ABSTRACT

Our studies indicate that tritiated 12-O-tetradecanoylphorbol-13-acetate ([3H]TPA) produced by the reduction of the C-20 aldehyde with sodium [3H]borohydride is recognized by the same cellular site as is unlabeled 12-O-tetradecanoylphorbol-13-acetate (TPA). None of the concentrations of TPA used in these studies had an effect on the cell number and viability of human peripheral blood lymphocytes (HPBL) when incubated up to 1 hr at temperatures of 37 and 4°C as compared to untreated controls. [3H]TPA was not significantly metabolized by these cells after 1 hr at 37°C. Examination of the binding of [3H]TPA with simultaneous examination of uptake of tritiated thymidine ([3H]dThd) in parallel cultures demonstrated a close correlation between the apparent binding constant \((0.94 \times 10^8 \text{ M}^{-1})\) and the activation constant for TPA stimulation of [3H]-dThd incorporation \((0.95 \times 10^{-8} \text{ M})\). Binding of [3H]TPA was examined in two experimental conditions in which TPA-induced mitogenesis was inhibited: (a) preincubation of HPBL at 37°C for 24 hr causes a decrease of [3H]dThd uptake of 50% and an apparent loss of binding sites for [3H]TPA; and (b) glucocorticoid inhibition of [3H]dThd uptake in HPBL by 50%, however, did not reduce [3H]TPA binding. Our data suggest that cellular receptors either at the membrane or in the cytoplasm exist for TPA in HPBL. Alterations in binding of TPA to these receptors may account for the decrease in mitogenic response in preincubation experiments.

INTRODUCTION

The phenomenon of tumor promotion by croton oil or its phorbol ester components in mouse skin (3, 4, 10), the effects of TPA \(^3\) in many other cell systems (5–8, 28, 29), and emergence of promoters in model systems other than mouse skin \((11, 20)\) have caused an explosion of interest in the action compounds which participate as active agents in this experimental model. It is clear from work \(in vitro\) that the most active and best studied agent in croton oil, TPA, is acting in many senses as a hormone \((5, 29)\); i.e., it has a multiplicity of biological and biochemical effects on a multiplicity of cell types and has a low concentration of the agent necessary for the effect. Recently, the demonstration of cellular receptors for a variety of hormones (both peptide and nonpeptide) on cell surfaces and within cells has opened new areas for understanding of these agents and for considering their mode of action.

Since TPA acts much as a hormone, it is possible that it also might have a receptor of some type. Preliminary studies of binding of [3H]TPA to whole cells correlated well with the amount of [3H]dThd incorporated \((5)\). This correlation and the presence of [3H]TPA-labeled cells as indicated by autoradiography encouraged us to examine TPA binding to intact HPBL in a systematic manner, hoping to find correlations or lack of them with binding and biological effects of the compound. In this report, we describe the methods utilized to assay binding to HPBL and data on the correlation of [3H]TPA binding to whole cells and lymphocyte mitogenesis.

MATERIALS AND METHODS

Media and Reagents. Growth medium was minimal essential medium with Earle’s salts, 9% AB serum with 1% glutamine, and 1% penicillin-streptomycin. Media and tissue culture reagents were obtained from Grand Island Biological Co. (Grand Island, N. Y.). Serum was obtained fresh from normal AB donors, defibrinated, and treated at 56°C for 1 hr. Subsequently, the serum was filtered through 0.2-μm Nalgene filters, aliquoted, and used after being frozen and thawed once.

Unlabeled TPA and [3H]TPA were obtained from Dr. P. Borchert (University of Minnesota, Minneapolis, Minn.). [3H]TPA was prepared by reduction of the C-20 aldehyde of unlabeled TPA utilizing sodium [3H]borohydride. Purification of the radiochemical product by high-pressure liquid chromatography resulted in a single peak of radioactive TPA with a specific activity of 8 Ci/mmol. In separate experiments, unlabeled sodium borohydride reduction of the C-20 aldehyde TPA was followed by purification of the product by preparative thin-layer chromatography and then compared to a TPA standard and produced an identical dose response in [3H]dThd uptake in HPBL. In addition, labeled and unlabeled TPA produce the same amount of aggregation of lymphocytes at the same concentrations, another correlate of biological activity. By these tests, the [3H]-TPA was considered to be identical to the unlabeled material. [3H]TPA was used within 1 month or rechromatographed.

[3H]dThd was obtained from Schwarz/Mann (Orangeburg, N. Y.). All other chemicals were reagent grade. All cultures were performed in Falcon microtiter plates (Falcon Plastics, Oxnard, Calif.).

