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Calcium-mobilizing effectors inhibit P-*enol*pyruvate carboxykinase gene expression in cultured rat hepatocytes

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Incubation of primary cultures of hepatocytes from fed and fasted rats with calcium ionophore strongly decreased glucose production from pyruvate. Like insulin, calcium ionophore A23187, phenylephrine, vasopressin, and prostaglandins E_2 and $F_{2\alpha}$ caused a significant reduction (50–60%) in basal concentrations of mRNA for P-*enol*pyruvate carboxykinase (PEPCK), the main regulatory enzyme of gluconeogenesis. Phenylephrine, prostaglandin E_2 and calcium ionophore A23187 were also able to counteract the induction of PEPCK gene expression by Bt_2cAMP. These effects were similar to those exerted by both vanadate and phorbol ester TPA. The decrease in extracellular calcium by the addition of the calcium-chelating agent EGTA to the incubation medium caused an increase in PEPCK mRNA levels. This effect was additive to that of Bt_2cAMP and was counteracted by vanadate.

Gene expression; P-*enol*pyruvate carboxykinase; Calcium ionophore A23187; Phenylephrine; Insulin

1. INTRODUCTION

Hormones that act via changes in intracellular Ca^{2+} levels (adrenergic agonists, vasopressin, prostaglandins, etc.) interact with their own specific plasma membrane receptors to generate two intracellular messengers, myoinositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol [1]. Diacylglycerol has been shown specifically to activate protein kinase C in a calcium-dependent manner [2], whereas IP_3 mediates the release of free Ca^{2+} from specific intracellular pools [3]. However, calcium release accounts for only part of the overall rise induced in cytosolic Ca^{2+}, an important part originating from Ca^{2+} influx through the plasma membrane [4]. The rise in cytosolic Ca^{2+}, in combination with calmodulin and/or other effectors, leads to the activation of a number of Ca^{2+}-linked protein kinases, including Ca^{2+}/calmodulin-dependent protein kinases, phosphorylase kinase and protein kinase C [5–7]. These enzymes catalyze the phosphorylation of a number of protein substrates that cause changes in gluconeogenic and glycolytic flux [8]. While much is known about the short-term mechanisms by which calcium controls cytosolic processes, little has been reported about the long-term mechanisms by which calcium regulates glucose metabolism and gene expression.

The cytosolic form of P-*enol*pyruvate carboxykinase (PEPCK), which catalyzes the rate-limiting conversion of oxalacetate to phospho*enol*pyruvate in hepatic gluconeogenesis, is regulated by changes in the transcription rate of its gene [9]. Glucocorticoids and cAMP increase PEPCK gene expression, whereas insulin and TPA have the opposite effect [10–18]. Like phorbol ester TPA, the synthetic diacylglycerol *sn*-1,2-dioctanoylglycerol also inhibits PEPCK gene transcription, indicating that the stimulation of protein kinase C results in a decrease in PEPCK gene expression [11]. Although both TPA and insulin repress PEPCK gene transcription through different pathways [11,14,19], they share a common DNA element [14,19]. Vanadate is also able to inhibit PEPCK gene expression both in cultured hepatoma cells [20] and 'in vivo' in diabetic animals [21]. We have previously shown sequences responsive to vanadate between sites −109 and −68 in the 5′-flanking region of the PEPCK gene [20]. The most likely target for vanadate in this region is the cAMP-responsive element (CRE), which maps from −91 to −84 [20]. This would indicate a major difference in the site of action of vanadate and insulin, since an insulin-responsive element has been located between −416 and −407 on the PEPCK promoter [19]. Vanadate and insulin may initiate a common signal, for example, via phosphorylation of the insulin receptor, since vanadate inhibits phosphotyrosine phosphatases [22,23]. Vanadate has other effects, e.g. it increases the formation of inositol phosphates [24] and the uptake of Ca^{2+} [25], which could further alter the transcription of the PEPCK gene. Recently, it has been suggested that calcium may mediate the regulation of gene expression by insulin [26].

