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Surface Properties of Phorbol Esters and Their Interaction with Lipid Monolayers and Bilayers[1]

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SUMMARY

The potent tumor-promoting agent 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) is surface active and was found to occupy a limiting area of 62 sq Å/molecule in monolayers at the air-water interface. The interfacial tension of aqueous TPA solutions is decreased by increasing the bulk-phase TPA concentrations up to 2×10^{-6} M, beyond which no further decreases were observed. This concentration is in agreement with the apparent solubility limit previously obtained. The apparent aqueous solubility limit of the more hydrophobic phorbol-didecanoate is 5×10^{-8} M.

Interaction of TPA with egg phosphatidylcholine monolayers at the air-water interface was shown by an increase in the surface pressure of the monolayer from 22 dynes/cm, initial film pressure, to 34 dynes/cm 90 min after introduction of TPA into the aqueous subphase. It was shown by gel filtration chromatography that a similar phorbol derivative, tritiated phorbol-didecanoate, binds to phospholipid vesicles. Differential scanning calorimetry also indicated that the addition of either TPA or an inactive stereoisomer, 4-α-phorbol-didecanoate, to phospholipid bilayers results in a marked reduction of the enthalpy of the minor transition of dipalmitoylphosphatidylcholine liposomes. Several fluorescence polarization probes for membrane fluidity indicate that TPA does not affect this membrane parameter. Further, the presence of TPA induces no measurable change in the cation permeability of phospholipid vesicles, the conductance of planar bilayer membranes, or the electrophoretic mobility of negatively charged liposomes. The lack of a specific effect with bilayers alone, combined with the documented physiological effects at low TPA concentrations, point to the possibility of a specific membrane component as the receptor for TPA at the plasma membrane.

INTRODUCTION

The cell membrane is a likely organelle for regulation of cell growth (4) possibly through alterations in transport properties (9) or membrane enzyme activities regulated by membrane fluidity (7, 14). On the basis of several studies, it can be inferred that a possible site of interaction for cocarcinogenic phorbol esters is at the cell plasma membrane. Baird and Boutwell (1) have demonstrated that only those phorbol esters with lipophilic side chains are active as promoters. Belman and Troll (2) have observed that cyclic adenosine 3′:5′-monophosphate levels are reduced upon treatment of mouse epidermis with phorbol esters. Sivak *et al.* (21) have reported that active phorbol esters such as TPA[3] alter cell membrane enzyme activity, although others (3, 22) have failed to confirm the finding that the $Na^+ + K^+$-ATPase is stimulated by TPA in mouse skin. Instead, at concentrations of TPA greater than 5×10^{-6} M, TPA has been observed to inhibit this enzyme. Further, Wenner *et al.* (22) have observed that the active promoter, TPA, at 10^{-6} M, but not an inactive stereoisomer, α-PDD, decreases cell electrophoretic mobility by 15%. Also, Zucker *et al.* (23) have reported that TPA at 10^{-9} M induces platelet aggregation. Finally, an upswing in cyclic guanosine 3′:5′-monophosphate level was observed within minutes after TPA administration to mouse embryonic fibroblasts (BALBc/3T3 cells) similar to the effect of mitogenic plant lectins which are capable of binding at the cell surface (6). Intracellular membranes may also be sites of action for TPA as the promoter cosediments with liver microsomal and nuclear membranes in CsCl equilibrium density gradients (15). This evidence stimulated our interest in studying the surface activity of several phorbol esters and characterizing their interaction with phospholipid monolayers and bilayers.

MATERIALS AND METHODS

Reagents. TPA and PDD were obtained from Consolidated Midland Corp., Brewster, N. Y. α-PDD and [^{3}H]PDD were generous gifts of Dr. E. Hecker and Dr. R. Boutwell, respectively. TPA and α-PDD purity were checked by thin-layer chromatography (8). TPA preparations initially displaying a single spot (R_F about 0.30 on Brinkman Silica Gel F-254 plates) at 25° using a solvent of 75% CH_2Cl_2/25% acetone (v/v) were stable for at least 5 months when stored in ethanolic stock solutions under

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[3] The abbreviations used are: TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; α-PDD, 4-α-phorbol-12,13-didecanoate (inactive stereoisomer); [^{3}H]PDD, tritiated PDD; PDD, phorbol-12,-13-didecanoate (active stereoisomer); PC, egg phosphatidylcholine; PS, beef brain phosphatidylserine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; DPH, 1,6-diphenyl-1,3,5-hexatriene.

nitrogen at 10 mg/ml at −5° and in the dark. [^{3}H]PDD was further purified by preparative thin-layer chromatography. Its concentration was determined by comparing the area that an aliquot occupied at the air-water interface in a Langmuir trough to that occupied by a known amount of PDD. The similarity of surface pressure *versus* area isotherms for the [^{3}H]PDD and PDD monolayers provided evidence for the purity of the [^{3}H]PDD preparation.

