Vol. 86, No. 4, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1979 Pages 1063-1068

TUMOR PROMOTERS INDUCE MEMBRANE CHANGES DETECTED BY FLUORESCENCE POLARIZATION

Paul B. Fisher, Michael Flamm, David Schachter and

I. Bernard Weinstein

Institute of Cancer Research,
Departments of Physiology and Medicine,
and Division of Environmental Sciences,
Columbia University College of Physicians and Surgeons
701 West 168th Street, New York, New York 10032

Received January 10,1979

SUMMARY: The probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) was used to determine if tumor promoting agents alter cell membranes. The active tumor promoters TPA (12-0-tetra-decanoyl-phorbol-13-acetate), PDD (phorbol-12,13-didecanoate) and PDB (phorbol-12,13-dibenzoate) were found to decrease fluorescence polarization of DPH in rat embryo cells, whereas the inactive tumor promoting compounds phorbol and 4α-PDD failed to induce this change.

In mouse carcinogenesis (1) a single application of a subcarcinogenic dose of an "initiating carcinogen" followed by repeated applications of a "promoting agent" result in the induction of multiple skin tumors. The most potent promoting agent for mouse skin is 12-0-tetradecanoyl-phorbol-13-acetate (TPA) and related plant diterpenes (2-4). TPA has been shown to induce changes in the growth and morphology (5,6), cell surface properties (7-9), 2-deoxyglucose transport (10) and plasminogen activator production (7,8,11) of cells in culture which mimic those changes associated with cell transformation. Although the biologically important interactions between tumor promoters and cells are not known, evidence implicating the cell membrane as a critical target has been presented (5,12-17). In the present study, fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH)

Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; TPA, 12-0-tetra-decanoyl-phorbol-13-acetate; PDD, phorbol-12,13-didecanoate; PDB, phorbol-12,13-dibenzoate; DMEM-10, Dulbecco's modified Eagle's medium with 10% fetal calf serum; DMEM-2, same as preceding except 2% fetal calf serum; DMSO, dimethylsulfoxide.

0006-291X/79/041063-06$01.00/0

(18-20), a hydrocarbon fluorophor, was used to determine if tumor promoting agents perturb the lipid components of cell membranes.

METHODS Experiments were performed using second to fifth passage rat embryo cultures established from 14 day old Sprague Dawley rat embryos (21). Confluent monolayers ($\sim$4-5x10^6 cells/9 cm tissue culture plate) were grown in Dulbecco's modified Eagle's medium (22) with 10% fetal calf serum (DMEM-10) and were exposed to 0.01% DMSO (solvent control) or the test compound in 10 ml of DMEM with 2% fetal calf serum (DMEM-2) for 1 to 5 h. Cells were then dispersed into a single cell suspension with trypsin (0.2%), washed three times in phosphate-buffered saline (23) free of calcium and magnesium, suspended in 2 ml of phosphate-buffered saline ($\sim$3-4x10^6 cells/ml) containing 10^{-6}M DPH, and incubated for 1-2 h at 37° C (19,20). Thereafter, estimations of fluorescence polarization were made at 25° C as previously described (24) using an SLM fluorescence polarization spectrophotometer (SLM Instruments, Urbana, Illinois) with an activating wavelength of 365 nm. The polarization of fluorescence was expressed as the fluorescence anisotropy, $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized, respectively, parallel and perpendicular to the direction of polarization of the exciting beam. Results were also expressed in terms of an anisotropy parameter (19,20,24), $[(r_o/r)-1]^{-1}$, where r_o is the maximal limiting anistropy, which has an experimental value of 0.362 for DPH (25). Total fluorescence intensity, $F = I_{\parallel} + 2I_{\perp}$, was also monitored to detect changes in the mean lifetime of the excited state of the probe.

RESULTS As shown in Fig. 1, TPA levels as low as 0.1 ng ml^{-1} ($1.6x10^{-10}$M) induced a decrease in fluorescence polarization of the fluorescence probe in confluent rat embryo cells. The maximum change in DPH $[(r_o/r)-1]^{-1}$ resulted after exposure to 25 ng ml^{-1} of TPA and concentrations of 50-200 ng ml^{-1} gave no further significant decrease. The decrease observed was greater when rat embryo cells were treated with TPA in DMEM-2 rather than DMEM-10 and in confluent rather than subfluent cultures.

