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Mechanism of tumor promoter inhibition of cellular binding of epidermal growth factor

(phorbol esters/receptors/carcinogenesis/cell surface)

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ABSTRACT In previous studies we demonstrated that the tumor-promoting agent 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) and related macrocyclic diterpenes are potent inhibitors of the binding of epidermal growth factor (EGF) to its cell surface receptors in HeLa cells. The present study explores the specificity and mechanism of this effect. We have found that the same effect is observed in various cell types derived from mice, rats, or humans. In HeLa cells TPA inhibits the initial binding of EGF and also accelerates the loss of previously bound EGF from cells. The released EGF is recovered largely intact in the medium, indicating that TPA does not induce increased proteolysis or increased cellular internalization and degradation of EGF. The TPA effect on EGF receptors is mediated by a highly temperature-dependent process because TPA inhibition of EGF binding, and TPA-induced release of prebound EGF, are much greater at 37°C or 22°C than at 4°C. A curious feature is that when cells are grown in TPA for one or more days they escape or become refractory to TPA inhibition of EGF binding. Taken together, these results suggest that TPA inhibits EGF binding not by binding directly to the "active site" of the EGF receptor but by indirectly altering the conformation or inducing the clustering of EGF receptors. These and other membrane effects of this tumor promoter suggest that it is a valuable probe for elucidating complex aspects of membrane structure and function.

We have previously reported (1) that the potent tumor promoter 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), in the range of 0.1–10 nM, caused a rapid and marked inhibition of cellular binding of epidermal growth factor (EGF) to cell surface receptors of HeLa cells. There was a correlation between the ability of a series of related macrocyclic diterpenes to exhibit this effect and other biologic effects of these compounds, including tumor promotion on mouse skin. We were led to this observation by the hypothesis that this class of compounds might exert their tumor-promoting effect by mimicking the action of endogenous factors controlling growth and differentiation (2, 3).

EGF was of particular interest to this hypothesis because it shares a number of biologic effects with TPA. These include: stimulation of proliferation of both epidermal and mesodermal cells, increase in deoxyglucose transport, and induction of ornithine decarboxylase and prostaglandin synthesis (4–9). EGF has been reported to promote tumor induction on mouse skin (10) and we have found that, like TPA, EGF is a potent inducer of plasminogen activator synthesis in cell cultures (11). Mouse EGF has been extensively characterized. It is a 6100-dalton polypeptide containing 53 amino acids of known sequence (12–15).

Like several other polypeptide hormones, EGF binds to specific cell membrane receptors (6, 7, 15). The physiological role of EGF is not fully understood and it is not clear whether the action of EGF, or of other polypeptide hormones, results entirely from their binding to cell surface receptors. Nor is it clear whether binding of a polypeptide to its receptor triggers cellular effects via some type of "transmembrane signal," stimulation of the production of a second messenger (e.g., cyclic AMP, in response to glucagon), or more complex membrane perturbations. Recent studies indicate that the hormone–receptor complexes for insulin (16, 17) and for EGF (17–19) are actually taken up by cells, presumably by endocytosis, and may appear in lysosomal vesicles. The latter results suggest that the polypeptide hormone or its derivative(s) may exert its action on intracellular sites, either cytoplasmic or nuclear (19). On the other hand, studies with very low concentrations of EGF and very short exposure periods, in conjunction with anti-EGF antiserum, have suggested the existence of high-affinity cell surface receptors that are not internalized and yet produce the full biological effects (20). Todaro and DeLarco (21) have found that cells transformed by a murine sarcoma virus elaborate a polypeptide, "sarcoma growth factor," that appears to bind specifically to EGF receptors. Thus, changes in the EGF effector systems might play an important role not only in the process of tumor promotion but also in the maintenance of the transformed state.

