Heterogeneity of $[^3H]$Phorbol 12,13-Dibutyrate Binding in Primary Mouse Keratinocytes at Different Stages of Maturation

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ABSTRACT

Mouse keratinocytes respond heterogeneously to phorbol esters with distinct subpopulations stimulated to proliferate or induced to differentiate. The maturation state of the epidermal cell at the time of exposure may determine its response. The binding of phorbol esters to primary mouse keratinocytes was studied under culture conditions selecting for proliferating cells or differentiating cells. [20-3H]12-Deoxyphorbol 13-isobutyrate ($[^3H]$DPB) bound to both types of cells at one class of binding sites. The dissociation constant ($K_d$) for $[^3H]$DPB in the proliferative cells (epidermal cells grown in Eagle’s minimal essential medium containing 8% fetal calf serum (Chelex treated) and 0.05 mM CaCl$_2$) was 69 nM and the binding at saturation ($B_{max}$) was 1.3 pmol/mg of protein. The corresponding values in the differentiative cells (epidermal cells grown in Eagle’s minimal essential medium containing 8% fetal calf serum and 1.2 mM CaCl$_2$) were 96 nM and 1.5 pmol/mg of protein, respectively. In contrast to the results obtained with $[^3H]$DPB, 20-3H]phorbol 12,13-dibutyrate ($[^3H]$PDBU) bound to both cell types in a heterogeneous fashion. Analyzed by the Ligand Program with a two-site model, the $K_a$ values for the two binding sites in the cells grown in the medium containing 0.05 mM CaCl$_2$ were 5.5 and 100 nm and the $B_{max}$ values were 0.9 and 1.7 pmol/mg of protein, respectively. The site for $[^3H]$DPB binding seemed to correspond to the higher affinity $[^3H]$PDBU binding site. The major difference in the cells grown in the medium containing 1.2 mM CaCl$_2$ was an increase (approximately two-fold, depending on details of the analysis) in the $B_{max}$ of the lower affinity binding site with the other three parameters remaining similar. The state of epidermal differentia
tion thus appears to modulate the amount of the lower affinity binding sites for phorbol esters. Three Ca$^{2+}$-resistant cell lines have been developed from carcinogen-treated primary keratinocytes. These cells are not tumorigenic and demonstrate characteristics consistent with their being initiated cells. In the medium containing 1.2 mM CaCl$_2$, $[^3H]$PDBU bound to these cells at one class of binding sites with affinities similar to that of the higher affinity sites for $[^3H]$PDBU in cells grown in the medium containing either 0.05 or 1.2 mM CaCl$_2$.

INTRODUCTION

The biological effects of phorbol ester tumor promoters on mouse skin have been well documented (1–3). The phorbol esters are potent inflammatory and hyperplastic agents which give rise in initiated skin to papillomas and carcinomas. The biochemical events following a single exposure of phorbol esters to mouse skin suggest simultaneous responses characteristic of both the differentiative and proliferative programs of epidermis (4). Studies from this laboratory utilizing cultured primary basal keratinocytes from newborn mice (5) suggested that this mixed response reflected heterogeneity at the cellular level. Basal keratinocytes cultured in media in which the concentration of Ca$^{2+}$ was less than 0.1 mM (6) responded in two different fashions to PMA. A fraction of the cell population stopped growing and differentiated following exposure; the remainder were refractory to the differentiative signal of PMA and continued to proliferate (5). Studies in intact skin keratinocytes by Reiners and Siaga (7) confirmed that phorbol esters both induce a subpopulation of basal cells to undergo terminal differentiation, as well as accelerate the maturation of cells already committed to differentiate.

We have proposed that the cellular basis for divergent responses among basal cell populations was the state of maturation at the time of exposure to PMA (5, 8). This idea was based on the following observations. (a) Cells resistant to the differentiative influences of a single PMA exposure resisted terminal differentiation upon subsequent exposures if the interval between exposures was short; these cells responded to PMA in a proliferative mode. (b) If long intervals were used between exposures, the population again responded heterogeneously, suggesting that the proliferatively responding cells were the progenitor population of the differentiating responders. (c) If cells were put into a more advanced state of differentiation at the time of PMA exposure by increasing the extracellular Ca$^{2+}$ to >0.1 mM, the differentiation program was markedly accelerated while the ability to respond in the proliferative mode was rapidly lost (8, 9). Similar conclusions could be reached from in vivo studies in which multiple exposures to PMA spaced at appropriate intervals enhanced or accelerated several proliferation associated responses, including the induction of ornithine decarboxylase, DNA synthesis (where the initial inhibition of synthetic activity was abolished), and the elevation of cyclic GMP levels (10, 11). These results might be expected if a proliferative population were being selected by multiple promoter exposures.

