Insulin-mimetic actions of phorbol ester in cultured adult rat hepatocytes
Lack of phorbol-ester-elicted inhibition of the insulin signal

Armin QUENTMEIER,* Haleh DANESCHMAND,† Harald KLEIN,† Kirsten UNTHAN-FECHNER* and Irmelin PROBST†‡

*Institut für Biochemie, Fachbereich Medizin, Georg-August-Universität Göttingen, Humboldtallee 23, D-3400 Göttingen,
and † Klinik für Innere Medizin, Medizinische Universität zu Lübeck, Federal Republic of Germany

The actions of the phorbol ester phorbol 12-myristate 13-acetate (PMA) on glucose metabolism, amino acid transport and enzyme inductions were studied in primary cultures of adult-rat hepatocytes and compared with the effects of insulin. PMA and insulin stimulated glycolysis 5- and 7-fold respectively. The half-maximal effective dose of PMA was 60 nM. Stimulation of glycolysis was accompanied by an insulin- or PMA-dependent and okadac acid-sensitive activation of 6-phosphofructo-2-kinase and pyruvate kinase, as well as by an increase in fructose 2,6-bisphosphate. Glucose production from glycogen was decreased to 50% by PMA and to 15% by insulin, whereas glycogen synthesis was stimulated 2- and 7-fold respectively. PMA also increased aminoisobutyrate uptake, induced ornithine decarboxylase and counteracted the glucagon-dependent induction of phosphoenolpyruvate carboxykinase. PMA strongly antagonized the hormonal activation of glycogen synthesis, but all other insulin actions assayed were not decreased by the phorbol ester. Whereas additive effects of PMA and insulin were not detected, PMA and a simultaneous increase in the glucose concentration had additive effects on glycysis and glycogen metabolism. Cell exposure to insulin resulted in receptor autophosphorylation and a more than 10-fold activation of the receptor tyrosine kinase. PMA did not alter these effects, and also had no effect on the receptor phosphorylation status in the absence of insulin. Long-term (15 h) pretreatment of the cells with PMA abolished all PMA effects, but not the insulin effects. It is concluded that PMA does not generally antagonize the action of insulin in differentiated adult hepatocytes, and that insulin and PMA may use related signal-transduction pathways.

INTRODUCTION

It has been well established that phorbol esters evoke insulin-mimetic effects in muscle and adipose tissue. Phorbol 12-myristate 13-acetate (PMA) stimulated glucose uptake in both tissues [1-5], enhanced amino acid uptake in cultured myocytes [2] and increased lipogenesis in adipocytes [2,6]. Phorbol esters, however, did not mimic all the actions of insulin [2,7], but instead have also been shown to inhibit selectively some insulin effects, such as insulin-dependent glucose uptake and lipogenesis in adipocytes [1,6] and amino acid uptake in muscle [7]. Whereas this insulin-antagonistic action of PMA in adipocytes could in part be due to inhibition of the insulin-receptor tyrosine kinase [8], inhibition of this enzyme does not appear to be involved in the attenuation of the insulin signal in muscle [7]. These contradictory actions of PMA are also evident in the study of liver systems, although available data are scarce and in most investigations liver tumour cells have been used. Insulin-antagonistic PMA effects were demonstrated on glycogen synthesis in FAO [9] and Zajdela [10] hepatoma cells as well as on tyrosine aminotransferase (TAT; EC 2.6.1.5) induction [9]. The finding that exposure to PMA decreased the insulin-stimulated tyrosine kinase activity of the hepatoma insulin receptor through serine/threonine phosphorylation [11,12] provided an accepted explanation. These reports, however, are at odds with data obtained with H4IIE and H35 hepatoma cells; in these cells PMA acted insulin-mimetic with respect to induction of ornithine decarboxylase (ODC; EC 4.1.1.17) and inhibition of phosphoenolpyruvate (PEP) carboxykinase (EC 4.1.1.32) gene expression [13-16], with the combined effects of PMA and insulin being additive or synergistic. No information is available for differentiated hepatocytes from adult animals apart from two reports on the stimulatory effect of PMA on lipogenesis [17,18].

