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Stimulation of adipocyte phospholipid methyltransferase activity by phorbol 12-myristate 13-acetate

Differential regulation of phospholipid methyltransferase and lipolysis

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The present studies demonstrate that treatment of rat adipocytes with the phorbol ester phorbol 12-myristate 13-acetate (PMA) causes a dose-dependent stimulation of phospholipid methyltransferase (PLMT) activity. The stimulatory effect of PMA was not additive with that of isoprenaline or forskolin. The sensitivity of stimulated PLMT activity to inhibition by insulin, however, was decreased in the presence of PMA. The inhibitory effect of a maximal concentration of insulin on PLMT was unchanged in the presence of PMA. In contrast with the effects on PLMT, the lipolytic response of adipocytes to isoprenaline and the anti-lipolytic response to insulin were unaffected by PMA. These data suggest that PLMT is, whereas hormone-sensitive lipase is not, an intracellular target for the action of PMA. The lack of effect of PMA on lipolysis suggests that PLMT and hormone-sensitive lipase can be regulated by separate mechanisms. Furthermore, phorbol esters do not interfere in the regulatory pathway whereby insulin inhibits PLMT or lipolysis.

INTRODUCTION

Phospholipid methyltransferase (PLMT) is an ubiquitous mammalian enzyme [1], sensitive to regulation by hormones controlling cellular metabolism [2–6]. In hepatocytes [7–9], adipocytes [4] and Leydig cells [5], the activation of PLMT occurs in response to hormones that increase cyclic AMP. Glucagon administered *in vivo* has been shown to increase the phosphorylation of PLMT [10], and this effect was mimicked *in vitro* by cyclic AMP-dependent protein kinase [11]. PLMT is also activated by a Ca^{2+}-dependent mechanism [3,12–15]. In hepatocytes, the activation of PLMT by angiotensin is associated with phosphorylation of PLMT [16] on a residue different from that phosphorylated by cyclic AMP-dependent protein kinase, and in adipocytes the stimulatory effect of oxytocin on PLMT is additive with that caused by isoprenaline [12].

Insulin, the major anabolic hormone, inhibits basal PLMT activity as well as that stimulated by hormones acting through either cyclic AMP or Ca^{2+} [4,12]. The biological steps that follow the interaction of insulin with its receptor and lead to inhibition of PLMT remain to be elucidated. On the basis of the mechanisms whereby PLMT is activated, and the effect of insulin to dephosphorylate other enzymes [17–19], it has been postulated that insulin inhibits PLMT activity by causing dephosphorylation of the enzyme.

The present study was undertaken to determine if phorbol esters, which have been reported to modulate the activity of certain enzymes [20–22], could alter PLMT activity in adipocytes. The phorbol ester tumour promoters have been shown to activate directly a Ca^{2+}/phospholipid-dependent protein kinase, termed protein kinase C [23]. The cellular receptor for phorbol esters co-purifies with protein kinase C [24], and evidence suggests that the activation of this protein kinase and subsequent phosphorylation of specific proteins could mediate many, if not all, of the actions of tumour-promoting phorbol esters.

EXPERIMENTAL

Materials

Male Sprague–Dawley rats were obtained from Hilltop Animals, Scottdale, PA, U.S.A. Collagenase was from Worthington, and albumin (fraction V) was from U.S. Biochemicals. Phosphatidyl-*N*-monomethylethanolamine was from Avanti Polar Lipids. Forskolin was from Calbiochem. Phorbol 12-myristate 13-acetate (PMA) was from Sigma. Insulin was a gift from R. Chance of Eli Lilly. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (Ado[^{3}H]Met) was purchased from New England Nuclear. *S*-Adenosylmethionine (AdoMet; chloride salt) was purchased from Boehringer–Mannheim. All other chemicals and biochemicals were purchased from standard sources.

Isolation and incubation of adipocytes

Isolated fat-cells were prepared by the collagenase-digestion method of Rodbell [25] from epididymal pads of fed male Sprague–Dawley rats (125–175 g). Krebs–Ringer bicarbonate buffer, pH 7.4, containing 2.5 mM-Ca^{2+} and 4% (w/v) bovine serum albumin was used. Fat-cells (1×10^6 cells/ml) were incubated as 1 ml batches in separate polyethylene vials gassed with O_2/CO_2 (19:1), with shaking at 37 °C. After 30 min of preincubation, samples of Krebs–Ringer bicarbonate buffer or buffer containing test agents were added, and the incubation was continued. After incubation with the test agents, a 0.5–1.0 ml sample of cells was removed for measurement of PLMT activity and/or glycerol release (as a measure of lipolysis). PMA was dissolved in a final

Abbreviations used: PLMT, phospholipid methyltransferase; AdoMet, *S*-adenosyl-L-methionine.

concentration of 0.05% dimethyl sulphoxide, and an equivalent concentration of dimethyl sulphoxide was added to matched control cells. Forskolin was dissolved in a final concentration of 0.1% dimethyl sulphoxide, and an equivalent amount of dimethyl sulphoxide was added to control cells.

