Phorbol esters stimulate phosphatidylinositol 3,4,5-trisphosphate production in 3T3-L1 adipocytes: implications for stimulation of glucose transport

Barbara T. NAVÉ, Kenneth SIDDLE and Peter R. SHEPHERD*

Department of Clinical Biochemistry, University of Cambridge, Cambridge, U.K.

The effects of insulin and phorbol 12-myristate 13-acetate (PMA) on the levels of cellular phosphoinositides were investigated in 3T3-L1 adipocytes. Stimulation for 4 min with PMA (1 μ M) or insulin (10 nM) increased levels of PtdIns(3,4,5) P_3 approx. 2-fold and 6-fold respectively. PMA also had a small effect on the cellular levels of PtdIns4P, whereas insulin had no effect on PtdIns4P levels; levels of PtdIns(4,5) P_2 and PtdIns3P were not significantly affected by either agent. Insulin increased the levels of the p85 α subunit of phosphoinositide (PI) 3-kinase associated with membranes, whereas PMA decreased levels of membrane-associated p85 α . PMA did not increase PI 3-kinase activity in

anti-phosphotyrosine or anti-p85 immunoprecipitates. The stimulation of glucose transport by insulin or PMA was blocked by 100 nM wortmannin or 10 ng/ml LY294002, indicating that PI 3-kinase is essential for stimulation by both agents. In summary, these results demonstrate: (1) that PMA and insulin stimulate PtdIns(3,4,5) P_3 production by distinct mechanisms in 3T3-L1 adipocytes, and (2) that stimulation of PtdIns(3,4,5) P_3 production by PMA is likely to be important in signalling pathways leading from PMA stimulation to end-point responses such as glucose transport.

INTRODUCTION

Phorbol esters are tumour-promoting agents that are thought to act largely through stimulation of the protein kinase C family of serine/threonine protein kinases [1]. End-point responses stimulated by phorbol 12-myristate 13-acetate (PMA) overlap to a large extent with responses that are activated by growth factors such as insulin. Responses activated by both PMA and insulin include gene expression [1], membrane trafficking events such as glucose transporter translocation [2–4], membrane ruffling [5] and patterns of phosphorylation of cellular proteins [6]. The effects of PMA and insulin on glucose transport are not additive in fibroblasts [7] or adipocytes [3,4]. Further, despite the fact that the effects of insulin and exercise on glucose transport in muscle are additive, the effects of PMA and insulin are not [8]. However, the available evidence suggests that insulin does not signal to downstream events via protein kinase C [7,9–11]. Therefore it seems likely that the signalling cascades initiated by insulin and PMA converge at some point to produce similar responses.

Previous studies indicate that stimulation of phosphoinositide 3-kinase (PI 3-kinase) and the subsequent transient increase in the intracellular levels of phosphatidylinositol 3,4,5-trisphosphate are necessary for insulin stimulation of a range of processes, including stimulation of glucose transporter translocation [12,13] and induction of membrane ruffling [5]. This significant overlap with PMA-induced responses led us to consider the possibility that at least some of the effects of PMA may be mediated by stimulation of PI 3-kinase.

MATERIALS AND METHODS

Analysis of phosphoinositide levels

3T3-L1 adipocytes were serum-starved overnight, then incubated for 90 min at 37 °C in phosphate-free medium containing 800 μ Ci/ml [32 P] P_i (du Pont-NEN). Cells were washed twice in buffer A (5 mM KCl, 25 mM Hepes, 0.85 mM MgSO $_4$, 1.8 mM

CaCl $_2$, 115 mM NaCl, 25 mM glucose, 0.1 % fatty-acid-free BSA, 1 mM NaHCO $_3$, pH 7.4), then incubated in buffer A at 37 °C for 2 min before addition of stimulants (10 nM insulin, 1 μ M PMA or carrier) for 4 min. The reaction was stopped with ice-cold 1 M HCl. Lipids were then extracted, deacylated and analysed by HPLC as previously described [14].