Collection and Preparation of Lymphocytes. Lymphocytes were obtained from the peripheral blood of normal adults. Mononuclear cells, approximately 90% small lymphocytes and 10% macrophages, were obtained by centrifugation over Ficoll-Hypaque, as described by Thorsby and Bratlie \((27)\). The cells were then resuspended and counted (Coulter Counter, Model ZBI, Coulter Electronics, Franklin Park, Ill.) in growth medium.

Binding Experiments. Each concentration of [3H]TPA in growth media, as described above, with a final concentration

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; [3H]TPA, tritiated 12-O-tetradecanoylphorbol-13-acetate; [3H]dThd, tritiated thymidine; HPBL, human peripheral blood lymphocytes.

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of 0.1% dimethyl sulfoxide was added to each of 4 Beckman Biowells (Beckman Instruments, Inc., Fullerton, Calif.). To each vial was added either 1 ml of lymphocytes at $4 \times 10^6$ cells/ml or 1 ml of media (both warmed to $37^\circ$). The cells were incubated at $37^\circ$ for 30 min and then centrifuged at 1200 rpm for 5 min ($37^\circ$). An aliquot was removed from the supernatant of each vial for counting, and the disposal tip was included for counting. The remaining supernatant was decanted from the vials, and the vial blooted on a gauze sponge to remove the liquid remaining on the lip of the vials. Dioxane-based scintillation fluid was added to each vial and counted for 1 min on the $^3$H channel of the Beckman Model LS-250 scintillation counter.

Four replications of each concentration of $[^3H]$TPA were also added to vials, and these served to quaniticate the amount of $[^3H]$TPA added to each other vial. In separate tubes, $[^1C]$inulin (New England Nuclear, Boston, Mass.) was used to estimate media entrained with cell pellets. Media containing $[^1C]$inulin were placed in tubes with and without cells, and the residual volume of media was estimated in quadruplicate. This procedure demonstrated that there was no residual in tubes without cells and that 5 to 10 μl of fluid entrained with cells.

The amount of $[^3H]$TPA bound to cells was determined by subtracting the amount bound to tubes without cells and the average of counts in entrained media from the amount bound to vials containing cells. The result was considered to be the amount bound to the cells. The amount of radioactivity in an aliquot of supernatant was multiplied by the total volume of the supernatant to give the total cpm in the supernatant. The total count of each tube was compared to the amount of cpd added, and the recovery was calculated. The results were considered valid only if the recoveries were within 10% of the amount added.

Scatchard plots are derived from the binding data according to the method of Müller and Crothers (17), where $r$ is molecules $[^3H]$TPA bound per cell, and $m$ is the concentration of free $[^3H]$TPA. The apparent number of binding sites per cell ($B_{app}$), and the apparent binding constant ($K_{app}$), are determined from a plot of $r/m$ versus $r$ with slope equal to $K_{app}$ and intercept $r$ equal to $B_{app}$ at $r/m$ equal to 0.

$[^3H]$dThd Uptake. TPA in media with a final culture concentration of 0.1% dimethyl sulfoxide was added to 5 wells of a round-bottomed microtiter plate. Then 0.2 ml lymphocytes at $0.5 \times 10^6$ cells/ml was added to the wells. Cells were incubated for varying periods (42 and 66 hr) at $37^\circ$ in 5% CO$_2$ and pulsed with $[^3H]$dThd (1 μCi/well) and incubated at $37^\circ$ for another 6 hr. The plates were frozen at $-20^\circ$ to terminate the assay. Cells were harvested with a Titertek cell harvester (Skatron A. S., Liebyren, Norway) onto filter paper, which were placed into biowells and counted on the $^3$H channel of a Beckman Model LS-250 scintillation counter.

Extraction of $[^3H]$TPA. Lymphocytes and disposable glass 16- x 100-mm test tubes containing media were prepared as for the binding assays and incubated with $[^3H]$TPA (10 ng/ml). The cells were incubated at $37^\circ$ for 1 hr in a CO$_2$ incubator, the cells were pelleted, and the supernatant media were withdrawn. This concentration of $[^3H]$TPA usually gave approximately 10,000 to 16,000 cpm in the cell pellet. Cells and medium supernatants were extracted with chloroform:methanol:water (2:2:1.6) according to the methods described by O’Brien and Diamond (18) and were analyzed by thin-layer chromatography using a methylene chloride:acetone (3:1) solvent system, as described by the same authors.

