Comparison of the effects of insulin and okadaic acid on phosphoenolpyruvate carboxykinase gene expression

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Many hormones regulate the rate of synthesis of phosphoenolpyruvate carboxykinase (PEPCK), the enzyme that governs the rate-limiting step in gluconeogenesis. In H4IIE rat hepatoma cells, glucocorticoids, retinoic acid and cyclic AMP (cAMP) increase PEPCK gene transcription whereas insulin and phorbol esters have the opposite effect. Insulin and phorbol esters are dominant as they prevent cAMP- and glucocorticoid-stimulated PEPCK gene transcription. In contrast, insulin and phorbol esters both stimulate transcription of gene 33 in the same H4IIE cells, with the same time course as seen for their inhibitory effect on PEPCK gene transcription. We now report that the protein phosphatase inhibitor, okadaic acid, mimics the action of insulin and phorbol esters on expression of both gene 33 and PEPCK gene in H4IIE cells. Okadaic acid stimulates gene 33 mRNA accumulation whereas it inhibits cAMP- and glucocorticoid-

stimulated PEPCK mRNA accumulation. The effect of okadaic acid on the PEPCK gene is mediated through the PEPCK promoter as, in a cell line, HL1C, stably transfected with a PEPCK–chloramphenicol acetyltransferase (CAT) fusion gene, okadaic acid inhibits cAMP- and glucocorticoid-stimulated CAT expression. Desensitization of the protein kinase C pathway by exposure to phorbol 12-myristate 13-acetate for 16 h abolishes the subsequent action of the phorbol ester but does not markedly affect the inhibition of cAMP- and glucocorticoid-stimulated CAT expression by insulin or okadaic acid. Even though insulin and okadaic acid appear to repress PEPCK gene expression through a pathway initially distinct from that used by phorbol esters, transient-transfection studies show that the final target of the action of okadaic acid, insulin and phorbol ester is the same DNA element.

INTRODUCTION

The rate of transcription of the rat liver cytosolic phosphoenolpyruvate carboxykinase (PEPCK) gene is stimulated by glucagon [acting via cyclic AMP (cAMP)], retinoic acid, thyroid hormone and glucocorticoids but is inhibited by insulin and phorbol esters (for reviews, see refs. [1,2]). Detailed analysis of the PEPCK promoter by transfection of PEPCK–chloramphenicol acetyltransferase (CAT) fusion genes has shown that multiple *cis*-acting elements that collectively form hormone-response units are required to manifest the full response individually to all of these factors. This arrangement allows for the efficient integration of multiple signals in what we refer to as a hormone- or metabolic-response domain. For example, a *cis*-acting element within the cAMP-response unit is required for the full response to glucocorticoids [1]. Similarly, an intact glucocorticoid-response unit is required for the response to retinoic acid (D. Scott and D. K. Granner, unpublished work). (For reviews on the relative location of the elements that comprise the hormone-response domain in the PEPCK gene promoter, see refs. [1,2].)

The dominant negative effect of phorbol esters on glucocorticoid-stimulated PEPCK gene transcription appears to be mediated through the same *cis*-acting element as used by insulin [3,4]. Thus, despite the differences in the initial pathways of insulin and phorbol ester action, their effects converge on the same DNA element. Phorbol esters are known to act through the activation of specific members of the protein kinase C family [5]. Similarly, even though the insulin receptor is itself an insulin-stimulated tyrosine kinase, it appears that many of the actions of insulin are ultimately brought about through a cascade that

involves serine/threonine phosphorylation/dephosphorylation [6,7]. Insulin is known to activate multiple serine kinases, although in most cases the connection between a particular kinase and a specific physiological substrate has yet to be established [6,7].

