The nuclear-associated endoplasmic reticulum is an early target for the action of the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate in C3H/10T½ fibroblasts

Ian F. Pryme, Johan R. Lillehaug, Anders Fjose and Kjell Kleppe

Department of Biochemistry, University of Bergen, Årstadveien 19, 5000 Bergen, Norway

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12-*O*-Tetradecanoyl-phorbol-13-acetate (TPA) had a dual effect on the cellular membranes of C3H/10T½ cells in that it caused both a stimulation of [^{3}H]choline incorporation and an enhancement of the solubilization of choline from prelabelled cells. Subfractionation studies showed that the release of [^{3}H]choline occurred almost exclusively from nuclear-associated endoplasmic reticulum. The release was dependent on the presence of Mg^{2+} and Ca^{2+}, indicating an enzyme-mediated reaction. In vivo, TPA stimulated the incorporation of [^{3}H]choline into all subcellular fractions. The data indicate that the nuclear-associated endoplasmic reticulum represents an early target for TPA action.

1. INTRODUCTION

The tumor promoter, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), is highly lipophilic [1], and various studies indicate that early changes in the physical properties of the lipid phase of the cellular membranes take place when cells are exposed to TPA [2–6].

A recent report [7] has provided evidence of the TPA-mediated stimulation of the reaction catalyzed by CTP:phosphocholine cytidyltransferase in HeLa cells. Circumstantial evidence suggests that TPA treatment did not stimulate either choline kinase or CDP-choline:1,2-diacylglycerol phosphocholinetransferase activity.

In [8] it was shown that choline was incorporated into membranes of C3H/10T½ fibroblasts. They also showed that TPA stimulated the rapid release of choline from prelabelled cells, possibly through the activation of membrane-associated phospholipase(s).

Most investigations related to altered membrane behaviour caused by TPA have either been restricted to studies of the cellular membrane or to gross changes in total cellular phospholipid content. We have recently developed a technique which has enabled us to study 3 subfractions of ER membranes (heavy rough-HR, light rough-LR and smooth-S subfractions) in MPC-11 cells [9,10]. We have reported that the HR fraction appears in C3H/10T½ cells after a 24-h period of incubation with TPA but not in acetone-treated control cells [11]. The data presented here indicate that the nuclear-associated endoplasmic reticulum in C3H/10T½ cells represents an early target for TPA action.

Abbreviations: TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; ER, endoplasmic reticulum; NAER, nuclear-associated endoplasmic reticulum; PBS, phosphate-buffered saline 8.0 g NaCl: 0.3 g KCl: 0.75 g Na_2HPO_4: 0.2 g KH_2PO_4: 1.0 liter distilled H_2O (pH 7.2)

2. MATERIALS AND METHODS

2.1. *Chemicals and medium*

12-0-tetradecanoyl phorbol-13-acetate (TPA) was from PL-Biochemicals. Heat-inactivated foetal calf serum and Basal Medium Eagle (BME) were from Gibco, [methyl-^{3}H]choline chloride (15 Ci/mmol) and [methyl-^{14}C]choline chloride (52 mCi/mmol) were from the Radiochemical Centre (Amersham).

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2.2. *Cells and culture conditions*

Mouse embryo fibroblast C3H/10T½C18 cells were cultured as in [12].

2.3. *Cell disruption and isolation of subcellular fractions*

Procedures for cell disruption by nitrogen cavitation [13] and subcellular fractionation [14] have been described previously.

2.4. *Determination of radioactivity*

Radioactivity was measured either by collecting trichloroacetic acid insoluble material on glassfiber filters or by mixing aqueous aliquots with a water soluble scintillation liquid and counting the samples in a 460 CD Packard scintillation counter.

3. RESULTS

We found that [^{3}H]choline was rapidly incorporated into trichloroacetic acid precipitable material by the C3H/10T½ cells as reported in [8]. However, the degree of TPA stimulation varied with the growth state of the cells. The reproducibility of this experiment was best with cells 2–3 days after they had reached confluence. At this time, the TPA-stimulated cells incorporated choline 2–4-times faster than did acetone-treated control cells 15–20 min after initiation of exposure to TPA. The level of incorporated [^{3}H]choline in confluent cells after 24 h of TPA treatment was 20–50% higher than in acetone controls (not shown).

