Vol. 100, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1981 Pages 370-376

TPA-RESISTANCE IN FRIEND ERYTHROLEUKEMIA CELLS: ROLE OF MEMBRANE LIPID FLUIDITY

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Received March 27,1981

SUMMARY: Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene was used to examine membrane lipid dynamics in clones of Friend erythroleukemia cells displaying sensitivity or resistance to the tumor promoting agent 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Two resistant clones yielded higher fluorescence anisotropy values, indicative of decreased lipid fluidity, as compared to two sensitive clones. This difference in fluorescence anisotropy was abolished by treatment of the sensitive clones with cholesteryl hemisuccinate. The treatment also removed another phenotypic difference between the sensitive and resistant clones. Cell adherence to plastic culture dishes in the presence of TPA, a property of sensitive but not of resistant clones, was markedly diminished when the former were enriched with the cholesteryl ester. No significant differences were observed in the binding of a phorbol ester to high affinity saturable receptors between sensitive versus resistant cells. These results implicate membrane lipids as determinants of the actions of phorbol ester tumor promoters.

INTRODUCTION

The carcinogenic process is complex and often proceeds through numerous steps subject to the influence of environmental factors (1). Studies of mouse skin provide experimental evidence for at least two stages termed, respectively, "initiation" and "promotion" (2-4). Chemicals capable of "initiation" are usually mutagenic and yield electrophiles that bind covalently to DNA, whereas tumor promoting agents do not exhibit these properties (5-7). The precise mode of action of potent tumor promoting agents such as 12-O-tetra-

Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; FELC, Friend erythroleukemia cells; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; TR, TPA-resistant FELC clones; TS, TPA-sensitive FELC clones; PDBu, phorbol-12,13-dibutyrate; CHS, cholesteryl hemisuccinate; DMEM-10, Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum; PBS, Ca^{2+} and Mg^{2+} free phosphate-buffered saline; DMSO, dimethyl sulfoxide.

0006-291X/81/090370-07$01.00/0

decanoyl-phorbol-13-acetate (TPA) and related plant diterpene esters (2-7) remains unknown, although TPA has been shown to induce a spectrum of changes in cultured cells, including alterations in membrane functions, enhancement of the expression of phenotypic markers of transformation induced by oncogenic viruses or chemical carcinogens, and modulation of cell differentiation (for reviews see 8,9).

Studies of the mechanisms of tumor promoter action have been facilitated by the isolation of Friend erythroleukemia cell (FELC) clones either sensitive (TS) or resistant (TR) to the effects of TPA (10-13). Among the actions of TPA observed in the sensitive but not the resistant clones are inhibition of differentiation, induction of cell adherence to plastic tissue culture plates and release of arachidonic acid and prostaglandins E_2 and $F_{2\alpha}$ (12,13). In the present studies we examined the possibilities that resistance to TPA involves a change either in the dynamics of membrane lipids or in the binding of phorbol esters to high-affinity saturable receptors present in these cells.

METHODS

Two TPA-sensitive (TS19-6 and TS19-10) and two -resistant (TR19-4 and TR19-9) FELC clones, isolated as previously described (10-13), were grown in suspension in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (DMEM-10) and subcultured into fresh DMEM-10 at a density of 2×10^5 cells/ml twice weekly. For the studies described below, logarithmically growing cultures which had been subcultured 24 or 48 hrs previously were used. Cells were labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) by adding 0.5µl of a 1.5 mM solution of DPH in absolute ethanol to 1 ml of PBS containing $\sim 2.5 \times 10^6$ cells. Steady-state fluorescence polarization was determined after incubation of the cells for 1 hr at 37°C, using an SLM fluorescence polarization spectrophotometer (SLM Instruments, Urbana, Illinois), as previously described (14,15). Results are expressed in terms of the anisotropy parameter, $[(r_0/r)-1]^{-1}$ (15,16), which varies inversely with lipid fluidity; r the maximal limiting anisotropy of DPH was taken as 0.362° (17). Cells were enriched with cholesteryl hemisuccinate (CHS) by a modification of the method of Shinitzky et al. (18). The enrichment medium was prepared by dropwise addition of a solution of 50µg/ml of CHS (Sigma) in absolute ethanol to a solution of 3.5% (wt/vol) of polyvinylpyrrolidone (Sigma: mol. wt. 40,000) and 1% (wt/vol) bovine serum albumin in phosphate-buffered saline (PBS), pH 7.4. TS or TR cells ($\sim 2 \times 10^7$) were sedimented at 1500g for 4 min at room temperature, washed 3 times with PBS and finally suspended in 20 ml of the enrichment medium and incubated for 30-60 min at 37°C. Control cells were treated identically with a medium lacking CHS. After incubation, the cells were washed 3 times with PBS, viability was confirmed by trypan blue dye exclusion and aliquots were taken for DPH studies and for adhesion assays as previously described (12). Specific binding of [20-^{3}H]-

Table 1: Fluorescence Anisotropy of Diphenylhexatriene in TPA-Resistant (TR) and TPA-Sensitive (TS) Friend Erythroleukemia Cells (25°C).

