole derivative compound was a very strong inhibitor. At 8 mM glucose (low physiological concentration), the IC50 of GPI (at 1 mM Pi) was reduced by one-half to 0.05 µM. The IC50 for CP-403700 at 5 mM Pi is ~0.3 µM.

Effect of Combinations of Effectors Plus CP-403700 on Phosphorylase a Activity

In subsequent studies, we determined HLGPa activity using a combination of all known endogenous effectors in the presence of GPI.

Combination 1 is a mixture of 0.3 mM AMP, 6 mM ATP (in the form of MgATP), 0.5 mM UDP-glucose, 0.3 mM G-6-P, 0.3 mM F-1-P, and 8 mM glucose. Combination 2 is the same combination of effectors, but the glucose concentration has been increased to 20 mM. ADP was also added at concentrations [3 mM (Fig. 2, A and B) vs. 0.1 mM (Fig. 2, C and D)] that correspond to the total vs. free concentrations reported for rat and human liver cells (3–5, 7, 9, 23, 27, 33, 46). Glucose concentrations were selected to cover the ranges reported for fasting and highest intracellular liver glucose concentrations under physiological conditions (6, 34); 8 mM represents the basal glucose concentration in rat liver intracellular fluid (ICF). We have previously reported that fasting glucose concentration in the rat liver ICF was 9 µmol/ml water. After a glucose load, the glucose concentration in the liver ICF increased up to 17 µmol/ml water. Therefore, 8 and 20 mM represent extremes of glucose concentration in the liver ICF. In the liver, the intracellular concentration of glucose is always greater than the extracellular fluid concentration (34).

In the absence of CP-403700, both combinations 1 and 2 modestly inhibited human GPα activity, as reported previously (Fig. 2, A–D), and inhibition was glucose dependent (9). In the present study, we were surprised to find that, in the presence of 0.1 µM CP-403700 (equal to the IC50 concentration), the inhibition of human liver phosphorylase a activity at both 1 and 5 mM Pi concentrations was the same as that in its absence. This was true both for combinations 1 and 2 regardless of the ADP concentration (Fig. 2, A–D, columns 3 and 4). Thus addition of this strong inhibitor (CP-403700) at its IC50 concentration did not further increase the inhibition by a combi-
nation of modulators at their presumed physiological concentrations. Furthermore, despite the presence of 0.1 μM CP-403700, the inhibition by combinations 1 and 2 continued to be less when the ADP concentration was lowered to 0.1 mM (Fig. 2, C and D), as noted previously (9).

The IC₅₀ of CP-403700 in the presence of combination 1 effectors at a 5 mM P₁ concentration was 1.7 μM (Table 1), i.e., 17-fold higher than the IC₅₀ in the absence of any effectors.

In the presence of combination 1 effectors, CP-403700 at a 1 μM concentration reduced phosphorylase activity to 12% of control at 1 mM P₁ and to 20% of control at 5 mM P₁ (Fig. 2A, columns 5 and 6). At a 10 μM concentration, the activity was reduced to 3 and 10%, respectively (Fig. 2A, columns 7 and 8). When CP-403700 was added to combination 2, the inhibition was greater, as expected (Fig. 2B). The inhibition in the presence of combination 2 was essentially maximal at a 1 μM CP-403700 concentration and was almost complete (Fig. 2B, columns 5 and 6).

When the ADP concentration in the combination was lowered to 0.1 mM (Fig. 2, C and D), the inhibition at high concentrations of CP-403700 was similar to those in the presence of 3 mM ADP (Fig. 2, A and B).

**Glucose/GPI interaction.** When the glucose effect was defined as the net difference between combinations 1 and 2 at a given ADP concentration, there was no interaction with GPI concentrations and the glucose effect. In the post hoc analysis, the glucose effect increased at 1 μM GPI concentration, suggesting potentially increased effect of GPI at higher glucose concentrations (P < 0.05). However, there was no interaction with glucose and GPI at 0.1 and 10 μM GPI concentrations (P > 0.05). The latter is likely because of almost complete inhibition of combination 2 at a 1 μM GPI concentration.