The aim of this study was to investigate the action of PMA alone, as well as its interaction with insulin on several short- and long-term insulin-sensitive parameters in the hepatocyte. We chose the adult primary hepatocyte culture because this system in vitro is the only differentiated adult liver system in which a variety of acute effects of insulin on glucose metabolism is readily detectable [19-21] and which also lends itself to the study of enzyme inductions [22]. It is shown here that PMA acted as an insulin-mimetic agent on both short- and long-term parameters. Its inability to suppress the biopotency of insulin was in accord with its failure to alter the phosphorylation status or the kinase activity of the insulin receptor.

EXPERIMENTAL

Materials

Enzymes, M199 medium, collagenase A, melittin, staurosporine and okadac acid were obtained from Boehringer (Mannheim, Germany). Ham's F-12 medium, bovine insulin and glucagon were from Serva (Heidelberg, Germany). NCTC-135 medium was from Gibco (Eggenstein, Germany). PMA, polymyxin B, sphenoginse, wheat-germ agglutinin—agarose and dexamethasone were from Sigma (Taufkirchen, Germany). Acridine Orange was obtained from Aldrich (Steinheim, Germany). D-[U-14C]Glucose was from New England Nuclear (Dréeihe, Germany), 3-O-methyl-D-[1-3H]glucose, 2-amino[1-14C]isobutyrate ([14C]-AIB) and [33P]P, were from Amersham (Braunschweig, Germany). M, standards were obtained from BioRad (München, Germany). A stock solution of PMA (10 mM) was made in dimethyl sulph-
oxide; before use it was diluted 1:100 in M199 medium containing 0.2% BSA. Okadaic acid was dissolved in dimethyl sulfoxide and further diluted with medium M199 to yield a 0.1 mM stock solution with 20% dimethyl sulfoxide. The FAO cell line was kindly provided by Dr. M. C. Weiss (Gif-sur-Yvette, France) through Dr. F. J. Wielbel (Gesellschaft für Strahlen- und Umweltforschung, München, Germany).

Cell culture
Male Wistar rats (180–250 g) were kept on a 12 h-day/-night rhythm and were allowed free access to the standard diet sniff R 15 (sniff, Soest, Germany). Hepatocytes were isolated by recirculating collagenase perfusion in situ and cultured in M199 medium on 60 mm plastic dishes [21]. For the first 3 h (attachment phase) medium contained 4%, newborn-calf serum, 1 nM insulin and 0.1 μM dexamethasone. After the first change of medium (2.5 ml/dish), serum was omitted and the cells were cultured for the next 43 h in the presence of 0.1 μM dexamethasone and insulin concentrations as stated in the legends. Medium was changed at 22 and 30 h. The gas atmosphere was CO₂/O₂/N₂ (5:17:78). For determination of glucose production from glycogen, glycogen was labelled with [¹⁴C]glucose (1.5 μCi/ml) from 24 to 46 h in the presence of 20 mM glucose (M199 plus 15 mM additional glucose) and 10 nM insulin. FAO cells were cultivated either in Ham’s F-12 or in Ham’s F-12/NCTC-135 medium (1:1, v/v), both with 5% fetal-calf serum.

Cell experiments
Primary culture
After 46 h, dishes were washed twice and then incubated in M199 (2.5 ml/dish) containing 0.1 μM dexamethasone for 1 h. Medium was changed again to one containing dexamethasone and 2 mM lactate (2 ml/dish). When glycogen synthesis and glycolysis were determined medium was additionally supplemented with [¹⁴C]glucose (0.8 μCi/ml). After a 30 min preincubation, zero-time samples were taken and the experiment was started by addition of agonists to the dishes. The incubation was terminated by rapidly aspirating the medium and immersing the dishes in liquid N₂. Glucose transport was measured in M199 medium as outlined above by the uptake of methyl-[1-³H]glucose (final concn. 0.1 mM, 0.8 μCi/ml) for 20, 40 or 60 s and 60 min. For determination of amino acid transport, 0.5 mM [¹⁴C]Ala (0.03 μCi/ml) was added 1 h after insulin or PMA; uptake was then monitored for 30 min, and was linear for at least 60 min. When transport rates were assayed, dishes were washed three times with ice-cold 0.9% NaCl before freezing. For the receptor phosphorylation experiments, cells cultured in 100 mm dishes were washed four times with phosphate-free MEM medium supplemented with 0.1% serum albumin, 15 mM Hepes, 0.1 μM dexamethasone and 2 mM lactate. Cells were then incubated in this medium with 1.1 μCi of [³²P]P₄/ml. After 2.5 h, 0.1 μM insulin or 1 μM PMA was added and the incubation was continued for 20 min. The experiment was terminated by rapidly aspirating the medium and immersing the dishes in liquid N₂.

