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# Phorbol esters inhibit inositol phosphate and diacylglycerol formation in proliferating HL60 cells

## Relationship to differentiation

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Phorbol 12-myristate 13-acetate (PMA) induces the differentiation of the human promyelocytic cell line, HL60, towards adherent macrophage-like cells within 2 days. We have examined the early effects of PMA on inositol phosphates and on diacylglycerol production, two second messengers derived from inositol lipids. In proliferating HL60 cells, PMA induced a time- and concentration-dependent decrease in inositol phosphate levels. Maximal effects were seen after 1 h at 10 nM PMA. PMA also induced the translocation of protein kinase C from the cytosol to the membrane. Comparison between the differentiating effects of several phorbol esters and of 1-oleoyl-2-acetylglycerol with their ability to inhibit inositol phosphate formation suggests that the two effects are correlated.

Protein kinase C; Inositol phosphate; Diacylglycerol; Phorbol 12-myristate 13-acetate; Differentiation; (HL60 cell)

## 1. INTRODUCTION

It is now well established that polyphosphoinositide phosphodiesterase (PPI-pde) activation is one route for generating intracellular signals. A wide range of receptor-directed agonists are coupled to this signalling system and this includes some growth factors and mitogens [1]. The products of PPI-pde activation are $IP_3$ and DG and both have second messenger functions. $IP_3$ mobilises intracellular $Ca^{2+}$ and DG activates a calcium- and phospholipid-dependent protein kinase (protein kinase C). Both of these second messengers are involved in the regulation of numerous functional responses of the cell including cell proliferation.

The tumour promoter, PMA, induces the differentiation of HL60 cells, a human promyelocytic cell line, towards an adherent macrophage-like cell [2,3]. Evidence of macrophage differentiation is detectable within 24 h and the process is completed within 48 h. Differentiation is accompanied by arrest of proliferation [2,4]. PMA is also known to activate directly protein kinase C and to translocate the enzyme from the cytosol to the plasma membrane [5,6]. The respective roles of protein kinase C activation and of the translocation of this enzyme into plasma membranes in the process of differentiation are not yet clear. Indeed, molecules which activate protein kinase C such as OAG or byrostatin do not induce differentiation of HL60 cells [6-8].

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*Abbreviations:* OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; PRA, phorbol 12-retinoate 13-acetate; 4$\alpha$PDD, 4$\alpha$-phorbol 12,13-didecanoate; IPs, total inositol phosphates; $IP_3$, inositol trisphosphate; DG, diacylglycerol

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Here, we investigate the role of PMA on inositol phosphate production in HL60 cells during the initial stages of differentiation. If the levels of $IP_3$ and DG are responsible for proliferation, we would predict that PMA may cause cessation of proliferation by inhibiting the formation of the second messengers, $IP_3$ and DG. We demonstrate that PMA does decrease the level of inositol phosphates and DG during the reversible step which precedes cell commitment towards a macrophage-like cell.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Human transferrin, bovine insulin, PMA, PDBu, PRA, 4$\alpha$PDD and OAG were purchased from Sigma. The purchase of all other materials has been described [9].

### 2.2. *Methods*

Culturing and labelling of HL60 cells with [$^3$H]inositol have been described previously [9]. After labelling, cells were washed 2–3 times in RPMI and then resuspended at $25 \times 10^6$ cells/ml in RPMI containing 15% fetal bovine serum (FBS) and LiCl (10 mM). PMA (or other related agents) was then added to the cells for the indicated times as detailed in the figure legends.

Aliquots of cells (typically 200 $\mu$l) were taken from control or PMA-treated cells at the indicated times and the reaction quenched with chloroform/methanol (1 : 2, v/v). Analysis of inositol phosphates was carried out as in [10]. In brief, the aqueous phase obtained after extraction of the lipids was loaded onto Dowex columns. Total inositol phosphates (IPs) were eluted with 1 M ammonium formate in 0.1 M formic acid. When the inositol lipids were also analysed, extraction of lipids was carried out with acidified chloroform/methanol and the lipids separated by TLC as in [11]. Lipids were localised by autoradiography. After evaporation of the solvent, the plates were sprayed with EN$^3$HANCE and exposed to Kodak X-Omat film for 24 h at −70°C. The individual spots containing the lipids of interest were scraped off and transferred into scintillation vials. Methanol (250 $\mu$l) was added to elute the lipids before addition of scintillant.

