Vol. 129, No. 3, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 28, 1985 Pages 721-726

ACUTE EFFECTS OF TUMOR-PROMOTING PHORBOL ESTERS ON HEPATIC INTERMEDIARY METABOLISM

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Received April 18, 1985

SUMMARY: In hepatocytes isolated from meal-fed rats, phorbol 12-myristate 13-acetate as well as phorbol 12,13-didecanoate stimulated *de novo* fatty acid synthesis in a dose-dependent manner. Moreover, phorbol 12-myristate 13-acetate inhibited ketogenesis from exogenous oleate, but slightly enhanced oleate esterification. The stimulation of esterification was more pronounced with endogenously synthesized fatty acids. In hepatocytes from 24h-starved rats a moderate stimulation of gluconeogenesis and ureogenesis was observed with glutamine as substrate. It is concluded that tumor-promoting phorbol esters mimick the short-term effects of insulin on hepatic fatty acid metabolism.

Despite a vast number of publications on metabolic effects of insulin, the precise mechanism of action of this hormone largely remains an enigma (cf. ref. 1). However, it has been firmly established that insulin conveys its message to target cells in a cyclic AMP-independent fashion. This may indicate involvement of protein kinase C and/or Ca^{2+}-calmodulin-dependent protein kinase in insulin signal transduction.

Data from the literature do not lend much support to the latter possibility. Ca^{2+}-mobilizing agonists affect hepatic metabolism differently from insulin (2,3), and no clear-cut effects of insulin on cytosolic Ca^{2+} levels have been reported as yet. On the other hand, a certain similarity between protein kinase C and long-term effects of insulin is apparent: (a) protein kinase C has been claimed to play a crucial role in the stimulation of cell proliferation by various compounds (4), and (b) insulin is well-known as an obligatory factor in the maintenance and growth of cell cultures in serum-free media. This led us to investigate the possibility of acute insulin-mimicking effects by protein kinase C.

Tumor-promoting phorbol esters like phorbol 12-myristate 13-acetate (PMA) were used to manipulate protein kinase C activity *in situ*. Not only do these esters belong to the most potent co-carcinogens currently known (5), but they also bind to cell surface receptors co-purifying with pro-

Abbreviation: PMA, 4β-phorbol, 12β-myristate, 13α-acetate (also known as 12-*O*-tetradecanoyl-phorbol-13-acetate, TPA).

0006-291X/85 $1.50

tein kinase C (6) and they substitute for diacylglycerol in the activation of this enzyme (7). As most, if not all, biological effects of phorbol esters appear to be mediated through this activation (4), these esters are convenient probes of the effects of protein kinase C.

The present communication describes the influence of tumor-promoting phorbol esters, especially of PMA, on intermediary metabolism in freshly isolated rat liver cells. Part of this work has been presented earlier in abstract form (8). Acute changes in glycogen metabolism caused by PMA treatment have not been studied as those were recently reported by several research groups (9-12). These reports, though partly conflicting, point to a depletion of hepatic glycogen in response to PMA.

METHODS

Freshly isolated hepatocytes (13) from male Wistar rats (200-250 g) were used in suspension. The basic reaction mixture consisted of Krebs-Ringer bicarbonate buffer (pH 7.4) with 2.5 mM $CaCl_2$, 10 mM glucose and 1%(w/v) defatted and dialysed bovine serum albumin; cell concentration, 2-5 mg protein/ml. Glucose was not included in the medium when hepatocytes from 24h-starved rats were used. Other incubation procedures and measurement of *de novo* fatty acid synthesis were as in (13). Incubations were performed either in triplicate (Fig. 1; Table 1, part A) or in duplicate. Standard methods were used for extraction and separation of glycerolipids (14) and for enzymatic assays of glucose (15), ketone bodies (16) and urea (17). Stock solutions of phorbol esters (Sigma Chemical Co.) were prepared in dimethylsulfoxide (DMSO) and the final DMSO concentration in the incubations was kept at 0.1%(v/v). No significant influence of DMSO on hepatocyte metabolism was observed at this concentration. Controls had the corresponding DMSO content. Statistical analysis of PMA effects was performed using paired *t*-testing.