When C3H/10T½ cells were prelabelled for 24 h with [^{3}H]choline we observed a small but reproducible decrease in trichloroacetic acid precipitable radioactivity early after TPA treatment (fig.1A). This effect was also observed when trichloroacetic acid soluble radioactivity was determined as demonstrated in fig.1B. The data in fig.1B suggested that about 5% of total trichloroacetic acid precipitable labelled material was solubilized within 1 h. Double-labelling experiments in which 3-day confluent C3H/10T½ cells were prelabelled for 24 h with [^{3}H]choline, and then both TPA and [^{14}C]choline were added simultaneously, showed that degradation of [^{3}H]choline metabolites occurred at the same time as TPA-stimulated [^{14}C]choline incorporation 150% relative to acetone control cultures 30 min

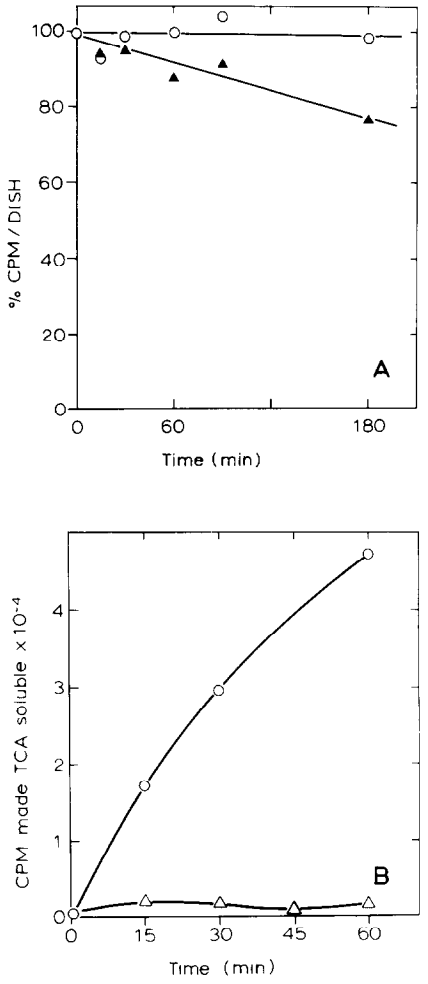

Fig.1. Solubilisation of radioactive choline from prelabelled cells. C3H/10T½ cells which had been confluent for 3 days were used. (A) The cells were grown for 24 h in medium containing 1.2 μCi/ml [^{3}H]choline. At zero time, the labelled medium was removed, cells were washed with PBS and fresh medium added: (—○—) acetone control; (—▲—) 0.17 μM TPA. At the time points indicated, duplicate dishes were harvested for trichloroacetic acid precipitation; (B) Cells labelled with 0.06 μCi/ml [^{14}C]choline for 24 h. The medium was changed and fresh medium with and without TPA was added. At the times indicated, to parallel dishes containing 2 ml of medium, 100 μl of 100% trichloroacetic acid was added. The cells were scraped off and the homogenate centrifuged at 5000 rev./min for 10 min in a SS 34 rotor. The radioactivity in 1 ml of each supernatant was determined: (—△—) acetone control; (—○—) 0.17 μM TPA. Total radioactivity was ~10^6 cpm/dish. There were about 6.10^5 cells/dish.

after initiation of TPA treatment (not shown).

The solubilizing effect of TPA on choline metabolites was studied in more detail in an attempt to determine whether or not the release was specifically associated with either the cytosol or a membrane-containing subcellular fraction. From the results presented in fig.2, it was concluded that essentially all of the degradation occurred in the membrane/nuclear fraction while only negligible release of choline metabolites took place in the cytosolic fraction. Analysis of the mitochondrial fraction (which contained the bulk of plasma membranes) was also performed and no release of radioactivity was observed (not shown). It was also observed in separate in vitro experiments that the degradation was dependent on the presence of the divalent cations Mg^{2+} and Ca^{2+}, indicating that the process was at least in part enzyme-mediated. Total release of choline metabolites from the membrane/nuclear fraction after 50 min was 3–4% of total trichloroacetic acid precipitable radioactivity, this accounts for most of the release found in vivo.

