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Epidermal growth factor inhibits phosphoenolpyruvate carboxykinase gene expression in rat hepatocytes in primary culture

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Epidermal growth factor (EGF) decreased the basal, and blocked the dibutyryl cyclic AMP (Bt_2cAMP)-induced, expression of P-enolpyruvate carboxykinase (GTP) (PEPCK) and tyrosine aminotransferase (TAT) genes in both rat hepatocytes in primary culture and the FTO-2B hepatoma cell line. Treatment of hepatocytes with EGF in combination with phorbol ester (TPA) resulted in an additive decrease of PEPCK mRNA levels. Overnight pretreatment of hepatocytes with TPA, which is known to downregulate protein kinase C, abolished the TPA and reduced the EGF-mediated inhibition of PEPCK gene expression. These results suggested that EGF caused its effect, at least in part, through protein kinase C.

Epidermal growth factor; Phorbol ester; P-enolpyruvate carboxykinase; Gene expression; Hepatocyte

1. INTRODUCTION

Epidermal growth factor (EGF) is a polypeptide that increases skin growth of newborn mice and may also act as a mitogen in a variety of cell types [1,2]. EGF, like insulin, has a receptor that exhibits tyrosine kinase activity [3]. Liver membranes and isolated hepatocytes bind EGF specifically. Both the EGF-receptor number and tyrosine kinase activity decrease during starvation in the rat liver [4], suggesting that EGF may have a physiological role in the liver. EGF has a profound effect in different cellular processes including alteration of gene expression. It has been reported that EGF increased the levels of c-*fos* mRNA in 3T3 cells [5], in H4IIE hepatoma [6], and in A431 epidermoid carcinoma [7] cells. In A431 cells, EGF also induced c-*myc* mRNA production [7]. In addition, it has been shown that EGF modifies the carbohydrate metabolism in different cell types [8–10]. The intracellular signals involved in EGF action are not well understood. However, a mechanism for the effect of EGF in these diverse processes has been linked to the activation of the phospholipase C-τ (PLC-τ) as a result of phosphorylation on tyrosine residues by EGF receptor tyrosine kinase [11,12]. The activation of PLC-τ enhances the breakdown of phosphatidilinositol 4,5-bisphosphate in a variety of cell types to yield two putative second messengers: 1,2-diacylglycerol and myo-inositol 1,4,5-triphosphate (IP_3) [13]. Diacylglycerol has been shown to specifically activate protein kinase C in a calcium-dependent manner [14], whereas IP_3 mediates the release of free Ca^{2+} from specific intracellular pools [15]. EGF effects mediated by both protein kinase C activation [16,17] and calcium mobilization [18] have already been described. However, other factors, like neural growth factor (NGF) or basic fibroblast growth factor (FGF), also stimulate phosphorylation of PLC-τ in tyrosine [19], and, although many growth factors elicit similar early responses, the long-term phenotypic responses to these diverse ligands vary. In this regard, NGF and basic FGF cause differentiation of PC12 cells, whereas EGF is mitogenic [20].

Liver glycolysis is induced by insulin [21], EGF [17], vanadate [22] and phorbol ester (TPA) [17], while gluconeogenesis is blocked [23–25]. The cytosolic form of P-enolpyruvate carboxykinase (PEPCK) governs the rate-limiting step in gluconeogenesis. The activity of the enzyme is mainly regulated by changes in the transcription of its gene. Insulin, TPA and vanadate decrease the PEPCK gene transcription whereas glucocorticoids and cAMP have the opposite effect [26–32].

The aim of this work was to study whether EGF could inhibit PEPCK gene expression either in hepatoma cells and hepatocytes in primary culture, and whether this effect could be related to an activation of protein kinase C.

2. MATERIALS AND METHODS

2.1. *Materials*

The random primer labeling kit, all restriction enzymes and Bt_2cAMP were obtained from Boehringer Mannheim. [α-^{32}P]dCTP (3,000 Ci/mmol) came from Amersham Corp. GeneScreen Plus was purchased from DuPont-New England Nuclear. All media and sera

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were obtained from Gibco Laboratories. EGF and TPA were obtained from Sigma. The other reagents used were of the best grade commercially available. The P-enolpyruvate carboxykinase cDNA was provided by Dr. Richard W. Hanson, Case Western University, Cleveland, Ohio, USA. The tyrosine aminotransferase cDNA was a gift from Dr. Günter Schutz, Institut für Zell und Tumorbiologie, Heidelberg, Germany. Animal studies were conducted in accord with the 'Guidelines for Care and Use of Experimental Animals'.