Analytical procedures
Rates of glucose formation from glycogen and of glycolysis were estimated by the rates of glucose and lactate release into the culture medium. Labelled glucose was separated from labelled lactate by chromatography of 100 μl of medium on Dowex 1X8 (formate form) as outlined in [23]. The rate of glucose production was calculated from the specific radioactivity of glycogen [24] and is expressed as μmol of glycosyl units/mg of DNA. The rate of glycogen synthesis was determined by extracting and quantifying the [¹⁴C]-labelled glycogen from one dish as described by Fleig et al. [24]. For the determination of methylglucose and AIB transport, cells were scraped into 1.25 ml of 0.2 M NaOH and counted for radioactivity.

For measurement of [³²P]P incorporation into the β-subunit of the insulin receptor, cells from one 100 mm dish were processed. Partial purification of the receptor was performed as described by Klein et al. [25], by using wheat-germ agglutinin chromatography and immunosioilation. Samples were electrophoresed on SDS/7.5%-polyacrylamide gels under reducing conditions, blotted on to polyvinylidene difluoride (0.8 mA/cm², 60 min) and detected by autoradiography using preflushed Hyperfilm-MP with an intensifying screen for 24 h.

Measurement of insulin-receptor kinase activity after exposure of intact cells to insulin was performed as described by Klein et al. [25]. Cells from two 140 mm dishes were processed; kinase activity was determined in vitro by incorporation of phosphate into histone 2B. 6-Phosphofructo-2-kinase (PFK 2; EC 2.7.1.105) was determined as described by Bartrons et al. [26], by using the enzyme assays for the active form at pH 6.6 and for the total form at pH 8.5. Data are expressed as the activity ratio of the active form to the total form, which correlates with the phosphorylation state of PFK 2. Changes of the activity ratio were stable to salt fractionation by 15% poly(ethylene glycol). Fructose 2,6-bisphosphate (Fru(2,6)P₂) was determined as described by Van Schaftingen et al. [27]. Cells of single dishes were processed as outlined in [19]. For pyruvate kinase (EC 2.7.1.40) determinations the cell material from two dishes was pooled and processed with (NH₄)₂SO₄ as described [19], and activity was assayed as described by Feliu et al. [28]. PEP carboxykinase was determined by the method of Seubert and Huth [29]. TAT was assayed as described by Granner and Tomkins [30]. ODC activity was estimated by ¹⁴CO₂ production from L-[¹⁴C]ornithine in Warburg flasks. Cells from four dishes were pooled. Then 900 μl of cell cytosol in 50 mM Hepes (pH 7.1)/5 mM dithiothreitol/0.5 mM pyridoxal 5'-phosphate and 0.1 mM EDTA were mixed with 100 μl of 1 mM ornithine (0.2 μCi); the reaction proceeded for 2 h at 37 °C.

DNA was measured as detailed elsewhere [31].

RESULTS
Modulation of glucose metabolism by PMA
When cells had been cultured with 1 mM insulin, basal glycolysis in the presence of 5 mM glucose was low (0.6 μmol/h per mg of DNA; Figure 1a). Addition of 0.1 μM insulin or 1 μM PMA gradually increased glycolysis, which attained its maximally stimulated rate after a 30 min lag period; stimulation was 5- and 7-fold respectively. The hal-maximal effective dose of PMA was 60 nM (Figure 1b). The combined addition of PMA and insulin did not lead to a decrease in the insulin signal, but instead consistently resulted in slightly higher absolute values; in 4 out of 7 experiments the actual glycolytic rate did not differ from the corresponding insulin-stimulated rate. Parallel increases in lactate formation were then observed with insulin alone and with

FAO cells
Nearly confluent cells were washed twice with serum-free Ham’s F-12 medium. The experiments were conducted in Ham’s F-12 medium (supplemented with 10 mM Hepes and 0.1% BSA) as described in detail for primary cultures.
Table 1 Activation of PFK 2 and pyruvate kinase by insulin and PMA