At the biochemical level, considerable insight into the initial steps in the action of the phorbol esters has been obtained. Specific phorbol ester receptors are present in intact cells and particulate preparations (see Ref. 12 for a review). In addition a phorbol ester aporeceptor (which requires phospholipids for activity) has been identified in cytosol (13–16). The cytosolic aporeceptor and at least a portion of the membrane receptor copurify with protein kinase C (17). The evidence that the phorbol ester receptors mediate biological responses to these agents
deserves in substantial part from comparison of structure-activity relations. In general, the structure-activity relations for binding agree well with those for induction of a variety of biological effects (12).

Detailed analysis, however, suggests heterogeneity in biological responses to the phorbol esters. A number of compounds exist which have been shown to possess only a fraction of the properties of the typical phorbol esters. These include the highly inflammatory but poorly promoting agent resiniferatoxin (18), the inflammatory short chain 12-deoxyphorbol esters (19), and the second stage tumor promoter mezerein (20). The same phorbol ester, moreover, may be required in different concentrations to induce different responses in the same cell type. For example, Jaken et al. (21) have reported a 20-fold difference in the potency of mezerein to inhibit epidermal growth factor binding and to release prostaglandin E₂ from cultured G-292 cells (21). In several systems binding analysis likewise indicates heterogeneity. We have reported that [³H]DPB bound selectively to a subset of PDBU receptors in whole mouse skin particulate preparations (22). By competition analysis we demonstrated that the affinities of a large series of phorbol esters at the major [³H]DPB site correlated well with their inflammatory potencies, while the affinities at the remaining PDBU binding site correlated better with the promoting activity. Using intact cell systems, a number of other laboratories have also reported heterogeneity in phorbol ester binding (23–25).

In the present study we wished to determine whether the heterogeneity in phorbol ester binding to skin particulate preparations extended to intact keratinocytes and, if so, whether it depended on the state of differentiation of the cell population. We report that in the primary keratinocytes Ca²⁺-induced differentiation generates a significant change in the [³H]PDBU binding characteristics of these cells as compared with their proliferating counterparts. In contrast, over the range of ligand concentrations examined, the specific binding of [³H]DPB to both proliferative and differentiative cells was similar. Possible interpretations of the heterogeneity of [³H]PDBU binding to differentiated keratinocytes are presented.

MATERIALS AND METHODS

Materials. [³H]PDBU was obtained from New England Nuclear (Boston, MA; 10.8–13.4 Ci/mmol); [³H]DPB (9.8 Ci/mmol) and DPB were prepared from 12-deoxyphorbol 13-isobutyrate 20-acetate as previously described (22). 12-Deoxyphorbol 13-isobutyrate 20-acetate and PDBU were obtained from LC Services Corp. (Waltham, MA) and Sigma Chemical Co. (St. Louis, MO), respectively.

Cell Culture. Epidermal cells were isolated from newborn BALB/c mice by the trypsin flotation technique (6) and were plated in 12-well Costar (Cambridge, MA) tissue culture dishes at an initial density of 1 × 10⁶ cells/well in Eagle’s minimal essential medium containing 8% Chelex-treated fetal calf serum and 0.05 μM Ca²⁺. The cells were washed in Ca²⁺-, Mg²⁺-free Dulbecco’s phosphate-buffered saline (0.2 g KCl, 0.2 g KH₂PO₄, 8 g NaCl, and 2.16 g NaHPO₄·2H₂O/liter) and were refed daily with 1 ml of low Ca²⁺ medium; they become confluent by 72 h. Within 12 h after reaching confluency in low Ca²⁺ medium, approximately one-half of the cells for each experiment were refed with high Ca²⁺ medium to induce terminal differentiation. Cell lines were studied in some experiments. These lines were isolated by virtue of their resistance to Ca²⁺-induced terminal differentiation and have been previously described (26).

