Phorbol Ester-induced Alteration of Differentiation and Proliferation in Human Hematopoietic Tumor Cell Lines: Relationship to the Presence and Subcellular Distribution of Protein Kinase C

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ABSTRACT

The intracellular translocation of protein kinase C (PKC) from the soluble to the membranous fraction has been shown previously to correlate with biological activity of phorbol esters in several systems. In this paper, we describe that PKC translocation was a general phenomenon in all PKC containing cell types when five 12-O-tetradecanoylphorbol-13-acetate (TPA) responsive and nonresponsive hematopoietic tumor cell lines were investigated. The nonresponsive cell line U-266 contained undetectable levels of PKC. The dose of TPA required for translocation was similar to the TPA concentration necessary to suppress erythroid differentiation in K-562 cells and to induce macrophage differentiation in U-937 cells, but 100-fold higher than that required for suppression of proliferation in K-562 and U-937 cells. By contrast, PKC translocation and TPA induced proliferation inhibition exhibited a similar dose dependence in a subline of U-937 (U-937 RES) adapted to growth in the presence of $10^{-8}$ M TPA. It is suggested that U-937 RES is deficient in a TPA dependent but PKC independent signal pathway.

INTRODUCTION

The phorbol esters, and especially TPA, are known to have profound effects on general cellular metabolic functions as well as at the level of cell differentiation and proliferation status (1-3). Depending on the cell system investigated, the effects observed are inductive or suppressive with regard to metabolism and proliferation and positive or negative with respect to the alteration of the level of differentiation. These observations have stimulated an intense search for the mechanisms of action of the phorbol esters (cf. Ref. 4).

The phorbol esters are active at low concentrations, indicating a specific interaction with high affinity receptors. Castagna et al. (5) showed that the Ca$^{2+}$ and phospholipid dependent protein kinase (PKC) was directly activated by TPA, thereby mimicking a number of physiological signals normally transmitted by PKC. Purification of PKC has confirmed that the enzyme is a high affinity receptor for TPA (6, 7).

PKC is, in many types of cells, found in a soluble form. One of the first observed changes after TPA treatment is a rapid translocation of PKC to a particulate, pelletable cell homogenate fraction (8). The mechanisms behind and the significance of this phenomenon are incompletely understood. In a previous study, we found that the effects of phorbol ester on isolated rat adipocytes correlated, with respect to the concentration dependence, with the apparent subcellular redistribution of PKC from the soluble to the particulate fraction of the cells (3). The ability of various phorbol esters to promote the intracellular translocation of PKC, as well as to stimulate the rate of glucose oxidation and lipid biosynthesis in these cells, was proportional to their potency as tumor promoters.

We have recently studied various means of affecting the level of differentiation in a number of established human hematopoietic tumor cell lines. The monoblastic U-937 cell line was shown to undergo further differentiation to a monocyte-macrophage-like cell upon treatment with TPA, retinoid acid, vitamin D$_3$, and mixed leukocyte culture supernatants (9-11). The erythroleukemia cell line K-562, in contrast to U-937 but similar to Friend erythroleukemia cells (12), dedifferentiated when exposed to TPA, using heme synthesis as erythroid marker (13, 14). The U-698 and U-266 cell lines, finally, represent B-cell tumors arrested at the B-lymphocyte and plasma cell stage of the B-cell differentiation lineage, respectively (15), and cannot be induced to alter their stage of differentiation by TPA.

In this study, we have used this panel of tumor cell lines, known to be affected differently by TPA with respect to differentiation, and a low dose ($10^{-8}$ M) TPA resistant subclone of U-937 to examine the relationship between the presence of PKC, the TPA induced intracellular translocation of PKC, and the proliferative response of the cells to TPA treatment.

MATERIALS AND METHODS

Cells and Cell Culturing

The following established human tumor cell lines were used. U-837. U-937 was established from a human histiocytic lymphoma. Its phenotypic properties conform with those of immature monocytic cells (16). The experiments presented in this paper were performed with the subline U-937 GTB.