Measurement of PLMT activity

PLMT activity of adipocyte infranatant was assayed as previously described [4]. A 0.5–1.0 ml suspension of adipocytes (1×10^6 cells/ml) was transferred to a Microfuge tube, and cells were separated from the incubation medium by centrifugation for 0.5 min at 10000 *g*. The incubation medium was removed by aspiration, and the cells were disrupted by the addition of 0.25 ml of homogenization buffer (125 mM-Tris/HCl, 2 mM-KF, 2 mM-EGTA, 2 mM-EDTA pH 8.5). The cells were vigorously shaken and the homogenate was micro-centrifuged. The resulting infranatant was transferred free of lipid to another tube, and kept on ice. For assay of PLMT, 0.125 ml of fat-cell infranatant (0.81 ± 0.03 mg of protein/ml) was used for assay in a reaction mixture containing (final volume 0.5 ml): 125 mM-Tris/HCl, pH 8.5, 4 mM-dithiothreitol, 5 mM-$MgCl_2$, 0.5 mM-KF, 0.5 mM-EGTA, 0.5 mM-EDTA, 10 μM-AdoMet, 2 μCi of Ado[^{3}H]Met (71 Ci/mmol) and 40 μg of phosphatidyl-*N*-monomethylethanolamine (0.1 mM). Solutions of phosphatidyl-*N*-monoethylethanolamine were dried under N_2 and dispersed by sonication in 1% propylene glycol in 125 mM-Tris/HCl, pH 8.5. The reaction was initiated by the addition of AdoMet. Samples were incubated for 20 min at 37 °C. The reaction was stopped by addition of 3 ml of acidified (0.05 M-HCl) chloroform/methanol (1:2, v/v). Phospholipids were extracted by the method of Bligh & Dyer [26] as modified by Lapetina & Michell [27]. A sample of the chloroform phase was transferred to a counting vial, evaporated to dryness, and resuspended in 3 ml of OCS (New England Nuclear) for determination of radioactivity. Under these standard assay conditions, *methyl*-^{3}H in the organic phase was incorporated predominantly into phosphatidyl-*NN*-dimethylethanolamine, with lesser incorporation into phosphatidylcholine and lysophosphatidylcholine as determined by t.l.c. [4]. Specific activity of the enzyme was expressed as pmol of [^{3}H]methyl incorporated into phospholipids/min per mg of protein.

Measurement of lipolysis

Lipolysis was routinely measured as the net release of glycerol from adipocytes over the incubation period, and was linear for at least 20 min incubation. Lipolysis was terminated by the addition of 0.5 ml of cell suspension (0.5×10^6 cells/0.5 ml of Krebs–Ringer bicarbonate buffer) to 3 M-$HClO_4$. The acid extracts were centrifuged at 1000 *g* and neutralized with 2 M-$KHCO_3$. Glycerol content was determined fluorimetrically by the method of Wieland [28].

RESULTS

Effect of PMA concentration on PLMT activity

When isolated adipocytes were incubated with increasing concentrations of PMA in the absence of any stimulus, PMA had a slight stimulatory effect (to 122% of control) on basal PLMT activity that was maximal at 10 ng of PMA/ml (Fig. 1). Treatment of adipocytes with either 100 nM-isoprenaline or 10 μM-forskolin for 10 min caused a 2-fold increase in PLMT activity [12]. In the presence of isoprenaline or forskolin the stimulatory effect of PMA was obscured, except in the presence of a high concentration of PMA (100 ng/ml). Under these conditions, there was an additive effect of PMA and isoprenaline on PLMT activity.