2.2. *Preparation and incubation of hepatocytes*

Male Sprague–Dawley rats, weighing 200–250 g and fed ad libitum, were used in all studies. Hepatocytes were isolated as previously described [33]. After removing nonparenchymal cells and debris, hepatocytes were resuspended in Dulbecco's minimal essential medium (DMEM) containing 0.2% albumin and 10% fetal calf serum. 5.5×10^6 cells were plated in 10 ml of this medium on collagen-coated dishes and maintained at 37°C under a CO_2 atmosphere. After 4 h, the medium was removed and replaced by 10 ml of fresh DMEM medium containing 0.2% albumin without fetal calf serum. Then, one half of the cells were pretreated for 16 h with 1 μM TPA. Afterwards, cells were incubated with either 0.5 mM Bt_2cAMP, 50 nM insulin, 1 μM TPA, 33 nM EGF, or with different combinations of the effectors.

2.3. *Northern analysis*

Total RNA was extracted from confluent FTO2B cultures and primary hepatocytes by the guanidine isothiocyanate method [34], and the RNA samples (20 μg) were electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. Northern blots were hybridized to ^{32}P-labeled cDNAs. The PEPCK cDNA probe corresponded to a 1.1 kb *Pst*I–*Pst*I fragment from the 3′ end of the PEPCK cDNA [35]; tyrosine aminotransferase cDNA corresponded to a 600-base pair *Pst*I–*Pst*I fragment which included the 3′ end of the tyrosine aminotransferase cDNA [36]; the β-actin probe corresponded to a 1.3 kb *Eco*RI–*Eco*RI fragment of β-actin cDNA [37]. Hybridization and washing conditions have been described previously [24]. The membranes were exposed to Kodak XAR-5 film, and densitometric analysis of autoradiograms was performed at non-saturating exposures with a scanning densitometer. The actin signal was used to correct for loading inequalities, because the actin RNA levels were not appreciably changed by the various treatments.

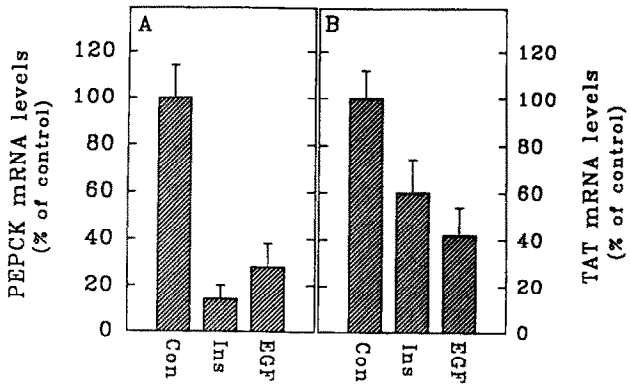

Fig. 1. Effects of EGF on PEPCK and TAT mRNA levels in rat hepatocytes in primary culture. Hepatocytes in primary culture were treated for 4 h with medium (no hormones added) (Con), with 50 nM insulin (Ins) of 33 nM EGF (EGF). RNA was extracted, electrophoresed (20 μg/sample) and probed for PEPCK (panel A) and TAT (B) as described in section 2. Signal intensity was quantitated by densitometry, and the readings obtained in experimental cultures were expressed as a percentage of readings in paired control cultures after correction for loading inequalities with the β-actin signal. Each bar represents the mean ± S.E.M. of the observations made in five independent experiments.

2.4. *Presentation of the data and statistical analysis*

In studies of individual RNA samples the correlation coefficient between increasing amounts of input RNA and signal intensity was 0.99 for all three transcripts. To analyze the effects of perturbations independently of variability in basal gene expression, the densitometric readings of experimental samples were expressed as a percentage of readings in paired control samples (always tested in the same Northern blot) after correction for loading differences with the actin signal. The data are expressed as the mean ± 1 S.E.M. Statistical analysis was by the Wilcoxon signed-rank test.

