Presence of Specific Binding Sites for Phorbol Ester Tumor Promoters in Human Epidermal and Dermal Cells in Culture but Lack of Down Regulation in Epidermal Cells

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ABSTRACT

The presence of specific binding sites for phorbol esters was demonstrated in human epidermal and dermal cells in culture by assay of binding of \([^{3}H]\)phorbol-12,13-dibutyrate (PDBU) to intact cells. The specificity of the binding was shown by displacement of the binding with biologically active tumor promoters, such as 12-O-tetradecanoylphorbol-13-acetate, teleocidin B, and mezerein, but not with inactive derivatives. The equilibrium binding data were analyzed by the Scatchard method and fitted by a straight line to the model of a single class of binding sites. Human epidermal cells bound PDBU with a \(K_d\) of 28 nM at 3.7 \(\times\) 10^8 molecules per cell, while human dermal cells bound PDBU with a \(K_d\) of 27 nM at 2.1 \(\times\) 10^8 molecules per cell. These values were compared with those of epidermal and dermal cells of mice. Although mouse cells showed the same affinity as did human cells, mouse epidermal cells bound one-third as much as human epidermal cells, and mouse dermal cells bound one-fifth as much as human dermal cells. When precultured with unlabeled PDBU for 24 hr, \([^{3}H]\)PDBU binding decreased time dependently in all cells except human epidermal cells. Thus, the binding of phorbol esters to human epidermal cells is unique in that there are a large number of binding sites compared with mouse epidermal cells, and there is no down regulation.

INTRODUCTION

Tumor promoters are compounds that are not themselves mutagenic or carcinogenic but promote the formation of tumors when applied repeatedly after a subcarcinogenic dose of a carcinoagen (1, 2). The process of tumor promotion has been studied in detail in mouse skin with phorbol ester tumor promoters isolated from croton oil. Of the phorbol esters, TPA3 is the most potent and therefore has been used extensively to study mechanisms of tumor promotion. In addition, TPA and related tumor promoters have pleiotropic effects on various cells in culture (37).

Biochemical and biological studies have suggested that the primary sites of action of phorbol esters are on the cell surface membrane (7), although a nuclear receptor for phorbol ester was reported recently by Perrella et al. (31). Indeed, specific saturable binding sites for phorbol esters have been demonstrated in the membrane fraction of chicken embryo fibroblasts (9), mouse skin (5), mouse epidermis (5), and mouse brain (10, 11) by using radioactive PDBU, which is much less hydrophobic than is TPA and shows less nonspecific binding. \([^{3}H]\)PDBU binding sites in intact cells have also been demonstrated in all cells so far examined except erythrocytes (35). The cells examined included mink lung cells (35), rat pituitary cells (22, 23), rat embryo cells (21), mouse fibroblasts (35), mouse epidermal cells (4, 38), and mouse Friend leukemia cells (41).

Although there have been many studies on TPA and other tumor promoters, in almost all of them, nonhuman materials, mostly mouse cells, were used. There have been few studies on human materials, especially human epidermal cells, and only recently was it found that TPA inhibits growth (13, 19, 30) but stimulates differentiation (13, 19, 30) of human epidermal keratinocytes in culture and stimulates growth of human melanocytes (13) and unidentified epidermal cells (19). There has been no report on receptors of phorbol ester tumor promoters in normal human epidermal cells, although recently, Mufson et al. (28) reported the presence of a receptor in SV40-infected human epidermal keratinocytes. Human lymphocytes and leukemia cells were reported to have specific binding sites to phorbol esters (14, 33, 39).

We are using human epidermal cells in studies on chemical carcinogenesis because of the importance of human cells, especially human epithelial cells. We focused attention first on the metabolism of benzo(a)pyrene, especially in relation to variation among species, individuals, and cell types (24, 26). We now use this system to study action of TPA and related compounds on human cells, because human epidermal cells might be a target tissue if TPA is a human promoter.

In the present study, we demonstrated the presence of specific binding sites for phorbol ester tumor promoters in intact human epidermal keratinocytes and dermal fibroblasts but the absence of down regulation of the binding sites in human epidermal cells.

