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Tumor promoters block tyrosine-specific phosphorylation of the epidermal growth factor receptor

(phorbol esters/indole alkaloids/polyacetates/protein kinase C)

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ABSTRACT **Tyrosine-specific phosphorylation of the epidermal growth factor (EGF) receptor in hormonally stimulated A431 cells is blocked by three chemically distinct classes of tumor promoters. Tumor-promoting esters of the diterpene phorbol (phorbol 12-myristate 13-acetate, β-phorbol 12,13-dibutyrate, and β-phorbol 12,13-didecanoate), indole alkaloids (teleocidin and lyngbyatoxin A), and polyacetates (aplysiatoxin and debromoaplysiatoxin) all inhibited EGF-stimulated phosphorylation of the receptor. Non-tumor-promoting analogs (β-phorbol, α-phorbol 12,13-didecanoate, and hydrolyzed teleocidin) had no effect on the levels of receptor phosphorylation. The ED_{50} values of the inhibitory effect (0.1–3 ng/ml) reflected the relative tumor-promoting abilities of these compounds *in vivo*. None of the tumor promoters tested significantly decreased the overall specific binding of ^{125}I-labeled EGF to A431 cells. Scatchard analysis, however, revealed two apparent EGF receptors in this cell type. The dose-responses for tumor-promoter inhibition of EGF receptor tyrosine phosphorylation and high-affinity EGF binding were similar, suggesting that the same initial event is responsible for both effects. This demonstrates a correlation between modulation of EGF receptor binding and phosphorylation of tyrosine by tumor promoters. The data suggest a possible role for protein kinase C, the putative cellular receptor for these tumor promoters, in the mechanism of action.**

Tumor promoters can modulate the action of epidermal growth factor (EGF) by reducing EGF receptor binding (1–4) and internalization (5, 6) and by potentiating the mitogenic activity of EGF in quiescent cells (7, 8). Upon binding to cells, EGF stimulates the tyrosine phosphorylation of its receptors (9, 10) via a receptor-associated kinase activity. The potential regulatory role of tyrosine kinase activity is suggested by its association with the action of other growth factors (insulin and platelet-derived growth factor) (11–13) and its apparent requirement for oncogenic transformation by a number of retroviruses (14, 15). This led us to examine whether the effects of tumor promoters on the action of EGF involved changes in receptor phosphorylation.

We have found that three chemically distinct classes of tumor promoters [esters of the diterpene phorbol, indole alkaloids (16), and polyacetates (17)] block tyrosine phosphorylation of EGF receptors in human epidermal carcinoma (A431) cells. The loss of EGF receptor tyrosine phosphorylation correlates with loss of EGF binding to the apparent high-affinity EGF receptor. These results, which have been reported in preliminary form (18), suggest a possible role for protein kinase C, the putative tumor promoter receptor, in the regulation of EGF-stimulated tyrosine kinase activity.

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MATERIALS AND METHODS

Materials. Esters of the diterpene phorbol were purchased from Sigma. Teleocidin, lyngbyatoxin A, aplysiatoxin, and debromoaplysiatoxin were prepared as previously described (16, 17). Mouse EGF (Collaborative Research, Waltham, MA) was iodinated by the IODO-GEN method (Pierce) (19) using $Na^{125}I$ (Amersham). The specific activity of the iodinated EGF was approximately 30 Ci/mmol (1 Ci = 37 GBq).

Cell Culture. A431 cells (20) were grown in Dulbecco's modified Eagle's medium (DME medium) supplemented with 10% heat-inactivated fetal calf serum.

$^{32}P_i$ Labeling and Detergent Extraction of Cells. Low-density cultures (10^5 cells per 35-mm well) of A431 cells were preincubated with $^{32}P_i$ at 0.5 mCi/ml in phosphate-free DME medium plus dialyzed heat-inactivated fetal calf serum for 3–4 hr at 37°C. Dimethyl sulfoxide (Me_2SO; final concentration 1%), tumor promoters, or nonpromoting analogs (dissolved in Me_2SO) were added for 10 min. EGF was then added for an additional 40 min. The cultures were placed on ice, the binding media were removed, and the cells were scraped and extracted with Tris-buffered Triton (10 mM Tris·HCl/1% Triton X-100/10 mM NaCl/5 mM EDTA/1 mM phenylmethylsulfonyl fluoride/0.1% bovine serum albumin, pH 8).

