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The tumor promoter tetradecanoylphorbol-13-acetate elicits the redistribution of heavy metals in subcellular fractions of rabbit thymocytes as measured by plasma emission spectroscopy

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Prolonged (90 min) incubation with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) causes no significant changes in the elemental composition of whole rabbit thymocytes (25 elements included). The only exception is the amount of magnesium, where a significant increase can be detected. However TPA preincubation elicits the redistribution of heavy metals (mainly Zn and to a much lesser extent Fe and Cu) from the nuclear and large granular (mitochondrial–lysosomal) fraction to the cytosol and microsomes. A significant translocation of calcium and phosphorus from the large granular fraction mainly to the nucleus can also be observed. The TPA-induced heavy metal depletion of the nucleus might play a role in the antiproliferative activity of TPA, the arrest of G_1-S phase transition. On the other hand the increased amount of heavy metals in the cytosol might be linked to the TPA-induced block of intercellular communication and the desensitization of TPA-treated cells towards extracellular calcium which are well-known properties of neoplastic cells.

Introduction

12-*O*-Tetradecanoylphorbol-13-acetate (TPA*) is a very potent, well-known tumor promoter (1–3). Since the discovery reported by Castagna *et al.* (4) that active phorbol esters can directly activate protein kinase C, TPA is considered to exert its effects via protein kinase C-induced protein phosphorylations. However, the mechanism of the action of TPA is far from being fully elucidated.

The block of intercellular communication and the desensitization towards changes in the extracellular calcium concentration are well-known properties of tumor cell growth (1–3). TPA elicits such effects in several cell types tested (5,6). On the other hand, TPA is reported to block agonist-induced Ca-fluxes via the phosphorylation of Ca-channels or the receptor→N protein→phospholipase C system (7,8). The TPA-induced block of intercellular communication and the desensitization towards changes in calcium concentration of the medium might be linked to the ability of TPA to block calcium signals and/or Ca-fluxes.

In previous work we have investigated the effect of TPA on the Ca-ionophore-induced rise in intracellular calcium concentration (9). It was found that prolonged incubation with TPA sharply decreases the ionophoretic activity of Ca-ionophores. The optimal conditions of this TPA-induced desensitization needed a 90 min preincubation with TPA concentrations higher than 10 nM. This finding was surprising since it can not be explained by assuming TPA-induced protein phosphorylation. Looking for possible explanations, mechanisms of the phenomenon we have observed that the intracellular heavy metal chelator, TPEN restores the ionophoretic activity of Ca-ionophores after TPA treatment. This observation led to the hypothesis that TPA may cause changes in the heavy metal content of different subcellular fractions. To test this hypothesis and to examine which heavy metal ion(s) are responsible for the diminution of the effect of Ca-ionophores in this work the changes in heavy metal content of subcellular fractions upon previously optimalized (90 min, 20 nM) TPA treatment were investigated by plasma emission spectroscopy.

*Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TPEN, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine.

Materials and methods

Materials

TPA and Hepes were from Sigma. Dimethyl-sulfoxide was obtained from Fluka. Cell culture medium ingredients were Gibco products. HNO_3 was a Carlo-Elba ultrapure product. All other chemicals used were of best analytical purity.

Isolation and TPA treatment of cells

Rabbit thymocytes were isolated by the method of Kleiman *et al.* (10). Cells were incubated with 20 nM of TPA or with the solvent, dimethyl-sulfoxide at a final concentration of 0.05% (v/v; control cells) at 37°C for 90 min at a cell density of 10^7 cells/ml in Eagle's minimum essential medium supplemented with 10 mM Hepes (pH 7.4), essential amino acids, streptomycin and penicillin (100–100 U/ml). Viability of the cells was never <95% as judged by trypan blue exclusion.

