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EVIDENCE THAT PHORBOL ESTER-ACTIVATED PATHWAYS ARE NOT DIRECTLY INVOLVED IN THE ACTION OF INSULIN IN RAT ADIPOCYTES

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SUMMARY: The effects of 12-phorbol 13-myristate acetate (PMA) on glucose transport, glucose metabolism and lipolysis in rat adipocytes were examined. Alone, PMA (100 ng/ml) stimulated 2-deoxyglucose transport, glucose oxidation and lipogenesis by an amount corresponding to about 30-50% of the maximal insulin effect. The effect of PMA on the insulin-stimulated processes was additive at all insulin concentrations. Adenosine deaminase-stimulated lipolysis was enhanced by about 50% by PMA (100 ng/ml). Thus, in contrast to insulin, PMA had a lipolytic effect. The antilipolytic effect of insulin was unaffected by the presence of PMA, both ED_{50} and maximal inhibition of insulin were unchanged. In conclusion, it is suggested that phorbol esters may activate pathways other than those activated by insulin in rat adipocytes.

Phorbol esters bind to and activate protein kinase C both in adipocytes and in other cells, and they have been shown to have "insulin-like" effects on glucose transport (1,2), on glucose oxidation (3), and on lipogenesis (2,3,4) in rat adipocytes. This suggests that some of the events initiated by insulin and by phorbol esters may be the same. Protein kinase C is activated by the naturally-occurring diacylglycerol, produced in the plasmamembrane by the hydrolysis of inositol phospholipids by phospholipase C (5). A possible role of protein kinase C in insulin action is also suggested by the observation that insulin stimulates rat adipose tissue phospholipase C (6,7) and the synthesis of diacylglycerol and phospholipids (7,8,9). Although PMA activates protein kinase C, long-term treatment of adipocytes with PMA renders these cells deficient in protein kinase C (10). However, these studies provide only indirect evidence for the involvement of protein kinase C, and other

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studies have shown dissimilarities between the action of phorbol esters and that of insulin. Treatment of adipocytes by phorbol ester, but not by insulin induces translocation of protein kinase C from the soluble to the particulate fraction of the cells (11) and phosphorylation of the glucose transporter (12). In addition, protein kinase C has been found to activate adipocyte adenylate cyclase (13), which suggests that protein kinase C and insulin may have different effects on lipolysis. In the light of these observations, we have investigated the effects of phorbol ester on lipolysis and on glucose metabolism in rat adipocytes.

MATERIALS

12-phorbol 13-myristate acetate (PMA) and adenosine deaminase (ADA) were supplied by Sigma Chemicals (USA). 2-Deoxy-D-[1-^{14}C] glucose (59 mCi/mmol) and [U-^{14}C]glucose (270 mCi/mmol) were obtained from Amersham (UK). All other reagents were obtained as described in references (14) and (15).

METHODS

Adipocytes were prepared from male Wistar rats as described in reference (15) and incubated in buffer containing 10 mM Hepes, 135 mM NaCl, 4.8 mM KCl, 1.7 mM $MgSo_4$, 2.5 mM $CaCl_2$, 0.2 mM NaH_2PO_4, 1 mM Na_2HPO_4, 0.5 mM glucose and 5% human serum albumin (pH 7.4). Insulin binding, lipogenesis, glucose oxidation and lipolysis were measured essentially as described in reference (14). In lipogenesis and glucose oxidation studies, adipocytes were pre-incubated for 30 min. in the presence of insulin and PMA in combination, as described in the legends to Figures 1-3; thereafter, [U-^{14}C] glucose was added and the incubation was continued for another 60 min. In the lipolysis studies, the adipocytes were pre-incubated with and without PMA for 15 min and then for 15 min with insulin. Subsequently, lipolysis was stimulated with 0.5 U/ml adenosine deaminase (ADA) for 90 min.

The uptake of 2-Deoxy-D-[1-^{14}C] glucose was measured as described in reference (14), but with the following changes: 40 µl aliquots of adipocytes (1.6 x 10^5 cells) were pre-incubated for 30 min with insulin in the presence or absence of PMA. Then 0.1 µCi 2-Deoxy-D-[1-^{14}C] glucose (10µl) was added; the cell suspensions were mixed immediately by shaking the test tubes and were then incubated for 2 min.

