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Dual effect of the phorbol ester TPA on arachidonic acid release from HeLa cells

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The tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induces release of arachidonic acid (AA) from HeLa cells with a maximum at 2–3 h. Subsequently the extracellular level of AA decreases. Cycloheximide (CH, 10^{-5} M) does not influence the release of AA, however, it causes the AA level to remain elevated. In the presence of TPA and CH (i) re-uptake of AA is not altered, (ii) re-incorporation of AA into phosphatidylinositol (and phosphatidylethanolamine) is largely increased, and (iii) the level of lysophosphatidylinositol is elevated. The latter two phenomena can be prevented by fluocinolone acetonide (10^{-8} M), i.e. by inhibition of phospholipase A_2 (PLA_2). These data point to a continuously elevated PLA_2 activity in the presence of TPA and CH. The phorbol ester appears to induce a proteinaceous principle which diminishes PLA_2 activity.

Tumor promoter; Phospholipid metabolism; Phospholipase A_2; Arachidonic acid

1. INTRODUCTION

The tumor promoter TPA has been shown to cause numerous metabolic changes in various cells, particularly with regard to membrane constituents [1,2]. Besides stimulation of PC turnover [3–8], probably as a consequence of the activation of a PC-specific phospholipase C [9,10], increased deacylation of phospholipids by PLA_2 has been observed [11–13] which yields AA. Metabolites formed from AA may be responsible for alterations in the genome [14–17].

This study was performed in order to learn more about the 'strategy' of the tumor promoter TPA in influencing AA metabolism in HeLa cells. This cell represents a suitable model system for the analysis of TPA-effected alterations of membrane metabolism [3,18,19]. Evidence is presented which indicates that, in response to TPA, AA release is turned on and off in a stepwise fashion.

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Abbreviations: AA, arachidonic acid; CH, cycloheximide; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

2. MATERIALS AND METHODS

TPA was a gift from Professor E. Hecker (German Cancer Research Center). It was kept in acetone as a 5×10^{-3} M stock solution at −70°C. Fluocinolone acetonide (FA), PGE_2, phospholipids, 1,2-dioctanoylglycerol, AA (Sigma, München), CH (Serva, Heidelberg), bovine serum albumin (BSA, electrophoretically pure; Biomol, Ilvesheim, FRG) were obtained from the sources quoted. [1-^{14}C]AA (2.2 MBq/μmol) as well as [1(3)-^{14}C]glycerol (1.11 MBq/μmol) were from Amersham Buchler (Braunschweig).

Cloned HeLa cells were cultivated as monolayers as described [20]. For experiments 9×10^5 cells were transferred to plastic Petri dishes (Falcon 3.5 cm diameter). Prelabeling with [1-^{14}C]AA (7.4 kBq/ml) was as described [21]. For analysis of lysophospholipids 6×10^5 cells were cultured for 24 h prior to prelabeling with [1(3)-^{14}C]glycerol (74 kBq/ml) in 1.5 ml complete medium for another 18 h period. The radioactive medium was replaced by 2 ml fresh medium containing 10% calf serum, if not otherwise indicated, as well as the compounds to be assayed. For determining the reincorporation rate of AA after

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induced AA release, unlabeled cells were challenged with the compounds to be tested. After the times indicated [1-^{14}C]AA (7.4 kBq/ml) was added for 0.5-h periods. Analysis of lipids from the medium as well as from cells was performed as in [21,22]. Extraction of lysophospholipids was carried out with *n*-butanol [23]. Separation of lysophospholipids was performed by two-dimensional chromatography [24].

Radioactivity was determined by use of a Linear Analyzer (Berthold, Wildbad, FRG). Experiments were performed at least in duplicate with comparable results. Values given are means of 3 dishes ± SD.

3. RESULTS AND DISCUSSION

HeLa cells were prelabeled with [^{14}C]AA as described [21] and then treated with TPA under various conditions. TPA, over a wide range of concentrations [21], induced the release of AA from HeLa cells with a peak at 2–3 h (shown for 10^{-8} M TPA in fig.1A). The release of AA occurred in the absence as well as presence of 10^{-5} M CH (fig.1A); in the latter case it even increased. This concentration of CH is sufficient to inhibit the incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material of HeLa cells within less than 1 h for at least 6 h by approx. 90%. These data, therefore, suggest that the TPA-induced release of AA in HeLa cells does not require de novo protein synthesis. Probably a PLA_2 becomes effective due to inactivation of an inhibitor [25–27]. The glucocorticoid fluocinolone acetonide (FA) at 10^{-8} M was capable of preventing the liberation of AA almost completely (fig.1A).