**Glucose/ADP interaction.** There was no significant interaction between glucose effect and ADP at all GPI concentrations (P < 0.05).

### The In Vitro IC₅₀ Values for Three Additional Indole-Site Ligands

We next determined IC₅₀ values for highly related indole-site inhibitors CP-316819, CP-320626, and CP-380867 in the presence of combination 1 effectors (Table 1). The assays were performed both in the direction of glycogen synthesis and glycogenolysis for comparison. The IC₅₀ values for these compounds, as well as for CP-403700, were ~10- to 60-fold higher in the presence of combination 1 effectors than those in the presence of glucose only.

### The In Vivo IC₅₀ Values for the Indole-Site Ligands

We next determined the in vivo IC₅₀ values for CP-316819, CP-320626, and CP-380867 inhibition of forskolin-stimulated glycogenolysis in human liver-derived SK-HEP-1 cell cultures (Table 1). The in vivo IC₅₀ values for these compounds were ~12- to 14-fold higher than those in vitro, consistent with the reduction in potency observed by addition of endogenous phosphorylase effectors. Thus presence of the known effectors of the enzyme corresponds with the in vivo vs. in vitro potency differences observed for these compounds.

#### Concentration-Response for CP-316819, CP-320626, and CP-380867 in Perfused Rat Liver Preparations

Rat liver perfusion studies were performed under basal and glucagon-stimulated conditions. CP-316819 at a 10 μM concentration reduced glucose effect increased at 1 μM GPI concentration, suggesting potentially increased effect of GPI at higher glucose concentrations (P < 0.05). However, there was no interaction with glucose and GPI at 0.1 and 10 μM GPI concentrations (P > 0.05). The latter is likely because of almost complete inhibition of combination 2 at a 1 μM GPI concentration.

### Table 1. IC₅₀ (μM) for various indole-site inhibitors

<table>
<thead>
<tr>
<th>In Vitro</th>
<th>In Vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen synthesis direction</td>
<td>Glycogenolytic direction</td>
</tr>
<tr>
<td>Glucose (7.5 mM)</td>
<td>Combination of all effectors</td>
</tr>
<tr>
<td>Glucose</td>
<td>Combination of all effectors</td>
</tr>
<tr>
<td>CP-316819</td>
<td>0.04</td>
</tr>
<tr>
<td>CP-320626</td>
<td>0.16</td>
</tr>
<tr>
<td>CP-380867</td>
<td>0.06</td>
</tr>
<tr>
<td>CP-403700</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Results are means of triplicate determinations from two or more independent experiments. Results are expressed as %inhibition of the forskolin-induced glycogenolysis in the SK-HEP-1 cells. Combination of effectors, 8 mM glucose, 0.3 mM AMP, 3 mM ADP, 6 mM ATP, 0.3 mM glucose 6-phosphate, 0.3 mM fructose 1-phosphate, 0.5 mM UDP-glucose. The assay in the glycogenolytic direction was performed at 5 mM P₁ concentration. Assay method is described in MATERIALS AND METHODS. *Ability of CP-316819, CP-320626, and CP-380867 to inhibit forskolin-stimulated glycogenolysis in human liver-derived SK-HEP-1 tissue culture cells. †Data from Ref. 18.
concentration weakly decreased hepatic glucose output in a perfused rat liver preparation, where in contrast this concentration inhibited forskolin-stimulated glycolysis in SK-HEP-1 cells by 95% (results not shown). However, at 30 μM, all compounds decreased hepatic glucose output by ~50%. Higher concentrations were not tested because of solubility limitations in the perfusate medium. The reduction in glucagon-stimulated glycolysis in perfused rat liver is consistent with suppression of hepatic glycolysis demonstrated in vivo by the related compound CP-91149 (30). We did not measure the liver glycogen concentration as a part of all the perfusion experiments reported in Fig. 3; however, glycogen concentration was measured at the end of the 120-min perfusion for a limited subset (n = 2); liver glycogen concentration in the controls and CP-316819 (10 μM) perfused livers were 86 ± 26 and 155 ± 38 μmol/g, respectively (J. L. Treadway, personal communication). Thus the measured glycogen levels are consistent with GP inhibition, as reported by glucose production in Fig. 3.

