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Specific binding of phorbol ester tumor promoters

(carcinogenesis/receptors/chicken embryo fibroblasts)

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ABSTRACT [20-^{3}H]Phorbol 12,13-dibutyrate bound to particulate preparations from chicken embryo fibroblasts in a specific, saturable, reversible fashion. Equilibrium binding occurred with a K_d of 25 nM; this value is very close to the 50% effective dose (ED_{50}), 50 nM, previously determined for the biological response (induction of fibronectin loss) in growing chicken embryo fibroblasts. At saturation, 1.4 pmol of [20-^{3}H]phorbol 12,13-dibutyrate was bound per mg of protein (approximately 7×10^4 molecules per cell). Binding was inhibited by phorbol 12-myristate 13-acetate (K_i = 2 nM), mezerein (K_i = 180 nM), phorbol 12,13-dibenzoate (K_i = 180 nM), phorbol 12,13-diacetate (K_i = 1.7 μM), phorbol 12,13,20-triacetate (K_i = 39 μM), and phorbol 13-acetate (K_i = 120 μM). The measured K_i values are all within a factor of 3.5 of the ED_{50} values of these derivatives for inducing loss of fibronectin in intact cells. Binding was not inhibited by the inactive compounds phorbol (10 μg/ml) and 4α-phorbol 12,13-didecanoate (10 μg/ml) or by the inflammatory but nonpromoting phorbol-related diterpene esters resiniferatoxin (100 ng/ml) and 12-deoxyphorbol 13-isobutyrate 20-acetate (100 ng/ml). These data suggest that biological responses to the phorbol esters in chicken embryo fibroblasts are mediated by this binding activity and that the binding activity corresponds to the phorbol ester target in mouse skin involved in tumor promotion. Binding was not inhibited by the nonphorbol promoters anthralin (1 μM), phenol (1 mM), iodoacetic acid (1.7 μM), and cantharidin (75 μM), or by epidermal growth factor (100 ng/ml), dexamethasone acetate (2 μM), retinoic acid (10 μM), or prostaglandin E_2 (1 μM). These agents thus appear to act at a target distinct from that of the phorbol esters.

Tumor promoters are agents which, although not themselves carcinogenic, induce tumors in animals previously exposed to a subthreshold dose of a carcinogen. The phenomenon of tumor promotion has been investigated in most detail in the mouse skin system (1, 2). In that system, the most potent class of tumor promoters is that of the phorbol esters and related diterpene derivatives (3).

Recent *in vivo* studies have indicated that the phorbol esters can also promote tumors in various tissues other than skin (4, 5). *In vitro*, the phorbol esters have been shown to possess potential biological activity on many different cell types (6, 7). Three generalizations have emerged from our studies and those of others: (*i*) the phorbol esters cause normal cells to assume a partially transformed phenotype (8–10); (*ii*) the phorbol esters cause superexpression of transformation-sensitive properties (10, 11); and (*iii*) the phorbol esters modify differentiation and differentiated cell functions (12, 13). At least certain of these responses may reflect interaction of the phorbol esters at a target homologous to that involved in tumor promotion. In the case of chicken embryo fibroblasts (CEF), we have reported a close quantitative correlation between the structure–activity requirements for induction of fibronectin loss in these cells and the inflammatory and tumor-promoting activities of the phorbol esters *in vivo* (14).

Attempts to identify the target for the phorbol esters directly by specific binding of ^{3}H-labeled phorbol 12-myristate 13-acetate ([^{3}H]PMA) have met with difficulty. The general observation has been that [^{3}H]PMA binds to cells in a nonsaturable and noncompetitive manner (15). This behavior may result from the substantial lipophilicity of PMA (16), which causes it to partition strongly into membranes. As an alternate approach, we have examined the binding of phorbol 12,13-dibutyrate [P(Bu)$_2$], a phorbol derivative that is still highly active *in vivo* (17) and *in vitro* (18) but is much less lipophilic than PMA (19). Using this derivative, we have been able to demonstrate and characterize specific binding of the phorbol esters to CEF particulate preparations.

