Interactions of Phorbol Esters with Cellular Membranes in Vitro

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SUMMARY

Esters of phorbol, a family of tumor-promoting agents, interact in vitro with cellular membranes. In CsCl equilibrium density gradients, isotopically labeled phorbol diester cosediments with microsomal and nuclear membranes prepared from rat liver. The interaction between these membranes and the promoting compound is enhanced in the presence of native or denatured DNA. Moreover, changes in membrane density and in the amounts of radioactive DNA associated in vitro with the membrane are seen in CsCl in the presence of several unlabeled phorbol esters. The possibility that alterations of cellular membranes are related to tumor promotion is considered.

INTRODUCTION

Carcinogenic chemicals interact in vivo with cellular membranes (5, 8, 18, 26, 27), and this interaction can be further studied in vitro by the technique of equilibrium density gradient centrifugation in CsCl (12-14). Following the in vitro exposure of cellular membranes to chemical carcinogens, changes in the density of the membrane and in the amounts of nucleic acids associating with it were observed (12, 14). It seemed interesting to us to investigate the effects of compounds that by themselves are not carcinogenic but that are able to induce neoplasms (“promotion”) in cells formerly treated with carcinogens at subcarcinogenic levels (“initiation”). Moreover, work done in several laboratories suggested that cellular membranes may be an early target for tumor promoters (11, 20, 22, 25).

The agents chosen for our experiments were esters of phorbol, as well as the parent chemical itself. In order of decreasing potential for tumor promotion in mouse skin (3), TPA is followed by PDD and PDB. Phorbol-12,13-dipropionate (data to be published) and phorbol-12,13-diacetate, as well as free phorbol, are inactive. The role of these compounds in tumor induction in experimental animals has been summarized in recent articles from this and other laboratories (2, 3, 7).

MATERIALS AND METHODS

Methods of isolation and purification of 32P-labeled Escherichia coli Q13 DNA were previously described (15). The nuclear and microsomal membranes from rat liver were purified as described by Kashnig and Kasper (9). This method calls for treatment of the membrane with citrates, sonic dispersion, and separation on discontinuous sucrose gradients. The membrane was collected at the interface between sucrose layers of density 1.16 and 1.14 g/ml. “Crude microsomal membrane” was prepared without the treatment with citrates and presumably contained some proteins that normally are removed by the citrate-sucrose washes. Such membrane preparations bind rRNA’s and tRNA’s (unpublished data), in addition to DNA, thus differing in their specificity from the purified microsomal membrane (13). The erythrocyte “ghosts” were prepared by the method described by Dodge et al. (4).

Calf thymus histone and calf thymus DNA were purchased from the Worthington Biochemical Company, Freehold, N. J. This DNA sedimented in sucrose density gradients as a homogeneous material at the rate of about 22 S. Yeast RNA (high molecular weight) was purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio, and was further purified by 2 successive rounds of cold phenol extraction and ethanol precipitation. Methyl-esterified bovine serum albumin was prepared according to the method of Mandell and Hershey (16).

Phorbol and its esters were synthesized as previously described (2) and dissolved in dimethyl sulfoxide at a concentration of 10 mg/ml immediately before use. One of these esters, PDD, was custom labeled by the Amersham/Searle Corporation, Arlington Heights, Ill., by means of a mild tritium-exchange procedure. After labeling, PDD-3H was purified by thin-layer chromatography (2). All other chemicals used for this study were of analytical grade.

Technical details of the cesium chloride density gradient centrifugation of cellular membranes and DNA-membrane complexes have been reported (13). Briefly, membrane and labeled DNA were mixed in a buffered salt solution (0.1 M NaCl, 0.01 M MgSO4, and 0.02 M phosphate buffer, pH 7.2) and incubated for 30 min at 40°. After the incubation, samples were diluted, supplemented with a saturated solution of CsCl (up to a density of about 1.18 g/ml; refractive index between 1.351 and 1.352), and centrifuged in a Spinco SW 41 rotor for 22 hr at 28,000 rpm. The tubes were subsequently viewed, the positions and the appearance of membrane bands were recorded, and the contents were collected dropwise from the bottom. The refractive
indices of several fractions were measured and the densities of CsCl were calculated (1). Radioactivity in individual fractions was determined by routine methods.