PC and PS were prepared as previously described (17). The synthesis of DPPC has also been described (10). Phosphatidyl[^{14}C]choline was obtained from ICN Pharmaceuticals, Inc., Irvine, Calif. The fluorescence probes used were perylene and 1,6-diphenylhexatriene (Aldrich Chemical Co., Milwaukee, Wis.), and all-*trans*-retinol (Sigma Chemical Co., St. Louis, Mo.).

Methods. The monolayer techniques (13) and bilayer conductivity measurements (12) are described elsewhere. Phospholipid vesicle permeability measurements (16), differential scanning calorimetry measurements (10), and fluorescence polarization measurements (11) have also been previously described. Electrophoretic mobilities were measured on unsonicated lipid dispersions composed of PC and PS in a 9/1 molar ratio following the method of Wenner *et al.* (22). Specific experimental details are given in the chart legends.

All lipid dispersions were prepared in 10 or 100 mM NaCl buffered to pH 7.4 using 2 mM histidine and 2 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid and containing 0.1 mM EDTA. Sonication, where required, was performed as previously described (16) for 1 hr at 25°.

For a demonstration of PDD binding to phospholipid bilayers, sonically treated and centrifuged vesicles (100,000 × *g* for 1 hr at 20°), composed of PC and PS in a 9/1 molar ratio (7.15 μmoles/ml, total phospholipid), were incubated with 5.6×10^{-7} M [^{3}H]PDD for 12 hr at 4°. The suspension was then passed over a Sephadex G-50 column having a bed volume of 65 ml and a height of 30 cm. Under such conditions, the vesicles elute in the void volume (17) and could be located by incorporation of <0.001 mole per 100 ml phosphatidyl[^{14}C]choline as well as absorbance at 300 nm. Tritium counting was performed in a Beckman LS-230 counter.

RESULTS

TPA Solubility in Aqueous Media

TPA has a strong affinity for the air-water interface. This is shown in Chart 1 where the surface tension of the air-water interface at 25° is seen to be progressively reduced by TPA, until a concentration of about 2 μM is reached. This concentration corresponds closely to the apparent aqueous solubility limit of 3 μM obtained by radioactive techniques similar to that described below for [^{3}H]PDD (B. L. Van Duuren, private communication). Visible turbidity occurs in the range of 10 to 30 μM.

We have examined the apparent solubility of [^{3}H]PDD in the following way. Aqueous solutions of [^{3}H]PDD were prepared by adding the promoter in less than 0.5% (v/v) ethanol to 10 ml of distilled water. Following mixing, the solutions were allowed to equilibrate for 16 hr at 25°. Then aliquots were removed for scintillation counting. The radioactivity remaining in the aqueous phase increased linearly with concentration from 5×10^{-9} to 5×10^{-8} M; beyond 5×10^{-8} M only slight further increases in radioactivity were observed. In the linear region, aqueous-phase recovery was 90% complete. Thus, 5×10^{-8} M is an apparent solubility limit in the sense that material added above this concentration forms insoluble aggregates which settle out. The presence of small "micellar structures" below 5×10^{-8} M cannot be excluded. The lower apparent solubility limit for [^{3}H]PDD compared to TPA would be expected on the basis of the greater number of methylene groups in the alkyl chain moieties for [^{3}H]PDD.

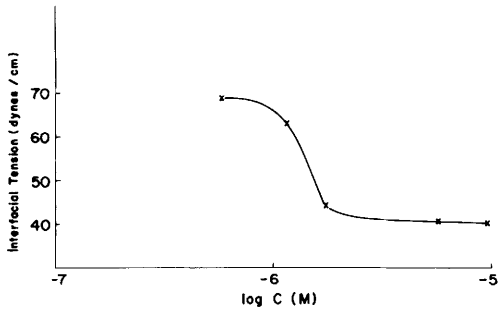

Chart 1. Interfacial tension as a function of TPA concentration. TPA in 5 μl ethanol was placed in the stirred distilled water subphase at 27°, and the interfacial tension was allowed (over 2 hr) to reach an equilibrium value. Trough volume was 60 ml; film area was 30 to 35 sq cm.