Although the *trans*-acting factors that mediate the action of insulin and phorbol esters on the PEPCK gene have yet to be identified, the general importance of changes in phosphorylation state in the regulation of transcription factor activity is now well documented (see refs. [8,9] for review). Protein kinase C and insulin both alter the phosphorylation state of transcription factors and nuclear matrix proteins [8–11]. However, the *c-fos* gene is the only example for which specific kinases have been associated with the action of insulin on the transcription of a specific gene [12,13]. However, as with PEPCK, the *trans*-acting factor that mediates the effect of insulin on *c-fos* gene transcription has not been identified. In contrast, cAMP is thought to mediate its stimulatory effect on PEPCK gene transcription through the activation of protein kinase A [14]. The subsequent phosphorylation and activation of the cAMP-response-element-binding protein (CREB) may be important for mediating the effect of cAMP on PEPCK gene transcription [15].

To initiate an assessment of the role of protein phosphorylation on PEPCK gene expression we chose to study the action of okadaic acid, a phosphatase inhibitor that can specifically inhibit both the cytosolic and nuclear forms of protein phosphatases 1 and 2A [16,17]. In the H4IIE rat hepatoma cell line, okadaic acid mimics the effect of insulin on PEPCK mRNA accumulation through a pathway initially distinct from that used by protein kinase C. However, the effects of okadaic acid, insulin and

Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase; CAT, chloramphenicol acetyltransferase; CREB, cyclic AMP-response element-binding protein; PMA, phorbol 12-myristate 13-acetate; TK, thymidine kinase; IRS, insulin-response sequence.

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phorbol esters all converge on the PEPCK insulin-response sequence (IRS).

MATERIALS AND METHODS

Materials

Okadaic acid was purchased from Calbiochem Corp. Radioisotopes were obtained from Amersham or ICN. Insulin was purchased from Collaborative Bioproducts, and 8-(4-chlorophenylthio)-cAMP from Boehringer-Mannheim. Phorbol 12-myristate 13-acetate (PMA) and dexamethasone were obtained from Sigma Chemical Company. All other chemicals were of the highest grade available.

Synthesis and labelling of oligonucleotides for primer extension

Three oligonucleotides, 5'-GGAGAGAGGCAGGGACTCTG-GTGCCACC-3' (PC28), 5'-TCTCTTTCTTTGCTTGTTTCG-AGGCTGTGA-3' (P33.P16) and 5'-AGCCTCAGCCGCCG-CCCAGTGC-3' (CAM23), were synthesized on a model 380A Applied Biosystems DNA synthesizer. They were designed to be complementary to the region of the PEPCK gene located between +102 and +129 relative to the transcription start site, to gene 33 between +57 and +86 and to the rat calmodulin gene located between +46 and +69 relative to the most 5' transcription start site reported respectively [18,19]. PC28, P33.P16 and CAM23 were 5' end-labelled with [γ - 32 P]ATP (> 5000 Ci/mmol; Amersham) to a specific radioactivity of about 10^8 c.p.m./ μ g [18].

RNA isolation and primer-extension analysis

RNA isolation and primer-extension analysis were performed as previously described [18].

Plasmids

The construction of thymidine kinase (TK)-CAT fusion genes containing either the wild-type or mutant insulin-response sequence (IRS) has been previously described [3,4]. The native vector is designated TKC-VI, whereas the vectors containing the wild-type and mutant IRS are designated PC42 and PC4M respectively [3,4]. All plasmid constructs were sequenced using the USB Sequenase kit and purified by centrifugation twice through caesium chloride gradients [20].

Transient transfection

H4IIE rat hepatoma cells were grown to confluence in Dulbecco's modified Eagle's medium containing 2.5% (v/v) newborn calf serum and 2.5% (v/v) fetal calf serum, diluted the day before transfection and replated in 75 cm² culture dishes (2×10^6 to 3×10^6 cells per dish). Attached cells were transfected, after removal of medium, by incubation for 30 min at room temperature with 2 ml of a calcium phosphate/DNA co-precipitate containing 30 μ g of PEPCK-TK-CAT plasmid DNA and 20 μ M chloroquine. This solution was then diluted by addition of 8 ml of medium supplemented with 5% (v/v) of both the 0.25 M CaCl₂/40 μ M chloroquine solution and Hepes-buffered saline (pH 7.10), which were used to prepare precipitates [3]. The cells were then returned to a humidified 5% CO₂/95% air atmosphere