RESULTS AND DISCUSSION

The influence of PMA on the utilization of added [1-^{14}C]oleate was studied in liver cells from meal-fed rats. PMA at 10^{-6} M caused a slight stimulation of oleate esterification and a concomitant decrease in the rate of oleate oxidation. The inhibition of oleate oxidation was most marked in terms of ketogenesis (Table 1, part A). As shown in Table 1, part B, the PMA-induced increase in esterification was more pronounced for endogenously synthesized fatty acids. These observations strongly resemble those obtained earlier with insulin using hepatocyte monolayer cultures (18).

In hepatocytes from 24h-starved rats PMA, like insulin (19), did not cause a significant change in the rate of glucose formation from lactate. Moreover, gluconeogenesis as well as ureogenesis from proline were not affected by PMA (Table 1, part C). Similar results (not shown here) were obtained using the substrate combination of lactate (10 mM) + ornithine (2 mM) + NH_4Cl (10 mM) + oleate (0.5 mM).

Only with glutamine as precursor a moderate stimulation by PMA of gluconeogenesis and ureogenesis occurred (Table 1, part C), suggesting that the

Table 1. Effect of PMA on fatty acid metabolism, gluconeogenesis and ureogenesis in isolated rat hepatocytes

	Substrate	Metabolic parameter	Control	+ PMA (% of control)
(A)	Oleate	Esterification	39.4 ± 7.7	113.1 ± 3.9 [a]
		Oxidation	25.2 ± 8.0	72.4 ± 3.6 [a]
		$^{14}CO_2$ production	10.3 ± 2.5	79.6 ± 8.5 [b]
		Acid-soluble products	14.9 ± 5.9	68.5 ± 3.6 [a]
		Ketogenesis	16.5 ± 2.1	59.5 ± 6.5 [a]
(B)	Acetate	Esterification	36.8 ± 2.3	128.7 ± 1.6 [a]
(C)	Lactate	Gluconeogenesis	429.0 ± 22.8	105.9 ± 4.1
		Ureogenesis	71.1 ± 4.8	99.8 ± 6.7
	Proline	Gluconeogenesis	79.2 ± 1.5	106.9 ± 6.3
		Ureogenesis	158.1 ± 3.0	101.9 ± 1.4
	Glutamine	Gluconeogenesis	100.5 ± 0.6	129.0 ± 0.2 [c]
		Ureogenesis	410.1 ± 39.9	118.5 ± 1.7 [d]

(A) and (B): Hepatocytes from meal-fed rats were incubated with (A) 0.5 mM [1-^{14}C]oleate (0.05 Ci/mol) or (B) 10 mM [1-^{14}C]acetate (0.1 Ci/mol) in the absence or presence of 1.0 µM PMA. Incubation time, 30 min (oxidation, ketogenesis) or 60 min (esterification). Esterification: sum of fatty acid incorporated into diacylglycerols, triacylglycerols and phospholipids. Oxidation: sum of $^{14}CO_2$ production and formation of acid-soluble products. Ketogenesis: net formation of ketone bodies (measured enzymatically). Control values are expressed as nmol fatty acid utilized/h per mg protein.
(C): Hepatocytes from 24h-starved rats were incubated with or without 1.6 µM PMA and, as indicated: 10 mM L-lactate (+ 0.5 mM oleate); 5 mM L-proline (+ 2 mM L-ornithine); 5 mM L-glutamine (+ 2 mM L-ornithine). Metabolic rates (nmol product/h per mg protein) were calculated from a linear time-interval (t = 20 - 40 min).
Shown are means ± S.D. from 3 (B, C) or 4 (A) experiments. Versus control: [a] $P < 0.001$; [b] $P < 0.005$; [c] $P < 0.01$; [d] $P < 0.05$.

phorbol ester affects cellular glutamine uptake (20) and/or glutaminase activity (21). Corresponding data on effects of insulin are not available. At any rate, PMA acts differently from vasopressin as this latter hormone was reported to stimulate gluconeogenesis and ureogenesis both from proline and from glutamine (22).

By far the largest effect of PMA was observed with *de novo* fatty acid synthesis in hepatocytes from meal-fed rats. Addition of 10^{-6} M PMA increased the rate of fatty acid synthesis to 140-180% (13 separate cell preparations) of the corresponding control value. Figure 1 shows a typical dose-response curve in this respect. Again, PMA resembles insulin (13,23) in its stimulatory behavior. It remains to be seen whether PMA, like insulin, exerts this effect at the level of acetyl-CoA carboxylase (EC 6.4.1.2).