We have now examined the effect of calcium-mobiliz-

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ing agents on glucose production and PEPCK gene expression in rat hepatocytes in primary culture, in comparison with the effects of insulin, phorbol ester TPA and vanadate. We found that calcium-mobilizing agents inhibit basal and Bt_2cAMP-induced PEPCK gene expression, suggesting that the activation of calcium/calmodulin-dependent protein kinases could be involved in this process.

2. MATERIALS AND METHODS

2.1. *Materials*

The random-primed DNA labeling kit and Bt_2cAMP were obtained from Boehringer Mannheim. [^{32}P]dCTP (3000 Ci/mmol) came from Amersham Corp. GeneScreen Plus was obtained from DuPont-New England Nuclear. All media and sera were obtained from Gibco Laboratories. TPA, vasopressin, phenylephrine, prostaglandins and calcium ionophore A23187 came from Sigma. Insulin was obtained from Calbiochem. The other reagents used were of the highest purity available. P-*enol*pyruvate carboxykinase cDNA was a gift from Dr. Richard W. Hanson, Case Western Reserve University, Cleveland, Ohio, USA. Tyrosine aminotransferase (TAT) cDNA was obtained from Dr. Günter Schütz, Institute of Cell and Tumor Biology, German Cancer Research Center, Heidelberg, Germany.

2.2. *Preparation and incubation of hepatocytes*

Male Sprague–Dawley rats, weighing 200–250 g were used in these studies. They were fed 'at libitum' unless otherwise indicated. Hepatocytes were isolated as previously described [27]. After removal of non-parenchymal 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. Four hours later, medium was removed and replaced by 10 ml of fresh DMEM medium containing 0.2% albumin without fetal calf serum. Hepatocytes were maintained in this medium for 16–18 h before treatment with different effectors. Then, cells were incubated with medium (no serum or hormones added) or with 50 nM insulin, 10 μM phenylephrine, 100 nM vasopressin, 25 μM prostaglandin E_2, 20 μM prostaglandin $F_{2\alpha}$, 5 μM calcium ionophore A23187, 0.5 mM Bt_2cAMP plus 1 mM theophylline, 1 μM phorbol ester TPA, 1 mM vanadate, or with different combinations of the effectors. The concentration of EGTA used in individual experiments is indicated in the text.

2.3. *Isolation and analysis of cellular RNA*

Total RNA was extracted from primary cultures of hepatocytes and from tissues by the guanidine isothiocyanate method [28], and 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 [29]; the tyrosine aminotransferase (TAT) cDNA probe corresponded to a 600 bp *Pst*I–*Pst*I fragment which included the 3′ end of the tyrosine aminotransferase cDNA [30]; the β-actin probe corresponded to a 1.3 kb *Eco*RI–*Eco*RI fragment of β-actin cDNA [31]. These probes were labeled using [α-^{32}P]dCTP, following the method of random oligopriming as described by the manufacturer. Specific activity of the DNA probe labelled in this manner was approximately 10^9 cpm/μg DNA. Membranes were placed in contact with 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. Presentation of the data and statistical analysis were performed as indicated previously [32]. Differences were considered statistically significant at $P < 0.05$.

3. RESULTS

3.1. *Effects of calcium-mobilizing agents on the rate of gluconeogenesis by hepatocytes in primary culture*

Primary cultures of hepatocytes isolated from fed and 48-h starved rats were incubated for up to 24 h in DMEM without glucose and supplemented with 20 mM pyruvate as a gluconeogenic precursor, with or without calcium ionophore A23187, prostaglandin E_2, or the combination of both effectors. An aliquot of the incubation medium was removed at different times, and glucose production was measured. Both calcium ionophore and prostaglandin E_2 inhibited glucose production in hepatocytes from fed (80% and 25% reduction, respectively, at 24 h), and from starved, rats (80% and 40% reduction, respectively, at 24 h) (Fig. 1A and B). When both effectors were added together, 90% inhibition of glucose production was detected. These results indicate that calcium-mobilizing agents are able to inhibit gluconeogenesis by hepatocytes in primary culture.