U-937 RES. A subline of U-937 clone 3 was adapted to growth in TPA containing medium by successively increased doses of TPA in the culture medium. The cell line is continuously grown in $10^{-8}$ M TPA in our laboratory. We refer to this subline as U-937 RES. The characteristics of this subline are stable for at least 4 weeks in normal medium. The line, established by Lozzio and Lozzio (17), has erythroid characteristics (18). The cell line has been claimed to have features of bipotent stem cells with the capacity to develop either megakaryocyte or erythroid differentiation markers upon induced differentiation (19).

Received 2/26/85; revised 8/26/85; accepted 8/27/85.

1 Supported by grants from the OE and Edla Johansson Foundation, the Magnus Bergwall Foundation, the Swedish Cancer Society, and the Swedish Medical Research Council.

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3 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; EGTA, ethylene glycol bis(iso-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
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U-698. This line is from a B-lymphocytic lymphoma arrested at a stage of differentiation corresponding to that of slightly immature B-cells (20).

U-288. U-266 is an IgC myeloma cell line with features of terminally differentiated B-cells (plasma cells) (21, 22).

All cell lines, tested and found to be free of Mycoplasma infection, were kept under standard culture conditions (22). Ham’s F-10 medium (23) supplemented with 10% newborn calf serum (GIBCO, Paisley, Scotland) was used.

In the experiments, cells were harvested from optimally growing stock cell cultures and washed in phosphate buffered saline. Determination of cell numbers and viability was done by counting the cell suspensions in hemocytometers after incubation in trypsin blue solution. The initial doubling times for the different cell lines under the TPA free culture conditions used were 26 h for U-937 GTB, 30 h for U-937 RES, 42 h for K-562, 28 h for U-698, and 50 h for U-266.

The cell densities used in the experiments were 0.2–4 million cells/ml. It was ascertained, using [3H]TPA, that the concentrations of TPA in the cell free medium were not influenced by more than 10% by the amount of cells used.

Phorbol Ester Stock Solution

TPA (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol and kept at –20°C at a concentration of 10^{-6} M. Additions of ethanol never exceeded 2 μl to 1 ml culture medium, which was found to have no effect on the systems tested.

Preparation of Cytosol and Solubilized Membranes

Cells in logarithmic growth were used in all experiments. Cell viability always exceeded 90%. Cells received either TPA or an equivalent amount of solvent, 2 μl ethanol/ml of medium. They were then washed twice with 0.9% NaCl solution (37°C) and centrifuged at 1000 × g for 5 min. The washed cell pellet was suspended in 3 ml ice-cold 4 mM EDTA-1 mM EGTA (pH 7.5), homogenized with ten strokes in a Teflon-glass homogenizer, and treated for 2 × 10 s in a sonicator water bath. The lysed suspension was then mixed with an equal volume of 1 M Tris-HCl-0.66 mM sucrose-0.1 mM mercurioethanol (pH 7.5) and centrifuged at 1000 × g for 5 min. The supernatant was centrifuged at 100,000 × g in a Ti 45.5 rotor (34,000 rpm) for 60 min at 4°C. The resulting high speed supegrant is referred to as the cytosol. In some experiments, the resulting pellet was suspended in 6 ml 20 mM Tris-HCl-0.33 mM sucrose-2 mM EDTA-0.5 mM EGTA-50 mM mercurioethanol (pH 7.5) containing 1% Triton X-100 (v/v). After 45 min, the solubilized membrane suspension was clarified by centrifugation at 100,000 × g for 60 min. The resulting supernatant is referred to as the solubilized membranes. The cell recovery during incubation and washing was controlled by measurements of lactate dehydrogenase activity in the cytosol fraction. The assay solution contained 0.2 mM NADH, 1 mM pyruvate, and 0.19 mM Tris-HCl (pH 7.3). The formation of NAD^+ was measured at 30°C by following the decrease of the absorbance at 340 nm. Using this method, less than 10% variation in cell yield between the different cell suspension aliquots was observed. No correction was made for this variation.

