Cell signalling and the hormonal stimulation of the hepatic glycine cleavage enzyme system by glucagon

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The glycine cleavage enzyme system (GCS) is found in mitochondria. In liver it is activated by glucagon and other hormones but it is not known how the hormonal signal is transmitted to the mitochondria. We found that the cell-permeant protein phosphatase inhibitor okadaic acid stimulated flux through GCS and could induce a significant increase in the sensitivity of GCS and of glycogenolysis to glucagon. Half-maximal stimulation of GCS by glucagon occurred at 3.2 ± 0.6 nM, whereas it was fully activated at 0.3 nM in the presence of 1 μM okadaic acid. The protein kinase A agonist adenosine-3′,5′-cyclic monophosphorothioate, Sp isomer (10 μM) stimulated the GCS flux by approx. 100 %. This stimulation was inhibited by the protein kinase A antagonist 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp isomer (Rp-8-Br-cAMPS). Although Rp-8-Br-cAMPS significantly inhibited glucagon-stimulated glycogenolysis it had no effect on the glucagon-stimulated GCS flux. These results indicate that a cytoplasmic phosphorylated protein is involved in transmitting glucagon's effect to the mitochondria. However, protein kinase A does not have a necessary role in transmitting glucagon's signal. We also examined the role of protein kinase C because angiotensin II also stimulated flux through GCS. However, the phorbol ester PMA had no effect on either GCS or on glycogenolysis.

INTRODUCTION

Glycine is a dietary non-essential amino acid that can be readily synthesized from common metabolic intermediates in all organisms. In addition to its role in protein synthesis, glycine has multiple roles in many synthetic reactions such as gluconeogenesis, purine, haem and chlorophyll synthesis and bile acid conjugation. Glycine is also a major source of one-carbon units with its α-carbon being transferred to tetrahydrofolate to give N^5,N^{10}-methylene-tetrahydrofolate. The major pathway for glycine catabolism is through the hepatic glycine cleavage enzyme system (GCS) [1,2]. GCS is loosely bound to the inner mitochondrial membrane. The overall reaction catalysed is:

$$\text{glycine} + \text{THF} + \text{NAD}^+ \rightarrow \text{CO}_2 + \text{NH}_3 + \text{NADH} + \text{H}^+ + \text{CH}_2\text{-THF}$$

where THF and CH_2-THF represent tetrahydrofolate and N^5, N^{10}-methylene-tetrahydrofolate respectively.

Many studies have shown that the flux through GCS is stimulated by hormones that act via cAMP as well as by hormones known to act by increasing the intracellular Ca^{2+} concentration. Jois et al. [3] were the first to report the stimulation of GCS by glucagon. Dibutyryl-cAMP was also effective in stimulating GCS flux; a significant correlation was observed between increased cellular cAMP levels induced by glucagon and stimulation of the flux through GCS by glucagon [4]. It is clear that glucagon acts by the stimulation of GCS itself rather than by the activation of transport. In isolated hepatocytes, glucagon increases neither the intracellular glycine concentration nor its specific radioactivity [3]. It is also evident that stimulation of glycine uptake by mitochondria cannot be the underlying mechanism, because the mitochondrial uptake of glycine is much too rapid to present a rate limitation [5]. It is not known exactly which signalling pathway is reponsible for the activation of this mitochondrial enzyme, or how the hormonal signal is transmitted to the mitochondria. A single glucagon receptor cloned from rat liver transduces signals that give rise to two separate second messengers, cAMP and Ca^{2+}, probably by interaction with separate G-protein [6]. Therefore we examined this question with the help of various agents that specifically stimulate or inhibit certain parts of signalling pathways.

MATERIALS AND METHODS

Materials

[1-^{14}C]Glycine and Omnifluor were obtained from DuPont–New England Nuclear (Mississauga, Ontario, Canada). Collagenase CLS2 was from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). Okadaic acid was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). Adenosine-3′,5′-cyclic monophosphorothioate, Sp isomer (Sp-cAMPS) and 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp isomer (Rp-8-Br-cAMPS) were purchased from Biolog Life Science Institute (La Jolla, CA, U.S.A.). Vasopressin and angiotensin II, PMA and PMA 4-*O*-methyl ether were from Sigma (St. Louis, MO, U.S.A.). Thapsigargin was obtained from LC Services Corporation (Boston, MA, U.S.A.). Hexokinase and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim (Laval, Quebec, Canada).