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Okadaic acid</th>
<th>PFK 2 activity ratio (active/total form)</th>
<th>Pyruvate kinase activity ratio (V/\nu_{max})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>0.58 ± 0.02</td>
<td>0.073 ± 0.015</td>
</tr>
<tr>
<td>+</td>
<td>0.53 ± 0.09</td>
<td>0.60 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>--</td>
<td>0.98 ± 0.05</td>
<td>0.183 ± 0.013</td>
</tr>
<tr>
<td>+</td>
<td>0.57 ± 0.01</td>
<td>0.058 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td>--</td>
<td>0.94 ± 0.05</td>
<td>0.134 ± 0.010</td>
</tr>
<tr>
<td>+</td>
<td>0.57 ± 0.04</td>
<td>0.063 ± 0.004</td>
<td></td>
</tr>
<tr>
<td>Insulin + PMA</td>
<td>--</td>
<td>0.99 ± 0.06</td>
<td>0.178 ± 0.016</td>
</tr>
<tr>
<td>+</td>
<td>0.62 ± 0.04</td>
<td>0.076 ± 0.007</td>
<td></td>
</tr>
</tbody>
</table>

Insulin + PMA. In these cases the higher absolute values with insulin + PMA resulted from a shortening of the lag phase. The slight increase in the glycolytic rate with insulin + PMA compared with insulin alone (Figure 1a) was not significant.

In cultured hepatocytes, stimulation of lactate production by insulin is preceded by a sequential activation of PFK 2, elevation of the Fr(2,6)P2 level and activation of pyruvate kinase, all of which occur during the lag phase described above [19]. These three parameters were assayed for their responsiveness towards PMA. PMA increased the activity ratios of both interconvertible enzymes by 60% (Table 1). Whereas there was no significant difference in the effects of PMA and insulin on PFK 2, PMA could not increase the pyruvate kinase activity ratio to the same extent as did insulin (100%). PMA could also not decrease the higher insulin effects. Activation of the two enzymes was completely inhibited by the protein phosphatase inhibitor okadaic acid. However, okadaic acid did not modulate the steady-state basal activity ratios, which indicates that the enzymes were mainly in their phosphorylated inactive forms in cultured hepatocytes. This finding is consistent with an increase in cyclic AMP and activation status of protein kinase A during culture of adult rat hepatocytes [32].

Consistent with the activation of PFK 2, PMA increased Fr(2,6)P2 (Figure 2). As previously described [19], the basal Fr(2,6)P2 level slowly increased to stable values around 1.5 nmol/mg of DNA. Insulin or PMA elevated the rate at which the Fr(2,6)P2 level rose between 10 and 20 min from 19 to 70 pmol/min per mg of DNA (3.7-fold); final levels reached were not significantly different from each other. The initial increase elicited by PMA always showed a prominent lag phase. When the two agonists were supplied in combination, neither inhibitory nor additive effects could be detected.

Since insulin-antagonistic effects of PMA with respect to stimulation of glycogen synthesis have been described for two hepatoma lines [9,10], synthesis and degradation of glycogen were studied (Table 2). Insulin and PMA stimulated glycogen synthesis 7- and 2-fold respectively. Although PMA exhibited a stimulatory effect on basal synthesis, it decreased the effect of insulin by 65%. PMA also attenuated glycogen breakdown to glucose (Figure 3); inhibition was less pronounced than that evoked by insulin (50 versus 85%). The decrease in glycogen degradation by insulin was not relieved by simultaneous PMA addition, even when PMA was added 30 min before insulin or when a 10-fold lower insulin concentration (10 nM) was used.

The increase by PMA of glycogenesis and glycogen synthesis could not be explained by an increase in the glucose transport rate. Uptake of Methylglucose amounted to 20.1 ± 2.1 nmol/min per mg of DNA for control cells and to 19.1 ± 2.2 for cells exposed to PMA for 1 h.
Table 2  Modulation of glycogen synthesis and glycogenolysis by insulin and PMA

