Tumour-promoting phorbol esters increase basal and inhibit insulin-stimulated lipogenesis in rat adipocytes without decreasing insulin binding

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In isolated rat adipocytes, tumour-promoting phorbol esters caused (1) dose-dependent stimulation of lipogenesis in the absence of insulin and (2) inhibition of the lipogenic effect of submaximal concentrations of insulin, but without affecting insulin binding. The possible involvement of protein kinase C in insulin action is discussed.

Receptors for hormones, neurotransmitters and growth factors use a variety of mechanisms to transmit their message across the plasma membrane. Amongst them, the generation of cyclic AMP by β -adrenergic and glucagon receptor stimulation is well documented. However, recent work has focused on the stimulation of phosphoinositide metabolism as a cyclic AMP-independent post-binding event for a large number of agonists, including angiotensin, vasopressin, acetylcholine, 5-hydroxytryptamine, platelet derived growth factor and epidermal growth factor (see Berridge, 1984). The mechanism for this method of signal transduction is now better understood. An increase in phosphoinositide metabolism, induced by hormone or agonist receptor interaction, results in the increased hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce inositol 1,4,5-trisphosphate and diacylglycerol which both act as separate messengers: inositol trisphosphate causes an increase in intracellular calcium, while diacylglycerol triggers the activation of a calcium- and phospholipid-dependent protein kinase (protein kinase C) (see Nishizuka, 1984a,b).

In addition to its physiological activation by diacylglycerol, protein kinase C is also activated by tumour-promoting phorbol esters, which share some structural similarities with diacylglycerol (Castagna *et al.*, 1982; see also Kikkawa *et al.*, 1984). There is good evidence that many of the esters' effects, including the tumour-promoting activity, are mediated through this activation (Nishizuka, 1984a,b). Recently it has been proposed that protein kinase C may play an

important role in modulating the affinity of some receptors for their hormones (Jacobs *et al.*, 1983). For example, the active phorbol esters, i.e., those acting through this kinase, have been shown to inhibit the binding of epidermal growth factor to its receptor (Brown *et al.*, 1979; Shoyab *et al.*, 1979), to stimulate the phosphorylation of the insulin receptor (Jacobs *et al.*, 1983) and to reduce insulin binding (Grunberger & Gorden, 1982; Thomopoulos *et al.*, 1982; Robert *et al.*, 1984).

In the present study, we investigated the effects of phorbol esters on insulin binding and on basal and insulin-stimulated lipogenesis in isolated adipocytes. We found that while the active phorbol esters specifically stimulate lipogenesis, they inhibit the lipogenic effect of submaximal concentrations of insulin, but without reducing receptor binding.

Materials and methods

Preparation and incubation of adipocytes

Isolated adipocytes were prepared from the epididymal fat pads of 150–200 g male Wistar rats. Finely cut tissue pieces were incubated for 45 min at 37°C with collagenase (168 units/mg; Worthington) at a concentration of 1 mg/ml. Digestion was terminated by filtration followed by two washes, each with 70 ml of buffer. Krebs–Ringer bicarbonate buffer, pH 7.4, with 3% (w/v) bovine serum albumin (Armour Pharmaceutical) and glucose (0.25 mg/ml) was used during the collagenase digestion, the preincubation and the assays. Buffer without bovine serum albumin was used for the wash. Washed cells were preincubated at 37°C in insulin-free buffer for 30 min before studies proceeded.

Abbreviation used: PMA, 4 β -phorbol 12 β -myristate 13-acetate.

Lipogenesis

Lipogenesis was measured by incubating $(0.59 \pm 0.04) \times 10^5$ cells in a volume of 1 ml of buffer in polyethylene counting vials in the presence of $[3\text{-}^3\text{H}]\text{glucose}$ ($0.2\mu\text{Ci/vial}$) and varying concentrations of porcine insulin, with or without phorbol esters (Sigma). All the esters were dissolved in dimethyl sulphoxide, in a final concentration ranging from 0.001% to 0.01%. All experiments were performed with the appropriate control containing dimethyl sulphoxide. After 1 h, the reaction was terminated by the addition of toluene based scintillant which allows radioactivity in the lipid phase to be counted directly (Moody *et al.*, 1974). Duplicate blanks containing insulin, tracer, and cells were prepared with each assay, and the counts subtracted from those obtained in the other reaction vials. Details of individual experiments are given in the legends to the Figures.