Isolation of cells

Hepatocytes were isolated from fed male Sprague–Dawley rats (300–400 g) as described previously [7]. The rats were permitted free access to water and Purina rat chow. More than 95 % of the isolated hepatocytes cells excluded 0.2 % Trypan Blue.

Mitochondria were isolated from livers of male Sprague–Dawley rats as described previously [3]. Mitochondrial protein was determined by the biuret method with BSA as a standard [8].

Abbreviations used: GCS, glycine cleavage enzyme system; Rp-8-Br-cAMPS, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp isomer; Sp-cAMPS, adenosine-3′,5′-cyclic monophosphorothioate, Sp isomer.

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Measurement of flux through GCS

Flux through GCS was obtained by measuring $^{14}CO_2$ production from 0.3 mM [1-^{14}C]glycine [3]. This concentration was chosen because it approximates the glycine concentration in the hepatic portal vein [9]. Hepatocytes were preincubated for 20 min before incubations were performed, in triplicate, in a total volume of 1 ml of Krebs–Henseleit medium, gassed with O_2/CO_2 (19:1) at 37 °C in 25 ml Erlenmeyer flasks. The final incubations contained 6–10 mg dry weight of cells/ml. The preincubation period was followed by a 30 min incubation period with [1-^{14}C]glycine. $^{14}CO_2$ production was measured as described [3] and glycogenolysis was determined by measuring glucose production [10] during the 30 min incubation (any glucose produced during the 20 min preincubation period was determined and subtracted from that found after the 30 min incubation).

Statistical analysis

All values are expressed as means ± S.D. for three separate experiments. Statistical analysis was done by Student's *t* test and analysis of variance (ANOVA); $P < 0.05$ was taken as indicating statistical significance. Maximal stimulation and half-maximal stimulation by different hormones and agonists were calculated by fitting the data to the equation for a rectangular hyperbola, which describes the binding of a ligand to a receptor as a function of its concentration:

$$Y = AX/(B+X)$$

where Y represents the rate of flux through GCS, X the hormone or agonist concentration, A the maximal stimulated rate and B the hormone or agonist concentration that gives half the maximal stimulated rate. The curves are well described by this equation, as indicated by r^2 values of more than 0.95.

RESULTS

Protein phosphorylation and flux through GCS

The possibility of the involvement of phosphorylated proteins in the stimulation of GCS was examined by using okadaic acid, which is a potent cell-permeant inhibitor of protein phosphatases PP1 and PP2A [11]. Okadaic acid stimulates the flux through GCS and glycogenolysis significantly in isolated hepatocytes (Figure 1). The maximal effect was obtained at 1 μM okadaic acid. Okadaic acid had no effect on GCS when assayed in isolated mitochondria (results not shown). This suggests that the stimulation found in hepatocytes is due to the inhibition of cytosolic protein phosphatases, with a consequent increase in the phosphorylation state of cytosolic proteins.

We examined the glucagon response curve for the activation of GCS in the presence and the absence of okadaic acid. A glucagon dose–response curve in the presence of a low concentration of okadaic acid (0.01 μM) was not significantly different from one with glucagon alone. Half-maximal stimulation occurred at 3.5 ± 1.3 compared with 2.0 ± 0.1 nM (results not shown). Similar results were obtained for glycogenolysis. However, when 1 μM okadaic acid was used there was a marked leftwards shift in the cells' response to glucagon without affecting the maximal stimulation by glucagon (Figure 2a). Half-maximal stimulation of the flux through GCS by glucagon occurred at 3.2 ± 0.6 nM, whereas full activation was evident at 0.3 nM in the presence of okadaic acid ($P < 0.05$. Glycogenolysis was already substantially stimulated by this concentration of okadaic acid (Figure 2b) so that addition of glucagon in the presence of okadaic acid had little effect.

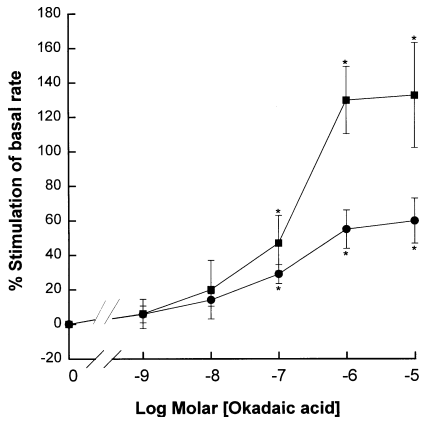

Figure 1 Effect of okadaic acid on the flux through GCS and glycogenolysis

Okadaic acid was added to the hepatocytes at the beginning of the 20 min preincubation at 37 °C, after which 0.3 mM [1-^{14}C]glycine was added for a further 30 min. Okadaic acid was dissolved in DMSO. Control incubations with DMSO alone were performed. The basal rate for GCS flux (●) was 1.1 ± 0.3 nmol/30 min per mg dry weight and for glycogenolysis (■) was 235 ± 38 nmol/30 min per mg dry weight. Results are means ± S.D.; $n = 3$. *$P < 0.05$ compared with control, paired *t* test.

