Phorbol ester inhibits phosphatidylserine synthesis in human promyelocytic leukaemia HL60 cells

Possible involvement of free radicals and correlation with phosphorylation of nuclear protein 1b

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Treatment of human promyelocytic leukaemia HL60 cells in conditioned medium with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) for 4 h resulted in 25–30% inhibition of labelling of phosphatidylserine (PS) with [^{14}C]serine. PS labelling was 40% lower, and no inhibitory TPA effect was observed when the experiments were performed in fresh medium. Cycloheximide or puromycin also inhibited PS labelling by 38–44%; their inhibitory effects were non-additive with that of TPA and occurred only in conditioned medium. Catalase (CAT) and superoxide dismutase (SOD), both free-radical scavengers, and H7, a protein kinase C inhibitor, reversed to various extents the inhibitory effect of TPA on PS synthesis. On the other hand, chlorobenzoic acid, a free-radical-generating agent, also inhibited PS synthesis by 22% after 4 h treatment when conditioned medium was used. When ethanolamine was added to cells in conditioned medium to quench PS formation through the exchange of free serine with the ethanolamine moiety of phosphatidylethanolamine (PE), PS labelling was decreased by 33% and the inhibitory TPA effect was significantly decreased. On the other hand, ethanolamine had marginal quenching effect on PS labelling when added to cells in fresh medium. TPA increased the phosphorylation of various proteins in the cells, including protein 1b (M_r 80000; pI 5.5) shown to be localized mainly in the nuclear fraction. Chlorobenzoic acid selectively stimulated the phosphorylation of protein 1b, whereas CAT and SOD specifically attenuated the TPA-stimulated phosphorylation of this protein. All these agents affected phosphorylation of protein 1b only if conditioned medium was used. The findings suggested that net synthesis of PS through the base-exchange mechanism was stimulated in HL60 cells by cell products present in the conditioned medium. TPA inhibited this stimulated PS synthesis by a mechanism which appeared to involve active oxygen species and protein synthesis and might be related to the phosphorylation of protein 1b.

INTRODUCTION

Protein kinase C (PKC) has been shown to regulate many cellular functions including cell growth and differentiation [1,2]. In many cell lines [3–6], including HL60 cells [7], the enzyme is distributed predominantly in the cytosol as an inactive form. Treatment of the cells with phorbol esters such as TPA, or with agents shown to elevate endogenous levels of diacylglycerol, facilitate binding of the enzyme to plasma membranes [5,8–14]. Activation of the enzyme occurs through the formation of a quaternary complex on the membrane surface involving PKC, Ca^{2+} , PS and diacylglycerol [15–17]. Phorbol esters can substitute for diacylglycerol, the physiological activator of enzyme, formed mostly by stimulus-coupled breakdown of phosphatidylinositol [18].

Activation of PKC requires a very low Ca^{2+} concentration in the presence of diacylglycerol or TPA, so that an increased intracellular Ca^{2+} seems unnecessary for the enzyme activation *in vivo* [19–21]. In comparison, the presence of biomembranes or PS is essential for maximal enzyme activity [16,17]. It is plausible that agents that affect PS synthesis could potentially modulate PKC

activity and other membrane functions in cells. Studies addressing this specific problem appear lacking, probably because PS metabolism is not likely to be modified, at least acutely, by agents. In the present paper we report a long-term effect of TPA in HL60 cells on PS synthesis which appeared to be related to a rapid phosphorylation of an 80 kDa nuclear protein, 1b.

MATERIALS AND METHODS

Materials

TPA, CAT, SOD, BHT and PS were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; OAG was from Avanti Polar Lipids, Birmingham, AL, U.S.A.; ampholites (pH ranges 3.5–10 and 5–7) were from LKB Instruments, Gaithersburg, MD, U.S.A.; [^{32}P]orthophosphate (carrier-free) and L-[^{14}C]serine (135 mCi/mmol) were from ICN Radiochemicals, Irvine, CA, U.S.A.

