

Phorbol ester stimulates the synthesis of sphingomyelin in NIH 3T3 cells

A diminished response in cells transformed with human *A-raf* carrying retrovirus

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The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulated the synthesis of sphingomyelin (CerPCho) from a [¹⁴C]choline-labelled phosphatidylcholine (PtdCho) pool in NIH 3T3 cells. Maximal stimulation (68%) of CerPCho synthesis, accompanied by an increase (38%) in its cellular content, required only 2 nM TPA. Higher concentrations of TPA (2–100 nM) had progressively less effect on CerPCho synthesis which correlated with increased hydrolysis of precursor PtdCho. In cells transformed with human or mouse *A-raf* carrying retroviruses TPA-stimulated PtdCho hydrolysis, but not CerPCho synthesis, suggesting independent regulation of these processes by the TPA-stimulated signal transduction system.

Phorbol ester; Sphingomyelin synthesis

1. INTRODUCTION

Sphingomyelin is a major phospholipid of mammalian cell membranes [1]. Its synthesis occurs by the transfer of the phosphorylcholine group from PtdCho to ceramide [2–10], catalyzed by PtdCho:ceramide phosphotransferase. The main, although not exclusive, site of CerPCho synthesis both in fibroblasts and baby hamster kidney cells is the plasma membrane [5–9]. This correlates with predominant plasma membrane localization of CerPCho [11–13].

CerPCho and PtdCho show markedly different behaviour in bilayer membrane systems. Thus, CerPCho increases while PtdCho decreases the

viscosity of biological membranes ([1] and references therein). In addition, CerPCho has a particularly high affinity for cholesterol [14–16], another membrane rigidifying agent. Furthermore, various metabolites of CerPCho appear to have important regulatory functions. Notably, sphingosine was shown to exhibit multiple biological actions including inhibition of PKC [17], stimulation of PKC-independent phosphorylation of EGF receptor [18], an increase in the binding affinity of EGF receptor [18], and inhibition of thyrotropin-releasing hormone binding to pituitary cells [19]. These effects of sphingosine, or its derivatives, may be related to various disorders, such as Niemann-Pick disease, atherosclerosis and certain types of cancer, known to be associated with altered sphingolipid metabolism [1].

Because of a possible interrelationship between PKC and CerPCho metabolism, studies were initiated to determine if PKC activation might be involved in regulating the synthesis or degradation of

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Abbreviations: CerPCho, sphingomyelin; PtdCho, phosphatidylcholine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; EGF, epidermal growth factor

this important lipid. The potent phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was used to activate PKC within intact cells. This tumor promoter binds to and stimulates PKC, the primary cellular phorbol ester receptor which mediates the effects of TPA [20–22]. A second approach was to determine CerPCho metabolism in NIH 3T3 cells transformed with human or mouse *A-raf* carrying retroviruses. *Raf* genes encode cytoplasmically located proteins which possess serine/threonine-specific protein kinase activity [23–25]. Importantly, the c-*raf* and A-*raf* proteins show significant primary and secondary amino acid sequence homology to PKC [26]. In this communication we have assessed whether TPA treatment of NIH 3T3 cells modulates CerPCho synthesis and whether this response is altered in cells transformed with A-*raf* and v-*raf*.

2. MATERIALS AND METHODS

2.1. Materials

TPA, PtdCho and CerPCho were purchased from Sigma; [*methyl*-¹⁴C]choline chloride (50 μ Ci/mmol) was from Amersham.

2.2. Cell culture

Untransformed NIH 3T3 clone 7 and its transformed variants were used. Transformations were performed with retroviruses carrying either human A-*raf* (NIH 3T3/6A-*raf* MSV; [26]) or mouse A-*raf* (NIH 3T3/9iV-*raf* MSV; [27]) oncogenes. Cells were cultured continuously in DMEM medium (Gibco) supplemented with 10% fetal calf serum, penicillin-streptomycin (50 U/ml and 50 μ g/ml, respectively), and glutamine (2 mM). Cells (passages 16–34) were in the logarithmic growth phase in each experiment.

2.3. Labelling of CerPCho and PtdCho with [¹⁴C]choline

Cells were incubated with [*methyl*-¹⁴C]choline (0.1 μ Ci/ml) for 4 h in the absence or presence of varying concentrations of TPA. At the end of incubation the ¹⁴C label-containing medium was removed and 4 ml of chloroform/methanol (1:1, v/v) was added directly to the plates. After 1 min the extracts were transferred to glass tubes and the plates washed with a chloroform/methanol mixture (4 ml). Phospholipid extraction was continued for at least 1 h and then CerPCho and PtdCho were separated by thin-layer chromatography as described earlier [28,29].