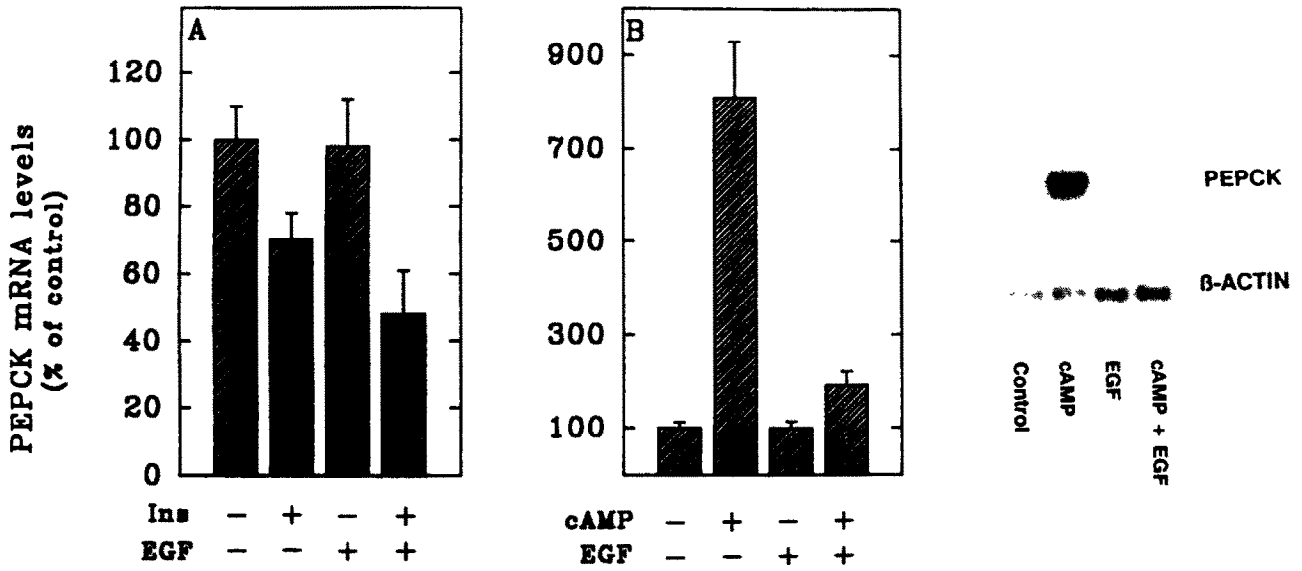

Fig. 2. Effects of EGF on PEPCK mRNA levels in FTO-2B rat hepatoma cells. FTO-2B cells were treated with 0.5 mM Bt_2cAMP and 1 mM theophylline, 33 nM EGF, 50 nM insulin or with combinations of these effectors for 4 h. Experimental procedures were described in the legend to Fig. 1. Each bar represents the mean ± S.E.M. of the observations made in three independent experiments. A representative Northern blot is presented.

3. RESULTS AND DISCUSSION

3.1. *Effects of EGF on PEPCK and TAT gene expression*

The effect of EGF on the regulation of PEPCK gene expression has been studied using rat hepatocytes in primary culture and FTO-2B rat hepatoma cells. Treatment of hepatocytes for 4 h with EGF caused a strong reduction on the PEPCK mRNA basal concentration, which decreased to 28 ± 10% of control ($P < 0.01$) (Fig. 1A), while no significant inhibitory effect of EGF on the basal expression of PEPCK was detected in FTO-2B cells (98 ± 14% of control) (Fig. 2A). However, in these cells a 50% inhibition of basal PEPCK mRNA levels was observed when EGF and insulin were added together into the incubation media (48 ± 13% of control, $P < 0.01$) while insulin alone only caused a 30% reduction (Fig. 2A). As expected, incubation of both hepatocytes and FTO-2B cells with Bt_2cAMP increased the levels of PEPCK mRNA. EGF blocked this induction both in hepatocytes in primary culture and FTO-2B hepatoma cells (Figs. 3A and 2B). In hepatocytes, EGF, like insulin, caused a 40% inhibition of the Bt_2cAMP induction of the PEPCK mRNA concentrations (from 1,360 ± 155% to 795 ± 106, $P < 0.01$), while the blockade was stronger in hepatoma cells, where EGF produced a 75% reduction of the cAMP induction of PEPCK mRNA levels.

Since tyrosine aminotransferase (TAT) is also involved in gluconeogenesis and the expression of its gene is regulated in a similar manner to the PEPCK gene [38–40], we have studied, in rat hepatocytes in primary culture, the effects of EGF on the basal TAT gene expression, in comparison to the effects of insulin. EGF