PKC Assay

In order to measure PKC activity in a cell extract, endogenous interfering substances must be removed. Each supernatant was therefore applied to a 0.3-ml bed volume DEAE-Sepharose (Pharmacia, Uppsala, Sweden) column equilibrated in 20 mM Tris-HCl, 2 mM EDTA, and 0.5 mM EGTA (pH 7.5). The column was washed with 0.5 ml equilibrating buffer containing 50 mM NaCl. PKC was eluted with 0.6 ml equilibrating buffer containing 150 mM NaCl. Pilot experiments showed that less than 5% of the PKC activity was eluted at 50 mM NaCl. The major part distributed between the fractions obtained by elution with 150 and 500 mM NaCl. Routinely the two later fractions were assayed for enzymic activity and more than 75% of the activity observed was eluted at 150 mM NaCl. PKC activity was determined according to the method of Takai et al. (24) as described previously (25). Our incubation scheme gives final concentrations of 10 μM ATP, 8.5 mM free Mg^2+, and 0.4 mM free Ca^2+.

These values are calculated from dissociation constants given by Sillen and Martell (26) assuming negligible binding of divalent cations to lipids and protein. Kinase activity in the presence of Ca^2+ but in the absence of phosphatidylethanolamine was routinely subtracted to calculate PKC activity. Enzyme activity is expressed as pmol 32P incorporated into histone per min and per 10^6 cells.

Assay of Influence of TPA on Proliferation Rates

Cell cultures were set up on cluster plates (Costar, Cambridge, MA; 0.5 × 10^6 cells/well; 2.5 ml F10 medium supplemented with 10% newborn calf serum). TPA was added in graded doses from 10^{-10} to 10^{-4} M (duplicates) and incubation was carried out at 37°C in a humidified 5% CO_2 atmosphere for 24 h. The cellular proliferation was assayed by measurement of the incorporation of [methyl-3H]thymidine (specific activity, 5 Ci/mmol; 1 μCi/well; Amersham International, plc, Amersham, Buckinghamshire, England) during 4 h incubation. The cultures were harvested on glass fiber filters (Whatman GF-A, England). The radioactivity was measured by scintillation counting in Supersolve scintillation fluid (Koch-Light, Haverhill, England) in a Packard Tri-Carb scintillation counter. Parallel untreated cultures were run as controls.

Each dose-response experiment was run at least three times. The cpm values were normalized with the control values as 100%, and the arithmetic means and standard deviations were calculated.

Preliminary studies on U-937 GTB cells showed an exponential decrease of [methyl-3H]thymidine incorporation after TPA application (t_1/2, 6.6 h).

Assay of Iron Uptake and Heme Synthesis in K-562 Cells

Human apotransferrin (Sigma) was labeled with 59Fe by incubation in a molar excess of 59FeCl_3 (Amersham International; 3–20 mCi/mg iron) in the presence of excess citrate and bicarbonate at neutral pH. Unreacted reagents were removed by desalting on a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column.

K-562 cells were treated with TPA (dose range, 10^{-10} to 10^{-4} M) for 2 days in cluster plates (Costar; 2 × 10^6 cells/well; 2 ml F10 medium). The incubation was terminated by an overnight addition of transferrin bound 59Fe (approximately 500,000 cpm/well). The cells were washed three times in ice-cold phosphate buffered saline before the total amount of accumulated radioactivity was measured in a Beckman 8000 gamma counter (Beckman Instruments, Fullerton, CA). The heme bound iron was extracted by the acid butanone method from Triton X-100 (0.5%) lysates of the cell pellet, slightly modified after the method of Teale (27).

Extraction from the incubation medium alone gave a background of approximately 5% of the total radioactivity.