All incubations were performed at 37^{o}C.

PMA was dissolved in dimethylsulphoxide (DMSO) and stored at -20 ^{o}C. Before use it was heated for 30 min. at 37 ^{o}C. The final concentration of DMSO in the experiments never exceeded 0.01 % vol/vol.

Results are given as mean $\pm$ SEM. Statistical significance was determined using Student's two-tailed t test (paired form); $P < 0.05$ was considered significant. No statistically significant difference = NS.

RESULTS

As shown in Table 1, the maximal stimulation by insulin (2.5 nM) and PMA (100 ng/ml) of 2-deoxyglucose transport was 145 ± 27% and 49 ± 10% above basal value, respectively. The maximal stimulation by insulin and PMA acting together was 203 ± 19% above basal value. Thus the effects of PMA and insulin in stimulating glucose transport are additive.

For maximally effective concentrations of insulin (2.5 nM) and PMA (100 ng/ml), lipogenesis was stimulated by respectively 121 ± 10% and 63 ± 8% above basal value (Fig 1A). For the two acting simultaneously, the maximal effect was 197 ± 9% above basal value. The half maximally effective concentration of insulin was about 0.12 nM both with and without PMA. The relative insulin responsiveness with and without PMA was 135 ± 13% and 121 ± 10% (NS) respectively. Consequently, the effect of PMA on insulin-stimulated lipogenesis was additive at all insulin concentrations tested. A similar additive effect of PMA was obtained on the insulin-stimulated glucose oxidation (Fig 1B).

In order to investigate whether this additive effect of PMA on insulin action was dependent on the PMA concentration, we examined the effect of PMA both in the absence and in the presence of insulin at its maximally effective concentration (2.5 nM) (Fig 2). The relative PMA responsiveness with and without insulin was unchanged (64 ± 16% vs. 76 ± 14% (NS)) and the ED_{50} value of PMA was about 20 ng/ml in both cases. Similar

TABLE 1. Effect of PMA on insulin-stimulated 2-deoxyglucose transport

Treatment	2-Deoxyglucose transport (% of basal value)
No (basal value)	100
Insulin (2.5 nM)	245 ± 27
PMA (100 ng/ml)	149 ± 10
Insulin (2.5 nM) + PMA (100 ng/ml)	303 ± 19

Adipocytes were pre-incubated with insulin (2.5 nM), with PMA (100 ng/ml) or with both. The assay of 2-Deoxyglucose transport assay was performed as described in METHODS.
Data are expressed as percentage of basal value (no addition) and are given as mean ± SEM for 5 paired experiments.