The ability of TPA to affect the function of EGF receptors could provide a valuable probe for clarifying some of the above questions. In addition, further studies of this effect might shed light on the mechanism of action of tumor promoters at the cellular level. The present studies were undertaken to provide information on whether TPA binds directly to the EGF receptor and perhaps usurps its function or whether its effects on EGF binding by cells reflect a more generalized change in membrane structure induced by TPA, with secondary effects on the function of the EGF receptor.

MATERIALS AND METHODS

EGF and ^{125}I-labeled EGF (^{125}I-EGF) (see figure and table legends for specific activity) were purchased from Collaborative Research (Waltham, MA). TPA and other phorbol esters were from Consolidated Midland Chemical (Brewster, NY). Mezerein was kindly supplied by M. Kupchan. Cell culture materials were from GIBCO. All other chemical reagents were from Sigma. The phorbol esters and mezerein were dissolved in dimethyl sulfoxide (Me_2SO) at 1 mg/ml and stored at −20°C.

Cell Culture and EGF Binding Assays. HeLa cells were maintained in growth medium consisting of Eagle's minimum essential medium supplemented with 10% calf serum, 10 units

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Abbreviations: TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; EGF, epidermal growth factor; Me_2SO, dimethyl sulfoxide.

of penicillin per ml, and 10 units of streptomycin per ml, and the cells were shown to be mycoplasma free. Cultures were grown at 37°C in a humidified 95% air/5% CO_2 atmosphere. The other cell types listed in Table 1 were grown under similar conditions as described (2, 3). The binding assays were performed with subconfluent cell culture monolayers in 5-cm tissue culture petri dishes (Nunc, Roskilde, Denmark) as described (1). Briefly, the cells ($\approx 6 \times 10^5$ cells per dish) were seeded in growth medium 72 hr before the binding assay. At the time of the assay the cell monolayer was washed twice with 2.5-ml aliquots of minimal essential medium lacking serum and then incubated with mouse ^{125}I-EGF (for concentration and specific activity, see each experiment) in 1.5 ml of binding buffer per dish, with or without the indicated concentrations of TPA. Control samples were exposed only to the solvent (Me_2SO). Binding buffer consisted of Dulbecco's modified Eagle's medium containing 1 mg of bovine serum albumin per ml and 50 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid, pH 6.8. After incubation for 50 min at 36°C (unless specified otherwise), the above medium was removed and the cell monolayer was washed three times with 5-ml aliquots of cold Dulbecco's modified Eagle's medium. The cells were then solubilized with 1% Triton X-100 and 1% sodium dodecyl sulfate and assayed for radioactivity in a liquid scintillation counter programmed for counting ^{125}I. Nonspecific binding was measured by the amount of cell-bound radioactivity when binding was done in the presence of a vast excess of unlabeled EGF (1000 ng per plate). It represented about 50–100 cpm above the instrument background counts and the value was subtracted from all binding assay data. Cell counts on replicate cultures were done by trypsinization and counting with a Coulter Counter model Z_f. All assays were done in duplicate and the values given are the means. Variations between duplicates were less than 10%.

To assess the amount of radioactive material present in the medium, the collected medium was centrifuged at 10,000 × *g* for 10 min to remove any suspended particulates, lyophilized, and resuspended in 1 ml of 50 mM ammonium acetate buffer, pH 5.6. The Bio-Gel column (0.5 × 50 cm) was pre-equilibrated with the same buffer and loaded with the sample. Fractions of 1 ml each were collected and assayed for radioactivity.

RESULTS

EGF receptors are found in many but not all types of cell cultures. Table 1 shows that in four very different cell lines (HeLa, 3T3, XP, and K22) and in secondary rat embryo cell cultures, all of which have appreciable levels of EGF receptors, exposure to TPA (33 ng/ml) resulted in a marked reduction (50–90%) in the cellular binding of ^{125}I-EGF. Thus, mouse, rat, and human cells and both fibroblastic (3T3 and XP) and epithelial cells (HeLa and K22) are affected. The other four cell cultures examined (CHO, B16, HTC, and CEF) had negligible levels of EGF receptors in the absence of TPA and, therefore, no significant effect of TPA on ^{125}I-EGF binding could be detected.