The Hypoglycemic Response Resulting from Oral Dosing of CP-316819 in Diabetic ob/ob Mice

CP-316819 was administered to diabetic ob/ob mice to examine its glucose-lowering activity. The mean plasma glucose concentration in these animals was 332 ± 14 mg/dl (n = 78). The results demonstrate that doses of 5, 10, 25, and 50 mg/kg po were active (P < 0.05), resulting in glucose lowering relative to vehicle of 28, 33, 50, and 40%, respectively (P < 0.05). These results were similar to those reported previously for CP-320626 except that for the latter compound the dose of 5 mg/kg was not active (22). When we determined the liver drug concentration in mice that received a modestly active or inactive concentration (5 mg/kg po) of these compounds, we demonstrated that liver drug concentration over the 4-h time period was on average 5.81 and 17.1 μM for CP-316819 and CP-320626, respectively. Therefore, at 5 mg/kg where CP-316819 showed modest glucose lowering, the measured drug concentration in the liver was ~100-fold higher than the in vitro IC50 values. For CP-320626, our results indicate that liver drug concentrations in excess of those in Table 2 (9–28 μM) are required for efficacy. These concentrations are also in marked excess of the in vitro enzyme IC50 value. It is likely that the in vivo potency of the indole-site GPIs is markedly affected by endogenous ligands in addition to other hepatic factors (e.g., protein binding) not present in the in vitro assay conditions.

DISCUSSION

On discovering the indole inhibitor site, we sought to characterize its interactions with all the allosteric sites on human liver GP to understand its potential role in vivo. The indole-site ligand CP-403700 has been reported to strongly inhibit phosphorylase a, and the inhibition was increased in the presence of 2.5–7.5 mM glucose (36). In the present study, we have confirmed that, at 8 mM glucose, the IC50 for CP-403700 (at 1 mM Pi) is reduced by 50% (from 0.1 to 0.05 μM; Fig. 1). The mechanism for the effect of various effectors on GP may be by each stabilizing its most thermodynamically favored structure. The GPI-favored structure for GP may be closest to the glucose-favored structure.

However, we were surprised to find that, when we added this strong inhibitor at the same IC50 concentration to a combination of other known GP effectors at their physiological concentrations, the inhibitor displayed reduced potency, i.e., the net effect was no change in phosphorylase a activity compared with that in the absence of CP-403700 under the same testing conditions. In the presence of all effectors of the enzyme, the concentration of CP-403700 required to achieve ~60–70% inhibition was ~20-fold higher than the IC50 concentration in 0.05).

Table 2. Drug concentration in PV, VC, and liver after 5 mg/kg oral administration of CP-316819 and CP-320626 to diabetic ob/ob mice

<table>
<thead>
<tr>
<th>Time, h</th>
<th>PV (μM)</th>
<th>VC (μM)</th>
<th>Liver (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.17</td>
<td>4.79</td>
<td>1.67</td>
<td>16.6</td>
</tr>
<tr>
<td>0.5</td>
<td>0.98</td>
<td>0.62</td>
<td>4.26</td>
</tr>
<tr>
<td>1</td>
<td>2.07</td>
<td>1.07</td>
<td>4.03</td>
</tr>
<tr>
<td>2</td>
<td>0.42</td>
<td>0.22</td>
<td>4.32</td>
</tr>
<tr>
<td>4</td>
<td>0.23</td>
<td>0.15</td>
<td>0.97</td>
</tr>
<tr>
<td>AUC 0–4, μMh</td>
<td>4.02</td>
<td>1.96</td>
<td>14.73</td>
</tr>
</tbody>
</table>

PV, portal vein; VC, vena cava; AUC 0–4, area under the curve from 0 to 4 h; n, no. of mice.
the presence of glucose alone (0.05 vs. 1.0 μM; Fig. 2A). Inhibition at higher concentrations of CP-403700 (i.e., 1 and 10 μM) remained glucose dependent (Figs. 2 and 3, A vs. B), suggesting a potentially increased effect of CP-403700 in diabetes.