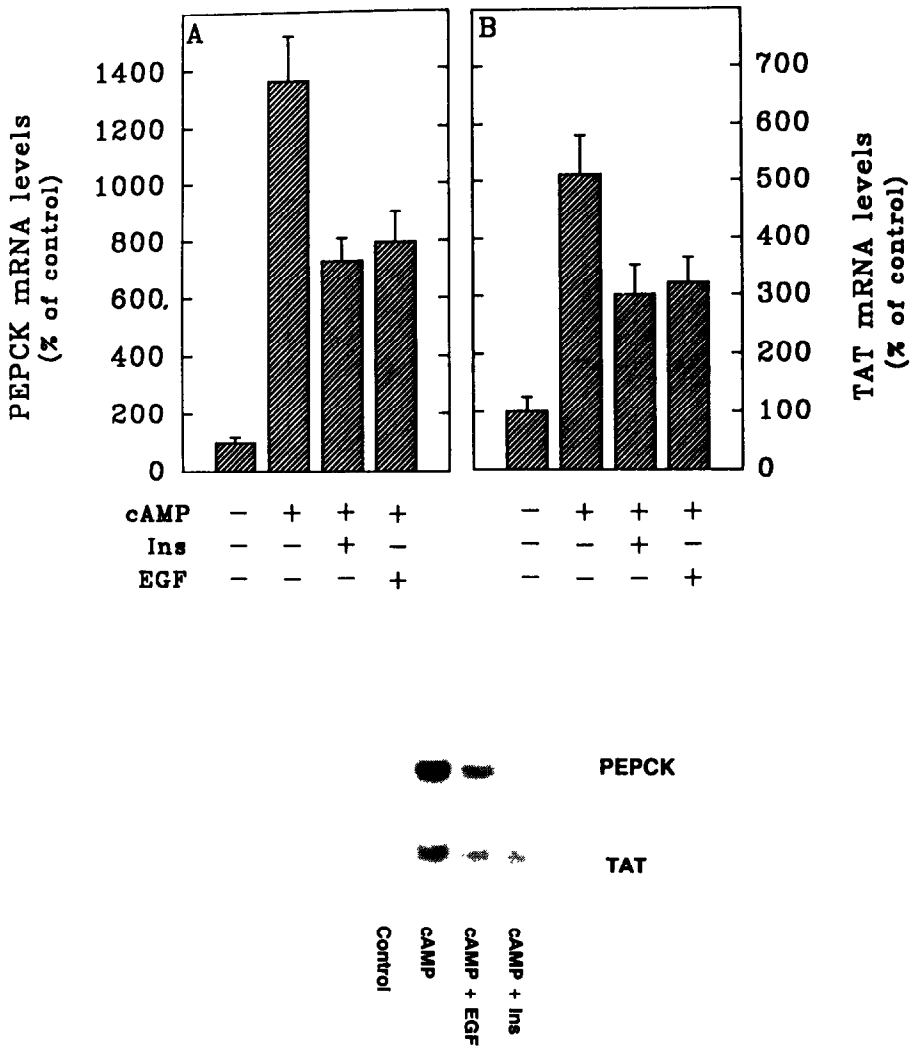

Fig. 3. Effects of EGF on cAMP induction of PEPCK and TAT mRNA levels in rat hepatocytes in primary culture. Hepatocytes in primary culture were treated for 4 h with 0.5 mM Bt_2cAMP, and in combination of 50 nM insulin or 33 nM EGF. Experimental procedures and presentation of data are as described in the legend to Fig. 1. Results represent the mean ± S.E.M. of five different experiments. A representative Northern blot is presented.

