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EVIDENCE FOR A NEW ACTIVATOR OF RAT LIVER PHOSPHOFRUCTOKINASE

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SUMMARY

A low molecular weight compound that activates purified rat liver phosphofructokinase has been isolated and partially purified from rat hepatocyte extracts. It can be separated from both fructose bisphosphate and AMP on DEAE-Sephadex. Incubation of rat hepatocytes with glucagon lowers the level of this activator, and this accounts for the inhibition of phosphofructokinase that was observed in hepatocyte extracts (S. Pilkis, et al. (1979) Biochem. Biophys. Res. Commun. 88, 960-967). Other characteristics of this activator are described which suggest that it is not any of the known effectors of rat liver phosphofructokinase.

INTRODUCTION

Hepatic phosphofructokinase (PFK) has been identified as a site of glucagon action (1-4). The hormone stimulates ^{32}P-incorporation into the enzyme *in vivo* (3) and in isolated hepatocytes (4), and the cyclic AMP-dependent protein kinase catalyzes the phosphorylation of purified PFK *in vitro* (5). From these results, it has been suggested that glucagon causes inhibition of the enzyme by stimulating its phosphorylation (2,3). However, we recently have shown that the inactivation of the enzyme by glucagon observed in crude hepatocyte extracts is due to a change in the level of an unidentified effector(s) rather than to phosphorylation of the enzyme (4). In this report, we describe some characteristics of the effector that is responsible for the inhibition of PFK by glucagon. The results suggest that it is not one of the known effectors of rat hepatic PFK.

METHODS

Preparation of Hepatocyte Extracts and Filtrates. Isolated hepatocytes were prepared from fed rats as described previously (4). After incubation for 10 min with or without 10 nM glucagon, heated extracts from 5 ml aliquots of the cell suspension (50 mg liver/ml) were prepared as described previously (4)

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except that the cells were homogenized in 1.5 ml of buffer that contained 30 mM potassium phosphate, pH 7.5, 100 mM NaF, 1 mM EDTA, and 20 mM β-mercaptoethanol. Filtrates were prepared by filtering the heated extracts through YM-5 membranes (Amicon) in an Amicon ultrafiltration device. The filtrates had no PFK activity and were essentially free of protein as determined by Lowry protein analysis.

Purification of Rat Liver PFK. Rat liver PFK was purified by a modification (5) of the method of Kemp (6). It had a specific activity of 95 U/mg protein, and was homogeneous as determined by sodium dodecyl sulfate disc gel electrophoresis (data not shown).

Assay of PFK. PFK activity was determined with the aldolase-coupled assay as described previously (1,4). Activation of PFK by the filtrate was determined by pre-incubating an aliquot of the filtrate and PFK in 1 ml of the assay mixture for 3 min and then starting the reaction with 0.2 mM fructose-6-phosphate. The rate of the reaction at a given substrate concentration (v) was expressed as a fraction of the maximum rate (V_{max}). The maximum activity of PFK was determined in the presence of 4 mM fructose-6-phosphate plus 1 mM AMP. In no instance did addition of the filtrate affect the maximum activity of PFK measured under these conditions.

RESULTS

Previously, we presented evidence that strongly suggested that the inhibition of PFK by glucagon observed in hepatocyte extracts was mediated by a change in the level of an effector(s) of the enzyme (4). However, the identity of this effector had not been established. In order to aid its identification, we prepared protein-free filtrates by passing heated extracts of basal and glucagon-treated cells through a YM-5 membrane. The effect of these filtrates on the activity of purified rat liver PFK is shown in Fig. 1. When PFK was assayed in the presence of filtrate from basal cells, the $S_{0.5}$ for fructose-6-phosphate was 0.12 mM, while the $S_{0.5}$ in the presence of filtrate from glucagon-treated cells was 0.52 mM. When only homogenizing buffer was added to the enzyme, the $S_{0.5}$ was 1.4 mM. These results are identical to those obtained when PFK was assayed in heated extracts from cells incubated in the absence and presence of glucagon (4) . Similar results were obtained using extracts that had been passed through a UM-2 membrane (MW cut off, 1000) which retains the stabilizing factor that was reported by Dunaway and Segal (7) to be an activator of the enzyme.