The time course (1-5 h) of change in fluorescence polarization of DPH in control and TPA treated rat embryo cells is illustrated in Fig. 2. Control cultures exposed to the solvent alone, (0.01%) DMSO, showed a small decrease during a 4-5 h exposure. The addition of 4α-PDD (200 ng ml^{-1}), a phorbol ester analog that lacks tumor promoting activity (3), gave a result similar to that of the solvent control. On the other hand, TPA (200 ng ml^{-1}) caused a significant decrease in the $[(r_o/r)-1]^{-1}$ values as compared to the controls. The decrease was apparent within 1 h, maximal at 3-4 h, and partially reversed at 5 h. Addition of actinomycin D (10 μg ml^{-1}) at time zero

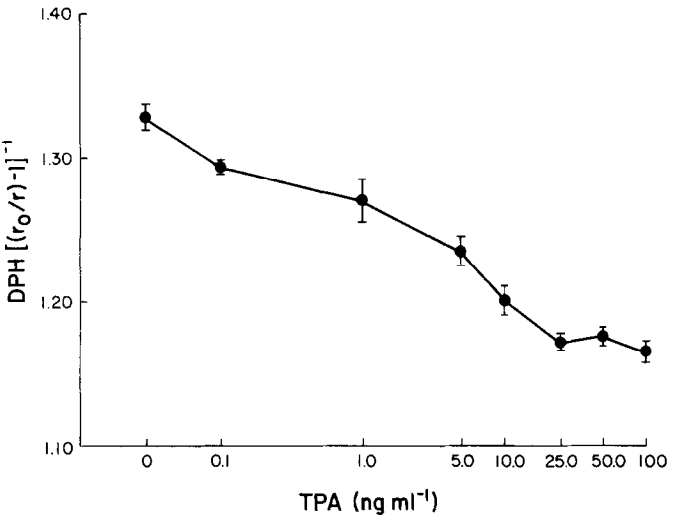

Fig. 1. Dose effect of TPA on rat embryo cells. Confluent cultures ($\sim 5 \times 10^6$ cells per 9 cm. dish) were refed with DMEM-2 and treated with the indicated concentrations of TPA for 4 h. Cells were dispersed with trypsin, washed 3 times with phosphate-buffered saline, resuspended in 1 ml of phosphate-buffered saline and incubated for 2 h with 10^{-6}M DPH. Fluorescence polarization of DPH was determined and expressed as $[(r_o/r)-1]^{-1}$, as described in the text. Values are means±SE for 4 determinations of each sample. TPA 0 refers to DMSO (0.01%) treated rat embryo cells.

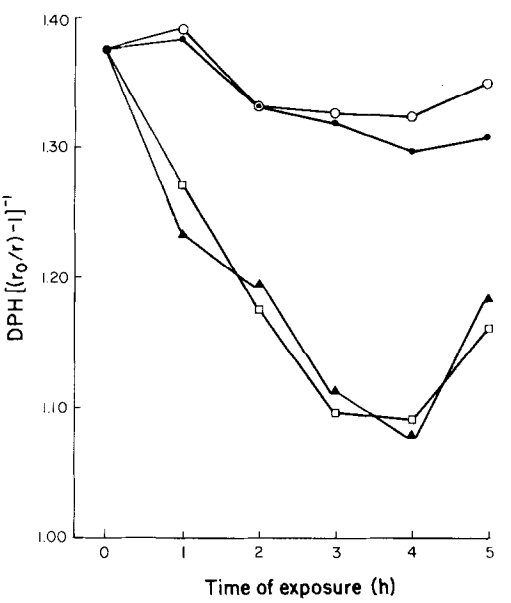

Fig. 2. Time course of DPH $[(r_o/r)-1]^{-1}$ change in rat embryo cells. Agents were added in DMEM-2 at time zero to confluent rat embryo cultures. Cells were collected at the indicated times and assayed for DPH fluorescence polarization as described in Fig. 1. ●, solvent control (DMSO, 0.01%) treated cells; o, 4α-PDD (200 ng ml^{-1}) treated cells; ▲, TPA (200 ng ml^{-1}) treated cells; □, TPA (200 ng ml^{-1}) and actinomycin D (10 μg ml^{-1}) treated cells.