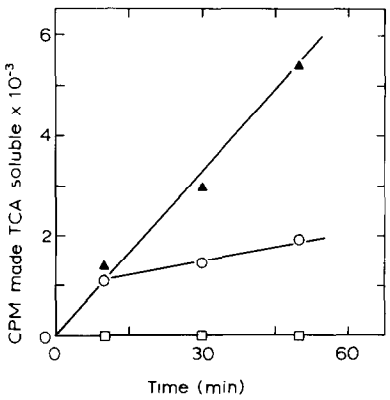

Fig.2. In vitro release of radioactivity from prelabelled cellular components. The cells were labelled with [^{3}H]choline for 24 h, then harvested and disrupted by nitrogen cavitation [13]. The homogenate was centrifuged at 27000 × *g* (Sorvall SS 34 rotor) for 10 min and the supernatant constituted the cytosol fraction and the pellet the membrane/nuclear fraction. Cell disruption was performed in a Mg^{2+} and Ca^{2+} free buffer. In vitro incubation at 37°C was commenced after the simultaneous addition of $MgCl_2$ and $CaCl_2$ to final concentrations of 10 and 2 mM respectively, and either acetone or 0.17 μM TPA. Aliquots of 200 μl were withdrawn at the time indicated and trichloroacetic acid precipitation carried out. Trichloroacetic acid insoluble material was pelleted by centrifugation and radioactivity in 200 μl of the supernatant was determined: (—○—) membrane/nuclear fraction control containing 0.2% acetone; (—▲—) membrane/nuclear fraction plus 0.17 μM TPA; (—□—) cytosolic fraction with and without TPA. Total radioactivity in the membrane/nuclear fraction was about 150000 cpm/assay.

Table 1 shows the incorporation of [^{3}H]choline into trichloroacetic acid insoluble material in various cell fractions during a 2-h incubation of C3H/10T½ cells with TPA in acetone or acetone alone. After 15 min incubation in the presence of TPA there was a marked increase in radioactivity in the cytosol (3.4-times), with smaller increases being observed in the mitochondrial fraction and in nuclear-associated endoplasmic reticulum. At 30 min the most significant observation was the continued large increase in radioactivity in the nuclear-associated endoplasmic reticulum. Between 30 and 60 min of incubation there were large increases in incorporated radioactivity in all fractions, both in control and TPA-treated cultures. With the exception of the nuclear fraction there was about twice as much radioactivity in all subcellular fractions after 60 min of TPA treatment.

4. DISCUSSION

When compared to the control, the maximum stimulation of [^{3}H]choline incorporation after the addition to TPA to C3H/10T½ cells occurred within 30 min ([8] and unpublished). It was therefore expected that should TPA have a specific target site (e.g., a particular enzyme system) then this perhaps could be localized by performing a subcellular fractionation at various times after the addition of [^{3}H]choline and TPA to cell cultures, and then comparing the amount of incorporated radioactivity into the subfractions. From table 1 it is evident that the most significant stimulation of [^{3}H]choline incorporation within 30 min occurred in the cytosol and the nuclear-associated endoplasmic reticulum, suggesting that these represent the major early sites of TPA action. After 120 min there was an increase in [^{3}H]choline incorporation in all subfractions isolated from the TPA-treated culture ranging from 28% in the nuclear fraction to over 100% in the microsomes and the nuclear-associated endoplasmic reticulum. Thus,

Table 1

Distribution of [^{3}H]choline in various subfractions of C3H/10T½ cells

	Radioactivity in subfractions (cpm)									
	Cytosol		Nuclei		Microsomes		Mitochondria		NAER	
Min	A	T	A	T	A	T	A	T	A	T
15	540	1836	825	805	156	164	328	712	864	1416
30	828	1872	595	836	264	300	2044	1904	2538	6066
60	4932	1276	3136	3276	1608	3112	6616	12516	11718	21618
120	16632	25056	5348	6872	2508	5100	13256	18288	33684	67932

A = Acetone control
T = TPA-treated
NAER = Nuclear-associated endoplasmic reticulum

The cells were simultaneously given medium containing [^{3}H]choline and 0.17 μM TPA or 0.2% acetone. The cells were washed with cold PBS containing 10 mg/ml unlabelled choline and scraped off the dishes at the time indicated. The cells were disrupted by N_2-cavitation [13] and subfractionation was performed by differential centrifugation [14]. The NAER fraction was prepared by treatment of the washed nuclear pellet with 0.5% Nonidet P-40. After low speed centrifugation the supernatant constituted the NAER fraction and the resuspended pellet the nuclei

TPA has exhibited the greatest effect on those cellular subfractions containing membranes of the endoplasmic reticulum, perhaps suggesting a stimulation of proliferation of the ER system. It would appear that TPA does not exhibit a major effect on choline-containing constituents of the plasma membrane. Firstly, no release of radioactivity was observed in the plasma membrane-rich fraction after addition of TPA to prelabelled cells, and secondly, there was no rapid increase in [^{3}H]choline incorporation in this fraction at early times after TPA treatment.

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