Mechanism of glucagon stimulation of fructose-1,6-bisphosphatase in rat hepatocytes

Involvement of a low-M_r activator

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Isolated rat hepatocytes were incubated in the absence or presence of glucagon and the activity of fructose-1,6-bisphosphatase was measured in cell extracts. After glucagon treatment the V_{max} was increased (20–50%) whereas the K_m remained unchanged. The stimulation was complete at 5 min after addition of glucagon. The glucagon concentration needed for maximal stimulation was 10^{-9} M. After gel filtration the fructose-1,6-bisphosphatase activity in extracts of glucagon-treated cells was lowered to the control level. The effect of glucagon could not be completely mimicked by dibutyryl cAMP. The data indicate that in addition to the possible regulatory role of enzyme phosphorylation, a positive effector is involved in the stimulation of fructose-1,6-bisphosphatase activity by glucagon.

Fructose-1,6-bisphosphatase Glucagon Low-M_r activator (Rat hepatocyte)

1. INTRODUCTION

Fructose-1,6-bisphosphatase (EC 3.1.3.11; FBPase) is part of the regulatory important fructose 1,6-bisphosphate/fructose 6-phosphate substrate cycle, and is thought to be regulatory for the gluconeogenic/glycolytic pathway [1]. Rat liver FBPase can be phosphorylated in vitro by cAMP-dependent protein kinase [2,3] and in hepatocytes its phosphorylation is increased by glucagon [4]. FBPases from mouse, rabbit and ox liver as well as from pig kidney cannot be phosphorylated [3,5], since they lack a C-terminal extension, containing the phosphorylation site [6]. The effect of phosphorylation on the kinetic properties of the enzyme is under dispute. An increase in V_{max} [2], a decrease in K_m [7], both an increase in V_{max} and a decrease in K_m [8] or no change in V_{max} and K_m [2,6] have been reported. Phosphorylated FBPase has also been reported to be less sensitive to inhibition by fructose 2,6-bisphosphate than the unphosphorylated enzyme [9,27]. Phosphorylation of FBPase is not generally accepted as playing an important role in the regulation of gluconeogenesis and glycolysis [10].

Allosteric regulation of FBPase can be performed by several metabolites. FBPase is inhibited by AMP [11] and fructose 2,6-bisphosphate [12,13]. Since the fructose 2,6-bisphosphate level in hepatocytes is lowered after glucagon treatment [14,15], fructose 2,6-bisphosphate has been put forward as the main factor controlling the activity of FBPase in vivo [10] through a relief of enzyme inhibition. However, Corredor et al. [16] found that micromolar concentrations of fructose 2,6-bisphosphate can also stimulate FBPase activity.

Administration of glucagon in vivo leads to increased activity of rat [17,18] and mouse [19] liver FBPase. Since mouse liver FBPase cannot be

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phosphorylated [3,6] it is unlikely that phosphorylation plays a decisive role in determining the activity of FBPase.

Here, we show that treatment of hepatocytes with glucagon leads to a rapid increase in V_{max} of FBPase. This increase is not due to phosphorylation of the enzyme or to a change in fructose 2,6-bisphosphate concentration, but is caused by a low-M_r activator.

2. MATERIALS AND METHODS

Male Wistar rats (250 g) were anesthetized with 18 mg Nembutal given intraperitoneally. Parenchymal liver cells were isolated by perfusion with collagenase by the method of Seglen [20]. 2% albumin was added to the collagenase buffer and washing buffer. Cells were incubated in Krebs-Ringer with a protein concentration of 10 mg/ml. The cells were kept in suspension by shaking in a water bath at 37°C and gassed with 95% O_2, 5% CO_2. Viability of the cells was usually over 90% as judged by phase-contrast microscopy. Incubations were stopped by cooling in ice and after addition of 1 mM 2-mercaptoethanol samples were immediately homogenized. Homogenates were centrifuged for 10 min at 10000 × g. In the supernatant, FBPase activity was assayed immediately after centrifugation and an Aminco DW2 double-beam spectrophotometer was used to monitor the assay at 340/400 nm. The assay mixture consisted of 10 mM potassium phosphate buffer (pH 7.5), 25 mM 2-mercaptoethanol, 1 mg/ml albumin, 2.5 mM $MgSO_4$, 0.4 mM NADP, 7 units glucose-6-phosphate dehydrogenase and 3.5 units phosphohexose isomerase (both enzymes were desalted on Sephadex G-25); final volume 2.3 ml. The mixture was preincubated with 200 μl sample for 3 min at 30°C and the reaction initiated by the addition of 100 μl fructose 1,6-bisphosphate solution. In the samples some 6-phosphogluconate dehydrogenase activity was present, but it was verified that under the present conditions it did not interfere with our measurements. The FDPase activity was determined between 1 and 3 min after starting the reaction, when V was nearly constant. Where indicated, aliquots of the supernatant fraction were desalted on Sephadex G-25 medium (equilibrated with H_2O) by the method of Penevsky [21]. The low-M_r fraction was obtained by elution and subsequent lyophilisation. Protein concentrations were determined by the method of Lowry et al. [22]. L-type pyruvate kinase activity was determined as in [23]. Collagenase type I, bovine serum albumin fraction V, fructose 1,6-bisphosphate and fructose 2,6-bisphosphate were from Sigma.

