Inactivation of Phosphofructokinase by Glucagon in Rat Hepatocytes*

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Kinetic evidence of a time- and dose-dependent inactivation of phosphofructokinase by glucagon in isolated rat hepatocytes is reported. This inactivation, which persists after gel filtration of a cell-free extract on Sephadex G-25 and after 400-fold purification of the enzyme on agarose-ATP, is observed when the enzyme activity is measured at subsaturating concentrations of fructose-6-phosphate, while there is no change in \( V_{\text{max}} \). Phosphofructokinase inactivation by glucagon parallels the known inactivation of pyruvate kinase L and activation of glycogen phosphorylase a. Exogenous cyclic AMP mimics the effect of this hormone. Half-maximal effect for both phosphofructokinase and pyruvate kinase L is caused by a similar dose of glucagon (1 \( \times 10^{-10} \) M). The inactivation of phosphofructokinase by nonsaturating concentration of glucagon is reversed spontaneously within 40 min of incubation and this reversion is accelerated by insulin.

A decrease in the metabolic flux through phosphofructokinase by the action of glucagon has been deduced from isotopic studies in isolated rat hepatocytes (1, 2). Söling and co-workers (3, 4) have concluded from their experiments that rat liver phosphofructokinase can be phosphorylated by a cAMP-independent protein kinase and dephosphorylated by a phosphatase with, respectively, increase and decrease of the \( V_{\text{max}} \) of the enzyme. These results cannot easily explain the decrease in the metabolic flux through phosphofructokinase by glucagon as this hormone acts mainly by a cAMP-dependent mechanism. Furthermore, a clear evidence of a change in the kinetic properties of the liver enzyme produced by glucagon has not been reported (5, 6).

We have found a modification of the kinetic regulatory properties of phosphofructokinase from isolated rat hepatocytes treated with glucagon which produces the inactivation of the enzyme measured at subsaturating concentrations of fructose-6-P. This inactivation, antagonized by insulin and mimicked by cAMP, could account for the decrease in the metabolic flux through phosphofructokinase by glucagon.

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**EXPERIMENTAL PROCEDURES**

Isolation and Incubation of Hepatocytes—Hepatocytes were isolated from normally fed Wistar rats (250 to 300 g) by the method of Berry and Friend (7) as modified by Hue et al. (8). Livers were perfused at 37°C with 200 ml of Krebs-Henseleit bicarbonate medium without calcium (9) and afterwards perfused 15 min with 100 ml of the same medium to which approximately 9000 units of collagenase (Sigma, No. C-2139) was added. Continuous oxygenation of the perfusate was achieved by a mixture of 95% O₂ and 5% CO₂. After disruption of the liver, the cell suspension was filtrated through cheese cloth, centrifuged 1 min at 50 x g, and washed in Krebs-Henseleit bicarbonate medium. Cells were centrifuged again and resuspended in the last medium to give a final concentration of 30 to

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**FIG. 1. Time course of the effect of glucagon on phosphofructokinase, pyruvate kinase L, and glycogen phosphorylase a activities of rat hepatocytes. Control hepatocytes, open symbols; glucagon (10\(^{-6}\) M)-treated hepatocytes, closed symbols. Panel a, phosphofructokinase activity; Panel b, pyruvate kinase activity; Panel c, glycogen phosphorylase a activity. Incubations of hepatocytes and assays of enzyme activities were carried out as described under "Experimental Procedures." At zero time, saline or glucagon were added. Values for Panels a and b are means ± S.D. from three experiments, each one with incubations by triplicate. Values for Panel c are an average of two experiments.
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Fig. 2. Saturation curves of phosphofructokinase for fructose-6-P. Control hepatocytes; Glucagon (10−8 M)-treated hepatocytes. Panel a, saturation curve of phosphofructokinase from crude extracts; Panel b, saturation curve of partially purified phosphofructokinase. Aliquots of hepatocytes were taken 10 min after addition of saline or glucagon. Incubations of hepatocytes and assays and purification of phosphofructokinase were carried out as described under “Experimental Procedures.” Insets show Hill plots of the saturation curves.

**Table 1**

| Incubation Conditions | Before Sephadex G-25 | After Sephadex G-25 |
|-----------------------|----------------------|---------------------|
| **Control**           |                      |                     |
| V<sub>max</sub>       | 12 ± 2               | 11 ± 2              |
| (V<sub>max</sub>/V<sub>burst</sub>)<sup>a</sup> | 50 ± 3               | 45 ± 5              |
| **Glucagon-treated**  |                      |                     |
| V<sub>max</sub>       | 12 ± 2               | 11 ± 2              |
| (V<sub>max</sub>/V<sub>burst</sub>)<sup>a</sup> | 7 ± 3                | 8 ± 2               |

*<sup>a</sup> 50 mg wet weight of liver cells/ml of suspension. All measurements of v<sub>max</sub> were done at room temperature. The viability of isolated hepatocytes were evaluated by trypan blue exclusion; usually 80 to 90% of cells excluded the stain.