RESULTS

Effect of Phorbol Esters on Binding of DNA to Rat Liver Microsomal Membrane. E. coli DNA mixed with purified microsomal membrane from rat liver forms stable complexes which can be detected by the technique of equilibrium density gradient centrifugation in CsCl (13). The labeled DNA is found associated with the membrane band at the density characteristic for the membrane (ca. 1.2 g/ml), which is much lower than that of pure E. coli DNA (1.71 g/ml). The membrane forms in the gradient a narrow band composed of white, amorphous material.

The effects of phorbol and its esters on the density of the membrane and on its ability to bind DNA are summarized in Table 1. In accordance with our earlier observations (13), we consider as significant differences in density equal to or larger than 3 mg/ml and differences in radioactivity associated with the membrane band equal to or larger than plus or minus 10%. Except for free phorbol, all esters irrespective of their promoting ability changed one or more of the parameters measured, namely, the density of the membrane, its macroscopic appearance, and the amounts of DNA associated with the band. In one case (PDB) the alterations in the density were so extensive that 2 bands, instead of 1, were visible, and DNA associated with only 1 of them. Binding of DNA to the membrane is strongly inhibited by TPA, an effective promoter. However, the 2nd-strongest compound, PDD, significantly enhances the DNA-membrane association.

Binding of Tritiated PDD to Microsomal Membrane. One nM of labeled PDD (specific activity, 117 mCi/mmole) was mixed with 400 µg of microsomal membrane in a buffered NaCl solution (0.1 M NaCl, 0.01 M MgSO₄, 0.02 M phosphate buffer, pH 7.2), incubated for 30 min at 40°, diluted with more of the buffered saline, and centrifuged in CsCl for 22 hr. More than 25% of the total radioactivity was associated with the membrane band (Chart 1). An additional 2 to 3% of the radioactivity was distributed evenly through the gradient, while less than 1% was found in the paraffin oil used to cover the gradients. The balance of the radioactive material firmly associated with the walls of the nitrocellulose tubes.

The distribution of the radioactivity was constant, irrespective of the amounts of radioactive PDD added. However, the increase in the concentration of microsomal membrane beyond 500 µg/sample did not further enhance the binding of PDD (Chart 2). The significance of this observation is not clear.

Effects of pH, Ionic Composition, and Conditions of Incubation on Binding of Tritiated PDD to Microsomal Membrane. The association between the microsomal membrane and PDD occurred over a wide range of pH, with the maximum between 7.2 and 7.5. Nevertheless, more than 80% of the radioactivity was recovered with the membrane

![Chart 1](chart1.png)

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Change in membrane density (mg/ml)</th>
<th>Change in binding of labeled E. coli DNA (control = 100)</th>
<th>Macroscopic description of the band</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phorbol</td>
<td>No change</td>
<td>No change (96)</td>
<td>Unchanged</td>
</tr>
<tr>
<td>PDB</td>
<td>+5: +29</td>
<td>No change (103)</td>
<td>Two bands: upper, granular; lower, hazy. Labeled DNA associated only with the granular band</td>
</tr>
<tr>
<td>PDD</td>
<td>No change</td>
<td>Increased (128)</td>
<td>Unchanged</td>
</tr>
<tr>
<td>PDP°</td>
<td>-6</td>
<td>Decreased (80)</td>
<td>Heavy, granular band</td>
</tr>
<tr>
<td>TPA</td>
<td>-14</td>
<td>Decreased (20)</td>
<td>Clumps of membrane material above the main band</td>
</tr>
<tr>
<td>PDA</td>
<td>-20</td>
<td>Increased (114)</td>
<td>Intense band composed of small uniform granules</td>
</tr>
</tbody>
</table>

*PDP, phorbol-12, 13-dipropionate; PDA, phorbol-12, 13-diacetate.*
Effects of Phorbol, Phorbol Esters, and Various Macromolecules on Association between Microsomal Membrane and PDD. In the experiments discussed in this paragraph, microsomal membrane was preincubated for 20 min with various compounds before the addition of radioactive PDD. In separate experiments we have established that 20 min are sufficient to fully charge the membrane with all the compounds studied. Effects of phorbol and several phorbol esters are summarized in Table 2. The unlabeled esters and the parent compound itself were in 500 to 1000 molar excess over the radioactive PDD. The 2 most potent promoters, TPA and PDD, inhibited most strongly the radioactive PDD-membrane interaction, while PDB, a less effective promoter, competed less efficiently. No decrease in the amounts of PDD bound to the membrane band was seen in the presence of free phorbol, a nonpromoting compound. As noted in a previous paragraph, PDB fractionated the membrane into 2 bands, easily separable in the gradient. The radioactive PDD and DNA associated with the same band (Table 1).