Isolation of subcellular fractions

After incubation cells were centrifuged and resuspended in the isolation buffer containing 140 mM KCl, 0.25 mM $MgCl_2$ and 20 mM Hepes pH 7.0 at a cell density of 5×10^8 cells/ml in a final volume of 10 ml. Isolation of four subcellular fraction (nuclear pellet, large granules, mirosomes and cytosol) of rabbit thymocytes disrupted by nitrogen cavitation was performed as described earlier (11–13). Considering the lipid composition of different subcellular fractions and the distribution of various marker enzyme activities (11,13) we can say that in our preparations the nuclear pellet contains only trace amounts of large granules and microsomes (derived from a minor population of partially disrupted cells), in the large granular fraction there is a very small amount of aggregated microsomes and the microsomal and cytosolic fractions contain no cross-contaminations. After centrifugation the nuclear pellet was suspended in 5 ml, the large granular and the microsomal fractions each in 1 ml of isolation buffer.

Plasma emission spectroscopy measurements

Whole cell suspensions and the subcellular fractions were lyophylized and digested with 2 ml of concentrated HNO_3 at 120°C for 2 h in quartz tubes. The digested samples were diluted with distilled water to 5 ml and their elemental composition was determined using an ICAP-9000 simultaneous ICP spectrometer (Fischer Scientific Co.). Data have been corrected for the amount of different elements in appropriate 'blank' samples. (The appropriate amount of isolation buffer plus HNO_3 in case of whole cells, nuclear pellet, large granules and microsomes; and the isolation buffer carried through all the steps of isolation in case of cytosol.) The correction was never >10% of the total amount of the actual element with three exceptions. In the case of Fe the correction was approximately 50% of the total Fe content of the samples because of the relatively high Fe content of HNO_3. In the case of Ca and Mg content the correction was 10–20% and 40–80% of the total value respectively because of the Ca-impurities and the Mg content of the isolation buffer.

Results

Examining the elemental composition of rabbit thymocytes and their subcellular fractions we could not detect any significant amount of As, Co, Ga, Hg, Li and Se. Al, B, Ba, Cd, Cr, Mn, Mo, Ni, Pb, Sr, Ti and V were present in trace amounts in our samples and TPA did not cause significant ($P > 0.05$; Student's

Table I. The amount and distribution of different elements in control and TPA-treated rabbit thymocytes

Element	Elemental composition (nmol/5 × 10^9 cells)[a]										Recovery (%)[b]	
	Whole cell		Nuclear fraction		Large granules		Microsomes		Cytosol			
	Control	+TPA	Control	+TPA	Control	+TPA	Control	+TPA	Control	+TPA	Control	+TPA
Ca	2010	2000	238	457[c]	451	198[c]	81	79	1020	1056	89	90
	(180)	(190)	(20)	(53)	(58)	(19)	(10)	(13)	(70)	(59)		
Cu	177	177	65	59	20	13[c]	18	13	54	70[c]	89	88
	(12)	(12)	(6)	(4)	(2)	(1)	(1)	(0.6)	(3)	(3)		
Fe	165	138	99	81[d]	15	12	1.3	3.6[c]	25	19	85	84
	(51)	(59)	(4)	(4)	(1)	(1)	(0.4)	(0.6)	(6)	(1)		
Mg	2100	2230[d]	483	481	175	140	96	171[c]	1080	1170	87	88
	(40)	(70)	(24)	(22)	(18)	(15)	(4)	(3)	(60)	(70)		
P	40 000	39 300	22 600	23 800[d]	6300	3660	2200	3200[c]	7190	7220	96	96
	(900)	(1000)	(500)	(500)	(160)	(190)	(50)	(60)	(140)	(120)		
Zn	1060	1000	700	469[c]	64	31	20	35[c]	153	395[c]	88	93
	(60)	(100)	(42)	(36)	(3)	(1)	(2)	(2)	(7)	(10)		

[a]Preparation of the samples and mesurement of their elemental composition was done as described in Materials and methods. Data are means and (in parentheses) + S.D.s of five separate experiments.
[b]Percent of recovery was calculated summarizing the amount of different elements in the four subcellular fractions (columns 2–5) and expressed as a percentage of the total amount of the same element in whole cells (column 1).
[c–d]Significant difference between control and TPA treated sample; level of significance; [c]$P > 0.001$, [d]$P > 0.01$.

t test) changes in the amount of elements listed. K and Na were present in great amounts, however, their total concentration and subcellular distribution did not change significantly upon TPA incubation (20 nM, 90 min, 37°C) (data not shown).