Hepatocytes were incubated as follows: 2 ml of cell suspension were shaken (150 strokes/min) in stoppered 20-ml vials at 37°C, in the presence of 10 mM glucose. The gas phase was 95% O<sub>2</sub> and 5% CO<sub>2</sub>. After 30 min of preincubation, glucagon, cAMP, insulin, or saline (0.9% NaCl solution) were added unless otherwise indicated. For the determination of pyruvate kinase and glycogen phosphorylase activities, 0.1 ml of homogenization buffer was added to the frozen aliquots, then vigorously shaken in a mixer until complete thawing of the sample. Further homogenization of the cells was not necessary and did not increase the phosphofructokinase activity. The resultant homogenate was centrifuged at 30,000 × g for 30 min at 4°C. Usually 50 μl of the supernatant were used for enzyme assay. The incubation mixture contained in a final volume of 1 ml: 50 mM Hepes at pH 7.0, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 1.5 mM MgATP, 0.15 mM NADH, 5 mM P<sub>i</sub>, 0.1 mM AMP, 1 mM NAD<sub>C</sub>, 0.5 units of both aldolase and α-glycerophosphate dehydrogenase, 5 units of triose phosphate isomerase, 1 unit of phosphoglucone isomerase and fructose-6-P plus glucose-6-P in a relation of concentrations of 1:3 to achieve the actual concentration of fructose-6-P indicated. All auxiliary enzymes were freed of ammonium sulfate by centrifugation of the suspension at 30,000 × g for 30 min and extensive dialysis against 10 mM Tris-HCl, pH 7.5, at 4°C. The activity of phosphofructokinase was measured at 0.25 mM (t<sub>0.05</sub>) and at 5 mM (V<sub>burst</sub>) fructose-6-P. The activity of the enzyme is given as V<sub>max</sub> or as (t<sub>0.05</sub>/V<sub>burst</sub>) × 100. Pyruvate kinase was assayed as described by Feliu et al. (10) but, using Hepes instead of glycylglycine, the activity was measured at 0.15 mM (t<sub>0.15</sub>) and at 5 mM (V<sub>burst</sub>) P-enolpyruvate and expressed as (t<sub>0.15</sub>/V<sub>burst</sub>) × 100. Glycogen phosphorylase α was assayed according to Stalumans and Hirs (11).

Sephadex G-25 gel filtration was achieved in columns (1 × 20 cm) equilibrated with homogenization buffer and 0.2 ml of a cell-free extract was applied. Main protein fraction was collected and used for enzyme assay.

Partial purification of phosphofructokinase was achieved by affinity chromatography on Agarose-ATP<sup>2</sup> (Sigma, No. A-9254). The general procedure consists of application of cell extracts prepared in homogenization buffer plus 10% glycerol, to 1-ml columns equilibrated with 50 mM potassium phosphate, pH 7.6, 0.5 mM MgCl<sub>2</sub>, 5 mM 2-mercaptoethanol, and 10% glycerol. After washing, the enzyme is eluted with equilibration buffer plus 0.1 mM fructose-6-P and 0.1 mM ATP. Phosphofructokinase elutes as a single peak and the recovery of the enzyme is around 90%, both for extracts from control and glucagon-treated hepatocytes. The purification of phosphofructokinase obtained has less than 0.5% of the following enzymatic activities: adenylate kinase, fructose-1,6-bisphosphatase, and adenosine triphosphatase.

The concentration of fructose-6-P and ATP used for the elution of phosphofructokinase does not interfere in the assay providing that a 5% more detailed description of the enzyme purification and kinetic data of the purified enzyme is in preparation. J. G. Castillo and A. Nieto, manuscript in preparation.

<sup>1</sup> The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid.
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50-fold dilution is achieved in the reaction mixture. Care was taken to maintain the final concentration of phosphate at 5 mM in the assay of the partially purified enzyme.

Protein was measured according to Lowry et al. (12). Specific activity of the enzymes is expressed as nanomoles of substrate transformed per min per mg of protein, at 25°C.

Biochemicals were from Sigma or Boehringer. Other reagents were of analytical reagent grade.

Results are expressed as means ± S.D. from three experiments, unless otherwise indicated.

RESULTS

Treatment of isolated hepatocytes with glucagon (10^-7 M) causes a marked inactivation of phosphofructokinase measured at 0.25 mM fructose-6-P (Fig. 1), while no change was observed at saturating concentration (5 mM). V_max was 12 ± 2 nmol×min^-1×mg of protein^-1, both in control and glucagon-treated hepatocytes. The inactivation of phosphofructokinase parallels the inactivation of pyruvate kinase and activation of glycogen phosphorylase a (Fig. 1).

The addition of cAMP (1 X 10^-4 M) to the isolated hepatocytes also inactivates phosphofructokinase, decreasing the ratio (V_{0.25}/V_{max}) × 100 from 50 ± 3 to 9 ± 2 after 10 min of incubation and also without affecting V_max.

Gel filtration on Sephadex G-25 of cell-free extracts from control or glucagon-treated hepatocytes does not affect either the V_max or the ratio (V_{0.25}/V_{max}) × 100 of the enzyme (Table I).