For glycogen-synthesis determinations, cells were cultured with 1 nM insulin and 0.1 μM dexamethasone, whereas for glycogenolysis experiments cells additionally received 10 nM insulin and 15 mM extra glucose (= 20 mM final conc.) from 24 to 46 h. The experiments were performed with 5 mM glucose in the medium as detailed in Figure 1. Data are means ± S.D. of four cultures (n.d., not determined); * significantly different from the control (P < 0.005), by Student's t test for paired data.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Glycogen synthesis (μmol/h per mg of DNA)</th>
<th>Glycogenolysis (μmol/h per mg of DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.13 ± 0.06</td>
<td>28.5 ± 4.0</td>
</tr>
<tr>
<td>PMA (0.1 μM)</td>
<td>n.d.</td>
<td>17.3 ± 2.5</td>
</tr>
<tr>
<td>PMA (1.0 μM)</td>
<td>0.27 ± 0.08*</td>
<td>14.5 ± 2.1</td>
</tr>
<tr>
<td>Insulin (0.1 μM)</td>
<td>0.85 ± 0.08</td>
<td>4.3 ± 1.5</td>
</tr>
<tr>
<td>Insulin (0.1 μM) + PMA (1.0 μM)</td>
<td>0.38 ± 0.06</td>
<td>6.2 ± 0.9</td>
</tr>
</tbody>
</table>

Table 3  Additive actions of glucose and PMA on glucose-dependent metabolic rates

Cells were cultured for 46 h and the experiments were performed as detailed in the legends to Figure 1 and Table 2. The experiments were started by the single or simultaneous addition of 1 μM PMA and 5 mM glucose (= elevation of glucose conc. from 5 mM [M199 medium] to 10 mM final conc.). Glycolysis and glycogen synthesis were measured for 2 h, and glycogenolysis was measured for 4 h, and data are expressed as [14C]lactate production, [14C]glucose incorporation into glycogen and glucose production from 14C-labelled glycogen, respectively. Data are means ± S.D. of four different cultures; * significantly different from the corresponding glucose values (P < 0.025), by Student's t test for paired data.

<table>
<thead>
<tr>
<th>Metabolic parameter</th>
<th>Control</th>
<th>Glucose</th>
<th>PMA</th>
<th>Glucose + PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolysis</td>
<td>0.52 ± 0.1</td>
<td>3.0 ± 0.6</td>
<td>3.7 ± 0.5</td>
<td>8.5 ± 0.2</td>
</tr>
<tr>
<td>Glycogen synthesis</td>
<td>0.33 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>1.5 ± 0.3*</td>
</tr>
<tr>
<td>Glycogen degradation</td>
<td>19.52 ± 2.6</td>
<td>12.2 ± 1.7</td>
<td>14.0 ± 2.1</td>
<td>9.9 ± 1.3*</td>
</tr>
</tbody>
</table>

Table 4  Increase in AIB transport and ODC activity and inhibition of the glucagon-dependent increase in PEP carboxykinase activity by insulin and PMA

Cells were cultured for 46 h. AIB transport was measured after cells had been exposed to insulin for 1.5 h and induction of ODC was monitored for 3 h; insulin and PMA concentrations were 0.1 and 1 μM, respectively. PEP carboxykinase was induced with 1 nM glucagon for 4 h in the absence (= control) or presence of 10 nM insulin or 1 μM PMA. When both agents were supplied together, PMA was added 2 min before insulin. Data are means ± S.D. from four different cultures; * control represents the difference between the un-induced basal activity and the activity after glucagon treatment (absolute values: 0.28 ± 0.06 versus 1.1 ± 0.2 units/mg of DNA).

<table>
<thead>
<tr>
<th>Agonist</th>
<th>AIB uptake (μmol/h per mg of DNA)</th>
<th>ODC activity (m-unit/mg DNA)</th>
<th>PEP carboxykinase activity (% of glucagon-inducible activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.26 ± 0.04</td>
<td>0.07 ± 0.03</td>
<td>100*</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.66 ± 0.04</td>
<td>0.16 ± 0.03</td>
<td>27.5 ± 8</td>
</tr>
<tr>
<td>PMA</td>
<td>0.42 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>55.2 ± 12</td>
</tr>
<tr>
<td>Insulin + PMA</td>
<td>0.73 ± 0.1</td>
<td>0.19 ± 0.03</td>
<td>20.2 ± 6</td>
</tr>
</tbody>
</table>

Additive actions of PMA and glucose on glucose metabolism

Glycolysis and glycogen metabolism are also subject to regulation by the glucose concentration. It was therefore decided to test for additive or antagonistic effects when cells were simultaneously exposed to PMA and a rise in the glucose concentration (5–10 mM; Table 3). Glycolysis was stimulated 6-fold by glucose and 7-fold by PMA; combined addition resulted in a little more than additive effect (16-fold increase). PMA was not capable of antagonizing the glucose-dependent activation of glycogen synthesis; on the contrary, additive effects were observed. Glycogen degradation, which was inhibited by glucose and PMA by 38 and 28%, respectively, was further decreased to 50% by their combined addition.