Cell culture

The human promyelocytic cell line HL60 [22], obtained through the American Type Culture Collection (Rockville Pike, MD, U.S.A.) and having a doubling time of

Abbreviations used: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; OAG, *sn*-1-oleoyl-2-acetyl-glycerol; CAT, catalase; SOD, superoxide dismutase; BHT, butylated hydroxytoluene; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PMSF, phenylmethane sulphonyl fluoride.

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about 28 h, was continuously cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY, U.S.A.) supplemented with 20% (v/v) heat-inactivated fetal-calf serum, penicillin (50 units/ml), streptomycin (50 µg/ml) and glutamine (2 mM). Cells were harvested for experiments at a density of $(0.6\text{--}0.8) \times 10^6/\text{ml}$.

Radiolabelling of cellular proteins

Unless stated otherwise, incubations were carried out in conditioned medium. Cells in a Falcon flask (at a density of $0.8 \times 10^6/\text{ml}$) were incubated in RPMI 1640 medium with [^{32}P]P_i (0.5–0.6 mCi/ml) for 4 h. Aliquots (1 ml) of the cells were incubated in Eppendorf tubes containing CAT (25 µg/ml), SOD (25 µg/ml) or BHT (10 µM) during the last 1 h of incubation period. Some samples were also incubated with TPA (100 nM) or OAG (25 µg/ml) during the last 30 min. The ^{32}P incorporation was terminated by pelleting (10000 g, 5 s) then lysing the cells with 50 µl of the gel sample buffer [23]. Stock solutions of CAT, SOD, BHT and OAG were made in RPMI medium. TPA was dissolved in dimethyl sulphoxide; the concentration of the solvent in the incubation mixtures never exceeded 0.05% (v/v) and had no effect on protein synthesis or PS synthesis.

Two-dimensional polyacrylamide-gel electrophoresis

The procedures were those of O'Farrell [24] modified by Steinberg & Coffino [23]. The first-dimension isoelectric-focusing gel contained 2% ampholytes of pH ranges of 3.3–10 (Figs. 1 and 2 below) or 5–7 (Figs. 3–5 below). Portions (30–50 µl) of the samples corresponding to 0.8×10^6 cells (30 µg of protein) were applied. Total acid-precipitable radioactivity $[(1\text{--}2) \times 10^5 \text{ c.p.m.}]$ in various samples from the same experiment differed from each other by less than 10%. The first-dimension gels were run for 7000 V · h. The second-dimension separation was performed using sodium dodecyl sulphate-polyacrylamide (10%) gels. The proteins were stained with Coomassie Blue and the ^{32}P -labelled proteins were detected by autoradiography of the dried gels by exposing Kodak XAR-5 films for 5–6 days at -20°C using Kodak Omat intensifying screens. For the determination of the pH gradient, the entire first-dimension gel was cut into 0.2 cm slices, homogenized in ion-exchanged water and, after centrifugation, the pH values of the supernatants were measured. The validity of this approach was confirmed by using standard proteins with known pI values.

Measurements of radioactivity in specific proteins

In order to quantify the changes in ^{32}P content of phosphoproteins, they were located on the dried gels by autoradiography, excised from the gels and counted for radioactivity in a liquid-scintillation counter. Pieces of the gels of the same sizes were also excised from the radioactive-free areas and served as blanks. The radioactivity in some major proteins, whose ^{32}P -labelling was unaltered by TPA, was also measured and served as controls to assess a possible experimental variability. Since ^{32}P -labelling of each of these control proteins differed only slightly (less than 10%) in all experiments, we did not correct the radioactivity of proteins 1a and 1b reported in Table 3 (below).

Alkali treatment of ^{32}P -labelled proteins

To screen for the presence of phosphotyrosine residues,

the two-dimensional electrophoretograms were treated with 1M-KOH at 55°C for 2 h as described by Cooper *et al.* [25].