in a Queue cell culture incubator. After 4–6 h, cells were treated with 20% (v/v) dimethyl sulphoxide in serum-containing medium for 5 min, washed in serum-containing and then serum-free medium, and then incubated for 18 h in serum-free medium in the presence or absence of 100 nM okadaic acid as described in Table 4. Cells were then harvested and CAT activity was assayed as described below. CAT activity was corrected for protein concentration in the cell lysate, as measured by the Pierce bicinchoninic acid assay.

Stable transfection

Isolation of the HL1C cell line stably transfected with a PEPCK reporter construct containing PEPCK promoter sequence from –2100 to +69 ligated to the CAT reporter gene has been described previously [18].

CAT assay

Cells were harvested by trypsin digestion and sonicated in 300 μ l of 250 mM Tris/HCl at pH 7.8. After sonication the extracts were heated for 10 min at 65 °C and cell debris was removed by centrifugation. CAT assays were performed on the supernatants as follows: a 100 μ l portion of supernatant was incubated in a final volume of 250 μ l containing (final concentrations) 200 mM Tris/HCl, pH 7.8, 6 mM MgCl₂, 75 mM KCl, 0.4 mM CoA, 3 mM ATP, 1 mM chloramphenicol, 0.03 unit of acetyl-CoA synthetase and 0.34 mM sodium [3 H]acetate (0.5 Ci/mmol). This mixture was incubated for 2 h at 37 °C, extracted with benzene and transferred to scintillation vials. After removal of the benzene by evaporation, the sodium [3 H]acetate incorporated into chloramphenicol was quantified by liquid-scintillation spectroscopy. The radioactivity in a lysate-free blank was subtracted and CAT activity was corrected for variations in protein concentration as described above.

Statistics

Statistics were analysed by repeated-measure analysis of variance using the StatView II program from Abacus Concepts Inc. When significant *F* ratios were found, *post hoc* analysis was by Fisher Protected Least Significant Difference. The level of significance was $P < 0.05$ (two-sided test).

RESULTS

Okadaic acid has opposite effects on PEPCK and gene 33 mRNA accumulation in the same cell

We utilized the rat hepatoma H4IIE cell line to study the effect of okadaic acid on PEPCK gene expression. After a 16 h period of serum starvation, H4IIE cells were treated with various hormones for 3 h and PEPCK mRNA was measured by primer-extension analysis (Figure 1). A combination of dexamethasone (a synthetic glucocorticoid) and 8-(4-chlorophenylthio)-cAMP (a cAMP analogue) stimulated PEPCK mRNA accumulation, an effect that was blocked by insulin, as previously reported [1] (Figure 1). Okadaic acid mimicked this dominant inhibitory effect of insulin on PEPCK mRNA accumulation, and also inhibited basal PEPCK mRNA expression, as did insulin [1] (Figure 1). As okadaic acid can be cytotoxic [21] and could therefore inhibit PEPCK mRNA accumulation non-specifically, the effect of this agent on the expression of another liver-specific gene that is normally stimulated by insulin was investigated. In H4IIE cells, transcription of gene 33 is stimulated by both insulin and glucocorticoids [22,23]. Okadaic acid by itself also stimulated

Figure 1 Primer-extension analysis of the multihormonal regulation of PEPCK and gene-33 mRNA expression in H4IIE cells

After 16 h of serum starvation, H4IIE cells (approx. 8×10^6 per 75 cm² dish) were placed in fresh serum-free medium in the presence or absence of various hormones. After 3 h, total RNA was isolated from four dishes of control cells (lane 1), cells exposed to 800 or 400 nM okadaic acid (OA; lanes 2 and 3 respectively) or cells exposed to dexamethasone (Dex; 500 nM) plus 8-(4-chlorophenylthio)-cAMP (cAMP; 0.1 mM) alone (lane 4) or in the presence of okadaic acid (OA; 800 nM) (lane 5) or insulin (10 nM) (lane 6). PEPCK, gene 33 and calmodulin mRNA were measured by primer-extension analysis as described in the Materials and methods section. The positions of the single PEPCK, two gene-33 and multiple calmodulin (CAM) start-site products are indicated by the arrows and were of the expected size. An experiment representative of two other similar experiments is shown.

