Proc. Natl. Acad. Sci. USA
Vol. 78, No. 4, pp. 2549–2553, April 1981
Medical Sciences

Specific binding of phorbol ester tumor promoters to intact primary epidermal cells from Sencar mice

(tumor promotion/phorbol ester binding sites/down regulation)

VIRENDRA SOLANKI AND THOMAS J. SLAGA

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

Communicated by Charles Heidelberger, December 1, 1980

ABSTRACT **The binding of [20-^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDB) to intact living epidermal cells in monolayer culture was characterized. At 37°C, the maximum specific [^{3}H]PDB binding (binding displaceable by 30 μM unlabeled PDB) was attained in 15–20 min and was followed by a rapid decrease (down regulation) of radioactivity bound to the cells. The activity lost by the cells during this decrease was found in the incubation medium. Prior exposure of cells to phorbol 12-myristate 13-acetate (PMA; 12-*O*-tetradecanoylphorbol 13-acetate) but not to phorbol for 2 hr at 37°C caused ≈55% reduction in the number of measurable binding sites for [^{3}H]PDB. The down regulation was temperature sensitive; there was no loss of radioactivity after 1 hr at 4°C. The specific binding of [^{3}H]PDB at 4°C reached equilibrium in 15–20 min and was saturable and freely reversible. At equilibrium, epidermal cells contained 1.2×10^5 binding sites per cell, and binding sites had a K_D of 10 nM. Specificity of binding was shown by the observation that the biologically active phorbol esters PMA and 12-deoxyphorbol 13-decanoate inhibited the binding, whereas the inactive parent compound phorbol and the nonphorbol tumor promoter anthralin did not have any effect. The abilities of these compounds to inhibit [^{3}H]PDB binding directly correlates with their tumor promoting activities. Epidermal cells exposed to retinoic acid or fluocinolone acetonide for 24 hr had similar [^{3}H]PDB binding characteristics as untreated cells suggesting that inhibition of tumor promotion induced by these compounds is not mediated through alterations in the phorbol ester binding sites.**

Tumor-promoting agents are a class of weakly carcinogenic or noncarcinogenic compounds that enhance the formation of tumors when repeatedly applied to mouse skin that has been previously treated with a subcarcinogenic dose of a carcinogen. This process is known as the two-stage model (initiation and promotion) for the development of skin tumors in mice (1, 2). A wide range of chemical compounds have been shown to have promoting activity (3). Phorbol 12-myristate 13-acetate (PMA; 12-*O*-tetradecanoylphorbol 13-acetate) is the most potent tumor promoter among the 25 phorbol 12,13-diesters isolated from croton oil. The results of investigations of the metabolism of phorbol esters and their chemical requirements for promotion have been discussed (3–5).

Tumor promoters have pleiotropic effects and modify many cellular and biochemical responses (3, 6, 7). It is not known which of these are specific to tumor promotion and which represent secondary events. In the past decade, one of the main strategies for examining the specificity of responses has been correlating the degree of response to graded doses of the promoters or their nonpromoting derivatives. Because correlation does not imply causation, however, this approach has not greatly increased our understanding of the actual mechanism of promotion. Thus, the critical events in tumor promotion remain unresolved. Recently, it has been reported that promotion can be clearly divided into two stages (8).

Current evidence suggests that the primary interaction of PMA is with the cell surface (9–11). Attempts have also been made to identify specific binding sites for phorbol ester tumor promoters in several tissues (12, 13), including mouse skin (14). Investigations using the phorbol derivative [20-^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDB), which is much less lipophylic than PMA itself, have been successful in identifying specific binding sites in particulate fractions of whole cells or tissues. In the present study, we examined the specific binding of [^{3}H]PDB to intact living primary epidermal cells from newborn Sencar mice.

MATERIALS AND METHODS

Chemicals. [^{3}H]PDB (specific activity 6.4 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and unlabeled PDB were purchased from Lifesystem (Newton, MA). PMA, 4-*O*-methyl PMA, and phorbol were obtained from P. Borchert, University of Minnesota, Minneapolis, MN. Mezerein was a generous gift from S. M. Kupchan, University of Virginia, Richmond, VA and J. D. Dowios, National Cancer Institute, Bethesda, MD. 12-Deoxyphorbol 13-decanoate (DPD) was kindly supplied by G. Furstenberger, German Cancer Centre, Heidelburg, Federal Republic of Germany. Dihydroteleocidin B (DHT-B) was donated by T. Sugimura, National Cancer Centre Research Institute, Tokyo, Japan. Anthralin was purchased from Pfaltz and Bauer (Stamford, CT), fluocinolone acetonide (FA) was obtained from Syntex (Palo Alto, CA); and retinoic acid (RA), cadaverine, and methylamine were from Sigma.