Various kinetic aspects of this TPA effect were examined in HeLa cell cultures. In control cultures, after the addition of ^{125}I-EGF there was rapid binding which was linear for about 30 min, reached a plateau at about 50 min (Fig. 1*A*), and began to decline after 90 min (Fig. 1*B*). When TPA (30 ng/ml) was added simultaneously with the EGF (Fig. 1*A*) there was rapid inhibition of ^{125}I-EGF binding, which was apparent within 15 min and persisted for at least 150 min. The preincubation of cells with TPA 10 min prior to the addition of EGF gave results similar to those obtained when the two were added simultaneously.

Table 1. Effects of exposure of various cell cultures to TPA on binding of ^{125}I-EGF to cellular receptors

Cell cultures*	^{125}I-EGF bound, cpm/10^6 cells		% inhibition
	−TPA	+TPA	
HeLa	4714	1059	78
3T3 (mouse fibroblast)	1086	142	87
XP (human fibroblast)	3669	1698	54
K22 (rat liver)	636	115	82
2°RE (secondary rat embryo)	3232	1296	60
CHO (chinese hamster ovary)	25	22	—
B16 (mouse melanoma)	26	24	—
HTC (rat hepatoma)	20	19	—
CEF (chicken embryo fibroblast)	66	33	—

* The sources and growth of these cell types have been described (1–3). Binding assays were performed at 37°C for 50 min. Cells were grown to near confluence in 5-cm dishes and binding assays were then done in the absence or presence of TPA (33 ng/ml). The total cpm of ^{125}I-EGF added per plate was 58,580 (specific activity, 80 μCi/μg; 1 Ci = 3.7×10^{10} becquerels).

The effects of delayed addition of TPA are also shown in Fig. 1*A*. The addition of TPA either during the phase of linear binding of ^{125}I-EGF or during the plateau phase resulted in a rapid decline of ^{125}I-EGF binding. This effect was apparent within 5–10 min after addition of the TPA. In a separate experiment cells were incubated with ^{125}I-EGF for 50 min to

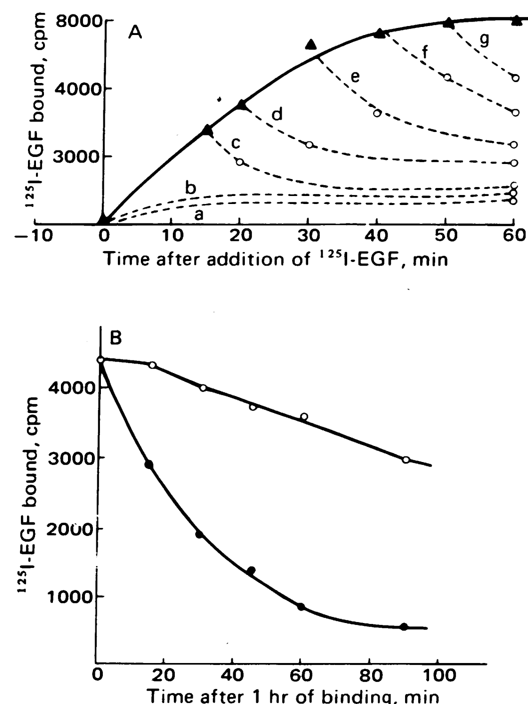

FIG. 1. Time course of binding of ^{125}I-EGF to HeLa cells at 37°C and the effects of addition of TPA at various times. (*A*) ^{125}I-EGF was added at time zero and cell-associated material was determined at various time intervals in the absence (▲) or presence of 33 ng of TPA per ml (○). The TPA was added at the following times: a, 10 min; b, 0; c, 15 min; d, 20 min; e, 30 min; f, 40 min; g, 50 min. All plates received 11,488 cpm of ^{125}I-EGF (specific activity, 77 μCi/μg). (*B*) After a 50-min incubation of HeLa cells with ^{125}I-EGF at 37°C, 33 ng of TPA per ml (●) or 0.003% Me_2SO (○) was added in assay buffer. Incubation at 37°C was continued for various times and the amount of cell-associated ^{125}I-EGF was determined. All plates received 7939 cpm of ^{125}I-EGF (specific activity, 80 μCi/μg).