Phospho Amino Acid Analysis. Phospho amino acid analysis was carried out by acid hydrolysis and two-dimensional thin-layer electrophoresis as described (21).

Immunoprecipitation with Antibody to Phosphotyrosine. Detergent extracts of ^{32}P-labeled cells were dialyzed against Tris-buffered Triton, incubated with bovine serum albumin coupled to Sepharose 4B to eliminate nonspecific binding proteins, and then absorbed by monoclonal anti-phosphotyrosine coupled to Sepharose 4B. The phosphotyrosine-containing proteins were eluted with buffer containing 40 mM phenylphosphate, denatured in 1% sodium dodecyl sulfate/0.128 M 2-mercaptoethanol for 10 min at 100°C, separated by electrophoresis on 0.1% sodium dodecyl sulfate/7.5% polyacrylamide gels, and visualized by autoradiography (21).

Binding of ^{125}I-Labeled EGF (^{125}I-EGF) to Cells. A431 cells in DME medium containing 0.5% bovine serum albumin were pretreated with tumor promoters or Me_2SO at 37°C. ^{125}I-EGF was added to the cultures at the indicated concentrations, and incubation was continued for 40 min. Radioactivity was measured in binding media, first wash, second wash, and cells.

Scatchard Analysis. The values for EGF receptor binding affinity and receptor number were determined by analysis of the EGF binding data using LIGAND, a computer program for fitting multiple binding site data developed by Munson

Abbreviations: EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; Me_2SO, dimethyl sulfoxide.

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and Rodbard (22) and adapted to the IBM PC computer by R. Rosner and M. R. Rosner.

RESULTS

Initially, we determined whether tumor promoters altered the total amount of phosphotyrosine in EGF-stimulated cells. Preliminary studies had shown that EGF alone caused a 4- to 6-fold increase in the ratio of phosphotyrosine to phosphoserine, in agreement with observations of others (10). Pretreatment of cells with β-phorbol did not alter this ratio (Fig. 1 *a* and *b*). In contrast, phorbol 12-myristate 13-acetate (PMA) inhibited the EGF-stimulated tyrosine phosphorylation by about 80% (Fig. 1 *c* and *d*).

To determine if phosphorylation of the EGF receptor itself was inhibited by tumor promoters, EGF receptors with phosphorylated tyrosine residues were isolated by affinity chromatography on monoclonal anti-phosphotyrosine antibody (21) coupled to Sepharose-4B beads. The EGF receptor protein was easily identified by molecular weight (170,000) and increased phosphorylation after EGF binding