In the presence of various concentrations of TPA the level of released AA started to decrease by 2–3 h ([21]; shown for 10^{-8} M TPA in fig.1A) and reached control values by approx. 20 h [21]. In order to analyze whether this decrease was due to elevated uptake of AA by the cells or reduced liberation of AA, the following experiment was performed. HeLa cells were treated with 10^{-8} M TPA and pulsed at various times afterwards with radioactive AA for 30-min periods. HeLa cells incorporate AA linearly for almost 1 h (the amount of unlabeled AA released due to the action of TPA appears to be negligible in terms of isotope dilution). The data (fig.1B) demonstrate that the rate of incorporation of AA into cellular lipids was not significantly altered by pretreatment with TPA,

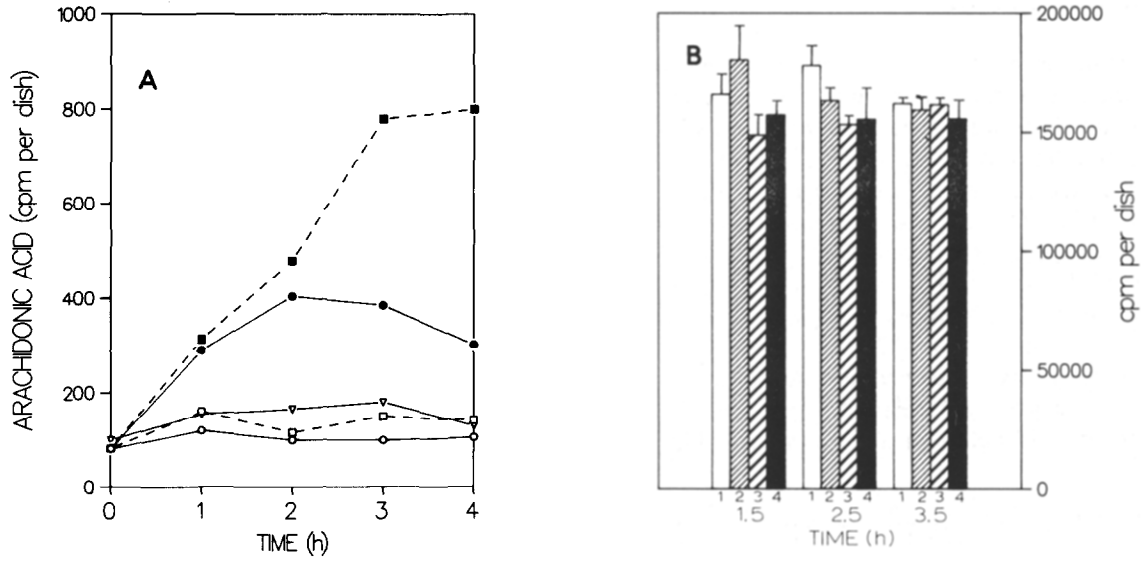

Fig.1. Influence of TPA on release (A) and incorporation (B) of AA by HeLa cells. (A) TPA-induced AA release and influence of CH. HeLa cells were prelabeled for 5 h with [1-^{14}C]AA and pretreated with CH (10^{-5} M) or FA (10^{-8} M) before replacing the radioactive medium by fresh complete medium containing acetone/PBS (○), acetone/CH (10^{-5} M) (□), TPA (10^{-8} M)/PBS (●), TPA (10^{-8} M)/CH (10^{-5} M) (■), or TPA (10^{-8} M)/FA (10^{-8} M) (▽). At the indicated time points the medium was analyzed for AA. (B) AA incorporation into cellular lipids of TPA-treated HeLa cells and influence of CH. Unlabeled HeLa cells were treated at zero time with acetone/PBS (1), acetone/CH (10^{-5} M) (2), TPA (10^{-8} M)/PBS (3), or TPA (10^{-8} M)/CH (10^{-5} M) (4). After 1, 2 or 3 h [1-^{14}C]AA was added for 30 min. Radioactivity in cellular lipid extracts was determined by liquid scintillation counting.

and certainly not stimulated. Therefore, a decrease of the extracellular AA level 2–3 h after addition of TPA appeared to be the result of reduction of AA release.