Insulin binding

Insulin binding studies were performed at two temperatures (22°C and 37°C). Approx. $(0.62 \pm 0.08) \times 10^5$ adipocytes were incubated in 0.5 ml of buffer for 30 min at 22°C with 60 pg of ^{125}I -insulin tracer (A14 human insulin; Amersham International) and various concentrations of porcine insulin (Novo), with or without 1000 ng of PMA/ml. Mixing was achieved by constant rotation of the capped polythene tubes on a slow rotator. At 30 min, an 0.3 ml aliquot was removed

from each tube and the adipocytes were separated from the medium by centrifugation through 0.2 ml of chilled dinonyl phthalate oil. When binding studies were performed at 37°C , the cells were incubated in 1 ml of buffer for 15 min in polyethylene vials and mixed by gentle shaking. At the end of the incubation, cells were separated from medium as described, and the cell pellets were counted. Non-specific binding (which did not exceed 28% at either temperature) was estimated as the binding obtained in the presence of 2 units of unlabelled insulin/ml and subtracted from the total binding. Insulin degradation was assessed by solubility in the presence of 10% (w/v) trichloroacetic acid (final concentration). At 22°C , 4% of the insulin tracer was degraded compared with 8% when the assay was performed at 37°C .

Results

Effect of phorbol esters on lipogenesis in the absence of insulin

Fig. 1(a) shows the dose-dependent stimulation of lipogenesis in isolated adipocytes by PMA. No stimulation could be detected at concentrations of 10 ng/ml or below, and maximal stimulation was achieved at 1000 ng/ml. The specificity of this effect was next examined, and, as shown in Fig. 1(b), stimulation only occurred with those esters having tumour-promoting activity (PMA and 4 β -phorbol 12 β , 13 α -dibutyrate) but not with the

Fig. 1. *Effect of phorbol esters on lipogenesis in the absence of insulin*

Adipocytes were isolated as described in the Materials and methods section and incubated for 60 min with or without increasing concentrations of PMA as indicated (a), or with PMA (A), 4 β -phorbol 12 β , 13 α -dibutyrate (B), 4 α -phorbol, 12 β , 13-didecanoate (C), 4 β -phorbol 13 α -monoacetate (D) (all at 1000 ng/ml), or with 0.01% dimethyl sulphoxide (DMSO) (b). Lipogenesis is expressed as a change in the incorporation of $[3\text{-}^3\text{H}]\text{glucose}$ into lipids from that in adipocytes incubated without phorbol esters or dimethyl sulphoxide. Basal lipogenesis was 10.8 ± 2.1 c.p.m./cell. Values are means $\pm$ S.E.M. for four different cell preparations each assayed in duplicate. Lipogenesis was significantly changed from control by $P < 0.05$ (paired Student's *t* test) in the presence of 100–1000 ng of PMA/ml (a) and by $P < 0.01$ (unpaired comparison) in the presence of esters (A) and (B) (b).

Fig. 2. Effect of phorbol esters on insulin-stimulated lipogenesis

The same conditions as in Fig. 1 were used, except that insulin was added at the indicated concentrations to the incubation medium of the adipocytes for 60 min; that the concentration of the phorbol esters in (b) was 10 ng/ml; and that lipogenesis is expressed as absolute values. (a) Shows a representative experiment out of a series giving similar results. Values in (b) are means $\pm$ S.E.M. for three different cell preparations each assayed in duplicate. Lipogenesis in the presence of esters (A) and (B) in (b) was significantly different from control by $P < 0.0025$ (unpaired comparison).

Fig. 3. Dose-response of PMA on basal and insulin-stimulated lipogenesis

The same conditions as in Fig. 1 were used. Values are expressed as change above basal (10.8 ± 2.1 c.p.m./cell), and for the insulin-stimulated lipo-

genesis represent the mean $\pm$ S.E.M. of three different cell preparations each assayed in duplicate. Lipogenesis was significantly changed from control by at least $P < 0.05$ (paired comparison) in the presence of 100–1000 ng of PMA/ml.

Effect of phorbol esters on insulin-stimulated lipogenesis

Fig. 2(a) shows the effects of different concentrations of PMA on the dose-response curve of lipogenesis for insulin. PMA inhibited submaximal but not maximal doses of insulin, indicating that the phorbol ester reduced the sensitivity of lipogenesis to insulin. Fig. 2(b) demonstrates that the inhibition of insulin-stimulated lipogenesis by phorbol esters (each at 10 ng/ml) was, like their lipogenic effect, specific for those with tumour-promoting activity. Dimethyl sulphoxide at a final concentration of 0.001% had no inhibitory effect on lipogenesis. The inhibition by PMA of insulin-stimulated lipogenesis was dose-dependent, as illustrated in Fig. 3, where the effect on basal lipogenesis (data of Fig. 1a) is also shown for comparison. Although there was some variability in sensitivity of lipo-

Fig. 4. Effect of PMA on insulin binding to adipocytes. Insulin binding to adipocytes was measured at 22°C as described in the Materials and methods section. Adipocytes were incubated with (●) or without (○) 1000 ng of PMA/ml.

genesis to insulin from one preparation of adipocytes to the other (Fig. 2a versus Fig. 3), the observations were qualitatively the same.