2.4. Measurement of CerPCho synthesis and PtdCho degradation during the chase period in cells prelabelled with [¹⁴C]choline

In the experiments shown in figs 2 and 3, cells were incubated for 24 h with [*methyl*-¹⁴C]choline (0.1 μ Ci/ml), washed twice with 5 ml of fresh medium, followed by further incubation (8 h) in the presence of 25 mM unlabelled choline to dilute the en-

dogenous free pool of ¹⁴C-labelled choline and its nonlipid intermediates. In fig. 3, the last 5 h of incubation is considered to be the chase period. At the end of the incubation period, cells were processed to determine the [¹⁴C] content of phospholipids as described above.

2.5. Measurement of degradation of CerPCho during the chase period in cells prelabelled with [¹⁴C]serine

Cells were incubated with [U-¹⁴C]serine (1 μ Ci/ml) for 48 h to label the sphingosine portion of CerPCho. Cells were washed and first incubated for 3 h to eliminate non-lipid [¹⁴C]labelled intermediates of serine. Cells were washed again, and then reincubated in the absence or presence of TPA (0.5–100 nM) for 4 h.

3. RESULTS

In the continuous presence of [*methyl*-¹⁴C]-choline, radiolabelling of both PtdCho and CerPCho was stimulated in NIH 3T3 cells exposed to TPA (fig. 1). In both cases significant effects were observed at TPA concentrations as low as 0.5 nM, although the labelling of CerPCho was increased to a greater extent than that of PtdCho. The most likely explanation for this labelling pattern was that TPA stimulated CerPCho synthesis, so pulse-chase experiments were performed to study this possibility.

Since CerPCho synthesis occurs by an intramembrane mechanism [9], (i.e. membrane-bound substrates are preferentially utilized [8,9]), this method of radiolabelling with [*methyl*-¹⁴C]choline seemed to be more suitable than *in vitro* methods using external substrates. However, to utilize radiolabelled endogenous PtdCho as a substrate it was necessary to first determine the end point of the pulse period, i.e. when no further labelling of PtdCho occurred. In the case of NIH 3T3 cells, replacement of [¹⁴C]choline with a high concentration of unlabelled choline resulted in a significant decrease in PtdCho labelling only after the 3rd hour of chase (fig. 2). However, during the subsequent 5 h period, when no labelled choline and choline intermediates remained in cells (not shown), both the decrease in PtdCho labelling and the increase in CerPCho synthesis were constant (fig. 2).

Under the experimental conditions established in fig. 2, studies were carried out to determine the effect of increasing concentrations of TPA on CerPCho synthesis in control and A-*raf*-transformed NIH 3T3 cells (fig. 3). CerPCho synthesis in NIH 3T3 was stimulated 36% by TPA at concentrations

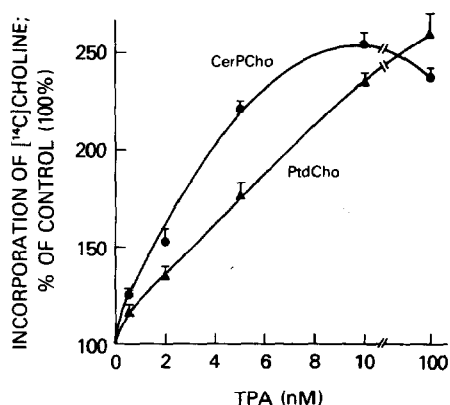


Fig. 1. Stimulatory effect of TPA on the incorporation of [14 C]choline into sphingomyelin and phosphatidylcholine in NIH 3T3 cells. Cells were incubated with [14 C]choline for 4 h in the absence or presence of varying concentrations of TPA as indicated. Each point represents the mean $\pm$ SE of three determinations of [14 C]choline-labelled CerPCho ($\bullet$ - $\bullet$) and PtdCho ($\blacktriangle$ - $\blacktriangle$). Similar results were obtained in two other experiments.

as low as 0.5 nM (half-maximal effect), while a full stimulatory effect (68%) required only 2 nM TPA. At concentrations above 2 nM, TPA was either without apparent effect (10 nM) or was even inhibitory (100 nM). In NIH 3T3 cells transformed with the human *A-raf* carrying retrovirus TPA had no significant stimulatory effect at low concentrations but retained its apparent inhibitory effect toward CerPCho synthesis at higher concentrations (fig. 3). Similarly, transformation of NIH 3T3 cells by either mouse *A-raf* or *v-raf* carrying retroviruses also resulted in the loss of a stimulatory effect on CerPCho synthesis by low concentrations of TPA, while the inhibitory effect (60–70%) of 100 nM TPA was retained (not shown).