Table 1. Effects of Phorbol Esters on Fluorescence Polarization of Diphenylhexatriene in Rat Embryo Cells

Compounds*	No. of Samples†	r (mean±SE)	DPH[$(r_o/r)-1]^{-1}$ (mean±SE)	P††	Tumor-promoting activity in mouse skin§
Dimethyl sulfoxide (DMSO)	15	0.202±0.001	1.268±0.020		0
Phorbol	6	0.202±0.002	1.242±0.010	n.s	0
4α phorbol-12,13-didecanoate (4α-PDD)	6	0.201±0.003	1.255±0.035	n.s	0
Phorbol-12,13-dibenzoate (PDB)	3	0.198±0.001	1.217±0.010	<0.05	+
Phorbol-12,13-didecanoate (PDD)	9	0.192±0.003	1.125±0.035	<0.001	++
12-0-tetradecanoyl-phorbol-13-acetate (TPA)	15	0.187±0.002	1.069±0.020	<0.001	++++

*DMSO (0.01%); phorbol, 4α-PDD, PDB, PDD and TPA (100 and 200 ng ml^{-1}).

†Samples were confluent rat embryo cell plates(~4-6x10^6 cells per 9 cm plate) exposed to test compound for 3-4 h.

††P values as calculated by the paired t test for differences from DMSO controls in each experiment.

§Refer to Boutwell (3).

together with TPA failed to block the TPA effect (Fig. 2) and cycloheximide (100 μg ml^{-1}) was similarly ineffective. Hence the change in DPH fluoresence polarization induced by TPA does not seem to require a new synthesis of RNA or protein.

The effects of mouse skin tumor promoters (TPA, PDD, PDB), and of structurally related compounds inactive in tumor promotion (phorbol and 4α-PDD), on DPH fluorescence polarization in confluent rat embryo cultures are compared in Table 1. Phorbol and 4α-PDD (100 ng ml^{-1}) failed to alter the fluorescence polarization significantly. (Phorbol was similarly ineffective when tested at concentrations as high as 1000 ng ml^{-1}). In contrast, the active tumor promoters TPA, PDD and PDB decreased the control

values of DPH $[(r_o/r)-1]^{-1}$ by 15.7% ($P<0.001$), 11.3% ($P<0.001$) and 4.0% ($P<0.05$), respectively. The rank order of these values corresponds to tumor promoting activity (TPA>PDD>PDB) on mouse skin (3). Although the DPH $[(r_o/r)-1]^{-1}$ values of control, DMSO-treated rat embryo cells varied somewhat in different isolates, in different passage numbers and in growing versus confluent cells, TPA and PDD consistently caused a significant decrease when compared to the corresponding controls. It is significant that the decreases in fluorescence polarization of DPH induced by TPA, PDD and PDB were not associated with changes in total fluorescence intensity. Thus the effect on fluorescence polarization is apparently not due to a change in the mean lifetime of the excited state.

DISCUSSION The present results provide further evidence that TPA and related tumor promoters affect the dynamic organization of cell membranes (5, 12-17). Inasmuch as the changes observed occur within 1-2 h of exposure and do not require new protein or RNA synthesis, they appear to result from primary effects of the tumor promoters on cell membranes. A decrease in fluorescence polarization of DPH unaccompanied by a change in excited state lifetime of the probe has been interpreted as an increase in lipid fluidity of cell membranes (10,20,24,25). Recent studies with model membranes (26,27) suggest that the DPH fluorescence polarization may also be influenced by additional structural parameters which may restrict free rotation of the probe in the membrane. Hence further studies employing purified cell membranes and time-resolved fluorescence polarization (26) or differential phase fluorometry (27) are required to interpret more precisely the physical basis for the effects of the tumor promoters. Extensions of the present approach may provide further insights into the possible role of membrane changes in the mechanism of tumor promotion. In addition, the ability to detect effects of tumor promoting agents by fluorescence polarization may be useful in screening for potential tumor promoters in the environment.

ACKNOWLEDGMENTS The technical assistance of Mr. Scott Sinclair is appreciated. We also thank Dr. R. A. Mufson for helpful discussions. This research was supported by Contract Number NO-1-CP-2-3234 from the National Cancer Institute, NIH Grants HL-16851 and AM-01483, a career award of the Irma R. Hirschl Trust (D.S.), and the Columbia University Medical Scientist Training Grant GM07367(M.F.).

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