3.2. *Effects of calcium-mobilizing agents on PEPCK gene expression*

PEPCK is the main regulatory enzyme of gluconeogenesis, and its activity is regulated at the level of expression of the gene [9]. The effects of hormones that act via changes in intracellular calcium levels on the regulation of the PEPCK mRNA levels were studied in rat hepatocytes in primary culture. Effectors belonging to different groups (catecholamines, peptides and prostaglandins) were used, but all raise cytosolic calcium levels when added to the incubation medium of liver parenchymal cells [1]. Treatment of hepatocytes for 4 h with calcium ionophore A23187, phenylephrine, vasopressin, prostaglandins E_2 and $F_{2\alpha}$ led to a decrease in PEPCK mRNA levels (50–60% reduction), as determined by Northern blotting (Fig. 2A). This effect was similar to that noted following treatment of hepatocytes with insulin (Fig. 2A). We also investigated whether the expression of the gene for tyrosine aminotransferase (TAT), which is related to gluconeogenesis and regulated in a manner similar to PEPCK [33–35], was modified by these hormones. Like for PEPCK, TAT mRNA levels were decreased by treatment of hepatocytes with the different calcium-mobilizing agents used (Fig. 2B).

Incubation of hepatocytes with Bt_2cAMP plus theophylline increased the level of PEPCK mRNA (17-fold), while phenylephrine, prostaglandin E_2 and calcium ionophore A23187 blocked this induction (40–50% reduction) (Fig. 3). The effect of insulin was slightly stronger than that of the calcium-mobilizing agents and caused a 60% reduction of the Bt_2cAMP-induced PEPCK mRNA levels (Fig. 3).

Phorbol ester TPA and vanadate also decreased PEPCK mRNA levels induced by Bt_2cAMP in primary culture of rat hepatocytes (Fig. 4A). The inhibitory effect of these two effectors was additive, and hepatocytes

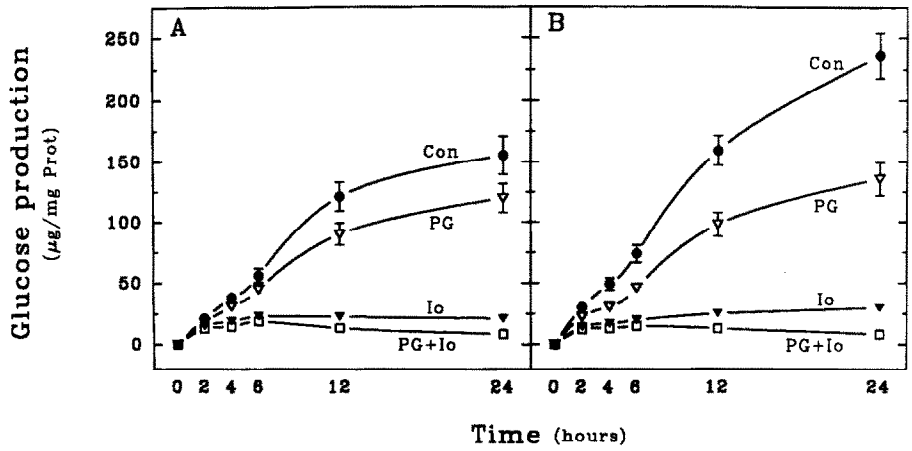

Fig. 1. Effects of the calcium ionophore A23187 and prostaglandin E_2 on glucose production from pyruvate in hepatocytes in primary culture. Hepatocytes in primary culture, isolated from fed (A) and 24-h fasted rats (B), were incubated in a medium with 20 mM pyruvate and lacking glucose, in the absence (●) or presence of 5 μM calcium ionophore A23187 (▼), 25 μM prostaglandin E_2 (▽), or combinations of both effectors (□). At the indicated time, glucose concentration was measured in the incubation medium of the cells as indicated in section 2. The results are mean ± S.E.M. of four different experiments.

cultured in the presence of TPA plus vanadate completely blocked PEPCK gene expression, in contrast to hepatocytes treated with the effectors alone, which only showed 70% reduction (Fig. 4A). However, overnight pretreatment of hepatocytes with 1 μM TPA, which downregulates protein kinase C [36], did not modify the effect of vanadate on PEPCK mRNA levels (Fig. 4A). We have already demonstrated that further addition of the phorbol ester to TPA-pretreated hepatocytes does not cause any significant reduction on Bt_2cAMP-induced PEPCK mRNA levels [32]. Similar effects of vanadate and TPA were noted on TAT mRNA concentration (Fig. 4B). These results suggest the mechanism of inactivation of PEPCK gene expression by vanadate is different from that of TPA, and that protein kinase C is probably not involved in vanadate action.