achieve plateau binding. Then either TPA (33 ng/ml) or Me_2SO was added, and the rate of loss of radioactivity was measured during a subsequent 90-min period. In the control culture there was a gradual decline, whereas in the TPA-treated culture there was a rapid decline, in cell-associated radioactivity (Fig. 1*B*). Thus, TPA is capable of reversing the initial binding of ^{125}I-EGF to cells and also enhances the loss of cell-associated EGF that normally occurs at later time points.

Because the above studies demonstrated that TPA not only inhibits the inital binding of EGF to cells but also accelerates the cellular loss of prebound EGF, it was of interest to determine whether the EGF that was lost was released in an intact or degraded form. Cells were incubated with ^{125}I-EGF at 37°C for 40 min, washed, and then incubated with either Me_2SO (0.003%), TPA (30 ng/ml), or nonradioactive EGF (100 ng/ml) at 37°C for an additional 60 min. The medium was then collected and analyzed on a Bio-Gel P-6 column for determination of the amount of radioactive material present in the medium. Table 2 indicates that with Me_2SO 90% of the radioactivity present in the medium was low molecular weight material—i.e., degraded EGF. On the other hand, at least 50% of the radioactivity released to the medium in the presence of either TPA or EGF had the same molecular weight as intact EGF. In a second experiment TPA or Me_2SO was added together with ^{125}I-EGF at time zero and the medium was assayed at 30, 60, and 90 min to determine the amount of ^{125}I-EGF remaining in the medium (Table 2). It is apparent that in the absence of TPA there was more rapid destruction of the ^{125}I-EGF than there was in the presence of TPA. These results provide evidence that TPA does not exert its effect on cellular binding of EGF by enhancing the degradation of EGF via either direct proteolysis or enhanced cellular internalization and proteolysis.

The influence of temperature on the ability of TPA to affect EGF binding is summarized in Table 3. As described (8, 18), we found that the binding of ^{125}I-EGF to cellular receptors was temperature dependent. During a 1-hr incubation the binding at 37°C was about 2.4 times greater than at 4°C. It is of interest that, although TPA caused about a 77% inhibition of ^{125}I-EGF binding at 37°C, at 22°C the inhibition by TPA was 60% and at 4°C it was only 26% (when compared to the control values at the same temperatures). Additional studies (not shown here) indicate that, when ^{125}I-EGF binding was studied over a time course of several hours at either 22°C or 4°C in the absence or presence of TPA, the percentage inhibition by TPA was greater at all time points at 22°C than at 4°C. Although TPA had only a small effect on EGF binding when added to cells at 4°C, if cells were preincubated with TPA at 37°C (in the absence of EGF), there was a marked inhibition in their capacity to subsequently bind ^{125}I-EGF at 4°C when compared to the binding obtained at 4°C with cells not previously exposed to TPA at 37°C.

Table 2. Effects of TPA on release and degradation of EGF in HeLa cell cultures

	% of total radioactivity in medium	
	Intact ^{125}I-EGF	Degraded ^{125}I-EGF
Experiment I		
^{125}I-EGF for 40 min, wash, then add medium plus		
TPA (30 ng/ml) 60 min	51	49
Me_2SO (0.003%) 60 min	10	90
EGF (100 ng/ml) 60 min	58	42
Experiment II		
^{125}I-EGF plus		
TPA (30 ng/ml): 30 min	97	3
60 min	95	5
90 min	89	11
Me_2SO (0.003%): 30 min	92	8
60 min	69	31
90 min	50	50

Cells were grown to near confluency and binding assays were then done at 37°C under the conditions indicated in the table. The medium samples were then collected and the extent of degradation of ^{125}I-EGF was determined by gel filtration on Bio-Gel P-6 columns.