Cell Culture. The primary epidermal cells were derived from newborn Sencar mice raised at the Oak Ridge National Laboratory, Oak Ridge, TN. The procedure for the isolation and culture conditions has been described (15). In these experiments, horse serum instead of the previously described fetal bovine serum was used in a modified Waymouth's MS 752/1 medium. Binding assays were performed on confluent monolayer cultures 4 or 5 days after plating of 1×10^6 cells per 35-mm-diameter plastic Petri dish (Falcon).

Trypsinization of Cells for Counting. Confluent cell cultures were washed with two 1.5-ml portions of phosphate-buffered saline (P_i/NaCl) (pH 7.4) and then incubated with trypsin/EDTA solution (0.2% of each in P_i/NaCl) for 10–15 min at 37°C. Detached cells were flushed from the Petri dishes by using a sterilized Pasteur pipette and were counted by using a hemocytometer. Cell viability was determined by trypan blue exclusion.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: [^{3}H]PDB, [20-^{3}H]phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate (12-*O*-tetradecanoylphorbol 13-acetate); DPD, 12-deoxyphorbol 13-decanoate; FA, fluocinolone acetonide; RA, retinoic acid; EPP, ethyl phenyl propiolate; DHT-B, dihydroteleocidin B; P_i/NaCl, phosphate buffered-saline.

Assay of [^{3}H]PDB Binding to Intact Primary Epidermal Cells. Medium from the confluent monolayer cultures was removed by aspiration, and the cells were washed with two 1.5-ml portions of Waymouth's MB 752/1 medium (without serum) before final transfer into 1 ml of this medium. [^{3}H]PDB (≈27 nM) and other compounds were then added, and incubation was carried out at 4°C for 45 min. The reaction was terminated by washing the cells with five portions of P_i/NaCl (pH 7.4) over a period of 5 min. Finally, the cells were dissolved in 1.5 ml of 1% $NaDodSO_4$/10 mM dithiothreitol and incubated at 37°C for 1–2 hr. The $NaDodSO_4$ mixture was transferred to scintillation vials containing 10 ml of ACS scintillation fluid. Radioactivity was counted at 40–50% efficiency in a Tri-carb Packard spectrometer (model 3255). In these experiments, specific [^{3}H]PDB binding is defined as the difference between [^{3}H]PDB bound in the absence and presence of 30 μM unlabeled PDB. The specific binding, 55–70% of the total binding, is expressed as cpm per 10^6 cells.

RESULTS

General Characteristics of [^{3}H]PDB Binding. In initial experiments, [^{3}H]PDB binding was carried out at 37°C. At this temperature, the specific binding of [^{3}H]PDB to intact primary epidermal cells reached a maximum 15–20 min after addition of labeled PDB and then decreased substantially (Fig. 1). This apparent loss (down regulation) appeared due to the dissociation of bound [^{3}H]PDB from the epidermal cells. A progressive increase in the amount of radioactivity in the medium was observed following the maximum binding (Table 1). This increase paralleled the decrease in the [^{3}H]PDB bound to cells. Pretreatment of epidermal cells with 1 μM PMA for 2 hr at 37°C reduced the specific binding of [^{3}H]PDB to about 55% that of control (Fig. 2). In addition, cells pretreated with PMA displayed no down regulation of radioactive label over a period of 1 hr at 37°C. However, prior incubation of cells with 1 μM phorbol for 2 hr at 37°C had no effect on the specific [^{3}H]PDB binding sites or on the down regulation of bound [^{3}H]PDB.