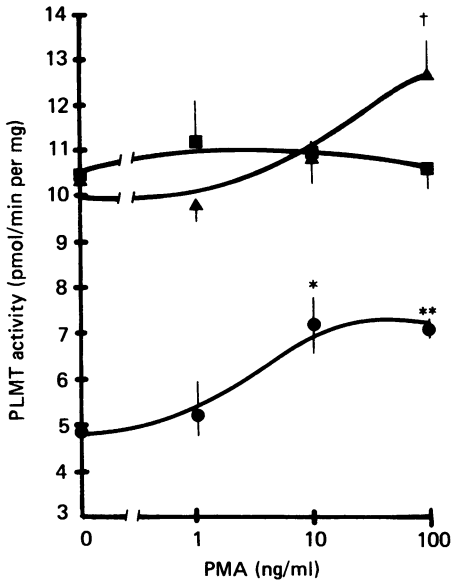

Fig. 1. Effect of PMA concentration on PLMT activity

Adipocytes were incubated with increasing concentrations of PMA in the absence (○) or in the presence of 100 nM-isoprenaline (▲) or 10 μM-forskolin (■) for 10 min. Each determination represents the mean (±S.E.M.) PLMT activity from three independent experiments performed in triplicate: $*P < 0.05$, $**P < 0.025$ versus control; $\dagger P < 0.05$ versus isoprenaline alone.

Effect of PMA concentration on inhibition of PLMT activity by insulin

It has previously been demonstrated that treatment of adipocytes with physiological concentrations of insulin causes a dose-dependent inhibition of both basal and hormone-stimulated PLMT activity [4]. A submaximal concentration of insulin (10 μunits/ml) inhibited the stimulatory effect of 100 nM-isoprenaline by 47% (Fig. 2*a*). The addition of PMA caused a dose-dependent reversal of the inhibitory effect of insulin. The inhibitory effect of 10 μunits of insulin/ml was completely overcome in the presence of 10 ng of PMA/ml. Additionally, in the presence of 100 ng of PMA/ml, the effect of PMA was additive with that of isoprenaline in stimulating PLMT, as was the case in the absence of insulin.

A similar series of experiments was carried out with 10 μM-forskolin (Fig. 2*b*). A submaximal concentration of insulin (10 μunits/ml) inhibited the stimulatory effect of forskolin (10 μM) by 46%. PMA (1 or 10 ng/ml) completely antagonized the inhibitory effect of insulin. In the presence of insulin, there was an additive effect of 100 ng of PMA/ml on forskolin-stimulated PLMT

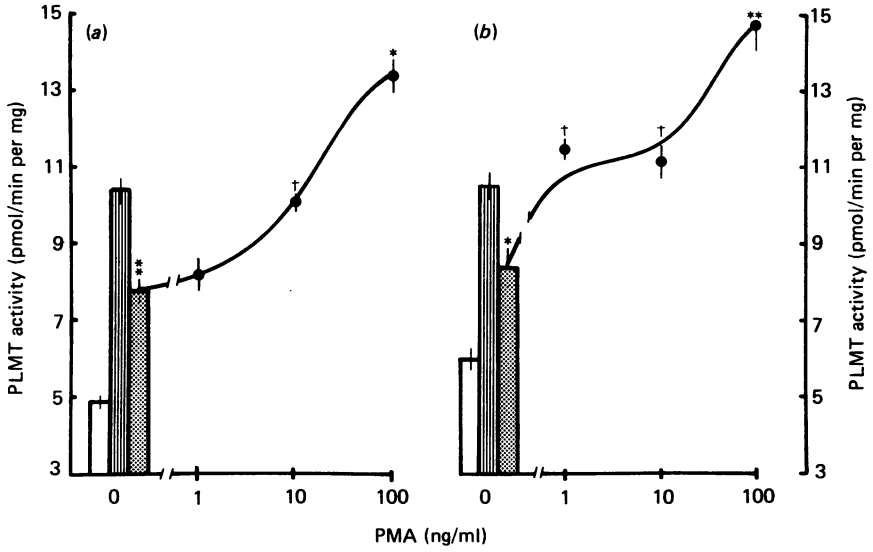

Fig. 2. Effect of PMA concentration on inhibition of PLMT activity by insulin

Adipocytes were incubated in the absence of any stimulus (□) or (*a*) in the presence of 100 nM-isoprenaline (■) plus 10 μunits of insulin (▨)/ml and increasing concentrations of PMA for 10 min. $*P < 0.05$ and $**P < 0.005$ versus isoprenaline alone, and $\dagger P < 0.005$ versus isoprenaline plus insulin. In (*b*), isoprenaline was replaced with 10 μM-forskolin. $*P < 0.025$ and $**P < 0.005$ versus forskolin alone, and $\dagger P < 0.005$ versus forskolin plus insulin. Each determination represents the mean (±S.E.M.) PLMT activity from three independent experiments performed in triplicate.

activity that was not evident when these same concentrations of forskolin and PMA were incubated in the absence of insulin.

