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Epidermal-growth-factor stimulation of gluconeogenesis in isolated rat hepatocytes involves the inactivation of pyruvate kinase

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Preincubation of rat hepatocytes with EGF (epidermal growth factor) caused a stimulation of gluconeogenesis from alanine. The effect was maximal after preincubation of 20 min, and a half-maximal effect of EGF was obtained at 10 nM. EGF also stimulated gluconeogenesis from lactate and asparagine, but not from glutamine or from proline. Preincubation of hepatocytes with EGF caused a stable inactivation of pyruvate kinase, which may account, at least in part, for the observed effects of EGF on gluconeogenesis.

INTRODUCTION

The liver cell plasma membrane contains large numbers of epidermal growth factor (EGF) receptors [1], and the similarities between these receptors and the insulin receptor [2], together with the fact that both EGF and insulin stimulate cell growth [3], has prompted a comparison of the effects of insulin and EGF on isolated hepatocytes. Some effects of EGF are similar to those of insulin, e.g. stimulation of phosphorylation of acetyl-CoA carboxylase and ATP citrate lyase [4], stimulation of glycogen synthesis at high glucose concentrations [5] and inhibition of the increase in cyclic AMP caused by glucagon [5]. EGF, however, failed to stimulate glycolysis and to inhibit ketogenesis in both hepatocyte suspensions and cultures [6].

Other effects of EGF on hepatocytes differ from those of insulin. EGF increases the rate of Na^+ influx and Na^+/K^+-ATPase activity [7]. EGF has also been shown to exert a short-term stimulation of Na^+-dependent amino acid transport, which has been attributed to membrane hyperpolarization [8]. Soley & Hollenberg [9] have shown that 100 nM-EGF stimulates gluconeogenesis from pyruvate in isolated mouse hepatocytes when measured over a period of 1 h. In contrast, Chowdhury & Agius [6] observed no stimulation by 10 nM-EGF of incorporation of [^{14}C]pyruvate into glucose in 24 h cultures of hepatocytes.

Since EGF stimulates alanine transport [8], and alanine transport under some conditions can be a rate-limiting step in alanine metabolism (see, e.g., [10]), we investigated the ability of EGF to stimulate the production of glucose from alanine and from other substrates in rat hepatocytes. The results presented indicate that EGF stimulates gluconeogenesis from alanine and lactate, and that this stimulation is associated with an inactivation of pyruvate kinase.

MATERIALS AND METHODS

Materials

EGF (receptor grade) was obtained from Sigma Chemical Co., Poole, Dorset, U.K. Collagenase for hepatocytes isolation and dibutryryl cyclic AMP (dbc-AMP) were purchased from Boehringer, Mannheim. The cyclic AMP radioimmunoassay kit was purchased from Amersham International. All other reagents were of analytical grade.

Methods

Hepatocytes were isolated from male Wistar rats as described previously [11]. Cells were incubated at 10–15 mg of protein/ml in Krebs–Henseleit bicarbonate buffer [12] containing 2% (w/v) dialysed bovine serum albumin at pH 7.4 and 37 °C under an atmosphere of O_2/CO_2 (19:1).

For experiments involving the measurement of gluconeogenesis, cells isolated from 24 h-starved rats were incubated in the presence of hormones for the appropriate times before additions of substrate. Glucose production was measured in neutralized $HClO_4$ extracts after a further 10 min [13].

Pyruvate kinase activity was assayed in cell extracts as described by Denton *et al.* [14]. After pretreatment with hormones of cells isolated from fed rats, the cell suspension was centrifuged at 100 *g* for 30 s and the pellet rapidly frozen within 15 s in liquid N_2. Cell pellets were then sonicated for 5 s with a 3 mm probe (Dawe Soniprobe; 40W) in ice-cold extraction buffer [0.1 M-Tris/HCl (pH 7.5)/50 mM-NaF/5 mM-EDTA] and centrifuged at 10000 *g* for 10 min. The supernatants were frozen in liquid N_2 and stored at −20 °C until assayed.

Cyclic AMP was determined in neutralized $HClO_4$ extracts of cells pre-treated with hormones by radioimmunoassay kit according to the manufacturer's instructions.