3.3. *Effect of calcium-chelating agent EGTA on PEPCK gene expression*

Since the action of agents that increase the cytosolic calcium concentration resulted in inhibition of the PEPCK gene expression, we next investigated whether a decrease in extracellular calcium could modify the expression of this gene. Incubation of hepatocytes in

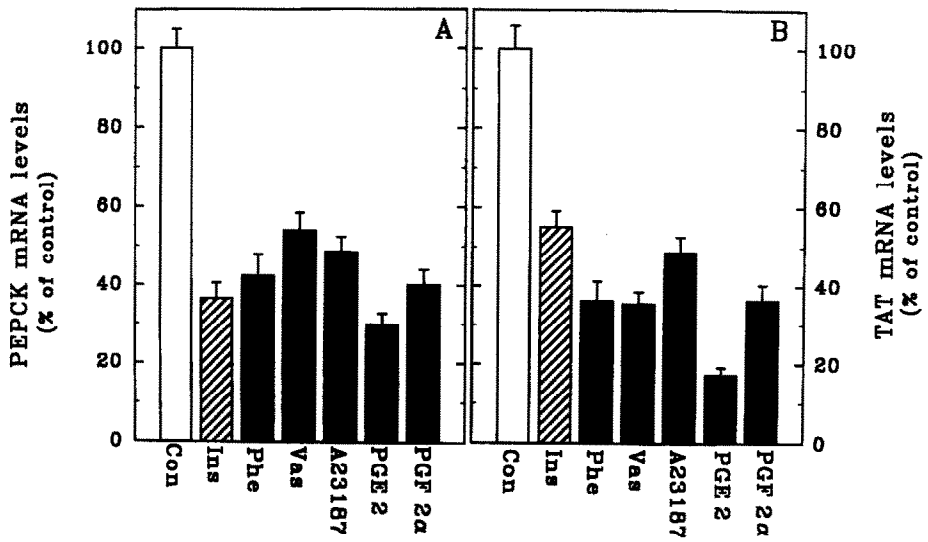

Fig. 2. Effects of calcium-mobilizing agents on PEPCK and TAT mRNA levels. Hepatocytes in primary culture were incubated with a medium (no serum or hormones added) (Con), 50 nM insulin (Ins), 10 μM phenylephrine (Phe), 100 nM vasopressin (Vas), 5 μM calcium ionophore A23187 (A23187), 25 μM prostaglandin E_2 (PGE_2) and 20 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) for 4 h. RNA was extracted from the cells, and the concentration of PEPCK (A) and TAT (B) mRNA was determined by Northern blotting using PEPCK and TAT cDNAs as hybridization probes. The data are expressed as a percentage of non-treated control cells, and results are mean ± S.E.M. of three independent experiments.