To measure DG, cells were labelled for 48 h in RPMI supplemented with FBS by adding 2 $\mu$Ci/ml of [$^3$H]glycerol to the culture medium. After labelling, the cells were washed and resuspended at $25 \times 10^6$ cells/ml in fresh culture medium. After treatment with PMA, the reaction was quenched with chloroform/methanol (1:2, v/v). After lipid extraction, DG was separated by TLC as in [12] and visualised by autoradiography as described above.

Protein kinase C activity was measured in membrane and cytosol fractions prepared from control HL60 cells and cells pretreated with PMA. After incubation of the cells with PMA (10 nM) for 1 h, the cells were washed, sonicated and the membranes separated from the cytosol by ultracentrifugation as described [13]. Protein kinase C activity was measured in both fractions as in [14].

## 3. RESULTS

The levels of inositol-containing phospholipids and of inositol phosphates in HL60 cells were measured after labelling the cells for 48 h. On the assumption that the cells had reached isotopic equilibrium, the distribution of radioactivity in the individual inositol-containing compounds would give a measure of the relative amounts present. The distribution of [$^3$H]inositol radioactivity among the inositol lipids and inositol phosphates was phosphatidylinositol (84%), phosphatidylinositol phosphate (5%), phosphatidylinositol bisphosphate (2.5%), $IP_1$ (inositol monophosphate) (6.4%), $IP_2$ (inositol bisphosphate) (1.8%) and $IP_3$ (0.3%). This shows that phosphatidylinositol is the major inositol-containing compound and the inositol phosphates represent less than 10% of the total inositol-containing components.

$IP_1$ can be derived either directly from PI or from sequential hydrolysis of $IP_3$ via $IP_2$. In intact cells, degradation of $IP_3$ back to inositol is rapid and this cycle can be interrupted by adding $Li^+$

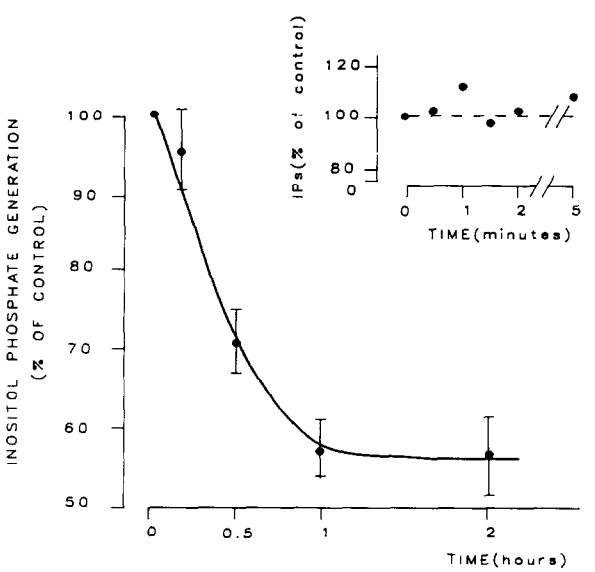


Fig.1. Level of inositol phosphates in HL60 cells after incubation with PMA for different times. [$^3$H]Inositol-labelled cells were incubated with 10 nM PMA and at the indicated times, aliquots were removed for the determination of inositol phosphates. Results are expressed as the percentage of inositol phosphates in control cells incubated under the same conditions. Results are means ± SD of 5 independent experiments done in triplicate. Inset represents the time period between 0 and 5 min.

which blocks the conversion of $IP_1$ back to inositol. Therefore, all experiments were performed by measuring the total inositol phosphates (IPs) without further separation.