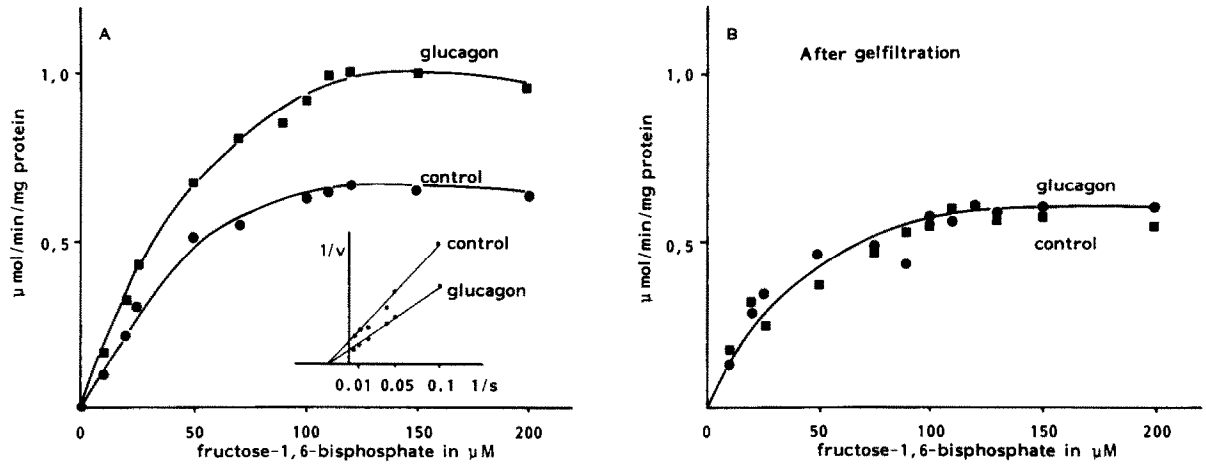

Fig.1. Effect of glucagon on the FBPase activity. FBPase activity was measured in supernatants of control (●) and glucagon (10^{-7} M, 10 min) treated cells (■), before (A) and after (B) gel filtration. Inset: double-reciprocal plot of substrate curves of control and glucagon stimulated FBPase. Results shown are from a typical experiment (n = 7).

3. RESULTS AND DISCUSSION

Substrate curves of FBPase from control and glucagon-stimulated parenchymal cells are given in fig.1A. After glucagon treatment the activity of FBPase is increased. The double-reciprocal plot indicates that the V_{max} is increased by glucagon treatment whereas the K_m (40 μM) remains unchanged. Routinely we found 20–40% stimulation of FBPase upon glucagon addition, although occasionally up to 70% stimulation was observed. To determine whether the increase in activity is caused by covalent modification or by the presence of an effector, the FBPase activity was determined in gel-filtered samples (fig.1B). Upon gel filtration the activity of control samples was unchanged, while the activity in the glucagon-treated samples was lowered to the control level. This indicates that the increase in FBPase activity after glucagon treatment is not caused by phosphorylation but probably mediated by an effector.

In our experiments however, an increased phosphorylation state of FBPase was indicated by the finding that in gel-filtered glucagon-treated samples FBPase was less sensitive to fructose 2,6-bisphosphate inhibition than control FBPase, similar to the findings in [9]. Since the activation of FBPase by glucagon can be abolished by gel filtration, a low-M_r activator is suspected as being responsible for the observed difference. Readdition of the low-M_r fraction from glucagon-treated samples indeed leads to an increase in FBPase activity (table 1). The nature of this activator is however unclear. Activation of FBPase cannot be explained by decreased inhibition by fructose 2,6-bisphosphate since gel filtration would then lead to increased FBPase activity of the control samples. Although we found, as did Corredor et al. [16], that 1 μM fructose 2,6-bisphosphate could stimulate FBPase after gel filtration, fructose 2,6-bisphosphate could not be responsible for activating FBPase after glucagon treatment because the concentrations of fructose 2,6-bisphosphate in the assay can be calculated to be about 2 nM for glucagon-treated and 20 nM for control samples, which are too low to stimulate FBPase. Calculations were based on data from [24]. Moreover, in the presence of fructose 2,6-bisphosphate at inhibiting concentrations, added low-M_r fraction stimulates the FBPase activity, indicating that the activator is not fructose 2,6-bisphosphate. To characterize further the kinetics of the stimulation of FBPase activity by glucagon we studied the dose and time dependency of the effect. Fig.2 shows that the effect is almost complete at 5 min after addition of glucagon. Fig.3 indicates that 10^{-9} M glucagon is needed for maximal activation of FBPase activity, while half-maximal activation occurs at about 10^{-11} M, which is well within the range of the glucagon dose needed for other gluconeogenic effects [10].