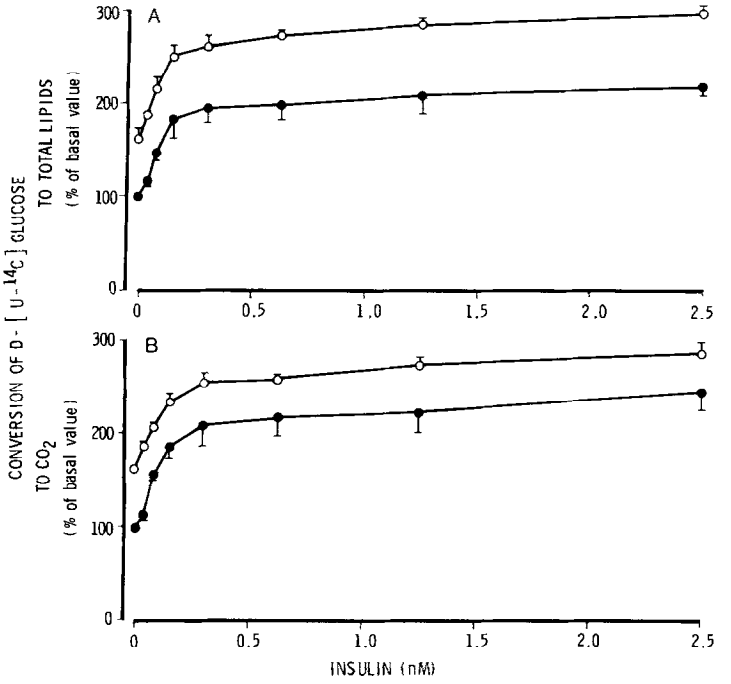

FIG. 1
Effect of PMA on the dose-response curve for insulin-stimulated lipogenesis (A) and glucose oxidation (B).
Adipocytes were incubated with insulin in the presence (O-O) or absence (●-●) of PMA (100 ng/ml) for 30 min. [U-^{14}C] glucose was then added and assays were performed as described in METHODS.
Data are expressed as percentage of the basal value (i.e. the value obtained with no addition) and are given as mean ± SEM for 3-7 paired experiments.

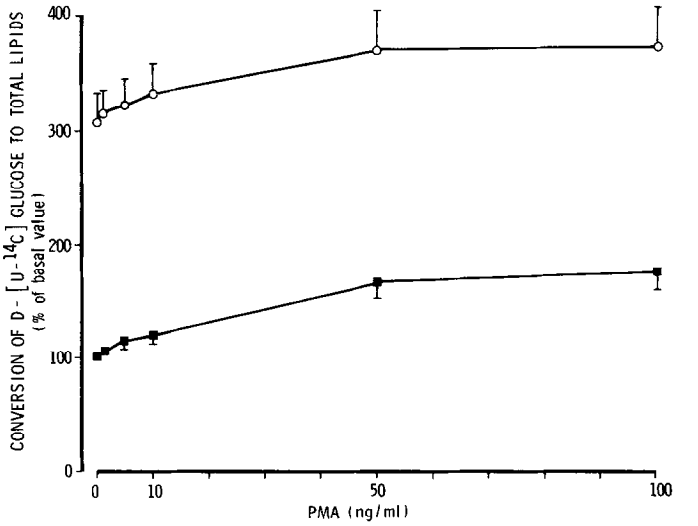

FIG. 2
Effect of insulin on the dose-response curve for PMA on lipogenesis.
Adipocytes were incubated with PMA in the presence (O-O) or absence (■-■) of insulin (2.5 nM). [U-^{14}C] glucose was added and the assay was performed as described in METHODS.
Data are expressed as percentage of the basal value (no addition) and are given as mean ± SEM for 5 paired experiments.

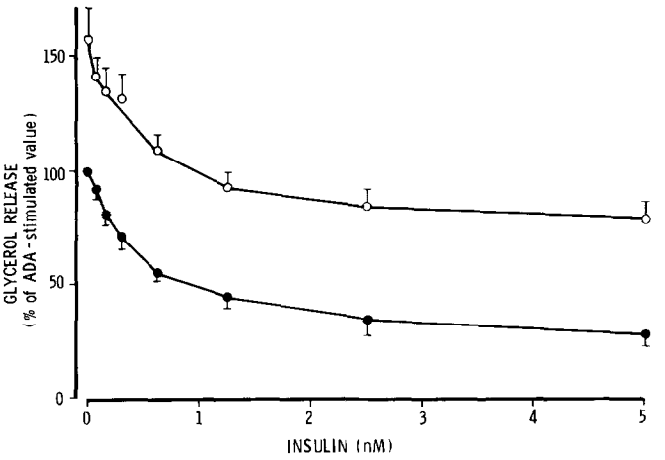

FIG. 3
Effect of PMA on the antilipolytic effect of insulin. Adipocytes were pre-incubated for 15 min with (O-O) or without (●-●) PMA (100 ng/ml). Insulin was added and after a further 15 min. lipolysis was stimulated with ADA (0.5 U/ml) for 90 min. Data are expressed as percentage of the ADA-stimulated value and are given as mean ± SEM for 3 to 6 paired experiments.

results were obtained, for the PMA-stimulated oxidation of glucose (data not shown).

In the lipolysis studies, it was found that addition of PMA (100 ng/ml) increased the ADA-stimulated lipolysis by 57 ± 14%, but did not affect the dose-dependent inhibitory effect of insulin on the ADA-stimulated lipolysis (Fig 3). The ED_{50} of insulin with and without PMA was o.43 ± 0.10 nM vs. o.52 ± 0.11 nM, (NS). In addition, the maximal relative insulin-induced inhibition of lipolysis was not influenced by the presence of PMA (85 ± 7% vs. 77 ± 5%, (NS)). Moreover, PMA did not change the ^{125}I-insulin binding to the adipocytes (data not shown).