Influence of PMA on long-term insulin-sensitive parameters

To answer the question whether the mode of PMA–insulin interaction differed between acute regulation of metabolism and regulatory processes involving gene transcription, three long-term insulin-sensitive parameters were assayed for PMA responsiveness: activation of amino acid transport ([33]; system A), induction of ODC ([16,34]) and inhibition of glucagon-dependent induction of PEP carboxykinase ([22,35]). Compared with insulin, PMA elicited lower maximal effects on all three activities (Table 4). AIB uptake and ODC activity were both increased about 2.5-fold by insulin and 1.6–1.8-fold by PMA. Suppression of glucagon-induced PEP carboxykinase activity
was 70\% by insulin, but only 45\% by PMA when the action of 1 nM glucagon was to be antagonized. In neither case could an additive or synergistic effect be demonstrated after addition of both agonists. The PEP carboxykinase experiments lent themselves to a more detailed study of possible inhibitory or additive effects of PMA on insulin action, since insulin can counteract low glucagon doses (0.01–0.1 nM) at equally low doses [22]. Even at low (0.1–1 nM) insulin concentrations, PMA exerted neither inhibitory nor additive effects when supplied 5, 10 or 30 min before insulin (results not shown).

### Activation of the insulin-receptor tyrosine kinase

It was tested whether the kinase activity was modulated when cultured hepatocytes had been incubated with PMA, insulin or PMA + insulin. FAO hepatoma cells were investigated also, since the widely accepted view that phorbol esters inhibit insulin-receptor autoprophosphorylation in the liver stems from results obtained with this cell line [9]. PMA did not change basal tyrosine kinase activity in adult hepatocytes (Table 5). Exposure of the cells to insulin led to a 13-fold increase in the kinase activity and this effect of insulin was not altered by prior incubation of the cells with 1 μM PMA for 2, 5 or 30 min. Exposure of FAO cells to insulin resulted in an 8-fold increase of the receptor tyrosine kinase activity. PMA did not alter basal kinase activity in FAO cells, and it could also not decrease the effect of insulin. This result is in contrast with the finding by Takayama et al. [9] that PMA decreased the insulin-dependent autophosphorylation of the receptor in this cell line. When we measured the induction of TAT, the parameter chosen by Takayama et al. [9] for the biological action of insulin, we could exactly reproduce their data: PMA inhibited the 2.7-fold increase in the insulin-provoked induction by 65\% (Table 5). When the FAO cells were cultured in Ham’s F-12/NCTC 135 medium (1:1), cell attachment and growth improved. Under these culture conditions there was no change in the results obtained for the receptor kinase. However, antagonism by PMA or insulin-elicted TAT induction was less pronounced, and in some experiments was not observed at all (results not shown).

### Phosphorylation of the insulin receptor in the intact cell

When adult hepatocytes were incubated with insulin for 20 min after pre-loading the cells with [32P]Pi, the autoradiogram revealed an increase in the [32P]P labelling of the receptor β-subunit (M, 99000). This value agrees with the M, of 101000 observed by Burant et al. [36] for the rat liver receptor β-subunit. Densitometric scanning showed that phosphorylation of the β-subunit was stimulated 2-fold by insulin. PMA did not alter [32P]P incorporation into the receptor in the absence of insulin, nor did it alter the insulin effect. This is consistent with the data obtained for activation of tyrosine kinase (Table 5).