RESULTS

Before TPA binding could be studied, a number of experiments were necessary to ensure that the intact cell system was free from some obvious potential problems. The first experiments were done to determine whether or not the labeled and unlabeled material were recognized by the same binding sites on the HPBL. Additional experiments were performed to determine whether or not cells were lysed or killed under the conditions utilized in binding experiments. Finally, we determined whether HPBL were capable of metabolizing TPA under conditions utilized for binding.

Identity of $[^3H]$TPA with unlabeled TPA. Cells were incubated with $[^3H]$TPA (10 ng/ml) at $37^\circ$ for 30 min or at $4^\circ$ for 18 hr with varying amounts of unlabeled TPA (both present at the time of the addition of cells). The results seen in Charts 1 and 2 indicate that $[^3H]$TPA is diluted as one would expect if it were recognized in the same way by the cells as unlabeled TPA. For instance, about 50% of the label is diluted by 10 ng of unlabeled TPA, while about 10% remains at a cold:labeled TPA ratio of 10:1.

Cell Number and Viability. Cell numbers and viabilities were...
examined at concentrations of unlabeled TPA of 1 to 1000 ng/ml after incubations of 1 hr at 37° and overnight at 4°. These experiments uniformly indicated that, under all incubation conditions as outlined above, cell numbers remained the same. Viabilities of control cells and cells exposed to TPA were assayed by uptake of 0.4% trypan blue and ranged from 95 to 100% and usually were above 98%. Additionally, cells exposed to TPA overnight at 4° responded by taking up [3H]dThd identically to TPA-treated, freshly isolated cells. By these criteria of morphology and function, TPA was not producing a toxic or cytobiotic reaction which might easily account for altered binding of the molecule (results not shown).

Metabolism of TPA. Cells and media exposed to TPA for 1 hr at 37° were extracted with chloroform:methanol, and the extracts of both cells and media supernatants of these cultures were chromatographed on thin-layer plates. When lymphocytes were incubated at 37° for 1 hr, no breakdown to products soluble in organic solvent of TPA was detected. There was also no loss of TPA to a water-soluble form as assayed by counting the extracted pellets or media. (The results for supernatant TPA were identical and are not shown.) Thus, HPBL or supernatant media did not contain detectable metabolites of TPA, and the extracted radioactivity migrated with authentic TPA as demonstrated in Chart 3.

Binding Time Course at 37°. The binding of [3H]TPA (10 ng/ml) to whole lymphocytes over a time period of 1 hr can be seen in Chart 4. For the first 10 min, the binding shows a linear increase; by 30 and 60 min, no further increase was seen. We chose to do the remainder of our binding experiments at 37° for a 30-min period for 2 reasons: (a) TPA was not toxic to nor was it metabolized by the cells for up to 1 hr; and (b) at 30 min, the binding reached a plateau.

Correlation of Binding and [3H]dThd Uptake. As illustrated in Chart 5a, concentrations of TPA which produced linear increases in binding with increasing concentrations of TPA at 37° for 30 min also produced a linear increase in [3H]dThd uptake at 72 hr. The response is typical of several different donors in many experiments. The maximum response of [3H]dThd uptake usually is found between 10 and 40 ng TPA per ml. The results of the binding data shown are also typical in that saturation is never achieved. The [3H]dThd uptake curve indicates that at about 6 ng TPA per ml produce a half-maximal [3H]dThd uptake or a Kapp of 0.94 x 10^{-8} M for [3H]dThd uptake. A Scatchard plot of the above data can be seen in Chart 5b. This plot of the data indicates that at least 2 classes of binding sites probably exist, a high-affinity class with a Kapp of 0.94 x 10^{-8} M and a Bapp of 4.7 x 10^{5} sites/cell. In several experiments with different human donors, these low-
affinity sites varied from $1.3 \times 10^7$ to $1.8 \times 10^7 \text{ M}^{-1}$ for the $K_{\text{app}}$ to $1.3 \times 2.5 \times 10^6$ for the $B_{\text{app}}$.

Since the probability exists that any experiment at $37^\circ$ may involve processing of label or receptors in a number of ways, binding of $[^{3}H]TPA$ at $4^\circ$ for 18 hr was done. At this temperature, pinocytosis should be eliminated and other processes should be minimized. The results shown in Chart 6 illustrate that the binding response is similar in both instances; $K_{\text{app}}$, $1.6 \times 10^6 \text{ M}^{-1}$; $B_{\text{app}}$, $7.5 \times 10^8$. The $[^{3}H]dThd$ uptake is consistent with that at $37^\circ$ in that a similar dose-response relationship for $[^{3}H]dThd$ is obtained when cells are maintained overnight in the concentration of TPA, then warmed to $37^\circ$ and incubated for 48 hr. The magnitude of the mitogenic response to TPA is the same whether cells are incubated immediately or after 24 hr at $4^\circ$ (data not shown).