Two proteins, calf thymus histone and methyl-esterified bovine serum albumin, both known to interact with microsomal membrane (13), did not interfere with the binding of PDD (Table 2). However, preincubation of the membrane with either native or denatured DNA significantly increased the association. This enhancement did not seem to be due to a direct binding of PDD to DNA. In separate velocity centrifugation experiments, we have observed that labeled PDD did not penetrate into sucrose gradients after extended incubation with native or denatured DNA (as well as RNA). Under these conditions, DNA and RNA were recovered from the lower one-third of the gradients. Similarly, no association of PDD-3H with DNA band was observed when DNA was centrifuged (15) until equilibrium in CsCl density gradients.

Table 2  
Effects of various compounds on microsomal membrane-PDD-3H interaction (control = 100)

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>% radioactivity associated with the band</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal membrane, control</td>
<td>100</td>
</tr>
<tr>
<td>+ phorbol</td>
<td>400</td>
</tr>
<tr>
<td>+ PDB</td>
<td>400</td>
</tr>
<tr>
<td>+ PDD</td>
<td>400</td>
</tr>
<tr>
<td>+ TPA</td>
<td>400</td>
</tr>
<tr>
<td>+ calf thymus histone</td>
<td>100</td>
</tr>
<tr>
<td>+ methyl-esterified bovine serum albumin</td>
<td>100</td>
</tr>
<tr>
<td>+ DNA, calf thymus</td>
<td>80</td>
</tr>
<tr>
<td>+ DNA, calf thymus denatured*</td>
<td>80</td>
</tr>
<tr>
<td>+ yeast high-molecular-weight RNA*</td>
<td>500</td>
</tr>
</tbody>
</table>

* By heating for 3 min at 100° and rapidly cooling in an ice-water bath.
* Assayed with the "crude microsomal membrane": see "Materials and Methods."
No substantial change in PDD-membrane interaction was seen when high-molecular-weight yeast RNA reacted with the crude microsomal membrane, although the density of the membrane was significantly increased (by more than 15 mg/ml).

These results were quite reproducible when different batches of membranes prepared under identical conditions were used.

**Binding of PDD to Other Cellular Membranes.** Some other cellular membranes were tested for their ability to interact with PDD. Nuclear envelope from rat liver was almost as efficient as microsomal membrane (Table 3), and preincubation with DNA dramatically increased the binding. However, no association was seen between PDD and erythrocyte ghosts from 2 different sources.

**Table 3**

<table>
<thead>
<tr>
<th>Membrane</th>
<th>% radioactivity associated with the band</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear envelope</td>
<td>72</td>
</tr>
<tr>
<td>Nuclear envelope + DNA, calf thymus*</td>
<td>272</td>
</tr>
<tr>
<td>Erythrocyte ghosts (rat)</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Erythrocyte ghosts (human)</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

* At the concentration of 80 µg/sample.

Since it was becoming increasingly evident that PDD binds only to those membranes (or membrane fractions) that are capable of binding DNA (13), we further tested this possibility by using a batch of microsomal membrane that spontaneously splits in CsCl into 2 independent bands. Such membranes arise as rare and usually inadvertent artifacts of preparation; a significant contamination with a different cellular membrane creates hybrids between the 2 membranes (10). Such hybrid membranes, banding at a density different from that of the normal endoplasmic reticulum, often differ from the latter in their affinity toward DNA. We tested 1 such membrane lot which in CsCl density gradient fractionated into 2 bands of approximately equal turbidity. As already suspected, DNA and PDD bound preferentially to the same membrane fraction. The membrane banding at the density of 1.18 g/ml had associated with 84 and 78% of the total DNA and PDD recovered in the gradient, respectively. The other band, seen at the density of 1.165 g/ml, contained 17 and 22% of DNA and PDD, respectively.