Cell Type	Agent	Treatment time (h)	DPH$[(r_0/r)-1]^{-1}$ (mean ± SE)*
TR19-4	none	0	2.45 ± 0.04
	DMSO+	4	2.12 ± 0.01
	TPA++	4	2.01 ± 0.03
TR19-19	none	0	2.15 ± 0.03
	DMSO	4	2.02 ± 0.03
	TPA	4	2.06 ± 0.02
TS19-6	none	0	1.92 ± 0.02
	DMSO	4	1.78 ± 0.07
	TPA	4	1.74 ± 0.02
TS19-10	none	0	1.62 ± 0.02
	DMSO	4	1.42 ± 0.05
	TPA	4	1.47 ± 0.03

*Cells were loaded with DPH and fluorescence anisotropy estimated as described in Methods. Means of two determinations are listed.

+Exposure to 0.01% DMSO vehicle alone for 4h at 37°C

++Exposure to 100 ng/ml of TPA in 0.01% DMSO for 4h at 37°C.

phorbol-12,13-source dibutyrate (^{3}H-PDBu; 6.4 Ci/mmol;) was determined on intact cells (9,19). Approximately 2.5 x 10^7 TR19-4 or TS19-10 cells were pelleted, washed once with PBS and suspended in 25 ml of "assay" buffer (2 volumes DMEM diluted with 1 volume PBS and containing 1 mg/ml bovine serum albumin). One-ml aliquots of the suspension were incubated in 1.5 ml Eppendorf tubes containing 0.05μCi ^{3}H-PDBu plus sufficient unlabeled PDBu to yield final concentrations ranging from 4nM to 50μM. PDBu was added from a 20 mM stock solution in dimethylsulfoxide (DMSO). The cells were shaken at 37°C for 30 min, pelleted by centrifugation at 2000g for 10 min at 4°C, washed once with 1 ml of ice-cold assay buffer and the final pellet solubilized in 0.5 ml of 1% (wt/vol) sodium dodecylsulfate (SDS). After standing in the SDS overnight at 37°C, the preparations were washed into counting vials and counted in 10 ml of Hydrofluor (New England Nuclear). Nonspecific binding was measured in parallel assays that contained a thousand fold excess of unlabeled PDBu and the values obtained were subtracted from the total binding to obtain specific binding (19).

RESULTS AND DISCUSSION

As shown in Table 1, the values of the DPH anisotropy parameter in two TR clones were 2.45 & 2.15. These values are significantly higher than the values of 1.92 & 1.62 obtained with the two TS clones. Additional studies (not shown here) also revealed differences in fluorescence anisotropy with a membrane-impermeant fluorophor, glutathione-pyrene II (20)(Cogan, Fisher and Schachter, unpublished data); the $[(r_0/r)-1]^{-1}$ values at 25°C for TR19-4 and TS19-10 were

Table 2: Effect of Cholesteryl Hemisuccinate (CHS) Treatment on the Adhesion of TPA-Sensitive (TS19-6) and TPA-Resistant (TR19-4) FELC Clones in the Presence of TPA.

Cell Type	Experiment Number	% of Cells Attached to Culture Dish* (1 hr)	% of Cells Attached to Culture Dish (4 hrs)
TS19-6	1	60	65
	2	75	80
TS19-6+CHS	1	25	10
	2	28	16
TR19-4	1	20	18
	2	18	12
TR19-4+CHS	1	18	20

*The cell adhesion assay was performed as previously described (12). CHS modification of cells is described in Methods and reference 18. The values given are the means of duplicate determinations. In the absence of TPA the % of cells attached at 1 hr or 4 hrs, with or without the CHS treatment, was less than 5%.

2.06 and 1.08, respectively. These results provide evidence that motional freedom ("fluidity") of membrane lipids is greater in the TS than in the TR clones. Exposure of the FELC clones to DMSO (0.01%) or TPA (100ng/ml in 0.01% DMSO) for 4 hrs at 37^{o}C caused approximately equivalent reductions in the DPH anisotropy parameter. Prior studies have demonstrated that TPA does increase the membrane lipid fluidity of rat embryo cells (15) and of certain human lymphocyte and lymphoblastoid cell lines (21), and that this is not simply due to the DMSO solvent. It is possible that the apparent lack of a similar TPA effect in the FELC clones may relate to the relatively short time period studied; since although TPA induction of arachidonic acid release and prostagladin synthesis in fibroblast cultures occurs within the first few hrs (22-24), in TS sensitive FELC cells these TPA responses require 24-48 hrs (13).