Phorbol Ester Monolayers at the Air-Water Interface

Chart 2 shows surface pressure-area curves for TPA, PDD, and α-PDD at the air-water interface at 25°. The ability of these phorbol esters to exist in monolayers at high surface pressure suggests considerable surface activity.

Although PDD and α-PDD pack quite differently at low pressures, at higher pressures (presumably analogous to the

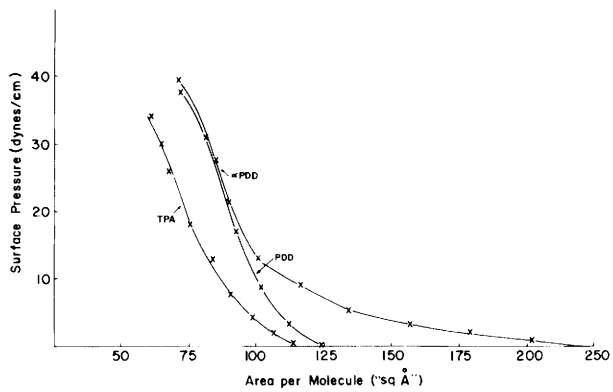

Chart 2. Force-area curves for TPA, PDD, and α-PDD monolayers at the air-water interface. The aqueous subphase consisted of 0.5 M KCl. The solvent used for dissolving phorbol esters was absolute ethanol. Less than 5 μl were added in each case.

membrane situation) both have similar packing areas and collapse pressures. This would suggest that the inactivity of α-PDD is not due to simply a lack of surface activity.

The limiting area of TPA at a collapse pressure (34 dynes/cm) is about 62 sq Å/molecule. This area fits a molecular conformation with the plane of the phorbol ring system parallel to the interface. PDD and α-PDD occupy a greater area near collapse (about 72 sq A/molecule) probably because the adjacent hydrocarbon chains at positions 12 and 13 cannot be packed normal to the ring system unless the molecule occupies more area than TPA.

Interaction of TPA with Phospholipid Monolayers and Bilayers

TPA Penetrates PC Monolayers. Chart 3 shows the kinetics of TPA (1.5 μM) penetration from a distilled water subphase into a PC monolayer initially at a pressure of 22 dynes/cm. At a TPA concentration of 1.5 μM, a final pressure of 34.5 dynes/cm is reached within 90 min. At 2.5 μM TPA (not shown), a final pressure of 38 dynes/cm is obtained. Since these pressures exceed the TPA equilibrium spreading pressure obtained in this subphase (29.6 ± 2 dynes/cm, mean of 4 experiments), we conclude that TPA interacts with and is dispersed within the monolayer.

[^{3}H]PDD Binds to Phospholipid Vesicles. When 5.6×10^{-7} M [^{3}H]PDD was incubated with sonicated phospholipid vesicles composed of PC and PS in a 9/1 molar ratio at 4° for 12 hr, 98% of the total [^{3}H]PDD could be eluted with the vesicles in the void volume of a Sephadex G-50 column, suggesting that the [^{3}H]PDD had become incorporated into the phospholipid vesicles (Chart 4). The remaining [^{3}H]-PDD eluted as a free molecule between 52 and 70 ml of eluting volume. Similar results were obtained when [^{3}H]-PDD was incubated with pure PC vesicles. Since the PDD concentration in this experiment exceeded its solubility limit, it is possible that the phorbol was eluting as an aggregate. For investigation of this possibility, 5.6×10^{-7} M [^{3}H]PDD was incubated for 12 hr at 4° in buffer containing no vesicles. This sample was then eluted from a similar column with the result that less than 4% of the PDD eluted in the void volume, and 28% was recovered in the free molecule peak. The remainder was presumed to have adhered to glass surfaces. This property of PDD was also observed in the solubility experiments at concentrations above the solubility limit.