MATERIALS AND METHODS

Reagents. Reagents used and their sources were: prostaglandin E_2, retinoic acid, and dexamethasone acetate (Sigma); epidermal growth factor (Collaborative Research, Waltham, MA); sodium boro[^{3}H]hydride (specific activity, 5 Ci/mol; 1 Ci = 3.7×10^{10} becquerels; Amersham). Nonphorbol promoters (20) and phorbol esters (14, 18) were obtained as indicated. Mezerein was a generous gift of John Douros (National Cancer Institute). Resiniferatoxin was generously provided by E. Hecker and F. J. Evans. [^{3}H]P(Bu)$_2$ was labeled at position 20 by the methods of Kreibich and Hecker (21); specific activity was determined to be 1.38 Ci/mmol by measurement of the UV absorbance at 230 nm [assuming an extinction coefficient of 5000 (22)] and assay of an aliquot of this solution in Scintiverse. The efficiency of counting was determined by using a [^{3}H]toluene internal standard. The radiochemical purity exceeded 97% as shown by analysis by thin-layer chromatography and high-pressure liquid chromatography.

Cell Cultures. Primary CEF were prepared from 10-day chicken embryos (Spafas, Norwalk, CT) as described (9). They were grown in roller bottles for 3 days in medium 199 containing 2% tryptose phosphate broth, 1% calf serum, and 1% chicken serum. The medium was then changed to medium 199 supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% chicken serum, and the cells were grown for an additional 2 days before they were harvested. Secondary CEF were prepared by trypsinizing primary cultures and replating the cells at 5×10^5 cells per 100-mm culture dish in medium 199 containing 10% tryptose phosphate broth and 5% chicken serum. All cultures were kept in 5% CO_2/95% air.

Preparation of Particulate Fractions. The crude particulate fraction used for binding studies was prepared from primary or secondary CEF as follows. Cells were scraped from the monolayer into Ca^{2+},Mg^{2+}-free phosphate-buffered saline and were pelleted by low-speed centrifugation. The pellet was

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Abbreviations: CEF, chicken embryo fibroblast(s); PRDE, phorbol-related diterpene ester; P(Bu)$_2$, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; ED_{50}, concentration yielding half-maximal response; $NaDodSO_4$, sodium dodecyl sulfate.

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resuspended in 0.05 M Tris·HCl at pH 7.4, and the cells were disrupted in a Potter–Elvehjem homogenizer. The cell lysate was centrifuged at 100,000 × *g* for 60 min. As an alternative method, the cell pellet from the low-speed centrifugation was resuspended in Ca^{2+},Mg^{2+}-free phosphate-buffered saline, the cells were disrupted by nitrogen cavitation (750 psi; 5.2 megapascals), and the cell lysate was centrifuged at 35,000 × *g* for 60 min. In either case, the particulate fraction from the high-speed centrifugation was resuspended in 0.05 M Tris·HCl at pH 7.4 and stored frozen at −80°C. This fraction contained the microsomes, mitochondria, and nuclei. Of the total cellular protein, 68–75% was found in the 100,000 × *g* pellet.

