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STIMULATION BY A TUMOR-PROMOTING PHORBOL ESTER OF ACETYL-CoA CARBOXYLASE ACTIVITY IN ISOLATED RAT HEPATOCYTES

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SUMMARY: Acetyl-CoA carboxylase (EC 6.4.1.2) in hepatocytes from meal-fed rats was activated by phorbol myristate acetate (PMA) in a time- and concentration-dependent fashion. This activation can account for the PMA-induced stimulation of *de novo* fatty acid synthesis. Purified rat-liver acetyl-CoA carboxylase was found to be phosphorylated and activated by protein kinase C, thus providing a possible mechanism for the metabolic action of PMA in intact hepatocytes.

In the course of studies on short-term metabolic effects of growth factors, we recently observed that phorbol esters such as phorbol 12-myristate 13-acetate (PMA), mostly known as potent tumor promoters, stimulate fatty acid synthesis in freshly isolated rat-liver cells (1). The site of action of this metabolic effect of PMA is unknown. Moreover, it seems important to know whether protein kinase C (Ca^{2+}- and phospholipid-dependent protein kinase) is involved in this PMA action since most, if not all, biological effects of phorbol esters are mediated by stimulation of protein kinase C activity (reviewed in 2).

The reaction catalysed by acetyl-CoA carboxylase (ACC) is the first committed step in *de novo* fatty acid synthesis, and it is generally considered to be the prime target for acute regulation of fatty acid synthesis (3-6). Yet, depending on the precursor used, regulation may occur at several other steps during the conversion of carbon precursors into fatty acids (7). Therefore, our first aim was to establish a possible effect of PMA on ACC activity in intact hepatocytes. Next we sought to obtain information on the mechanism underlying this PMA effect. ACC is subject to allosteric regulation by citrate and long-chain acyl-CoA (reviewed in 6,8) as well as to covalent phosphorylation/dephosphorylation (reviewed in 7-9). PMA could exert its metabolic effect via either mechanism. However, it is widely held that agonist-induced phosphorylation of ACC corres-

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Abbreviations: ACC, acetyl-CoA carboxylase; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PDD, 4α-phorbol 12,13-didecanoate; PMA, 4β-phorbol 12β-myristate 13α-acetate.

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ponds with inactivation of the enzyme (10-13). In other words, if PMA activates ACC by covalent modification, protein kinase C could only be involved in some indirect manner.

In the present report we show that ACC activity, as measured in digitonin-permeabilized hepatocytes, is indeed increased by pretreatment of the cells with PMA. In our opinion, this time- and concentration-dependent effect of PMA, which was also observed in conditions of increased substrate supply, is sufficient to explain the PMA-induced stimulation of fatty acid synthesis in rat hepatocytes. Furthermore, we demonstrate a reversible phosphorylation and concomitant activation of highly purified rat-liver ACC by protein kinase C, thus providing evidence: (i) that the effect of phosphorylation on ACC activity, if any (cf. 14, 15), is not necessarily an inhibitory one (cf. 11-13); and (ii) that activation by PMA of protein kinase C in hepatocytes may be directly responsible for the PMA-provoked increase in ACC activity.

MATERIALS AND METHODS

Materials: Alkaline phosphatase (calf intestine, grade II) and BSA were from Boehringer; PMA, PDD and other biochemicals were from Sigma. Stock solutions of phorbol esters were prepared in dimethylsulfoxide. BSA was defatted and dialysed before use. ^3H_2O (5 Ci/ml), $NaH^{14}CO_3$ (57 Ci/mol) and [γ-^{32}P]ATP (3 x 10^6 Ci/mol) were supplied by Amersham International, and [1-^{14}C]acetyl-CoA (54.3 Ci/mol) by New England Nuclear.

Hepatocyte incubations: Freshly isolated hepatocytes (4) from meal-fed, male Wistar rats (200-250 g) were used in suspension. The basic incubation medium consisted of Krebs-Henseleit bicarbonate buffer (pH 7.4) with 10 mM glucose, 2.5 mM $CaCl_2$ and 1%(w/v) BSA. The final dimethylsulfoxide concentration was 0.1%(v/v) in all incubations. Cell concentration, 5-6 mg protein/ml; incubation temperature, 37^{0}C. Protein was determined by the Lowry method.

Fatty acid synthesis: Incorporation of ^3H_2O (0.5 mCi/ml) into saponifiable fatty acids (4) was used to monitor the rate of *de novo* fatty acid synthesis. This measure offers a meaningful index of lipogenesis (16), whereas incorporation of radioactive acetate or glucose is prone to label dilution.