Glucagon treatment of hepatocytes changes the kinetic properties of phosphofructokinase, decreasing the affinity for fructose-6-P and raising the cooperativity of the enzyme for this substrate, both in crude extracts and in the partially purified enzyme (Fig. 2).

The dose-dependent inactivation of phosphofructokinase and pyruvate kinase is shown in Fig. 3. Half-maximal inactivation for both enzymes is achieved approximately at 1 × 10^-8 M glucagon.

The inactivation of phosphofructokinase by nonsaturating concentration of glucagon (3 × 10^-10 M) is spontaneously reversible without change in V_max. Addition of insulin (10^-8 M) clearly accelerates this reactivation (Fig. 4). Insulin alone (10^-7 M) has no effect on the enzyme activity either measured at 0.25 or 5 mM fructose-6-P (Fig. 4). This reversion has also been reported for pyruvate kinase L inactivation by glucagon (10).

Fig. 3. Effect of various concentrations of glucagon on phosphofructokinase and pyruvate kinase L activities of rat hepatocytes. Panel a, phosphofructokinase activity; Panel b, pyruvate kinase activity. All assays of hepatocytes for enzyme assays were taken 10 min after addition of glucagon. Incubations of hepatocytes and assays of enzyme activities were carried out as described under "Experimental Procedures." Values are means ± S.D. of triplicate incubations from a single experiment.

Fig. 4. Time course of the action of glucagon and insulin on phosphofructokinase activity of rat hepatocytes. At zero time, 10^-8 M insulin (O) or 3 × 10^-10 M glucagon (■) were added. X, 3 × 10^-10 M glucagon added at zero time plus 10^-8 M insulin added 5 min later, as indicated by the arrow. Control (C) with saline was constant along the incubation (data not shown). Incubations of hepatocytes and assays of phosphofructokinase activity were carried out as described under "Experimental Procedures." Values are means ± S.D. from three experiments, each one with incubations by triplicate.

DISCUSSION

It seems clear that the pair fructose-1,6-bisphosphatase-phosphofructokinase is one of the sites of action of glucagon in the regulation of hepatic gluconeogenesis (1, 2). We have found a short term regulation of phosphofructokinase activity of isolated hepatocytes by glucagon, which involves an increase in S_{0.5} and n_H for fructose-6-P without change in V_max. As a result of these kinetic changes, an inactivation of phosphofructokinase is detected when the enzyme activity is assayed at subsaturating concentration of fructose-6-P (0.25 mM).

The fact that this inactivation persists after gel filtration of a cell-free extract on Sephadex G-25 and that a 400-fold purified phosphofructokinase preparation, from control and glucagon-treated hepatocytes, maintains kinetic properties similar to their respective crude extracts, clearly indicates that the changes observed by the action of glucagon on phosphofructokinase activity are due to a stable modification of the enzyme and not to changes in metabolite concentrations or in other enzymes.

As shown in Fig. 4, the inactivation of phosphofructokinase is spontaneously reversible; therefore, a proteolytic mecha-
nism for the short term regulation of the enzyme activity by glucagon can be discarded. The inactivation of phosphofructokinase seems to be a cAMP-mediated process because: (a) cAMP added to isolated hepatocytes also inactivates the enzyme; (b) phosphofructokinase inactivation parallels the known cAMP-dependent inactivation of pyruvate kinase L and activation of glycogen phosphorylase a by glucagon; and (c) the dose of glucagon that produces the half-maximal inactivation of phosphofructokinase and pyruvate kinase is similar.

Besides the experiments of Söling and co-workers (3, 4, 6) on phosphorylation of the liver enzyme, several reports indicate that phosphofructokinase from other tissues is also susceptible of being phosphorylated (13–16). Therefore, the most obvious explanation for the inactivation of liver phosphofructokinase by glucagon would be a phosphorylation or dephosphorylation of the enzyme by a CAMP-dependent process. In any case, the consequence is a decrease of the enzyme activity at subsaturating concentrations of fructose-6-P without change in $V_{\text{max}}$, similar to the CAMP-dependent inactivation of pyruvate kinase L that is only detected at subsaturating concentrations of P-enolpyruvate (10, 17, 18). This is in contrast with the decrease in $V_{\text{max}}$ of phosphofructokinase proposed by Söling et al. (6) as explanation for the action of glucagon and cAMP.

The inactivation of phosphofructokinase by glucagon that can attain 80% in our assay conditions would diminish the recycling between fructose-6-P and fructose-1,6-P$_2$ raising the net gluconeogenic flux. The glucagon-insulin antagonism that is implicated in the short term regulation of hepatic gluconeogenesis is also manifested in the regulation of phosphofructokinase activity. These facts support a relevant role of phosphofructokinase in the control of the gluconeogenic pathway as it has been shown for pyruvate kinase (10). The coordinate hormonal control of these two major regulatory glycolytic enzymes could explain, at least in part, the increase of hepatic gluconeogenesis by glucagon, both for substrates entering at the pyruvate and the triose phosphate levels.

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