When we tested three additional indole-site ligands, the IC_{50} values for all of these compounds were significantly higher in the presence of a combination of effectors in vitro (Table 1). The intracellular IC_{50} values in the presence of a combination of all effectors were generally similar to the cellular IC_{50} values in hepatocytes (Table 1). This suggests that a combination of known effectors of phosphorylase significantly decreased the inhibitory effect of the indole-site ligands in vivo as well as in vitro.

Our results also suggest that there is a significant difference between the effective concentrations in cell-based assays and those in animal models. For example, CP-316819 at 10 μM was maximally inhibitory in human SK-HEP-1 cells (results not shown) but only modestly effective in decreasing hepatic glucose output from perfused liver preparations. Significant inhibition of glucagon-stimulated hepatic glucose production in the perfused rat liver was observed at 30 μM CP-316819 and other GPI compounds (Fig. 3). Furthermore, we demonstrated that oral doses of these compounds that were either inactive or moderately effective for lowering blood glucose concentration (5 mg/kg) in ob/ob mice were associated with liver drug concentrations that were 100-fold higher than the in vitro IC_{50} values. This further confirms that a large in vitro vs. in vivo potency difference exists for these indole-site ligands.

The results of this study suggest that major contributors to the in vitro vs. in vivo difference are the physiological ligands of GP found endogenously in cells. Other factors, not studied here, that could also attenuate indole-site GPI potency in vivo include protein binding, partitioning or compartmentalization of compound, and/or yet unidentified endogenous ligands at the indole site. In diabetic ob/ob mice, reduced in vivo effectiveness may in part be the result of systemic effects of GPI compounds in the muscle. However, we have not been able to demonstrate significant changes in muscle glycogen concentration with acute administration of GPI compounds (results not shown). Therefore, skeletal muscle contribution to in vivo effectiveness of GPI compounds likely is minimal. Other unidentified factors that reduce in vivo effectiveness of the GPI compound cannot be completely excluded.

Total phosphorylase and phosphorylase a activities have been reported to be high in type 2 diabetes animal models (db/db, ob/ob, and falfa). However, the phosphorylase activity ratio (ala + b) was not changed. Thus increased interconversion from b to a form of the enzyme did not occur (39). This makes our results more interesting, since it implies that the increased phosphorylase a could potentially be further inhibited by allosteric effectors. In type 1 diabetes animal models (streptozotocin induced and BB rat), both phosphorylase a and total phosphorylase activities were decreased. The ratio again was unchanged (13).

The complete mechanism by which phosphorylase a activity is inhibited in vivo remains to be determined. We hypothesize a mechanism where another, as yet unidentified, endogenous indole-site inhibitor is present which increases GP inhibition by glucose. This hypothesis still needs to be proven by identification of the endogenous ligand. In the present study, we have demonstrated that, in the presence of all known endogenous effectors of the GP enzyme, the potency of indole-site ligands is reduced. However, high concentrations of the indole-site GPIs almost completely inhibit phosphorylase activity and retain a glucose concentration dependence. Thus the effectiveness of an endogenous compound in vivo likely will depend on its concentration in the liver. The natural ligand(s) for the indole site is unknown. The present data also indicate that intracellular concentrations of the synthetic indole-site ligand most likely must be in the micromolar range for it to be an effective inhibitor in vivo. Preliminary data indicate that endogenous purines, which presumably bind to the purine site, may also interact with glucose in regulating phosphorylase a activity, adding to the complexity of the regulation (11).

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GRANTS

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DISCLOSURES

P. E. Generex, E. M. Gibbs, C. B. Levy, and I. L. Treadway are employed by Pfizer, Inc., and hold stock in that company. Y. Kwon holds stock in Pfizer, Inc.

REFERENCES

EFFECTS OF INDOLE-SITE INHIBITORS ON LIVER PHOSPHORYLASE