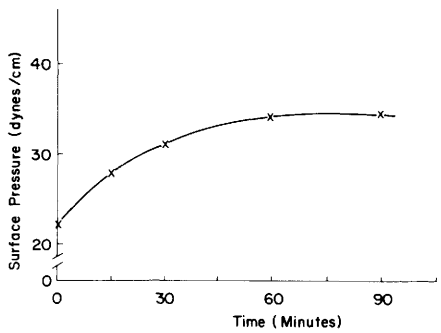

Chart 3. Kinetics of TPA penetration into a PC monolayer. Initial PC film pressure, 22 dynes/cm. TPA was added to the stirred distilled water subphase in less than 5 μl absolute ethanol to give an initial concentration of 1.5 μM. Other conditions as in Chart 1.

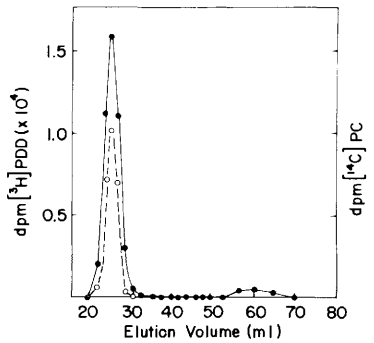

Chart 4. Sephadex G-50 elution profile of PC and PS sonically treated vesicles (9/1 molar ratio, 7.15 μmoles total phospholipid per ml) incubated with 5.6×10^{-7} M [^{3}H]PDD for 12 hr at 4°. ●, [^{3}H]PDD counts; O, phosphatidyl [^{14}C]choline counts.

Effect of TPA on the Thermotropic Phase Transition of DPPC Bilayers

The influence of TPA on the structure of bilayers is demonstrated in DSC studies of the gel-to-liquid crystalline phase transition of DPPC dispersions. Thermograms are given in Chart 5; Chart 5, *curve a* shows the "melting" of DPPC dispersions. The main transition occurs at about 42° with enthalpy of melting of 7.7 ± 0.5 kcal/mole; a minor transition occurs at about 37° and has a heat of 1.2 ± 0.2 kcal/mole (10). The main phase transition is associated with the melting of the hydrocarbon chain region, while the minor transition appears to be associated with a change in average orientation or "tilt" of the acyl chains (19). At

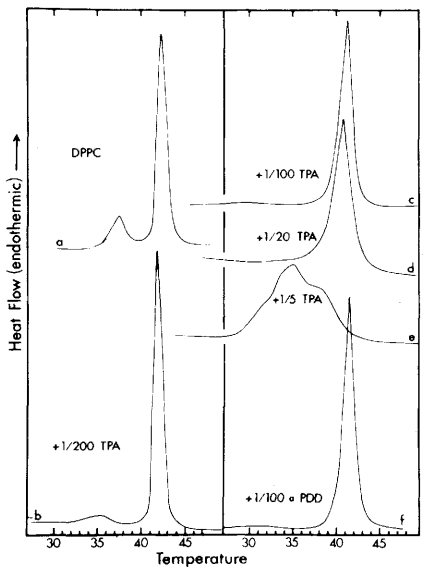

Chart 5. DSC of DPPC dispersions with the indicated mole ratios of TPA or α-PDD added in organic solvent prior to dispersion of the lipid. Heating rate, 5°/min. See Ref. 11 for other details of suspension preparation and calorimetry.

molar ratios (TPA/DPPC) of 1/200 and 1/100, assuming that all added TPA is membrane bound, the heat associated with the minor transition peak at about 34° is strongly diminished (Chart 5, *Curves b* and *c*, respectively). The main phase transition is not greatly affected, except that the midpoint temperature is diminished slightly by about 0.5 and 1°, respectively. At TPA/DPPC ratios of 1/20 (Chart 5, *Curve d*) the minor transition is abolished and the main transition is broadened from 1.2° (no TPA) to 2.0°. Still higher TPA/DPPC ratios completely alter the phase transition (Chart 5, *Curve e*). The inactive isomer, α-PDD, causes a similar effect at 1/100 molar ratios (Chart 5, *Curve f*). Thus, in comparing *Curves c* and *f* of Chart 5, it appears that at low molar ratios both TPA and α-PDD have a similar effect on the melting behavior of DPPC membranes, indicating an effect not specific to tumor promotion.