If TPA affected only one of the sites of CerPCho synthesis then increased labelling of this phospholipid might not truly reflect changes in its actual cellular content. To quantitate changes in the amount of CerPCho, cells were labelled with [14 C]choline for 48 h to reach a radioisotopic equilibrium among various choline intermediates. Treatment of these prelabelled cells with 2 nM TPA for 4 h increased the amount of CerPCho from 8.3 ± 0.8 to 11.4 ± 0.5 nmol/ 10^6 cells ($n=4$). In contrast, TPA again failed to significantly elevate the cellular content of CerPCho in the *raf*-

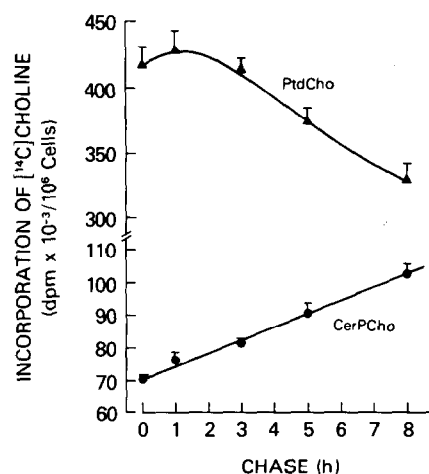


Fig. 2. Continuous increase of incorporation of [14 C]choline into sphingomyelin during the chase period in NIH 3T3 cells. Cells were pulse-labelled with [14 C]choline, washed, and reincubated (chase period) for up to 8 h. Each point represents the mean $\pm$ SE of three determinations. ($\bullet$ - $\bullet$), CerPCho; ($\blacktriangle$ - $\blacktriangle$), PtdCho.

transformed cells. These data indicate that stimulation of synthesis is associated with the observed increase in the cellular content of CerPCho.

Several different mechanisms might be considered to explain the inhibitory phase of the TPA effect on CerPCho synthesis. To examine if TPA treatment altered the degradation of CerPCho, control and *A-raf*-transformed cells were first preincubated with [14 C]serine to equilibrium to label the sphingosine portion of CerPCho. The half-life time of this labelled CerPCho was determined to be 12 and 16 h in the control and transformed cells, respectively. Exposure of these [14 C]serine-labelled cells to increasing concentrations (0.5–100 nM) of TPA was found not to alter the degradation rate of CerPCho (not shown). In another study [30] TPA also failed to stimulate the breakdown of CerPCho in GH₃ pituitary cells. To elucidate further the mechanism of TPA inhibition of CerPCho synthesis, cells were incubated with [14 C]choline for 48 h until radioisotopic equilibrium was attained and then the cells were treated with 100 nM TPA. As shown in fig. 4, TPA enhanced the loss of 14 C-labelled PtdCho and 14 C-labelled CerPCho to a similar extent in both the control and *raf*-transformed cells, during a 6 h chase period. In other experiments it was found

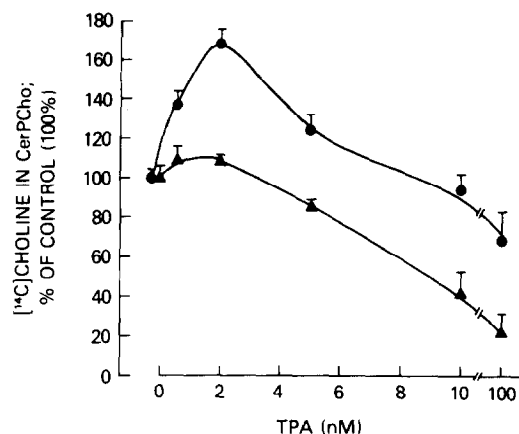


Fig. 3. Concentration-dependent effect of TPA on sphingomyelin synthesis in NIH 3T3 and NIH 3T3/6A-raf MSV cells. NIH 3T3 (●-●) or NIH 3T3/6A-raf MSV cells (▲-▲) were pulse-labelled with [*methyl*-¹⁴C]choline, washed, incubated for 3 h in the absence of TPA and then for 5 h in the presence of varying concentrations of TPA. Each point represents the mean $\pm$ SE of three determinations. Similar results were obtained in two other experiments.

