ABSTRACT

The potent mouse skin tumor promoter phorbol 12-myristate 13-acetate (PMA) has been previously shown to stimulate increased deoxyglucose transport and to cause loss of the large external transformation-sensitive glycoprotein (LETSP) from the surface of normal chicken embryo fibroblasts. We have now obtained dose-response data for loss of LETSP in chicken embryo fibroblasts induced by six other phorbol esters. With one exception, these derivatives all caused a comparable maximal decrease in LETSP, whereas their half-maximally effective doses (ED_{50}s) varied over a range of four orders of magnitude. Good quantitative correlation was obtained between these ED_{50}s in the chicken embryo fibroblast system and the known inflammatory potencies (quantity causing ear inflammation in 50% of a group of treated mice, ID_{50}s) of these derivatives in the mouse ear assay. With respect to stimulation of deoxyglucose transport, the phorbol esters examined fell into two categories. PMA, phorbol 12,13-diacetate, and phorbol 12,13,20-triacetate showed ED_{50}s for stimulating deoxyglucose transport which closely matched their ED_{50}s for causing loss of LETSP and which thus also correlated well with the quantity causing ear inflammation in 50% of a group of treated mice, ID_{50}s, in the mouse ear assay. Others, phorbol 12,13-didecanoate, phorbol 12,13-dibenzooate, and 4-O-methylphorbol 12-myristate 13-acetate, were 4- to 9-fold more potent in stimulating deoxyglucose transport than in causing LETSP loss. Unlike the phorbol esters, the parent diterpene phorbol and free myristic acid had little effect on deoxyglucose transport or LETSP levels at concentrations up to 3 mM (phorbol) or 0.2 mM (myristic acid), above which toxicity was observed. For PMA, the relation between stimulation of deoxyglucose transport and toxicity was examined. In contrast to an ED_{50} for stimulation by PMA of 3.7 nM, inhibition of this stimulation occurred only with an ED_{50} of 3.3 μM.

INTRODUCTION

PMA and many other phorbol derivatives elicit multiple skin tumors when chronically applied to mice that have been pretreated with a single subthreshold dose of a carcinogen (2, 6, 9, 44). If either treatment is omitted or if the order of treatments is reversed, no tumors result. On the other hand, delay of up to a year between exposure to the carcinogen and the subsequent exposure to the phorbol derivative do not prevent tumor appearance. The reversibility of the action of the phorbol esters and their lack of mutagenicity (33) and carcinogenicity (6) clearly distinguish these compounds from carcinogens. Understanding of their mechanism of action is thus of considerable interest and importance.

Because mouse skin is a complex, heterogeneous organ, tissue culture systems provide a means for simplifying the study of phorbol ester action at the cellular level. The phorbol esters show diverse biological effects on many different types of cells in culture (for reviews, see Refs. 6 and 45; also see Ref. 39). Our laboratory has shown that PMA induces in CEF a series of phenotypic changes that strongly resemble changes induced in CEF by oncogenic transformation with RSV (see ‘‘Discussion’’). We have hypothesized that the ability to induce partial mimicry of the transformed phenotype may account for the tumor-promoting effects of the phorbol esters in vivo (4). The CEF system has also been used to examine other aspects of the tumor promotion phenomenon. We found that compounds unrelated to phorbol which are both inflammatory agents and tumor promoters on mouse skin do not mimic the effects of PMA on CEF (12). These results suggested that the phorbol esters may have a unique mechanism of action.

A crucial issue regarding the use of CEF or other in vitro systems for studying the mode of action of the phorbol esters is the relevance of these systems to the in vivo phenomena in the mouse. Detailed comparison of structure-activity relationships for phorbol derivatives in in vitro systems and in the mouse would thus be desirable. In this paper, we report the effects of various phorbol derivatives on surface LETSP levels and on DG transport in CEF. The activities that we found show excellent quantitative correlation with the inflammatory activities of the same compounds determined in the mouse ear assay by Hecker et al. (20). These results argue for homologous targets for the phorbol esters in the 2 systems.

MATERIALS AND METHODS

Chemicals and Media. Chemicals used and their sources were: [3H]DG (specific activity, 8 Ci/mmol), 125I, and Aquasol (New England Nuclear Corp., Boston, Mass.); chicken serum that was heat inactivated for 30 min at 56°, and calf serum (Flow Laboratories, Rockville, Md.); trypsin, Medium 199, and Eagle’s minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.); Tryptose Phosphate Broth (Difco, Detroit, Mich.).