Table 1 Primer-extension analysis of the effect of okadaic acid on glucocorticoid-stimulated PEPCK mRNA expression in H4IIE cells

After 16 h of serum starvation, H4IIE cells (approx. 8×10^6 cells per 75 cm² flask) were placed in fresh serum-free medium in the presence of dexamethasone (500 nM) or dexamethasone plus okadaic acid (675 nM). After 4 h, total RNA was isolated from individual flasks and PEPCK mRNA was then quantified by primer-extension analysis and densitometric scanning of autoradiographs as described in the Materials and methods section. Results are expressed relative to PEPCK mRNA accumulation in dexamethasone-treated cells and represent the mean $\pm$ S.E.M. of four independent experiments. Statistical differences were measured as described in the Materials and methods section: * $P < 0.05$.

Treatment	PEPCK mRNA (% of dexamethasone-induced value)
Dexamethasone	100
Dexamethasone + okadaic acid	16.2 $\pm$ 4.6*

gene-33 mRNA accumulation and, like insulin, did not significantly alter the stimulatory effect of a dexamethasone and 8-(4-chlorophenylthio)-cAMP combination (Figure 1). Thus okadaic

Figure 2 Effect of okadaic acid and insulin on PEPCK mRNA decay

After 16 h of serum starvation, H4IIE cells (approx. 8×10^6 cells per 75 cm² flask) were placed in fresh serum-free medium supplemented with dexamethasone (500 nM). After a 4 h preincubation there was no addition (control) or insulin (10 nM) or okadaic acid (675 nM) were added. After the times indicated, total cellular RNA was isolated from individual flasks and PEPCK mRNA was then quantified by primer-extension analysis as described in the Materials and methods section. (a) Representative autoradiograph; (b) densitometric scan (means $\pm$ S.E.M. of three to five independent experiments).

acid mimics the action of insulin on the expression of two genes in the same cell: both agents inhibit PEPCK and stimulate gene 33. Unlike insulin, okadaic acid (at 800 nM) also has a stimulatory effect on expression of the calmodulin gene [18] (Figure 1).

Effect of okadaic acid on glucocorticoid-stimulated PEPCK mRNA accumulation is delayed in onset

Although the inhibitory effect of okadaic acid on glucocorticoid-stimulated PEPCK mRNA accumulation is clearly apparent at 4 h (Table 1), this effect, unlike that of insulin, appears to be delayed in onset. Studies on the time course of glucocorticoid-stimulated PEPCK mRNA accumulation showed little effect of okadaic acid before 60–90 min (results not shown). This delayed effect of okadaic acid is more easily visualized after elevation of PEPCK mRNA by dexamethasone. The subsequent decay of PEPCK mRNA after the addition of insulin or okadaic acid can then be easily followed (Figure 2). H4IIE cells, starved of serum for 16 h, were treated with dexamethasone for 4 h before addition of either insulin (10 nM) or okadaic acid (625 nM) for the times indicated. After isolation of total cellular RNA, PEPCK mRNA was quantified by primer-extension analysis (Figure 2). In the absence of insulin or okadaic acid, PEPCK mRNA remained constant over the time course of the experiment (Figure 2). After addition of insulin, PEPCK mRNA began to decrease immediately whereas in okadaic acid-treated cells it only began to decrease after 90 min (Figure 2). This is probably not due to

some limitation in okadaic acid entry into the nucleus as it has rapid effects on the expression of other genes [21]. Despite this delayed action of okadaic acid on glucocorticoid-stimulated PEPCK gene expression, a dramatic effect on PEPCK mRNA was seen by 3 h (Figure 1).