Measurement of PS synthesis in intact cells

Cells were suspended either in the same medium in which they were growing for 3 days (conditioned medium) or in fresh RPMI 1640 medium and incubated with [$\text{U-}^{14}\text{C}$]serine (1 µCi/ml) at a density of $1 \times 10^6/\text{ml}$ for 4 h. The cells were then pelleted as described above, and phospholipids were extracted with a mixture of chloroform/methanol (2:1, v/v), separated by silica-gel t.l.c., and the radioactivity in PS was determined as reported previously [26,27]. Before chromatography, 2 µg of PS was added to each sample to aid identification of the lipid.

Measurement of PS synthesis in cell homogenates

Untreated cells or cells treated with TPA (100 nM) for 4 h were pelleted and resuspended in 20 vol. of ice-cold buffer consisting of 10 mM-Tris/HCl, pH 7.4, 240 mM-sucrose, 2 mM-EGTA, 2.5 mM-MgCl₂, 10 mM-mercaptoethanol, 0.5 mM-leupeptin and 1 mM-PMSF (freshly prepared). The cell suspension was passed through a syringe (25 G 5/8 needle) 20 times to rupture the cells, and the homogenates were used immediately for PS-synthesis experiments. Incubation conditions employed had been predetermined to be optimal for PS synthesis via the base-exchange reaction [27]. Briefly, the assay mixture (0.2 ml) contained 1 µM-[$\text{U-}^{14}\text{C}$]serine (0.5 µCi), 20 mM-Tris/HCl, pH 7.4, 0.7 mM-CaCl₂, 0.2 mM-EGTA, 0.1 mM-ATP, 2 mM-MgCl₂, 1 mM-hydroxylamine, 10 µg of sonicated PE, 24 mM-sucrose, 1 mM-mercaptoethanol, 0.05 mM-leupeptin, 0.1 mM-PMSF and homogenate equivalent to 100 µg of protein. Some incubation mixtures also contained 5 µg of sonicated PS plus 100 mM-TPA. Incubations were carried out at 37°C for 10 min and were terminated by the addition of 5 ml of chloroform/methanol (2:1, v/v).

Separation of ^{32}P -prelabelled subcellular fractions

Cells were incubated with [^{32}P]P_i (0.5 mCi/ml) for 6 h in the absence or presence of 100 nM-TPA, which was added for the last 1 h of the incubation period. Cells were pelleted and washed essentially free of [^{32}P]P_i, followed by homogenization as described above. Subcellular fractionation of the cells was carried out as described by Perrella *et al.* [28], except that the homogenization buffer contained 50 mM-KF to inhibit phosphoprotein phosphatase activity. Briefly, the homogenates, containing essentially no intact cells, were centrifuged at 1000 g for 5 min, followed by three washes of the pellets (nuclear fraction) using the same buffer. The original supernatants (postnuclear fraction) and the washes were combined and were centrifuged at 20000 g for 30 min to yield the crude membrane fraction (pellet) and cytosol plus endoplasmic-reticulum fraction (supernatant). Proteins in these fractions, along with the nuclear fraction, were precipitated with 10 vol. of ice-cold acetone and resuspended in 50 µl of gel sample buffer [23]. Portions of the samples, corresponding to 1.5×10^6 cells for the individual subcellular fractions and 0.75×10^6 cells for the total homogenates, were used for the two-dimensional electrophoresis and subsequent autoradiography as described above.

Table 1. Effects of TPA, cycloheximide, antioxidant enzymes and H7 on PS labelling in HL60 cells

PS labelling was measured by incubating the cells for 4 h with [^{14}C]serine as described in the Materials and methods section. CAT (25 $\mu\text{g/ml}$), SOD (25 $\mu\text{g/ml}$) and H7 (50 μM) were added to the cell culture 30 min before the addition of [^{14}C]serine, but TPA (100 nM) and cycloheximide (4 $\mu\text{g/ml}$) were added together with the radioactive serine. The results presented are means $\pm$ S.E.M. for three determinations. The findings were confirmed in two other separate experiments. Statistical significance: *significantly different from control ($P < 0.05$); **significantly different from TPA alone ($P < 0.05$).