Binding Assay. The binding assay was carried out in 400-μl high-density polyethylene microcentrifuge tubes (Brinkman, no. 2236-530-4) containing, in a total volume of 250 μl, 50 mM Tris·HCl at pH 7.4, 0.6–0.7 mg of the particulate protein preparation, [^{3}H]P(Bu)$_2$ at ≈30 nM, 0.15% dimethyl sulfoxide, and other ligands as specified. The tubes were maintained at 0°C while the additions were made. The components were then thoroughly mixed and incubated at 39°C for 30 min. After the incubation, the tubes were immediately centrifuged at 17,500 × *g* (12,500 rpm in the HB-4 rotor of a Sorvall RC-5 centrifuge; the rotor was equipped with custom-made adapters having a total capacity of 24 tubes) for 45 min at 4°C. After centrifugation, a 100-μl aliquot of the supernatant was withdrawn for scintillation counting to determine the actual concentration of free [^{3}H]P(Bu)$_2$. As much as possible of the remaining supernatant was removed with a pasteur pipet. The residue was drawn off by capillary action from the twisted end of a Kimwipe or from a cotton swab. For most of the experiments, the pellet was suspended in 100 μl of 1% sodium dodecyl sulfate (NaDodSO$_4$) and transferred to a counting vial, and the tube was rinsed twice with additional 100-μl portions of 1% NaDodSO$_4$. A faster method proved to be to cut off the tip of the tube, dislodge the pellet from the tip with the head of a pin, and transfer the tip of the tube, the pellet, and the pin to a counting vial. Radioactivity was determined in Scintiverse (Fisher) at efficiencies of 36–48%. Specific binding represents the difference between total and nonspecific bindings. The latter was measured in the presence of 30 μM nonradioactive P(Bu)$_2$. In each experiment the partition coefficient of [^{3}H]P(Bu)$_2$ under the conditions of that experiment was determined, and nonspecific binding for each experimental tube was then calculated by using this partition coefficient and the actual amount of [^{3}H]P(Bu)$_2$ measured in the supernatant of that tube.

Identity of the Bound and Free Ligand. The supernatants and, separately, the pellets from 14 tubes of a typical binding experiment were combined, and the radioactivity was extracted with ethyl acetate containing excess unlabeled carrier P(Bu)$_2$. Under the conditions of this experiment, 18% of the [^{3}H]P(Bu)$_2$ in the pellet was bound specifically. Recovery of radioactivity was 105% for the pellet and 89% for the supernatant. After concentration, the extracts were analyzed by thin-layer chromatography on silica gel with a hexane/ethyl acetate, 25:75 (vol/vol), solvent system. At least 93% of the radioactivity from the pellet and 96% of the radioactivity from the supernatant migrated with P(Bu)$_2$. No other radioactive peaks were found.

RESULTS

Characterization of Binding. [^{3}H]P(Bu)$_2$ bound to particulate preparations from CEF in a saturable, competitive manner (Fig. 1). Both double-reciprocal and Scatchard plots of the data, determined for reversible binding under equilibrium conditions, displayed a single slope corresponding to a K_d for P(Bu)$_2$ of 25 nM (Fig. 2). This value is close to the concentration yielding half-maximal response (ED$_{50}$), 50 nM, previously determined for induction of fibronectin loss by P(Bu)$_2$ in CEF (18). At saturation, 1.4 pmol of [^{3}H]P(Bu)$_2$ was bound per mg of protein, or ≈75,000 molecules per CEF cell.

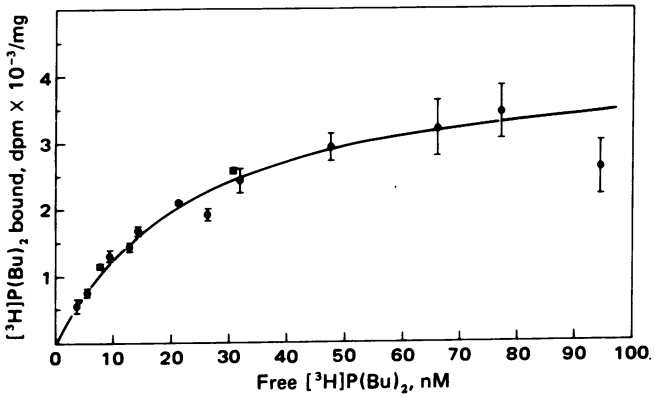

FIG. 1. Specific binding of [^{3}H]P(Bu)$_2$ to the particulate fraction from CEF. Each point represents the mean for four separate tubes; SEM is indicated by the error bars. Nonspecific binding was determined in the presence of excess nonradioactive P(Bu)$_2$ (30 μM) and was measured separately for each concentration of [^{3}H]P(Bu)$_2$. The levels of non-specific binding ranged from 57% of total binding at 3.8 nM [^{3}H]P(Bu)$_2$ to 79% at 77 nM. The curve was calculated by using the values for the dissociation constant and the amount of binding at saturation determined from the Scatchard plot of the data (see Fig. 2). Similar results were obtained in two experiments with separately prepared particulate fractions.