In many types of cells (16–18), the internalization of a ligand-receptor complex is preceded by surface clustering of receptors into specific regions of the plasma membrane. This phenomenon has been shown to be prevented at lower temperatures (18) or by treatment of the cells with alkylamines such as methylamine or cadaverine (18–20). As shown in Fig. 1, down regulation of [^{3}H]PDB binding in epidermal cells was temperature sensitive; at 4°C, no loss of bound [^{3}H]PDB occurred for up to 60 min after addition of the ligand. The down regulation of [^{3}H]PDB binding at 37°C was also modified by the inclusion of 0.1 mM cadaverine in the assay mixture (Fig. 3). However, the inhibitory effect of cadaverine was transient and only delayed the time of down regulation. A major loss of radioactive label occurred at 45–60 min in the presence of cadaverine in contrast to 20–45 min in the absence of cadaverine (see Fig. 1). The reason for this insensitivity of the epidermal cells to cadaverine after 45 min is unknown. In another experiment, the down regulation in cells after 30 min of exposure at 37°C to 10 mM methylamine or 0.1 mM dansylcadaverine was examined. The decrease in specific [^{3}H]PDB binding in these cells began 45–60

Table 1. Release of bound [^{3}H]PDB from epidermal cells into the medium

Time (after addition of medium), min	[^{3}H]PDB bound, cpm per 10^6 cells	[^{3}H]PDB released, cpm per 10^6 cells
0	11,735 ± 1273	
15	3,515 ± 256	8,001 ± 573
30	1,835 ± 118	9,398 ± 759
60	1,525 ± 107	10,003 ± 1,236
120	1,508 ± 121	10,114 ± 1,435

Each plate contained 2 × 10^6 cells and ≈54 nM of [^{3}H]PDB, and the reaction was carried out at 37°C. After maximum binding at 20 min, the medium containing labeled PDB was removed by aspiration and the cells were washed with five 2-ml portions of warm P_i/NaCl. Finally, 1.5 ml of fresh medium was added to each plate, and further incubation was carried out at 37°C. The loss of [^{3}H]PDB bound to epidermal cells into the medium was followed with time. Each value is the mean ± SE of determinations from six separate plates.

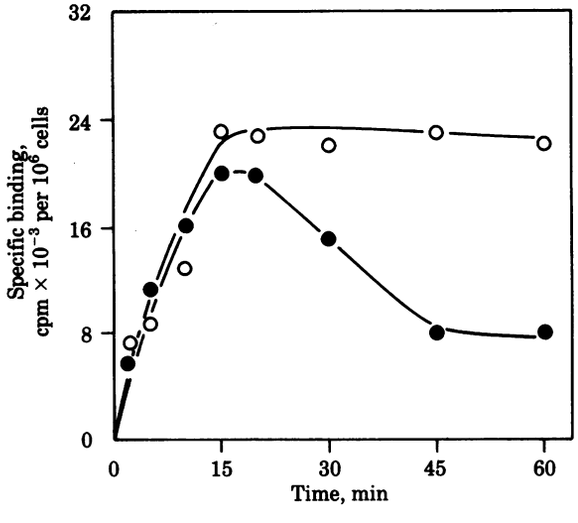

FIG. 1. Time course of specific [^{3}H]PBD binding to intact primary epidermal cells from Sencar mice at 4°C (○) and at 37°C (●). Each point is the mean of triplicate assays carried out on three separate plates.

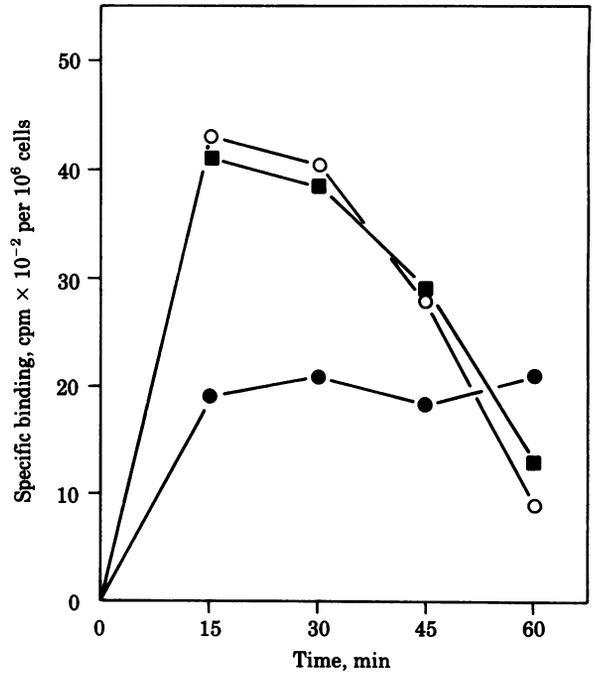

FIG. 2. Time course of specific binding to intact primary epidermal cells that had been pretreated with P_i/NaCl (○), 1 μM PMA (●), or 1 μM phorbol (■) for 2 hr at 37°C. After 2 hr of treatment, cells were washed with three 1.5-ml portions of warm medium, and further [^{3}H]PDB binding assay was carried out at 37°C. Each point is the mean of triplicate assays carried out on three separate plates.