Addition	[^{14}C]PS (c.p.m./ 10^6 cells)
None (control)	3124 $\pm$ 31
TPA	2197 $\pm$ 83*
Cycloheximide	1750 $\pm$ 48*
TPA + cycloheximide	1580 $\pm$ 41*
CAT	3066 $\pm$ 34
SOD	2951 $\pm$ 47
H7	3077 $\pm$ 69
TPA + CAT	3183 $\pm$ 26**
TPA + SOD	2943 $\pm$ 39**
TPA + H7	2951 $\pm$ 47**

Statistical methods

The unpaired t test was used. P values of < 0.05 were considered significant.

RESULTS

Effects of TPA, cycloheximide, H7 (an inhibitor of PKC [36]) and antioxidant enzymes on PS labelling in HL60 cells incubated in conditioned medium

Treatment of HL60 cells with TPA for 4 h in conditioned medium resulted in a 30 % inhibition of PS labelling, as measured by incorporation of [^{14}C]serine into PS (Table 1). A shorter treatment (1 h) with TPA had no effect, whereas 2 h treatment caused 12 % inhibition (results not shown). TPA appeared to have no effect on PS degradation, because a 4 h incubation of the cells prelabelled with the radioactive serine in the absence or presence of TPA yielded a similar decrease (40 %) in the amount of radioactive PS (results not shown). The observations were consistent with the hypothesis that TPA regulated PS labelling at the level of base-exchange, a reaction involved in PS synthesis and turnover in mammalian cells [29–32]. Cycloheximide also inhibited PS synthesis by 44 % and the inhibition was only slightly higher (50 %) in the presence of TPA (Table 1), suggesting that TPA, like cycloheximide, might affect the synthesis of the base-exchange enzyme or a modulator protein. A longer incubation of the cells with some of the agents was not feasible in the present studies because, for example, an 8 h treatment with cycloheximide caused a 20–30 % cell death and a 6 h treatment with TPA caused some cells to adhere to substratum, a characteristic of differentiating cells. In the latter situation the uptake of [^{14}C]serine was decreased. It has been reported that TPA induces the synthesis of ornithine decarboxylase, and this effect is suppressed by antioxidant agents

Table 2. PS synthesis in homogenates of HL60 cells treated, or not treated, with TPA

PS synthesis was measured for 10 min in homogenates prepared from HL60 cells treated, or not treated, with 100 nM-TPA for 4 h. The homogenates were assayed for the incorporation of [^{14}C]serine into PS in 10 min in the presence or absence of TPA (100 nM) + PS (25 $\mu\text{g/ml}$). Other experimental conditions were as indicated in the Materials and methods section. The results presented are means $\pm$ S.E.M. for three experiments, with each assay being performed in duplicate. *Indicates significantly different from the control ($P < 0.05$).

Treatment	Addition ...	[^{14}C]PS formation (pmol/10 min per mg of protein)	
		None	TPA + PS
None (control)		11.4 $\pm$ 0.5	13.1 $\pm$ 0.7
TPA		6.3 $\pm$ 0.6*	7.8 $\pm$ 0.5

such as CAT, SOD and BHT [33,34], indicating the involvement of active oxygen species. We found here that CAT and SOD, although without effect when present alone, markedly reversed the inhibitory effect of TPA on PS synthesis (Table 1). Because uptake of the enzymes by HL60 cells might be slow [35], for a significant effect to occur it was necessary to pretreat the cells with them for 30 min before the addition of TPA. The inhibitory effect of TPA was also reversed by H7 (Table 1). H7 is an inhibitor [36] and TPA is an activator [1,2] of PKC; it is likely, therefore, that the enzyme might play a role in inhibition of PS synthesis.