Binding Assay. The binding of either [³H]PDBU or [³H]DPB to both differentiative and proliferative cells was measured by incubating various concentrations of either radioligand in cell culture medium containing serum as described above, in triplicate, with each cell type for 30 min at 37°C. In general, 10 concentrations ranging from 1 to 150 nM for [³H]-PDBU and 1 to 500 nM for [³H]DPB were chosen. Following incubation, 100 μl of the culture medium were removed from each well and radioactivity was quantitated to determine the concentration of the free ligand. Cells were then placed on ice, rinsed three times with ice-cold medium containing serum, and lysed in 0.5 ml of 0.1 n NaOH. The radioactivity associated with each well was determined by counting 0.45 ml of the lysate using a liquid scintillation counter. Nonspecific binding was determined exactly as described above by including 30 μM of either nonradioactive PDBU or DPB in a single well for each concentration of radioactive ligand. Specific binding represents the difference between the total binding and the nonspecific binding for each well. Protein concentrations were determined according to the method of Lowry et al. (27) from quadruplicate wells treated identically, except that cells were washed using ice-cold Dulbecco’s phosphate-buffered saline instead of the assay medium. Scatchard plots (28) were used in analyzing the binding data and the binding parameters (Kₐ and B_max) were determined by computer fitting using the Ligand Program (29). The F values were calculated (29) and compared with the tabulated F values (30) to determine if a two-site model was justified.

RESULTS

Binding of [³H]DPB to Keratinocytes. [³H]DPB bound in a specific and saturable fashion to intact keratinocytes cultured in the presence of low Ca²⁺ (Chart 1A). Specific binding ranged from 88% of the total at 5 nM [³H]DPB to 46% at 150 nM. Because of the nonspecific binding, higher concentrations of [³H]DPB could not be used. Analysis of the binding data by the method of Scatchard yielded a straight line, consistent with a single binding affinity (Chart 1B). The dissociation constant for [³H]DPB was 69 ± 6 nM; the B_max was 1.3 ± 0.2 pmol/mg of protein (mean ± SE of five experiments). At the K_d for [³H]DPB, the specific binding accounted for 57% of the total.

The specific binding of [³H]DPB to intact keratinocytes cultured in the presence of high Ca²⁺ for 3 days closely resembled that for the cells grown in the presence of low Ca²⁺ (Chart 1, C and D). The dissociation constant was 96 ± 26 nM; the B_max was 1.5 ± 0.4 pmol/mg of protein (mean ± SE of three experiments). These values for K_d and B_max were similar to those we obtained for phorbol ester binding site 2 in mouse skin particulate preparations (Table 1).

Binding of [³H]PDBU to Keratinocytes. In contrast to the results with [³H]DPB, [³H]PDBU bound to low Ca²⁺ keratinocytes in a heterogeneous fashion (Chart 2). The results illustrated represent the combined data from 12 experiments. Each data point is the mean value for triplicate wells containing the same concentration of [³H]PDBU within a single experiment. In these measurements specific binding ranged from 88% of the total at 1 nM [³H]PDBU to 60% of the total at 150 nM [³H]PDBU.

The data were analyzed with both one-site and two-site models using the Ligand Program, which gives a nonlinear least squares fit together with a measure of the quality of the fit. Whether a two-site model provided a better statistically significant fit than a one-site model was then determined by an F ratio test (29). In the case of low Ca²⁺ cells, the calculated F ratio (11.5) was greater than the critical F ratio (4.9) for P < 0.01 (Table 2), indicating that a two-site model fit the data appreciably better than did a one-site model. A three-site model was not
HETEROGENEITY OF PDBU BINDING IN KERATINOCYTES