Effect of PMA on the dose–response curve and time course of PLMT activity in response to isoprenaline and insulin

To determine whether PMA decreased the sensitivity of PLMT to insulin by interacting in the regulatory pathway through which insulin inhibits PLMT, or by a direct effect on PLMT, the following experiments were performed. Adipocytes stimulated with 10 μM-forskolin were treated with increasing concentrations of insulin in the absence and presence of 10 ng of PMA/ml. PMA decreased the effect of submaximal, but not of maximal, concentrations of insulin to inhibit PLMT (Fig. 3). These data suggest that PMA decreased the sensitivity of PLMT to insulin without altering the mechanism whereby insulin mediates this action.

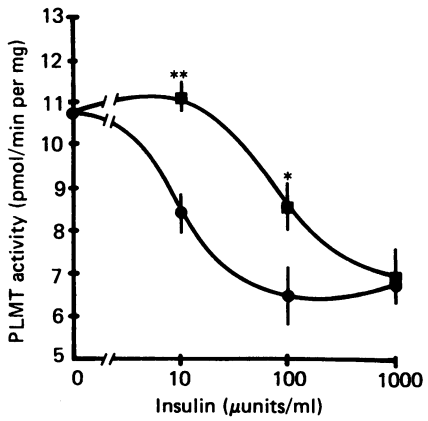

Fig. 3. Effect of PMA on the dose–response curve for inhibition of PLMT activity by insulin

Adipocytes were incubated with 10 μM-forskolin and increasing concentrations of insulin in the absence (●) or presence of 10 ng of PMA/ml (■). Basal activity was 6.3±0.4 pmol/min per mg. Each determination represents the mean (±S.E.M.) PLMT activity from at least three independent experiments performed in triplicate: $*P < 0.05$ and $**P < 0.01$ versus isoprenaline plus insulin.

The kinetics of the effect of PMA on insulin inhibition of PLMT were studied to determine how the effect of insulin was antagonized. The time course of the effect of PMA (10 ng/ml) on isoprenaline-stimulated PLMT activity in the absence and presence of insulin is shown in Fig. 4. Isoprenaline (100 nM) caused a time-dependent increase in PLMT activity that reached a plateau after 20–30 min. As previously demonstrated (Fig. 1), 10 ng of PMA/ml had no effect on the stimulation of PLMT in response to isoprenaline. Treatment of adipocytes with a submaximal concentration of insulin (10 μunits/ml) inhibited the stimulatory effect of isoprenaline. In the presence of 10 ng of PMA/ml, the inhibitory effect of insulin on PLMT activity in response to isoprenaline was antagonized, but the time-dependent effect of insulin to return PLMT activity to basal values was unchanged.

Effect of PMA concentration on isoprenaline-stimulated lipolysis and insulin-mediated anti-lipolysis

Incubation of adipocytes with isoprenaline and increasing concentrations of PMA had no effect on lipolysis (Fig. 5), except at a very high concentration of PMA, 100 ng of PMA/ml, there was a slight decrease in the stimulatory effect of isoprenaline. There was, however, no effect at any concentration of PMA, to alter the inhibitory effect of insulin on lipolysis. These data are in agreement with the findings of two other laboratories

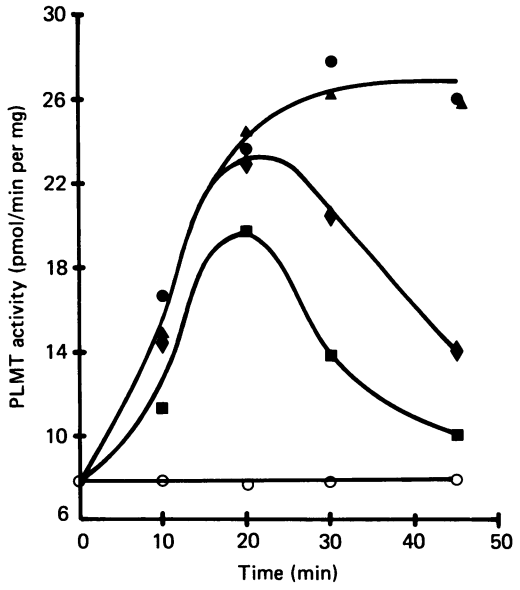

Fig. 4. Effect of PMA on the time course of PLMT activity in response to isoprenaline and insulin

Adipocytes were incubated with vehicle (○) or with 100 nM-isoprenaline alone (●), or in the presence of 10 ng of PMA/ml (▲), 10 μunits of insulin/ml (■), or PMA+insulin (◆). Each determination represents the mean of two to four independent experiments performed in triplicate.