Okadaic acid inhibits PEPCK gene expression through the PEPCK promoter

Having established that okadaic acid inhibits PEPCK mRNA accumulation, it was of interest to determine its site of action. Okadaic acid could inhibit transcription, reduce primary transcript processing or decrease mRNA stability. Insulin primarily inhibits the initiation of PEPCK gene transcription but it also has a significant, but smaller, effect on transcript elongation [1]. To investigate whether okadaic acid, like insulin, acts through the PEPCK promoter we utilized an H4IIE-derived cell line stably transfected with a PEPCK-CAT fusion gene. The fusion gene contains the PEPCK promoter sequence from -2100 to +69, relative to the transcription-start site, and is designated HL1C [18]. Previous studies [18] showed that hormonal regulation of the transgene in this cell line mimics that of the endogenous PEPCK gene. HL1C cells were starved of serum for 16 h and then treated with various hormones for 3 h, at which time cells were harvested and CAT activity was quantified (Table 2). A combination of dexamethasone and 8-(4-chlorophenylthio)-cAMP stimulated CAT expression, an effect that was blocked by insulin and phorbol esters, as previously reported [3,4,18]. Okadaic acid, like insulin and phorbol esters, caused a dose-dependent inhibition of CAT expression (Table 2) at concentrations consistent with its ability to inhibit protein phosphatase 1 and 2A in intact cells [16,24]. Thus the response of the PEPCK-CAT fusion gene to okadaic acid mimics that of the endogenous PEPCK gene. We conclude from this result that okadaic acid inhibits PEPCK gene expression through the PEPCK promoter, as previous studies suggest that okadaic acid does not affect the rate of translation or stability of CAT mRNA [21,25-27]. However, only nuclear run-on assays can formally prove that this is an effect on the rate of CAT transcription. Although okadaic acid can alter glucocorticoid receptor phosphorylation and its ability to interact with transcription complexes [28], the data presented in Table 4 demonstrate that this cannot explain the action of okadaic acid on PEPCK gene expression (see below).

Okadaic acid inhibits PEPCK gene expression through a pathway distinct from that utilized by phorbol esters

There is considerable controversy as to the role of protein kinase C in insulin action (see refs. [29,30] for review). However, it is clear that a prolonged (16 h) treatment of H4IIE cells with phorbol esters abolishes the inhibitory effect of a subsequent phorbol ester treatment on PEPCK gene expression, whereas the effect of insulin is unaltered [31]. Even though such a pretreatment with phorbol esters is now known to down-regulate only some protein kinase C family members [32], this result still indicates that insulin and phorbol esters act through a different pathway with regards to PEPCK gene expression. The question as to whether insulin inhibits PEPCK gene transcription through a 'non-classical' [5] phorbol ester-insensitive form of protein kinase C that is not down-regulated remains to be addressed.

Pretreatment of HL1C cells with phorbol esters abolishes the inhibitory effect of a subsequent phorbol ester application on CAT expression, whereas the effect of insulin is unchanged (Table 3). This result mirrors that previously described for the

Table 2 Multihormonal regulation of a PEPCK-CAT fusion gene in the stably transfected HL1C cell line

HL1C cells (approx. 8×10^6 per 75 cm² dish) were starved of serum for 16 h and then placed in fresh serum-free medium in the absence of hormones (control) or serum-free medium supplemented with dexamethasone (Dex; 500 nM) plus 8-(4-chlorophenylthio)-cAMP (cAMP; 0.1 mM) alone or with 200 nM, 400 nM, 800 nM okadaic acid (OA), 10 nM insulin or 1 μ M PMA. After 3 h cells were harvested and CAT activity was assayed and corrected for protein concentration as described in the Materials and methods section. The results from two independent experiments, each of which was performed in duplicate, are expressed relative to CAT activity in untreated cells. {Dexamethasone and 8-(4-chlorophenylthio)-cAMP stimulate a time-dependent linear increase in CAT accumulation [18] which explains the smaller induction of CAT activity (30-fold) at 3 h than at 18 h (> 100-fold) as previously reported [4].}