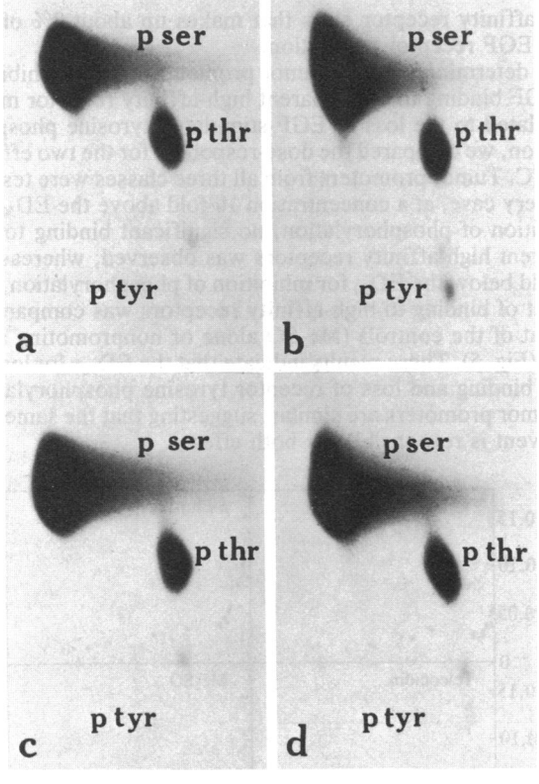

FIG. 1. Phospho amino acid analysis of cell extracts from A431 cells treated with either the non-tumor-promoter β-phorbol or the tumor promoter PMA in the presence or absence of EGF. $^{32}P_i$-labeled A431 cells were incubated with Me_2SO, tumor promoters at 100 ng/ml, or non-tumor-promoting compounds (added in Me_2SO). Samples were then analyzed by two-dimensional (pH 1.9, pH 3.5) thin-layer electrophoresis. (*a*) β-Phorbol at 100 ng/ml; (*b*) β-phorbol at 100 ng/ml plus 12.5 nM EGF; (*c*) PMA at 100 ng/ml; and (*d*) PMA at 100 ng/ml plus 12.5 nM EGF. Locations of phosphoserine (p ser), phosphothreonine (p thr), and phosphotyrosine (p tyr) standards are indicated on the autoradiograph. The autoradiograph is a mosaic of single film exposed for 12 hr. Phosphorylated amino acids were then scraped from the thin-layer sheet and radioactivity was determined by liquid scintillation counting. Quantitation of the radioactive spots yielded the following results, all in cpm: (*a*) Phosphoserine, 39,134; phosphothreonine, 3335; phosphotyrosine, 77. (*b*) Phosphoserine, 46,436; phosphothreonine, 3050; phosphotyrosine, 316. (*c*) Phosphoserine, 53,809; phosphothreonine, 3895; phosphotyrosine, 74. (*d*) Phosphoserine, 40,535; phosphothreonine, 3725; phosphotyrosine, 94.

(Fig. 2, lanes a and b), and precipitation with antibody to EGF receptor.

Tumor promoters blocked the enhancement of receptor phosphorylation by EGF, whereas non-tumor-promoting analogs of these compounds had no effect. The action of representative tumor promoters and non-tumor-promoting analogs (100 ng/ml) on growth factor-induced phosphorylation of receptor is shown in Fig. 2, lanes c–f. In the absence of EGF, neither tumor promoters alone nor Me_2SO stimulated the tyrosine phosphorylation of the receptor.

Dose–response curves for the three classes of tumor promoters (Fig. 3) showed that phosphorylation of tyrosine in the EGF receptor exhibited a strong dependence on tumor promoter concentration. For each tumor promoter, 60–85% of the inhibition occurred over a 100-fold concentration range. This permitted us to determine the 50% inhibitory doses (ED_{50}s) for each compound. The ED_{50}s for all of these agents are extremely low (ranging from 0.1 to 3 ng/ml) and correlate well with their abilities to promote tumors in the two-stage mouse skin model of carcinogenesis (Table 1).

To determine whether the loss of EGF-stimulated tyrosine phosphorylation due to tumor promoters could be a consequence of reduced EGF binding to the receptor, we determined the extent of ^{125}I-EGF binding to A431 cells in the presence and absence of the tumor promoters noted above. Cells were treated under identical conditions (same density, temperature, and time of incubation) for binding studies as for phosphorylation studies. In contrast to a recent report indicating no change (25), we did note a slight reduction in EGF binding to A431 cells after treatment with each of the classes of tumor promoters. This effect is more dramatically illustrated when the data are analyzed by Scatchard plots