Since the glucagon effect on gluconeogenesis can be mimicked by dibutyryl cAMP [10], we compared the effect of dibutyryl cAMP with that of glucagon. Although dibutyryl cAMP was equally active as glucagon in inactivating L-type pyruvate kinase [25], we observed only a marginal effect of dibutyryl cAMP on FBPase (table 2). This implies

Table 1

Effect of low-M_r fraction on the activity of FBPase

	Cells incubated without glucagon		Cells incubated with glucagon	
	FBPase (pmol/min per mg protein)	% stimulation	FBPase (pmol/min per mg protein)	% stimulation
Control	553 ± 51	–	519 ± 31	–
20 μl activator	623 ± 33	13	589 ± 39	14
40 μl activator	666 ± 9[a]	21	671 ± 15[a]	29

[a] Significant difference from control ($P < 0.05$, Student's t-test, tested for equal variances)

Low-M_r fraction ('activator') was isolated and concentrated from a glucagon-stimulated sample. Different amounts (20, 40 μl) were added to assays of untreated and glucagon-treated gel-filtered samples. Values are given ± SD ($n = 3$)

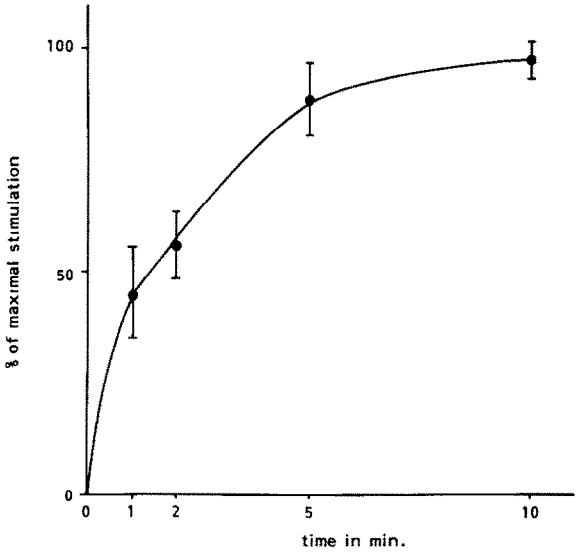

Fig.2. Time dependency of the FBPase activation by glucagon. FBPase activity was measured at 100 μM fructose 1,6-bisphosphate, in supernatants of cell stimulated with glucagon (10^{-7} M) for different periods of time. Values are given ± SE (n = 4).

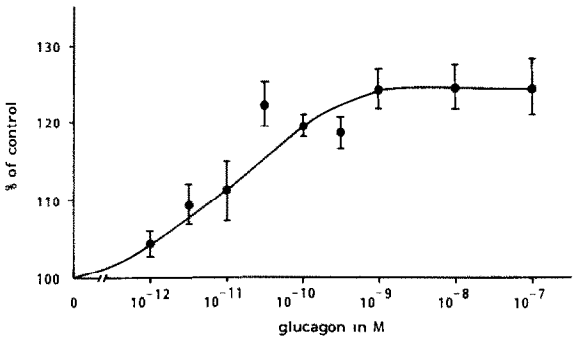

Fig.3. Dose dependency of the FBPase activation by glucagon. FBPase activity was measured at 100 μM fructose 1,6-bisphosphate in supernatants of cells stimulated with different doses of glucagon for 10 min. Values are given ± SE (n = 5).

that besides cAMP other second messengers might be involved, perhaps Ca^{2+}, which is known to increase after glucagon treatment [26]. The difference in the effects of glucagon and dibutyryl cAMP is a further indication against the involvement of cAMP-dependent phosphorylation or

Table 2

Influence of glucagon and dibutyryl cAMP on the fructose-1,6-bisphosphatase and L-type pyruvate kinase activity

	FBPase (pmol/min per mg protein)	% stimulation	Pyruvate kinase (v/V_{max})	% inhibition
Control	545 ± 7	–	0.66 ± 0.10	–
Glucagon	675 ± 6[a]	24	0.34 ± 0.09[a]	51
Dibutyryl cAMP	581 ± 27	7	0.33 ± 0.07[a]	50

[a] Significant difference from control ($P < 0.01$, Student's t-test tested for equal variances)

FBPase and pyruvate kinase activity were measured in supernatants of cells stimulated with glucagon (10^{-7} M) or dibutyryl cAMP (10^{-4} M) for 10 min. FBPase activity was measured at 100 μM fructose 1,6-bisphosphate. Pyruvate kinase was measured at 2 mM phospho*enol*pyruvate in the absence (v) and presence (V_{max}) of 50 μM fructose 1,6-bisphosphate. Values are given ± SD (n = 4)

fructose 2,6-bisphosphate in the activation of FBPase in rat hepatocytes. Our data indicate that a low-M_r activator is involved in the activation of FBPase by glucagon in rat hepatocytes.

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