Treatment	Fold induction of CAT activity	
	Expt. 1	Expt. 2
Control	1	1
Dex + cAMP	31.0	34.4
Dex + cAMP + OA (200 nM)	21.4	20.6
Dex + cAMP + OA (400 nM)	10.4	11.5
Dex + cAMP + OA (800 nM)	5.7	9.8
Dex + cAMP + insulin	4.7	4.9
Dex + cAMP + PMA	2.2	2.0

Table 3 Multihormonal regulation of a PEPCK-CAT fusion gene in the stably transfected HL1C cell line after preincubation with phorbol ester

HL1C cells (approx. 8×10^6 per 75 cm² dish) were incubated for 16 h with PMA (1 μ M) in serum-free medium. Cells were then placed in fresh serum-free medium in the absence of hormones (control) or serum-free medium supplemented with dexamethasone (Dex; 500 nM) plus 8-(4-chlorophenylthio)-cAMP (cAMP; 0.1 mM) alone or with 800 nM okadaic acid (OA), 10 nM insulin or 1 μ M PMA. After 3 h, cells were harvested and CAT activity was assayed and corrected for protein concentration as described in the Materials and methods section. Results are expressed relative to CAT activity in control cells and represent the mean $\pm$ S.E.M. of three independent experiments. Statistical differences were measured against the fold induction of CAT activity in the presence of dexamethasone and cAMP, as described in the Materials and methods section: * $P < 0.05$.

Treatment	Fold induction of CAT activity
Control	1
Dex + cAMP	20.0 $\pm$ 1.7
Dex + cAMP + OA	8.1 $\pm$ 1.5*
Dex + cAMP + insulin	2.44 $\pm$ 0.4*
Dex + cAMP + PMA	25.2 $\pm$ 5.7

endogenous gene [31]. As with insulin, the inhibitory effect of okadaic acid is substantially unaffected by this pretreatment, which suggests that okadaic acid, like insulin, works through a pathway initially distinct from that activated by phorbol esters (Table 3).

Okadaic acid inhibits PEPCK gene expression through the IRS

Multiple *cis*-acting elements are required to mediate the negative effect of insulin on PEPCK gene transcription [3,18]. Only one such element, designated the distal IRS, has so far been defined [3,4]. As this IRS (located at -416 to -407 relative to the transcription-start site) coincides with a *cis*-acting element critical for the response to glucocorticoids, it is ideally positioned to mediate the dominant negative effect of insulin on glucocorticoid-stimulated PEPCK gene transcription [1]. This element also mediates the negative effect of phorbol esters so, even though

Table 4 Okadaic acid inhibits PEPCK gene expression through the PEPCK IRS

H4IIE cells were transiently transfected, as described in the Materials and methods section, with a TKCAT vector or with the same vector containing a mutant or wild-type IRS. Transfected cells were incubated in serum-free medium in the presence or absence of 100 nM okadaic acid. After 18 h cells were harvested and CAT activity was assayed and corrected for protein concentration. The results shown represent the ratio of CAT activity in okadaic acid-treated versus control cells (expressed as percentage change) and are means $\pm$ S.E.M. of nine independent transfections for each construct. To compare the percentage change in CAT activity seen in cells treated with okadaic acid, after transfection with the vector or the same vector containing the wild-type IRS, statistical differences were measured as described in the Materials and methods section. * $P < 0.05$.

Construct	CAT activity (% change)
Vector	+58 $\pm$ 6
Wild-type	-16 $\pm$ 8*
Mutant	+57 $\pm$ 13

insulin and phorbol esters initially activate different pathways, their actions converge at this point [4].