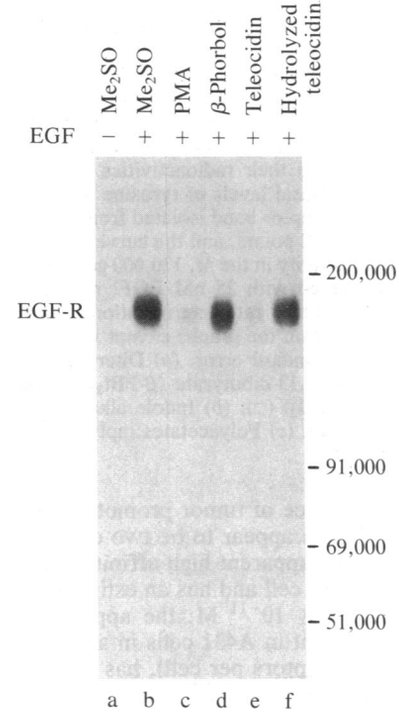

FIG. 2. Effects of tumor promoters on the tyrosine phosphorylation of EGF receptors (EGF-R). $^{32}P_i$-labeled A431 cells were incubated with Me_2SO alone or tumor promoters or non-tumor-promoting compounds at 100 ng/ml in the presence or absence of 25 nM EGF. Phosphotyrosine-containing proteins were then extracted and purified by immunoprecipitation with anti-phosphotyrosine antibody. Lane a, no EGF, Me_2SO; lane b, EGF, Me_2SO; lane c, EGF, PMA; lane d, EGF, β-phorbol; lane e, EGF, teleocidin; lane f, EGF, hydrolyzed teleocidin. The autoradiograph is a mosaic of a single gel and film exposure. Molecular weights are indicated on the right.

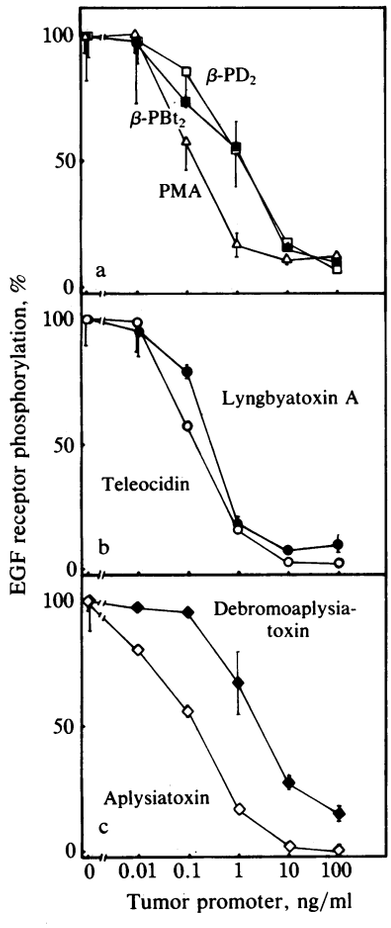

FIG. 3. Inhibition of the tyrosine-specific phosphorylation of the EGF receptor as a function of tumor promoter concentration. Tyrosine-phosphorylated proteins were extracted and purified from A431 cells. Gel slices corresponding to the EGF receptor (the M_r 170,000 band) were excised and their radioactivities were measured in a scintillation counter. Basal levels of tyrosine phosphorylation (the amount in the EGF receptor band isolated from unstimulated cells) were subtracted from all points, and the curves were normalized to the amount of radioactivity in the M_r 170,000 gel band that occurred when cells were treated with 25 nM EGF, no tumor promoters. Points are the means of triplicate determinations. Standard errors of the mean are indicated on the graphs except where the size of the symbol exceeds the standard error. (*a*) Diterpene phorbol esters: PMA (△), β-phorbol 12,13-dibutyrate (β-PBt_2) (■), and β-phorbol 12,13-didecanoate (β-PD_2) (□). (*b*) Indole alkaloids: teleocidin (○) and lyngbyatoxin A (●). (*c*) Polyacetates: aplysiatoxin (◇) and debromoaplysiatoxin (◆).

(Fig. 4). In the absence of tumor promoters or in the presence of Me_2SO, there appear to be two classes of EGF receptors at 37°C. The apparent high-affinity class consists of 1×10^5 receptors per cell and has an estimated dissociation constant K_d of 5.3×10^{-11} M; the apparent low-affinity class, which is present in A431 cells in an abnormally high amount (4×10^6 receptors per cell), has an apparent K_d of 3.5×10^{-8} M. Although these values were determined at 37°C, when the system is not at equilibrium because of down-regulation, similar receptor classes were recently reported for glutaraldehyde-fixed A431 cells, which could not take up EGF (26). We have also detected both apparent affinity classes at 4°C (27), where down-regulation does not occur. In the presence of tumor promoters, almost all EGF binding to the apparent high-affinity class disappeared; binding to the low-affinity class, however, remained effectively unchanged (Fig. 4). Thus, in A431 cells, tumor promoters appear to inhibit EGF binding exclusively to an apparent high-affinity receptor class that makes up about 2% of the total EGF receptor population.