The results of Fig. 1 suggest that the hormone effect is due to different

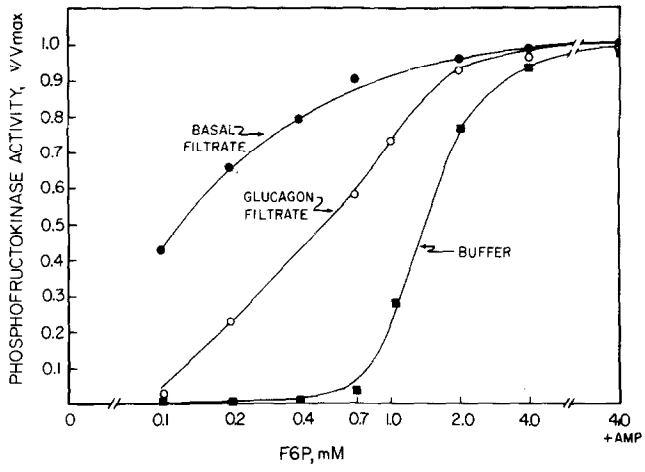

Fig. 1. Effect of filtrates from basal and glucagon-treated cells on the fructose-6-phosphate (F6P) dependency of purified rat liver PFK. Filtrates and pure PFK were prepared as described under Methods. PFK activity (20 mU) was measured in the presence of 40 µl filtrate or homogenizing buffer.

amounts of an activator(s) in the filtrates. This is supported by the results in Fig. 2 where purified PFK was assayed with increasing amounts of filtrate from cells incubated with or without glucagon. About 3 times more filtrate from glucagon-treated cells than from basal cells was needed to half-maximally activate PFK. When the filtrates were treated with charcoal in order to remove AMP and other nucleotides, the amount of filtrate from basal cells that was needed to half-maximally activate PFK increased from 8 µl to 16 µl (Fig. 2). The amount of filtrate from glucagon-treated cells dramatically increased from 25 µl to 65 µl. These results suggest that the activator was not AMP. They also suggest that the inhibition of PFK activity by glucagon seen in heated extracts (4) was due to a substantial decrease in the amount of activator in the heated extracts from glucagon-treated cells.

Further purification of the PFK effector from a charcoal-treated filtrate of basal cells was achieved by DEAE-Sephadex chromatography (Fig. 3). The fractions that were able to activate PFK were clearly separated from those that contained [^{32}P]P_i or the monophosphate, [^{3}H]AMP. The activator was eluted in the sugar diphosphate region, but was slightly separated from

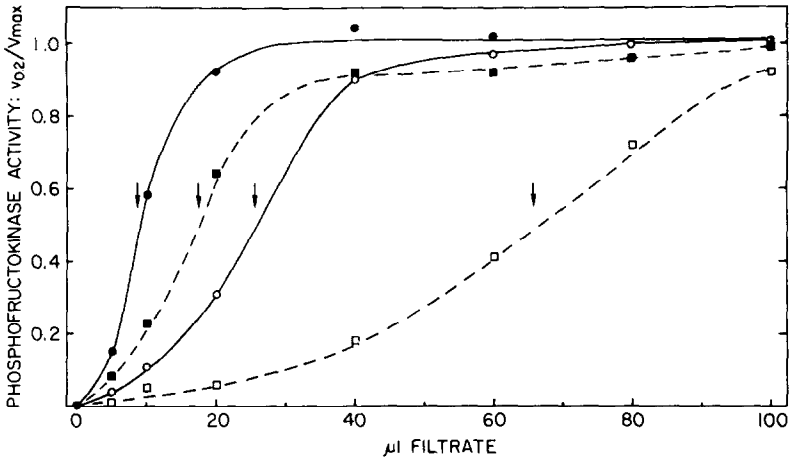

Fig. 2. Effect of increasing amount of filtrate on the activity of purified rat liver PFK. PFK was assayed with 0.2 mM F6P in the presence of filtrate from basal (●,■) or glucagon-treated cells (o,□). The filtrates were either not treated (●,o) or treated with charcoal (■ ,□) prior to the assay. The arrows represent the amount of filtrate needed to half-maximally activate PFK. The values are basal, non-treated (●): 8 µl; basal, treated (■): 16 µl; glucagon, non-treated (o): 24 µl; glucagon, treated (□): 65 µl.