Chart 1. Binding of [3H]DPB to intact keratinocytes. A, specific binding to low Ca\textsuperscript{2+} cells as a function of the concentration of free [3H]DPB. Data were fitted by a theoretical curve derived from the parameters obtained in B. B, Scatchard plots of [3H]DPB binding data shown in A. A one-site model was used in the analysis of the binding data. C, specific binding to high Ca\textsuperscript{2+} cells as a function of the concentration of free [3H]DPB. D, Scatchard plots of [3H]DPB binding to high Ca\textsuperscript{2+} cells. Data shown in C and D were fitted by the identical methods as described in A and B, respectively. The results shown were obtained from one representative experiment. Four other experiments performed with low Ca\textsuperscript{2+} cells and two with high Ca\textsuperscript{2+} cells gave similar values. The statistics of the binding parameters are summarized in Table 1.

justified by the data. The dissociation constants derived for the two-site model were 5.5 and 100 nM; \( B_{\text{max}} \) values were 0.9 and 1.7 pmol/mg of protein, respectively, for a total of 2.6 pmol/mg of protein (Table 3). As we had observed previously for mouse skin particulate preparations, not only was additional heterogeneity observed with the keratinocytes for [3H]PDBU binding compared to that for [3H]DPB, but the total number of binding sites detected was greater.

[3H]PDBU bound to the high Ca\textsuperscript{2+} keratinocytes, as to the low Ca\textsuperscript{2+} keratinocytes, in a heterogeneous fashion (Chart 2). The binding characteristics were similar in cells exposed to high Ca\textsuperscript{2+} medium for 1 or 3 days (results not shown). After 1 day, culture medium was switched to one containing high Ca\textsuperscript{2+} concentrations, total protein increased about 50%, and both cell number and DNA content increased about 40% (results not shown) as compared to the low Ca\textsuperscript{2+} cells. The results illustrated in Chart
HETEROGENEITY OF PDBU BINDING IN KERATINOCYTES

2 represent the combined data from eight experiments with high Ca\textsuperscript{2+} cells 1 day after switch of medium. The level of specific binding was greater in the high Ca\textsuperscript{2+} keratinocytes than in the low Ca\textsuperscript{2+} cells, particularly at the higher [\textsuperscript{3}H]PDBU concentrations, and the Scatchard plots revealed greater curvature (see Table 3 for binding parameters).

A general methodological problem in the analysis of multisite binding is that, because of the number of degrees of freedom, it is difficult to obtain unique values for the different binding parameters unless the binding sites differ quite substantially in their $K_d$/$B_{\text{max}}$ ratios. In order to better quantitate the differences in the binding of [\textsuperscript{3}H]PDBU to the low and high Ca\textsuperscript{2+} keratinocytes, we consequently adopted an approach which had proven of value on a one-site model, which bound with similar dissociation constants and number of receptors to a subclass of the sites in both high and low Ca\textsuperscript{2+} cells. These sites appear to correspond to the higher affinity [\textsuperscript{3}H]PDBU site (site 1); as illustrated in Chart 3, at a low free [\textsuperscript{3}H]PDBU concentration (15 nm) which would predominantly occupy site 1, DPB was able to inhibit [\textsuperscript{3}H]PDBU binding in low Ca\textsuperscript{2+} cells. The calculated dissociation constant for DPB, determined by inhibition, was 62 nm, similar to the value obtained from direct binding. We therefore fixed the value of the dissociation constant for site 1 of the high Ca\textsuperscript{2+} cells to 5.5 nm, the same as that for the corresponding sites in the low Ca\textsuperscript{2+} cells, and used the Ligand Program to derive the other three parameters (Table 3). This analysis suggested that the major difference in the high Ca\textsuperscript{2+} cells was an increase in the $B_{\text{max}}$ for site 2. A comparable result was obtained if the $B_{\text{max}}$ for site 1 of the high Ca\textsuperscript{2+} cells was fixed and the other parameters were determined (Table 3).

The increase in the lower affinity [\textsuperscript{3}H]PDBU binding site reflected a change upon growth of the cells in the high Ca\textsuperscript{2+} medium rather than a direct effect on the binding assay itself. No differences in binding parameters were found when [\textsuperscript{3}H]PDBU binding to the high Ca\textsuperscript{2+} cells was assayed in low Ca\textsuperscript{2+} medium (results not shown).