The specific binding of [^{3}H]P(Bu)$_2$ represents the difference between total binding and that occurring in the presence of a large excess, 30 μM, of nonradioactive P(Bu)$_2$. Under the conditions used for characterizing the specificity of binding, it accounted for 28 ± 5% (±SD) of the total [^{3}H]P(Bu)$_2$ bound, depending on the concentration of [^{3}H]P(Bu)$_2$ and the specific binding activity of the particulate preparation. The residual binding in the presence of 30 μM P(Bu)$_2$ was linear with the concentration of [^{3}H]P(Bu)$_2$ and of protein (not shown). Because the fraction of total bound [^{3}H]P(Bu)$_2$ that was specific decreased with increasing [^{3}H]P(Bu)$_2$ concentration, binding assays were routinely carried out at [^{3}H]P(Bu)$_2$ concentrations below saturation, 30 ± 8 nM (±SD).

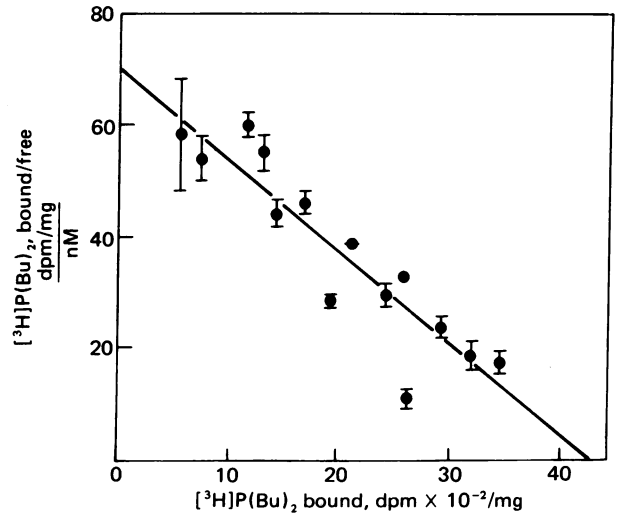

FIG. 2. Scatchard plot of specific binding of [^{3}H]P(Bu)$_2$. Data are from the experiment of Fig. 1. Error bars indicate ±SEM. The lines were determined by linear regression. Bound/free is in (dpm/mg)/nM.

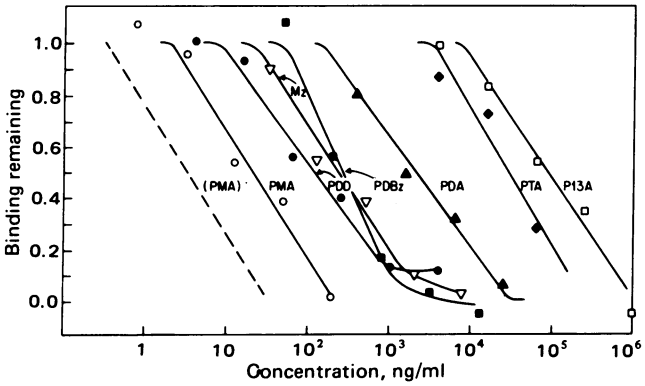

FIG. 3. Dose–response curves for inhibition of [^{3}H]P(Bu)$_2$ binding by nonradioactive PRDEs. Specific binding of [^{3}H]P(Bu)$_2$ was determined in the presence of the indicated concentrations of other PRDEs. Dilutions of PRDEs were made in 0.75% dimethyl sulfoxide. Each point represents the mean for two or three separate tubes in a single experiment. All experiments were performed at least twice. As determined in separate experiments, the presence of added ligands did not affect the nonspecific binding of [^{3}H]P(Bu)$_2$. The dotted line indicates the shift in the curve for inhibition of binding by PMA upon correction of the PMA concentrations for nonspecific partitioning into the particulate fraction, measured directly with [20-^{3}H]PMA. ○, PMA; ●, phorbol 12,13-didecanoate (PDD); ■, phorbol 12,13-dibenzoate (PDBz); ▽, mezerein (Mz); ▲, phorbol 12,13-diacetate (PDA); ◆, phorbol 12,13,20-triacetate (PTA); □, phorbol 13-acetate (P13A).