Protein kinase A is a major kinase responsible for phosphorylating cytosolic proteins in response to glucagon. Therefore the question arises as to whether protein kinase A is responsible for the activation of GCS. Sp-cAMPS, a cell-permeant agonist for protein kinase A, and Rp-8-Br-cAMPS, a cell-permeant antagonist of protein kinase A, were therefore used to examine the regulation of glycogenolysis and gluconeogenesis in isolated hepatocytes [12–15]. Sp-cAMPS was a potent stimulator of the flux through GCS and glycogenolysis, whereas Rp-8-Br-cAMPS at concentrations up to 100 μM had no effect on GCS flux or glucose production. Half-maximal stimulation of the flux through GCS occurred at 215 ± 70 nM Sp-cAMPS, with a maximal stimulation of approx. 100 % at 100 μM Sp-cAMPS. A control experiment showed that neither Sp-cAMPS nor Rp-8-Br-cAMPS had an effect on the flux through GCS in isolated mitochondria (results not shown).

We therefore examined whether Rp-8-Br-cAMPS could inhibit the Sp-cAMPS-stimulated GCS flux in isolated hepatocytes. Isolated hepatocytes were preincubated with different concentrations of Rp-8-Br-cAMPS before the addition of [1-^{14}C]glycine and 10 μM Sp-cAMPS. Rp-8-Br-cAMPS was found to inhibit the Sp-cAMPS stimulation of flux through both GCS and glycogenolysis, with virtually complete inhibition at 10 μM Rp-8-Br-cAMPS (Figure 3). When we preincubated isolated hepatocytes with 100 μM Rp-8-Br-cAMPS before challenge with different concentrations of Sp-cAMPS we found a significant decrease in the sensitivity of the GCS flux to stimulation by Sp-cAMPS (Figure 4a). The concentration of Sp-cAMPS required for half-maximal stimulation increased from 190 ± 133 to 902 ± 133 nM ($P < 0.05$). A similar inhibition of Sp-cAMPS-stimulated glycogenolysis was observed in these experiments (Figure 4b). For glycogenolysis the concentration of Sp-cAMPS required to effect a half-maximal stimulation increased from 220 ± 33 to 804 ± 155 nM. Thus both Sp-cAMPS and Rp-8-Br-cAMPS were effective in these experiments. Sp-cAMPS is sufficiently cell-permeant and insensitive to phosphodiesterase to activate GCS and glycogenolysis, whereas Rp-8-Br-cAMPS is sufficiently cell-permeant and insensitive to phosphodiesterase to inhibit protein kinase A.

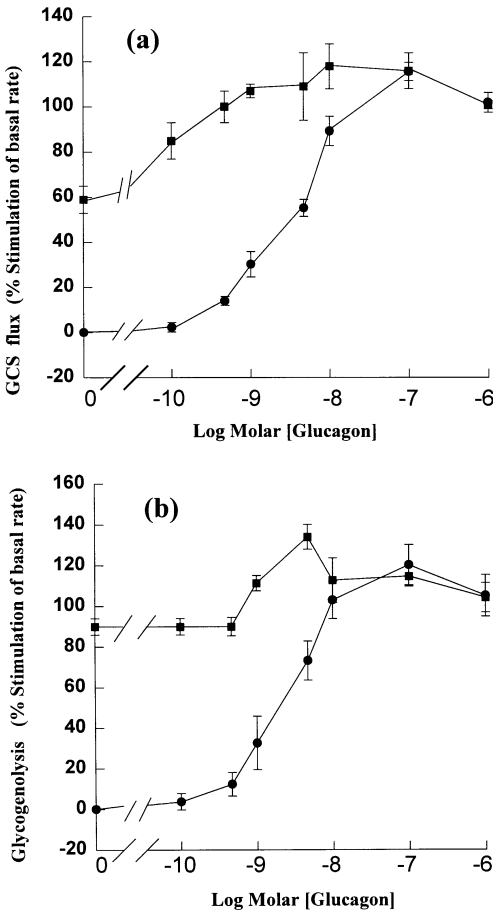

Figure 2 Effect of okadaic acid on glucagon-stimulated GCS flux (a) and glycogenolysis (b) in isolated hepatocytes