Fig.1 shows the time course of generation of IPs after the addition of PMA (10 nM), the concentration which has been shown to induce differentiation of about 90% of cells in 2 days. No significant change in levels of IPs was observed for at least 5 min (see inset to fig.1). After 10 min, the level of IPs begins to decline, reaching about 55% of the control level after 1 h and then plateaus. Since the ratios of the individual inositol lipids or their respective amounts were not changed after 1 h treatment with PMA (not shown), the effect of PMA is likely to be exerted at the level of formation of IPs. Fig.2 illustrates the concentration dependence of PMA (measured after 1 h incubation). A significant decrease in generation of IPs is observed with 3 nM and is maximal by 10 nM.

It would be expected that a decline in IPs would be accompanied by a decrease in DG levels. Therefore, we measured the DG levels in cells pretreated with PMA (10 nM). As reported in fig.3, the DG levels declined to 40 ± 3% of the control level after 1 h and remained at that level for another hour.

We also studied the effect of molecules related to PMA on production of IPs after 1 h incubation

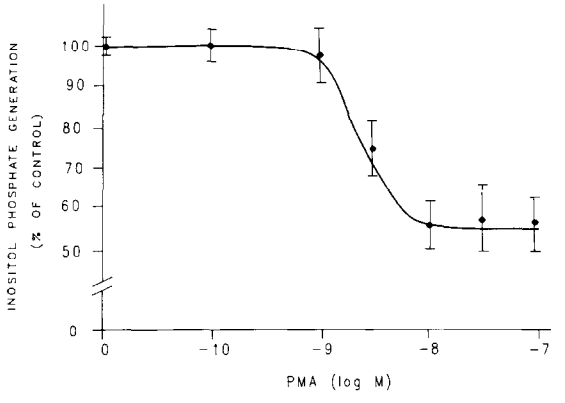


Fig.2. Level of inositol phosphates in HL60 cells after incubation with different concentrations of PMA. [$^3$H]Inositol-labelled cells were incubated for 1 h with different concentrations of PMA followed by the determination of inositol phosphate levels. Results are expressed as the percentage of inositol phosphates in control cells incubated under the same conditions. Results are means ± SD of 3 independent experiments done in triplicate.

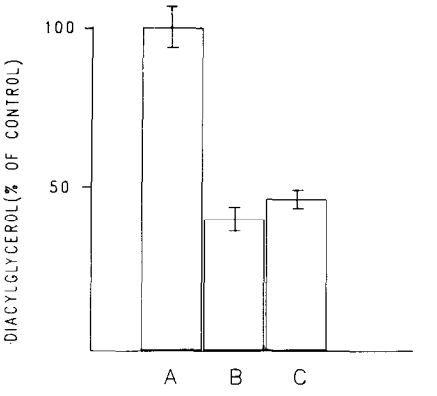


Fig.3. Level of diacylglycerol in control and PMA-treated HL60 cells. [$^3$H]Glycerol-labelled cells were incubated for 1 and 2 h without or with PMA (10 nM) and the radioactivity in DG was measured. A, control cells (100%) after 1 and 2 h incubation (1377 ± 88 cpm); B,C, percentage of control levels after incubation with PMA (10 nM) for 1 and 2 h, respectively. Results are means ± SD of 2 similar experiments done in triplicate.

(table 1). It is clear that molecules such as PDBu, which acts in the same way as PMA, produce the same degree of inhibition of production of IPs even if higher concentrations are necessary to achieve the same decrease. PRA also decreases production of IPs but is less potent than PMA, whereas 4$\alpha$PDD and OAG have no effect on their production.