Immunoprecipitations and PI 3-kinase activity

3T3-L1 adipocytes were serum-starved overnight and then treated with insulin or PMA for 4 or 10 min. Cells were lysed in buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl $_2$, 1 mM CaCl $_2$, 0.5 mM Na $_3$ VO $_4$, 10 μ g/ml leupeptin, 0.2 mM PMSF, 10 % (w/v) glycerol and 1 % Nonidet P-40. Cleared lysates were immunoprecipitated with the monoclonal anti-phosphotyrosine antibody PY-20 or with a polyclonal antibody raised against the N-terminal SH2 domain of the p85 subunit of PI 3-kinase.

PI 3-kinase enzymic activity was assayed by measuring the incorporation of 32 P from [γ - 32 P]ATP into PtdIns. Phospholipid product was resolved by TLC as described [14] and quantified using a Fuji Bas 2000 phosphorimager.

Preparation of total cell membranes

3T3-L1 adipocytes were serum-starved overnight and treated with PMA for the indicated times. Cells were disrupted by 10 passages through a 25 gauge needle in Tris/sucrose buffer (0.25 M sucrose, 20 mM Tris/HCl, pH 7.4, 1 mM Na $_3$ VO $_4$, 1 mM PMSF, 10 μ g/ml leupeptin, 200 kallikrein-inhibitory units/ml aprotinin, 1 μ g/ml antipain, 400 μ g/ml benzamidin and 1 μ g/ml pepstatin). Nuclei and unbroken cells were removed by centrifugation at 3000 g for 5 min. Total membrane fractions were prepared by centrifugation of the 3000 g supernatant for 30 min at 350 000 g using a Beckman TL100 centrifuge.

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; PI 3-kinase, phosphoinositide 3-kinase.

* To whom correspondence should be sent, at present address: Department of Biochemistry and Molecular Biology, University College London, Gower St., London WC1E 6BT, U.K.

Hexose transport

2-Deoxy[^3H]glucose uptake was determined as previously described [15]. Cells were serum-starved for 3 h, then transferred to Krebs–Ringer phosphate buffer (pH 7.4). Wortmannin or vehicle was added 5 min prior to stimulation and cells were incubated a further 15 min, at which stage 2-deoxy[^3H]glucose uptake was measured over 10 min.

RESULTS AND DISCUSSION

Treatment of 3T3-L1 adipocytes with PMA for 4 min caused a significant (~ 2 -fold) increase in cellular levels of $\text{PtdIns}(3,4,5)\text{P}_3$ (Table 1). Insulin caused a greater increase in $\text{PtdIns}(3,4,5)\text{P}_3$ levels (~ 6 -fold) but had no statistically significant effect on the levels of the other phosphoinositide products (Table 1). Previous studies in other cell types have reported that phorbol esters stimulate increases in cellular levels of $\text{PtdIns}(3,4)\text{P}_2$ [16,17]. Reports also exist of PMA-induced increases in $\text{PtdIns}(3,4,5)\text{P}_3$ levels in fibroblasts [18] and neutrophils [16], although these effects were much less than seen in the present study.

The results also show that PMA treatment caused a significant rise in the cellular levels of $\text{PtdIns}4\text{P}$. The effect of PMA on $\text{PtdIns}4\text{P}$ levels is consistent with the PMA-induced stimulation of PI 4-kinase that has been observed in other cell types [14,16,19]. While the percentage increase in $\text{PtdIns}4\text{P}$ was small, it should be noted that in absolute terms the amount of $\text{PtdIns}4\text{P}$ produced in response to PMA was around two orders of magnitude greater than the amount of $\text{PtdIns}(3,4,5)\text{P}_3$ produced following the same stimulation (Table 1), and this is therefore likely to have relevance for signalling pathways downstream of PI 4-kinase. Levels of $\text{PtdIns}(4,5)\text{P}_2$ were also increased in most experiments, although this was not statistically significant (Table 1).