Table 3. Effect of temperature on TPA inhibition of EGF binding

Temperature, °C	^{125}I-EGF bound, cpm/10^6 cells −TPA	+TPA	% of control
4	3110	2295	73.8
22	6634	2612	39.4
37	7499	1687	22.5

HeLa cells were incubated with ^{125}I-EGF (22,727 cpm; specific activity, 57 μCi/μg) plus or minus TPA (33 ng/ml) for 60 min at the indicated temperature, and the amount of cell-bound material was measured.

To determine the effect of temperature on the ability of TPA to enhance the loss of previously bound EGF from cells, we first allowed cells to bind ^{125}I-EGF at 37°C for 50 min, shifted the cells to either 4°C or 22°C, added either Me_2SO or TPA, and then measured the rate of loss of cell-associated ^{125}I-EGF (Fig. 2). The 22°C temperature was chosen because previous studies (18) indicated that this temperature minimizes loss of cell-bound EGF due to internalization and degradation. It is apparent that at 22°C TPA induced a rapid loss of cell-associated ^{125}I-EGF. At 4°C, however, there was no significant loss of ^{125}I-EGF in the presence of TPA. Thus, the ability of TPA to induce the loss of cell-associated ^{125}I-EGF is also markedly temperature dependent. Control studies done without adding TPA indicated that at 4°C there was no loss of cell-associated ^{125}I-EGF over a 12-hr time period and at 22°C no loss was observed over a 5-hr period.

During the course of these studies we have observed that, after prolonged exposure to TPA, HeLa cells "escape" the TPA-induced inhibition of EGF binding. This is shown in Table 4. HeLa cells were grown in monolayer cultures in the absence or presence of TPA (20 ng/ml) over a 4 day period, and each day replicate plates were assayed for EGF binding in the ab-

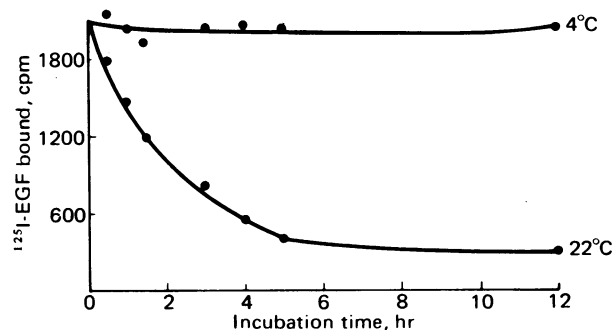

FIG. 2. Effect of temperature on TPA-induced release of cell-bound ^{125}I-EGF. HeLa cells were incubated with ^{125}I-EGF (6660 cpm per plate; specific activity, 67 μCi/μg) for 55 min at 37°C. The cells were then shifted to either 4°C or 22°C, 33 ng of TPA per ml was added, and the amount of cell-associated radioactivity was measured in replicate plates during the subsequent 12 hr.

Table 4. Cellular escape from TPA-induced inhibition of EGF binding during growth in TPA

Growth condition	Assay condition	^{125}I-EGF bound (cpm/10^6 cells) on day: 0	1	2	3	4
Me_2SO	Me_2SO	3486	2292	1322	979	930
Me_2SO	TPA	106	209	170	121	130
TPA	Me_2SO	106	1776	1342	1258	1246
TPA	TPA	106	1004	1085	1072	1171

On day 0 HeLa cells were seeded at 7×10^5 per 5-cm dish in complete growth medium in the presence of TPA (20 ng/ml) or Me_2SO (0.002%) and grown at 37°C. During the subsequent 4 days, replicate plates were used to determine cell counts and ^{125}I-EGF binding. The binding assays were done for 55 min at 37°C with 11,900 cpm of ^{125}I-EGF per plate (specific activity, 67 μCi/μg) plus or minus freshly added TPA (33 ng/ml) or Me_2SO (0.003%). By day 4 the cell counts in the Me_2SO-containing cultures were $5.3 \pm$ (SD) 0.3×10^6 cells per plate and that in TPA-containing cultures were $3.9 \pm 0.5 \times 10^6$ cells per plate.