Table 1. Inhibition of EGF receptor phosphorylation

Compound	ED_{50}, ng/ml	Promoting activity
PMA	0.1	+++
Teleocidin	0.1	+++
Aplysiatoxin	0.1	+++
Lyngbyatoxin A	0.3	+++
β-Phorbol 12,13-didecanoate	1	++
β-Phorbol 12,13-dibutyrate	1	+
Debromoaplysiatoxin	3	++
4α-Phorbol 12,13-didecanoate	>100	−
β-Phorbol	>100	−
Hydrolyzed teleocidin	>100	ND

The abilities of members of the three different chemical classes of tumor promoters to inhibit EGF receptor tyrosine phosphorylation and to promote tumors in the two-stage mouse skin model of carcinogenesis are tabulated. The ED_{50}s were determined from the data presented in Fig. 2. The *in vivo* promotional activity of each agent relative to that of PMA is ranked on a scale of − to +++. ND, not determined. These data are from refs. 23 and 24. The relationship between the potencies of these compounds is closer to logarithmic than linear.

To determine whether tumor promoter-induced inhibition of EGF binding to the apparent high-affinity receptor might be related to the loss of EGF-stimulated tyrosine phosphorylation, we compared the dose-responses for the two effects at 37°C. Tumor promoters from all three classes were tested. In every case, at a concentration 10-fold above the ED_{50} for inhibition of phosphorylation, no significant binding to the apparent high-affinity receptors was observed, whereas, at 10-fold below the ED_{50} for inhibition of phosphorylation, the extent of binding to high-affinity receptors was comparable to that of the controls (Me_2SO alone or nonpromoting analogs) (Fig. 5). These results indicate that the ED_{50}s for loss of EGF binding and loss of receptor tyrosine phosphorylation by tumor promoters are similar, suggesting that the same initial event is responsible for both effects.

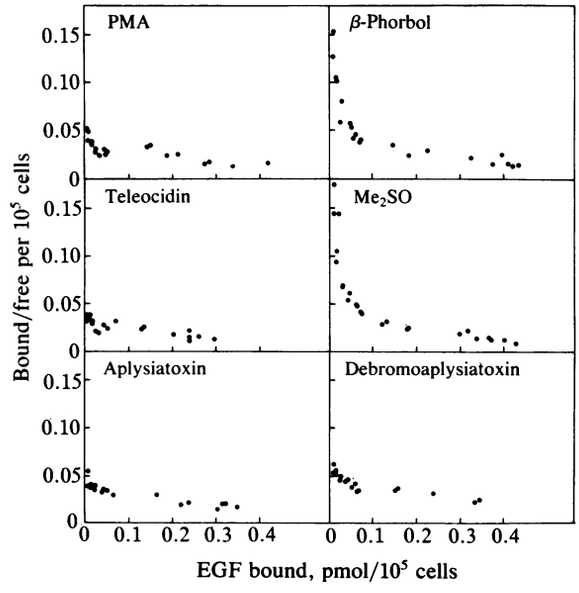

FIG. 4. Scatchard analysis showing the effect of tumor promoters on the specific binding of ^{125}I-EGF to A431 cells. Cells were pretreated at 37°C with tumor promoters or nonpromoting analog at 100 ng/ml or Me_2SO and the EGF binding activity of the cells was determined. Nonspecific binding, determined in the presence of unlabeled EGF at 1 μg/ml, has been subtracted from the total binding observed.