H4IIE cells were transiently transfected with a variety of TK-CAT fusion genes to determine whether okadaic acid also mediates its negative effect on PEPCK gene expression through this element. CAT expression directed by the parent vector, designated TKC-VI [3], is unresponsive to insulin but ligation of the PEPCK IRS into the *Bam*HI site of the TK promoter confers insulin-dependent inhibition of CAT expression [3,4]. H4IIE cells were transfected with the TKCAT vector alone or the vector containing the wild-type or mutant IRS sequence (Table 4). Okadaic acid, unlike insulin [3], clearly stimulated CAT expression directed by the vector alone or by the vector containing the mutant IRS. This observation may be explained by an effect of okadaic acid on an undefined *trans*-acting factor that binds the TK promoter. A similar non-specific stimulatory effect of protein kinase A on expression directed by TKCAT plasmids has been observed [33]. However, in the vector containing the wild-type PEPCK IRS this positive effect was abolished (Table 4). We therefore conclude that okadaic acid is capable of mediating a negative effect on PEPCK gene expression through the IRS. A greater inhibitory effect of okadaic acid is seen at higher concentrations but, owing to the prolonged incubation required in this type of experiment (18 h), cytotoxic effects of the inhibitor become apparent at these levels (results not shown).

DISCUSSION

The role of protein phosphorylation in a number of biological systems has been established, in part, by the use of okadaic acid to inhibit protein phosphatases in intact cells (see ref. [16] for review). The results presented in the present paper support the involvement of phosphorylation in the regulation of expression of two specific genes. Okadaic acid mimics the inhibitory effect of insulin on PEPCK gene expression and the stimulatory effect of insulin on gene-33 expression in the same cells over the same time span (Figure 1). Our results on the effect of okadaic acid on PEPCK gene expression in H4IIE cells are consistent with those of Morrison et al. [34], who employed primary hepatocytes. However, our results appear to contradict those of Wadzinski et al. [17], who showed that okadaic acid enhanced the response of a transfected PEPCK-CAT fusion gene to cAMP in HepG2 cells. cAMP is thought to mediate its stimulatory effect on PEPCK gene transcription through the activation of protein

kinase A and subsequently through the phosphorylation and activation of the cAMP-response-element-binding protein [14,15]. Thus the results of Wadzinski et al. [17] would be consistent with okadaic acid maintaining this protein in a phosphorylated state because its dephosphorylation by protein phosphatase 1 [35,36] or 2A [17,35] was inhibited. This discrepancy may be due to inherent differences related to studies in cells that express the endogenous PEPCK gene (H4IIE cells [1], primary hepatocytes [34]) and those that do not [HepG2 cells (D. K. Granner, unpublished work)]. In cells in which the regulation of the transgene mimics that of the endogenous gene, the same mechanism of hormone action is more likely to apply to both; unlike in H4IIE cells, insulin only slightly represses cAMP-stimulated PEPCK-CAT expression in the HepG2 cell line [37]. Alternatively, this discrepancy may reflect a species difference between the human hepatoma HepG2 cell line and the rat hepatoma H4IIE cell line/rat primary hepatocytes. Finally, Morrison et al. [34] suggest that the specific cell culture regimen before experimental manipulation may be of importance, in an undefined way, as in initial studies they also found that okadaic acid enhanced the response of the PEPCK gene to cAMP [38].

Over the past few years there have been several studies on the effect of okadaic acid on gene expression. Okadaic acid stimulates expression of members of the *jun* and *fos* families (see ref. [21] for review) and *Egr*-1 [25], whereas it inhibits expression of *MyoD*1 [26] and *c-myc* [27]. In some cases transient-transfection studies have begun to identify *cis*-acting elements through which okadaic acid mediates its effect [21,25-27]. In the PEPCK gene promoter, okadaic acid mediates its effect through a well-defined IRS (Tables 2 and 4).