Cell protein was determined by a biuret method [15], with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of preincubation of hepatocytes with EGF for different times on the rate of glucose production from alanine. Alanine (5 mM) was added 10 min before the end of the total incubation period. The stimulatory effect was maximal after hepatocytes had

Abbreviations used: EGF, epidermal growth factor; dbcAMP, dibutyryl cyclic AMP.

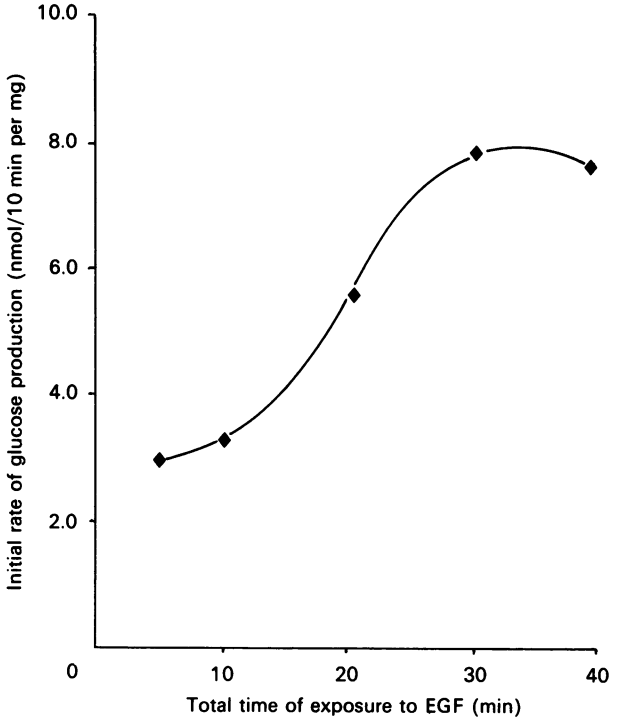

Fig. 1. Time course for stimulation of glucose production by EGF

Hepatocytes were incubated with EGF (50 nM) for the total times shown, with 5 mM-alanine added 10 min before the end of the incubation. Glucose production was measured over this 10 min period. For the 5 min time point shown, EGF and alanine were added simultaneously and glucose production was measured over a 5 min period.

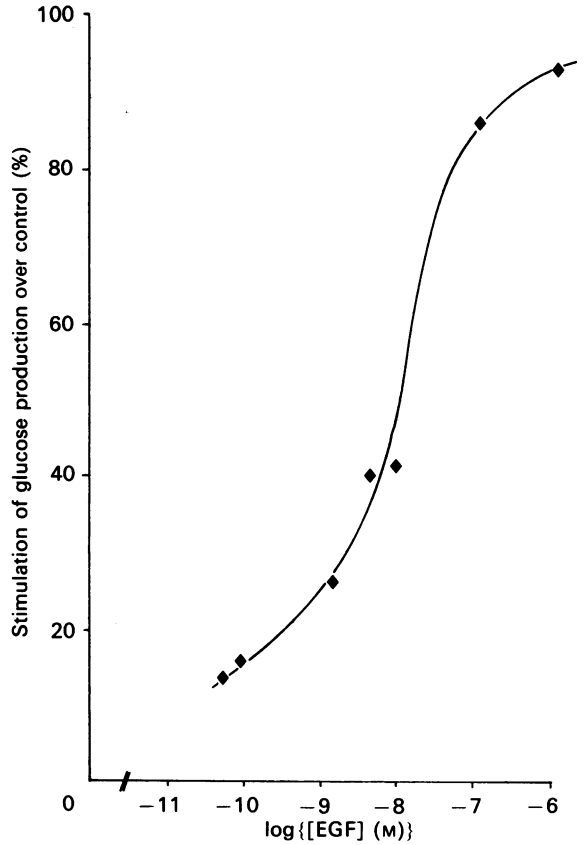

Fig. 2. Dose-dependence for effect of EGF on gluconeogenesis

Hepatocytes were incubated with the appropriate concentrations of EGF for 20 min; 5 mM-alanine was then added, and glucose production measured over the next 10 min.