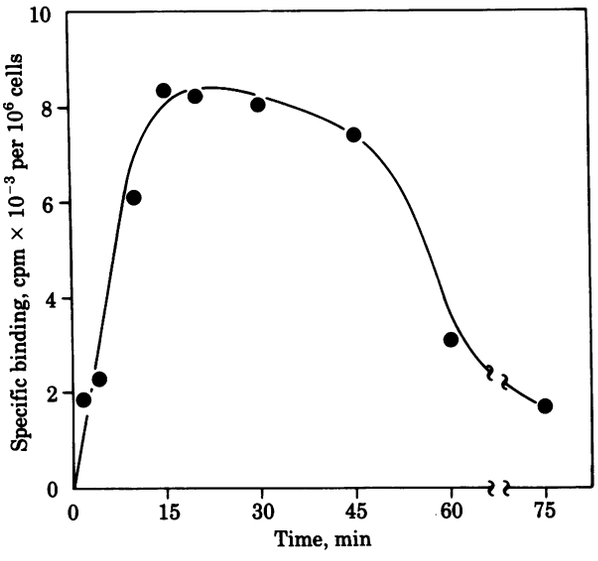

FIG. 3. Time course of specific [^{3}H]PDB binding to intact primary epidermal cells in the presence of 0.1 mM cadaverine. Cadaverine was included in the incubation mixture from time zero, and the reaction was carried out at 37°C for various times. Each point is the mean of triplicate assays carried out on three separate plates.

min after addition of the ligand (data not shown), suggesting that the effect of methylamine or dansylcadaverine was reversible.

Maximum specific [^{3}H]PDB binding to intact cells occurred within 15–20 min at both 4°C and 37°C (see Fig. 1). Specific binding of [^{3}H]PDB to intact cells at 4°C was fully reversible (data not shown). In this experiment, binding of labeled ligand was allowed to reach equilibrium (45 min at 4°C). An excess of unlabeled PDB (30 μM) was then added, and the displacement of label with time was followed. Complete displacement of specific [^{3}H]PDB binding was observed after 25–30 min.

The dependence of specific [^{3}H]PDB binding on the concentration of ligand was also determined (Fig. 4*A*). The specific [^{3}H]PDB binding to the intact cells increased with increasing concentrations of the ligand and appeared to be saturable. Scatchard analysis of the binding data (Fig. 4*B*) indicated a slope corresponding to K_D = 10 nM, in fair agreement with values reported for the 100,000 × *g* pellet from mouse skin (14) and chicken embryo fibroblasts (12). Saturation of binding sites (B_{max}) was obtained at ≈200 fmol of [^{3}H]PDB bound per 10^6 cells. The total number of saturable binding sites per cell was calculated to be ≈120,400.

Inhibition of [^{3}H]PDB Binding by Phorbol Ester Tumor Promoters. The relative abilities of various tumor promoters to inhibit the binding of labeled PDB to intact primary epidermal cells were compared. Data from a representative experiment (Fig. 5) shows that PMA and DPD competed very effectively with labeled PDB. However, PMA was more potent than DPD in inhibiting the binding of [^{3}H]PDB. Binding of [^{3}H]PDB was also inhibited by the resiniferonol derivative, mezerein, a diterpene similar to PMA in its biochemical and morphological effects and a potent stage II promoter (22, 23). 4-*O*-methyl PMA, which has been shown to be a stage I tumor promoter in mouse skin (see refs. 8, 22, and 23), also competed for about 20–25% [^{3}H]PDB binding sites at 1 μM concentration. DHT-B, a derivative of teleocidin B, which is similar to PMA in inducing biochemical events (24), also inhibited [^{3}H]PDB binding and has proved nearly as effective a promoter as PMA in a mouse skin tumor promoting assay (unpublished results). Unlike the biologically active phorbol ester PMA, the parent diterpene