Isolated hepatocytes were preincubated at 37 °C for 20 min with (■) or without (●) 1 μM okadaic acid before the addition of 0.3 mM [1-^{14}C]glycine and various concentrations of glucagon. The basal rate of GCS flux was 1.7 $\pm$ 0.4 nmol/30 min per mg dry weight, and that for glycogenolysis was 210 $\pm$ 21 nmol/30 min per mg dry weight. Results are means $\pm$ S.D. for three separate experiments.

We next examined whether Rp-8-Br-cAMPS could inhibit the stimulation by glucagon of GCS flux and glycogenolysis. The results presented in Figure 5 show that even very high concentrations of Rp-8-Br-cAMPS did not inhibit glucagon-stimulated flux through GCS, whereas they effectively inhibited glucagon-stimulated glycogenolysis. Glucagon dose–response curves were performed with and without preincubation of the isolated hepatocytes with 10 μM Rp-8-Br-cAMPS. Rp-8-Br-cAMPS had no effect on the sensitivity of the hepatocytes to glucagon stimulation of the flux through GCS. Half-maximal stimulation was unchanged at 2.7 $\pm$ 0.3 nM (Figure 6a). However, the concentration of glucagon required to give a half-maximal stimulation of glycogenolysis increased from 2.3 $\pm$ 0.2 to 5.0 $\pm$ 0.9 nM ($P < 0.05$) (Figure 6b).

Effect of angiotensin II, protein kinase C and thapsigargin on GCS

Jois et al. [3] showed that vasopressin stimulates GCS. We therefore examined whether angiotensin II, which also stimulates glycogenolysis by increasing intracellular Ca^{2+} and has no effect on cellular cAMP levels [16], could stimulate flux through GCS. Incubation of isolated hepatocytes with 100 pM–1 μM angio-

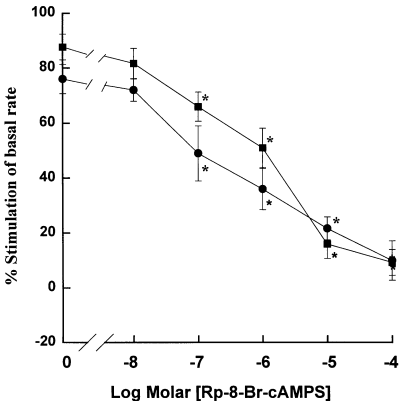

Figure 3 Effects of Rp-8-Br-cAMPS on the Sp-cAMPS-stimulated GCS flux and glycogenolysis

Isolated hepatocytes were preincubated with various concentrations of Rp-8-Br-cAMPS for 20 min before the addition of 0.3 mM [1-^{14}C]glycine and the challenge with 10 μM Sp-cAMPS. The basal rate for GCS flux (●) was 2.0 $\pm$ 0.2 nmol/30 min per mg dry weight and that for glucose production (■) was 120 $\pm$ 19 nmol/30 min per mg dry weight. Results are expressed as percentage stimulation of the basal rate and are means $\pm$ S.D. * $P < 0.05$ compared with control with no Rp-8-Br-cAMPS, using ANOVA.

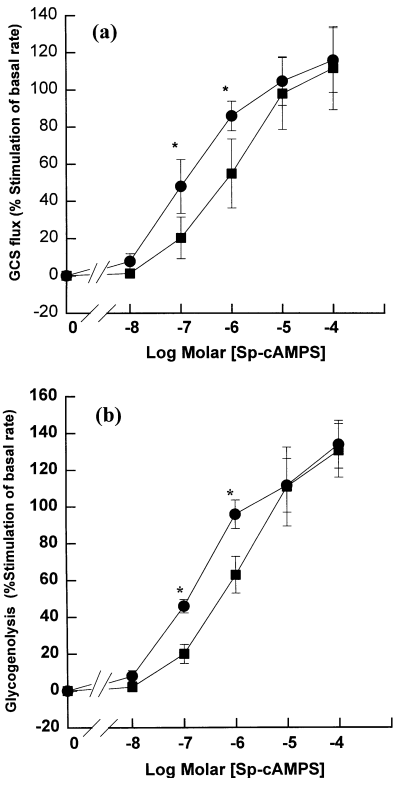

Figure 4 Effect of Sp-cAMPS on flux through GCS (a) and glycogenolysis (b) in the presence and the absence of Rp-8-Br-cAMPS