Table I shows the amount of Ca, Cu, Fe, Mg, P and Zn (expressed as nmol/5 × 10^9 cells) in whole cells and subcellular fractions of rabbit thymocytes. From the data showing the amount of different elements in control and TPA-treated whole cells (Table I, first column) it can be seen that there is no significant change in the concentration of Ca, Cu, Fe and Zn upon TPA incubation. (This result is not obvious since the cell culture medium contained substantial amounts even from Cu, Fe and Zn corresponding to 12, 148 and 220 nmol metals respectively in medium containing 5 × 10^9 cells; not shown. Thus in our conditions not only an efflux but an uptake of different elements would also have been possible.)

Mg is the only element of which total concentration is increased during TPA incubation. The increased amount of Mg can be found mainly in the microsomal fraction. Furthermore, from the data of Table I it can be see that there is a significant difference between the Ca-content of nuclear and large granular (mitochondrial-lysosomal) fractions of control and TPA-treated cells. The distribution of phosphorus shows similar changes with the addition that phosphorus content of microsomes has been also increased during TPA incubation.

The distribution of heavy metals (Cu, Fe and Zn) changes similarly after TPA treatment: a translocation from the nuclear and large granular fractions to microsomes and cytosol can be observed. The changes are less dramatic (though significant) in case of Cu and Fe and mostly expressed in case of Zn.

From the last column of Table I it can be seen that the recovery (i.e. the summarized amount of a given element in subcellular fractions expressed as the percentage of the total amount of the same element in whole cells) is all the time >80% and the difference between the recovery of TPA treated and corresponding control samples never exceeds 5%. Therefore the changes observed can not be attributed to the loss of the appropriate element.

Discussion

Our results, indicating that during TPA incubation a substantial Mg uptake occurs, are in good agreement with the results of Grubbs and Maguire (14). They have observed that TPA induces a significant Mg uptake in S49 lymphoma cells while the Ca-fluxes remained essentially unchanged upon TPA treatment.

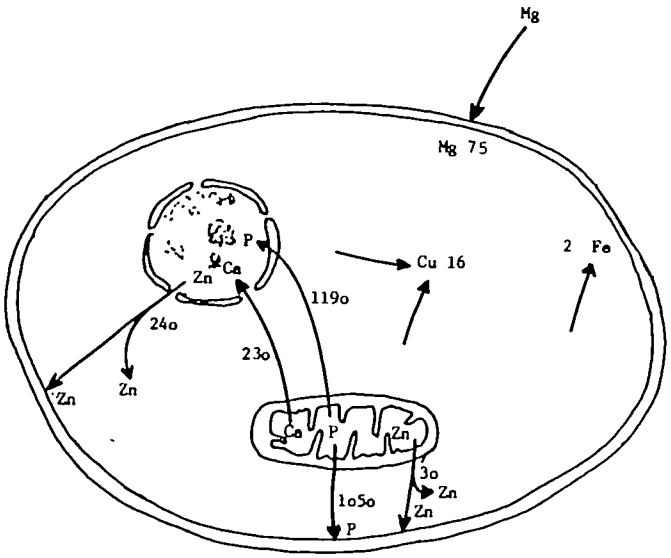

Fig. 1. Translocation of different elements after prolonged TPA incubation of rabbit thymocytes. The numbers were calculated from the data of Table I denoting the amounts of elements translocated in nmol/5 × 10^9 cells. For the sake of clarity in the figure the elemental content of the large granular fraction and microsomes is attributed solely to the mitochondria and plasma membrane respectively, the lysosomes and endoplasmic reticulum are not indicated.

The increase in phosphorus content of nucleus and microsomes might reflect (among others) the increased phosphorylation of the proteins in these subcellular compartments. The fact that the calcium content of microsomes is not affected during TPA incubation is consistent with the results of Cox and Carroll (15) who showed that TPA has no effect on the Ca-induced fluorescence of chlorotetracycline, i.e. the amount of mainly plasma membrane-bound calcium. The total calcium content of the cells is in good agreement with the value reported by Waller *et al.* (16).