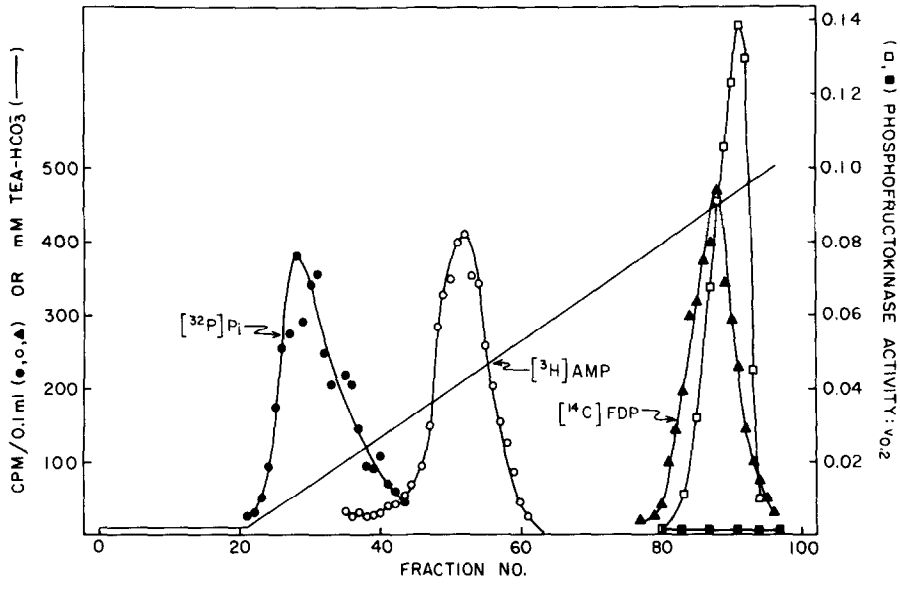

Fig. 3. DEAE-Sephadex chromatography of charcoal-treated filtrates. Tracer amounts of [^{32}P]P_i, [^{3}H]AMP, and [^{14}C]FDP were added to 20 ml of filtrate from either basal or glucagon-treated cells and the sample applied to a 0.9x10 cm DEAE-Sephadex column in 10 mM triethylammonium bicarbonate (TEA-HCO_3^-), pH 8.2. After washing with 25 ml of 10 mM TEA-HCO_3^-, the samples were eluted with a linear gradient (10-500 mM TEA-HCO_3^-). Aliquots (20 µl) of the fractions (1.3 ml) were assayed for their ability to activate PFK (□ ,■) as described in Fig. 2. □ , basal filtrate; ■ , glucagon filtrate.

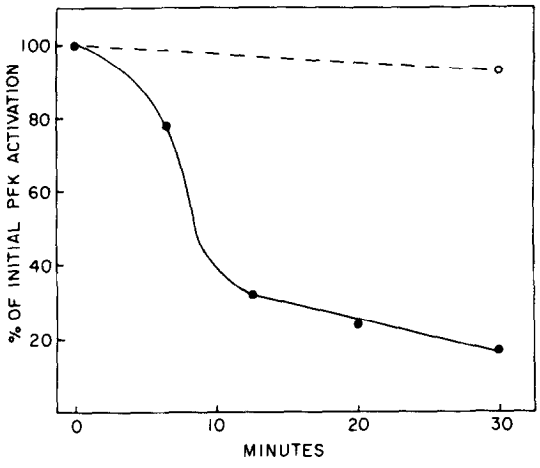

Fig. 4. The time course of inactivation of the PFK activator at pH 3. Charcoal-treated filtrate from basal cells was adjusted to pH 3 by the addition of 1N HCl and incubated at 25°. At the given times, an aliquot was withdrawn and the pH raised to 7 by the addition of 1 N NaOH. PFK activity was measured with 40 µl of the filtrate in the presence of 0.2 mM F6P.

fructose 1,6-bisphosphate. Both glucose-1,6-diphosphate and sedoheptulose-1,7-diphosphate, known activators of rat liver PFK (8), were eluted prior to fructose 1,6-bisphosphate (results not shown). When a charcoal-treated filtrate of glucagon-treated cells was chromatographed on an identical column, only about 10 percent as much PFK activator was found (Fig. 3).