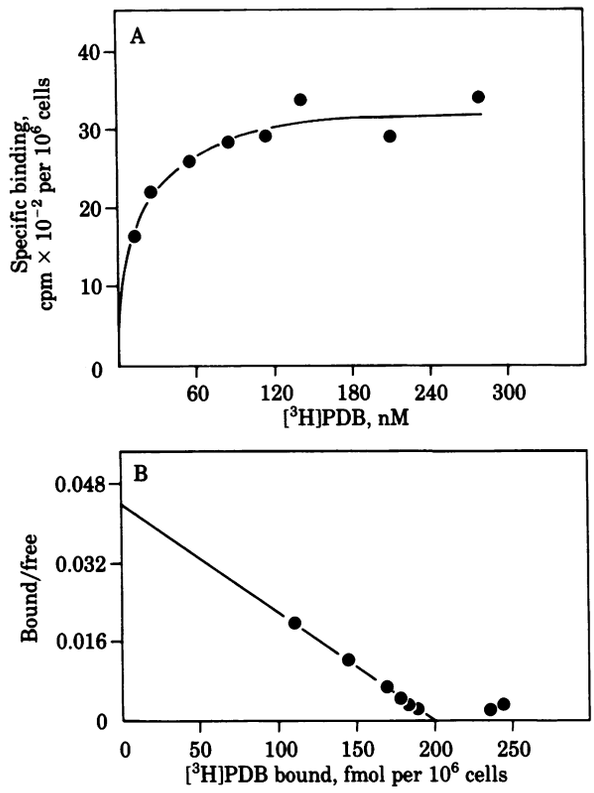

FIG. 4. Equilibrium specific [^{3}H]PDB binding to intact epidermal cells as a function of ligand concentration. The concentration of [^{3}H]PDB was adjusted by adding various amounts of the ligand to the culture plates (2.2 × 10^6 cells per plate). Incubation was carried out at 4°C in the absence or presence of 30 μM unlabeled PDB for 45 min. Each point is the mean of triplicate assays done on three separate plates. Scatchard (21) plot of (*B*) specific [^{3}H]PDB binding data.

phorbol did not compete for [^{3}H]PDB binding sites.

Another tumor promoter, anthralin, which is structurally different from the phorbol ester promoters, was unable to in-

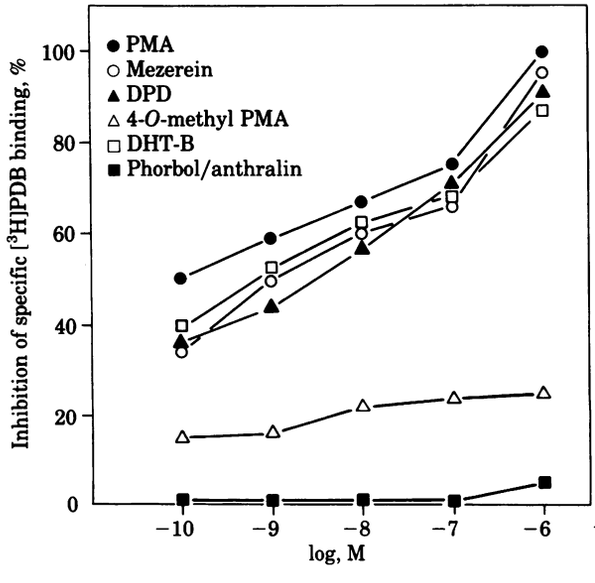

FIG. 5. Displacement of [^{3}H]PDB binding from intact primary epidermal cells. Each plate contained ≈27 nM of [^{3}H]PDB and various concentrations of promoters and related compounds. The reaction was performed at 4°C for a period of 45 min. Each point is the mean of three assays carried out on three separate plates.

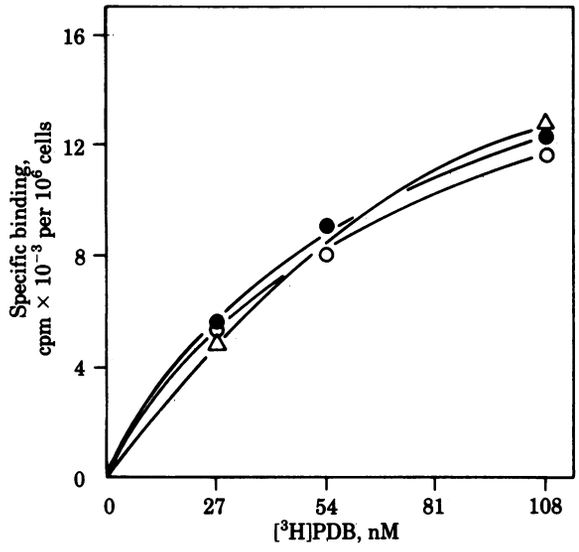

FIG. 6. Epidermal cells were exposed to P_i/NaCl (●), 10 μM RA (○), or 4 μM FA (△) for 24 hr. Cells were washed with three 1.5-ml portions of P_i/NaCl, and binding was carried out at 4°C as described in *Materials and Methods*.

