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Epidermal growth factor stimulates the phosphorylation of pyruvate kinase in freshly isolated rat hepatocytes

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Epidermal growth factor (EGF) has previously been shown to stimulate gluconeogenesis in rat liver by decreasing the activity of pyruvate kinase ((1988) Biochem. J. 255, 361–364). Here we investigate the mechanism underlying the inactivation of the enzyme. EGF was found to increase the incorporation of phosphate into pyruvate kinase, with maximal phosphorylation achieved only after 10 min in the presence of the growth factor. The increase in phosphorylation was not additive with that caused by cyclic AMP. Phosphoamino acid analysis of pyruvate kinase isolated from cells treated with EGF indicated that EGF increases phosphorylation solely on serine residues. The exact site of EGF-mediated phosphorylation has yet to be identified.

Pyruvate kinase; Epidermal growth factor; Cyclic AMP; Phosphorylation; Hepatocyte

1. INTRODUCTION

Hepatocytes contain large numbers of epidermal growth factor (EGF) receptors on their cell surface [1], and in addition to its well-characterised stimulation of DNA synthesis both in vivo and in cultured hepatocytes [2], EGF has a number of important short-term effects on liver cell metabolism. The similarities between the EGF receptor and that of insulin has led a number of groups to look for parallels in the actions of these two effectors. Although EGF does indeed have some effects in liver which mimic those of insulin (e.g. increased phosphorylation of acetyl-CoA carboxylase and ATP-citrate lyase [3], stimulation of fatty acid synthesis [3,4] and activation of glycogen synthase [5]), some differences have also been noted.

Recent work in this laboratory has shown that EGF exerts two distinct effects on hepatocytes which resemble those of glucagon rather than insulin. Firstly, both EGF and glucagon have been shown to increase the rate of Na^+/H^+ exchange in isolated hepatocytes [6]. The consequent increase in electroneutral Na^+ influx causes stimulation of the electrogenic Na^+, K^+-ATPase and plasma membrane hyperpolarisation, thus increasing the rate of electrogenic Na^+-dependent alanine transport [7]. Plasma membrane hyperpolarisation by EGF may be an important early event in liver regeneration. The mechanism by which cAMP and EGF stimulate the Na^+/H^+ exchanger is unknown, but stimulation by EGF is independent of any changes in the intracellular concentration of cAMP [8]. Secondly we have shown that EGF, like glucagon, produces a stable decrease in the activity of pyruvate kinase leading to an increase in the rate of gluconeogenesis from a number of glucose precursors including lactate, alanine and asparagine [8]. This effect of EGF is again independent of any change in the intracellular concentration of cAMP. The work described below shows that the inactivation of pyruvate kinase by EGF is accompanied by an increase in the incorporation of phosphate onto serine residues of the enzyme.

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Abbreviations: BSA, bovine serum albumin; ClPhScAMP, 8-(4-chlorophenylthio)adenosine 3',5'-monophosphate; EDTA, ethanediamine tetraacetic acid; EGF, epidermal growth factor; MOPS, *N*-morpholinopropane sulphonic acid; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Hepatocyte isolation, incubation and extraction*

Rat liver hepatocytes were isolated essentially as described previously [7] and suspended at 10–15 mg cell protein/ml in low-phosphate medium (140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.2 mM KH_2PO_4, 20 mM Mops, 2% w/v BSA). Cells were labelled with ^{32}P by incubation plus 100–1 mCi/ml ^{32}P at 37°C for 1 h prior to the addition of 30 nM EGF or 50 μM ClPhScAMP. After the appropriate time in the presence of effectors, cells were spun at 10000×*g* for 5 s and resuspended in an equal volume of ice-cold extraction buffer (50 mM KH_2PO_4 (pH 7.4), 100 mM KCl, 50 mM NaF, 5 mM EDTA, 2 mM benzamidine, 30 mM potassium pyrophosphate, 1% Triton X-100 plus 1 μg/ml leupeptin, antipain and pepstatin). After a further 2 min the extracts were spun at 10000×*g* for 10 min and the supernatant retained. Samples were kept at 0–4°C at all times.