sence or presence of TPA at 37°C for 50 min. During this time period cell counts indicated that the cultures underwent about three doublings and there was slight (about 20%) growth inhibition by TPA. The results obtained in the absence of TPA indicated that as the culture aged there was about a 1:3.5 decrease in the number of receptors per cell (Table 4). The reasons for this are not known, but it is possible that as cells become confluent their content of EGF receptors is decreased. We were surprised to find that, although initially TPA caused a marked inhibition of ^{125}I-EGF binding (day 0), within 1 day of continuous exposure to TPA the cells escaped from this TPA effect and remained resistant to TPA-induced inhibition for at least the subsequent 3 days. The escape can not be attributed to exhaustion or degradation of TPA from the growth medium because the cells grown in the presence of TPA remained resistant to TPA-induced inhibition of EGF binding even when fresh TPA was added directly to the binding assay (Table 4). Additional studies revealed that when cells were exposed to 10 ng of TPA per ml in complete growth medium the onset of escape could be detected within 4 hr, and within 24 hr EGF binding had almost returned to the control value. The ^{125}I-EGF binding observed when the cells had escaped was still quenched by an excess of cold EGF, indicating that the binding was specific and saturable. Additional studies are required, however, to determine whether the receptors are qualitatively and quantitatively similar to those found in HeLa cells never exposed to TPA.

DISCUSSION

Although the precise mechanism by which TPA inhibits the binding of ^{125}I-EGF to its cellular receptors is not clear at the present time, the results obtained in this study, together with the fluid mosaic model of cellular membranes and previous findings on receptor–ligand interactions suggest several possible mechanisms.

In theory, TPA could exert an inhibitory effect on the EGF receptors by a direct mechanism—i.e., binding directly to the same site occupied by EGF. Alternatively it could act indirectly by binding to other sites on the EGF receptors or by inducing changes in the lipid microenvironment in which the receptors are embedded. Consistent with the latter possibility is the fact that TPA induces several other early effects on membrane structure and function, including altered nutrient uptake (22), altered fluorescence polarization of the membrane probe diphenylhexatriene (23), altered phospholipid metabolism (9, 24, 25), and altered cell adhesion (26). Either indirect mechanism could lead to an altered conformation of the receptor or to receptor clustering with a resultant decrease in EGF binding and increased release from the receptor of previously bound EGF.

Several findings in the present study favor an indirect mechanism for the action of TPA on EGF receptors. These include our finding that with decreasing temperature there is a decrease in the ability of TPA to inhibit EGF binding. This effect has also recently been seen in 3T3 cells (8, 27) and suggests that the action of TPA is mediated by a temperature-dependent process, such as clustering of the receptors or a change in the structure of the membrane fluid mosaic. Receptor clustering is known to be highly temperature dependent (28, 29). The fact that during growth in TPA HeLa cells became refractory to the inhibitory effect of the agent on EGF receptors also suggests an indirect mechanism, because it is unlikely that cells could rapidly modify the binding specificity of the receptor. The exposure of cells to EGF usually results in a decrease in their level of EGF receptors (18). This "down regulation" of receptors has also been seen with various hormones (30, 31) and with low density lipoprotein (32) and is thought to be due to intracellular translocation of the receptor–ligand complex. The escape phenomenon described above with TPA is in a sense the reciprocal of down regulation but its mechanism is not clear at the present time. It will be of interest to determine the extent to which cells and tissues may become refractory to the other effects of TPA and the relevance of this phenomenon to tumor promotion.