A dose–response curve for the stimulation of GCS flux and glycogenolysis by Sp-cAMPS was performed with (■) or without (●) 10 μM Rp-8-Br-cAMPS. The basal rate for GCS flux was 2.2 $\pm$ 0.5 nmol/30 min per mg dry weight. Half-maximal stimulation occurred at 199 $\pm$ 100 nM Sp-cAMPS compared with 902 $\pm$ 133 nM Sp-cAMPS in the presence of Rp-8-Br-cAMPS ($P < 0.05$; paired t test). The basal rate for glycogenolysis was 159 $\pm$ 33 nmol/30 min per mg dry weight. Half-maximal stimulation occurred at 220 $\pm$ 33 nM Sp-cAMPS compared with 804 $\pm$ 155 nM Sp-cAMPS. *$P < 0.05$, paired t test.

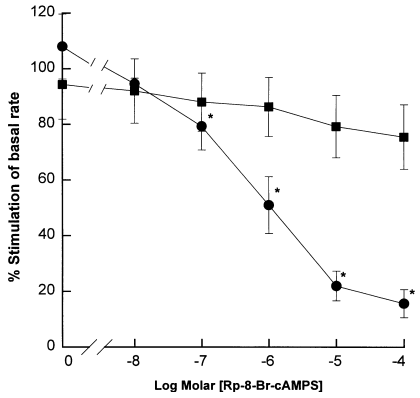

Figure 5 Effect of Rp-8-Br-cAMPS on the glucagon-stimulated flux through GCS and glycogenolysis in isolated hepatocytes

Isolated hepatocytes were preincubated with various concentrations of Rp-8-Br-cAMPS before they were challenged with 10 nM glucagon. The basal rate for GCS flux (●) was 2.0 ± 0.2 nmol/30 min per mg dry weight and that for glycogenolysis (■) was 156 ± 39 nmol/30 min per mg dry weight. Results are presented as percentage stimulation and are means ± S.D. for three experiments. $^*P < 0.05$ compared with control with no Rp-8-Br-cAMPS (paired *t* test).

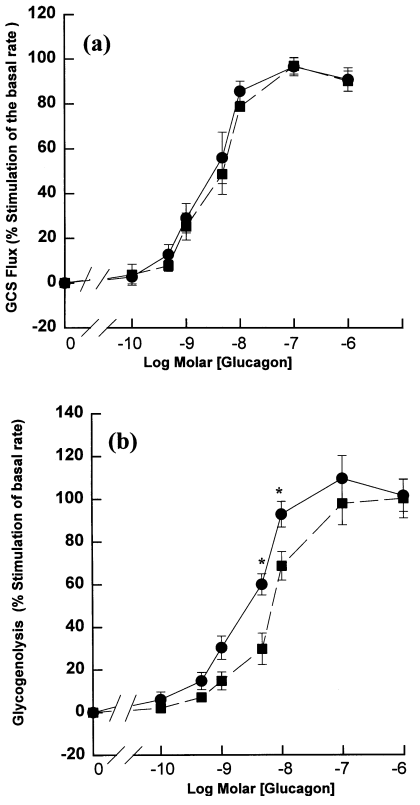

Figure 6 Effect of glucagon on GCS flux (a) and glycogenolysis (b) in the presence and the absence of Rp-8-Br-cAMPS

Dose–response curves for the glucagon stimulation of GCS (**a**) and glycogenolysis (**b**) were performed with (■) or without (●) 10 μM Rp-8-Br-cAMPS. The basal rate for GCS flux was 2.3 ± 0.9 nmol/30 min per mg dry weight and that for glycogenolysis was 205 ± 14 nmol/30 min per mg dry weight. Results are means ± S.D. for three separate experiments. $^*P < 0.05$ compared with control with no Rp-8-Br-cAMPS (paired *t* test).

tensin II stimulated the flux through GCS and glycogenolysis with a maximum stimulation of approx. 30% at 1 μM (results not shown). Because protein kinase C might be stimulated by glucagon [17] we determined whether activation of this enzyme would stimulate flux through GCS. PMA, a tumour-promoting phorbol ester, is a structural analogue of diacylglycerol and activates protein kinase C [17]. PMA 4-*O*-methyl ether interacts very poorly with protein kinase C and was used as a control [18]. Neither of these compounds, at concentrations of 1 nM to 10 μM, had any effect on glycogenolysis or GCS flux; nor did they affect glucagon's activation of GCS (results not shown). We also examined the effects of thapsigargin, an agent that increases intracellular Ca^{2+} levels in isolated hepatocytes [19] by inhibition of the endoplasmic-reticulum Ca^{2+}-ATPase. At 500 nM, thapsigargin significantly increased flux through GCS in hepatocytes (by 60%).