Pyruvate kinase was immunoprecipitated from cell extracts by mixing with an equal volume of rabbit anti-rat liver pyruvate kinase polyclonal IgG preparation. Immunoprecipitation reactions were carried out plus 20 μl protein-A Sepharose for 1 h at 4°C with continuous mixing. The protein-A Sepharose beads were then washed with 4 × 1 ml phosphate-buffered saline plus 0.05% Tween-20, 50 mM NaF, 5 mM EDTA and 1 μg/ml protease inhibitors. The immunoprecipitates were then solubilised and the proteins were separated by SDS-PAGE.

Depending on the radioactivity of the gel, the band corresponding to pyruvate kinase was either identified by conventional Coomassie brilliant blue staining or by autoradiography overnight using flash-preactivated X-Omat film (Kodak). The pyruvate kinase band was then excised and solubilised by heating in 1.5 ml of 60% v/v perchloric acid and 1.5 ml of 30% v/v H_2O_2 for 4 h at 80°C. Samples were cooled and the radioactivity was assessed by liquid scintillation counting in 15 ml Unisolve E (Koch-Light).

Pyruvate kinase activity was determined at 5 mM phospho-enolpyruvate as described previously [9]. One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 nmol of pyruvate from 5 mM phospho-enolpyruvate per minute at 37°C.

2.2. *Phosphoamino acid analysis*

Cell extracts were prepared from hepatocytes incubated with 1 mCi ^{32}P/ml, and pyruvate kinase was separated by immunoprecipitation and SDS-PAGE as described above. The band corresponding to pyruvate kinase was excised and the protein extracted by electroelution into 20 mM Tris-HCl (pH 8.0), 2 mM EDTA and 0.1% (w/v) SDS. The electroeluted protein was acetone-precipitated and samples were trypsinised overnight at 30°C with 0.1 mg trypsin/ml in 50 mM ammonium bicarbonate (pH 8.0). The tryptic digests were then hydrolysed in 100 μl of 6 M HCl for 1 h at 110°C. The samples were loaded onto 20 × 20 cm cellulose TLC plates (Kodak) along with phospho-serine, -threonine and -tyrosine as standards. Phosphoamino acids were separated by high voltage electrophoresis at pH 3.5 or pH 1.9 as described previously [10]. ^{32}P-Labelled amino acids were visualised by autoradiography for 7 days at −80°C using preflashed X-Omat S film and intensifier screens.

2.3. *Materials*

^{32}P was obtained from Amersham International (Amersham, Bucks, UK). EGF, ClPhScAMP, benzamidine, Triton X-100, EDTA and Tween-20 were all from Sigma Chemical Co. (Poole, Dorset, UK). Protease inhibitors were purchased from Cambridge Research Biochemicals (Cambridge, UK) and tosylphenylalanyl-chloromethane-treated trypsin was from Worthington Diagnostic Systems (Freehold, NJ, USA). Protein-A Sepharose was obtained from Bioprocessing Ltd. (Consett Co., Durham, UK) and collagenase was supplied by Boehringer-Mannheim (Lewes, East Sussex, UK).

3. RESULTS AND DISCUSSION

The effect of EGF on the phosphorylation of pyruvate kinase was determined both quantitatively and qualitatively by the immunoprecipitation of pyruvate kinase with a rabbit polyclonal anti-rat liver pyruvate kinase IgG preparation. Pyruvate kinase was the major phosphoprotein immunoprecipitated from hepatocyte cell extracts. Fig. 1 shows that EGF increased the incorporation of phosphate into pyruvate kinase over the basal control level. By measuring the pyruvate kinase activity remaining in the supernatant after immunoprecipitation, the degree of phosphorylation of the enzyme could be quantified. Since both cyclic AMP and EGF inactivate pyruvate kinase by increasing the K_m for phospho-enolpyruvate without altering the V_{max}

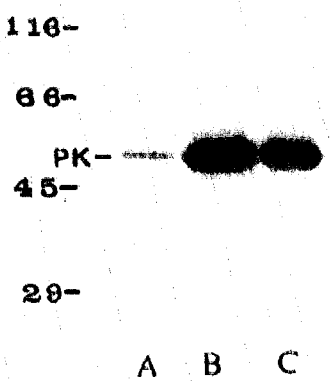

Fig. 1. The effect of EGF on the incorporation of ^{32}P into pyruvate kinase. Cell extracts were prepared from ^{32}P-labelled hepatocytes treated with EGF or cAMP for 10 min. Pyruvate kinase was immunoprecipitated and separated by SDS-PAGE. The degree of ^{32}P-incorporation was assessed by autoradiography using flash-preactivated X-Omat film (Kodak). Tracks A, B and C are immunoprecipitates from control, cAMP- and EGF-treated cell extracts, respectively. PK indicates the position of pyruvate kinase.