The specific binding of [^{3}H]P(Bu)$_2$ to the particulate preparations occurred rapidly and was reversible. No difference was observed in the amount of specific binding for times of incubation at 39°C, of 15–60 min (not shown); addition of excess nonradioactive (P(Bu)$_2$ to these tubes after a 30-min incubation at 39°C caused loss of all competitive binding within the next 30 min (not shown). As expected, the specific binding of [^{3}H]P(Bu)$_2$ was linear with protein.

Specificity of Binding. The structure–activity relationships for inhibition of [^{3}H]P(Bu)$_2$ binding by phorbol-related diterpene esters (PRDEs) were examined in detail. Binding was blocked by six phorbol esters and by the resiniferonol derivative mezerein (Fig. 3). The slopes of the dose–response curves for the different derivatives were similar in general, whereas the concentrations for 50% inhibition of binding spanned a range of 5 orders of magnitude. It should be noted that the concentrations indicated are based on the amounts added to the tubes. Nonspecific partitioning of PRDEs into the particulate fraction will decrease these values to a variable degree, depending on how lipophilic the specific compound is, and will thus shift the curves to lower concentrations. The magnitude of this partitioning has only been quantitated for the two radioactive derivatives we currently have available, [^{3}H]P(Bu)$_2$ and [20-^{3}H]PMA. The fraction of ligand remaining in the supernatant in the presence of 30 μM P(Bu)$_2$ under the conditions of the assay was 86% and 15% for [^{3}H]P(Bu)$_2$ and [^{3}H]PMA, respectively. Although the degree of partitioning of phorbol 12,13-didecanoate into the particulate fraction has not been determined, it should be at least as great as that for PMA.

The ED$_{50}$ values of the PRDEs for [^{3}H]P(Bu)$_2$ binding were compared with ED$_{50}$ values previously determined for induction of fibronectin loss in CEF by these derivatives (Table 1). In no case did these values differ by more than a factor of 3.5. Of particular interest is the observation that, although specific binding was measured with [^{3}H]P(Bu)$_2$, the most active phorbol derivative for inhibiting this binding was in fact PMA, the most potent known tumor promoter (3).

Unlike the above biologically active PRDEs, the parent diterpene phorbol and 4α-phorbol 12,13-didecanoate, the biologically inactive epimer of phorbol 12,13-didecanoate, did not block binding (Table 2). Binding was also not blocked by resiniferatoxin and 12-deoxyphorbol 13-isobutyrate 20-acetate. This result is of particular significance because these compounds are the best examples of a second class of PRDEs, those that are highly inflammatory but nonpromoting.

Binding of [^{3}H]P(Bu)$_2$ to membranes of *Bacillus subtilis* was examined on the assumption that these membranes might provide a control devoid of specific binding activity. This appears to be the case: binding per mg of protein was 5 ± 3% of that found for the CEF particulate fraction (±SEM; 14 determinations; four separate experiments; $P > 0.05$ for difference from 0). Specific binding of [^{3}H]P(Bu)$_2$ to mouse tissues and to other cultured cell types will be reported elsewhere.