DISCUSSION

Although the regulation of GCS in liver mitochondria by hormones has been established, the means whereby the signal is transmitted from the glucagon receptor through the cytoplasm into the mitochondria is not known. Phosphorylation/dephosphorylation events have important roles in metabolic regulation, including glucagon action. Therefore the possibility of the involvement of phosphorylated proteins in the regulation of GCS was examined by using the cytosolic protein phosphatase inhibitor okadaic acid. Okadaic acid inhibits protein phosphatase 1 and protein phosphatase 2A in the cytosol, leading to an increase in the level of phosphorylated proteins [11,20]. Our results (Figure 1) suggest that increased cytosolic protein phosphorylation, produced by okadaic acid, causes a significant increase in the flux through GCS and in glycogenolysis. That 1 μM okadaic acid was able to produce a leftwards shift of the glucagon dose–response curve suggests that cytosolic protein phosphorylation is an essential element in the glucagon stimulation of the flux through GCS.

The stimulation of GCS flux by Sp-cAMPS demonstrates that the activation of protein kinase A can result in a stimulation of flux through GCS. However, the failure of Rp-8-Br-cAMPS to antagonize glucagon's stimulation of GCS (even though it antagonized the stimulation brought about by Sp-cAMPS) is taken to indicate that protein kinase A activation is not necessary for glucagon's effect. Nevertheless Rp-8-Br-cAMPS did antagonize glucagon-stimulated glycogenolysis, which is consistent with results of Rothermel et al. [13]. A possible explanation for the failure of an inhibitory effect of Rp-8-Br-cAMPS on glucagon-stimulated flux through GCS could be the fact that glucagon stimulation results in an increase in both Ca^{2+} and cAMP levels in the liver [6,21] and that both of these intracellular messengers might independently activate the flux through GCS (i.e. redundant signalling mechanisms). Rp-8-Br-cAMPS would antagonize the cAMP mechanism (protein kinase A) but would not affect the Ca^{2+} signal. Indeed experiments from this laboratory have shown that submicromolar concentrations of Ca^{2+} can stimulate GCS in isolated liver mitochondria [22]. In addition, this paper reports experiments that show that thapsigargin, an agent that increases intracellular Ca^{2+} levels by inhibiting the endoplasmic Ca^{2+} reuptake mechanism, also activates GCS.

The observation that the phosphorylation of some glucagon targets can be stimulated by agents that increase intracellular Ca^{2+} concentration [23,24] raises the possibility that Ca^{2+}/calmodulin-sensitive protein kinases might also have a role in the response of hepatocytes to glucagon. It is also relevant that vasopressin [3] and angiotensin II (this study) activate GCS,

because these agents activate the Ca^{2+} signalling pathway but have no effect on cAMP levels [25,26]. Angiotensin II also stimulated glycogenolysis, which confirmed the results of Hems et al. [26].

Protein kinase C does not seem to be involved in the regulation of GCS or glycogenolysis. These results are in agreement with those of Corvera and Garcia-Sainz, who showed that 10 pM–1 μM PMA was unable to stimulate glycogenolysis in isolated rat hepatocytes [27]. In addition there was no effect of PMA on the glucagon stimulation of the flux through GCS or glycogenolysis. This is consistent with the results both of Corvera and Garcia-Sainz [27] and of Püschel et al. [28], who showed that the stimulation of glycogenolysis by glucagon was unaffected by PMA in isolated rat hepatocytes and in the perfused rat liver. It must be borne in mind that diacylglycerol produced in the cell might activate protein kinase C isoforms selectively, depending on its fatty acid composition, and might result in different effects from those caused by phorbol esters. However, at present the balance of our evidence is that protein kinase C does not activate either GCS or glycogenolysis.

In conclusion, our results indicate that phosphorylation of cytosolic proteins plays a role in the activation of GCS. Activation of protein kinase A can bring about the activation of the glycine cleavage enzyme, but antagonism of this enzyme does not interfere with glucagon's ability to activate it. This is most probably accounted for by redundant signalling pathways in the activation of GCS by glucagon.

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