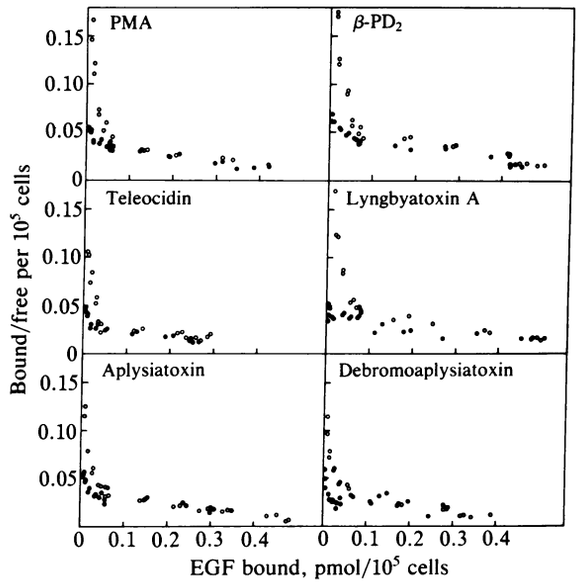

FIG. 5. Scatchard analysis showing binding of ^{125}I-EGF to A431 cells in the presence of tumor promoters at dosages 10-fold above and 10-fold below the ED_{50} for inhibition of tyrosine phosphorylation. Samples were treated as described for Fig. 4, except that the following concentrations of tumor promoters were added: PMA, 1 ng/ml (●) and 0.01 ng/ml (○); β-phorbol 12,13-didecanoate (β-PD_2), 10 ng/ml (●) and 0.1 ng/ml (○); teleocidin, 1 ng/ml (●) and 0.01 ng/ml (○); lyngbyatoxin A, 3 ng/ml (●) and 0.03 ng/ml (○); aplysiatoxin, 1 ng/ml (●) and 0.01 ng/ml (○); debromoaplysiatoxin, 30 ng/ml (●) and 0.3 ng/ml (○).

DISCUSSION

The use of the monoclonal anti-phosphotyrosine antibody permitted selective isolation of the subpopulation of EGF receptors that had become tyrosine-phosphorylated in response to EGF, enabling direct quantitation of the inhibition of receptor phosphorylation by tumor promoters. Although EGF receptors are phosphorylated at serine and threonine residues (10), the amount of radioactivity in the 170,000 M_r band appears to reflect the amount of phosphotyrosine in the purified receptor under the above conditions of labeling and EGF treatment. Studies with EGF receptor obtained through immunoprecipitation with polyclonal anti-EGF receptor antibody have shown similar inhibition of tyrosine phosphorylation by tumor promoters over the same dose range (unpublished results). These results also indicate that the antibody to phosphotyrosine extracts a representative population of EGF receptors.

To monitor the effect of tumor promoters on EGF receptor phosphorylation, A431 cells were chosen as the initial system of study because they have a large number of EGF receptors per cell (20, 28) and show marked enhancement in tyrosine phosphorylation after EGF treatment (10, 29). Several observations suggest that A431 cells are typical in their response to EGF in the presence or absence of tumor promoters. Even though high EGF concentrations are inhibitory to growth (29), it has recently been reported that A431 cells do respond mitogenically to low EGF concentrations (26) in the range of the K_d for high-affinity EGF binding. Further, as observed in other epithelial cell types (6, 30), we have noted a reduction in EGF binding in A431 cells after tumor promoter treatment that results from loss of apparent high-affinity but not low-affinity EGF binding. It should be noted that the apparent high-affinity receptor class may represent a distinct receptor population, may be interconvertible with the low-affinity class, or may result from negative cooperativity of EGF binding. Finally, we have shown that tumor promoters inhibit EGF receptor phosphorylation in preliminary experiments with a nontransformed human fibroblast cell line. Thus, the inhibition of EGF receptor tyrosine phosphorylation noted in A431 cells appears to be a more general phenomenon.

The fact that all tumor promoters tested modulate EGF receptor interactions in a similar manner suggests that they are acting through a common mechanism. We have found that the three classes of tumor promoters inhibit the specific binding of [^{3}H]PMA to a mouse particulate fraction (31). Therefore, the actions of phorbol esters, indole alkaloids, and polyacetates appear to be mediated through the same receptor system (31, 32), which differs from the receptor for EGF (2). Recent studies from a number of laboratories have suggested that a major receptor for tumor promoters is the Ca^{2+}, phospholipid-dependent protein kinase (C kinase), which becomes activated in the presence of PMA, teleocidin, debromoaplysiatoxin, or diacylglycerol and phosphorylates serine and threonine residues (33–35). We (unpublished data) and others (36) have observed that phorbol ester tumor promoters enhance serine and threonine phosphorylation of the EGF receptor. Moreover, the tumor promoter dosage that elicits this enhancement is similar to that for inhibition of high-affinity EGF receptor binding and inhibition of tyrosine phosphorylation (unpublished data). These results suggest that the same initial event is responsible for all three