The most likely explanation for the PMA-induced increase in $\text{PtdIns}(3,4,5)\text{P}_3$ levels is that there is an increase in the activity of a class I PI 3-kinase [20]. The mechanism by which growth factors stimulate PI 3-kinase has been extensively investigated. In the case of insulin, phosphorylation of specific tyrosine motifs on insulin receptor substrate-1 is induced; this is then able to recruit p85/p110 PI 3-kinase heterodimers via the SH2 domains of the p85 adapter subunit of this enzyme. This increases PI 3-kinase activity in two ways: by bringing the enzyme into proximity with the substrate and also by increasing catalytic activity [21]. We sought to determine whether PMA acted by a similar mechanism to stimulate PI 3-kinase. However, although

Table 1 Effects of PMA and insulin on cellular levels of phosphoinositides in 3T3-L1 adipocytes

Mature adipocytes were labelled with [^{32}P]P $_i$ as described in the Materials and methods section, then stimulated with 10 nM insulin or 1 μM PMA for 4 min. Lipid products were extracted, deacylated, separated by HPLC and quantified as described in the Materials and methods section. The data are means $\pm$ S.E.M. of duplicate determinations from a representative experiment. The results of three separate experiments were statistically analysed by Student's paired t test, and significant differences were found between basal and PMA-stimulated levels of $\text{PtdIns}4\text{P}$ ($P < 0.01$) and between basal and both insulin- and PMA-stimulated $\text{PtdIns}(3,4,5)\text{P}_3$ levels ($P < 0.01$).

Condition	$10^{-3} \times \text{Phosphoinositide levels (d.p.m.)}$			
	$\text{PtdIns}3\text{P}$	$\text{PtdIns}4\text{P}$	$\text{PtdIns}(4,5)\text{P}_2$	$\text{PtdIns}(3,4,5)\text{P}_3$
Basal	30.1 ± 1.9	761 ± 101	788 ± 41	1.24 ± 0.33
PMA	31.8 ± 2.9	878 ± 33	945 ± 171	2.75 ± 0.50
Insulin	32.6 ± 3.4	780 ± 58	805 ± 75	7.71 ± 1.45

Figure 1 Effect of PMA on levels of membrane-associated p85

3T3-L1 adipocytes were incubated for the indicated times with carrier or 1 μM PMA, harvested, and total membranes prepared as described in the Materials and methods section. Membranes were resuspended in Tris/sucrose buffer to about 5–10 μg of protein/ μl . Samples of 80 μg of protein were run on an SDS/PAGE gel and transferred on to nitrocellulose, which was blotted with a polyclonal anti-p85 antibody raised to the SH2 domain of the p85 subunit. Bands were visualized with ^{125}I -Protein A and quantified on a BAS2000 Fuji phosphorimager. Molecular masses (kDa) are shown. The results of a representative experiment carried out using duplicate wells are shown. Similar results were obtained in three separate experiments.

Table 2 PMA does not increase PI 3-kinase activity in anti-phosphotyrosine or anti-p85 immunoprecipitates

Cells lysates were prepared from 3T3-L1 adipocytes stimulated with either 10 nM insulin or 1 μM PMA for 10 min. Lysates were immunoprecipitated with the monoclonal PY20 anti-phosphotyrosine antibody or with the polyclonal anti-p85 antibody, and PI 3-kinase activity in immunoprecipitates was measured as described in the Materials and methods section. Data are presented as fold stimulation over basal (means $\pm$ S.E.M.) from a single experiment. Similar results were obtained in at least three separate experiments.

Condition	Stimulation (fold)	
	PY20 immunoprecipitates	Anti-p85 immunoprecipitates
Insulin	10.0 ± 0.8	1.3 ± 0.3
PMA	0.7 ± 0.1	0.6 ± 0.1

we and others have shown that insulin induces recruitment of p85 to membranes [22–23a], PMA actually caused a rapid decrease in membrane-associated p85 (Figure 1). Further, insulin caused a large stimulation of PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates, whereas PMA treatment did not increase PI 3-kinase activity in these immunoprecipitates (Table 2). It is also unlikely that PMA causes covalent modifications that result in increases in the catalytic activity of p85/p110, as the PI 3-kinase activity in p85 immunoprecipitates from whole-cell lysates was actually slightly decreased by PMA treatment (Table 2). These data demonstrate that PMA does not stimulate $\text{PtdIns}(3,4,5)\text{P}_3$ production by the same mechanisms as insulin.