hibit [^{3}H]PDB binding to epidermal cells. This observation suggests that this compound may have a different site of action than PDB. Similar results with anthralin have been obtained using mouse skin particulate preparations (14). Chrysarobin, which is closely related in structure to anthralin but 4 or 5 times more potent as a promoter in mouse skin, did not have any effect on [^{3}H]PDB binding (data not shown).

Lack of Inhibition of [^{3}H]PDB Binding by Antipromoting and Hyperplastic Agents. FA and RA, which are potent inhibitors of tumor promotion by phorbol esters in mouse skin (25–27), had no effect on [^{3}H]PDB binding to intact epidermal cells at concentrations up to 1 μM (data not shown), suggesting that these compounds do not interfere with the initial interaction of phorbol esters with the cell surface. Our findings are consistent with an earlier report (14) that there was no competition for binding sites between RA or FA and [^{3}H]PDB in the 100,000 × *g* particulate fraction from mouse skin. In addition ethyl phenyl propiolate (EPP), a purely hyperplastic agent, at 1 μM did not affect [^{3}H]PDB binding to epidermal cells (data not shown).

An attempt was also made to determine whether RA or FA treatment altered the characteristics of [^{3}H]PDB binding to intact cells; no measurable differences were observed (Fig. 6). Treatment of cells with RA or FA for 24 hr did not alter the specific [^{3}H]PDB binding to cells at ligand concentrations up to 108 nM.

DISCUSSION

Recent investigations (9–11) suggest that the primary interaction of phorbol ester tumor promoters is with the cell surface. In view of the fact that phorbol ester promoters have multifaceted effects in various biological systems (3, 6, 7), the specificity of this interaction has been questioned. Because of similarities between the biochemical changes induced by epidermal growth factor and by PMA (10), the growth factor receptor was predicted to be a candidate for PMA interaction. However, kinetic studies of the inhibition of growth factor binding by PMA have resulted in variable data (10, 11, 28–30), and the results to date have shown that the inhibition is not due to direct competition of PMA for the growth factor receptor. This report demonstrates specific binding of phorbol esters and related promoters to intact epidermal cells. These results are consistent with the recent report (14) that phorbol ester promoters have specific binding sites in the 100,000 × *g* particulate fraction from mouse skin.

The rapid down regulation of bound [^{3}H]PDB observed in these cells may be a general mechanism that also takes place in other cell types. A similar down regulation of [^{3}H]PDB was observed in human promyelocytic leukemia cells (31) and in a mouse epidermal cell line (unpublished results). Because PDB is not metabolized or inactivated by epidermal cells in 1 hr (unpublished results), the apparent loss of [^{3}H]PDB binding is not due to its metabolism. The loss of bound [^{3}H]PDB appears to result from conformational changes in the binding sites. Also, after down regulation, the measured radioactivity associated with the cells may be a consequence of interiorized ligand–receptor complexes. In addition, our data also show that PDB-induced down regulation in epidermal cells appears to be specific to the class of biologically active phorbol esters; prior exposure of cells to PMA, but not to phorbol, resulted in a reduction in the number of binding sites for [^{3}H]PDB. It should be remembered that phorbol does not compete for [^{3}H]PDB binding sites.

The down regulation of the binding of several hormones (16–20, 30), including epidermal growth factor (10, 28–30, 32, 33), occurs via a process of internalization of hormone–receptor complexes. The down regulation of [^{3}H]PDB binding sites is prevented at 4°C in a fashion similar to that of several other hormones (18, 28, 29) that have been shown to be internalized by a receptor uptake process. Because internalization occurs with epidermal growth factor and several hormones, it may be the case with phorbol esters but we do not have any evidence for this. The process of internalization in some cell types (18–20) but not in others (34) is prevented by alkylamines such as methylamine and cadaverine. Our data show that the down regulation of [^{3}H]PDB binding in epidermal cells is not prevented by these agents.