DISCUSSION

In agreement with our results, other studies have demonstrated that phorbol esters alone stimulate 2-deoxyglucose transport, glucose oxidation and lipogenesis (2,3,4). However, the reported effects of PMA on insulin action and binding are mutually inconsistent. In recent studies by Cherqui et al. (2) and Van De Werve et al. (4) it has been demonstrated that PMA inhibits insulin-stimulated glucose metabolism for submaximal insulin concentrations. However, in these studies PMA had no effect on the maximal insulin effect or on the insulin binding. In

contrast, Kirsch et al. (1) demonstrated that PMA is able to decrease both insulin binding and maximal insulin-mediated glucose transport in adipocytes.

In the present study it has for the first time been demonstrated that PMA has additive effects on insulin-stimulated glucose metabolism, both at submaximal and maximal insulin concentrations. In addition, the effect of PMA on the insulin action is now demonstrated for all PMA concentrations (Fig. 2). Moreover, PMA stimulated lipolysis without affecting the antilipolytic effect of insulin. As the insulin binding was unchanged, the observed effects of insulin and PMA might suggest that different pathways are being activated. Accordingly, it seems unlikely that phorbol ester-activated protein kinase C is directly involved in insulin-mediated glucose metabolism in adipocytes. This suggestion is supported by the following observations: Phorbol esters, but not insulin, promotes the depletion of cytosolic protein kinase C (11) and induces the phosphorylation of glucose transporters (12). In addition, phorbol esters inhibited insulin-activated recruitment of glucose transporters to the plasma membrane (16), and insulin-activated glucose transport in Swiss 3T3 cells was unaffected by depletion of the protein kinase C activity (17). However, prolonged treatment with PMA probably modulates the insulin effect on glucose metabolism, since Cherqui et al. (10) found that 3 hours' treatment with PMA induced an insulin-resistant state in rat adipocytes.

The discrepancies between our results and those of others concerning the effect of PMA on insulin action are unexplained, but might be due to methodological differences. The concentration of collagenase used in our experiments was 0.3 mg/ml and cells were subsequently washed four times, in which points our method differs from that used by Van De Werve et al. (4), where the collagenase concentration was higher (1 mg/ml) and cells were washed twice. The concentration of trypsin-like proteolytic enzymes in crude collagenase preparations varies from batch to batch (18,19), and insufficiently washed cell preparations might contain enough trypsin-like activity to affect the metabolism of the adipocytes. Trypsin digests insulin receptor proteins, and it may possess insulin-like effects on fatcell metabolism (20). Furthermore, buffer composition may play a role in the action of PMA and insulin. We used Hepes buffer, while others used Krebs-Ringer-Hepes buffer (1), Krebs-Ringer

bicarbonate buffer (4) and Krebs-Ringer phosphate buffer (2). Differences in activity and in the preparation of the phorbol esters used might also be of importance. In the experiments of Van De Werve et al. (4), a maximal stimulation of PMA on lipogenesis was achieved at 1000 ng/ml, while in our studies it was achieved at 100 ng/ml, in accordance with the results of Cherqui et al. (2).

In contrast to the "insulin-like" effect of PMA in stimulating glucose metabolism in adipocytes, we found that PMA enhanced lipolysis while insulin reduces it. In the presence of ADA, PMA activates lipolysis without affecting the antilipolytic effect of insulin. Activation of adenylate cyclase might be involved in the lipolytic action of PMA, as it has been found that protein kinase C could activate adipocyte adenylate cyclase (13).

In conclusion, the phorbol ester PMA has both insulin-mimetic effects and insulin-antagonistic effects in adipocytes. In the present study we have found that phorbol ester, presumably through activation of protein kinase C, has insulin-mimetic effects on glucose metabolism, but an effect opposite to that of insulin on lipolysis in rat adipocytes. The stimulatory effect of PMA on glucose metabolism seems, however, to be largely independent of the insulin-stimulated glucose metabolism, since the effects of PMA and insulin were additive both for submaximal and for maximal insulin concentrations.

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