Cellular ACC activity: Because the conventional $H^{14}CO_3^-$-fixation assay yields erroneous results with crude hepatocyte homogenates (17,18), ACC activity in hepatocytes was assessed as the incorporation by digitonin-permeabilized hepatocytes of [1-^{14}C]acetyl-CoA into long-chain fatty acids (19). Full details of this novel assay will be published elsewhere (C. Bijleveld *et al.*, in preparation).

Purification of rat-liver ACC: ACC was isolated from rats, starved 72 h and then refed a fat-free, high-sucrose diet for 48 h. Briefly, the $(NH_4)_2SO_4$-fraction (0-30%) of Nakanishi and Numa (20) was subjected to Sepharose-2B chromatography in the presence of citrate, followed by avidin-Sepharose affinity chromatography (13). Specific activity of ACC, 2.9 U/mg at 30^{0}C (units expressed as µmoles $H^{14}CO_3^-$ fixed into malonyl-CoA per minute) in the presence of 10 mM citrate and 12 mM Mg-acetate.

Phosphorylation of purified rat-liver ACC: Phosphorylation was measured after incubations carried out for 30 min at 30^{0}C in a medium containing 100 mM Hepes (pH 7.4), 2 mM Tris-HCl, 0.7 mM EDTA, 10 mM Mg-acetate, 15 µg/ml ACC, 0.4 mM EGTA, 1.2 mM $CaCl_2$, 5%(w/v) glycerol, 5 mM β-mercaptoethanol, 50 µg/ml phosphatidylserine, 3 U/ml protein kinase C (21), and 50 µM ATP or 50 µM [γ-^{32}P]ATP (480 Ci/mmol). As indicated (Fig. 4), some incubations with ACC and protein kinase C were continued for a further 10 min in the additional presence of alkaline phosphatase (17.5 U/ml).

Phosphate (^{32}P) incorporation into ACC was stopped using the filter-disk method, and was measured by liquid-scintillation counting.

To determine its effect on ACC activity, phosphorylation was quenched by a 10-fold dilution of the reaction mixture with 100 mM Hepes (pH 7.4)/ 2 mM EDTA/ 5 mM β-mercaptoethanol. This stops phosphorylation by chelating Mg^{2+}, and the resulting solution was then used to determine ACC activity. Before assay, ACC was preincubated at 30^0C in a medium comprising 100 mM Hepes (pH 7.4), 2 mM citrate, 5 mM β-mercaptoethanol and 0.1%(w/v) BSA. After 30 min preincubation, assay mixture was added to give final concentrations of 100 mM Hepes (pH 7.4), 2 mM citrate, 4 mM Mg-acetate, 4 mM ATP, 0.1%(w/v) BSA, 1 µg/ml ACC, 0.1 mM acetyl-CoA and 10 mM $NaH^{14}CO_3$ (20 Ci/mol). After 6 min at 30^0C assays were stopped and acid-stable radioactivity was counted as in (18).

RESULTS AND DISCUSSION

As reported previously (1), PMA enhanced the rate of fatty acid synthesis in isolated liver cells from meal-fed rats in a concentration-dependent manner (Fig. 1). Except perhaps for the highest PMA concentration employed (cf. Fig. 3), cellular ACC activity and rate of fatty acid synthesis were covariant (3-5), indicating that ACC set the pace for this anabolic pathway. Unlike PMA, the biologically inactive phorbol ester PDD had no effect (Fig. 1), pointing to the importance of protein kinase C activation (22) in this respect. The *in situ* activity of fatty acid synthase, the second enzyme of *de novo* fatty acid synthesis, was not significantly affected by PMA-treatment of the cells (not shown).