Lack of Inhibition by Nonphorbol Promoters and Other Agents. Various other agents have been hypothesized to interact or not to interact at the same target as do the phorbol esters. These hypotheses can now be tested directly. We had previously studied seven non-phorbol-related tumor promoters in the CEF system. On the basis of their failure to induce similar biological effects to those induced by the phorbol esters, we suggested that

Table 1. Comparison of binding inhibition by PRDEs with their biological activities in CEF

Compound*	Binding assay: ED$_{50}$, nM†	Binding assay: Calculated K_i, nM‡	Biological activity: ED$_{50}$, of fibronectin loss, nM§
PMA	31 ± 9 (n = 3)	13 ± 3 (1.9)¶	6.5
PDD	130 ± 11 (n = 2)	62 ± 5 (<9)‖	7
Mezerein	428 ± 170 (n = 4)	180 ± 66	56
PDBz	376 ± 79 (n = 2)	180 ± 3	84
PDA	3,800 ± 220 (n = 2)	1,700 ± 14	1,200
PTA	87,000 ± 16,000 (n = 2)	39,000 ± 5,300	12,900
P13A	290,000 ± 6,500 (n = 2)	120,000 ± 30,000	85,000

* Abbreviations as in Fig. 1.

† Number of separate experiments in parentheses. Range of values is indicated for n = 2; SEM is indicated for n > 2.

‡ The actual concentration of [^{3}H]P(Bu)$_2$ in each experiment was used to correct the measured inhibitory values of the PRDE for competition by [^{3}H]P(Bu)$_2$.

§ Induction of fibronectin loss was the activity tested (from ref. 14).

¶ K_i corrected to the actual concentration of free PMA in the tubes. Under the conditions of the assay, 85% of the added [20-^{3}H]PMA was found to partition nonspecifically into the particulate fraction.

‖ An upper limit for the actual K_i, determined by assuming that PDD partitions into the particulate fraction similarly to PMA.

Table 2. Lack of inhibition of [^{3}H]P(Bu)$_2$ binding by nonpromoting PRDEs

Derivative	Specific binding remaining, % of control
Inactive derivatives:	
Phorbol (10 μg/ml)	102 ± 5
4α-Phorbol 12,13-didecanoate (10 μg/ml)	104 ± 9
Inflammatory but nonpromoting derivatives:	
Resiniferatoxin (100 ng/ml)	106 ± 4
12-Deoxyphorbol 13-isobutyrate 20-acetate (100 ng/ml)	99 ± 8

Values are the mean ± SEM for four determinations in a single experiment. All experiments were performed at least twice.

the phorbol esters and the nonphorbol promoters may have different mechanisms of action (20). We have now examined four of these non-phorbol-related promoters for inhibition of [^{3}H]P(Bu)$_2$ binding. None was inhibitory (Table 3).

A number of agents—in particular, the retinoids and the glucocorticoids—are potent inhibitors of tumor promotion (23, 24), and at least the latter agents have been suggested to bind to the same target as do the phorbol esters (25). Neither retinoic acid nor dexamethasone acetate blocked [^{3}H]P(Bu)$_2$ binding (Table 4). These agents thus do not appear to be pharmacological antagonists of the phorbol esters and presumably act at a subsequent step in the pathway induced by the phorbol esters.

Like the glucocorticoids, the prostaglandins have been hypothesized to bind to the phorbol ester target (26). Prostaglandin E$_2$, like dexamethasone acetate, however, does not block [^{3}H]P(Bu)$_2$ binding (Table 4).

Epidermal growth factor has been reported to induce a number of responses similar to those to the phorbol esters (27). Binding of epidermal growth factor has been reported to be inhibited by the phorbol esters (28), although whether this effect is on the number (28) or affinity (29, 30) of the receptors remains controversial. In any case, epidermal growth factor does not block [^{3}H]P(Bu)$_2$ binding (Table 4).

DISCUSSION

In this report we have provided evidence that there is a specific target for the phorbol ester tumor promoters. A considerable body of evidence suggests that these receptors in CEF are homologous with those in mouse skin that are responsible for tumor promotion. Structure–activity comparisons indicate that the more potent of the phorbol esters tested here for inhibition of P(Bu)$_2$ binding—namely, PMA and phorbol 12,13-didecanoate—are also the most potent tumor promoters in the mouse (3, 17). The biologically inactive compounds phorbol and 4α-phorbol didecanoate do not inhibit binding. We have previously shown excellent correlation between the potency of

Table 3. Lack of inhibition of [^{3}H]P(Bu)$_2$ binding by nonphorbol-related tumor promoters

Compound	Specific binding remaining, % of control
Anthralin (1 μM)	110 ± 12
Phenol (1 mM)	92 ± 5
Iodoacetic acid (1.7 μM)	103 ± 6
Cantharidin (75 μM)	105 ± 7

Values are the mean ± SEM for four determinations in a single experiment. All experiments were performed at least twice.