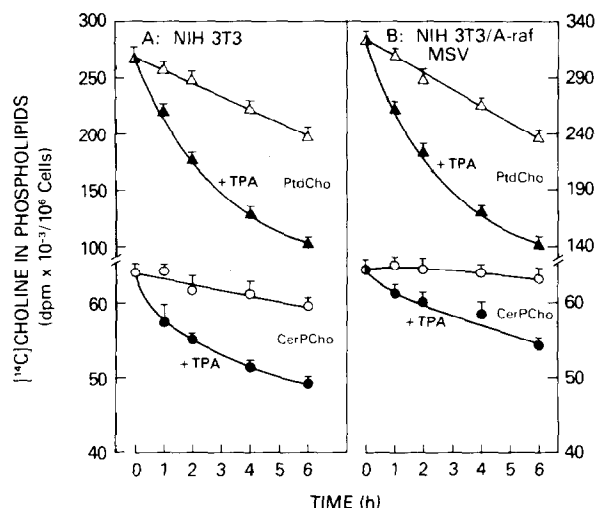


Fig. 4. TPA-induced loss of ¹⁴C-label from PtdCho and CerPCho during the chase period. Both NIH 3T3 (A) and NIH 3T3/6A-raf MSV (B) cells were labelled with [*methyl*-¹⁴C]choline for 48 h. Cells were washed, then incubated for 3 h at 37°C in cell culture medium, and washed again. Finally, the washed ¹⁴C-labelled cells were further incubated in the absence (open symbols) or presence (closed symbols) of 100 nM TPA for 0–6 h as indicated. Each point represents the mean $\pm$ SE of three determinations of CerPCho (circles) and PtdCho (triangles).

that 2 nM TPA had little effect on PtdCho hydrolysis, and that a maximal effect required more than 10 nM TPA. These data are compatible with a mechanism where a TPA-induced decrease of the prelabelled substrate (PtdCho) pool results in a proportional decrease in the synthesis of radiolabelled product (CerPCho).

4. DISCUSSION

The results presented indicate that treatment of NIH 3T3 cells with low concentrations of TPA stimulates the synthesis of CerPCho. Since PKC is the main cellular target of TPA [20], it is likely that this stimulatory effect of TPA on CerPCho synthesis is initially mediated through the activation of this protein kinase. The unusual sensitivity of CerPCho synthesis to low concentrations of TPA suggests that (i) only a small pool, or a special subtype, of PKC may be involved, and that (ii) a relatively small increase in diacylglycerol level, the physiological activator of PKC [20,21], may elicit a similar response. It is possible that the effect of TPA and/or diacylglycerol is self-potentiating, due to the concomitant formation of diacylglycerol during CerPCho synthesis, and is terminated only when other regulatory processes (e.g. substrate loss or downregulation of PKC) become dominant.

While this manuscript was in preparation, Wilson et al. [31] reported that in neutrophils TPA stimulated the synthesis of ceramide and, thereby, decreased the amount of free sphingosine. It is possible that at a low rate of PtdCho hydrolysis (i.e. at 0.5–2 nM TPA) ceramide was the rate limiting substrate for CerPCho synthesis in NIH 3T3 cells. In this case stimulation of ceramide synthesis could be reflected in a higher rate of CerPCho synthesis. The mechanism(s) by which the level of sphingolipids and their precursors is regulated is (are) of obvious interest since sphingosine and its derivatives may be natural inhibitors of PKC [17,32] and may also regulate the binding of various hormones to their receptors [18,19]. An interesting aspect of the TPA effect on sphingolipid metabolism is that a possible depletion of sphingosine stores may synergize with diacylglycerol activation of PKC. The significance of these postulated mechanisms would be that even small increases in diacylglycerol content could generate a relatively large pool of activated PKC molecules,

provided that the stimulatory mechanism for CerPCho synthesis exists. An exception may exist with GH₃ pituitary cells where TPA does not appear to stimulate either the hydrolysis of PtdCho or the synthesis of CerPCho [30]. On the other hand both processes were stimulated in human leukemic HL60 cells [33].

It is of interest that CerPCho synthesis, apparently regulated either directly or indirectly through activation of PKC, was not stimulated by TPA treatment of cells transformed by *A-raf* or *v-raf* carrying retroviruses.

c-raf is the cellular homologue of *v-raf*, the transforming gene of the murine sarcoma virus 3611 [34,35]. The role of the *raf* oncogene products appears to be that of serine/threonine-specific protein kinases with sequence homology to PKC [21,23-25]. In a mechanism similar to that for PKC [36-38], oncogenic activation of the *c-raf* and *A-raf* protein can occur by an amino-terminal truncation which removes a putative regulatory region [23,25,26]. This suggests that the kinase activity of *c-raf* and *A-raf* may be modulated by regulatory ligands similar to the regulatory properties of PKC.

The results presented in figs 3 and 4 show that transformation of NIH 3T3 cells with *A-raf* or *v-raf* carrying retroviruses negates the ability of TPA to enhance CerPCho synthesis. On the other hand, *raf* transformation of NIH 3T3 cells did not significantly alter the TPA-stimulated degradation of PtdCho. These results suggest a possible inter-relationship between PKC and *raf* kinase to regulate biological processes. Further, they indicate that the *raf* kinase may alter (either directly or indirectly) the metabolism of sphingomyelin and other phospholipids as part of the process involved in malignant transformation of cells.

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