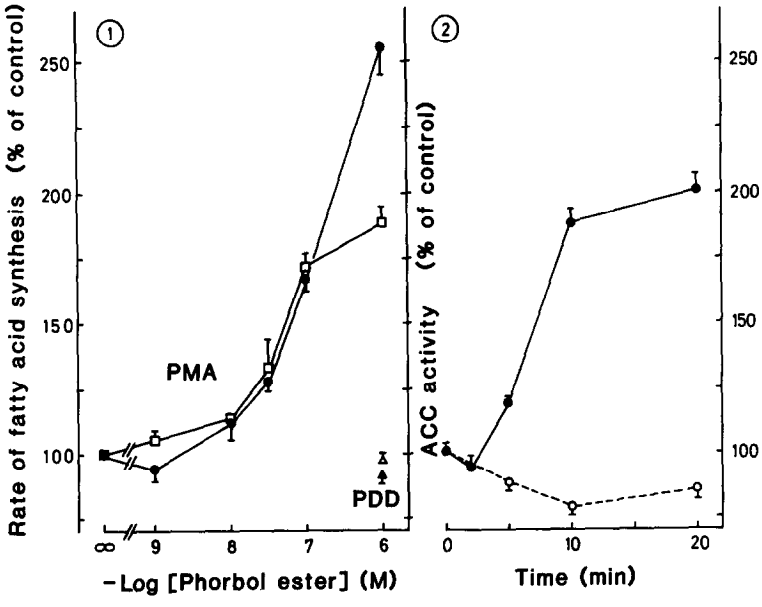

Fig. 1. Influence of phorbol ester on rate of fatty acid synthesis and on acetyl-CoA carboxylase activity in isolated rat hepatocytes. Cells were incubated for 20 min (ACC activity, ●—● and ▲) or 40 min (fatty acid synthesis, □—□ and △) in the basic medium supplemented with phorbol ester (circles and squares, PMA; triangles, PDD) and ^3H_2O (fatty acid synthesis). Data are taken from a representative experiment (means ± S.D. of triplicate incubations, expressed as % of control without phorbol ester).

Fig. 2. Time-course of PMA-induced activation of cellular acetyl-CoA carboxylase. Isolated rat hepatocytes were incubated in the basic medium with (●—●) or without (○--○) 10^{-7} M PMA. Values shown are means ± S.D. for duplicate assays of triplicate incubations from 2 hepatocyte preparations (expressed as % of zero time controls).

Isolated hepatocytes had to be exposed to PMA for at least 10 min to achieve optimal activation of ACC (Fig. 2). In comparison to the fast activation of protein kinase C by PMA (22) this response is strikingly slow. This lack of synchronism could either reflect the time lapse required for ACC to encounter active, i.e. membrane-bound, protein kinase C, or the need for protein kinase C to be converted into a soluble active form (23) before being able to phosphorylate and activate ACC. Such an interpretation tacitly assumes that, contrary to the common view (10-15), covalent activation of ACC does not necessarily mean dephosphorylation of the enzyme. On the other hand, PMA may exert its stimulatory effect equally well by promoting ACC dephosphorylation. Two options in the latter respect are: (i) PMA-induced inhibition of adenylate cyclase (24) resulting in an attenuation of cyclic-AMP-dependent protein kinase activity; and (ii) stimulation, by phosphorylation (cf. 25), of the appropriate protein phosphatases (26-28). Either mechanism would cause a lower phosphorylation level and, thereby, a de-inhibition of ACC *in situ*. Finally, PMA might remotely control ACC activity by affecting an enzymatic step located earlier in the lipogenic sequence. Increased substrate flux would lead to elevated cytosolic levels of the acetyl-CoA precursor citrate (29) and, as a consequence, to allosteric activation (6) of ACC.

To lower the number of possible explanations, we performed incubations in the presence of lactate and 2-chloropropionate. Exogenous lactate is an excellent lipogenic precursor, and 2-chloropropionate promotes the availability of acetyl-CoA by specific activation of pyruvate dehydrogenase (30). Addition of these two compounds serves to achieve conditions of optimal substrate provision and of high cytosolic citrate concentrations (cf. 29), and to by-pass possible effects of PMA on the supply of endogenous carbon precursors. The presence of lactate and 2-chloropropionate indeed increased basal rates of fatty acid synthesis and enhanced cellular ACC activity, presumably due to allosteric activation (Fig. 3). However, PMA was still able to cause an additional, though smaller, stimulation of fatty acid synthesis and a pronounced stimulation of ACC activity (Fig. 3). It follows then that a covalent modification of ACC is likely to be involved in this PMA effect.

The precise nature of the PMA-induced modification of ACC has yet to be established. Various protein kinases are able to phosphorylate ACC at multiple sites. Some of these sites affect catalytic activity (13,31) whereas others remain silent (14,14,32). *In vitro* phosphorylation of rat-liver ACC by cyclic-AMP-dependent protein kinase invariably led to a loss of activity (12,13). In addition, none out of several cyclic-AMP-independent protein kinases was found to stimulate ACC activity *in vitro* (14,32). This suggests that PMA-induced modification of cellular ACC represents dephosphorylation rather than phosphorylation (or both), but a definite answer has to await future studies with PMA-treated, ^{32}P-labelled hepatocytes.