The decrease in extracellular AA level beyond 2–3 h may have different causes. By that time appreciable amounts of AA become metabolized and are released into the medium as prostaglandins (fig.2A and [21]) and to a smaller extent as HETE products (not shown). The appearance of AA metabolites in the medium accounted only partly for the decrease in AA level therein beyond 2–3 h after TPA addition. The metabolism of AA is largely prevented by CH (fig.2A), thus indicating that it requires de novo protein synthesis. Under these circumstances the AA level in the medium of TPA-treated cultures continues to stay elevated beyond 2–3 h (fig.1A).

The AA metabolites missing in the presence of CH did not account for the total increase of AA in the medium. In the presence of CH the TPA-effected activation of PLA_2 appeared to last longer than in its absence, i.e. CH appeared to prevent a decrease of PLA_2 activity. CH, therefore, may interfere with the TPA-induced de novo synthesis of a PLA_2-diminishing principle, either of an inhibitor [25–27] or of a modulating enzyme (e.g. a protease).

Evidence in support of elevated PLA_2 activity in the presence of TPA plus CH is provided by investigation of the incorporation of AA into phospholipids. On addition of radioactive AA to cells pretreated with TPA, increased labeling of PI and PE (not of PS and PC) was observed (fig.3A,B) which may indicate that these are the phospholipids previously deacylated [28]. This idea is supported by the observation that on inhibition of TPA-effected AA release by FA, no increase in AA incorporation into these phospholipids occurred (fig.3B), i.e. they were probably not deacylated to such a degree and therefore less available as substrates for reacylation. Under these circumstances FA prevented AA release (fig.1A) but did not influence the rate of AA uptake (not shown). The further increase of AA incorporation in the presence of TPA plus CH (fig.3A) would appear to indicate that even more substrate, particularly lyso-PI, was generated. This is indeed the case for lyso-PI as shown in fig.3B (inset) but not for lyso-PC (not shown). The incorporation of AA into PI, which is usually 1/3 of that of PC, almost approaches the PC level in the presence of TPA

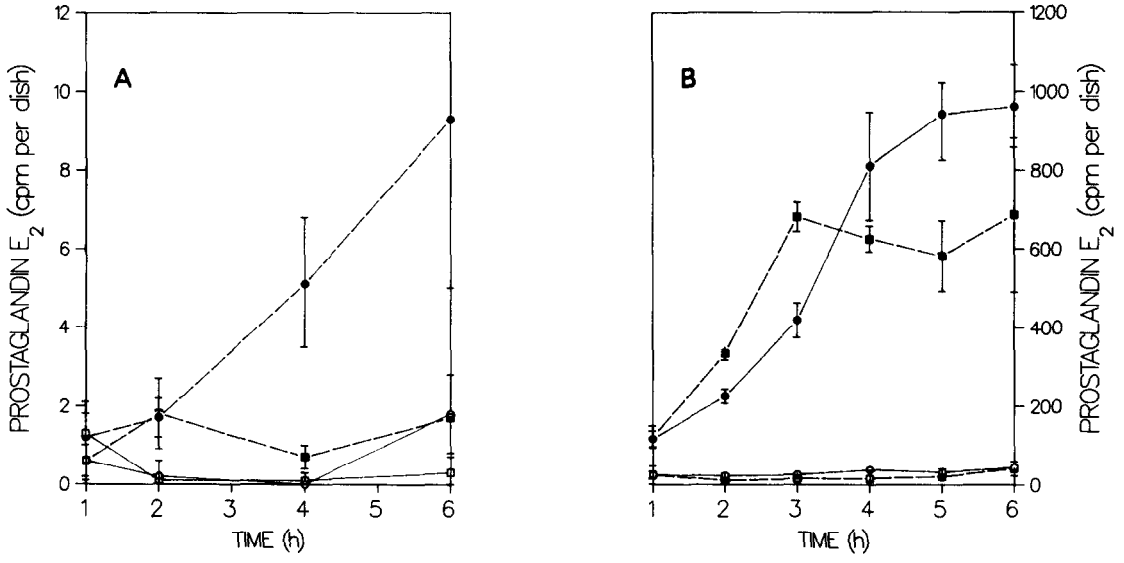

Fig.2. PGE_2 accumulation in the culture medium of HeLa cells under the influence of CH (A) and in the absence of serum (B). (A) At zero time prelabeled HeLa cells were treated with acetone/PBS (○), acetone/CH (10^{-5} M) (□), TPA (10^{-7} M/PBS (●) or TPA (10^{-7} M)/CH (10^{-5} M) (■), and the medium was analyzed for PGE_2. (B) The medium of prelabeled cells was replaced by fresh medium with (○,●) or without (□,■) 10% calf serum, and acetone (○,□) or TPA (3×10^{-8} M) (●,■) was added. At the indicated time points, the medium was analyzed for PGE_2.