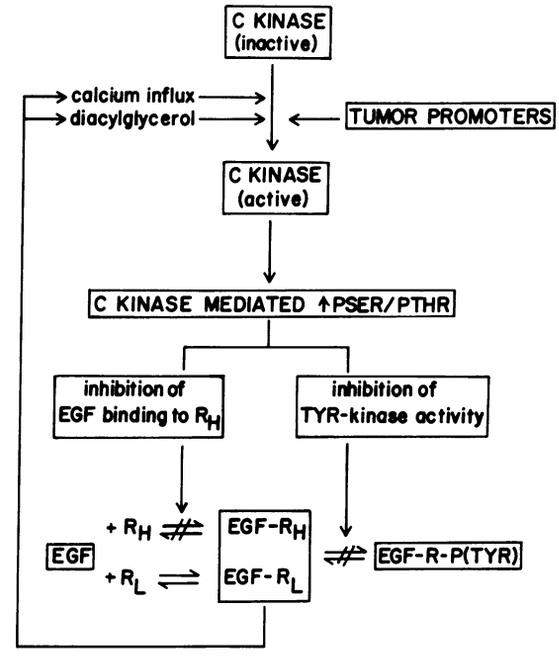

FIG. 6. Model describing the modulation of EGF receptors (high affinity, R_H; and low affinity, R_L) by tumor promoters. Tumor promoters bind to protein kinase C, stimulating direct or indirect serine and threonine phosphorylation of the EGF receptor. This or a related phosphorylation event could result in a conformational or steric change in the EGF receptor such that the high-affinity receptor can no longer bind to EGF and the EGF-stimulated tyrosine kinase is only partially activated, if at all. This loss of activation would result in inhibition of tyrosine phosphorylation of the EGF receptor and other substrates. Direct inactivation of substrates for tyrosine phosphorylation or activation of a tyrosine-specific phosphatase could lead to similar results. Thus, tumor promoters, through activation of the C kinase, would suppress the EGF-stimulated tyrosine kinase pathway. Similar sites of C kinase action may be involved in autoregulation of the EGF-stimulated tyrosine kinase. In this feedback pathway, EGF, through increasing Ca^{2+} and diacylglycerol, could transiently activate C kinase and thus inactivate the EGF-stimulated tyrosine kinase.

effects observed and suggest a possible role for C kinase in this pathway.

A model that describes the modulation of EGF receptors by tumor promoters is presented in Fig. 6. C kinase plays a central role in this mechanism. In addition to its activation by tumor promoters, C kinase may also be activated as a consequence of EGF binding. EGF induces enhanced Ca^{2+} influx and phosphatidylinositol turnover (yielding diacylglycerol) in A431 cells (37). The transient increase of the postulated endogenous C kinase activators, Ca^{2+} and diacylglycerol, may lead to feedback inhibition of the EGF-stimulated tyrosine kinase. This model of the interrelationship between EGF and tumor promoter action can be tested, and it may be applicable to other growth factor systems.