Morphological changes occurred rapidly following the shift of the keratinocytes to high Ca\textsuperscript{2+} medium and became visible by 2 h. Changes in binding activity were likewise apparent by 6 h (Chart 4). If, in the computer analysis, the $K_d$ values for [\textsuperscript{3}H]PDBU binding to the cells 6 h after Ca\textsuperscript{2+} shift were fixed at the same values as those either of the low Ca\textsuperscript{2+} cells or of cells incubated for 24 h in high Ca\textsuperscript{2+} medium, i.e., 5.5 and 100 nm, $B_{\text{max}}$ values for the corresponding sites were 1.1 ± 0.03 and 2.5 ± 0.1 pmol/mg of protein, respectively. Under the same constraints, $B_{\text{max}}$ values were 0.7 ± 0.04 and 1.8 ± 0.2 pmol/mg of protein, respectively, for low Ca\textsuperscript{2+} cells and 1.0 ± 0.1 and 5.6 ± 0.3 pmol/mg of protein, respectively, for high Ca\textsuperscript{2+} cells (Chart 4).

[\textsuperscript{3}H]PDBU Binding to Ca\textsuperscript{2+}-resistant Cell Lines. Several Ca\textsuperscript{2+}-resistant cell lines have been developed from carcinogen-treated primary keratinocyte cultures in this laboratory (26). In high Ca\textsuperscript{2+} medium, these cell lines maintain a high rate of proliferation. These cell lines are not tumorigenic and demonstrate characteristics consistent with their being initiated cells. [\textsuperscript{3}H]PDBU binding to three lines, line 308, line D, and line F, maintained in high Ca\textsuperscript{2+} medium was studied. Scatchard plots revealed only one class of binding sites in these cell lines cultured in high Ca\textsuperscript{2+} medium (Chart 5). A two-site model was not justified by the calculated $F$ ratios (Table 2). The $B_{\text{max}}$ and $K_d$ values were 1.0 ± 0.1 pmol/mg

\begin{table}[h]
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\begin{tabular}{|c|c|c|}
\hline
\textbf{Cell type} & \textbf{Assumptions} & \textbf{Site} & \textbf{Parameter} & \textbf{Value} & \textbf{Parameter} & \textbf{Value} \\
\hline
Low Ca\textsuperscript{2+} keratinocytes & Free fit & 1 & $K_d$ (nm) & 5.5 & $B_{\text{max}}$ (pmol/mg) & 0.9 \\
 & & 2 & & 1.7 & & 2.2 \\
 & & & & 100 & & 4.1 \\
High Ca\textsuperscript{2+} keratinocytes & Free fit & 1 & $K_d$ (nm) & 8.3 & $B_{\text{max}}$ (pmol/mg) & 1.8 \\
 & & 2 & & 800 & & 4.2 \\
 & & & & 100 & & 21,700 \\
 & & & & (5.5) & & 33 \\
 & & & & 100 & & 22,200 \\
 & & & & 4.8 & & 33 \\
Skin particulate & $B_{\text{max}}$ (sites 1 and 2) fixed & PBS-1 & $K_d$ (pmol/mg) & 0.7 & & 2.2 \\
 & & PBS-2 & & 10 & & 2.2 \\
 & & PBS-3 & & 53 & & 4.1 \\
\hline
\end{tabular}
\caption{Comparison of [\textsuperscript{3}H]PDBU binding parameters to intact low and high Ca\textsuperscript{2+} keratinocytes and skin particulate preparations}
\end{table}

\begin{thebibliography}{10}
\bibitem{ref1} For the comparison of the quality of the computer fitting in high Ca\textsuperscript{2+} cells.
\bibitem{ref2} Data from Ref. 22.
\bibitem{ref3} PBS-1, -2, -3, phorbol ester binding sites 1, 2, and 3.
\end{thebibliography}
HETEROGENEITY OF PDBU BINDING IN KERATINOCYTES

Chart 1. Competition of specific [3H]PDBU binding by DPB in low Ca²⁺ keratinocytes. The concentration of free [3H]PDBU was between 15 and 16 nM. Under this condition, 76 and 24% of total bound [3H]PDBU were associated with the high and low affinity PDBU binding sites, respectively. The solid line was a theoretical curve for competitive inhibition. The concentration of free [3H]PDBU at 50% inhibition was 15.3 nM. Two other experiments gave similar results.