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Broth (Difco, Detroit, Mich.); lactoperoxidase and bovine serum albumin (Calbiochem, San Diego, Calif.); glucose oxidase and croton oil (Sigma Chemical Co., St. Louis, Mo.); PMA, PDB, MPMA, and PDD (Consolidated Midland Co., Brewster, N. Y.); and PDA, PTA, P13A, and phorbol (Chemical Carcinogenesis, Eden Prairie, Minn.). Phorbol was also prepared from croton oil (21, 43), and PMA was also synthesized as described (7, 41). Stock solutions of phorbol and phorbol esters were made up in reagent grade DMSO and stored at —20°. Dilutions in DMSO were carried out in glass or polystyrene tubes and were made such that the final concentration of DMSO in the cultures was 0.1%. The purity of all phorbol derivatives was confirmed by reverse phase high-pressure liquid chromatography on a 30-cm µBondapak column (Waters Associates, Milford, Mass.) with methanol-water solvent systems.

**Cell Cultures.** Primary CEF were prepared from the body walls of 10-day-old embryos (Spafas, Norwich, Conn.) (48). Secondary CEF used for determination of LETSP levels were prepared by trypsinizing 3- to 6-day-old primary cultures and replating the cells at 1 x 10⁶/60-mm dish in Medium 199 containing 10% Tryptose Phosphate Broth and 5% chicken serum. Resting CEF for assay of DG transport were prepared by trypsinizing 3-day-old primary cultures, washing the cells 3 times by centrifugation and resuspension in Eagle's minimal essential medium containing 2% Tryptose Phosphate Broth and 0.5% chicken serum, and plating the cells at 1.3 x 10⁶/60-mm culture dish in Eagle's minimal essential medium containing 2% Tryptose Phosphate Broth and 0.5% chicken serum. No antibiotics were added to secondary cultures.

Stimulation by phorbol esters of deoxyglucose transport in resting CEF was assayed exactly as described previously (10). Under these conditions, transport is a linear function of time (27, 38). The decrease in LETSP in response to the phorbol esters was determined as before (5, 12). Briefly, CEF were incubated for 3 days in the presence of the phorbol ester, the cells were labeled by lactoperoxidase-catalyzed iodination, and the labeled proteins were fractionated by sodium dodecyl sulfate gel electrophoresis using the buffer system of Laemmli (30) with integrated gel apparatus (11). After the gel was dried, the radioactive band corresponding to LETSP was cut out and quantitated on a γ counter. An adjacent, equal area of the gel was also counted and subtracted as background.

**RESULTS**

**Phorbol Ester-induced Loss of CEF LETSP.** This laboratory has previously reported that CEF treated with PMA at 30 ng/ml for 3 days underwent loss of the surface protein LETSP to a level approximately 15 to 25% of control levels in cells treated with solvent only (4). LETSP loss occurred whether LETSP was detected by lactoperoxidase-catalyzed surface radiodination (4) or by staining of whole-cell proteins.⁵ We have now found that LETSP levels in CEF were similarly reduced when the cells were treated with other phorbol esters. The dose-response curves for induction of LETSP loss by 6 additional phorbol derivatives are presented in Chart 1. PDD, PMA, PDA, PTA, MPMA, and P13A all caused loss of LETSP 3 days after application to growing CEF to a similar extent, to levels approximately 20% of those in control cultures. Likewise, the linear portions of the dose-response curves are parallel for all but PDD. In the case of PDD, analysis may be complicated by its reported low aqueous solubility, approximately 50 nM (25). Such parallel slopes are consistent with similar modes of action. PDB also caused loss of LETSP in a dose-dependent fashion, but LETSP levels only declined to approximately 40% of control levels. At a PDB concentration of 1 µg/ml, a partial restoration of LETSP was observed in each of 4 experiments. The explanation for this unusual behavior is not known.