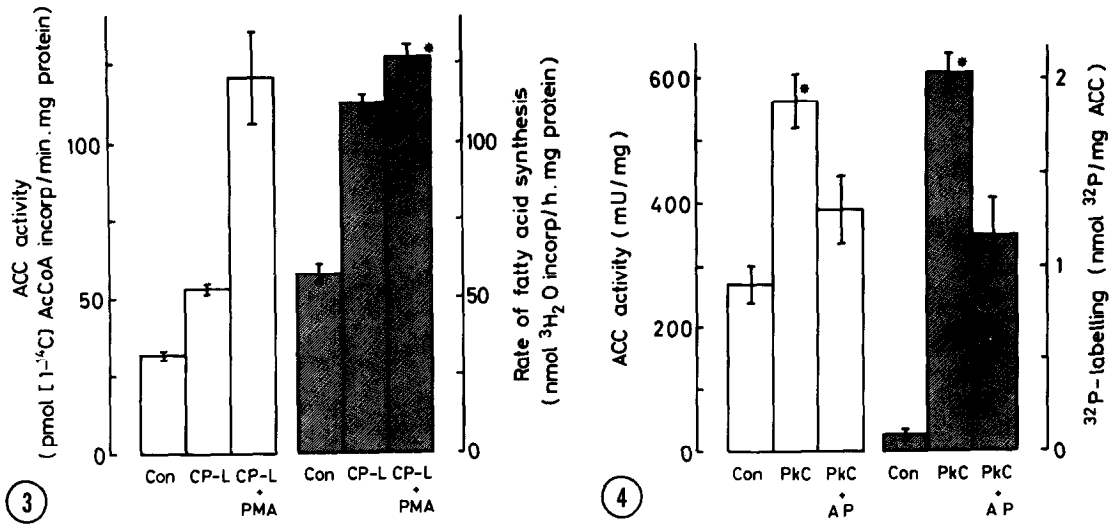

Fig. 3. Effect of PMA on cellular acetyl-CoA carboxylase activity and on fatty acid synthesis in the presence of excess lipogenic carbon precursor. ACC activity, open bars; rate of fatty acid synthesis, hatched bars. Con, controls (no further additions); CP-L, + 10^{-3} M 2-chloropropionate + 10^{-2} M L-lactate; PMA, + 10^{-6} M PMA. Data shown are means ± S.D. for triplicate incubations from 3 experiments. * indicates $P< 0.01$ as compared to incubations with chloropropionate + lactate (paired testing). Further experimental details, see Fig. 1.

Fig. 4. Activity and [^{32}P]phosphate-labelling of purified rat-liver acetyl-CoA carboxylase. Purified ACC was incubated for 30 min: Con, controls without protein kinase C or alkaline phosphatase, PkC, incubations in the presence of protein kinase C; or for 40 min: PkC + AP, incubations in the presence of protein kinase C and, after t = 30', of alkaline phosphatase. ACC activity, open bars; ^{32}P-incorporation from [γ-^{32}P]ATP, hatched bars. Experimental details, see Methods section (PkC + AP values: corrected for phosphatase-induced changes in control values in the absence of protein kinase C). Data represent means ± S.D. for 5 independent observations.
* indicates $P< 0.001$ as compared to controls (Con).

In any case, initial studies with purified rat-liver ACC (Fig. 4) demonstrate that phosphorylation of ACC by protein kinase C could explain ACC-activation by PMA in intact hepatocytes. Fig. 4 shows: (i) that protein kinase C is able to incorporate ^{32}P into purified ACC, (ii) that this phosphorylation is accompanied by an increase in catalytic activity, and (iii) that both ^{32}P-incorporation and activation are partially reversed by treatment with alkaline phosphatase. To our knowledge, Fig. 4 provides for the first time evidence that *in vitro* phosphorylation of rat-liver ACC is able to activate this enzyme. Hence, the functional consequences of ACC phosphorylation, if any (14,15,32), depend on the choice of protein kinase and, therefore, on the phosphorylation sites actually involved.

In conclusion, we feel that activation of ACC by covalent modification, though not necessarily excluding an additional contribution of allosteric mechanisms, can account for PMA-induced stimulation of fatty acid synthesis in intact rat hepatocytes. In principle, phosphorylation of ACC by protein kinase C may directly mediate this short-term metabolic effect of PMA.

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