**Binding and Inhibition of $[^{3}H]dThd$ Uptake.** Preincubation for 24 hr and the presence of glucocorticoids have been shown to cause a reduction in response to TPA (5). The results of binding on freshly isolated cells and cells incubated for 24 hr before binding can be seen in Table 1. In this table, it is apparent that preincubation of cells at $37^\circ$ for 24 hr affects the number of binding sites to a greater extent than does the binding constant.

Binding of $[^{3}H]TPA$ by cells is not altered when cells are incubated with concentrations of the glucocorticoid analog fluocinolone acetonide which inhibit the mitogenic response to TPA by approximately 50%. This glucocorticoid analog does not have an early effect on $[^{3}H]TPA$ binding despite the ability to inhibit TPA-stimulated thymidine uptake (Table 2).

**Retinoids and Human Lymphocytes.** Retinoic acid at $5 \times 10^{-7} \text{ M}$ inhibits $[^{3}H]dThd$ uptake in TPA- and PHA-treated bovine lymphocytes by 50% (14). However, retinoic acid did not inhibit TPA-induced $[^{3}H]dThd$ uptake from 72 to 144 hr in HPBL at concentrations of $10^{-10}$ to $10^{-9} \text{ M}$. Retinyl acetate, which was an inhibitor only above $10^{-9} \text{ M}$, and $\beta$-carotene, which was inactive in Kensler's and Mueller's (14) experiments, are also completely inactive in inducing changes in TPA-induced $[^{3}H]dThd$ uptake at retinoid concentrations of $10^{-6} \text{ M}$ (data not shown).

**DISCUSSION**

Isotope dilution data at 37 and $4^\circ$ indicate that the $[^{3}H]TPA$ is bound to cellular sites that recognize it as being similar or identical to unlabeled TPA. TPA does not destroy or lyse cells in 1 hr at $37^\circ$ which might produce a change in available binding sites. TPA metabolism by these human cells was not detected for a 1 hr period.

The results indicate that there is a class of binding sites which have a relatively high affinity for TPA. The $K_{\text{app}}$ is about $0.94 \times 10^5 \text{ M}^{-1}$. This resembles the $K_a$ for TPA stimulation of $[^{3}H]dThd$ uptake, which is $0.95 \times 10^{-6} \text{ M}$. The $K_{\text{app}}$ for TPA is similar to some of those found for peptide hormones (13); in this way, TPA also resembles known hormones.

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**Table 1**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$K_{\text{app}}$ (x $10^5$ sites/cell)</th>
<th>$B_{\text{app}}$ (x $10^8$ sites/cell)</th>
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<tr>
<td>Experiment 1</td>
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<td>$1.46 \times 10^9$</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>$5.6 \times 10^5$</td>
<td>$1.5 \times 10^9$</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>$8.7 \times 10^5$</td>
<td>$5.0 \times 10^9$</td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>PMA added (ng)</th>
<th>Fluocinolone acetonide</th>
<th>cpm bound to cells</th>
<th>% of inhibition of $[^{3}H]dThd$ uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0</td>
<td>2.125</td>
<td>55</td>
</tr>
<tr>
<td>1.0</td>
<td>+</td>
<td>2.038</td>
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</tr>
<tr>
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<td>0</td>
<td>3.880</td>
<td>55</td>
</tr>
<tr>
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<td>+</td>
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</tr>
<tr>
<td>5.0</td>
<td>0</td>
<td>5.821</td>
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</tr>
<tr>
<td>5.0</td>
<td>+</td>
<td>8.221</td>
<td>64</td>
</tr>
<tr>
<td>7.5</td>
<td>0</td>
<td>8.163</td>
<td>66</td>
</tr>
<tr>
<td>7.5</td>
<td>+</td>
<td>10.500</td>
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</tr>
<tr>
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<td>0</td>
<td>8.913</td>
<td>60</td>
</tr>
<tr>
<td>10.0</td>
<td>+</td>
<td>11.669</td>
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</tr>
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</table>