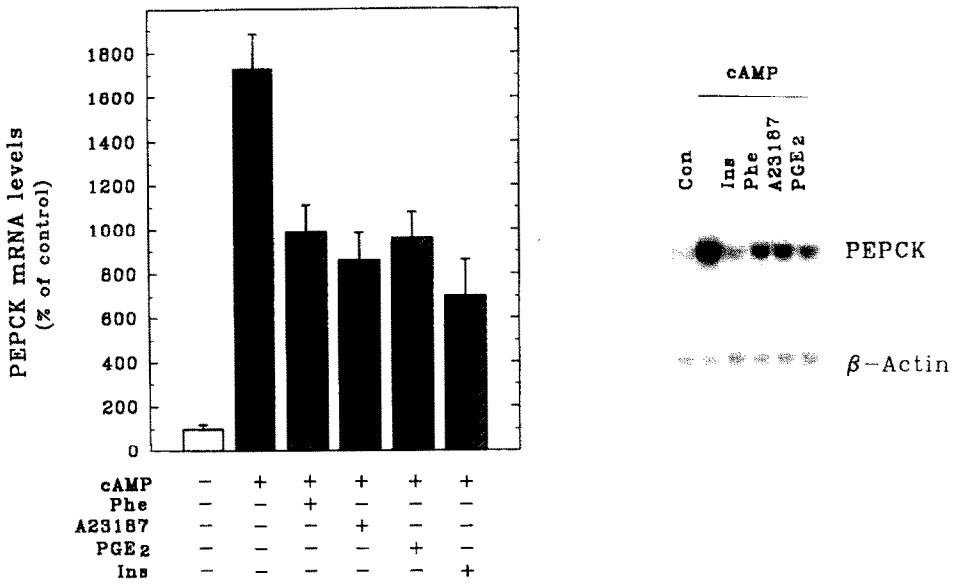

Fig. 3. Effects of calcium-mobilizing agents on Bt_2cAMP induced PEPCK mRNA levels. The level of RNA for PEPCK was determined by Northern analysis of total RNA isolated from hepatocytes treated for 4 h with 0.5 mM Bt_2cAMP plus 1 mM theophylline (cAMP) and with combinations of 0.5 mM Bt_2cAMP plus 1 mM theophylline and 10 μM phenylephrine (Phe), 5 μM calcium ionophore A23187, 25 μM prostaglandin E_2 (PGE_2) and 50 nM insulin (Ins). The data are expressed as a percentage of non-treated control cells, and results are mean ± S.E.M. of three independent experiments. A representative Northern blot is presented.

primary culture in DMEM with increasing concentrations of the calcium-chelating agent EGTA for 4 h led to an increase of PEPCK mRNA levels (Fig. 5A). This effect was detected at concentrations of EGTA of 2.5 mM or greater. At 1 mM EGTA no effect on PEPCK mRNA levels was noted, probably due to an inadequate reduction of the free unchelated calcium content of the incubation medium. A similar effect was noted when TAT mRNA levels were analyzed (Fig. 5B). The induction of TAT gene expression was even higher than that observed on PEPCK. The effect of 4 mM EGTA was also time-dependent (Fig. 5C and D). Hepatocytes incubated with 4 mM EGTA for different periods of time showed an increase in PEPCK mRNA levels, which was maximal after 2 h of incubation (Fig. 5C). Like for PEPCK, TAT mRNA levels also increased, but the maximal effect was noted after 4 h of incubation (Fig. 5D). These results suggest that the presence of calcium in the incubation medium maintains the expression rate of PEPCK and TAT low, since when extracellular levels of the cation are decreased, an induction of the mRNA concentration of both genes is detected. Thus calcium/calmodulin protein kinases may have a role in blocking the expression of these genes.

The effect of EGTA on PEPCK gene expression was additive to the induction provoked by Bt_2cAMP when cells were incubated with both effectors together for 2 h (Fig. 6), indicating that the decrease in extracellular calcium favours the cAMP-mediated induction of PEPCK gene expression. Vanadate was able to inhibit both the induction caused by EGTA alone and that caused by the combination of EGTA and Bt_2cAMP