We next examined the effect of TPA on PS synthesis from [^{14}C]serine in the homogenates of HL60 cells. Pretreatment of the cells with TPA for 4 h decreased PS synthesis by about 45 % in the cell-free system (Table 2), similar to that seen in the intact cell system shown above in Table 1. A combination of TPA and PS failed to inhibit PS synthesis in the homogenates of the cells treated or not treated with TPA (Table 2). The results clearly indicated that TPA had no direct effect on PS synthesis and that this effect was an indirect one that required treating the intact cells with TPA.

Comparative effects of TPA, chlorobenzoic acid, puromycin and ethanolamine on PS labelling in conditioned and fresh medium

If cells were washed twice and then resuspended in fresh medium, instead of conditioned medium, we observed 42 % decrease of PS labelling (Table 3) accompanied by the complete loss of the inhibitory TPA effect. Chlorobenzoic acid, a free-radical-generating agent, also inhibited PS labelling in the conditioned, but not in the fresh, medium. It should be added that chlorobenzoic acid had no significant inhibitory effect after 2 h treatment (results not shown). Puromycin, similarly to cycloheximide, decreased PS labelling by 38 % in the conditioned medium, but only 13 % in the fresh medium. Thus all these agents shared the common feature that they significantly inhibited PS labelling only in the conditioned medium.

We observed in the cell-free system that ethanolamine,

Table 3. Comparative effects of TPA, chlorobenzoic acid, puromycin and ethanolamine on PS labelling in conditioned or fresh medium

PS labelling was measured by incubating the cells for 4 h with [U- 14 C]serine as described in the Materials and methods section. The results presented are means $\pm$ S.E.M. of four determinations. The experiment was repeated twice with similar results. *Indicates significantly different from control ($P < 0.05$).

Addition	Medium ...	[14 C]PS (c.p.m./ 10^6 cells)	
		Conditioned	Fresh
None (control)		3004 $\pm$ 92	1752 $\pm$ 58
TPA (100 nM)		2128 $\pm$ 124*	1901 $\pm$ 65
Chlorobenzoic acid (10 μ M)		2356 $\pm$ 58*	1826 $\pm$ 76
Puromycin (1 μ g/ml)		1869 $\pm$ 37*	1523 $\pm$ 61
Ethanolamine (10 mM)		2006 $\pm$ 87*	1631 $\pm$ 47
Ethanolamine (10 mM) + TPA (100 nM)		1783 $\pm$ 49*	1710 $\pm$ 52

when present at concentrations 10-fold higher than those of serine, inhibited by more than 90 % the exchange of free serine with the ethanolamine moiety of PE (results not shown). When 10 mM-ethanolamine was added to cells in conditioned medium, we observed a 33 % decrease

of PS labelling, and no further decrease was noted in the presence of TPA (Table 3). Ethanolamine at this concentration did not affect the uptake of [14 C]serine (results not shown). In contrast, ethanolamine inhibited only 7 % of the labelling of cellular PS in fresh medium (Table 3). Choline at 20 mM did not affect PS synthesis (results not shown), indicating that PC was a poor substrate for the exchange enzyme.

We have attempted to quantify possible changes in PS content after treating the cells with TPA for 4 h in conditioned medium. In the five experiments performed we observed a slightly decreased (10 %) PS content in TPA-treated cells; however, owing to the relatively large experimental error (15 %), this change could not be considered significant.