Chart 2. Binding of [3H]PDBU to intact keratinocytes. A, specific binding to low Ca²⁺ cells (○) obtained from 12 experiments and high Ca²⁺ cells (●) from 8 experiments are compiled as a function of the concentration of free [3H]PDBU. Data were fitted by theoretical curves generated from the binding parameters obtained in B. B, Scatchard plots of [3H]PDBU binding to low (○) and high (●) Ca²⁺ keratinocytes. The experimental data were the same as shown in A. The concentrations of [3H]PDBU ranged from 1 to 150 nM. The Ligand Program was used for data fitting with a two-site model. Data from high Ca²⁺ cells was analyzed by fixing the dissociation constant of the higher affinity site at the same value as that of the corresponding site of the low Ca²⁺ cells. The results obtained from two other methods of analysis are summarized in Table 3.

Chart 3. Scatchard plots of [3H]PDBU binding to low Ca²⁺ keratinocytes. The concentration of free [3H]PDBU was between 15 and 16 nM. Under this condition, 76 and 24% of total bound [3H]PDBU were associated with the high and low affinity PDBU binding sites, respectively. The solid line was a theoretical curve for competitive inhibition. The concentration of free [3H]PDBU at 50% inhibition was 15.3 nM. Two other experiments gave similar results.

Chart 4. Kinetics of changes in binding characteristics of [3H]PDBU to low Ca²⁺ cells subjected to a switch to culture medium containing high Ca²⁺ concentration. The culture medium of low Ca²⁺ cells (○) was switched to a medium containing high Ca²⁺ for 6 h (●) or 1 day (▲). [3H]PDBU binding to these cells was performed as described in "Materials and Methods." The Scatchard plots shown were composite data from two independent experiments. For the purpose of comparison in changes of the number of PDBU binding sites between low Ca²⁺ cells and cells cultured for 6 h in high Ca²⁺ medium, the [3H]PDBU binding affinities to these cells were constrained (see text).

DISCUSSION

A variety of systems have proven of value for analyzing phorbol ester receptors and studying aspects of phorbol ester message transduction. To understand the mechanism of tumour promotion in skin, however, it is critical that the specific characteristics of the phorbol ester receptors be determined for the relevant cells. Initial studies by this laboratory had demonstrated that the binding of [3H]PDBU to particulate preparations from mouse skin yielded curved Scatchard plots consistent with receptor heterogeneity (22). Two uncertainties in the interpretation of the results were that the heterogeneity could either have been an artifact of cell disruption or else reflected the variety of cell types present in whole skin. The results reported here have indicated that there...
and the mouse skin particulate preparations suggests that the tinocytes. fit to a two-site model was not justified. A one-site model was used to fit the data. two experiments for line F yielded a slightly curved Scatchard plot, but a meaningful and the results were fitted by linear regressions. B, composite data obtained from the keratinocytes bears the same relationship to phorbol ester correspond to phorbol ester binding site 2 of the particulate really exists a continuum of different affinity states of the receptor validity of this assumption. An alternative possibility is that there direct biochemical analysis of the receptors will determine the is in fact heterogeneity in the binding properties of intact kera- tion of phorbol ester receptors in cells (12). In contrast to the commonly reported down modulation of phorbol ester receptors in cells (12), our finding that the total phorbol ester receptor increased upon Ca²⁺-induced differentiation in epidermal cells was unusual. The biochemical mechanisms for the increase in total phorbol ester binding are currently under investigation. Possible mechanisms include alterations in the rate of synthesis or processing. Among explanations for the shift in the proportionality of the two binding sites upon Ca²⁺-induced differentiation are: (a) high Ca²⁺ concentrations activate proteases and generate fragments of the receptor having lower affinity for the phorbol esters; (b) differentiation induces changes in the phospholipid compositions or association with membranes and, hence, alters the binding affinity (31); and (c) Ca²⁺ stimulates endogenous phospholipase C activity and generates diacetyl glycerols which compete for phorbol ester binding (32).