RESULTS

PKC Activity and Translocation in a Panel of Established Human Tumor Cell Lines. The PKC levels and distribution of the kinase activity between cytosol and membrane fractions were determined in a number of established cell lines of different histogenetic origins. The effect of a short term TPA incubation was also investigated. The results are summarized in Table 1. The cell line K-562 contained the highest levels of PKC activity, whereas in comparison U-937 GTB had about 50% of this PKC content. The subclone U-937 RES exhibited only about 50% of the PKC activity of the parental cell line. Of the B-cell tumor cell lines, U-266 contained undetectable levels of PKC activity, whereas in U-698 about 13% of the activity registered in the K-562 cells was found. The U-266 cell line contained similar...
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Table 1
Total PKC activity and effect of TPA on the distribution of the kinase activity between cytosol and solubilized membranes in hematopoietic tumor cell lines

The cells were incubated for 30 min with 1.6 μM TPA or an equivalent amount of solvent at 37°C before homogenization, ultracentrifugation, and DEAE-Sepharose fractionation as described under "Materials and Methods." Data are expressed as mean ± SD of the number of experiments indicated within parentheses.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>-TPA</th>
<th>+TPA</th>
<th>-TPA</th>
<th>+TPA</th>
<th>-TPA</th>
<th>+TPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-562 (3)</td>
<td>3.9 ± 2.0</td>
<td>2.5 ± 1.1</td>
<td>85 ± 13</td>
<td>7 ± 7</td>
<td>15 ± 13</td>
<td>93 ± 7</td>
</tr>
<tr>
<td>U-937</td>
<td>1.6 ± 0.8</td>
<td>0.86 ± 0.10</td>
<td>89 ± 12</td>
<td>9 ± 13</td>
<td>11 ± 12</td>
<td>91 ± 13</td>
</tr>
<tr>
<td>GTB (3)</td>
<td>0.75 ± 0.23</td>
<td>0.79 ± 0.15</td>
<td>72 ± 8</td>
<td>2 ± 2</td>
<td>28 ± 8</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>U-937 RES (4)</td>
<td>0.50 ± 0.21</td>
<td>0.28 ± 0.15</td>
<td>93 ± 5</td>
<td>8 ± 10</td>
<td>7 ± 5</td>
<td>92 ± 10</td>
</tr>
<tr>
<td>U-266 (2)</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
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* The activity is expressed as pmol of 32P incorporated into histone per minute and per million cells under the conditions used. The lower detection limit of the method was 0.05.

![Chart 1](chart1.png)

**Chart 1.** Dose dependence of TPA induced PKC translocation and decrease of the proliferation rate in U-937 GTB cells. O, proliferation of TPA treated cells in percentage of control (100% = 76,100 cpmp, three experiments); Cl, PKC activity, measured in the cytosol of cells treated with various concentrations of TPA for 30 min. The results were calculated as percentage of PKC activity remaining in the cytosol after TPA treatment compared to the activity in cytosol from control cells. The control cytosol value was 1.56 ± 0.81 pmol/min and 10^6 cells. For each point, 10^-20 x 10^6 cells were used. Each point is the mean of four experiments; bars, SD.

In all cell lines containing PKC, a rapid (within 20 min) and almost complete redistribution of the PKC activity from the soluble to the particulate fraction occurred subsequent to TPA treatment (Table 1). The recoveries of the PKC activity from the cells after TPA treatment were 50-75%, with the exception of U-937 RES, for which we obtained a 100% recovery.

Concentration Dependence of Effects of TPA. The effects of various TPA concentrations on proliferation and PKC translocation were tested in a number of experiments. Results are shown in Charts 1 (U-937 GTB) and 2A (K-562). Low doses of TPA caused a diminished rate of proliferation in both cell lines.