Treatment of the TPA sensitive FELC clones TS19-6 and TS19-10 with cholesteryl hemisuccinate increased their DPH anisotropy parameter by 31% and 30%, respectively, bringing the values to the levels observed in TR cells. Cell viability was not affected by CHS. The effects of this treatment on TPA-induced cell adhesion are given in Table 2. Under the conditions employed, in the absence of TPA less than 5% of the TS and TR clones became ad-

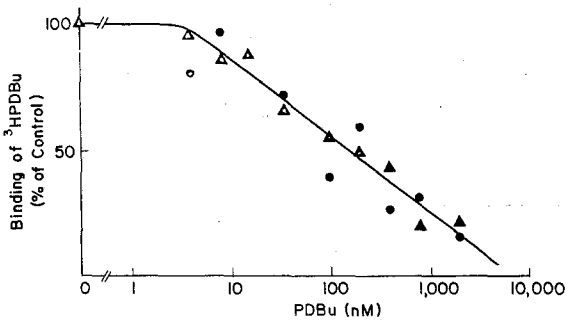

Figure 1: Inhibition of binding of ^{3}H-PDBu to TPA-sensitive (TS19-10) and TPA-resistant (TR19-4) Friend Erythroleukemia cells (FELC) by non-radioactive PDBu. Cultures were assayed for ^{3}H-PDBu binding as described in "Materials and Methods". Binding in the absence of unlabeled PDBu is expressed as 100%; the actual values for TS19-10 and TR19-4 cells were 678 and 617 cpm/10^6 cells, respectively. All data have been corrected for nonspecific binding, i.e. residual binding of radioactivity in the presence of 50μM unlabeled PDBu.
TS19-10 cells, ●; TR19-4 cells, ▲.

herent to the culture dish. After 4 hrs in the presence of TPA (100 ng/ml) 65-80% of the TS19-6 cells, but only 16-18% of the TR19-4 cells, adhered to the culture dishes. Following exposure to CHS only 10-16% of the TS19-6 cells adhered in the presence of TPA. CHS did not have a significant effect on the low adhesion obtained with the TR19-4 cells. Prior studies of TPA-induced adhesion of FELC lines (11,12) indicate that the phenomenon is temperature dependent but does not appear to require de novo RNA or protein synthesis (12). The present results indicate that TPA induction of cell adhesion is modulated by the lipid composition of cell membranes.

Recent studies have demonstrated the presence of saturable, high-affinity receptor sites for phorbol esters in crude membrane preparations of chick embryo fibroblasts (25) and mouse epidermal cells (26) and in various intact cells (9, 19,27). To determine if differences in TPA sensitivity between the FELC clones reflect a change in receptor number or affinity, ^{3}H-PDBu binding and the competitive inhibition of ^{3}H-PDBu binding by unlabeled PDBu were examined in the FELC clones TR19-4 and TS19-10. As shown in Fig. 1, the binding data for the sensitive and resistant clones are not significantly different. Scatchard plots of

these data were curvilinear and consistent with the presence of two classes of sites with affinity constants of about 8 nM and 800 nM, corresponding to 3 x 10^4 and 3 x 10^6 receptors per cell, respectively. These values were essentially the same in TR19-4 and TS19-10 cells.

Thus the acquisition of resistance to TPA by certain variants of FELC does not appear to be due to a decrease in number or affinity of phorbol ester receptors. In some cases TPA resistance may be due to an alteration in the lipid matrix of cell membranes, since we found that two TPA resistant clones had higher DPH anisotropy values than did two TPA sensitive clones. In addition, incubation of a TPA sensitive clone with CHS caused an increase in DPH anisotropy and made the cells resistant to TPA-induced cell adhesion. Although the underlying mechanisms are not known, it is of interest that the biologic effects of TPA are associated with changes in membrane lipids (for review see 9). It is tempting to speculate that specific alterations in the lipid composition of cell membranes, as a function of nutritional factors, might alter the sensitivity of tissues to tumor promoters in the intact animal.

ACKNOWLEDGEMENTS This research was supported by National Cancer Institute Grant CA-21111-04 and NIH Grants AM21238 and HL16851. The authors thank Dr. Hiroshi Yamasaki for providing the FELC clones used in the present study.

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