MATERIALS AND METHODS

Chemicals. \([^{20}\text{H}]\)PDBU (specific activity, 15.3 Ci/mmol) and unlabeled PDBU were purchased from New England Nuclear (Boston, Mass.) and Sigma Chemical Co. (St. Louis, Mo.), respectively. TPA and mezerein were purchased from Consolidated Midland Corporation, Brewster, N. Y., and EGF was from Collaborative Research, Waltham, Mass. 4a-PDD and phorbol were supplied by H. Yamazaki, International Agency for Research on Cancer, Lyon, France. Teleocidin B was a gift from K. Umezawa of this Institute. Cholera toxin was purchased from the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan. All these compounds, except EGF and cholera toxin, were dissolved in acetone, of which the final concentration in the medium was 0.5%.

Cell Culture. Human epidermal and dermal cells were isolated as described previously (24, 26). Briefly, dermato-mes-epithelial skin obtained as discarded material after plastic surgery was cut into small

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3 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PDBU, phorbol-12,13-dibutyrate; EGF, epidermal growth factor; 4a-PDD, 4a-phor- bol-12,13-didecanoate; PBS, phosphate-buffered solution containing 8 g NaCl, 0.2 g KCl, 0.2 g KH2PO4, and 1.15 g Na2HPO4 per liter; PDD, phorbol-12,13-didecanoate.
Down Regulation of [3H]PDBU Binding. Down regulation of [3H]PDBU binding to intact cells was measured in the presence of 100 nM unlabeled PDBU. Nonspecific binding was increased linearly with increase in concentration of [3H]PDBU. Specific binding of [3H]PDBU to intact human epidermal cells occurred rapidly, reaching a maximum after about 30 min and then remaining constant for at least 60 min (Chart 1). In dermal cells, the specific binding reached a maximum after 30 min, remained at a plateau for 30 min, and then decreased about 35% (Chart 1). These experiments, we therefore used a 30-min incubation period in the binding assay.

Specific [3H]PDBU binding to human epidermal and dermal cells increased linearly with increase in number of the cells in the range between 10^3 and 10^6 cells (data not included). The slopes of the lines for epidermal and dermal cells were parallel, and the apparent Kd values were calculated to be 28 nM for epidermal cells and 27 nM for dermal cells. At a saturating concentration of PDBU, about 6.1 pmol/10^5 cells (3.7 x 10^5 molecules/cell) and 3.5 pmol/10^5 cells (2.1 x 10^5 molecules/cell) were bound to epidermal and dermal cells, respectively. Thus, the affinity of PDBU for epidermal cells is the same as that for dermal cells, but the epidermal cells have 1.8-fold more binding sites than do dermal cells.

Inhibition of [3H]PDBU Binding by Other Tumor Promoters. The specificity of [3H]PDBU binding to human epidermal cells was examined by measuring the competitive bindings of other tumor promoters, namely TPA, mezerein, PDD, 4α-PDD, phorbol, and teleocidin B (Chart 4). Of these, TPA was the most potent inhibitor. High competition was also observed with mezerein, a reciniferanol derivative that is structurally related to phorbol.

RESULTS

Binding of [3H]PDBU to Human Epidermal and Dermal Cells. All experiments with human epidermal cells were performed in primary cultures after cultivation for 2 to 3 weeks. As reported previously (24, 26), the epidermal cultures consisted of tightly packed keratinocytes with a typical epithelial appearance with scarcely any fibroblasts but a few melanocytes distributed singly. The primary cultures of dermal cells consisted of mixed populations of fibroblasts and keratinocytes. Dermal cells were therefore usually used after selective transfer of dermal fibroblasts with 0.05% trypsin-0.02% EDTA solution.

Binding of [3H]PDBU to intact cells was measured in the presence and absence of excess (30 μM) unlabeled PDBU. Nonspecific binding was increased linearly with increase in concentration of [3H]PDBU, but was independent of the time of incubation. It was about 20% of the total binding in the standard assay conditions, i.e., 3 nM, and about 50% at 100 nM [3H]PDBU.

On incubation with [3H]PDBU at 37°C, specific [3H]PDBU binding to intact human epidermal cells occurred rapidly, reaching a maximum after about 30 min and then remaining constant for at least 60 min (Chart 1). In dermal cells, the specific binding reached a maximum after 30 min, remained at a plateau for 30 min, and then decreased about 35% (Chart 1). These experiments, we therefore used a 30-min incubation period in the binding assay.