**DISCUSSION**

Experiments summarized in this report indicate that phorbol esters are capable of reacting in vitro with cellular membranes. This reaction can be qualitatively and quantitatively analyzed by the technique of CsCl equilibrium density gradient centrifugation of the complexes. The membrane alterations include changes in density, in macroscopic appearance of the band, and in the ability of the membrane to bind DNA. The parental compound phorbol does not affect any of these characteristics of the microsomal membrane.

The diverse effects of the various phorbol esters on density and macroscopic appearance of the microsomal membrane are rather unexpected: chemically related compounds tend to change the properties of the microsomal membrane in a similar way (14).

Some of the phorbol esters modify (inhibit or enhance) the association of DNA with the membrane. An examination of Table 1 reveals no direct correlation between the degree of interference and the promoting activity of a compound. TPA, the strongest promoter, decreases 5-fold the amount of DNA bound, while PDD, the 2nd-strongest, increases this amount by about one-third. Nevertheless, these experiments demonstrate that the active promoters are capable of interfering with the membrane-DNA interaction. It should also be kept in mind that the associations detected with the model system of bacterial DNA and rat liver microsomal membrane are likely to be quantitatively different from those occurring in the mammalian nucleus. Thus, while the relevance of these observations to the biological activity in vivo remains to be established, the possibility that active promoters interfere with reactions between DNA and cellular membranes deserves further study.

Binding of one of the esters tested, PDD-3H, was followed by measurement of the amounts of tritium label associated with the membrane band. Evidence presented in this report seems to indicate that this ester binds at or close to the DNA "receptor" on the membrane and preferentially associates with the DNA-membrane complex, and that strong promoters may share the same (or adjacent) binding sites on the membrane. The following arguments favor these interpretations: (a) PDD binds only to those membranes capable of associating with DNA, namely, microsomal and nuclear membranes, but not to the erythrocyte ghosts (13). If a membrane fractionates into 2 or more bands in CsCl (after treatment with PDB or due to an artifact of purification) the labeled PDD is always found with the same band that, in a control tube, associates preferentially or exclusively with DNA. (b) Native and denatured DNA strongly enhance binding of PDD. No other macromolecule has been found thus far that alters the extent of binding, although the high-molecular-weight RNA, as well as the 2 basic proteins tested, interacts with microsomal membranes (13). (c) Other esters of phorbol inhibit binding of PDD, a good promoter, to microsomal membrane. Unlabeled PDD inhibits binding of the radioactive ester. Significant inhibition is observed in the presence of TPA, a strong promoter, while PDB and phorbol compete poorly or not at all. Thus, a rough correlation is seen between the promoting activity of a compound and its ability to compete with PDD.

Experiments are being done to isolate the DNA receptors from cellular membranes. The use of such purified receptors will help to establish more definitively the relationship (or lack of it) between the binding sites for DNA and the tumor-promoting compounds.

We do not know whether reactions similar to those observed in vitro occur in vivo and, if so, whether any of them is relevant to the tumor-promoting activity of these chem-
The association between membranes and phorbol esters evidently involves bonds strong enough to withstand prolonged exposure to high salt concentrations in the gradient, and the binding can take place under a wide variety of conditions (temperature, ionic composition, pH), thus suggesting that lasting bonds are likely to be produced in vivo. The research of Sivak and Van Duuren provides strong circumstantial evidence for an interaction in vivo of phorbol esters with cell membranes. They reported that in cell culture, TPA induces cell-shape (24) and permeability changes (22) and the release of cells from the density-dependent inhibition of growth (25). Furthermore, they reported TPA bound to a cell fraction having the properties of membranes (23). Also relevant are the observations that the incorporation of choline (11, 20) and P(1) (20) into phosphatidylcholine is stimulated within 2 hr after treatment of mouse skin with TPA. Mueller and Kajiwara (17) observed an inhibition of initiation of DNA synthesis after the exposure of HeLa cells to active phorbol esters, and Baird et al. (3) noted a similar effect after a single application of these compounds to mouse skin. The tentative conclusion from these observations is that TPA binds to membrane sites that control DNA synthesis and cell division, and this conclusion seems to be supported by the evidence presented in this report. The relationship of the binding reaction to the process of malignant transformation and to other known metabolic effects of phorbol esters (2, 3, 6, 19, 21, 24) must be established by future experimentation.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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