The mechanism whereby PMA stimulates PI 3-kinase remains to be established, although a number of possible explanations arise. It is possible that a readily reversible stimulation of p85/p110 PI 3-kinase is occurring which may not be detected in p85 immunoprecipitates. Such a case exists with stimulation of PI 3-kinase by interactions with Ras [24]. It is also possible that novel isoforms of PI 3-kinase are involved, such as the PI 3-kinases that are stimulated by the $\beta\gamma$ -subunits of heterotrimeric G-proteins [25,26]. Further, it remains a possibility that PMA is not directly stimulating PI 3-kinase activity and that the increase in $\text{PtdIns}(3,4,5)\text{P}_3$ may arise from a mass action effect consequent to the increase in $\text{PtdIns}4\text{P}$ and also perhaps $\text{PtdIns}(4,5)\text{P}_2$. In this regard, it has previously been established that PMA blocks the breakdown of $\text{PtdIns}(4,5)\text{P}_2$ [27], which could result in diversion of substrate to PI 3-kinase. It is also possible that PMA could inhibit $\text{PtdIns}(3,4,5)\text{P}_3$ 5'-phosphatase activity [28] and

Table 3 Measurement of glucose transport

Overnight serum-starved 3T3-L1 adipocytes were preincubated with DMSO, 100 nM wortmannin or 10 ng/ml LY294002 for 5 min prior to stimulation with either 10 nM insulin or 1 μ M PMA for a further 20 min. 2-Deoxy[3 H]glucose uptake was measured as described in the Materials and methods section. Data are shown as fold stimulation above glucose transport rate in the presence of vehicle or inhibitor alone (means $\pm$ S.E.M. of triplicate determinations). Similar results were obtained in three independent experiments.

Condition	Stimulation (fold)		
	+ Vehicle	+ LY294002	+ Wortmannin
Insulin	7.45 $\pm$ 0.04	0.82 $\pm$ 0.05	1.12 $\pm$ 0.04
PMA	1.81 $\pm$ 0.09	1.04 $\pm$ 0.05	1.08 $\pm$ 0.08

cause accumulation of PtdIns(3,4,5) P_3 , although preliminary experiments indicate that this is unlikely to be the case in this system (P. R. Shepherd and B. T. Navé, unpublished work).

Regardless of the mechanism used to stimulate PtdIns(3,4,5) P_3 production, the increased level of this phospholipid is likely to have important physiological consequences. We observed that the relative degree of stimulation of glucose transport in response to PMA and insulin (Table 3) was roughly proportional to the degree of stimulation of PtdIns(3,4,5) P_3 production (Table 1) induced in response to these two agents. This suggests a relationship between PI 3-kinase activity and the stimulation of glucose transport. Therefore the possibility that stimulation of glucose transport by PMA requires PI 3-kinase activity was investigated using the specific PI 3-kinase inhibitors wortmannin [29] and LY294002 [30] at concentrations of inhibitor thought to be specific for inhibiting PI 3-kinase. It has previously been demonstrated that wortmannin and LY294002 inhibit insulin-stimulated glucose transport by blocking recruitment of glucose transporters to the plasma membrane [13,31]. Wortmannin and LY294002 blocked insulin stimulation of glucose transport and also blocked the ability of PMA to stimulate glucose transport in 3T3-L1 adipocytes (Table 3). This demonstrates that PI 3-kinase activity is necessary for both insulin- and PMA-stimulated translocation of glucose transporters to the plasma membrane in 3T3-L1 adipocytes.

In summary, these results demonstrate that PMA and insulin stimulate PtdIns(3,4,5) P_3 production by distinct mechanisms in 3T3-L1 adipocytes. The ability of both insulin and PMA to increase PtdIns(3,4,5) P_3 production may represent a convergent signalling pathway utilized by both of these agents to stimulate glucose transport in the cell.

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