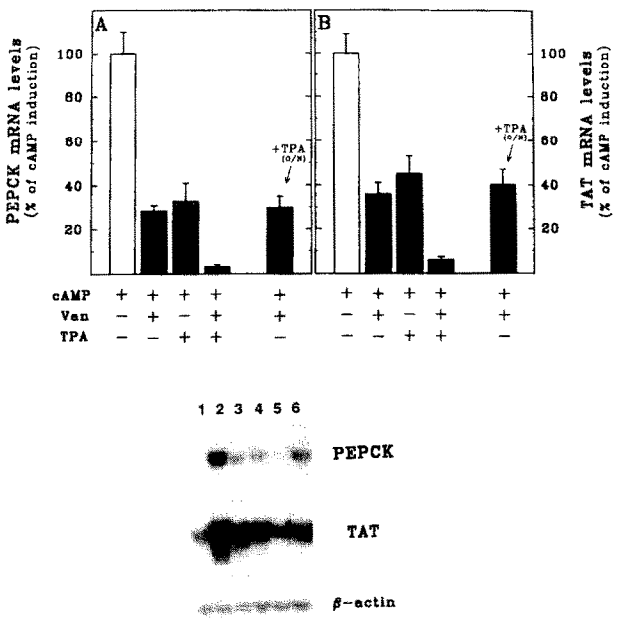

Fig. 4. Effects of combinations of vanadate and TPA on PEPCK and TAT mRNA levels. Hepatocytes were treated for 4 h with combinations of 0.5 mM Bt_2cAMP plus 1 mM theophylline (cAMP), 1 mM vanadate (Van) and/or 1 μM TPA. In addition, cells were pretreated for 18 h with 1 μM TPA (+ TPA O/N) and then treated for 4 additional hours with the combination of 0.5 mM Bt_2cAMP and 1 mM vanadate. The levels of PEPCK (A) and TAT (B) mRNA were determined by Northern analysis using RNA isolated from the hepatocytes. Data are expressed as a percentage of cAMP induction. Results represent the mean ± S.E.M. of three independent experiments. A representative Northern blot is presented. Lanes: 1, control; 2, cAMP; 3, cAMP + Van; 4, cAMP + TPA; 5, cAMP + Van + TPA; 6, TPA O/N + cAMP + Van.

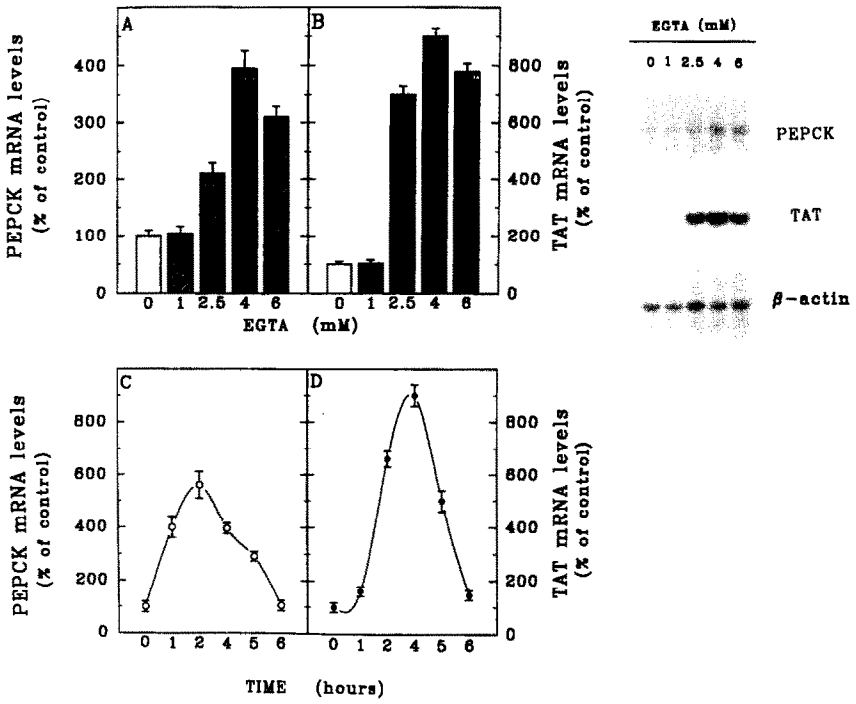

Fig. 5. Effect of the calcium chelating agent EGTA on PEPCK and TAT gene expression. Panel A and B, show the effect of 4 h treatment with increasing concentrations of EGTA on PEPCK and TAT mRNA levels in hepatocytes in primary culture. Panel C and D, indicate time-dependent effects of 4 mM EGTA on PEPCK and TAT mRNA levels. RNA was extracted from the cells, and the concentration of PEPCK and TAT mRNA was determined by Northern blotting using PEPCK and TAT cDNAs as hybridization probes. The data are expressed as a percentage of non-treated control cells. Results are mean ± S.E.M. of four independent experiments. A representative Northern blot is presented.