**Stimulation of DG Transport.** Resting or growing CEF show increased rates of sugar transport in response to stimuli such as addition of serum (29, 38) or transformation by RSV (28, 46). We have previously reported that PMA, PDB, PDA, MPMA, and PTA strongly stimulate DG transport in CEF assayed 5 hr after addition of the derivatives to the culture medium (10). These compounds all stimulated DG transport to similar maximal levels, although their potencies varied over a range of 4 orders of magnitude. The DG transport assay is a convenient measure of cell activation and provides a quantitative means for comparing in vitro activities of the phorbol esters. Chart 2 shows that PDD and P13A stimulated DG transport in CEF in a fashion analogous to our earlier results with PMA and the other phorbol derivatives. The linear portions of the dose-response curves were essentially parallel as before, and relative maximal levels of stimulation by the 2 new derivatives were compa-

⁵ P. E. Driedger and P. M. Blumberg, unpublished data.
The significance of this stimulation is uncertain, as the possible activity of free myristic acid on CEF was thus of interest, especially since some fatty acids, e.g., oleic and lauric acids, have been reported to possess weak promoting activity (23). As shown in Table 1, myristic acid at concentrations up to 0.2 to 0.4 mM had little effect on DG transport or LETSP levels. Such concentrations are orders of magnitude higher than those that might be generated by cleavage of PMA or other active derivatives at the concentrations used in this report.

Lack of Activity of Phorbol and Myristic Acid. The above phorbol derivatives, although varying greatly in potency, showed comparable maximal effects. The parent alcohol phorbol, in contrast, has almost without exception been toxic to the cells in the 3-day LETSP assay as shown by a decrease in protein per plate of greater than 25%.

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative rate of deoxyglucose transport</th>
<th>Relative LETSP levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulfoxide</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Phorbol</td>
<td>1.06</td>
<td>1</td>
</tr>
<tr>
<td>700 μM</td>
<td>1</td>
<td>1.06</td>
</tr>
<tr>
<td>2.8 mM</td>
<td>1.00</td>
<td>0.92</td>
</tr>
<tr>
<td>11.2 mM</td>
<td>1.38</td>
<td>b</td>
</tr>
<tr>
<td>28 mM</td>
<td>2.07</td>
<td>a</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>1.57</td>
<td>c</td>
</tr>
<tr>
<td>43 μM</td>
<td>0.85</td>
<td>d</td>
</tr>
<tr>
<td>110 μM</td>
<td>0.87</td>
<td>c</td>
</tr>
<tr>
<td>220 μM</td>
<td>0.87</td>
<td>c</td>
</tr>
<tr>
<td>430 μM</td>
<td>1.35</td>
<td>c</td>
</tr>
<tr>
<td>PMA (30 ng/ml)</td>
<td>6.5</td>
<td>0.20</td>
</tr>
<tr>
<td>10% chicken serum</td>
<td>6.8</td>
<td></td>
</tr>
</tbody>
</table>

a Toxic to the cells in the 3-day LETSP assay as shown by a decrease in protein per plate of greater than 25%.

b Above the solubility limit for myristic acid.

c From Refs. 10 and 12.

d Toxic at the concentrations used in this report.

**Chart 2.** Dose-response curves for phorbol ester-induced stimulation of deoxyglucose transport in CEF. Resting secondary CEF were plated at 1.3 x 10^6/60-mm plate in Eagle's minimal essential medium containing 2% Trypsin Phosphate Broth and 0.5% chicken serum (see "Materials and Methods"). Two days later, PDD or P13A at the indicated final concentrations were added, and [3H]DG transport was measured 4 to 5 hr later. Values are expressed relative to stimulation by 10% chicken serum and are normalized to protein per plate. The curves for PDD and P13A are averages of 4 and 5 experiments, respectively. The values of the EDDG_{50}'s from separate experiments were: PDD (△), 2.0 ± 0.44 mM; P13A, (○), 40.7 ± 8.8 μM. Values for PMA (●), taken from Ref. 10, are shown for comparison. - - - - , effect of 0.1% DMSO.
nation could have been detected, as shown by analysis of standard mixtures of the 2 compounds. Similarly, PTA was shown to be free of PDA, a potential contaminant of greater potency. These tests do not rule out the possibility of metabolic alteration of the compounds in culture.

Relation between PMA Activity and Toxicity. In several in vitro systems, toxicity at high concentrations of PMA has been observed (26, 34, 40). This toxicity may result from the amphipathic nature of the molecule, which contains a fatty acid side chain and a relatively polar head group. We have extended to high PMA concentrations the dose-response curve for stimulation of DG transport in CEF (EDD50) and inflammatory potency in the mouse ear assay (ED50). Values for the EDD50 (0) were determined as in Chart 2 for PDD and P13A and were taken from our previously reported results (10) for the other derivatives. For comparison with the results on LETSP loss, the points (B) and linear regression line of A are included in this graph. Numbers by each point, individual phorbol derivatives: 1, PDD; 2, PMA; 3, PDB; 4, PDA; 5, MPMA; 6, PTA; 7, P13A.