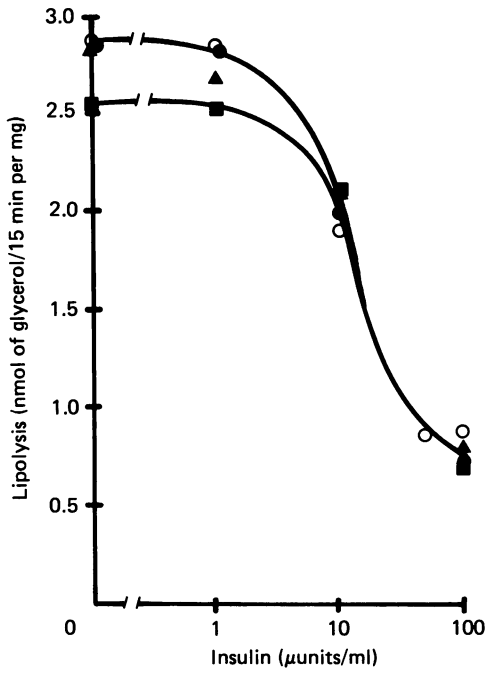

Fig. 5. Concentration effect of PMA on isoprenaline-stimulated lipolysis and insulin-mediated anti-lipolysis

Adipocytes were incubated with 100 nM-isoprenaline in the absence (○) or in the presence of 1 ng of PMA/ml (●), 10 ng of PMA/ml (▲), or 100 ng of PMA/ml (■), and increasing concentrations of insulin for 20 min. Basal glycerol release was 0.07 ± 0.03 nmol of glycerol/mg. Results are from a representative experiment performed in triplicate, and repeated three other times with similar effects.

[22,29]. The lack of effect of PMA on lipolysis and anti-lipolysis contrasts with the effect on PLMT.

DISCUSSION

An increase in PLMT activity in hepatocytes is associated with an increase in the phosphorylation state of the enzyme, mediated by either cyclic-AMP-dependent or Ca^{2+}-dependent kinases [10,16]. Adipocyte PLMT activity appears to be regulated in an analagous manner [4,12].

The results of this study demonstrate that PLMT is an intracellular target for the tumour-promoting phorbol ester, PMA. Current evidence suggests that protein kinase C, which is present in adipocytes [30,31], mediates the effect of phorbol esters on cells. The stimulation of PLMT activity in adipocytes occurred at a concentration of PMA equivalent to that which elicited protein phosphorylation in platelets [32], inactivation of glycogen synthase in hepatocytes [20], and inhibition of fatty acyl-CoA synthetase in adipocytes [22]. Consequently, it has been suggested that the effects of the PMA on PLMT are due to the activation of protein kinase C.

The weak stimulatory effect of PMA on PLMT activity was not additive with that caused by either isoprenaline or forskolin, except at a very high concentration of PMA, and only in the presence of isoprenaline. One might conclude either that PMA and isoprenaline stimulated PLMT activity by increasing phosphorylation of the enzyme at the same regulatory site, or at different regulatory sites, and the effect of PMA was over-ridden in the presence of isoprenaline. The latter hypothesis is supported by the observation that PLMT is phosphorylated *in vitro* by protein kinase C at a site different from that phosphorylated by cyclic AMP-dependent protein kinase [33].

An important aspect of the present study with regard to adipocyte metabolism is the observation that PLMT and hormone-sensitive lipase, both of which are activated by cyclic-AMP-mediated phosphorylation and inhibited by insulin, can be regulated independently. Although the activity of hormone-sensitive lipase is regulated by phosphorylation/dephosphorylation [34], the results from the present study support the hypothesis that hormone-sensitive lipase is not an intracellular target for the action of phorbol esters, or of protein kinase C [22,29]. In contrast, PLMT is an intracellular target for phorbol esters.

Protein kinase C does not appear to be involved in the regulatory pathway by which insulin inhibits PLMT or lipolysis. Although in the presence of PMA there was a loss of sensitivity to inhibition of PLMT by insulin, this appears to have been the result of a direct effect of PMA on PLMT, rather than an effect on the mechanism whereby insulin action is mediated.

The conclusions from this study are that PMA activates protein kinase C, leading to the phosphorylation of PLMT, thereby increasing its activity, and decreasing its sensitivity to inhibition by insulin. PMA does not interfere with the mechanism of insulin action. Although both hormone-sensitive lipolysis and PLMT activity are

increased by cyclic-AMP-dependent protein kinase, the activities of these two enzymes are dissociable, and they can be regulated independently.

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