Effects of TPA, antioxidant agents and chlorobenzoic acid on protein phosphorylation in HL60 cells

TPA increased phosphorylation in HL60 cells of several proteins, notably protein 1b (M_r 80 000; pI 5.5) and proteins 2–6, but it decreased phosphorylation of protein 1a (M_r 80 000; pI 5.7) (Fig. 1, cf. *a* and *b*). Unlike the stimulatory effects on proteins 2–6, the TPA-induced changes in the phosphorylation state of proteins 1a and 1b occurred only when cells were incubated in conditioned medium. The increased phosphorylation was presumably catalysed by PKC (a serine and threonine kinase) activated by TPA. Treatment of the slab gels with 1 M-

Fig. 1. Effects of CAT, SOD and BHT on TPA-stimulated protein phosphorylation in HL60 cells

Cells were labelled with [32 P] P_i for 4 h. CAT (25 μ g/ml), SOD (25 μ g/ml) and BHT (10 μ M) were added to the cell cultures for the final 1 h, whereas TPA (100 nM) was added for the final 30 min of the labelling period. Equal amounts of protein (30 μ g; 1.2×10^5 c.p.m. of acid-precipitable radioactivity) was loaded on to each electrophoresis gel. Autoradiographs were obtained by exposing the gels to X-ray film for 6 days. Cells were untreated (*a*) or were treated with TPA (*b*), TPA + CAT (*c*), TPA + SOD (*d*) or TPA + BHT (*e*).

Table 4. Effect of TPA and antioxidants on phosphorylation of proteins 1a and 1b in HL60 cells

The ^{32}P content of the proteins was directly measured as described in the Materials and methods section. CAT (25 $\mu\text{g}/\text{ml}$), SOD (25 $\mu\text{g}/\text{ml}$) and BHT (10 μM) were added to the cell cultures at the final 1 h, whereas TPA (100 nM) was added during the final 30 min of the 4 h labelling period. Results presented are means $\pm$ S.E.M. for three experiments. The values in parentheses are the actual radioactivities (c.p.m.) of three control samples, which were taken as '100%'. Statistical significance: *significantly different from control ($P < 0.01$ – 0.005); **significantly different from TPA alone ($P < 0.05$).

Addition	Protein ...	^{32}P labelling (% of control)	
		1a	1b
None (control)		100 $\pm$ 12 (174 $\pm$ 19)	100 $\pm$ 15 (64 $\pm$ 10)
TPA		32 $\pm$ 10*	435 $\pm$ 26*
TPA + CAT		85 $\pm$ 14**	160 $\pm$ 57**
TPA + SOD		111 $\pm$ 28**	215 $\pm$ 42**
TPA + BHT		76 $\pm$ 17**	198 $\pm$ 51**

KOH at 55 $^{\circ}\text{C}$ for 1 h resulted in a complete loss of ^{32}P in these proteins, indicating the apparent absence of tyrosine phosphorylation (results not shown). In addition, H7 inhibited the stimulatory effects of TPA (results not shown). It was observed that antioxidant agents CAT (Fig. 1c), SOD (Fig. 1d) and BHT (Fig. 1e) decreased the TPA-induced phosphorylation of protein 1b, but conversely increased that of protein 1a. As judged from their relative electrophoretic mobility and the identical apparent M_r , it was likely that proteins 1a and 1b were the same protein with different phosphorylation states, with protein 1b being more highly phosphorylated. The antioxidant agents, however, had only a very minimal effect on the TPA-stimulated phosphorylation of proteins 2–6. It should be noted here that the reversal effect of antioxidant agents on the opposite effects of TPA on phosphorylation of proteins 1a and 1b required a pretreatment of the cells with these

agents for 30 min, a situation similar to their reversal effect on the TPA-inhibited PS synthesis mentioned above in Tables 1 and 2. The actual radioactivity in proteins 1a and 1b under various incubation conditions, taken from three separate experiments such as shown in Fig. 1, are summarized in Table 4.

In order to establish the identity of proteins 1a and 1b, Coomassie Blue stainings of these and other proteins in the electrophoretic gels were examined. The amounts of proteins 1a and 1b were comparable in HL60 cells under the control condition (Fig. 2a). TPA increased the amount of protein 1b accompanied by a concomitant decrease in that of protein 1a (Fig. 2b) and, furthermore, CAT reversed the TPA effect (Fig. 2c). These findings are consistent with the effects of TPA on phosphorylation of proteins 1a and 1b and their reversal by CAT shown above in Fig. 1, indicating that the phosphoproteins represented two differently phosphorylated forms of the same protein.