been incubated with EGF for a total of 30 min. Little or no stimulation was observed if the cells were incubated for less than 10 min. A significant stimulation was sustained for period of up to 1 h (results not shown). The time course of stimulation is very similar to that of the stimulation of alanine transport by EGF [8], suggesting that the stimulation of gluconeogenesis from alanine may be simply a consequence of increased availability of intracellular alanine. Fig. 2 shows the dose-dependence of the stimulation of gluconeogenesis from alanine by EGF. A half-maximal effect was obtained at 10 nM-EGF, as compared with a half-maximal effect at 1 nM for stimulation of alanine transport by EGF [8]. These results indicated that events other than the increase in alanine transport may be responsible for the increase in gluconeogenesis observed.

Table 1 shows the substrate specificity of the stimulation of gluconeogenesis by EGF and by dbcAMP. Gluconeogenesis from lactate was stimulated by both effectors to similar extents. The stimulation of electrogenic Na^+-dependent alanine transport by dbcAMP is mediated by membrane hyperpolarization [16], and EGF has been proposed to stimulate alanine transport by a similar mechanism. Lactate transport into hepatocytes is thought to be an electroneutral process which would not be affected by changes in cell membrane potential [17]. This again indicates that EGF stimulates gluconeogenesis independently of any increase in intracellular substrate concentrations.

Gluconeogenesis from the C_5 substrates proline and glutamine was stimulated by dbcAMP, but not significantly by EGF. Glucose production from these precursors involves control points not encountered in gluconeogenesis from either lactate or alanine. Stimulation of gluconeogenesis from proline and glutamine by glucagon and by vasopressin has been shown to be a consequence of increased 2-oxoglutarate dehydrogenase activity [18, 19]. The inability of EGF to stimulate gluconeogenesis from C_5 precursors indicates that, unlike dbcAMP, EGF does not activate the mitochondrial enzyme 2-oxoglutarate dehydrogenase. The pathway of conversion of asparagine carbon into glucose need not involve any mitochondrial reactions. Table 1 shows that gluconeogenesis from asparagine was stimulated by EGF and dbcAMP to similar extents. It follows that the stimulation of gluconeogenesis from lactate, asparagine and alanine by EGF can be explained by stimulation of one or more rate-controlling cytoplasmic enzyme reactions which are common to all these pathways.

In order to determine whether EGF and dbcAMP stimulate gluconeogenesis by a common mechanism, the effect of adding both effectors together on glucose production from alanine was investigated. In experiments on three separate cell preparations, the control rate of gluconeogenesis from 5 mM-alanine was 3.67 ± 0.03 nmol/10 min per mg of protein (mean $\pm$ S.E.M.). Preincubation for 20 min with 0.1 mM-dbcAMP increased the rate to 8.01 ± 0.66 nmol/10 min per mg ($P < 0.01$

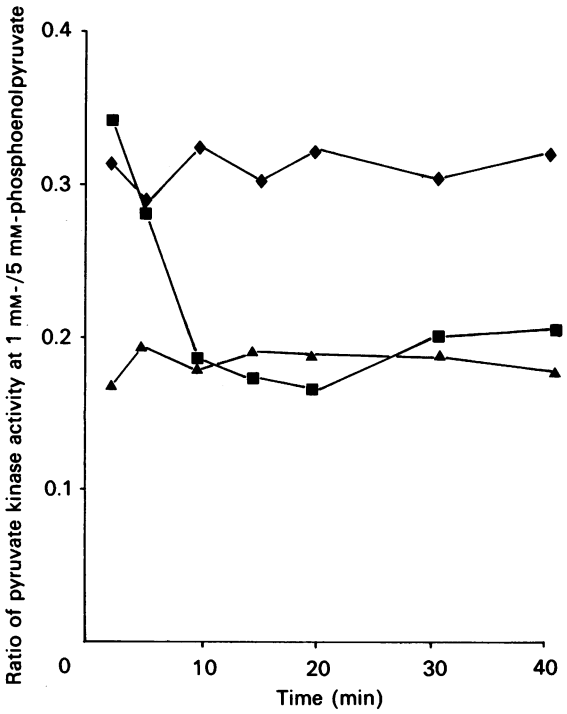

Fig. 3. Inactivation of pyruvate kinase by EGF

Cell extracts were prepared from hepatocytes incubated in the absence of effectors (◆) or in the presence of 50 nM-EGF (■) or 0.1 mM-dbcAMP (▲) for the times shown. Pyruvate kinase activity was determined at both 1 mM- and 5 mM-phosphoenolypyruvate. The results are expressed as the ratio of the activities at these two concentrations.