DISCUSSION

The phorbol esters are of considerable interest, both because of their ability to promote tumors in mice and because they elicit striking biological effects in a very wide variety of animal cell types. Studies on the cellular effects of the phorbol esters may contribute significantly to the understanding of the tumor promotion phenomenon. In particular, the papers of Boutwell (6), Weinstein et al. (47), and Wigler and Weinstein (50) have cited observations made in several cell systems that the phorbol esters can induce various normal or quasi-normal cell types to exhibit one or more properties thought to be characteristic of the transformed state. Our laboratory has shown that PMA can induce in CEF an extensive set of phenotypic alterations that overlaps substantially but not completely with the phenotype of RSV-transformed CEF, and we have hypothesized that this ability of phorbol esters to cause normal cells to resemble transformed cells may account for the tumor-promoting properties of the phorbol esters in mice (4). Alterations in the CEF phenotype which occur both in PMA-treated and in RSV-transformed CEF include: increases in metabolic alteration of the compounds in culture. To examine more closely the relevance of the properties of PMA in the CEF culture system to the inflammatory and tumor-promoting activity of PMA in mice, we have made quantitative structure-activity comparisons between the 2 systems, using a range of phorbol derivatives that had been previously studied in the mouse. We found excellent quantitative agreement between the EDD50 in our system and the ED50 in the mouse. This close correlation suggests that PMA may be interacting at homologous receptor sites in vivo and in vitro.
CEF. While extensive studies have not yet been carried out in other in vitro systems, those data currently available are consistent with homologous receptors being present in these systems as well (32, 35, 47, 49, 51). More detailed evidence for this generalization, however, would be highly desirable.

Our results here and those reported earlier (10) for DG transport indicate that the 7 active phorbol esters examined in this study all show very similar maximal stimulation of DG transport in CEF, i.e., they all show comparable efficacy. With the exception of PDB, the same was true for LETSP loss. These results agree with the findings of Hecker et al. (20) that the active phorbol esters by definition were all inflammatory (20), although their potencies varied over 4 orders of magnitude. The frequent observations in biochemical and in vitro studies that different derivatives caused graded effects may well arise from the use of single concentrations, often equimolar, of derivatives of differing potency. Such studies, while valuable, do not provide the information necessary to distinguish between efficacy and potency.

The potency of phorbol derivatives in the CEF system shows a correlation with the inflammatory potency of the derivatives in the mouse. For the compounds used in this study, moreover, a rank-order comparison shows general agreement between their inflammatory and tumor-promoting activities (Ref. 42; Ref. 10, Table 3). On the other hand, exceptions have been cited of phorbol derivatives which are inflammatory but only very weakly promoting. These include 12-deoxyphorbol derivatives with short-chain substituents (18, 22), unsaturated fatty acid derivatives of phorbol (14), and phorbol 12,13-dibutyrate (42). Determination of the response of CEF to these derivatives would be of particular interest. Our current hypothesis, however, is that inflammatory activity in the mouse provides an approximate measure of the intrinsic potency of phorbol derivatives, whereas the longer-term promotion assay is affected to a greater degree by other pharmacological properties of the derivatives such as rates of absorption, degradation, and excretion. In support of this hypothesis, the short-chain-substituted 12-deoxyphorbol derivatives were found to be toxic, causing substantial mortality during the course of the promotion experiments (22); and unsaturated fatty acid derivatives of phorbol were reported to be highly unstable (15). Similarly, PDB, which had initially been cited as demonstrating the independence of inflammatory and promoting activities (18), was subsequently found to be a good promoter if applied 4 rather than 2 times per week (1).

Several of the phorbol derivatives were 4- to 9-fold more potent in stimulating DG transport than they were in inducing LETSP loss. The reasons for this greater potency in the DG transport assay remain to be determined. Since the times of incubation of the derivatives with the cells in the 2 assays differ substantially (5 hr versus 3 days), the disparity may be due to the metabolism of some of the derivatives; a second possible factor may be the different serum concentrations used in the 2 assays. As yet, more complicated models, such as that the 2 sets of compounds are acting at different putative receptors or have differential effects on a single receptor, cannot be excluded. In any case, findings analogous to ours have been reported for the mouse ear inflammation assay. The limited studies performed so far indicate up to 10-fold lower potencies, depending on the derivative, when ID₅₀ values were determined at 24 hr (the standard assay) rather than at 4 hr (13).

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Quantitative Correlation between in Vivo and in Vitro Activities of Phorbol Esters

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