Since the free-radical-generating agent chlorobenzoic acid inhibited PS labelling in a similar fashion to TPA, we decided to compare its effect with that of TPA on protein phosphorylation in the same experiment. TPA (Fig. 3b) increased phosphorylation of several proteins, including protein 1b (4.0-fold), whereas chlorobenzoic acid specifically increased (2.5-fold) the phosphorylation of protein 1b (Fig. 3c). In additional studies we found that OAG, a membrane-permeable diacylglycerol and a potent activator of protein kinase C [37,38], increased phosphorylation of protein 1b as well as that of proteins 2–6, and decreased that of protein 1a, and that CAT reversed the effects of OAG on protein 1 phosphorylation (results not shown). These findings clearly indicated similar actions of OAG and TPA in intact HL60 cells.

Subcellular localization of protein 1b and other proteins

A fairly recent report [39] described a rapid phosphorylation of an 80 kDa nuclear matrix protein after treatment of HL60 cells with TPA. Since protein 1b has a similar M_r , and TPA did not appear to increase phosphorylation of any other proteins in this M_r range even when studied using a broader pH range (Fig. 1), protein 1b and the previously described nuclear protein [39] could be identical. To test this possibility, the subcellular localization of protein 1b and other phospho-

Fig. 2. Coomassie Blue staining of proteins 1a and 1b in HL60 cells

Incubation conditions of the cells and the protein patterns in the electrophoretic gels were as in Fig. 1. Cells were untreated (a) or treated with 100 nM-TPA (b) or 100 nM-TPA + CAT (25 $\mu\text{g}/\text{ml}$) (c).

Fig. 3. Comparison of the effects of TPA and chlorobenzoic acid on protein phosphorylation in HL60 cells

Cells (0.8×10^6 /ml) were labelled with [32 P]P_i for 6 h. Cells were untreated (a) or treated with 100 nM-TPA for the final 30 min (b) or with 10 μ M-chlorobenzoic acid for the final 1 h (c). Acid-precipitable radioactivity (30 μ g of protein; 2.4×10^5 c.p.m.) was loaded on to each gel. Autoradiographs were obtained by exposing the gels to X-ray films for 5 days.

Fig. 4. Subcellular distribution of protein 1b and other protein in HL60 cells

The 32 P-prelabelled cells were treated with 100 nM-TPA, and the subcellular fractions were prepared as described in the Materials and methods section. The homogenates were from the untreated (a) or the TPA-treated cells (b), whereas the nuclear (c), 20000 g pellet (d) and 20000 g supernatant (e) fractions were from the TPA-treated cells. r_1 , r_2 and r_3 are reference proteins.

proteins was examined in the TPA-stimulated HL60 cells prelabelled with [32 P]P_i. It was found that protein 1b phosphorylation in the homogenates was much higher in the TPA-treated cells (Fig. 4b) than in the control cells (Fig. 4a). Protein 1b was largely associated with the nuclear fraction (Fig. 4c) compared with the pellets (Fig. 4d) and supernatant fraction (Fig. 4e) obtained by centrifuging the postnuclear fraction at 20000 g . Dephosphorylation of protein 1b could occur in each fraction during the centrifugation steps, which could lead to misinterpretation of data shown in Fig. 4. However,

inspection of silver-stained gels, similar to those shown in Fig. 2, revealed that about 70% of protein 1b was associated with the nuclear fraction (results not shown), indicating that 50 mM-KF included in the homogenization buffer effectively inhibited dephosphorylation of this protein. The data suggested that protein 1b was likely to be identical with the 80 kDa TPA-sensitive nuclear protein reported by others [39]. Of the other proteins of interest, proteins 2 and 5 also appeared to be localized in the nuclear fraction, whereas protein 3 was mostly in the 20000 g pellets (crude membranes).