### Retention of insulin action in long-term PMA-treated cultures: use of protein kinase C inhibitors

The potential role of protein kinase C in the propagation of hormone signals is still commonly evaluated by the use of enzyme inhibitors and by down-regulation of the enzyme protein through long-term pretreatment of cells with phorbol esters. Therefore hepatocyte cultures were incubated with 16 μM PMA for 15 h (31–46 h), and various parameters (glycolysis, glycogen synthesis, glycogenolysis, AIB transport, ODC induction) were tested for their acute responsiveness towards 1 μM PMA and 0.1 μM insulin. The results were unequivocal: PMA effects were drastically decreased (< 20\% for glycolysis) or completely abolished (other parameters), whereas the responsiveness of the cell towards insulin persisted (results not shown). The half-maximal effective insulin concentration (ED50 = 0.5 nM) deduced from dose/response curves of glycolysis and AIB transport was not altered by long-term treatment with PMA. Since it has been reported that specific protein kinase C isozymes are retained after chronic phorbol ester treatment of cultured myocytes [37], the sensitivity of PMA- and insulin-regulated processes towards protein kinase C inhibitors was investigated next. Dose/response curves were obtained for five structurally different protein kinase C inhibitors. Basal and insulin-stimulated metabolic activities (glycolysis, glycogen synthesis, glycogenolysis, AIB transport) were measured under the conditions described in the Experimental section. Inhibitors were used at the following concentrations: 1–10 μM staurosporine, 1–100 μM polymyxin B, 10–100 μM sphingosine, 1–10 μg/ml melittin and 10–100 μM Acridine Orange. All inhibitors exerted pronounced effects on cell metabolism in the absence of insulin (results not shown).

Glycogen synthesis was stimulated 3-fold by staurosporine and inhibited by polymyxin B (50\%), melittin (50\%) and Acridine Orange (100\%). Glycogenolysis was increased 2-fold by polymyxin B and melittin, and was decreased by staurosporine (60\%). Polymyxin B, sphingosine and staurosporine increased glycolysis up to 10-fold. AIB transport was increased by 50\% by sphingosine and melittin. The effects of the inhibitors on insulin actions were contradictory. Staurosporine, polymyxin B, sphingosine and melittin completely blocked the stimulation of glycogen synthesis by insulin. Whereas these three inhibitors also relieved the insulin-dependent inhibition of glycogenolysis, staurosporine did not modulate the effect of insulin on this parameter. None of the inhibitors affected the activation of AIB uptake by insulin. Glycolysis was stimulated by insulin and protein kinase C inhibitors in an additive manner. Thus the data obtained allow no further conclusion than that protein kinase C inhibitors severely derange intermediary metabolism in cultured hepatocytes and that they should be used cautiously in whole-cell systems.
PMA effects in cells cultured for 3 h

It could be observed that sensitivity of intermediary metabolism towards PMA gradually evolved during the 48 h culture period and that it was not a property of the freshly isolated cell. Therefore the influence of PMA on glycolysis, glycogen synthesis and the level of Fru(2,6)P₂ was investigated in cells that had just adhered to the culture dish (3 h) (results not shown). PMA (1 μM) increased glycolysis between 1.5- and 2-fold. Insulin was again slightly more potent; stimulation was 2.5-3-fold and was not decreased by PMA. The Fru(2,6)-P₂ content was elevated 2-fold by PMA. Activation of glycogen synthesis by insulin was nearly completely abolished by PMA, which itself did not elevate the basal rate in these cells. From this we conclude that the probability of demonstrating non-liver-specific PMA effects in 48 h-cultured hepatocytes is low.

DISCUSSION

The data presented in this study show that the phorbol ester PMA elicits insulin-mimetic short- and long-term effects in primary hepatocyte culture and that it does not generally impair the action of insulin in this ‘in vitro’ liver system.

Insulin-mimetic effects of PMA on intermediary enzymes and metabolism

In accord with previously published data on adipose and muscle tissue, liver metabolism was subject to regulation by the protein kinase C activator PMA. None of the 10 metabolic parameters tested was refractory to PMA. Maximal effects of the phorbol ester were generally equal to or slightly lower than those provoked by insulin. Activation of glycogen synthesis constituted the only exception: a large discrepancy between the stimulatory potencies of insulin and PMA could be demonstrated (Table 2). It is well documented that exposure of isolated hepatocytes in suspension to PMA results in inactivation of glycogen synthase [38-40]. The small (2-fold) but unequivocal activation of glycogen synthesis by PMA described here hints at the possibility that under culture conditions a much higher stimulatory (= insulin-mimetic) PMA effect was counteracted by the inhibitory influence of PMA on glycogen synthase. Such a PMA-provoked inactivation of basal synthase would readily explain the inability of insulin to stimulate glycogen synthesis fully in the presence of PMA (Table 2), an effect that has also been reported for hepatoma cells [9,10]. Activation of synthase brought about by an increase in the glucose concentration from 5 to 10 mM could not be abolished by PMA (Table 3).