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were obtained for both epidermal and dermal cells. The regression lines obtained for epidermal and dermal cells of humans and mice. Primary cultures of epidermal and dermal cells isolated from newborn Sencar mice were assayed for [3H]PDBU binding under the same conditions as those used for the human cells. On Scatchard analyses of the binding, linear regression lines were obtained for both epidermal and dermal cells. The 4 regression lines obtained for epidermal and dermal cells of humans and mice were parallel (Chart 3), indicating the same affinity of PDBU for its receptors in these cells. However, the total number of saturable binding sites was more in human cells than in mouse cells (Table 2); human epidermal and dermal cells bound about 3 and 5 times, respectively, more than the corresponding mouse cells. This difference was not due to a difference in cell size, since a similar difference was observed in values for binding corrected for the cell surface area (Table 2).

**DISCUSSION**

We demonstrate here by [3H]PDBU binding assay that human epidermal and dermal cells contain a specific, saturable, high-affinity binding site for phorbol esters. The kinetics of binding in these cells was compared with that of the corresponding mouse cells under the same experimental conditions.

Scatchard plot analyses of the bindings to these cells gave straight lines, indicating a single class of binding sites. This observation is in keeping with most previous reports (5, 9, 10, 22, 38), except those on rat embryo fibroblasts (21) and SV40-infected human epidermal cells (28), which gave a biphatic curve.
Phorbol Ester Receptor in Human Cells

Charts. Down regulation of \(^{3}H\)PDBU binding in human epidermal cells (O, HUSKI-119K), human dermal cells (C, HUSKI-109F), mouse epidermal cells (E), and mouse dermal cells (D). After preincubation with 30 nM unlabeled PDBU at 37°C for 2 to 24 hr, bound PDBU was removed by 3 washings with PBS and incubation obtained with JB6 mouse epidermal cells (4). The specificity of PDBU binding to human epidermal and dermal cells was demonstrated by displacement of the binding; inactive 4a-PDD and phorbol. They also thank Professor T. Oszuka, Department of Plastic and Reconstructive Surgery, School of Medicine, Showa University, Tokyo, Japan, for supplying skin materials and N. Munezawa for secretarial assistance.

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The receptor for phorbol esters may be required for exertion of the biological and biochemical effects of these compounds but may not necessarily be an indication of sensitivity to phorbol esters. Cells with resistance to TPA have been selected but found not to lack the receptor. No difference was observed in the binding of TPA by sensitive and resistant variants of Friend leukemia cells (41), human leukemia HL-60 (39), and JB6 mouse epidermal cells (4). In contrast, a receptor deficiency for hormones was found to be associated with a number of clinical disorders, such as vitamin D-resistant rickets (12). In addition, Pruss and Herschman (32) reported that selection for EGF resistance yielded variants without EGF receptor.

A significant decrease in the number of binding sites after preincubation with the ligands has been shown previously for other receptors, such as those of EGF (3), insulin (17), thyrotropin-releasing hormone (20), \(\alpha\)-bungarotoxin (6), and concanavalin A (29) and also for PDBU binding to other cell types (4, 22, 39, 41). Down regulation of receptor-ligand complexes after binding has been postulated to be a means of reaching intracellular targets for expression of biological effect. Down regulation may result from conformational changes in the binding sites or from internalization of the binding sites. Solanki et al. (39) found that a TPA-resistant variant of HL-60 cells did not show down regulation of the phorbol ester receptor, suggesting the requirement of down regulation for expression of biological activity. However, the occurrence of down regulation of PDBU binding sites in TPA-resistant cells was reported by Colburn et al. for mouse epidermal cells (4) and by Yamasaki et al. for Friend leukemia cells (41). In this work, we found that human epidermal cells, but not the other cells, did not show down regulation of PDBU binding sites. This deficiency may not be explained by metabolic inactivation of PDBU, since Shoyab et al. (36) reported that human skin has little activity of phorbol-12,13-diester 12-ester hydrolase.

The functional significance of the lack of down regulation should be investigated further by studies on the biological activity of phorbol esters on this particular cell type. We are now investigating the response of human epidermal and dermal cells to phorbol esters.
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