The loss of specific [^{3}H]PDB binding in epidermal cells is one of the most rapid examples of down regulation thus far observed. The physiological significance of such a rapid loss of bound [^{3}H]PDB from these cells has yet to be determined. Also, 1-hr exposure of epidermal cells to PMA (followed by removal of the PMA from the medium) was sufficient to stimulate DNA synthesis and ornithine decarboxylase activity in a manner similar to that produced by long exposure to PMA (35). Thus, the observed down regulation may be a regular feature occurring when cells are exposed to phorbol diesters.

Despite the difficulties in making measurements at 37°C due to the rapid down regulation of specific [^{3}H]PDB binding, a satisfactory characterization of the [^{3}H]PDB binding was accomplished at 4°C. Specific binding of [^{3}H]PDB was rapid and fully reversible. Binding sites were saturable (K_D = ≈10 nM) and limited in number to ≈1.2×10^5 per cell.

The specificity of [^{3}H]PDB binding to epidermal cells was demonstrated by an experiment that showed that the biologically active phorbol esters PMA and DPD inhibited [^{3}H]PDB binding whereas 4-*O*-methyl PMA only partiallly inhibited it. Phorbol, the inactive parent diterpene, had no effect. The partial displacement of [^{3}H]PDB by 4-*O*-methyl PMA is important in view of the fact that this compound serves as a stage I promoter in the two-stage tumor-promotion protocol in mouse skin (8, 22, 23). Thus, there may be two separate classes of receptors, one for each stage of promotion. The stage I promotor 4-*O*-methyl PMA would only be expected to displace [^{3}H]PDB only from the sites specific for this stage.

Two other compounds, mezerein and DHT-B, that induce cellular and biochemical responses very similar to those pro-

duced by PMA (22–24), were potent inhibitors of [^{3}H]PDB binding. Naturally occurring promoters other than the phorbol esters may therefore initiate similar biochemical responses by binding to the same sites. DHT-B, a derivative of teleocidin B isolated from the mycellia of *Streptomyces* 2A 1563 (36), is nearly as potent as PMA in inducing tumors in mouse skin. Tumor promoters such as anthralin and chrysarobin, which are structurally unrelated to phorbol ester promoters, did not compete with [^{3}H]PDB. Thus, these compounds may have different sites of action than PDB. The possibility of promotion by a different mechanism by these agents cannot be ruled out. Furthermore FA- or RA-induced inhibition of tumor promotion (25–27) is not mediated through alterations in the phorbol ester binding sites. Our data also suggest that RA and FA and EPP, a hyperplastic agent, do not exert their effects by binding to the binding sites that bind the phorbol esters.

Comparison of the relative abilities of various phorbol esters and related promoters to compete for [^{3}H]PDB binding showed that PMA was a more effective competitor for the [^{3}H]PDB sites than DPD, mezerein, or DHT-B. The relative order of these promoters for the inhibition of [^{3}H]PDB binding was PMA > DHT-B > DPD ≥ mezerein > 4-*O*-methyl PMA. This is also the relative order of the tumor-promoting activities of these compounds in the one-stage promotion assay in mouse skin. In summary, these results suggest that phorbol ester-receptor binding and processing, perhaps by down regulation, may be involved in tumor promotion by these agents.

This research was supported by the Office of Health and Environmental Research, U.S. Department of Energy, under Contract W-7405-eng-26 with the Union Carbide Corporation. V.S. is a Postdoctoral Investigator supported by Subcontract 3322 from the Biology Division of Oak Ridge National Laboratory to the University of Tennessee.