---

Chart 6. $[^{3}H]Thd$ uptake and binding of $[^{3}H]TPA$ at $4^\circ$. a, $[^{3}H]dThd$ uptake; b, $[^{3}H]TPA$ binding. Binding of $[^{3}H]TPA$ over a concentration range from 1 to $1000 \text{ ng/ml}$ were carried out at $4^\circ$ for 18 hr. The data were plotted as in Chart 5, and the appearance of the curves was similar. The $[^{3}H]dThd$ uptakes were taken 48 hr after the cells were returned to $37^\circ$. Bars, S.E. b, The binding data at $37^\circ$ for 30 min as recorded in a, plotted as indicated in "Materials and Methods" to yield $K_{\text{app}}$ and $B_{\text{app}}$. |
Since our experiments use intact cells at a physiological temperature, many processes are probably occurring such as internalization of membrane, accumulation of label by pinocytosis of external membrane, loss of receptors by "shedding" into the medium, or other modulations of receptors. TPA may promote membrane internalization since examination of TPA-treated lymphoblasts (6) has shown that they have more vacuoles than cells stimulated with phytotemagglutinin. However, incubation overnight at 4°C which should reduce these processes, gives Kd and numbers of sites which are not unlike those seen when cells are incubated at 37°C for 30 min. Recently, it has been demonstrated that TPA can alter the epidermal growth factor binding to cells at 37°C but not at 4°C (22); it is unlikely that these ligands compete for the same site.

The preincubation of lymphocytes for 24 hr consistently produces a decrease in binding sites and usually with little or no change in the affinity of the sites for TPA. This result suggests that, because these sites are no longer available on the cells, the response to TPA is diminished. The TPA-binding sites may be shed from cells, as has been demonstrated for lectin receptors (12), or become unavailable by changes in the cell surface.

Another class of agents which causes a decreased response to TPA is glucocorticoids (1). Steroid hormones are thought to interact with a cytoplasmic receptor, migrate to the nucleus, and affect transcription of specific mRNA's (19). Such an action may or may not affect TPA binding, depending on the time of observation. One might also expect an immediate effect on TPA binding if the steroid altered the membrane or cytoplasmic TPA receptors directly. No evidence of the latter possibilities was observed. The action of the steroid to suppress mitogenesis induced by TPA in these cells is beyond this early interaction of TPA with the cell. However, subsequent interaction between TPA and the cell may be necessary for the mitogenic response, as has been demonstrated for other mitogens such as concanavalin A (21, 26), and steroids could interfere with such a later interaction of lymphocytes and TPA. Slaga et al. (25) have demonstrated previously that the same glucocorticoid analog used in our experiments had no effect on the binding of [3H]TPA to mouse skin although the steroid inhibited tumor promotion.

The lack of effect of retinoids on TPA-induced mitogenesis is at variance with data on "comitogenesis" of bovine lymph node lymphocytes (14). This difference may be explained by the facts that the data are derived from 2 differing populations of cells (lymph node as opposed to peripheral blood) and from different species (bovine and human). Further differences exist, for instance, the assay conditions are different in that Kensler and Mueller (14) precultured their cells overnight and show little effect of TPA alone as a mitogen. Additionally, 2 recent reports suggest that retinoids may have to be metabolized to inhibit TPA effects in the bovine lymphocyte (15, 30). Since human cells do not seem to metabolize TPA, the differences may be due to a differential ability to metabolize retinoid acid. In addition to the above, if TPA receptors are involved, then one must consider still another explanation. For example, cyclic guanosine 3',5'-phosphate has been found to be increased or not increased in differing cell types or tissues whose function is altered by TPA (2, 6, 16). We have suggested (28) that differing types of receptors for TPA may exist that are analogous to 2 or more receptor types that are found with other hormones such as α- and β-adrenergic receptors. Biochemical differences in cell responses in different cells or tissues may be accounted for by receptor differences.

We suggest that our experiments are consistent with the existence of a class of high-affinity cellular receptors for TPA on HPBL. Such receptors seem likely to be linked to the biological effects by beginning the biochemical events leading to mitogenesis. Although both cell membrane, or cytosol, or nuclear receptors are possible, the involvement of the cell membrane in early events has been pointed out (23, 24) and makes a cell membrane receptor seem somewhat more attractive. While membrane and cytosol receptors have been thought to act in a different manner, transport of insulin which has surface receptors into the cell and to the nucleus may indicate that another pathway of action may exist (9). The existence of different types of receptors also may partially explain the fact that differing cell types do not respond in a biochemically similar manner to a TPA stimulus. In utilizing such diverse tissues, we may produce information which has everything to do with the actions of TPA but little to do with tumor promotion in mouse skin, because different receptors are involved which may result in differing biochemical reactions.

REFERENCES


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Binding of $[^3\text{H}]12\text{-O}-\text{Tetradecanoylphorbol-13-acetate}$ to Intact Human Peripheral Blood Lymphocytes

Richard D. Estensen, Debra K. DeHoogh and Clair F. Cole