![Chart 2A](chart2a.png)

**Chart 2A.** Dose-response curves for effects of TPA on K-562 cells. A, translocation and proliferation decrease; O, proliferation of TPA treated cells in percentage of control measured by (methyl3H) thymidine incorporation (100% = 21,200 cpm, average of four experiments; bars, SD). Cl, PKC activity, measured in the cytosol of cells treated with various concentrations of TPA for 30 min. Results were calculated as percentage of PKC activity remaining in cytosol after TPA treatment compared to PKC activity in cytosol from control cells. The value for control cytosol was 4.3 ± 1.2 pmol/min and 10^6 cells. For each point, 10^-20 x 10^6 cells were used. Data are shown as means of three experiments; bars, SD.

The half-maximal effect was detected at about 5 x 10^-11 M. In contrast, almost a 100-fold higher concentration of TPA was required to promote the translocation of PKC. In these cases, half-maximal effects were registered at about 5 x 10^-9 M TPA. The effect of 10^-8 M TPA on translocation was reproducibly more pronounced in the U-937 GTB clone than in the K-562 cells. Prolonged incubation times (1, 2, 4, 8, and 24 h) with small concentrations of TPA (10^-20 M) did not promote any redistribution of PKC.

![Chart 2B](chart2b.png)

**Chart 2B.** Heme synthesis. The cells were treated for 2 days and at the end of the period the cells obtained an overnight Fe pulse. Heme synthesis values are expressed as acid butanone extractable counts in percentage of the total accumulated cpm. Results shown are means of three experiments; bars, SD.
proliferation was shifted upwards in the U-937 RES clone (Chart 3) as compared to the situation in the parental U-937 GTB clone (Chart 1). The half-maximal TPA concentration was about $5 \times 10^{-9}$ M and thus fell into the same range as the dose required for half-maximal PKC translocation (Chart 3).

The B-cell line U-698 responded to treatment with TPA (Chart 4). The rate of proliferation decreased by about 50% at $10^{-9}$ M TPA. However, the shape of the dose-response curve differed in a pronounced manner from the curves obtained using the other cell lines. It appeared difficult to identify any half-maximal TPA concentration.

The rate of proliferation of the U-266 cells was not significantly affected by TPA (Chart 4).

**Suppression of Erythroid Differentiation in K-562 Cells.** The TPA dose-response curve for the rate of heme synthesis after 48 h of treatment is shown in Chart 2B. The butanone extractable part of the cellular iron was diminished, with a half-maximal effect at about $5 \times 10^{-9}$ M TPA. Also total iron uptake was suppressed in a dose dependent manner with increasing TPA concentrations. The range of suppression was from one-half to less than one-third (28).

**DISCUSSION**

The data presented revealed the presence of a PKC activity in all TPA responsive hematopoietic tumor cell lines investigated. The PKC activity was redistributed from the soluble to the particulate fraction in response to added TPA, but at higher concentrations of the phorbol ester than required to inhibit proliferation in U-937 GTB and K-562 cells. On the other hand, the dose-response curves for the PKC translocation and TPA induced differentiation in K-562 cells agreed well.

The dose-response curve for K-562 showed a plateau at the intermediate TPA concentrations. This raises the possibility of different responsiveness of the cells on the two differentiation pathways described by Vainchenker et al. (19). However, we could not obtain any evidences for the presence of two cell populations.

The U-266 cell line did not respond to TPA treatment by alteration of immunoglobulin synthesis or proliferation and contained undetectable levels of PKC activity. The U-937 RES subline exhibited a 100-fold lower sensitivity to TPA than the parental U-937 GTB cell line with respect to the effect on the suppression of proliferation. However, this TPA resistant subline was not altered in TPA response with regard to the translocation of the PKC activity; i.e., in this subline, the TPA-induced cellular response and translocation of PKC coincided.