Effect of PMA on insulin binding to adipocytes

Fig. 4 shows the percentage specific binding of ¹²⁵I-insulin to adipocytes at 24°C in the absence and presence of increasing insulin concentration, with or without 1000 ng of PMA/ml. The phorbol ester did not alter the binding of tracer (even at 37°C and up to 120 min of preincubation with PMA; results not shown) or its displacement by unlabelled insulin.

Discussion

Stimulation of lipogenesis by phorbol esters in the absence of insulin

Our data show that tumour-promoting phorbol esters have an insulin-like effect to stimulate lipogenesis in adipocytes. This is consistent with recent work showing that these esters have other insulin-like actions; for example, phosphorylation of the insulin receptor in IM9 B lymphocytes (Jacobs *et al.*, 1983), stimulation of lactate production in 3T3 preadipocytes (Brown *et al.*, 1979) and inhibition of glucagon-activated adenylate cyclase in hepatocytes (Heyworth *et al.*, 1984). It has been known for many years that insulin stimulates phospholipid, and more precisely phosphoinositide, metabolism in white adipose tissue (De Torriontegui & Berthet, 1966; Stein & Hales, 1974; García-Sáinz & Fain, 1980; Farese *et al.*, 1982, 1984; Kelly *et al.*, 1984). Moreover, insulin was shown to activate endogenous adipocyte phospholipase C (which catalyses the

formation of diacylglycerol from phosphatidylinositol 4,5-bisphosphate) and pyruvate dehydrogenase in a parallel way (Koepfer-Hobelsberger & Wieland, 1984) and treatment of fat cells with exogenous phospholipase C provoked an insulin-like activation of pyruvate dehydrogenase (Honeyman *et al.*, 1983). It is thus possible that in adipocytes, insulin, through stimulation of phosphoinositide turnover and diacylglycerol production, triggers the same step (protein kinase C activation) as phorbol esters.

Although the specificity of these effects for those esters which have tumour-promoting activity (reviewed by Blumberg, 1980) suggest that they are mediated through activation of protein kinase C, it is possible that other receptors for phorbol esters which share the same specificity might be involved. The existence of such receptors has been demonstrated by Moon *et al.* (1984) in membranes of A431 epidermic carcinoma cells.

Inhibition of insulin-stimulated lipogenesis by phorbol esters

The present data also demonstrate that the active tumour-promoting phorbol esters inhibit the lipogenic effect of submaximal concentrations of insulin on isolated rat adipocytes. This inhibition was mediated without measurable change in insulin binding. These findings contrast with those of others, which have demonstrated that phorbol esters reduce insulin binding in some tissues, presumably through activation of protein kinase C (Grunberger & Gorden, 1982; Thomopoulos *et al.*, 1982) and have suggested (Jacobs *et al.*, 1983) that phosphorylation of the insulin receptor by phorbol esters leads to reduced affinity for insulin. On the other hand, our results are consistent with those of Shoyab *et al.* (1979) who reported that PMA did not reduce insulin binding to 3T3 cells. A similar finding was reported by Friedman *et al.* (1984) in A431 cells, where tumour-promoting esters blocked tyrosine-specific phosphorylation of the epidermal growth factor receptor without altering binding. These contradictory results might well be explained by the wide variation in the response of insulin receptors from different tissues to phorbol esters. Grunberger & Gorden (1982) have demonstrated this variation in three different human cells. While PMA induced 78% inhibition of insulin binding to IM-9 lymphocytes in 1 min, it required 30 min for a similar degree of inhibition in U-937 macrophages, and it only inhibited binding by 24% in fibroblasts after 60 min incubation.

We present here, for the first time, evidence that the presumed activation of protein kinase C can, in adipocytes, modulate insulin action without altering insulin binding. No conclusions can be drawn from these studies about the step(s) beyond

binding which protein kinase C modifies. It is interesting to note that both the insulin-like and anti-insulin effects of phorbol esters appear to be mediated through activation of protein kinase C. This feature could explain why optimal concentrations of PMA are not able to stimulate lipogenesis to the same extent as optimal concentrations of insulin (see Fig. 3), both putative components of PMA action (stimulatory and inhibitory to lipogenesis) possibly antagonizing each other. One can speculate that in adipocytes, as in other tissues, protein kinase C phosphorylates the insulin receptor. It would be of interest to know the sites which are phosphorylated by insulin and phorbol esters respectively. Phosphorylation of common sites might mediate the stimulation of lipogenesis we observed with both insulin and the esters, whereas phosphorylation of different sites might mediate opposite effects such as the inhibition of insulin-stimulated lipogenesis by the phorbol esters. Such dual intracellular regulation might modulate the final effects of insulin and growth factors and help preventing deleterious, overshooting actions of these anabolic hormones.

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