In HeLa cells the inhibition of ^{125}I-EGF binding by TPA is noncompetitive with physiologic concentrations of EGF (1). This is also true with inhibition of ^{125}I-EGF binding by mezerein or other TPA analogs (unpublished studies). Recent studies (8, 27), however, have reported that with mouse 3T3 cells TPA inhibition of EGF binding was reversed with very high concentrations of EGF. Further studies are required to determine whether this reflects a true difference between 3T3 and HeLa cells or differences in the conditions used to demonstrate competition. Although EGF binds specifically to isolated preparations of plasma membrane (33), EGF binding is not inhibited when TPA is added to this system (unpublished results). This also favors an indirect mechanism rather than direct competition for binding to the EGF receptor. The nature of this indirect process is not known but our unpublished inhibitor studies suggest that it does not require energy metabolism or macromolecular synthesis.

Certain previous results on hormone receptor–ligand interactions may be particularly relevant to the present study. De Meyts *et al.* (34) observed negative cooperativity in the binding of insulin to its cellular receptors—i.e., with increasing receptor occupancy there was a decrease in apparent receptor affinity—and reviewed evidence that this occurs with several other hormone receptor interactions. They suggested that insulin receptors can exist in at least two conformational states—one in which the receptor–ligand complex dissociates slowly and the other in which it dissociates rapidly—and that with increasing receptor occupancy there is an increase in the latter form. These two states may reflect a change in the conformation of the receptor or in its state of clustering. Ligand-induced clustering of receptors within the fluid mosaic membrane has been observed with a number of substances (29). At 37°C or 22°C TPA might induce similar changes in EGF receptors and thus impair their EGF binding capacity. On the other hand, at 4°C the mobility of the EGF receptors might be sufficiently limited that TPA could no longer induce their clustering and thus EGF binding would not be impaired. The fact that most of the EGF released into the medium in the presence of TPA was intact molecules suggests that the conformational changes or clustering of EGF-receptor complexes induced by TPA oc-

curs at the exterior of the cell surface. This result also provides evidence that TPA does not act simply by inducing a protease that destroys EGF; nor does it appear to act by inducing internalization and degradation of the EGF–receptor complex.

The effect of TPA appears to be preferential for the EGF receptors. We have found that TPA does not inhibit the binding of insulin to its receptor in HeLa cells. Nor does it inhibit nerve growth factor–receptor binding in neuroblastoma cultures (35). Its marked inhibitory affect on receptor binding also appears to be specific for EGF receptors in 3T3 cells (8, 27). Although TPA does not inhibit the binding of [^{3}H]propanolol to the β-adrenergic receptor in mouse skin, there is evidence that it causes an "uncoupling" of this receptor with respect to adenyl cyclase function (36). Further studies may reveal subtle effects of TPA on the function of other receptors. It is possible that the specific effects on EGF receptors are due to unique aspects of the membrane microenvironment of these receptors or a particular susceptibility of these receptors to conformational changes.

A related question is whether or not the altered state of EGF receptors induced by the phorbol ester tumor promoters triggers cellular responses similar to those that occur with EGF binding. The binding of antibodies to insulin receptors does evoke cellular responses similar to those induced when the ligand is insulin, presumably because the antibody induces receptor clustering (37). We have previously reviewed evidence that in certain cells TPA and EGF induce a number of similar phenotypic changes (1). With respect to induction of plasminogen activator, TPA and EGF show simple additive effects (11). On the other hand, there is evidence that some of the effects of TPA are exerted independently of EGF receptors (38). We have found that, although TPA is a potent inhibitor of differentiation in certain clones of murine erythroid leukemia cells (39), these cells lack detectable EGF receptors and do not respond to EGF (unpublished results). Perhaps, if cells have EGF receptors then they will be "engaged" by TPA; but TPA can also exert membrane and cellular effects that are not mediated by EGF receptors.

The present results indicate that TPA can exert specific and complex effects on the function of EGF receptors. These and other effects of TPA in cell culture systems (1–9) make TPA a valuable probe for elucidating aspects of membrane structure and function related to growth control and gene expression. In addition, further studies of these effects of TPA and related phorbol esters may shed light on their mechanism of action as tumor promoters.

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