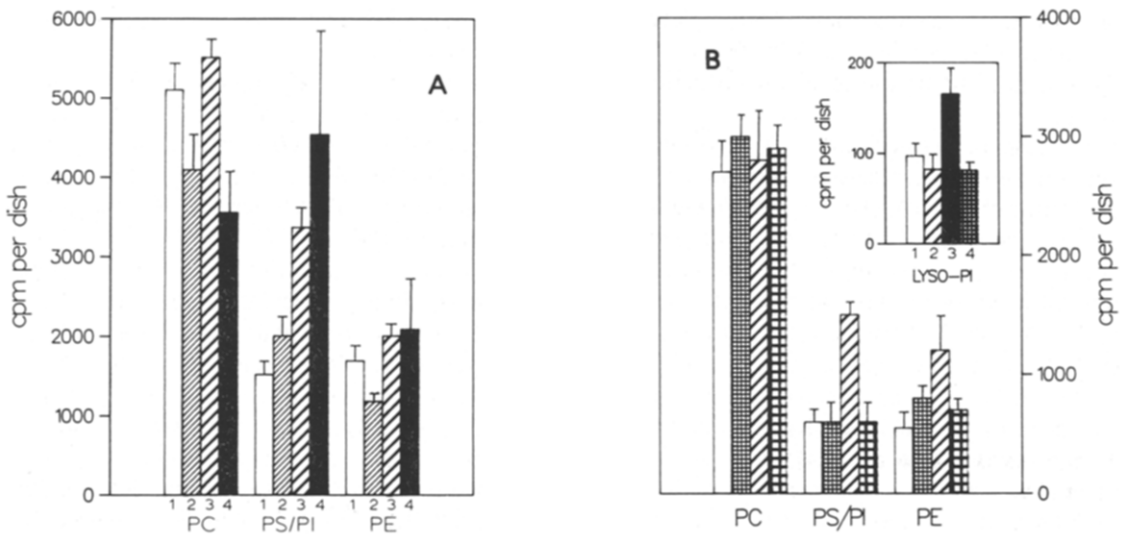

Fig.3. AA incorporation into cellular lipids of TPA-stimulated HeLa cells after treatment with CH (A) and FA (B). (A) Unlabeled HeLa cells were treated at zero time with acetone/PBS (1), acetone/CH (10^{-5} M) (2), TPA (10^{-8} M)/PBS (3), or TPA (10^{-8} M/CH (10^{-5} M) (4). After 2 h [1-^{14}C]AA was added for 30 min. The distribution of radioactive AA in the phospholipids was analyzed. (B) At zero time HeLa cells were treated with acetone (1), FA (10^{-8} M) (2), TPA (3×10^{-8} M) (3), or TPA (3×10^{-8} M)/FA (10^{-8} M) (4) for 2 h. Then [1-^{14}C]AA was added for 30 min and the labeling of phospholipids was analyzed. (Inset) For analysis of lysophospholipids cells were prelabeled for 18 h with [1(3)-^{14}C]glycerol and treated with acetone (1), TPA (10^{-8} M) (2), TPA (10^{-8} M)/CH (10^{-5} M) (3), or TPA (10^{-8} M)/FA (10^{-8} M) (4) for 2 h. Analysis of lysophospholipids was performed as described in section 2.

CH (fig.3A). These data argue for a more active state of a PLA_2 where CH was present.

Therefore, by the action of TPA the synthesis of a protein may become induced which decreases PLA_2 activity leading to decreased release of AA and as a consequence to a reduction of the AA level due to regular AA incorporation by the cells. In the presence of CH, however, the synthesis of this protein is prevented, thus allowing the PLA_2 to continue to liberate AA from cells leading to a plateau provided the release is balanced by the uptake.

Since all experiments described have been carried out in the presence of serum it was important to determine whether TPA was responsible for these cellular reactions. Therefore, TPA was applied in medium containing 0.5% BSA instead of serum. Under these conditions, increase and decrease in level of extracellular AA were observed also [21,28]. Similarly, prostaglandin release effected by TPA occurred in the presence of 10% serum as well as of 0.5% BSA or even in the absence of protein (fig.2B; under the latter condition AA release was not measurable; see [21]). These data demonstrate that the cellular responses were due to the action of TPA.

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