Effect of TPA on Membrane Fluidity, Cation Permeability, and Liposome Electrophoretic Mobility

In the following experiments, TPA was added to the aqueous suspensions of phospholipid vesicles in absolute ethanol (< 1% v/v). This is the method of addition when the effect of TPA on cellular properties is examined using *in vitro* cell systems. Measurement of the fluorescence polarization of lipophilic probes embedded in bilayer membranes leads to an estimation of membrane fluidity by calculating either probe rotation rate (11) or membrane "microviscosity" (20). Using 3 fluorescence polarization probes, perylene, retinol (18), and DPH (20), we have been unable to detect any fluidity changes upon addition of low concentrations of TPA ($\leq 1.6 \times 10^{-5}$ M or maximum TPA/PC of 1/50) either added to the sonically treated vesicles in the aqueous bathing solution or in organic solvent prior to the sonic treatment of PC dispersions. Experiments with 1 probe, DPH, were carried out with sonically treated PC suspensions at a lipid concentration of 0.01 μmole/ml, approximating the amount of plasma membrane lipid present in a fibroblastic cell suspension at 10^6 cells/ml. [Changes in the electrophoretic mobility of Ehrlich-Lettré ascites tumor cells were observed by incubating 10^6 cells/ml with 10^{-6} M TPA (22).] However, there was no significant effect of 1.6 μM TPA on the fluorescence polarization of DPH embedded in PC vesicles at 25° (polarization, 0.155 ± 0.005 with or without 1.6 μM TPA; excitation wavelength, 365 nm; emission wavelength, 460 nm.

Additions of TPA to sonically treated PS vesicles at concentrations of 0.16 to 32 μM did not alter ^{22}Na ion transport in this system to within experimental uncertainty (efflux rate = 0.8 to 2.0% captured ^{22}Na per hr). In agreement with this observation, no effect on membrane conductance was seen when 10 μM TPA was added to planar PC bilayers bathed in 0.1 N KCl and formed from solutions of PC in *n*-decane. Concentrations of either TPA or α-PDD less than 2 μM did not significantly affect the electrophoretic mobility (5.6 μm/sec cm/V) of negatively charged vesicles composed of PS and PC (1/9 molar ratio). At 3.2 μM TPA, there was a slight increase in mobility to 6.04 μm/sec cm/V opposite to the effect seen in Ehrlich-Lettré ascites tumor cells (22).

DISCUSSION

The binding studies, monolayer penetration, and DSC results show that TPA interacts with phospholipid monolayers and bilayers. Our tentative conclusion is that TPA is dispersed within the membrane, suggesting that promoters could laterally diffuse through biomembranes to distant receptors after binding to bilayer portions of the membrane. The interaction with DPPC at low mole fractions of TPA (and α-PDD) results in a marked decrease in the heat of the minor transition, which can be interpreted as TPA causing the average orientation of the chain axis to remain normal to the plane of the bilayer below the transition. In the absence of a number of impurities, the chains are tilted (β' conformation) below the minor transition and assume a vertical configuration above the minor transition (β conformation) (19). The β conformation is also associated with greater headgroup mobility (5). Other drugs also abolish the minor transition and it has been suggested that the vertical β chain conformation can more readily accommodate impurity molecules (5). The slight decrease in the main phase transition temperature is qualitatively expected when impurities are added to the acyl chain lattice. The width and temperature of the main phase transition of DPPC are not affected appreciably until large mole fractions of TPA are present in the bilayer.

Above the phase transition, the fluidity of the hydrocarbon region of the bilayer does not seem to be affected by the presence of TPA as judged by the lack of change in the fluorescence polarization of probes embedded in the membrane. Spin label studies of the TPA effect on bilayer fluidity have also produced this conclusion (R. E. Barnett, private communication). Further, cation permeability of phospholipid vesicles, conductivity of planar bilayer membranes, and vesicle electrophoretic mobility are unaffected by the presence of TPA. [The last parameter was checked because cellular electrophoretic mobility was altered by TPA (22).]

The DSC results show that similar bilayer interactions occur with the active and inactive phorbol esters; this suggests that *in vivo* these interactions, while they may be necessary, are not sufficient to cause the tumor promotion response. The extremely low concentrations of TPA (approximately 1 nM) required to induce platelet aggregation (23) suggest the existence of a TPA receptor for this response with a great affinity for the agonist. These possible TPA sites of action could be membrane proteins or protein/lipid complexes. Indeed, the dependence of the tumor promotion effect on lipophilicity of the phorbol derivative (1) suggests the possible involvement of lipid in the postulated membrane site.

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