Table 4. Lack of inhibition of [^{3}H]P(Bu)$_2$ binding by putative analogs and antagonists

Agent	Specific binding remaining, % of control
Retinoic acid (10 μM)	105 ± 14 (n = 3)
Dexamethasone acetate (2 μM)	95 ± 7 (n = 3)
Prostaglandin E$_2$ (1 μM)	90 ± 4 (n = 4)
Epidermal growth factor (100 ng/ml)	118 ± 17 (n = 3)

Values are the mean ± SEM for n experiments.

seven phorbol esters for causing loss of fibronectin in CEF and their inflammatory potency in the mouse (14). When tested for inhibition of P(Bu)$_2$ binding, these compounds all had ED$_{50}$ values within a factor of 3.5 of their ED$_{50}$ values for causing loss of fibronectin. We also have found that biological activity in the CEF system correlates with promotion rather than inflammation (18). The inflammatory but poorly promoting 12-deoxyphorbol derivatives (31) and the very highly inflammatory but nonpromoting resiniferatoxin (32) are orders of magnitude weaker for causing loss of fibronectin in CEF than expected from their inflammatory activity (18, 33). This relationship is confirmed by the lack of activity of these compounds in blocking [^{3}H]P(Bu)$_2$ binding. These results support our earlier conclusion that a separate target exists for these highly inflammatory but nonpromoting PRDEs and that this target is absent from CEF (or at least is not coupled to an identified biological response).

The binding studies may provide supportive evidence for a third class of targets for the PRDEs. In CEF, most of the phorbol esters examined showed similar potencies for induction of fibronectin loss and for stimulation of 2-deoxyglucose transport. In contrast, mezerein was 46-fold more potent in the latter assay (18), yielding an ED$_{50}$ of 1.2 nM, similar to the ED$_{50}$ of 3.7 nM for PMA. Mezerein is much less active in the binding assay, in agreement with its lower potency for inducing fibronectin loss. *In vivo*, mezerein is within 2-fold of PMA in its inflammatory potency; however, it is only a weak promoter (32). Although other explanations are possible, mezerein may thus represent a third category of PRDE—one that is inflammatory and nonpromoting but, unlike resiniferatoxin, possesses a sensitive target in CEF coupled to stimulation of 2-deoxyglucose transport.

The demonstration of specific binding activity for the phorbol esters has provided valuable information on a number of issues. It has been suggested that the side chains on the phorbol esters serve to facilitate transport of the phorbol esters to a target inside the cell (34). The similarity between the potencies of the PRDEs observed in the particulate preparations and *in vivo* provides no support for this hypothesis. Of particular interest in this regard is the lack of activity of phorbol.

Several studies have examined the *in vivo* metabolism of the phorbol esters (32, 35). Their objective has been to determine whether promotion by PMA is due to PMA itself or to a metabolite. No evidence for a more active metabolite has been found. The high binding potency of PMA and other phorbol esters to the particulate preparations is likewise consistent with these compounds being the "ultimate promoters," in analogy with carcinogens.

The phorbol esters have been reported to induce changes in membrane fluorescence polarization (36), and one hypothesis has been that the phorbol esters may act "by disturbing the structure and function of membranes" (37). The demonstration of binding activity for the phorbol esters suggests that their mechanism may be more specific.

The existence of specific binding activity for the phorbol

esters, natural products from plants, lends support to speculation current in the tumor promotion field that these compounds may interact at the receptor for an endogenous product. If such a compound exists, our competition studies indicate that it is not epidermal growth factor. The assay for phorbol ester binding, however, does provide a means of screening for such compounds.

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