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1. Lee, L.-S. & Weinstein, I. B. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5168–5172.
2. Lee, L.-S. & Weinstein, I. B. (1978) *Science* **202,** 313–315.
3. Shoyab, M., De Larco, J. E. & Todaro, G. J. (1979) *Nature (London)* **279,** 387–391.
4. Umezawa, K., Weinstein, I. B., Horowitz, A., Fujiki, H., Matsushima, T. & Sugimura, T. (1980) *Nature (London)* **299,** 411–412.
5. Magun, B. E., Matrisian, L. M. & Bowden, G. T. (1980) *J. Biol. Chem.* **255,** 6373–6381.
6. King, A. C. & Cuatrecasas, P. J. (1982) *J. Biol. Chem.* **257,** 3053–3060.
7. Dicker, P. & Rozengurt, E. J. (1979) *Supramol. Struct.* **11,** 79–93.
8. Frantz, C. N., Stiles, C. D. & Scher, C. D. (1979) *J. Cell. Physiol.* **100,** 413–424.
9. Cohen, S., Carpenter, G. & King, L., Jr. (1981) in *Control of Cellular Division and Development,* eds. Cunningham, D., *et al.* (Liss, New York), Part A, pp. 557–567.
10. Hunter, T. & Cooper, J. A. (1981) *Cell* **24,** 741–752.
11. Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M. & Kahn, C. R. (1982) *Nature (London)* **298,** 667.
12. Ek, B., Westermark, B., Wasteson, A. & Heldin, C.-H. (1982) *Nature (London)* **295,** 419–420.
13. Nishimura, J., Huang, J. S. & Deuel, T. F. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4303–4307.
14. Houslay, M. D. (1981) *Biosci. Rep.* **1,** 19–34.
15. Cooper, J. A. & Hunter, T. (1981) *J. Cell. Biol.* **91,** 878–883.
16. Fujiki, H., Mori, M., Nakayasu, M., Terada, M., Sugimura, T. & Moore, R. E. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3872–3876.
17. Fujiki, H., Suganuma, M., Nakayasu, M., Hoshino, H.-O., Moore, R. E. & Sugimura, T. (1982) *Gann* **73,** 495–497.
18. Rosner, M. R., Friedman, B., Frackelton, A. R., Ross, A. & Sugimura, T. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42,** 1905 (abstr.).
19. Markwell, M. A. & Fox, C. F. (1978) *Biochemistry* **17,** 4807–4817.
20. Fabricant, R. N., De Larco, J. E. & Todaro, G. J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 565–569.
21. Frackelton, A. R., Ross, A. H. & Eisen, M. N. (1983) *Cell. Biol.* **3,** 1343–1352.
22. Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107,** 220–239.
23. Shoyab, M. & Todaro, G. J. (1980) *Nature (London)* **288,** 451–455.
24. Fujiki, H. & Sugimura, T. (1983) in *Genes and Proteins in Oncogenesis,* eds. Weinstein, I. B. & Vogel, H. J. (Academic, New York), pp. 111–123.
25. Froscia, M., Tapley, P. M., Guy, G. R., Pappalardo, S., Jones, M. J. & Murray, A. W. (1982) *Carcinogenesis* **3,** 837–839.
26. Kamamoto, T., Sato, J. D., Le, A., Polikoff, J., Sato, G. H. & Mendelsohn, J. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1337–1341.
27. Friedman, B., Connors, J. M., McCaffrey, P. G., Fujiki, H., Sugimura, T. & Rosner, M. R. (1983) *J. Cell Biol.* **97,** 166a (abstr.).
28. Haigler, H. T., Ash, J. F., Singer, S. J. & Cohen, S. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3317–3321.
29. Gill, G. N. & Lazar, C. S. (1981) *Nature (London)* **293,** 305–307.
30. Lockyer, J. M., Bowden, G. T., Matrisian, L. M. & Magun, B. E. (1981) *Cancer Res.* **41,** 2308–2314.
31. Fujiki, H., Suganuma, M., Moore, R. E. & Sugimura, T. (1983) in *Human Carcinogenesis: Symposium,* eds. Harris, C. C. & Autrup, H. N. (Academic, New York), pp. 303–324.
32. Horowitz, A. D., Fujiki, H., Weinstein, I. B., Jeffrey, A., Okin, E., Moore, R. E. & Sugimura, T. (1983) *Cancer Res.* **43,** 1529–1535.
33. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 7847–7851.
34. Ashendel, C. L., Staller, J. M. & Boutwell, R. K. (1983) *Cancer Res.* **43,** 4333–4337.
35. Fujiki, H., Tanaka, Y., Miyake, R., Kikkawa, U., Nishizuka, Y. & Sugimura, T. (1984) *Biochem. Biophys. Res. Commun.,* in press.
36. Iwashita, S. & Fox, C. F. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42,** 1902 (abstr.).
37. Sawyer, S. T. & Cohen, S. (1981) *Biochemistry* **20,** 6280–6286.