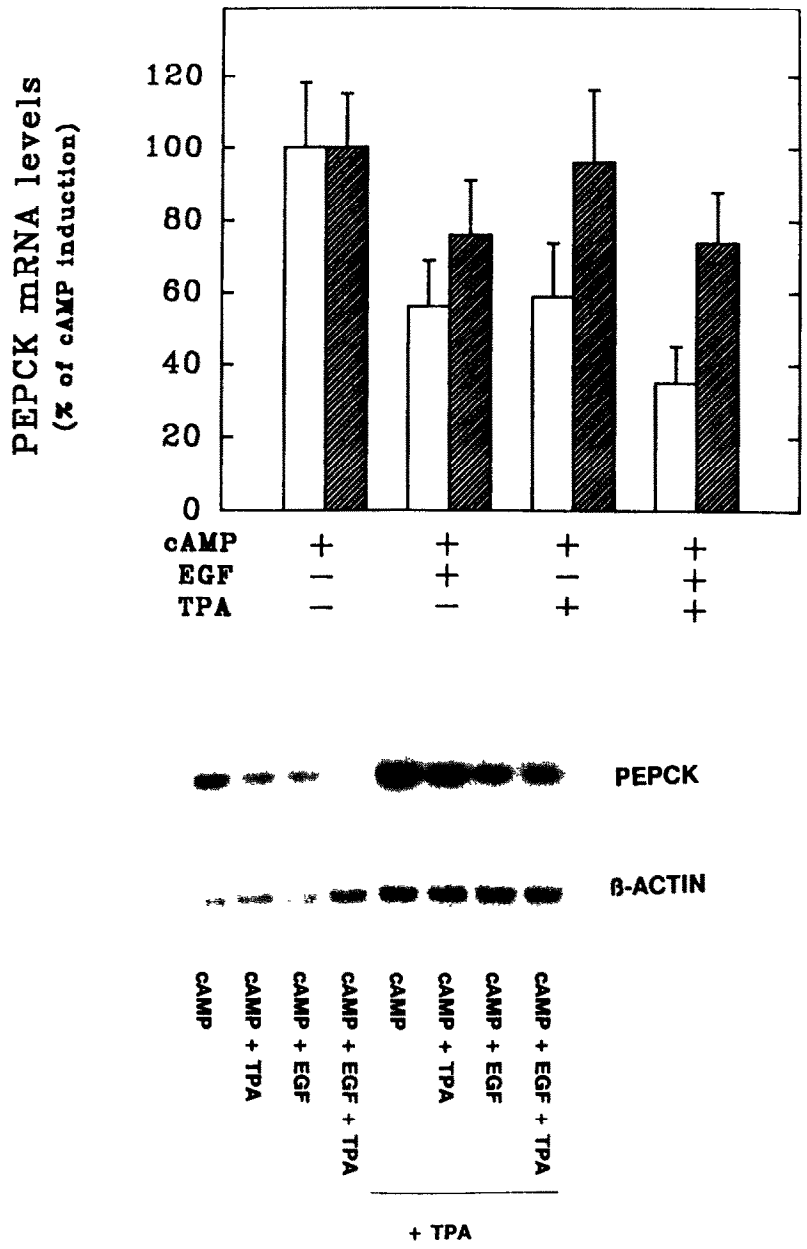

Fig. 4. Effects of combinations of EGF and TPA on PEPCK mRNA levels. Hepatocytes were treated for 4 h with combinations of 0.5 mM Bt_2cAMP, 33 nM EGF and/or 1 μM TPA (open bars). In addition (striped bars), cells were pretreated for 16 h with 1 μM TPA and then treated for 4 additional hours with combinations of 0.5 mM Bt_2cAMP, 33 nM EGF and/or 1 μM TPA. Experimental procedures were described in the legend to Fig. 1. Data are expressed as a percentage of cAMP induction. Results represent the mean ± S.E.M. of six independent experiments. A representative Northern blot is presented.

inhibited the TAT mRNA basal levels, with a decrease to 42 ± 12% of control ($P < 0.01$) (Fig. 1B), while insulin only reduced the TAT gene expression to 60 ± 14% of control (Fig. 1B). Treatment of hepatocytes with Bt_2cAMP induced the TAT gene expression. Induction was similarly reduced when these hepatocytes were incubated with either EGF or insulin (Fig. 3B).

3.2. *Effects of combinations of EGF and TPA on PEPCK gene expression*

In order to study the mechanism by which EGF inhibited the Bt_2cAMP induction of PEPCK gene expression, hepatocytes were treated with EGF, TPA and combinations of both effectors. As previously shown in hepatoma cells [25], TPA was able to decrease PEPCK mRNA levels induced by Bt_2cAMP in rat hepatocytes in primary culture (Fig. 4). When added together, EGF and TPA exerted a more pronounced inhibitory effect on PEPCK mRNA levels, which decreased to 35 ± 10% of cAMP-induced control ($P < 0.01$), than EGF or TPA added alone (Fig. 4). It has been described that phorbol esters activate protein kinase C and may stimulate var-

ious intracellular events that are mediated through this kinase. In order to determine if protein kinase C was involved in the EGF effect on PEPCK gene expression, hepatocytes were pretreated overnight with 1 μM TPA. Under these conditions, in which protein kinase C is down-regulated [41] a 20% reduction of EGF inhibition of Bt_2cAMP induction on PEPCK gene expression was observed (from 56 ± 13% of cAMP-induced control in non-pretreated hepatocytes to 76 ± 15% in treated hepatocytes, $P < 0.01$). In these conditions, EGF was clearly less effective in decreasing PEPCK mRNA levels but still had a significant effect (Fig. 4). In TPA pretreated hepatocytes, the addition of both TPA and EGF caused an inhibitory effect on Bt_2cAMP induced PEPCK gene expression (72 ± 14%) similar to that observed when EGF was added alone, while the addition of TPA in the pretreated hepatocytes did not produce a significant reduction on cAMP-induced PEPCK mRNA levels (94 ± 20% of cAMP-induced control) (Fig. 4). These results suggest that EGF could act on the expression of the PEPCK gene through, at least, two different mechanisms, one dependent and another independent of protein kinase C.

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