DISCUSSION

TPA has been shown to stimulate PC and PE synthesis in various cell types [40–45], including HL60 cells [46,47]. In the present studies we demonstrated that TPA conversely inhibited PS synthesis in HL60 cells without enhancing PS degradation. Incorporation of ^{14}C -labelled serine into PS has been shown to occur through exchange of free serine with either the ethanolamine or serine moiety of PE or PS [29–32], but only the former process leads to a net PS synthesis. Judged from the effect of ethanolamine on PS labelling in conditioned medium, it appeared that about 30–40% of the ^{14}C serine was incorporated into PS through an exchange reaction with PE and that most of the incorporation of ^{14}C serine reflected PS turnover. In fresh medium, the net synthesis of PS from PE was decreased, as concluded from a small inhibitory effect of ethanolamine (Table 2). Thus it seemed that the conditioned medium contained a cell-derived factor which specifically stimulated the synthesis of PS from PE. Since TPA inhibited only the ethanolamine-inhibitable portion of PS labelling and this inhibition was also observed for exogenous PE added to the homogenates, we concluded that TPA in fact counteracted the stimulatory effect of the putative cellular product.

Except for the likely involvement of PKC, the mechanism by which TPA exerts such inhibitory action is not known. The present data, however, indicated the involvements of active oxygen species, because antioxidant agents effectively attenuated, whereas the free-radical-generating chlorobenzoic acid mimicked, the TPA effect. Others have reported previously that antioxidant enzymes such as CAT and SOD suppressed the TPA effects on ornithine decarboxylase synthesis [33,34], chromosome breakage [48,49] and tumour promotion [50,51].

One primary effect of free radicals is on gene expression. The effect of TPA on PS synthesis, likely to be mediated through free radicals, might therefore occur in the nucleus. This contention is supported by the observations that (a) a significant inhibitory effect developed only after a 4 h treatment of the intact HL60 cells with TPA and (b) cycloheximide and puromycin mimicked the TPA action. In contrast with inhibition of PS synthesis, stimulation of protein phosphorylation was rapid in HL60 cells treated with TPA. Antioxidant agents decreased the TPA-stimulated phosphorylation of protein 1b, which may be identical with the 80 kDa TPA-sensitive nuclear phosphoprotein described previously [39]. Furthermore, chlorobenzoic acid, a free-radical-generating agent, stimulated phosphorylation of protein 1b in a manner similar to that in which TPA stimulated. Because of its occurrence in the nucleus, rapid phosphorylation of protein 1b might be related to gene expression, leading to subsequent inhibition of PS synthesis. Protein 1b appeared to be unique in this respect, since the antioxidant enzymes specifically inhibited its phosphorylation without affecting other proteins whose phosphorylation was also stimulated by TPA. It is unclear as to how the active oxygen molecules can stimulate phosphorylation of nuclear protein. It is likely, however, that they could mediate the effect of TPA on the phosphorylation of protein 1b, because significant accumulation of protein kinase C in the nucleus requires several hours of TPA

treatment (Z. Kiss, E. Deli & J. F. Kuo, unpublished work).

We observed in related studies that, in HL60 cells, TPA stimulated about 3-fold the incorporation of ^{14}C -ethanolamine or ^{32}P P_i into PE (Z. Kiss, E. Deli & J. F. Kuo, unpublished work). Because PE can serve as a substrate, one would expect an increased synthesis of PS in the presence of TPA. It appears that there is an inhibitory pathway in HL60 cells, initiated by PKC activators, which insures that cellular PS content will not increase drastically as the PE content increases. In addition, since multiple metabolic pools of phospholipids may exist [52], a sustained activation of PKC may result in a significant decrease of PS content in the metabolically more active compartments. Identification of the hypothetical inhibitory factor for PS synthesis would be helpful in the elucidation of the regulatory pathway.

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