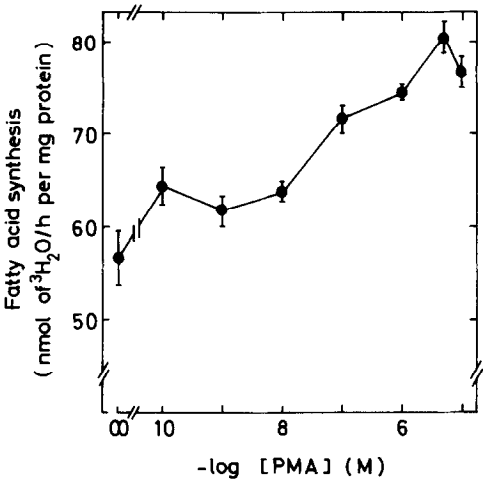

Fig. 1. Influence of PMA concentration on the rate of fatty acid synthesis in isolated hepatocytes from meal-fed rats. Fatty acid synthesis was monitored as incorporation of ^3H_2O (1.5 mCi/ml). Values shown are means ± S.D. for triplicate incubations in a representative experiment.

A possible mode of action of PMA could be activation of this enzyme via the inhibition of adenylate cyclase activity (EC 4.6.1.1) (24).

Using *in vitro* systems, phorbol esters such as PMA have been shown in the past to elicit various biological responses at concentrations of 10^{-6} to 10^{-8} M (25). As demonstrated in Figure 1, rapid metabolic responses of isolated hepatocytes occur at PMA levels in this same concentration range. Similar dose-response curves were reported for actions of PMA in other cell types, e.g., its stimulation of cytidylyltransferase activity in rat myoblasts (26), and of phosphatidate phosphohydrolase activity (27) and incorporation of [^{3}H]glucose into lipids (28) in rat adipocytes. Even though the optimal PMA concentration in Figure 1 lies in the micromolar range, a short preincubation with tumor-promoting esters (PMA, PDD) sufficed in other experiments to observe considerable effects at much lower concentrations (see Table 2). For reasons not yet understood PDD, being slightly weaker than PMA as promoter of mouse skin tumors (25) and as activator of purified protein kinase C (7), surpassed PMA in its stimulation of hepatic lipogenesis. More importantly, Table 2 also demonstrates that the parent compound 4β-phorbol and the biologically inactive ester 4α-PDD both failed to affect the rate of hepatic fatty acid synthesis, thus stressing the specificity of the PMA effects described here.

In conclusion, this report provides, for the first time, evidence that PMA is able to mimick acute effects of insulin related to hepatic fatty acid metabolism, viz., inhibition of ketogenesis (29) and stimulation of fatty acid synthesis (23). As to the underlying mechanism, all available

Table 2. Effect of phorbol esters on the rate of fatty acid synthesis in hepatocytes from meal-fed rats

Addition	Concentration (M)	Rate of fatty acid synthesis (% of control)
PMA	5.10^{-8}	144.3 ± 15.9 [a]
PDD	5.10^{-8}	164.5 ± 2.5 [b]
PMA	10^{-6}	164.8 ± 9.5 [a]
PDD	10^{-6}	181.9 ± 12.3 [a]
4α-PDD	10^{-6}	99.0 ± 2.7
4β-phorbol	10^{-6}	100.1 ± 1.1

Hepatocytes were preincubated for 10 min in the basic reaction medium with additions as indicated. PMA, 4β-phorbol 12β-myristate 13α-acetate; PDD, 4β-phorbol 12,13-didecanoate; 4α-PDD, 4α-phorbol 12,13-didecanoate. Subsequently ^3H_2O (1.5 mCi/ml) was added and its incorporation was followed for 1 h. Values represent the mean ± S.D. of 3 experiments (100%-control: 38.1 ± 1.5 nmol ^3H_2O incorporated/h per mg protein). Versus control: [a] $P < 0.01$; [b] $P < 0.001$.

evidence points to an involvement of protein kinase C in the action of PMA and, possibly, of insulin. Studies to directly prove this point are in progress. The apparent discrepancy between insulin and PMA in connection with glycogen metabolism (cf. ref. 9-12) remains to be clarified.

ACKNOWLEDGEMENTS

This research was supported in part by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO). The authors thank Professor S.G. van den Bergh for his critical reading of the manuscript.

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