(Fig. 6). Similar effects were observed in FTO-2B rat hepatoma cells treated with these effectors (data not shown). These results suggest that vanadate could counteract the EGTA effect either by releasing calcium from intracellular stores or through a mechanism independent of the cation.

4. DISCUSSION

Intracellular calcium causes a wide variety of effects in cellular processes. In the present study, we show that calcium-mobilizing agents are able to inhibit gluconeogenesis, at least in part by reducing the amount of PEPCK and TAT in rat hepatocytes in primary culture. In addition, they are able to counteract the induction of the mRNA concentration of these genes by Bt_2cAMP. This effect is similar to that described for insulin. Insulin and TPA block PEPCK gene transcription through different pathways, since preincubation of hepatoma cells with phorbol esters, which down-regulates protein kinase C, does not affect the inhibitory action of insulin on the PEPCK gene, while it attenuates the TPA-inhibition of PEPCK gene expression [11,19,38]. However, both insulin and TPA share a common DNA element in the PEPCK promoter [19]. On the other hand, changes in cytosolic calcium concentration can alter the induction of the p33 gene transcription caused by either insulin or calcium ionophore A23187 [26], suggesting

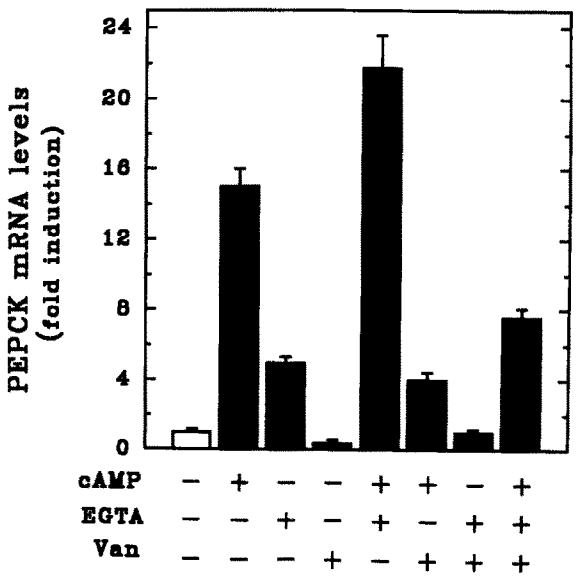

Fig. 6. Effect of combination of EGTA and Bt_2cAMP on PEPCK mRNA levels. Hepatocytes in primary culture were incubated with medium (no hormones or serum added), or with 0.5 mM Bt_2cAMP plus 1 mM theophylline (cAMP), 4 mM EGTA, 1 mM vanadate (Van) and with combinations of these effectors for 2 h. The levels of RNA for PEPCK were determined by Northern analysis. The data are presented as fold induction with respect to the control non-treated cells. Results are mean ± S.E.M. of four independent experiments.

that calcium might exert an important role in mediating insulin's regulation of gene expression. Our study also shows that vanadate, an insulin mimetic agent that increases the phosphorylation of the insulin receptor by inhibition of phosphotyrosine phosphatases, inactivates PEPCK gene expression through a mechanism that is independent of protein kinase C. Intracellular calcium concentration might be involved in the action of vanadate, since this effector is able to increase the uptake of calcium [25] and increase the formation of inositol phosphates [24]. We have shown that the effects of vanadate on glycogen phosphorylase activity in isolated hepatocytes are dependent on calcium in the medium, while the inactivation by vanadate of glycogen synthase is independent of the presence of the cation [37]. Similarly, the epidermal growth factor (EGF) causes the activation of the EGF receptor tyrosine kinase, which results in an induction of calcium influx in many cell types [38–40]. EGF is able to induce the expression of several genes and inhibit PEPCK gene expression through a mechanism involving calcium influx into the cell, in addition to a mechanism dependent on protein kinase C [32,40,41].

The inhibition of the expression of the PEPCK gene observed in this study suggests that calcium-mobilizing agents, insulin and vanadate probably cause activation of calcium/calmodulin-dependent protein kinases. This kinase(s) might be able to modify a set of specific transcription factors involved in the regulation of this gene. However, further research is needed before the mechanism(s) involved in this process can be understood fully.

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