We have reported earlier (29) that the U-937 cells were inducible to differentiation at TPA concentrations varying between $1.6 \times 10^{-11}$ and $10^{-8}$ M, with a half-maximum around $1.6 \times 10^{-9}$ M. The measured differentiation markers were the ability of the U-937 cells to function as effectors in antibody dependent cytotoxicity and the insensitivity to natural killer cells. These dose effects on differentiation fall in the same range as do the dose curves for the translocation of PKC observed in this paper.

The intracellular translocation of PKC has been shown previously to correlate with phorbol ester effects on, e.g., adipocytes (3), platelets (30), rat pancreatic acini (31), and A-431 cells (32). Since in this paper a strict correlation between the antiproliferative effect of TPA and the PKC translocation was not evident, it appears relevant to discuss the participation of PKC in all of the cellular events registered in the hematopoietic cells. Some recent reports suggest that the mechanisms behind the strong phorbol ester effects on cells are not simple consequences of the phorbols interacting with PKC as the only receptor. Of special interest in this respect are the recent findings that TPA, when membranes from U-937 cells are used, stimulates phosphorylation of tyrosine in a reaction independent of calcium, phospholipid, and diolein (33); in addition, TPA stimulates a tyrosine specific and $Ca^{2+}$ independent phosphorylation of the epidermal growth factor receptor in membranes from A-431 cells (34). Furthermore Pahlman et al. (35) have also suggested the existence of more than one mechanism for the action of phorbol esters (35). Accordingly it seems plausible that there exist principally different mecha-
nisms mediating the cellular effects of TPA, involving perhaps TPA inducible tyrosine phosphorylation by a presently unknown protein kinase but distinct from the PKC specific serine and threonine phosphorylation pathways. Such a cellular pathway could explain some of the antiproliferative effects of TPA on U-937 GTB and K-562 cells. In this respect it is interesting that TPA shares several biological activities of epidermal growth factor (36) known to preferentially induce phosphorylation of tyrosine. The U-937 RES subline exhibited a similar dose response to TPA with regard to antiproliferative effect and PKC translocation. One might thus speculate about a deficient high affinity pathway in these cells, possibly involving tyrosine phosphorylation. However, a reduced amount of a specific PKC substrate or other protein kinase activity, acting as a part of a protein kinase cascade initiated by TPA in these cells, cannot be excluded.

The involvement of PKC also in the suppression of proliferation can, nevertheless, presently not be excluded. It appears that PKC mediated phosphorylation and subsequent metabolic effects must not necessarily correlate to the intracellular translocation of the protein. Binding of, e.g., TPA in vitro to rat adipocyte PKC takes place at a 1000-fold lower concentration than is necessary to promote an increased rate of lipid biosynthesis or glucose oxidation in the cells (3), i.e., at a concentration similar to that of the registered antiproliferative effect of TPA on U-937 and K-562 cells. Furthermore if the translocation is necessary for the biological response, it may be sufficient with only a small portion of the kinase being translocated in order to affect phosphorylation-dephosphorylation equilibria and thus influence cellular signals.

Lower levels of PKC were recovered from TPA treated cells than from control cells, except when the U-937 RES clone was used. This cell line had from the start only about 50% of the parental U-937 GTB PKC activity. It seems reasonable that PKC is subject to a rapid TPA induced down-regulation as described by Rodriguez-Pena and Rosengurt (37) also in the hematopoietic cells studied here. The same type of PKC dependent down-regulation was registered in Friend erythroleukemia cells after prolonged exposure of TPA (12) and in a number of human cell lines (38). We have thus demonstrated that the TPA nonresponsive cell line (U-266), lacking PKC, and the U-937 RES, with an altered type of phorbol ester response, probably are deficient in one of the TPA dependent signal pathways. Further biochemical characterization of these cell lines might be of help to identify cellular systems of critical importance for control of differentiation and proliferation.

ACKNOWLEDGMENTS

We are indebted to Anita Dluzewski, Lillianne Karlsson, and Caroline Lilliehök for skilful technical assistance. We also thank Dr. Irene Ihved for providing the U-937 RES clone.

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