versus control), whereas preincubation with 50 nM-EGF increased the rate to 7.9 ± 0.35 nmol/10 min per mg ($P < 0.001$ versus control). When the cells were preincubated with both effectors at these concentrations, the rate of glucose production was 8.21 ± 0.93 nmol/10 min per mg ($P < 0.01$ versus control). Since the addition of one effector produced no significant additional stimulation in the presence of a saturating concentration of the other effector, it is probable that both effectors exert their actions on gluconeogenesis at the same control point. One of the primary mechanisms by which dbcAMP stimulates gluconeogenesis from lactate or alanine is via inactivation of pyruvate kinase [20]. The effect of EGF on pyruvate kinase activity in hepatocytes was therefore investigated.

Incubation of hepatocytes with EGF or dbcAMP for 30 min was found to increase the K_m of pyruvate kinase for phosphoenolpyruvate without changing the $V_{max.}$ (results not shown). Fig. 3 shows inactivation of pyruvate kinase as expressed by the ratio of activity at 1 mM-phosphoenolpyruvate to that at 5 mM-phosphoenolpyruvate. Although the effect of dbcAMP was maximal after 2 min, maximal inactivation of pyruvate kinase by EGF was attained only after 10 min. The time course of inactivation of pyruvate kinase is broadly consistent with that of the stimulation of gluconeogenesis shown in Fig. 1.

One possible explanation of these results is that EGF causes an increase in the cyclic AMP concentration in hepatocytes, and that this is responsible for the phosphorylation of pyruvate kinase via activation of cyclic-AMP-dependent protein kinase. The effect of EGF on cyclic AMP concentrations in hepatocytes isolaated from fed rats was therefore measured at intervals over a period of 1 h. During this time EGF failed to increase cyclic AMP over the control values (2.73 ± 0.18 pmol/mg of cell protein). Under the same experimental conditions glucagon increased cyclic AMP to 13.98 ± 1.03 pmol/mg within 2 min, and this returned to control values after 30 min. These results are consistent with the findings of others [5,6] who showed that EGF failed to increase cyclic AMP after 10 min.

The results presented in this paper indicate that EGF stimulates gluconeogenesis in rat hepatocytes simultaneously with a stable inactivation of pyruvate kinase, probably involving phosphorylation of this enzyme. This does not, however, rule out the possibility of further control at the level of phosphofructokinase/fructose-1,6-bisphosphatase. The time courses both of inactivation of pyruvate kinase and of stimulation of gluconeogenesis by EGF are considerably slower than the corresponding effects observed with glucagon or dbcAMP. The mechanism of action of EGF could involve a slow activation of cyclic-AMP-dependent protein kinase independent of any increase in cyclic AMP concentration or a slow activation of a different kinase acting at the same or at a different site on pyruvate kinase.

EGF has been shown to cause a transient increase in intracellular Ca^{2+} concentration, which returns to control values with 5 min [5,21]. There is evidence that increases in intracellular Ca^{2+} lead to phosphorylation of pyruvate kinase [22], possibly via a Ca^{2+}- and calmodulin-dependent kinase [23]. It is, however, unlikely that the effect of EGF on the stimulation of gluconeogenesis and on pyruvate kinase inactivation reported here is mediated by the reported increase in cell Ca^{2+}, since the intracellular Ca^{2+} concentration will have returned to control values before any effect on gluconeogenesis is observed. The lack of involvement of Ca^{2+} in the action of EGF on gluconeogenesis is also indicated by the failure of EGF to stimulate glucose production from those substrates whose metabolic pathways involve flux through the Ca^{2+}-sensitive enzyme 2-oxoglutarate dehydrogenase.

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