The effect of PMA on hepatic glycogenolysis and glycogen phosphorylase has been a matter of debate. Authors either reported no influence or described a stimulatory action [40], and references therein). In our culture system PMA decreased glycogenolysis (Table 2), in line with its insulin-mimetic properties on the other metabolic parameters. This decrease in the glucose output is accompanied in vitro by an increase in hepatic glucose degradation. Thus, mimicking the biological action of insulin, PMA elevated glycolysis and the Fru(2,6)P₂ level (Fig. 1). The only other hepatic model tested, chick-embryo hepatocyte culture [41], does not respond to PMA with an increase in the glycolytic rate, whereas in a number of different non-hepatic cell systems (chick-embryo fibroblasts, lymphocytes and colon adenocarcinomas [42-44]), phorbol esters have been reported to elicit the responses listed above.

With respect to the acute PMA effects on carbohydrate metabolism, the adult cultured hepatocyte system resembles the mouse muscle model investigated by MacLennan et al. [45]. In this system, however, PMA effects on glycolysis and glycogen synthesis can be at least partially explained by the increase in glucose transport; in the cultured hepatocytes, however, glucose uptake was not stimulated by PMA.

The interaction of insulin and phorbol ester has been studied extensively. Depending on the cell type, tissue and metabolic parameter tested, PMA can or cannot inhibit insulin-stimulated metabolic processes [4,6,7,45]. It is shown here that PMA did not generally decrease the metabolic potency of insulin. Thus the metabolic studies already indicated that in adult cultured hepatocytes PMA might not exert an insulin-antagonistic effect at the level of the receptor.

Phorbol-ester-dependent regulation of the insulin-receptor kinase

Autophosphorylation by insulin of the insulin-receptor kinase at tyrosine residues activates tyrosine kinase activity. In contrast, serine/threonine phosphorylation has been shown to decrease autophosphorylation and receptor kinase activity in a variety of tissues and cell lines, including the FAO hepatoma line [8,9,11,12,46]. Our data, however, do not support this theory in cultured adult hepatocytes, a well-differentiated insulin-sensitive liver system. PMA modified neither basal nor insulin-stimulated autophosphorylation and tyrosine kinase activity of the receptor when intact cells were exposed to the phorbol ester (Table 5, Fig. 3). Parallel evaluation of the insulin-stimulated kinase activity with and without PMA of the FAO hepatoma cell line under identical experimental and analytical conditions showed that, in contrast with [9], in our hands PMA did not antagonize the insulin signal at the receptor level in this cell line. At present we have no explanation for this discrepancy. Inhibition of the insulin-dependent induction of tyrosine aminotransferase by PMA was, however, exactly as reported [9].

Our data, however, agree with reports from several other groups: a lack of PMA-elicted inhibition of the insulin signal at the level of the receptor has been shown for skeletal muscle [7], NIH-3T3 cells over-expressing the human receptor [47], and Hep G2 and IM-9 cells [48].

Converging insulin and phorbol ester signal pathways

The finding that PMA exerted insulin-mimetic actions in cultured adult hepatocytes (the present study) and in adult hepatocytes in suspension [17,18], together with the failure to detect inhibitory effects of PMA on the insulin-receptor kinase, lends itself to the speculation that both agonists share a common or similar signal-transfer step, e.g. protein kinase C. A recent report on insulin's ability to increase the level of diacylglycerol and protein kinase C activity in isolated hepatocytes supports this hypothesis [49]. Down-regulation of protein kinase C by long-term PMA pretreatment and use of protein kinase C inhibitors results in strong decreases in insulin effects in some cells [2,4,50,51], but not in others ([10,13,14]; the present study). Many uncertainties, however, remain concerning the extent of kinase deprivation and specificity of inhibitors [7,37]. Therefore data obtained under these experimental protocols should be interpreted with caution. Our finding that the protein phosphatase inhibitor okadaic acid completely blocked the activation of pyruvate kinase and PFK 2 by insulin as well as that evoked by PMA points to the involvement of a phosphatase as a final common step in the two signal chains.

In conclusion, we have shown here that the phorbol ester PMA did not provoke an insulin-resistance-like state in cultured adult hepatocytes. Therefore this liver model seems to be suitable
for investigating converging insulin and phorbol ester signal pathways.

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