1. Berenblum, I. & Shukik, P. (1947) *Br. J. Cancer* **1**, 379–382.
2. Boutwell, R. K. (1964) *Prog. Exp. Tumor Res.* **4**, 207–250.
3. Boutwell, R. K. (1974) *CRC Crit. Rev. Toxicol.* **2**, 419–443.
4. Hecker, E. & Schmidt, R. (1974) in *Progress in the Chemistry of Organic Natural Products*, eds. Heiz, W., Grisebach, H. & Kirby, G. W. (Springer, New York), Vol. 31, pp. 377–367.
5. Hecker, E. (1978) in *Carcinogenesis: Mechanism of Tumor Promotion and Cocarcinogenesis*, eds. Slaga, T. J., Sivak, A. & Boutwell, R. K. (Raven, New York), Vol. 2, pp. 11–48.
6. Sivak, A. (1979) *Biochim. Biophys. Acta* **560**, 67–89.
7. Sivak, A. (1978) in *Carcinogenesis: Mechanism of Tumor Promotion and Cocarcinogenesis*, eds. Slaga, T. J., Sivak, A. & Boutwell, R. K. (Raven, New York), Vol. 2, pp. 553–564.
8. Slaga, T. J., Klein-Szanton, A. J. P., Fischer, S. M., Weeks, C. E., Nelson, K. & Major, S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2251–2254.
9. Blumberg, P. M. & Driedger, P. E. (1976) *Nature (London)* **264**, 446–447.
10. Lee, L. & Weinstein, I. B. (1978) *Science* **202**, 313–315.
11. Murray, A. W. & Fusenig, N. E. (1979) *Cancer Lett.* **7**, 71–77.
12. Driedger, P. E. & Blumberg, P. M. (1980) *Proc. Natl. Acad Sci. USA* **77**, 567–571.
13. Lee, L. S. & Weinstein, I. B. (1978) *J. Environ. Pathol. Toxicol.* **1**, 627–639.
14. Delclos, K. B., Nagle, D. S. & Blumberg, P. M. (1980) *Cell* **19**, 1025–1032.
15. Fischer, S. M., Viaje, A., Harris, K. L., Miller, D. R., Bohrman, J. S. & Slaga, T. J. (1980) *In Vitro* **16**, 180–188.
16. Goldstein, J. L., Anderson, R. G. & Brown, M. S. (1979) *Nature (London)*, **279**, 679–684.
17. Haigler, H. T., Mckenna, J. A. & Cohen, S. (1979) *J. Cell Biol.* **81**, 382–395.
18. Cheng, S., Maxfield, F. R., Robbins, J., Willingham, M. C. & Pastan, I. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3425–3429.
19. Maxfield, F. R., Willingham, M. C., Davies, P. J. A. & Pastan, I. (1979) *Nature (London)* **277**, 661–663.
20. Levitzki, A., Willingham, M. & Pastan, I. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2706–2710.
21. Scatchard, G. (1949) *Ann N.Y. Acad. Sci.* **51**, 660–672.
22. Slaga, T. J., Fischer, S. M., Nelson, K. & Gleason, G. L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3659–3663.
23. Mufson, R. A., Fischer, S. M., Verma, A. K., Gleason, G. L., Slaga, T. J. & Boutwell, R. K. (1980) *Cancer Res.* **39**, 4791–4800.
24. Fujiki, H., Mori, M., Nakayasu, M., Terada, M. & Sugimura, T. (1979) *Biochem. Biophys. Res. Commun.* **90**, 976–983.
25. Weeks, C. E., Slaga, T. J., Hennings, H., Gleason, G. L. & Bracken, W. M. (1979) *J. Natl. Cancer Inst.* **63**, 401–406.
26. Slaga, T. J. (1980) in *Carcinogenesis, Modifiers of Chemical Carcinogenesis*, ed. Slaga, T. J. (Raven, New York), Vol. 5, pp. 111–126.
27. Verma, A. K., Rice, H. M., Shapos, B. G. & Boutwell, R. K. (1978) *Cancer Res.* **38**, 793–801.
28. Brown, K. D., Dicker, P. & Rozengurt, E. (1979) *Biochem. Biophys. Res. Commun.* **4**, 1037–1043.
29. Weinstein, I. B. & Lee, L. S. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5168–5172.
30. Shoyab, M., DeLarco, J. E. & Todaro, G. J. (1979) *Nature (London)* **279**, 387–391.
31. Solanki, V., Slaga, T. J., Callaham, M. & Huberman, E. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1722–1725.
32. Schlessinger, J., Shechter, Y., Willingham, M. D. & Pastan, I. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2659–2663.
33. Magun, B. E., Matrisian, L. M. & Bowden, G. T. (1980) *J. Biol. Chem.* **255**, 6373–6381.
34. King, A. C., Hernaez-Davis, L. & Cuatrecasas, P. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3283–3287.
35. Yuspa, S. H., Lichti, U., Ben, T., Patterson, E., Hennings, H., Slaga, T. J., Colburn, N. & Kelsey, W. (1976) *Nature (London)* **262**, 402–404.
36. Takashima, M. & Sakai, H. (1960) *Bull. Agr. Chem. Soc. Jpn.* **24**, 647–651.