In Figure 1 the data of Table I are rationalized, showing the

redistribution of different elements in subcellular fractions of rabbit thymocytes during TPA incubation. Figure 1 shows that a substantial redistribution of heavy metals (mainly Zn) occurs from the nucleus and mitochondria (+ lysosomes) to the microsomes and cytosol during TPA incubation. Figure 1 also summarizes the TPA-induced decrease in the calcium content of large granules with the parallel increase of Ca in the nucleus. The TPA-induced 'exchange' of zinc to calcium in the nuclear fraction might activate certain DNA-endonucleases which are Ca-dependent and can be inhibited by zinc (17). The activation of endonucleases might play a role in the biological action of TPA.

As we have mentioned in 'Materials and methods' minor cross-contaminations of the nuclear and large-granular fractions can not be avoided during isolation. However, these contaminations do not change the validity of *comparative* data showing differences between the subcellular fractions of TPA-treated and control thymocytes, because these traces of cross-contaminations are dependent mainly on the method of isolation [appearing very similarly in the case of T lymphocytes of different origin (11,13) or in the case of EL_4 lymphoma cells (18)] and TPA preincubation does not change their appearance significantly (data not shown).

The TPA-induced redistribution of heavy metals is in accordance with our earlier data (9) which suggest an increased availability of heavy metals surrounding the plasma membrane after prolonged incubation with TPA. In an independent study we have detected changes in the heavy metal content of subcellular fractions of control and TPA-treated rabbit thymocytes using X-ray fluorescence. The qualitative evaluation of the X-ray fluorescence spectra and the semi-quantitative data calculated from the spectra recorded show very similar heavy metal redistribution to our present data (19). Our results showing the total Zn and Cu content of rabbit thymocytes are in the range of data published previously; 340–2450 and 20–185 nmol/5 × 10^9 cells respectively (reported mainly from human peripheral blood lymphocytes, 20–28).

The '*zinc depletion*' from the nucleus by incubation with the tumor promoter TPA resembles to the results of numerous studies demonstrating diminished nuclear Zn content in malignant lymphocytes compared to normal ones (29,30). The decrease of the Zn content in the nucleus and large granules may cause significant changes in the activity of several enzymes.

Besides its mitogenicity TPA has a well-documented antiproliferative effect too, which can be explained mostly with the arrest of $G_1 \rightarrow S$ phase transition (31). On the other hand, Zn seems to be essential for the activity of several enzymes necessary for RNA, DNA synthesis such as RNA, DNA polymerases and thymidine kinase (29). Zinc was shown to be necessary for the proliferation of lymphocytes (32) and zinc-deprivation leads to the arrest of $G_1 \rightarrow S$ phase transition (33). Therefore the TPA-induced depletion of zinc from the nucleus might be related to its antiproliferative activity.

As well as the zinc-depletion from nucleus and large granules there is a significant *increase in the zinc content of microsomes and cytosol*. Günther *et al.* (34) have found several Zn-binding proteins in the cytosol of normal and malignant lymphocytes. In recent reports it has been proved that the primary structure of several receptor proteins e.g. that of cortisol, oestradiol, thyroid hormone (35) and the phorbol ester receptor protein kinase C itself (36) shows the existence of putative Zn-binding sites. The increased availability of Zn in the cytosol might change the behaviour of these proteins.

Heavy metals exhibit a higher affinity for several calcium binding sites than calcium itself (37). Therefore an excess of heavy metal ions in the cytosol and microsomal fraction might occupy some of the calcium binding sites, channels attenuating the calcium fluxes essential in signal transduction. In this way the TPA-induced heavy metal redistribution might be linked to the TPA-induced block of the intercellular communication (5) and the desensitization of the TPA-treated cells towards extracellular calcium (6).

These complex changes in the heavy metal distribution upon TPA incubation might indicate a new route of action of TPA and help to explain the conflicting data about the need of heavy metals (mainly Zn) in tumor growth (30).

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