Elucidation of the mechanism through which okadaic acid mimics the effect of insulin on PEPCK gene expression is complicated by the potential existence of multiple pathways of insulin action [6,7]. Specifically, the 100-fold greater sensitivity of PEPCK gene transcription as compared with gene 33 suggests that alternative pathways of insulin action are in use in H4IIE cells [22]. Moreover, insulin dose-response curves for both genes have two components, which suggests that multiple pathways of insulin action are involved even in the regulation of the transcription of a single gene [22]. Recent reports have connected multiple steps in one such pathway that starts with the activation of the insulin receptor tyrosine kinase after insulin binding and ultimately results in activation of the mitogen-activated protein kinase family [7]. Mitogen-activated protein kinase has been shown to phosphorylate several transcription factors including *myc* and *jun* [8,9] but whether this kinase is involved in the action of insulin on the PEPCK gene or gene 33 is unknown. Roles for intermediates in the insulin receptor-mitogen-activated protein kinase pathway, including insulin receptor substrate-1, Ras and Raf-1 (and also phosphatidylinositol 3-kinase), have been demonstrated for insulin-stimulated *c-fos* transcription [12,39,40] but, unlike PEPCK, this may be partly accomplished through a protein kinase C-dependent pathway [13].

The involvement of protein kinase C in insulin action was initially proposed because phorbol esters mimic some of the actions of insulin, including stimulation of hexose transport [41] and lipogenesis [42]. Phorbol esters also mimic the action of insulin on several genes including PEPCK, gene 33 and *c-fos* (for review see ref. [4]). However, phorbol esters also antagonize some of the actions of insulin, for example, insulin-stimulated glycogen synthase and tyrosine aminotransferase enzyme activity [43]. Moreover, phorbol esters stimulate expression of the insulin-like-growth-factor-binding protein-1 gene, an effect that is dominant over the inhibitory effect of insulin on this gene [44]. Although several studies have shown that many of the actions of

insulin remain normal in cells in which protein kinase C has been down-regulated by prolonged exposure to phorbol esters, the validity of this approach has been challenged subsequent to the discovery of protein kinase C subtypes that are not affected by this treatment [32]. Furthermore, several studies have demonstrated insulin-stimulated translocation of protein kinase C from cytosolic to particulate fractions [30]. However, translocation does not necessarily indicate activation [29], and protein kinase C subtypes not down-regulated in one cell line may be efficiently down-regulated in another [32,45]. Finally, even though insulin can increase protein kinase C activity [30], the physiological significance of such activation has been challenged [29]. However, in situations where an effect of insulin is still present after prolonged phorbol ester pretreatment, it remains a formal possibility that insulin could act through a 'non-classical' form of protein kinase C that is phorbol ester insensitive [16,45]. With regard to the PEPCK gene, it is clear that the mechanism of action of insulin and okadaic acid on PEPCK gene expression is initially distinct from that of phorbol esters (Table 3). Comparisons of the effects of okadaic acid and phorbol esters on other systems have shown that they have a contrasting, although overlapping, spectrum of actions [46].

Several groups have investigated the effect of okadaic acid on other aspects of insulin action. Although okadaic acid itself stimulates glucose transport in adipocytes, the stimulatory effect of insulin is blocked in cells treated with the agent [26,47]. In contrast, in hepatocytes okadaic acid inhibits both basal and insulin-stimulated glycogen synthesis and insulin-stimulated glucokinase mRNA accumulation [48].

In summary, we have shown that okadaic acid, acting through the IRS in the PEPCK gene promoter, mimics the effect of insulin on PEPCK gene expression. As the effect is a dominant negative one, it is not possible to assess whether or not the combined influences of maximally effective concentrations of okadaic acid and insulin are additive. Thus the simplest explanation for these data is that protein phosphatase 1 or 2A is involved in attenuating insulin-regulated PEPCK gene transcription. Interestingly, the molecular mechanism by which insulin stimulates glycogen synthesis in mammalian muscle involves activation of protein phosphatase 1 [49]. An alternative explanation would implicate the existence of a third okadaic acid-sensitive pathway, in addition to the insulin and phorbol ester pathways, which, when activated, represses PEPCK gene transcription through the IRS. Purification and cloning of IRS-binding proteins and subsequent identification of potential phosphorylation sites should help to resolve these questions.

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