As it is known that PMA not only activates protein kinase C but also translocates it from the cytosol to the plasma membranes, we investigated

Table 1

Effect of phorbol esters and OAG on inositol phosphate production

| Concentration | PMA | PRA | PDBu | 4$\alpha$PDD | OAG |
|---|---|---|---|---|---|
| $10^{-8}$ M | 55 ± 5 | 102 ± 13 | 83 ± 4 | 113 ± 10 | nd |
| $10^{-7}$ M | 55 ± 6 | 78 ± 9 | 74 ± 6 | 105 ± 6 | nd |
| $10^{-6}$ M | nd | 74 ± 10 | 62 ± 1 | 115 ± 5 | nd |
| 50 $\mu$g/ml | nd | nd | nd | nd | 101 ± 3 |
| 100 $\mu$g/ml | nd | nd | nd | nd | 108 ± 3 |

HL60 cells, prelabelled with [$^3$H]inositol, were washed and incubated for 1 h at 37°C in the presence of the indicated concentrations of the different molecules. The level of inositol phosphates was determined as described in section 2. Results are expressed as percentage of IPs generated in control cells incubated under the same conditions. Results are means ± SD of 3 independent experiments all done in triplicate. nd, not determined

Table 2

Distribution of protein kinase C activity in control and PMA-treated HL60 cells

| | Protein kinase C activity (pmol histone phosphorylated/mg protein per min) | |
|---|---|---|
| | Cytosol | Membrane |
| Control cells | 430 | 158 |
| PMA-treated cells | 105 | 579 |

HL60 cells were treated with 10 nM PMA for 1 h at 37°C and then washed and disrupted by sonication. The particulate fraction was separated from the cytosol and protein kinase C activity was then determined in both fractions

whether translocation was effective after 1 h with 10 nM PMA (table 2). It is clear that the bulk of the activity of protein kinase C is located in the cytosol in untreated cells whereas most of the measured protein kinase C activity is recovered in membranes after the cells have been incubated for 1 h with 10 nM PMA. Moreover, after 1 h incubation in the presence of PMA, no loss of protein kinase C activity can be observed.

## 4. DISCUSSION

In this study, we demonstrate that PMA, which induces differentiation of HL60 cells towards macrophage-like cells within 48 h, leads to a decrease in IPs and DG after 1 h. The concentrations required for the induction of differentiation and the decline in IPs are similar, suggesting that the inhibition of PPI-pde activity is likely to play a role in the onset of differentiation itself. The ability of the different phorbol esters to induce differentiation is comparable to their ability to inhibit the level of IPs. For example, PRA and PDBu, which induce differentiation at higher concentrations and to a lesser extent than PMA [2,4,15], cause a smaller decrease in generation of IPs with higher drug concentrations than PMA does. In contrast, OAG and 4$\alpha$PDD, which cannot induce differentiation [2,4,8], are also unable to cause a decrease in IPs.

Phorbol esters exert their biochemical effect by activating protein kinase C leading to translocation of the enzyme to the membranes (table 2) but whether this accounts for the differentiating effect in HL60 cells is not clear. For example, both OAG and bryostatin can activate protein kinase C without causing differentiation of HL60 cells [6,8,16]. It is possible that directional translocation of protein kinase C towards plasma membranes and long-lasting increased membrane-bound protein kinase C activity might be the determining factors of whether an activator of protein kinase C can lead to differentiation. The importance of directional protein kinase C translocation and of its association to plasma membranes has been underlined in two studies. The first was performed on PMA-differentiation-resistant HL60 subclones and demonstrated that the translocation of protein kinase C to plasma membranes did not occur in the subclones when compared to the sensitive wild type [17]. The second study showed that in two cell lines which can be differentiated with PMA (HL60 cells and K561), protein kinase C was translocated to plasma membranes whereas in PMA-insensitive cell lines (CHO and E75KS), translocation of protein kinase C occurred towards nuclear membranes [18].

Retinoic acid and DMSO induce differentiation of HL60 cells towards granulocytes and both inhibit production of IPs before cell commitment (unpublished; and [19]). DMSO also induces the differentiation of Friend erythroleukemia cells [20] and PMA, that of a pre-B lymphocytic cell line [21], and in both cases, each agent provokes inhibition of inositol lipid metabolism very early in the differentiation process. Differentiation induced by all of these agents is preceded by a loss of self-renewal potential. Therefore, the observed inhibition in inositol lipid metabolism might well be a common signal for arrest of proliferation and/or initiation of differentiation.

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