[8], enzyme activities were measured at high phospho-enolpyruvate concentrations. In experiments on three separate cell preparations, the basal incorporation of phosphate into pyruvate kinase (expressed as mean cpm/U enzyme activity immunoprecipitated ± SE) was 91.1 ± 18.9. Incubation of hepatocytes with ClPhScAMP or EGF for 10 min increased the phosphorylation of the enzyme to 159.0 ± 17.8 ($P < 0.05$ vs control) and 186.0 ± 32.1 ($P < 0.01$ vs control), respectively. When added together, the two effectors did not produce an additive effect on phosphate incorporation into pyruvate kinase (163.0 ± 20.3; $P < 0.01$ vs control).

The results shown in Fig. 2 indicate that the time courses of the effects of EGF and cyclic AMP on pyruvate kinase phosphorylation differ significantly. While the phosphorylation of the enzyme in response to cAMP was apparent almost immediately (see also [11]), maximal phosphorylation by EGF was attained only after approximately 10 min. Under control conditions with no added effectors, the phosphorylation of pyruvate kinase did not alter significantly (results not shown). The time course for the phosphorylation of pyruvate kinase by EGF is broadly consistent with those for the stimulation of gluconeogenesis and inactivation of the enzyme by EGF (see [8]). The difference in the time courses for EGF and cyclic AMP, together with the fact that EGF does not increase the intracellular concentration of cyclic AMP [5,8], indicates that the two effectors alter the phosphorylation state of the enzyme via largely separate mechanisms.

In order to further investigate the EGF-mediated phosphorylation of pyruvate kinase, phosphoamino

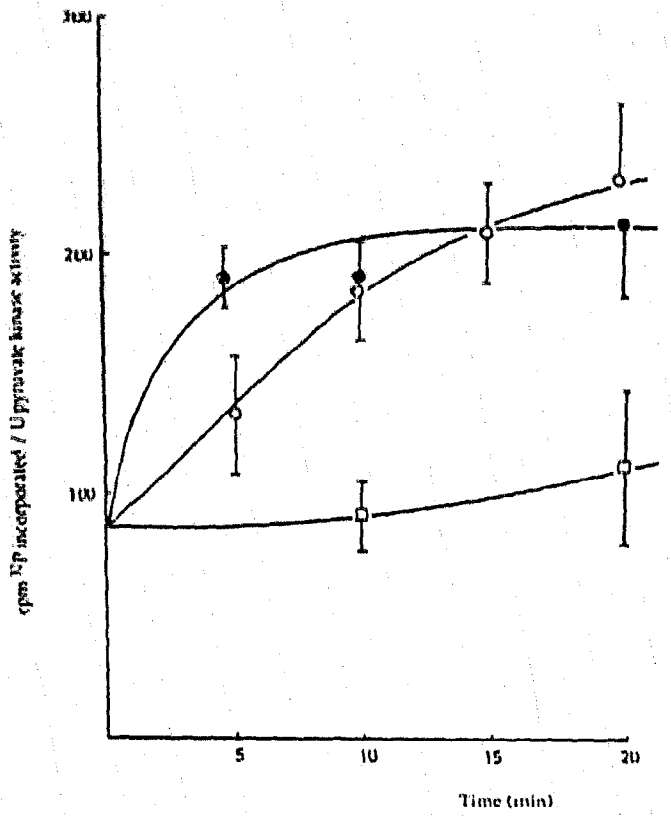

Fig. 2. Time course for the phosphorylation of pyruvate kinase by EGF. Cell extracts were prepared from ^{32}P-labelled hepatocytes incubated with no further additions (□), EGF (○) or cAMP (●) for the times shown. The degree of ^{32}P-incorporation into pyruvate kinase was assessed as described in the text. The results shown are the average of experiments on three separate cell preparations ±SE.