Figure 4 shows the time course of inactivation of the activator at pH 3 and 25°. Approximately half of the ability of the charcoal-treated filtrate to activate PFK was lost in 8 min and only 20 percent of the initial activity remained after 30 min. Under the same conditions, fructose 1,6-bisphosphate was almost completely stable for 30 min (Fig. 4). Glucose diphosphate and sedoheptulose 1,7-diphosphate gave results similar to fructose bisphosphate (results not shown). When charcoal-treated filtrates from basal and glucagon-treated cells were subjected to pH 3 for 30 min and then tested for their ability to activate PFK, no hormone effect was observed and the $S_{0.5}$ was 0.8 mM in both cases (Fig. 5). In contrast, the ability of the filtrate to activate PFK was insensitive to treatment with alkali (Fig. 5). In fact, the activator was unaffected by heating at 90° for 30

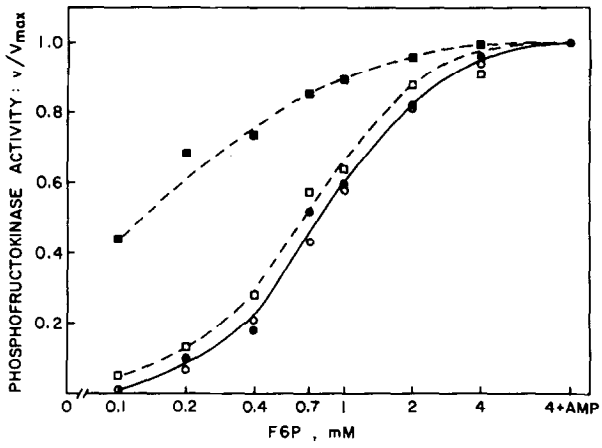

Fig. 5. Effect of acid and base treatment of filtrates on the activity of PFK. Filtrates from basal cells (●,□) or glucagon-treated cells (o,) were incubated at 25° for 30 min at pH 3 (●,o) or pH 11 (■,□). PFK activity was measured as described in Fig. 1.

min in 0.25 NaOH, conditions which degrade fructose 1,6-bisphosphate (9)(results not shown).

We have determined some other characteristics of this activator. It is unaffected by boiling at neutral pH for 3 min, or by treatment with proteases. It can be destroyed by treatment with alkaline phosphatase, and it can be precipitated with $BaCl_2$ in the absence of ethanol or with $Zn(Ac)_2/Na_3CO_3$.

DISCUSSION

The results we report here show that glucagon causes inhibition of PFK by lowering the level of a low molecular weight activator (Figs. 1-3). The activator is negatively charged. It is not likely a nucleotide or nucleoside since it is not readily adsorbed on charcoal and has no adsorption at 260 nm. It has some characteristics of a sugar diphosphate since it is precipitated by $BaCl_2$ in the absence of ethanol (10), and it can be eluted from DEAE-Sephadex in the sugar diphosphate region (Fig. 5). Of the known activators, only fructose 1,6-bisphosphate changes in response to glucagon in such a way as would result in inhibition of PFK (11-13). However, data presented here indicate that the activator is not fructose 1,6-bisphosphate.

It does not co-chromatograph with fructose 1,6-bisphosphate on DEAE-Sephadex (Fig. 3) and it is much more acid labile (Fig. 4). Also, the fact that the activation of PFK was measured with the aldolase-coupled assay, which measures fructose 1,6-bisphosphate production, tends to rule out fructose 1,6-bisphosphate as the activator.

Although inhibition of PFK by glucagon is explained in large part by a decrease in this activator, the stimulation of PFK phosphorylation (3,4) also may play a role in regulation of PFK flux and activity. The possibility that phosphorylation alters the sensitivity of the enzyme to activation by this effector is currently under investigation.

Some structural characteristics of the activator are suggested by its behavior in acid and base. The lability of the activator in acid (Fig. 4) but stability in base (Fig. 5) are characteristic properties of phosphate esters on hemiacetalic hydroxyl groups (15). If the activator is an hexose diphosphate, one of the phosphates must be located on the hemiacetalic hydroxyl group of either the aldohexose or the ketohexose. Work is in progress to elucidate the structure of what may be an as yet unidentified effector of the rat liver enzyme.

Note Added in Proof: Since the submission of this manuscript we have learned from the editors of this journal that E. Schaftingen and H.G. Hers have described a similar activator and identified it as 2,6-fructose bisphosphate. We have preliminary evidence for the same structure.

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