The heterogeneity in binding correlates with the responsiveness of the keratinocytes to the induction of differentiation by the phorbol esters whereby a greater proportion of cells are induced to differentiate when exposed in high Ca²⁺ medium (5, 8). Previous studies have also shown that differing maturation states in low Ca²⁺ cells dictate biological responses to phorbol esters. The proportions of subpopulations responding to PMA in proliferative and differentiative keratinocytes agree qualitatively with the proportion of lower affinity phorbol ester binding sites in these keratinocytes under both culture conditions. Likewise, for the Ca²⁺-resistant, initiated cell lines which respond slightly, if at all, to the differentiative signal of PMA (33), we found only a single class of binding sites with binding affinities similar to the higher affinity sites in the normal keratinocytes. The relation between binding sites and response is only correlative, however, and we do not have direct evidence as yet assigning the two classes of phorbol ester binding sites to the two differentially responding subpopulations or as regulating the two different response patterns.

Comparison of our binding data with that of other published studies indicates partial but not complete agreement. Solanki and Siaga (34, 35) and Chida and Kuroki (36) examined [³H]-PDBU binding to primary epidermal cells from SENCAR mice. The latter authors and Greenebaum et al. (24) measured [³H]-

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Chart 5. Scatchard analyses of [³H]PDBU binding to initiated cells. A, data shown for line D (○) and line 308 (●) were composite data from two experiments and the results were fitted by linear regressions. B, composite data obtained from two experiments for line F yielded a slightly curved Scatchard plot, but a meaningful fit to a two-site model was not justified. A one-site model was used to fit the data.

For the analysis of the keratinocyte binding data, we have assumed two distinct classes of phorbol ester binding sites. Only direct biochemical analysis of the receptors will determine the validity of this assumption. An alternative possibility is that there really exists a continuum of different affinity states of the receptor for which the two-site analysis yields averaged values. We have reported elsewhere that association of protein kinase C with different phospholipids generates different phorbol ester binding affinities and different structure-activity relations (31). Association of protein kinase C with membranes in cells so as to give a continuum in the compositions of the associated phospholipids could lead to a continuum of binding affinities. Although the fitted values of the binding parameters depended somewhat on the specific assumptions used in the data analysis, independent of the method of analysis we observed in the high Ca²⁺ cells an overall increase in the total phorbol ester binding sites. In addition, provided that the DPB binding in fact corresponded to the higher affinity sites as we have argued in "Results," then there is an increased proportionality in the lower affinity binding sites. This result suggests that the state of epidermal differentiation can modulate the amount of the lower affinity binding sites for phorbol esters. The modulation of the level of phorbol ester receptor has been reported both in animals and in cells. It has been shown that the level of phorbol ester binding increased as a function of development in several animal systems (12). In contrast to the commonly reported down modulation of phorbol ester receptors in cells (12), our finding that the total phorbol ester receptor increased upon Ca²⁺-induced differentiation in epidermal cells was unusual. The biochemical mechanisms for the increase in total phorbol ester binding are currently under investigation. Possible mechanisms include alterations in the rate of synthesis or processing. Among explanations for the shift in the proportionality of the two binding sites upon Ca²⁺-induced differentiation are: (a) high Ca²⁺ concentrations activate proteases and generate fragments of the receptor having lower affinity for the phorbol esters; (b) differentiation induces changes in the phospholipid compositions or association with membranes and, hence, alters the binding affinity (31); and (c) Ca²⁺ stimulates endogenous phospholipase C activity and generates diacylglycerols which compete for phorbol ester binding (32).

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PDBU binding to primary human keratinocytes. Several groups, including ourselves, have analyzed [3H]PDBU binding to epidermally derived cell lines. Our Ca2+-resistant cell lines, like the other epidermally derived lines, yielded only one class of binding sites for the phorbol esters with Kd and Bmax values similar to those of the other systems. Our results with primary cells agreed qualitatively with those of Greenbaum et al., who also observed two classes of binding sites, but not with the other two studies which reported only a single class of sites. We do not know the reason for the discrepancy. A major difference is that the cells were maintained in high Ca2+ medium (>0.1 mm) in the other studies, whereas our basal cells differentiate under similar conditions. The monophasic binding for our Ca2+-resistant cell lines argues strongly against the biphasic curves of the primary cells being an artifact of our assay procedure. The Kd values derived from our two-site model bracket the values of 11 and 32 nM, obtained in the other two studies on mouse keratinocytes. Our Bmax values are in the same range as those reported, 1.2 x 105 - 1.2 x 106 sites/cell.

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5546
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