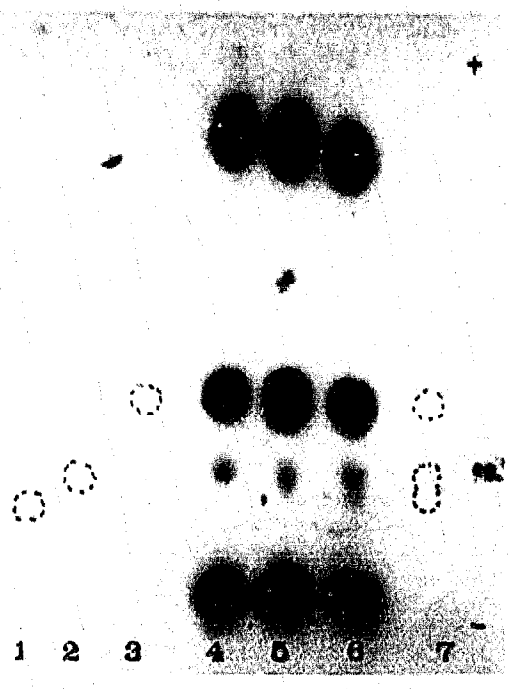

Fig. 3. Phosphoamino acid analysis of phosphorylated pyruvate kinase. Cell extracts were prepared from ^{32}P-labelled hepatocytes incubated with EGF or cAMP for 10 min. Phosphoamino acid analysis was carried out as described in section 2. Tracks shown on the autoradiograph are as follows: (1) phosphotyrosine; (2) phosphothreonine; (3) phosphoserine; (4), (5) and (6) acid hydrolysates of immunoprecipitates from control, cAMP and EGF cell extracts, respectively; and (7) all three standard phosphoamino acids together.

acid analysis of the enzyme was undertaken. Initial analysis of the acid hydrolystaes at pH 3.5 ruled out the possibility that EGF increased phosphate incorporation on tyrosine residues. High voltage electrophoresis at pH 1.9 showed that EGF, like cAMP, increased the phosphorylation of serine residues in pyruvate kinase (Fig. 3). Phosphothreonine was not detected.

The results presented here show that EGF inactivates hepatic pyruvate kinase by increasing the phosphorylation of the enzyme on serine residues. Pyruvate kinase is phosphorylated by cAMP-dependent protein kinase on serine-12 [12]. The enzyme has also been shown to be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase in vitro on serine-12 and, to a lesser extent, on threonine-17 [13]. In intact hepatocytes only phosphorylation on serine-12 is seen in response to Ca^{2+}-linked hormones [14]. Although we have not fully characterised the EGF phosphorylation site on pyruvate kinase, the lack of additivity with cyclic AMP and the fact that EGF has the same effect on the activity of the enzyme as cyclic AMP may suggest that EGF increases the phosphorylation of serine-12.

It is unlikely that the effect of EGF on pyruvate kinase is mediated via an increase in the activity of cAMP-dependent protein kinase as it has been established that EGF has no measurable effect on the intracellular concentration of cyclic AMP [5,8]. In hepatocytes, as in a number of other cell types, EGF-like vasopressin produces a rapid increase in intracellular Ca^{2+} concentrations. In contrast to vasopressin, however, the EGF-induced Ca^{2+} increases are smaller and transient, with levels returning to normal within 5 min [5,15]. The EGF-stimulated phosphorylation of pyruvate kinase is unlikely to be mediated by Ca^{2+}/calmodulin-dependent protein kinase as the increase in phosphorylation occurs predominantly after the Ca^{2+} transient has disappeared. In addition, the effect of EGF on pyruvate kinase phosphorylation is much slower than the effect of vasopressin which is maximal after only three minutes [14].

While effects of EGF on cAMP-dependent protein kinase or Ca^{2+}/calmodulin-dependent protein kinase cannot be entirely excluded at this stage, it seems likely that EGF exerts its effects on pyruvate kinase through activation of some other, as yet undefined, serine protein kinase. It is interesting to note that phosphoryla-

tion of the pyruvate kinase K-isozyme by a cAMP and Ca^{2+}/calmodulin-independent protein kinase has recently been reported in human glioma cells overexpressing the EGF receptor [16]. A similar protein kinase may be responsible for the EGF-mediated phosphorylation of hepatocyte pyruvate kinase described here.

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